

STUDIES WITH ANTIBODIES TO FENITROTHION AND ITS METABOLITES

THESIS SUBMITTED
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHARMACY
IN
PHARMACOLOGY

SUBMITTED BY
MILIND MANGESH PADKI

UNDER THE GUIDANCE OF
DR. M. B. BHIDE

DEPARTMENT OF PHARMACOLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
BOMBAY

Disclaimer

This research work belongs to

Mr Milind Mangesh Padki

No content of this work can be
circulated or used for any commercial
purpose without the consent of
respective researcher

**STUDIES WITH ANTIBODIES TO FENITROTHION
AND ITS METABOLITES**

A

THESIS

SUBMITTED TO

THE UNIVERSITY OF BOMBAY

FOR THE DEGREE OF

MASTER OF PHARMACY

FACULTY OF TECHNOLOGY

IN PHARMACOLOGY

615.33 : 632.957

BY

276

PAD

MILIND MANGESH PADKI

B.Pharm.

**DEPARTMENT OF PHARMACOLOGY
HAFFKINE INSTITUTE
BOMBAY.**

DECEMBER 1980.

ACKNOWLEDGEMENTS

I have neither the power of expression nor the vocabulary, to put into words the deep sense of gratitude that I feel for Dr M.B.Bhide, my teacher, mentor and guide. Suffice it to say that I have known many specialists; his speciality is omniscience. The inspiration and fire that he has kindled in me, I am sure, shall go a long way beyond this thesis.

My thanks are due to Dr. B.B.Gaitonde, Ex-Director, Haffkine Institute and Dr. K.D.Sharma, Director, Haffkine Institute, for all the facilities extended to me during the course of this work.

I have a special word of thanks for Mr. Satyajit Rath and Miss Vineeta Bal, not so much for helping me in my work, as much for forcing me to work. Without them, I am confident, this work would have taken eons longer than it did.

My sincerest thanks are due to my colleagues Mr P.Y.Naik, Mr U.M.Patil, Mr U.R.Phadke, Mr K.S.Pradhan, Mr R.E.Joshi, Mrs Arti Juvekar and Mr R.M.Gupta. I shall fondly remember forever the good times I have spent with them.

I am also deeply indebted to Mrs J.V.Iyer for the splendid job she has done in typing this thesis and to Mr D.V.Vangsarkar and Mr S.R.Mane for their excellent assistance in preparing the figures.

I thank all my colleagues in the Haffkine Institute for their co-operation.

Lastly, I thank the Indian Council of Medical Research for granting financial assistance to this work.


.....M.M.PADKI.

CONTENTS

Page No.

CHAPTER I : ORGANOPHOSPHATES :

AN INTRODUCTION

1.1.	Organophosphates : An Introduction	...	8
1.2.	Hazards of Pesticide use		10
1.3.	Mechanism of action of organophosphorus pesticides.		13
1.3.1.	Mechanism of action in mammals	...	13
1.3.1.1.	The mammalian nervous system	...	13
1.3.1.2.	Acetylcholinesterase (AChE)		16
1.3.1.3.	"Poisoning" of AChE		19
1.3.1.4.	"Aging" of AChE		18
1.3.2.	Mechanism of action in Arthropods	...	19
1.4.	Fenitrothion		20
1.4.1.	Chemistry		20
1.4.2.	Biological activity of Fenitrothion	...	21
1.5.	Toxicity and its treatment		22
1.5.1.	Manifestations of toxicity due to cholinesterase inhibition.	...	22
1.5.2.	Treatment		24
1.5.3.	Chronic Neurotoxicity		29

CHAPTER II : IMMUNOLOGICAL STUDIES

2.1.	Introduction to antihapten antibodies	...	31
2.2.	Principles of elicitation and characterization of antihapten antibodies.		38
2.2.1.	Methods of conjugation		38
2.2.2.	Choice of carrier		40
2.2.3.	Immunisation procedures		41

2.2.3.1.	Routes of inoculation	...	...	41
2.2.3.2.	Adjuvants	...	...	42
2.2.3.3.	Immunization schedules	...	...	44
2.2.3.4.	Choice of animal species and collection of serum	...	...	45
2.2.4.	Assessment of the antibodies	...	...	46
2.2.5.	Fractionation of the immunoglobulins	...	...	49
2.3.	Materials and methods	...	...	53
2.3.1.	Materials and animals	...	...	53
2.3.2.	Methods	...	...	54
2.3.2.1.	Coupling of Fenitrothion to bovine serum albumin	...	...	54
2.3.2.2.	Conversion of Fenitrothion to Fenitrooxon	...	...	56
2.3.2.3.	Anticholinesterase activity of Fenitrooxon	...	...	57
2.3.2.4.	Coupling of Fenitrooxon to bovine serum albumin	...	...	59
2.3.2.5.	Determination of hapten-carrier ratios in conjugates	...	...	59
2.3.2.6.	Immunization of animals	...	...	61
2.3.2.7.	Bleeding and collection of antisera	...	...	62
2.3.2.8.	Ammonium sulphate precipitation of the immunoglobulins	...	...	64
2.3.2.9.	Characterization of the antibodies	...	...	65
2.4.	Results	...	...	68
2.4.1.	Cholinesterase inhibiting activity of Fenitrooxon	...	...	68
2.4.2.	The conjugates	...	...	68
2.4.3.	Immunisation and collection of the plasma	...	...	68
2.4.4.	The antibodies	...	...	69

CHAPTER III : PHARMACOLOGICAL STUDIES

3.1.	Introduction to the electrical activity of the heart	...	72
3.2.	Effects of Fenitrooxon on the ECG	...	74
3.3.	Materials and methods	...	75
3.3.1.	Materials and animals	...	75
3.3.2.	Methods	...	76
3.3.2.1.	Determination of the minimum lethal dose of Fenitrooxon with ECG monitoring	...	76
3.3.2.2.	Effects of anti-fenitrooxon antibody on experimental poisoning by Fenitrooxon	...	76
3.4.	Results	...	77

CHAPTER IV : DISCUSSION

Discussion	...	80
Summary and conclusion	...	91

APPENDIX :

Bibliography	...	93
Synopsis	...	105
Statement	...	112

CHAPTER - I : ORGANOPHOSPHATES : AN INTRODUCTION

1.1. Organophosphates : An Introduction

Chemical crop protection offers the possibility of preventing a catastrophe or of obtaining a rapid increase in harvest in so far as pests or pathogens are the limiting factors. Plant varieties cultivated to give high yields are often particularly susceptible to pests and pathogens, so that the full yield is achieved only with the aid of chemical crop protection.

Indian rice production for 20 years has varied between 1000 & 1500 kg/ha with large deviations. Because of intensive cultivation measures, the Japanese yield is much more but still extreme variation in the yield used to occur due to the rice stem borer (Chilo suppressalis) and the rice blast (Piricularia oryzae). In 1953 there were considerable losses due to P. oryzae (see arrow in Fig. 1) which lead to the importation of parathion, an organophosphate insecticide, alongwith a mercurial leaf fungicide. Both pesticides were responsible for a steep rise in the rice production and monuments were erected to commemorate the event in Nangoku for Ceresan (the mercurial) and in Zentsuji for parathion (1). Many other examples can be cited.

Thus crop protection has become one of the major concerns of mankind. Positively formulated, the problem consists in developing and making available optimal agents according to activity, toxicity, economy and ecology.

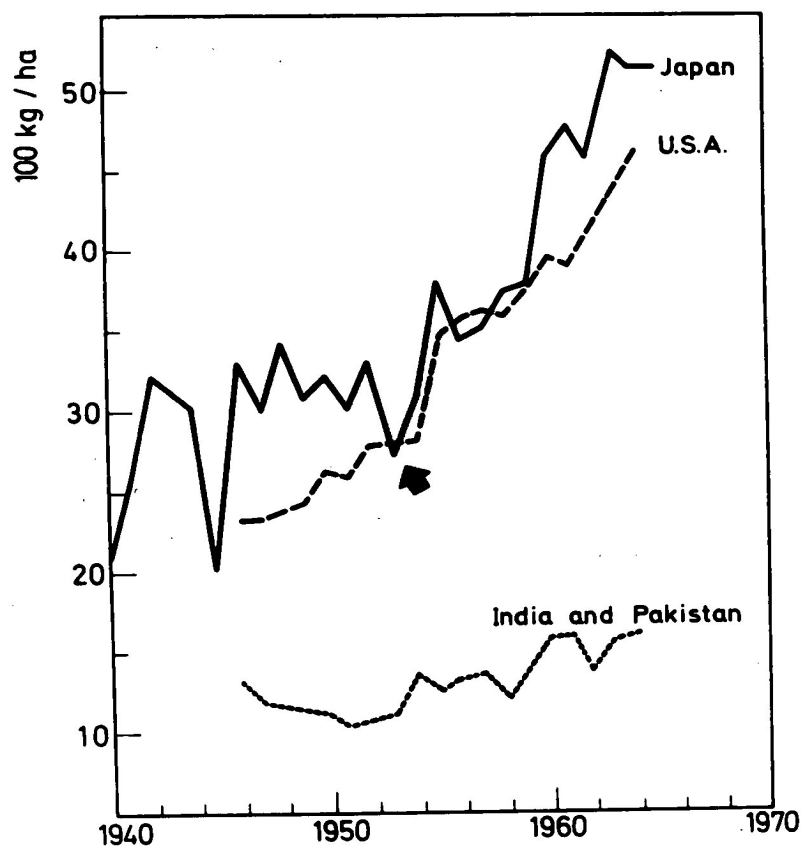
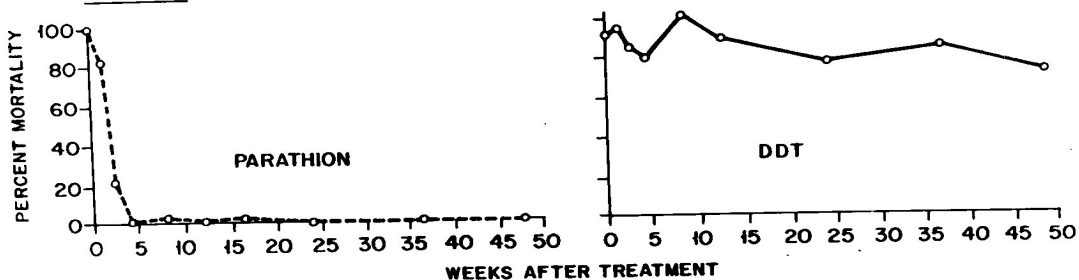


Fig. 1: Rice yields per hectare (1)

FIG. 2: PERSISTENCE OF BIOLOGICAL ACTIVITY OF INSECTICIDES IN SANDY LOAM SOIL (2).



In this respect, the phosphoric acid esters present a very favourable class of compounds, for, by combining four different substituents on the central phosphorous atom, it is possible to fit the properties of an active agent to the special practical requirements.

The insecticidal action of the very earliest compounds such as parathion and diazinon have set a standard which even 20-30 years after their discovery, is difficult to surpass. Toxicologically, this class of compounds has the advantage, due to their ester nature, of being metabolized in the living organism to inorganic phosphates. The price for this favourable property is a certain acute toxicity of the phosphoric acid esters, since, because of their mechanism of action, the toxicity for mammals and that for insects cannot basically be separated. However, the toxicologist prefers acute poisoning with its symptomatic treatment rather than chronic toxicity with ensuing irreversible damage. In the meanwhile, relatively non-toxic compounds have also been developed.

From an ecological point of view too, the organo-phosphates present a very interesting group. In contrast to certain chlorohydrocarbons, the problem does not consist in shortening persistence but on the contrary of extending the duration of action, particularly in the soil. A comparative picture of the persistence of biological activity is shown in Fig. 2.

The danger, therefore, of the accumulation of organophosphates in our environment upto a toxic level is very slight.

This class of compounds has also found use in other fields, although the emphasis will certainly remain on their use as insecticides. Esters such as para oxon or phosphoric esters of p-nitrophenol are known to have a marked miotic action and are used in human medicine to treat glaucoma. A series of less toxic compounds is also used in veterinary medicine. A broad development has begun in the field of fungicides. The first practically applicable herbicides and growth regulators are just becoming available and recently phosphonomycin, an antibiotic, has been introduced.

It would not be an exaggeration to claim the organophosphates to be the most fruitful class of compounds that man has found in his efforts to secure his physical existence.

1.2. Hazards of pesticide use :

There has been a very rapid increase in the use of pesticides in most developing countries, and in many instances, the safety services find themselves overwhelmed. Laws governing the importation, registration and handling of pesticides may be lacking and even when they exist, many governments lack the technical and administrative capacity to implement them. Those who apply pesticides

in the field may have very little notion of the dangers involved. The large number of cases of poisoning reported from certain countries indicates clearly that the technology of pesticides has outstripped the social and legislative development needed for their proper control.

Thus, for example, Hayes & Vanglin reported a total of 32 and 35 deaths due to pesticides in 1973 and 1974 respectively, in the United States (3). They also found that most poisonings in 1973 and 1974 were non-occupational and involved gross carelessness.

About 2500 cases of malathion poisoning occurred among spraymen in Pakistan, five of which proved fatal (4).

Dosage, whether due to single or repeated exposure, is the basic toxicological factor involved in all occupational and accidental exposure. Such exposure may result not only from the actual use of pesticides but also from poor storage, bad labelling, failure to recognise that the material is a poison and use of pesticide containers for food and drink. Illiteracy and low educational standards may also be important factors contributing to overexposure.

The next most important factor is the form in which the compound is used, as the materials used in formulation may contribute to its toxicity.

The route of exposure is also important. Almost everyone understands that ingestion of pesticides is dangerous. Paradoxically, this knowledge itself leads to their ingestion with suicidal intentions.

Exposure can also occur due to inhalation, and by absorption through the skin. The danger increases with temperature. In hot weather, absorption through the skin is more rapid, volatilization of the pesticide is also more rapid and the equilibrium concentration of a given compound in the air is higher. Therefore, both skin and respiratory exposure may be increased.

Animal studies show that toxicity may vary from one individual to another and also with strain, sex and age. In man, however, these factors seldom cause marked differences in susceptibility to these pesticides. The greater susceptibility of children to parathion is one of the exceptions to this rule. Also, so far there has been no example of human pesticide poisoning resulting from a genetic abnormality. People with abnormal plasma cholinesterase levels are not unusually susceptible to organophosphate pesticides.

Other factors such as nutrition, endocrinological status, disease, relative humidity, pressure and altitude, light, ionizing radiation and circadian rhythm are also influential in the occurrence of poisoning.

Impurities are sometimes introduced during manufacture or formed during storage as a result of interaction between the active ingredient and "inert" carriers or diluents. Improper handling can also lead to impurities. The poisoning incidence in Pakistan has brought into

focus the hazards of impurities that can increase the toxicity of a pesticide formulation(4,5).

In the Pakistan incident, isomalathion was implicated as the main cause of increased toxicity.

When considering the various factors that contribute to toxicity, their relative importance should be borne in mind. The most important way of reducing the danger is, of course, limitation of exposure.

1.3. Mechanism of Action of Organophosphorous Pesticides

1.3.1. Mechanism of Action In Mammals

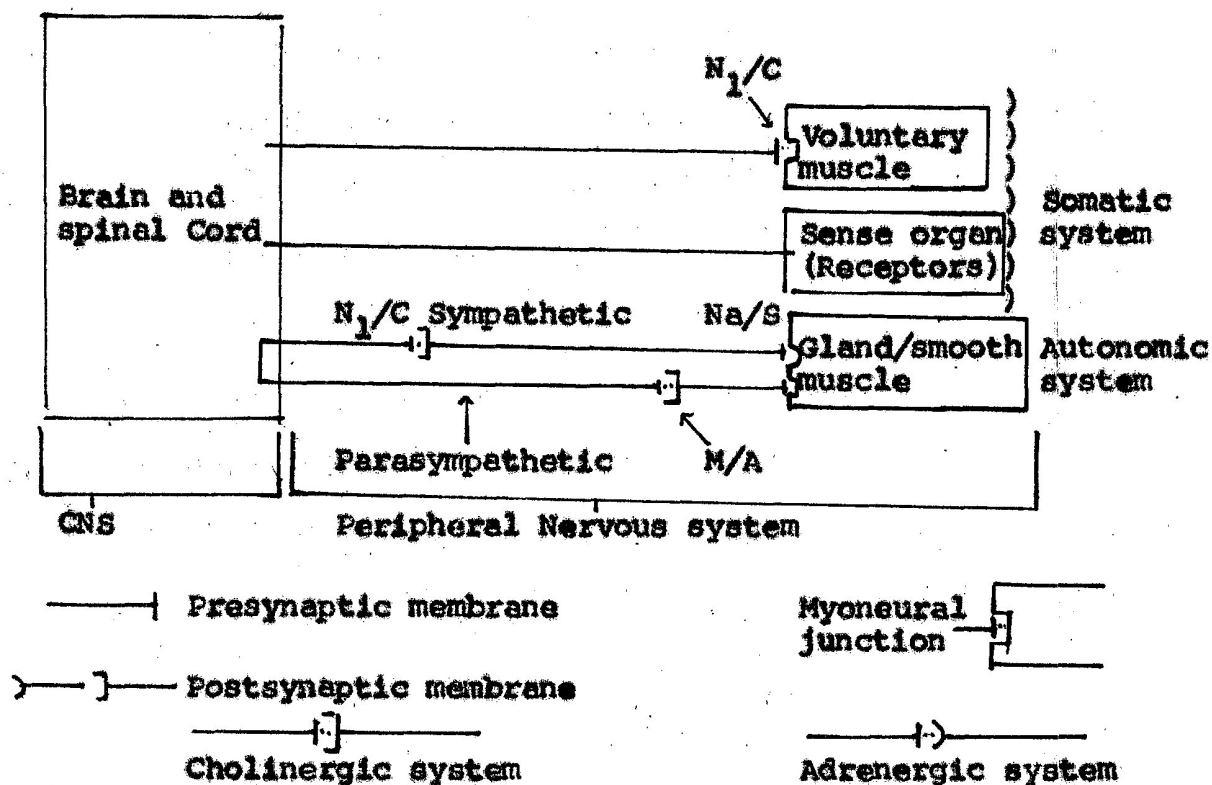
1.3.1.1. The Mammalian Nervous System

Organophosphates exert their biological action in mammals by attacking the system of neuronal transmission.

The basic structural unit of the nervous system is the neuron, essentially a cell having a nucleus and numerous fibres (dendrites) leading away from it. These fibres are responsible for the transmission of impulses from the cell body to other neurons or to the receptors. The simplest neural action in vertebrates involves the participation of several neurons which form a common functional path. The interneuronal transmission necessary for such participation is mediated through functional conjunctions called synapses. They consist of a presynaptic membrane and a post synaptic membrane which are 100-200 Å^o apart (the synaptic "gap"). The action potential (presynaptic impulse) is built up by selective Na⁺ and K⁺ concentration changes ("ion pump") and arriving at the

synapse induces a corresponding postsynaptic reaction. The impulse is transmitted in the synapse by a chemical mechanism. On the presynaptic side acetyl choline (ACh) or noradrenaline is liberated and is attached to receptors on the postsynaptic side, thus altering the permeability of the membranes to ions, which then results in the build up of a postsynaptic potential. The synapses and the myoneural junctions of the motor and the parasympathetic system, as well as the ganglia of the sympathetic system transmit the presynaptic impulse by means of acetyl choline, to the postsynaptic side. The neuromuscular junctions of the sympathetic system are stimulated by noradrenaline. It is possible that the adrenergic mechanism too is set in motion primarily by acetylcholine (6).

In order to bring the acetylcholine induced action potential back to the resting potential to facilitate the transmission of a new impulse, the stimulant in the synapse must be degraded. Degradation is effected by the enzyme acetylcholinesterase, which hydrolyses the acetylcholine to acetic acid and choline. A second enzyme, choline-O-acetyl transferase is capable of esterifying both compounds to acetylcholine again, with the help of ATP & Coenzyme A.



N_1/C : Agonist Nicotine Antagonist curare, M/A : Agonist muscarine Antagonist atropine

Na/S : Agonist adrenaline Antagonist sympatholytics.

Fig.3 - Scheme of Nervous system

(After Fest & Schmidt (7)).

The active organophosphates function by blocking acetylcholinesterase. This inhibition results in an accumulation of acetylcholine at the postsynaptic membrane which is then unable to return to its original (resting) state. Depending on the part of nervous system in which the synapses are thus kept in a state of permanent stimulation, symptoms of predominantly nicotinic, muscarinic or central poisoning are noted. In addition, toxicity unrelated to inhibition of Acetylcholinesterase is seen.

1.3.1.2. Acetylcholinesterase (AChE)

In classification of enzymes suggested by the enzyme commission this enzyme is known as acetylcholine acetylhydrolase (3.1.1.7.) (8).

Its molecular weight, determined by sedimentation equilibrium is found to be $2,60,000 \pm 10,000$ (9). The native molecule of AChE consists of four sub units of average molecular weight $64,000 \pm 4000$. This enzyme is a dimer, each protomer consisting of two nonidentical chains.

In addition to acetylcholinesterase a second cholinester hydrolyzing enzyme is found in the serum of mammals as well as in insects, viz. acetylcholine acylhydrolase (3.1.1.8). This hydrolyses butyrylcholine at a considerably faster rate than acetylcholine acetylhydrolase.

Both enzymes belong to a group of hydrolases (3.1.1.) which at their site of action contain similar amino acid sequences with participation of serine.

Enzyme E and substrate S form a complex "ES" which in the case in question splits of choline and changes to acetylated enzyme EAC. In the final stage, hydrolysis takes place, with reformation of enzyme and acetate which together with choline returns to the acetylcholine synthesis.

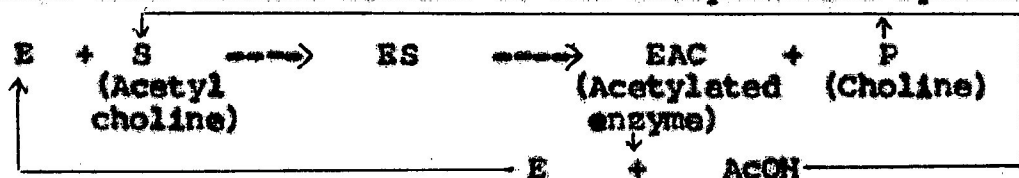


Fig. 4 Scheme of acetylcholinesterase function.

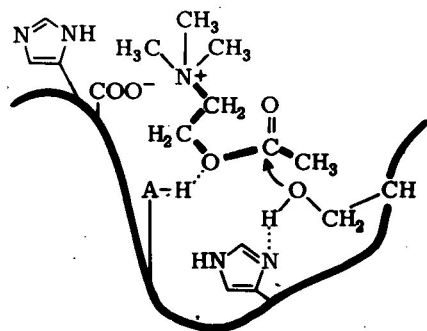


Fig.6a. Enzyme substrate complex (schematic) ("Michaelis complex")

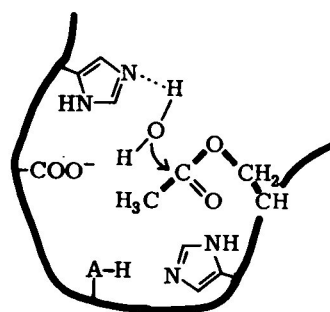
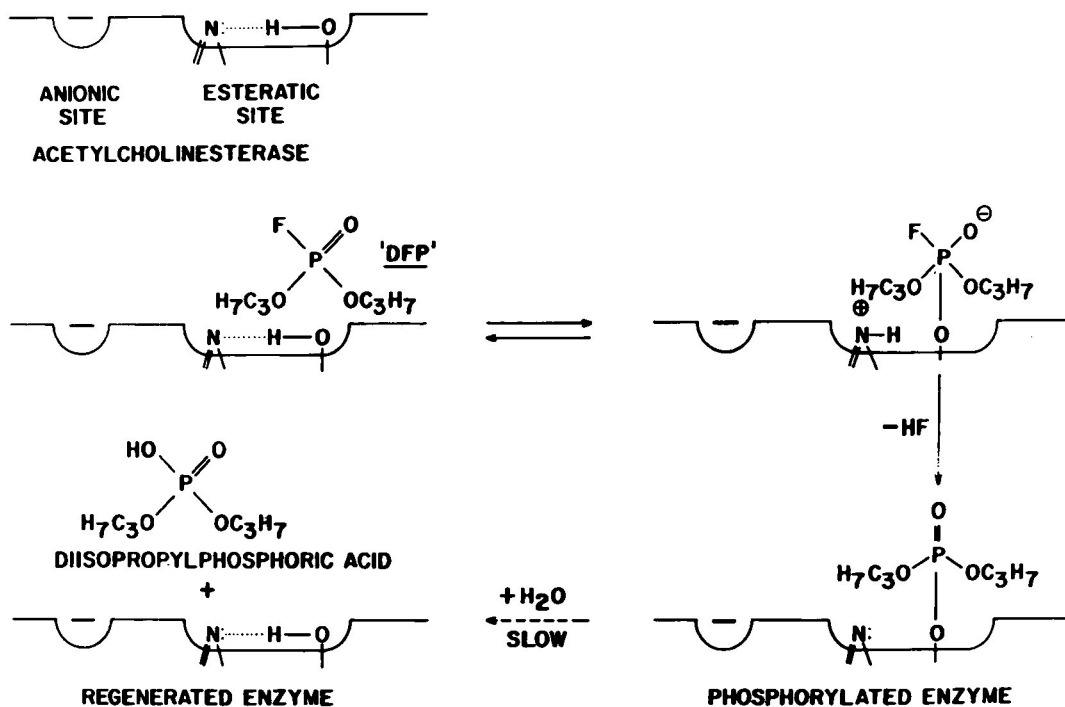


Fig.6b Hydrolysis of acetylated AChE (schematic)

FIG 7: POISONING OF AChE



1.3.1.3. "Poisoning" of AChE

The reaction between AChE and most organophosphate inhibitors occurs only at the esteratic site but proceeds in a comparable fashion. Here the resultant phosphorylated enzyme is extremely stable (See Fig. 7). If the attached alkyl groups are ethyl or methyl, significant regeneration of the enzyme by hydrolytic cleavage requires several hours. With isopropyl groups, virtually no hydrolysis occurs and the return of the AChE activity is dependent upon synthesis of a new enzyme, a process requiring days to months (11).

1.3.1.4. "Aging" of AChE

In the early stages, the phosphorylated AChE can be regenerated both in vitro and in vivo. This reactivatability decreases progressively until an irreversible stage of inhibition is reached in which substances such as - PAM no longer show a reactivating action. This is called the "aging" of AChE. The generally accepted chemical explanation for aging is that in phosphoryl serine one alkoxy group is hydrolyzed or de-alkylated. The resulting diester must be hydrolytically inert.

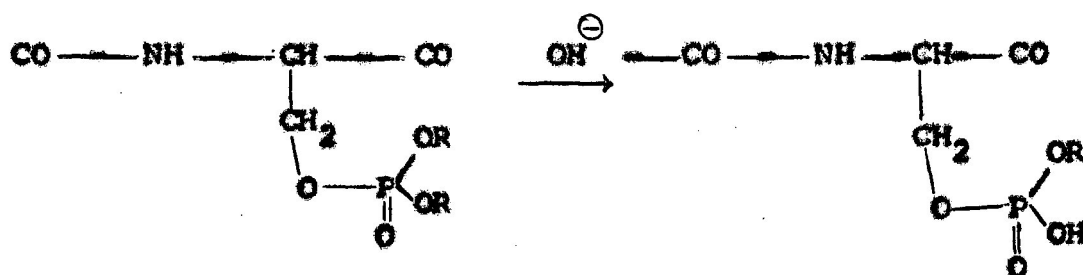


Fig.8 Aging of AChE

Banschop and Keijer were thus able to show that the rate determining step in the aging reaction consists of the unimolecular fission of the C-O bond in the alkoxy group(12).

1.3.2. Mechanism of action in Arthropods

Knowledge of the nervous system of insects, of its chemical function, and of the biochemical injuries caused by insecticides is not as soundly based as it ought to be, considering the economic importance of noxious insects. However, the most important hypotheses are the following (13):

1. The most reactive inhibitors effect a rapid accumulation of acetylcholine in the synapses. This high acetylcholine concentration, on one hand, protects cholinesterase against further inhibition and on the other increases the nervous activity and knock-down.

2. Organophosphates react not only with the enzyme acetylcholinesterase but also with the receptor site, whereby cholinesterase inhibition levels at knock-down can be influenced. Reaction at the receptor would antagonize the effect of ChE inhibition i.e. the accumulation of Ach.

3. Organophosphates evoke the liberation of endogenous substances which stimulate electrical activity and which effect an excess of acetylcholine and subsequent knock-down.

1.4. Fenitrothion

1.4.1. Chemistry :

Fenitrothion is O-O-dinethyl-O (3 methyl-4-nitrophenyl) Phosphorothioate, Bayer 41, 831, $C_9H_{12}NO_3PS$, Molecular weight 277.25, C-38.99%, H-4.36%, N-5.05%, O-28.85 %, P-11.17%, S-11.57%; a yellow oil bp $118^{\circ}C$, n_D^{25} 1.5528, d_4^{25} 1.3227 U.V. Max. 269.5 nm (ϵ 6756).

It is practically insoluble in water, has a low solubility in aliphatic hydrocarbons and is soluble in most organic solvents (14).

Fenitrothion, an organophosphorous pesticide, was described by Schrader (15). It is member of the "Parathion" series.

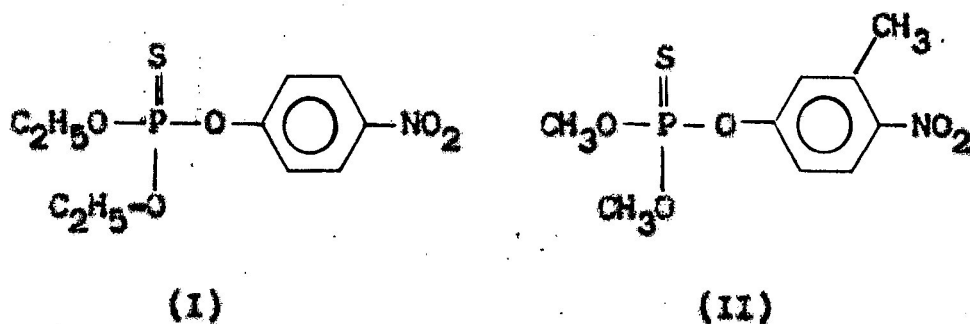


Fig. 9 I. Parathion II. Fenitrothion

The methyl substitution in the phenyl ring decreases toxicity without significant alteration in the biological activity. Parathion has LD_{50} in rats 3.6 - 13 mg/kg orally, whereas Fenitrothion has only 250 mg/kg orally (14). Stability towards hydrolytic influences is also improved (16).

1.4.2. Biological activity of Fenitrothion

Pure Fenitrothion has no inhibitory power towards acetylcholinesterase but is converted easily to its oxo form by enzymatic oxidation in vivo and this oxo form has high inhibitory power (16).

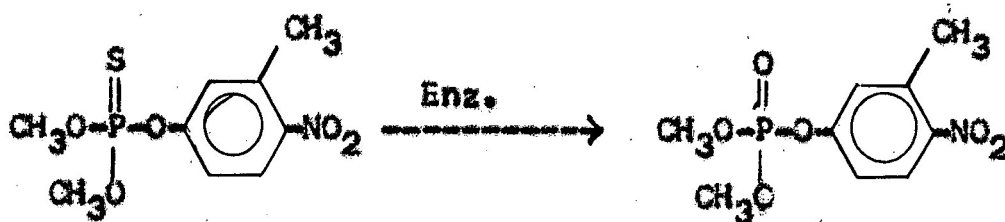


Fig. 10 Enzymatic oxidation of Fenitrothion.

Penitrothion has the following effect on various enzymes.

1. Depression of rat brain cholinesterase (17)
2. Depression of rat plasma cholinesterase (18)
3. Depression of rat RBC cholinesterase (18)
4. Depression of fish succinate dehydrogenase (19)
5. Stimulation of fish lactate dehydrogenase (19)

1.5. Toxicity and its treatment

1.5.1. Manifestations of toxicity due to cholinesterase inhibition

The effects of acute intoxication by anti-ChE agents are manifested by muscarinic and nicotinic signs and symptoms, and, except for compounds of extremely low lipid solubility, by signs referring to the CNS. Effects may be localized or generalized. Local effects are due to the action of vapours or aerosols at their site of contact with the eyes or respiratory tract, or to the local absorption after liquid contamination of the skin or mucous membranes, including those of the gastro-intestinal tract. General effects follow systemic absorption by any route. They appear most rapidly after inhalation of vapours or aerosols and severe effects may then be present within a few minutes. In contrast, after gastro-intestinal and percutaneous absorption, the onset of symptoms is delayed.

After local exposure to vapours or aerosols or after their inhalation, ocular and respiratory effects generally occur first. Ocular effects include marked miosis,

conjunctival congestion, ciliary spasm and brow ache, along with watery nasal discharge. Respiratory effects consist of tightness in the chest and wheezing respiration due to the combination of bronchoconstriction and increased bronchial secretion. Gastro-intestinal signs occur earliest after ingestion and include anorexia, nausea and vomiting, abdominal cramps and diarrhoea.

With percutaneous absorption of liquid, localized sweating and muscular fasciculation in the immediate vicinity are generally the earliest manifestations.

Muscarinic effects include extreme salivation, involuntary defaecation and urination, sweating, lacrimation, bradycardia and hypotension.

Nicotinic actions at the neuromuscular junctions of skeletal muscle usually consist of fatigability and generalized weakness, involuntary twitching, scattered fasciculation and eventually severe weakness and paralysis. A central component of action contributes to some of these effects. The most serious consequence is the paralysis of the respiratory muscles.

CNS effects include ataxia, slurred speech, loss of reflexes, Cheyne-Stokes respiration, generalized convulsions, coma and central respiratory paralysis.

The time of death after a single acute exposure may range from less than five minutes to nearly 24 hours, depending upon the dose, route, agent etc.

The cause of death is usually respiratory failure, usually accompanied by a secondary cardiovascular component. Muscarinic, nicotinic and central actions, all contribute to respiratory embarrassment. They include laryngospasm, bronchoconstriction, increased tracheobronchial and salivary secretion and peripheral and central respiratory paralysis.

In a recent study on mice Vadlamudi et al., have reported an oral LD₅₀ of Fenitrothion to be 884 mg/kg and the following symptoms - (20)

Respiratory distress, lacrimation, salivation, corneal opacity, shivering (muscular fasciculation) and clonic convulsions followed by death.

1.5.2. Treatment

History of exposure, characteristic signs and symptoms along with determination of plasma and erythrocyte ChE activities will generally establish diagnosis.

Atropine in sufficient doses effectively antagonizes muscaric actions at the receptor sites including the increased tracheobronchial and salivary secretions, the bronchoconstriction, the autonomic ganglionic stimulation and to a moderate extent, the central actions. It is virtually without effect against the peripheral neuromuscular activation and subsequent paralysis.

The last mentioned action of anti-ChE agents as well as other peripheral effects can be reversed by pralidoxime, a cholinesterase reactivator. The recommended adult dose of Pralidoxime is 1 gm, injected intravenously in not less than 2 minutes. If weakness is not relieved or if it occurs after 20 minutes again, the dose may be repeated. Early treatment is very important to ensure that the oxime reaches the phosphorylated AChE while it can still be reactivated. In late stages, the enzyme gets irreversibly damaged.

In addition, certain general measures may be necessary. These include -

1. Termination of exposure, by removal of the patient or application of a gas mask if the atmosphere is contaminated, copious washing of contaminated skin or mucous membrane with water or gastric lavage.
2. Maintenance of a patent airway
3. Artificial respiration if required
4. Oxygen administration
5. Alleviation of persistent convulsions by trimethadione (1 g, i.v. at every 15 minutes to a maximum of 5 gm. or Sodium pentothal - 2.5 % solution, 1 ml.) and
6. Treatment of shock

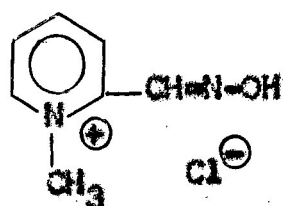
Atropine is used in heroic doses, far exceeding those used in normal practice. Thus, while the dose required for preanaesthetic medication and gastro-intestinal or renal

colic is 0.6 mg and toxic symptoms such as palpitations, dryness of mouth and blurring of vision are evident at a dose of 2 mg, the dose required in organophosphorous poisoning may be as high as 50 mg. Following an initial injection of 2 to 4 mg intravenously or intramuscularly, the 2 mg dose should be repeated every 3 to 10 minutes until muscarinic symptoms disappear and also if they reappear. A mild degree of atropinization is then maintained by the oral administration of 1 to 2 mg at intervals of several hours as long as symptoms are in evidence.

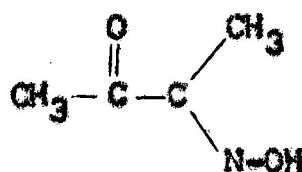
Cholinesterase reactivators :

While the phosphorylated esteratic site of AChE undergoes hydrolytic regeneration at a slow or negligible rate, Wilson found that the nucleophilic agent Hydroxylamine (NH_2OH) can reactivate the enzyme much more rapidly (21). In the search for more effective reactivators that followed, a large number of hydroxamic acids (RCO-NHOH) and Oximes (RCH=NOH) were shown to have this property. From the data that accrued, it was predicted that highly effective reactivation should be produced by a molecule containing both a quaternary N-atom and an oxime group, spaced at an appropriate distance.

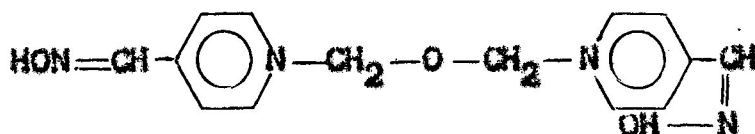
This goal was achieved to a remarkable degree by Wilson and Ginsberg with Pralidoxime (2-formyl-1-methylpyridinium chloride oxime) (22).



Pralidoxime (2-PAM)



Diacetylmonoxime (DAM)



Obidoxime chloride

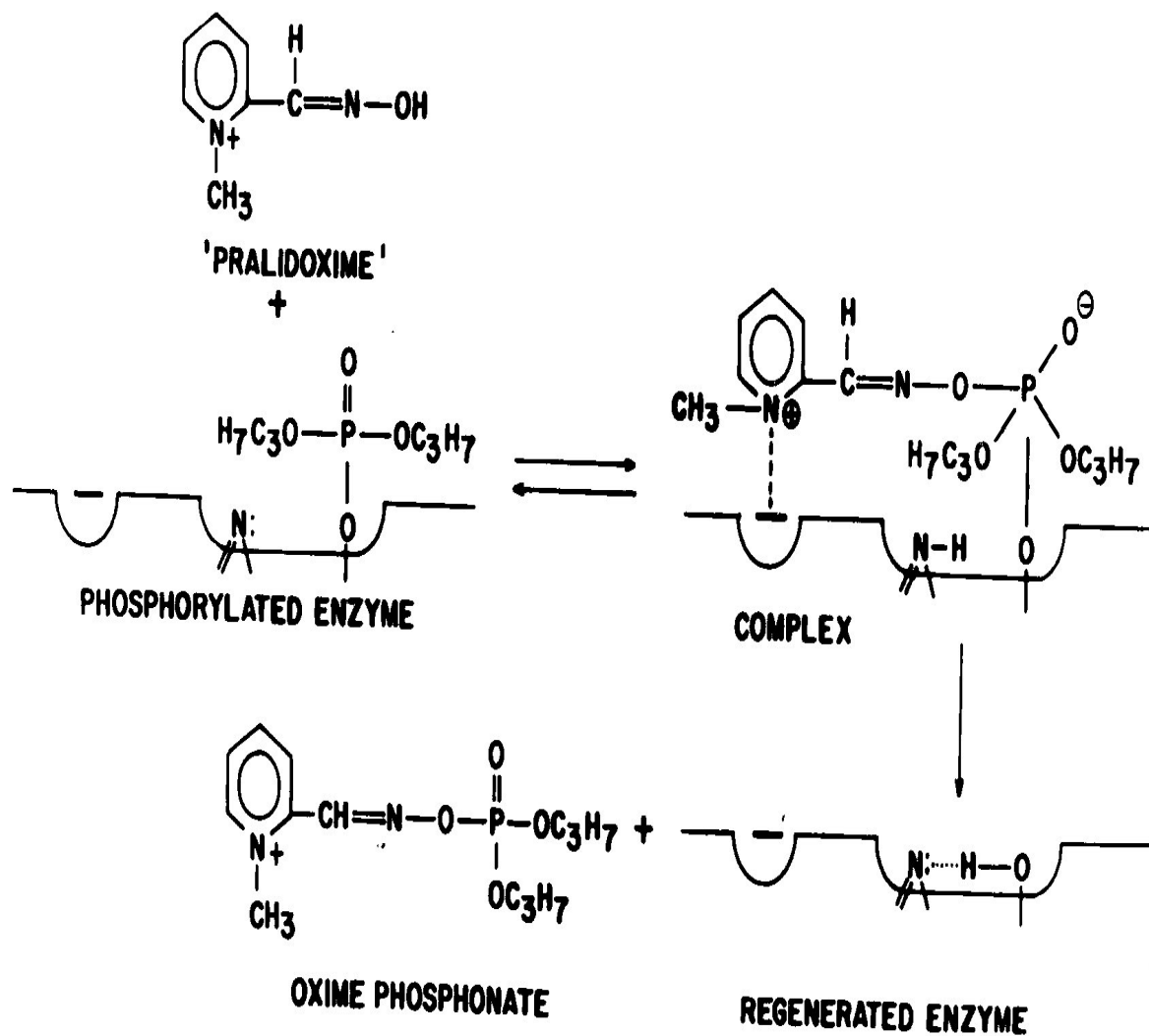
Fig. 11 Cholinesterase reactivators.

Certain bis-quaternary amines were shown to be even more potent as reactivators e.g. Obidoxime chloride (1,1' - (Oxydimethylene) bis (4-formyl pyridinium) dichloride dioxime (see Fig. 11).

Mechanism of action :

When the quaternary ammonium group of Pralidoxime is attracted electrostatically to the anionic site of the enzyme, the oxime group of the former is oriented optimally to exert nucleophilic attack on the electrophilic phosphorous atom of the phosphorylated esteratic site. The oxime phosphonate is then split off, leaving the regenerated enzyme (See Fig. 12).

FIG.12: REACTIVATION OF PHOSPHORYLATED AChE.



The velocity of reaction of reactivation by Pralidoxime varies with the nature of the phosphoryl group and in general follows the same sequence as the order for spontaneous hydrolytic reactivation i.e. dimethylphosphoryl - AChE > di-ethylphosphoryl AChE > di-isopropylphosphoryl AChE.

All types of phosphorylated AChE undergo a fairly rapid process of aging so that within the course of minutes or hours, they become completely resistant to the reactivators. The aging is probably due to the splitting off of one alkyl or alkoxy group leaving a much more stable monoalkyl or monoalkoxy phosphoryl AChE (12).

The reactivating action of oximes and hydroxamic acids in vivo is most marked at the skeletal neuromuscular junction, response being restored in a few minutes after intravenous injection. Effects are less striking at autonomic effector sites and insignificant in CNS (except with the nonquaternary compounds such as DAM).

High doses of Pralidoxime and related compounds can in themselves cause a neuromuscular blockade and other effects, including AChE inhibition.

If Pralidoxime is injected intravenously much more rapidly than the recommended 500 mg/min., it can cause mild weakness, blurred vision, diplopia, dizziness, headache, nausea and tachycardia.

The oximes as a group are largely metabolized by the liver and breakdown products are excreted by the kidney.

1.5.3. Chronic neurotoxicity

Fluorine containing alkylorganophosphates (e.g. DFP) and triarylphosphates (triorthocresylphosphate) induce a peculiar type of neuro toxicity. A severe polyneuritis begins several days after exposure to a sufficient amount of the toxic compound. It is manifested initially by weakness and ready fatigability of the legs and arms, reduced tendon reflexes, muscle twitching, fasciculation and tenderness. In severe cases, the weakness may proceed to complete flaccid paralysis that is often succeeded after weeks by a spastic paralysis with a concomitant exaggeration of reflexes. Muscles show a marked wasting. Recovery may require 2 or more years.

The pathologic lesion is characterized by axonal swelling, segmentation and eventual breakdown into granular debris. The marked demyelination is probably secondary to the axonal changes.

The pathways involved include the sciatic nerve, the spino-cerebellar tract, posterior columns and anterior tracts in the spinal cord and the lateral tracts in the medulla.

Neither the biochemical basis nor any specific therapy for the neurotoxic syndrome is known (23). However, it does not seem to be dependent on ChE inhibition.

CHAPTER II : IMMUNOLOGICAL STUDIES

2.1. Introduction to anti-hapten antibodies

It is well known that low molecular weight compounds, when linked to proteins or polypeptide carriers will elicit the production of antibodies capable of reacting specifically with the hapten used. The development of sensitive methods of detecting and quantitating reactions between hapten and antibody, and the perfection of techniques for obtaining highly purified preparations of anti-hapten antibodies, have played a significant role in the development of modern immunological concepts. Anti-hapten antibodies have proved particularly useful in the study of antigenic determinants, structural requirements for immunogenicity, the nature of antigen antibody reactions, and the chemical, physical and biological properties of antibody (24).

This branch of immunopharmacology achieved a major triumph when Smith and coworkers successfully reversed advanced digoxin intoxication with digoxin-specific antibodies in a human patient. They state " Our previous experience with digitalis intoxication of this magnitude, together with that of others leads us to believe that the present case would have had a fatal outcome without this intervention" (25).

Much information concerning complementarity of fit between antigen and antibody derives from the studies of antibodies against simple substances of known composition.

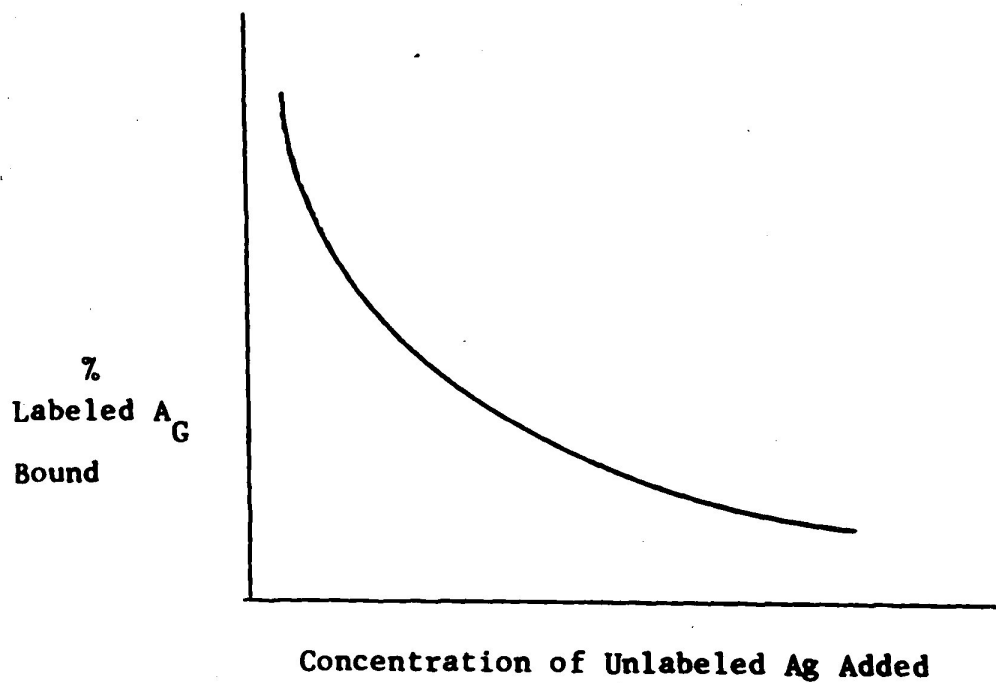


FIG.13: Standard Antibody-Antigen Curve

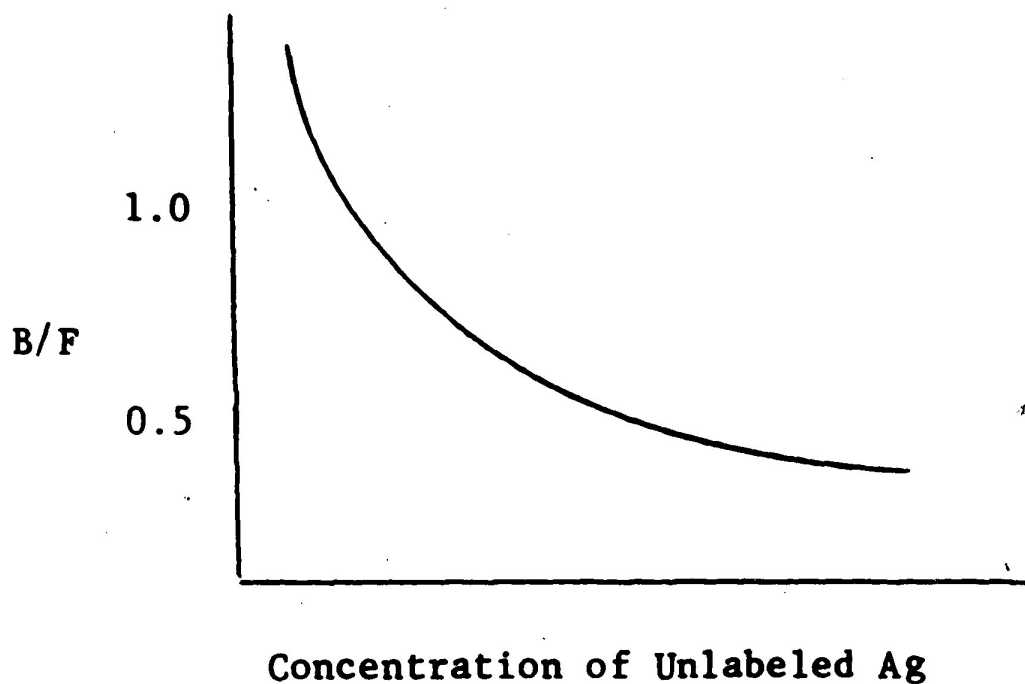


FIG.14. Standard Antibody-Antigen Curve

Antibodies against a hapten can be studied by their interaction with substances of known configuration which are structurally related to the original hapten. The structural features that are of importance to the interaction can thus be evaluated (26).

The specificity conferred on these antibodies makes them invaluable as pharmacological agents for research into the mechanisms of action of physiological and pharmacological agents. Thus Gupta and Ehde were able to demonstrate the role of 5-hydroxy tryptamine in acute inflammation and anaphylaxis, using an antibody specific to 5-hydroxytryptamine (27).

From a chemist's point of view, antibodies are important not only as naturally occurring substances with biochemical reactivity, but also as analytical reagents to identify a substance, to differentiate between very closely related substances, or even to determine quantitatively how much of a substance is present. Antibodies have an advantage in that they offer a means of preparing a large variety of specific chemical reagents by a single general method. It can even be labelled as with fluorescein or a radioactive isotope so that it can be located at a microscopic level (26).

The radioimmunoassay technique, developed by Berson and Yellow provides the pharmacologist with a powerful technique which can be utilized for pharmacokinetic analysis

of hormones and drugs, in various biological fluids. Labelled hapten is incubated in the presence of the specific antibody to form a labelled hapten-antibody complex.

Hapten* + Antibody ----- Hapten* + antibody complex

If to the incubation mixture serial additions of unlabelled antigen (hapten) are made, there occurs a competition for the available binding sites on the antibody. Thus two species of hapten antibody complexes are formed - a labelled hapten - antibody complex and an unlabelled hapten antibody complex.

Hapten* + Hapten + Antibody ----- Hapten* Antibody +
Hapten antibody

Consequently, the amount of label complexed to the antibody will be a function of the concentration of the unlabelled material. A curve shown in Fig. 13 is obtained.

One can also express the data on the basis of the ratio (B/F) of labelled antigen bound to antibody (B) to free labelled antigen (F) as a function of the concentration of unlabelled antigen, as in Fig. 14.

For the assay, a dilution of the antiserum is chosen which gives a B/F ratio of 0.5 - 0.7 in the absence of added unlabelled antigen. To obtain the standard curve, unlabelled hapten is then added in varying concentrations to the incubation mixture, containing the

diluted antiserum and a constant concentration of the labelled hapten. Unknown samples are added instead of the unlabelled hapten of the standard curve. Concentrations are then determined by comparison with the standard curve (28).

Thus, for accurate estimation of small amounts of drugs as well as neutralisation of drug actions, the antihapten antibodies form a specific, sensitive and therefore valuable tool.

Table 1

Biologically active molecules for which antibodies have been reported.

<u>Name of the compound</u>	<u>Reference No.</u>
<u>Mediators</u>	
Catecholamine	30, 31
Serotonin	32
Melatonin	33, 34
Histamine	35
β -endorphin	36
Prostaglandins	37, 38, 39
Angiotensin II	40
Bradykinin	41, 42
cGMP	43
cAMP	43, 44
Substance P	45

DrugsAnalgesics

Aspirin	46
Codeine	47
Pentanyl	48
Methadone	49
Morphine	50
Antipyrin	51

Antimicrobials

Adriamycin	52
Amikacin	53
Chloramphenicol	54
Daunomycin	52
Gentamycin	55
Isoniazid	56
Penicillin	57
Sulphonamides	58
Tetracyclin	59

Anticonvulsants

Phenytoin	60, 61
Sulthiame	62

Antihypertensives

Saralasin	63
-----------	----

Antineoplastic agents

Methotrexate	64, 65
--------------	--------

Barbiturates

Barbitone	66
Pentobarbitone	67
Phenobarbitone	68, 69

Cardiac glycosides

Digitoxin	70
Digoxin	71
Gitoxin	72
Ouabain	73

Hallucinogens

Cannabinoids	74
Lysergide	75
Mescaline	76

Pesticides

DDT	77
Paraquat	78
Parathion	79
Malathion	77, 80
S-bioallethrin	81, 82

Muscle Relaxants

Tubocurarine	83
--------------	----

Steroids

Aldosterone	84
Corticosteroids	85
Cortisol	86
Dexamethasone	87

Methoxyprogesterone	88
Ethinylestradiol	89
Testosterone	90
Mestranol	89
Estriol	91
5- α -dihydrotestosterone	92
Dimethylprednisolone	93
Dehydroepiandrosterone	94
Norethisterone	95
Estradiol	96
Norgestrol	97
Prednisone	98
Androstenedione	99

CNS Stimulants

Amphetamine	100
Strychnine	101

Alkaloids

Ergotamine and Ergochristine	102
Cotinine	103
Nicotine	104
N-nitrosomornicotine	105

Tranquillizers

Chlordiazepoxide	106
Chlorpromazine	107
Diazepam	108
Mephenteramine	109

Nonsteroidal hormones

Thyroxin	110
Chorionic gonadotrophine	111, 112
Thymopoetin	113
Lutropin	114
Calcitonin	115

Miscellaneous

5-methyltetrahydrofolate	116
ApApA	117
IgM Rheumatoid factor	118
N-N-dimethylindolalkylamines	119
Dihydroxycholecalciferol	120

.....

2.2. Principles of elicitation and characterisation of antihapten antibodies

2.2.1. Methods of conjugation

In order to elicit antibodies to small molecules of molecular weight 1000 or less, these molecules must be conjugated covalently to protein or polypeptide carriers. Thus the low molecular weight compound ("hapten") must possess at least one functional group that can be used for attachment to the available functional groups on the carrier, under chemical conditions which neither cause significant structural alterations in the hapten nor produce significant denaturation of the carrier to render it insoluble. Table 2 gives a summary of methods of covalently linking a drug to a carrier protein.

Table 2Methods of covalently linking a drug to a carrier protein

(After Landon (121)).

Functional group on drug	Method of conjugation
Carboxyl	1. Use of isobutyl chloroformate to form a mixed anhydride which will react directly with protein amino groups to form peptide bonds. 2. Use of carbodiimides ($R-N=C=N-R'$, where R and R' are alkyl or aryl groups). 3. Use of N-hydroxy succinimide in presence of dicyclohexylcarbodiimide to form the N-hydroxy succinimide ester, which will be more active than the free carboxyl group.
Aromatic amino	Direct diazotization to the active position of tyrosin tryptophan or histidine residues.
Aliphatic amino	1. Use of carbodiimides. 2. Use of glutaraldehyde to link amino groups. 3. Use of 2-Xylenediisocyanate or toluene 2-4 diisocyanate to link amino groups. 4. Use of 4-nitrobenzoylchloride to form 4-nitrobenzoyl amide which can then be reduced to 4-aminobenzoyl derivative and linked by diazotization.
Nitro	Reduction to an amino group and then as above.
Hydroxyl	1. Addition of equimolar proportions of anhydrides of dicarboxylic acids (e.g. succinic) to the drug to give half esters (hemisuccinate) and the free carboxyl group can then be linked as above. 2. Introduction of a carboxyl group using w-half substituted carboxylic acids. 3. Formation of a chloro carbonate with phosgene and use of the Schotten-Baumann reaction to link it directly to amino groups on the carrier molecule.

4. Use of oxidizing agents to convert the alcohol into a carboxylic acid.
- Carboxyl**
1. Formation of O- (Carboxy-methyl) oxime using O- (Carboxymethyl) hydroxylamine and reaction of the free carboxyl group as above.
 2. Introduction of a carboxyl group using 4-hydrazinobenzoic acid.
 3. Schiff-base formation directly with primary amino groups on the carrier molecule.
- Active hydrogen**
1. Direct conjugation using the Mannich reaction.
 2. Reaction with diazotized 4-aminobenzoic acid to give a derivative with free carboxyl group.
-

2.2.2. Choice of carrier

Because of their ready availability, low cost, excellent immunogenicity, high degree of solubility and relative resistance to denaturation under somewhat rigorous chemical conditions, serum albumins of various species have been used as carriers most frequently. Various serum globulin fractions, fibrinogen, ovalbumin and haemocyanin have also been used.

Recently, synthetic polypeptides have often been used as carriers of biologically active haptens. They are less immunogenic and much more expensive. However, they are totally free of natural products and thus do not elicit antibodies to albumins and other proteins that may impair the usefulness of the resulting antisera. The subject has been reviewed by Sela (122).

2.2.3. Immunization procedures

After removal of unconjugated hapten and other chemical by-products of the conjugation reaction, hapten carrier conjugates may be used to immunize experimental animals.

2.2.3.1. Routes of inoculation

- a) Intradermal: This route provides rapid access to the lymphatics and may be used with or without adjuvant.
- b) Subcutaneous: This is a convenient route and one from which the antigen will be absorbed relatively slowly. It is useful for booster injections in order to minimize the risk of anaphylactic shock. It should not be used for complete Freund's adjuvant since this leads to an ulcer, leading to loss of the depot.
- c) Intramuscular: The route of choice for complete Freund's adjuvant, it is also suitable for other adjuvants. It provides rapid access to the circulation and lymphatics but in the absence of an adjuvant, soluble antigens may become diluted out and lost. Oil emulsions rapidly break down into small drops that lie along fascial planes.
- d) Intravenous : This is the route of choice for particulate antigens, and antigens adsorbed onto alum. For alum adsorbed antigens a second subcutaneous depot

is advisable. Multiple emulsions can be given by this route if primary dispersed phase droplets are small and even in size. The response is rapid but not sustained.

e) Foot-pad : Complete Freund's adjuvant inoculated into the foot-pad causes severe swelling, ulceration and necrosis of the foot. It should be used only when absolutely necessary e.g. when the object is to examine the draining lymph node. Also, it should be put in only one foot of the animal, as the inoculation of more than one foot can cause great distress.

2.2.3.2. Adjuvants

Adjuvants are substances which when injected with antigens (or given prior to the antigen) non-specifically enhance or modify the immune response to that antigen.

The most widely used adjuvants are of three general types - 1) Antigen-depot adjuvants : Freund's incomplete and complete adjuvants and alhydrogel (Aluminium hydroxide gel, $Al(OH)_3$ (123)).

2) Bacterial adjuvants : Corynebacterium parvum, Bordetella pertussis and the synthetic adjuvant muramyl dipeptide (an analogue of a portion of the peptidoglycan of the bacterial cell wall)

3) Amphipathic and surface-active adjuvants : Saponin.

The most widely used adjuvant is the one introduced by Freund. In its incomplete form it consists of a neutral

detergent (Aracel 'A') in light paraffin oil. The complete form consists of paraffin oil, Aracel 'A' and killed mycobacteria. An aqueous solution of the antigen is emulsified with the adjuvant by a shearing effect, to give a water-in-oil emulsion.

The two components - paraffin oil and killed mycobacteria - serve different functions :

1) Paraffin oil :

Paraffin oil works by releasing the immunogen slowly over a course of weeks, thus preventing its rapid uptake into the circulation. This avoids rapid degradation of the immunogen by circulating enzymes, and in case of certain hormones such as insulin minimizes any direct physiological effect of the immunogen on the recipient.

2) Killed Mycobacteria :

Their use results in a greater antibody response than is stimulated by antigen in a simple water-in-oil emulsion and in some animals e.g. guinea pigs, there is an unusually potent production of some classes of immunoglobulin. Cell mediated immunity is enhanced, the mono-nuclear-phagocytic system is stimulated as judged by carbon clearance tests and macrophages are activated.

The adjuvant also stimulates the formation of local granulomatous lesions which may act as foci for antibody formation. However, it also leads to the formation of an abscess at the inoculation site.

2.2.3.3. Immunisation schedules

Principles of immunisation :

1. The first injections of antigen serve to 'load' the animal. Thus until the animal can clear the load by antibody production or normal catabolism, further injections may well be an embarrassment.

2. When antigen is administered in a long lasting form as when dispersed in Freund's adjuvant, enough antigen seems to persist to provide anamnestic recall over a long period of time with a sustained production of antibody.

3. In species that develop a high sensitization to tuberculin e.g. guinea pig and rabbits, reinjection of antigen in complete Freund's adjuvant leads to a violent inflammatory response to the mycobacterial component. Hence secondary "Booster" injections should have a reduced mycobacterial content.

4. The production of hapten-specific antibody by injection of artificially conjugated proteins is difficult in rabbits. Antibodies develop first to the whole complex and predominate throughout the course of immunization. Hapten specific antibodies appear late and in relatively few individuals to a satisfactory level.

Lader et al., have found the intradermal route to be quite economical of immunogen and time (124). They used 100 micrograms antigen for primary injections and

50 micrograms for the boosters. Aqueous solutions of the antigen were emulsified with Freund's adjuvant, and 40 injections of 50 μ l each were made on the shaved backs and limbs of each rabbit. The boosters were given on 70th day from the primary injection and the final bleeding was carried out on 84th day. Route used for boosters was the intramuscular route. This schedule compared favourably with another one in which intramuscular injections were made on the 0th, 42nd, 70th and 140th day and the final bleeding on 154th day.

However, recently, Dawson et al., have found 3 intramuscular injections at 2 week intervals followed by 5 intravenous boosters at 6 weeks intervals, to be a better method than the intradermal one(125).

The basis for suggesting guidelines for immunisation schedules consists of random observations and final evaluation must rest on orderly accumulation of data.

2.2.3.4: Choice of animal species and collection of serum

Choice of the animal species: The immune response depends upon both on the responsiveness of the animal and the properties of the injected material. The responsiveness to each antigen is genetically determined, probably as a single gene and is transmitted as a Mendelian dominant (126). Certain inbred strains of a species may produce antibodies to a particular antigenic determinant, while other strains may not (127). Consequently, it is better to use random-bred animals unless a particular strain is known to respond well to the immunogen under consideration.

The choice of species for immunization is usually restricted to rabbits and guinea pigs. Rabbits are easier to handle, are more hardy and 30-40 ml of blood can be taken at frequent intervals. Guinea pigs are less hardy and blood must be taken by heart puncture, with its attendant risks. Nevertheless they are more economic as regards initial cost and maintenance and need slightly less immunogen, making the use of a larger number possible.

If neither rabbits nor guinea pigs respond well to a particular antigen after an adequate trial, chickens may be tried. They are hardy and easy to bleed but there are differences between the physicochemical properties of their gamma globulins and those of mammalian globulins (128).

2.2.4. Assessment of the antibodies

An antibody is an immunoglobulin molecule secreted into the tissue fluids from lymphoid cells which have been exposed to a foreign substance, the antigen. The presence of several different antigenic determinants in most immunogens results in the production of several antibody populations, each originating from a single clone of cells and directed against a single determinant. Hence every serum sample including that from each bleed from an individual animal must be assessed carefully. Antibodies in the serum are usually detected by antigen-antibody reaction.

Parr's test : (129)

The test uses antigen labelled with ^{131}I iodine. This antigen is mixed with antibody containing serum and left to equilibrate. A solution of ammonium sulphate is then added to the mixture resulting in the precipitation of antibody together with any labelled antigen bound to it. The unbound antigen remains in solution. The quantity of isotope labelled antigen bound to the salt-precipitated antibody is then estimated by placing the washed precipitate in radioactive counting equipment. This test is the measure of the capacity of the antiserum to bind antigen.

Precipitin test : (130)

If a soluble antigen reacts with its antibody to form an insoluble complex, the reaction can be used for the detection of the antigen. The reaction may be carried out in an aqueous solution or a gel. For the precipitin reaction in the gel, wells are cut in a layer of agar on a slide or a petridish. Antigen and antiserum are placed in opposite wells and after allowing a few hours to pass for diffusion take place, precipitin bands will be seen to form at points where antigen and antibody meet in suitable proportions. The value of the method lies in its simplicity.

Agglutination reaction : (131)

In this reaction, the antigen is a part of the surface of some particulate material such as a red cell, a bacterium or perhaps a latex particle coated with the antigen.

Antibody added to a suspension of such particles combines with surface antigen and links the particles together, thus forming clearly visible aggregates. This test is much more sensitive than the precipitin test.

Complement Fixation : (132)

The fact that antibody, once it combines with the antigen is able to activate the complement system, is used to show the presence of a particular antibody in serum.

In a test, the antibody, complement and antigen are first mixed together and after a period of incubation the indicator system, viz. anti-sheep - RBC - antibody + sheep red cells, is added. If the reaction Antigen + Antibody + complement -----> adsorption of complement on antigen - antibody complex, has already taken place, it will prevent the reaction. RBC + Anti-RBC serum + complement -----> haemolysis from occurring. Thus a positive complement fixation test (presence of antibodies) is indicated by absence of lysis.

These tests are rather complex to perform but they are applicable to all kinds of antigens, soluble or insoluble.

Neutralization test :

This test is applicable only to biologically active antigens such as toxins, hormones and drugs and requires an antiserum that reacts with and thus neutralizes the biologically active part of the molecule.

The quantitative precipitin reaction :

The titre of the antibodies is generally determined by the quantitative precipitin test. The test is based on the observations that, upon the addition of increasing amounts of soluble antigen to a series of tubes containing a constant volume of the antiserum, the amount of precipitate formed increases to a maximum and then decreases. A measure of the antibody in absolute weight units can then be obtained by subtracting the protein content of the antigen from the total protein content of the precipitate at maximal precipitation. (145)

2.2.5. Fractionation of Immunoglobulins

Prior to the successful separation of any specific immunoglobulin, a preliminary fractionation procedure is usually made to obtain a concentrated solution. The immunoglobulins represent the most basic globulins of serum. Their solubilities, coupled with their relatively high isoelectric points, form the basis of most separations. Investigators who are concerned only with the separation of serum immunoglobulins may not wish to use the rather complicated and extensive fractionation conditions developed by Cornet al., for the isolation of a series of plasma proteins including gamma globulins (133). The present discussion is directed only to the separation in good yield of immunoglobulin concentrates by relatively rapid and

simple procedures, viz.,

- a) Ethanol fractionation
- b) Precipitation by neutral salts
- c) Use of complexing precipitants

A) Ethanol fractionation techniques :

Organic solvents greatly decrease protein solubility. This decrease probably arises from two effects, a decrease in the dielectric constant of the medium and dehydration. By decreasing the dielectric constant of the medium, the attractive forces between oppositely charged surface residues increase. This results in the formation of huge aggregates which precipitate out of solution. Since some organic solvents used dissolve in water and hence interact with water, protein dehydration occurs. In this regard organic solvents function in a manner similar to that described for salt precipitation. Three variables must be considered when using this method - 1) ionic strength of the medium from which precipitation occurs, 2) organic solvent used, 3) the temperature at which the precipitation will be carried out.

At low ionic strength increasing amounts of organic solvents severely decrease protein solubility. Moderately increasing the ionic strength, however, tends to increase protein solubility. Although decreasing ionic strength decreases protein solubility, it may also affect protein stability, especially of proteins composed of a large number of sub units.

The organic solvent most often used to bring about precipitation of immunoglobulins is ethanol.

The temperature at which precipitation occurs is an important factor to consider. Protein solubility in the presence of an organic solvent decreases markedly with temperature. Therefore, at low temperatures, (significantly below 0°C) less solvent is required to precipitate a given protein. Also, the freezing point is conveniently depressed, making such an approach possible. The most useful dividend of low temperatures (-20°C) is alleviation of the denaturing effects of most organic solvents. This is most easily done by cooling the protein solution to 0°C and then adding solvents which have been cooled to a much lower temperature.

A sample procedure for preparation of concentrates of immunoglobulin G and mixtures of immunoglobulins M and A is described by Deutsch (134).

B) Fractionation with neutral salts :

Salting-out procedures have certain advantages over ethanol fractionation for preliminary concentration and partial purification of immunoglobulins. Among these are simplicity and relatively less danger of denaturation. As the amount of salt molecules in a solution increases, less water is available to interact with any proteins that may be present. At some point there will no longer be sufficient water to maintain solution of a set of proteins and they precipitate. Salting-out of proteins as this is sometimes called, may actually be a dehydration process.

The salts commonly used for the fractionation of immunoglobulins are ammonium sulphate and sodium sulphate.

C) Use of Complexing precipitants :

The gamma-G-immunoglobulins have relatively high isoelectric points. It is possible, therefore, to form insoluble cation complexes with most of the other serum proteins at pH levels at which the gamma immunoglobulins do not form such complexes. When plasma or serum is mixed with 3 volumes of 0.4% Rivanol (2-ethoxy 6,9-diaminoacridine lactate) and the pH is adjusted to 7.6 to 7.8, a precipitate forms which contains most of ^{non-}gamma G protein (135). The supernatant is clarified by various means and the supernatant immunoglobulins may be precipitated by the addition of ethanol to a final concentration of 25% (temperature to -6°C), or by the addition of 20 gm of sodium sulphate per 100 ml of solution. The concentrate which consists of 60 to 98 % gamma globulins may be further purified by DEAE-cellulose chromatography.

Other procedures employing aluminium and zinc salts and polyphosphates have been used to obtain concentrates of gamma G-immunoglobulins. These methods depend in large measure on the formation of insoluble metal salts of either the gamma globulins or the other plasma proteins (136).

2.3. Materials and methods

2.3.1. Materials and animals

1. Chemicals:

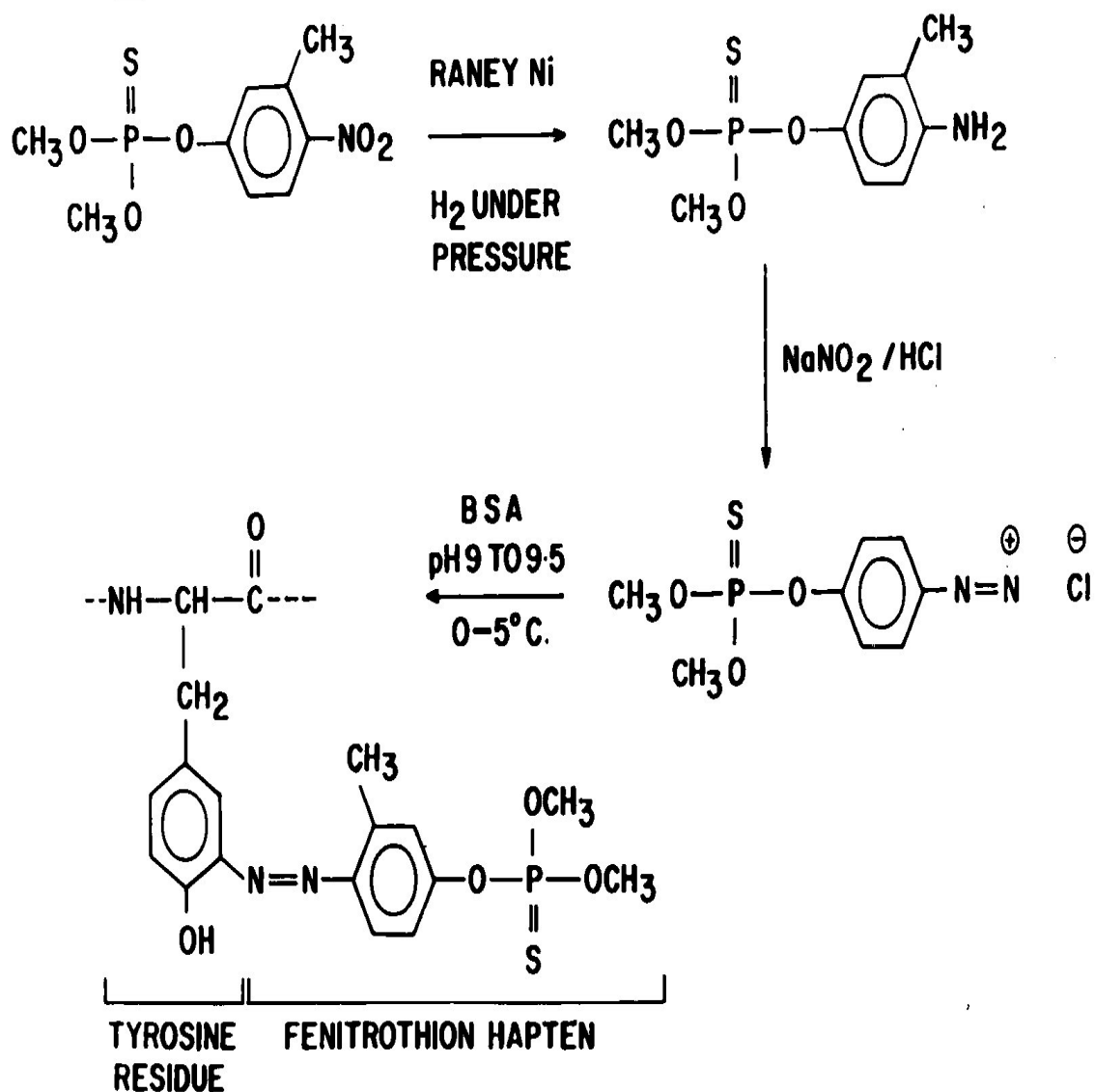
- a) Fenitrothion : Kindly supplied by Bayer (India) Ltd., India.
- b) Bovine serum albumin : Sigma Chemical Co., U.S.A.
- c) Acetylthiocholine iodide : Fluka AG, Switzerland.
- d) 5,5'-dithio-bis-2-nitrobenzoic acid : Koch-light Laboratories Ltd., England.
- e) Freund's adjuvant (complete) : Difco, U.S.A.
- f) Agar : Oxoid Ltd., England.
- g) 1,2,4-amino-naphthol-sulphonic acid : Sarabhai M. Chemicals, India.

All other routine chemicals used were of A.R. grade.

2. Animals :

- a) Albino rabbits - Norwegian strain.
- b) Albino mice - NIH strain

FIG.15: SCHEME OF CONJUGATION OF FENITROTHION TO A CARRIER PROTEIN.



2.3.2. Methods

2.3.2.1. Coupling of Fenitrothion to bovine serum albumin

Fenitrothion contains an aromatic nitro group which can be converted to an amino group by reduction. This amino derivative can then be diazotized and coupled to a protein (see Fig. 15). The coupling of diazonium salts of aromatic compounds has been extensively used as a means of introducing haptens into proteins. Classical immunological studies carried out with haptens coupled to proteins through azo linkages are exemplified by the work of Landsteiner and Pauling et al., (137, 138).

At slightly alkaline pH a diazonium salt will couple to the side chains of a protein molecule. If the number of moles of diazonium salt used is comparable to the number of moles of tyrosine, histidine and lysine residues in a protein, the reaction yield, mainly disubstituted lysine and monosubstituted tyrosine and histidine. Reaction mechanisms are discussed in detail by Howard and Wild (139).

Procedure

1. Purification of Fenitrothion :

One gram of Fenitrothion was dissolved in 50 ml of diethyl ether and extracted four times with 1% chilled aqueous sodium carbonate, in order to remove the P-nitro-m-cresol occurring as the main impurity (140). The ether was then evaporated by a gentle stream of air.

ii) Reduction of Fenitrothion :

The purified Fenitrothion was then dissolved in 25 ml ethanol and 1 gm of Raney Nickel was added to it. The bottle was mounted on an oscillator, hydrogen gas connected to the bottle at pressure of 28.51 b/in² and kept shaking for 24 hours. Then the bottle was removed and the alcohol was then removed on a flash evaporator. The amber coloured syrupy liquid gave the tests for aromatic amines.

iii) Preparation of Diazonium salt :

For this, 0.143 gram of amino-Fenitrothion was dissolved in 10 ml of 1 N HCl and was then diazotized by placing it in an ice bath and adding a solution of 36 mg of sodium nitrite in 4 ml of distilled water dropwise over a period of 15 minutes.

iv) Coupling procedure :

One gram of bovine serum albumin was dissolved in a buffer consisting of 0.13 M sodium chloride, 0.16 M Boric acid and sufficient sodium hydroxide to raise the pH to 9.0. The beaker containing the protein solution was placed in an ice bath, and its contents were stirred continuously by a magnetic stirrer.

The solution of the diazonium salt was added dropwise over a period of 1 hour and after each drop, the pH of the reaction mixture was maintained between 9.0 and 9.5 by addition of 0.5 N sodium hydroxide. When all the solution had been added, the contents were further stirred for 1 hour to ensure the completion of the reaction.

The reaction mixture was then dialysed against 0.1 M sodium chloride (at pH 7.0 to 7.5) for removal of reversibly bound dye. The dialysis fluid was changed daily, the last change being distilled water.

The conjugate solution was then distributed into ampoules and lyophilized.

2.3.2.2. Conversion of Fenitrothion to Fenitrooxon

Pure Fenitrothion has no inhibitory power towards acetylcholinesterase and is thus inactive in vitro. In the body, it is converted to its oxo-form and this metabolite, Fenitrooxon, has high inhibitory power towards acetylcholinesterase. With a view to prepare antibodies against this active metabolite, the synthesis and conjugation of the active metabolite was undertaken.

Procedure : (141)

Five grams of Fenitrothion was purified by extraction of its ethereal solution with 1% chilled aqueous sodium carbonate. This ethereal solution was then placed in a 30 ml long necked round bottom flask and evaporated to dryness with a gentle stream of air. The flask was then chilled in an ice-bath.

A mixture of concentrated and fuming nitric acids (1:1 v/v) was prepared and 45 ml of this reagent was

added drop by drop to the Fenitrothion in the flask. The flask was swirled to wet the walls and removed from the ice-bath. It was allowed to stand for five minutes and then about 25 ml of cold distilled water was added to it, and the contents were transferred to a 125 ml separating funnel. The separating funnel was well shaken and the aqueous layer was discarded. The ether layer was washed twice with 10 ml portions of chilled aqueous sodium bicarbonate till the washings were alkaline to litmus. Finally, the ether layer was washed with saturated sodium chloride and filtered through a small plug of dry cotton wool. The ether was then evaporated to get the Fenitrooxon.

2.3.2.3. Anticholinesterase activity of Fenitrooxon

The anticholinesterase activity of Fenitrooxon was studied on the whole blood cholinesterase of the mouse.

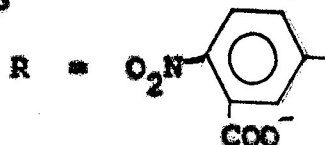
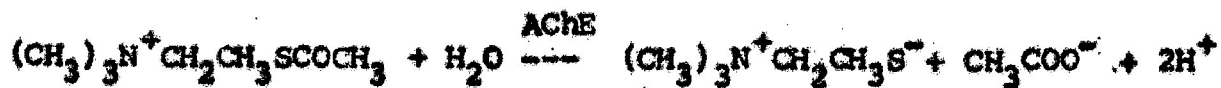
Determination of Acetylcholinesterase activity :

The acetyl cholinesterase activity of mouse blood was determined by the method of Ellman et al., (142).

Principle :

The substrate used for this enzyme assay is acetylthiocholine, which is specifically hydrolysed by acetylcholinesterase (E.C. 3.1.1.7.) to give thiocholine. This thiocholine formed during the hydrolysis reacts rapidly with the colour reagent 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) to produce a highly coloured 5-thio-2-nitrobenzoate anion which can be easily measured spectro-

photometrically at its absorption maximum of 405-420 nm.



Reagents :

1. DTNB-Buffer solution:

For this, 20 mg of DTNB was dissolved in phosphate buffer 0.1 M, pH 8.0, 150 ml containing triton X-100, 0.01 % w/v.

2. Acetylthiocholine Iodide (substrate):

For this, 4.33 mg of acetylthiocholine iodide were dissolved in 1 ml of phosphate buffer 0.1 M, pH 7.0.

3. Mouse whole blood (heparinized) :

This was collected by heart puncture from mice.

Procedure :

To 7 ml of the DTNB-buffer solution was added 0.01 ml of mouse blood. The mixture was thoroughly shaken and allowed to stand for 30 minutes to achieve complete haemolysis.

The actual enzyme reaction was carried out in quartz cuvettes. The reaction mixture contained -

DTNB-Buffer-blood mixture 3 ml

Acetylthiocholine iodide solution 0.1 ml

The substrate solution was added and the stop watch was started at once. After waiting for 5 minutes, to get a linear substrate hydrolysis, readings were taken every minute for the next 5 minutes and the change in optical density at 412 nm per minute was calculated.

To study the effect of Fenitrooxon on the enzyme activity, 0.01 ml of an alcoholic solution of Fenitrooxon was added to the DTNB-buffer-blood mixture and the enzymatic activity was determined as described above.

2.3.2.4. Coupling of Fenitrooxon to Bovine serum albumin

Conjugation of Fenitrooxon to bovine serum albumin was carried out in a manner exactly similar to that described for Fenitrothion (Section 2.3.2.1.).

2.3.2.5. Determination of hapten-carrier ratio in conjugates

Principle :

Since the hapten added contains phosphorus, an increase in the phosphorus content in the conjugate over that of the native carrier protein is an estimate of the number of haptenic groups added.

Method : (143)

Organically bound phosphorus was first converted to inorganic phosphate by ashing the compound at $600^{\circ}\text{C} \pm 20^{\circ}\text{C}$ in a muffle furnace. The ash so produced was dissolved in 0.1 N HCl and the phosphorus content of this solution was

determined by the Fiske and Subbarow method (144).

Determination of Inorganic phosphate :

Principle :

The inorganic phosphate is converted into the phospho-molybdic acid complex with molybdic acid. This is then reduced to molybdenum blue by 1,2,4-amino-naphthol-sulphonic acid and measured photometrically.

Reagents :

1. Acid molybdate solution (2.5 %) :

For this, 63 ml of concentrated sulphuric acid is added to 400 ml of water. In this is dissolved 25 grams of ammonium molybdate and the volume is made upto 1 litre.

2. Reducing agent ('ANSA' reagent) :

For this, 29.2 gram of sodium bi-sulphite and 1 gram of sodium sulphite are dissolved in 200 ml of water. To this is added 0.2 gram of powdered 1,2,4-amino-naphtholsulphonic acid, is shaken until dissolved. It should be stored in a brown or amber coloured bottle.

3. Phosphorus standard :

For this, 175.2 mg of potassium dihydrogen phosphate are made upto 1000 ml with water. It should be stored under toluene.

Procedure :

To each tube containing 1 ml of the phosphate solution is added 0.5 ml of acid molybdate solution and then 0.5 ml of the reducing agent. The tubes are shaken thoroughly and allowed to stand for 30 minutes, after which the optical density of the mixture is determined spectrophotometrically at 820 milli-microns.

2.3.2.6. Immunization of animals

Antibodies were produced in male healthy rabbits weighing 2 kg, using both Fenitrothion-BSA and Fenitrooxon-BSA conjugates. Six rabbits were used for each antigen. The primary doses for Fenitrothion-BSA and Fenitrooxon-BSA conjugates were 1 mg and 5 mg respectively, while the booster doses consisted of 1 mg of the conjugate in both the cases.

Aqueous solutions of the immunogen were emulsified with equal volumes of complete Freund's adjuvant. Following procedures were adopted for inoculation of the immunogens.

1. Multiple intradermal inoculation :

Prior to the injection, the backs of the rabbits were shaved and disinfected with 70% alcohol. Using a tuberculin syringe and a needle of 23 gauge, 10 injections each of 0.1 ml were made in the skin over the whole shaved area of the back. This method was used for primary inoculations in both cases.

2. Intramuscular injection : (146)

The thigh muscles of the hind limbs were employed for all booster injections. A suitable area of the thigh was shaved and disinfected prior to the injection. 0.5 ml of the emulsion containing the desired amount of conjugate was injected into the muscles.

Booster injections at monthly intervals were given until a high titre of antibodies was obtained.

2.3.2.7. Bleeding and collection of the antisera (147)

Animals were bled 10-14 days after booster injections. The following methods were used for the collection of blood from the immunized animals.

1. Ear vein puncture :

The lateral margin of the ear was dry shaved and cleaned with 70% alcohol and blood was collected from the vein using a heparinized syringe and needle. This method was employed for small trial bleedings of 5-10 ml.

2. Heart puncture :

The animal was restrained on a table with the flanks exposed. The thoracic area was cleared with 70% alcohol. The heart beats were palpated with the middle finger and the thoracic cage was penetrated at the site where the heart beats were felt most prominently. The needle was advanced in the chest for about 3 mm until it transmitted the heart

beats, and the blood was removed by applying a slight negative pressure. The blood was collected in siliconized glass bottles. The method was used for bleedings of 30-40 ml.

3. Terminal bleeding :

The animal was anaesthetized by injecting sodium pentobarbitone 30 mg/kg body weight intraperitoneally. The animal was taken onto an animal operation table and the throat region was shaved free of hair. Stretching the skin sideways, an incision was made a little to the left of the trachea. Fascia was carefully cut open and muscles were separated to visualize the carotid artery. The artery was clamped and cannulated with siliconized glass artery cannula. When the clamp was released, blood streamed out of the open end of the cannula and was collected in E.D.T.A. solution (final concentration 0.4 %) until the flow ceased. A maximum of 120 ml of blood could be obtained from each rabbit. The method was used for final collection of blood from the animals which had attained a high titre of antibodies.

Separation of plasma : (148)

The blood obtained was cooled immediately and centrifuged at 4°C at 1000 r.p.m. for 15 minutes. The supernatant was transferred to new tubes and the thrombocytes were separated from the plasma by centrifugation at 7000 r.p.m. for 30 minutes in a refrigerated centrifuge.

The plasma thus obtained was stored at -20°C.

2.3.2.8. Ammonium sulphate precipitation of the immunoglobulins

1. Preparation of saturated ammonium sulphate solution (SAS) :

For this, 550 grams of ammonium sulphate were weighed out and placed in a 1 litre beaker. 600 ml of distilled water was added to it and the mixture was heated slowly till all the salts dissolved. While the solution was still hot, it was filtered through coarse filter paper to remove insoluble matter. When the clarified solution reached room temperature the volume was brought to 990 ml with distilled water and concentrated ammonium hydroxide was added drop by drop to bring the pH to 6.5 (isoelectric point of gammaglobulins). The volume was then made upto one litre. Some undissolved salt is necessary to ensure saturation.

2. Fractionation of the immunoglobulin :

For this, 50 ml of saturated ammonium sulphate solution was added to 100 ml of the plasma. The precipitated proteins were removed by centrifugation, and dissolved in 50 ml of normal saline. 25 ml of SAS was added again, to this solution and the precipitate was removed by centrifugation. This process was repeated once.

The residual ammonium sulphate was removed by extensive dialysis against normal saline and then, at the end, against phosphate buffer 0.1 M, pH 7.4.

2-3.2.9. Characterization of the antibodies

Detection :

Antibodies were detected by the agar-gel diffusion method as described by Ouchterlony (149).

Principle :

When an antigen and its specific antibody are allowed to diffuse towards each other in a gellified medium, a precipitin line is formed.

Method :

1 % agar was prepared by heating it in normal saline. The agar was filtered while hot through moist cotton and sodium azide in a final concentration of 0.1 % was added to it as a preservative. 3 ml of agar, while still liquid, were layered carefully on cleaned, dry microscope slides. The agar was allowed to set and wells were cut in it using a standard template. The wells had a constant diameter of 3 mm and an inter-bore distance of either 3 mm or 10 mm.

Using a fine capillary pipette, the solutions of antibody and antigen were placed in the wells. The antigen concentrations used varied from 0.025 to 0.1 %.

The slides thus prepared were incubated in a moisture chamber for 24 hours and then inspected for the presence of precipitin lines.

Staining of slides :

After full development of the precipitin lines, the agar was washed for 3-4 days with normal saline to remove unreacted antigen and antibodies from the preparation. Finally, the gel was washed with distilled water and air dried by placing a whatman No.1 filter paper over it and allowing it to dry. The precipitin lines were then stained with 0.5% amino black 10 B in methanol-acetic acid-water (40:10:50) for 30 minutes (150). Excess stain was removed by washing with 1% acetic acid solution. The slides were then air dried.

The precipitin reaction in agar gel was performed for the following studies :

a) Screening of plasma for the presence of antibodies :

For experimental screening, plasma from individual immunized animals and the corresponding conjugates used for immunization, were used. Control studies were performed using plasma obtained from normal unimmunized rabbits.

b) Cross reaction of the immune plasma with the carrier protein bovine serum albumin :

Bovine serum albumin in 0.1 % solution in normal saline was reacted against the immune plasma.

c) To study specificity of the antibodies in the immune plasma or IgG fractions for Fenitrothion and Fenitrooxon.

Method :**Principle :**

When antibodies directed against the carrier are adsorbed on the carrier and thus removed from the solution, only the hapten specific antibodies remain in solution to react with the conjugate.

Incubation of this solution with an excess of hapten will result in the combining sites on the antibody being blocked by the hapten, thereby rendering them incapable of reacting with the conjugates. Thus no precipitin line will be observed in the agar gel precipitation test between plasma incubated with hapten and the conjugate.

Procedure :**1. Removal of anti-carrier antibodies : (150)**

To a series of tubes containing 0.5 ml of undiluted antibody solution, 0.5 ml of two-fold serially diluted 0.1 % bovine serum albumin solution in phosphate buffer, pH 7.4, was added. The tubes were then incubated for 1 hour at 37°C and 24 hours at 4°C. The resulting precipitates were centrifuged out and supernatants were tested for specificity to the conjugate.

2. Demonstration of specificity to hapten :

To 0.5 ml of the supernatant described above, 0.05 ml of a 0.01 % suspension of the hapten was added and the tubes were incubated for 30 minutes at 30°C followed by 15 minutes at 4°C and then reacted against the conjugate in the agar gel diffusion test (81).

2.4. Results

2.4.1. Cholinesterase inhibiting activity of Fenitrooxon :

Fenitrooxon was found to inhibit mouse blood cholinesterase activity in a dose dependent manner. The IC_{50} of Fenitrooxon in the system used was found to be 2.045×10^{-5} mM.

2.4.2. The conjugates :

a) Colour, solubility etc. :

The conjugates of Fenitrothion and Fenitrooxon with bovine serum albumin, prepared by using the diazo coupling procedure, were golden red in colour and after dialysis and lyophilization could be completely resolubilized in normal saline without giving rise to any turbidity.

b) Hapten-carrier ratios :

From the phosphorus content of the conjugates, it was calculated that -

i) Fenitrothion-BSA conjugate contained 5.626 groups of fenitrothion per molecule of BSA.

ii) Fenitrooxon-BSA conjugate contained 9.120 groups of Fenitrooxon per molecule of BSA.

2.4.3. Immunisation and collection of plasma

Individual bleedings from the rabbits were tested for the presence of antibodies to the appropriate immunogens by the

Fig. 16 : Precipitin lines showing antibodies specific to Fenitrothion.

A - Fenitrothion - BSA conjugate : 1 mg/ml

B - Anti-Fenitrothion Antibody.

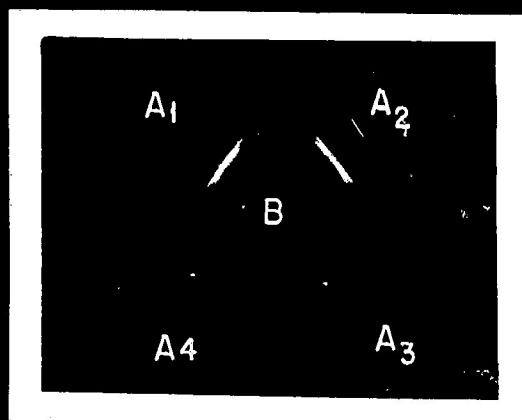


Fig. 17 : Precipitin lines showing antibodies specific to Fenitrooxon

A₁ - Fenitrooxon-BSA conjugate: 1 mg/ml

A₂ - Fenitrooxon-BSA conjugate: 0.5 mg/ml

A₃ - Fenitrooxon-BSA conjugate: 0.25 mg/ml

A₄ - Fenitrooxon-BSA conjugate: 0.125 mg/ml

B - Anti-Fenitrooxon Antibody

agar gel method. Precipitin lines developed in all the cases, while normal rabbit plasma taken as control showed no precipitin lines, for the conjugates. From this, it was concluded that all immunized rabbits have produced antibodies to the respective conjugates. Following amounts of plasma were obtained from each group :

For Fenitrothion-BSA :

- | | |
|------------------------------------|------------|
| 1. Number of rabbits bled |4 |
| 2. Average blood from each rabbit | ... 100 ml |
| 3. Total volume of plasma obtained | ... 160 ml |

For Fenitrooxon-BSA :

Heart bleeding was performed from time to time. Plasma obtained from rabbits No.2 and 3 on the 255th day was used in the biological protection experiments.

2.4.4. The antibodies :

a) Specificity to the hapten :

Adsorption of the immune plasma with the carrier protein bovine serum albumin, resulted in total removal of antibodies to the carrier.

Fig. No. 16 and 17 show the precipitin lines obtained by the reaction with the conjugate (no reaction with the carrier) for Fenitrothion and Fenitrooxon, respectively.

Further, these precipitin lines could be shown to be blocked after incubation with an excess of the requisite hapten.

From this, it was concluded that antibodies to both Fenitrothion and Fenitrooxon have been produced.

b) Absolute antibody titre :

This test was done only on the anti-fenitrooxon antibodies in order to get our estimate of the titre.

The quantitative precipitin test when performed with 0.5 ml of anti-fenitrooxon antibodies and two-fold serial dilutions of a 0.4 % solution of the Fenitrooxon-BSA conjugate, showed visible turbidity/flocculation in all the tubes. The tube containing 0.1 % concentration of Fenitrooxon-BSA conjugate showed maximum precipitation. The flocculated material from this tube showed a protein content of 21.33 mg/ml.

CHAPTER III : PHARMACOLOGICAL STUDIES

3.1. Introduction to the electrical activity of the heart

In the cycle of cardiac contraction the first event is the contraction of the atria that forces unoxygenated blood from the peripheral venous system and oxygenated blood from the pulmonary venous system into the relaxing right and left ventricles respectively. As the atria begin relaxing the ventricles contract, thus feeding the oxygenated blood to the systemic arterial system, and the unoxygenated blood to the pulmonary arterial system for oxygenation. During this time the atria have been relaxing and have accumulated blood from their respective venous connections. As the ventricles relax the atria once again contract, thus initiating a new cardiac cycle.

This sequential contractile activity of the heart is initiated by the electrical impulse originating in the cardiac pacemaker, at the base of the right atrium, the sino-atrial node, which has an inherent property of rhythmic electrical discharges. The impulse so generated here travels over the atria to the atrio-ventricular node, stimulating the atria to contract. From the atrio-ventricular node, the impulse is distributed to the ventricles along the bundle of His in the inter-ventricular septum and the specialized conducting tissue - the Purkinje fibres. This is the signal for the ventricles to contract.

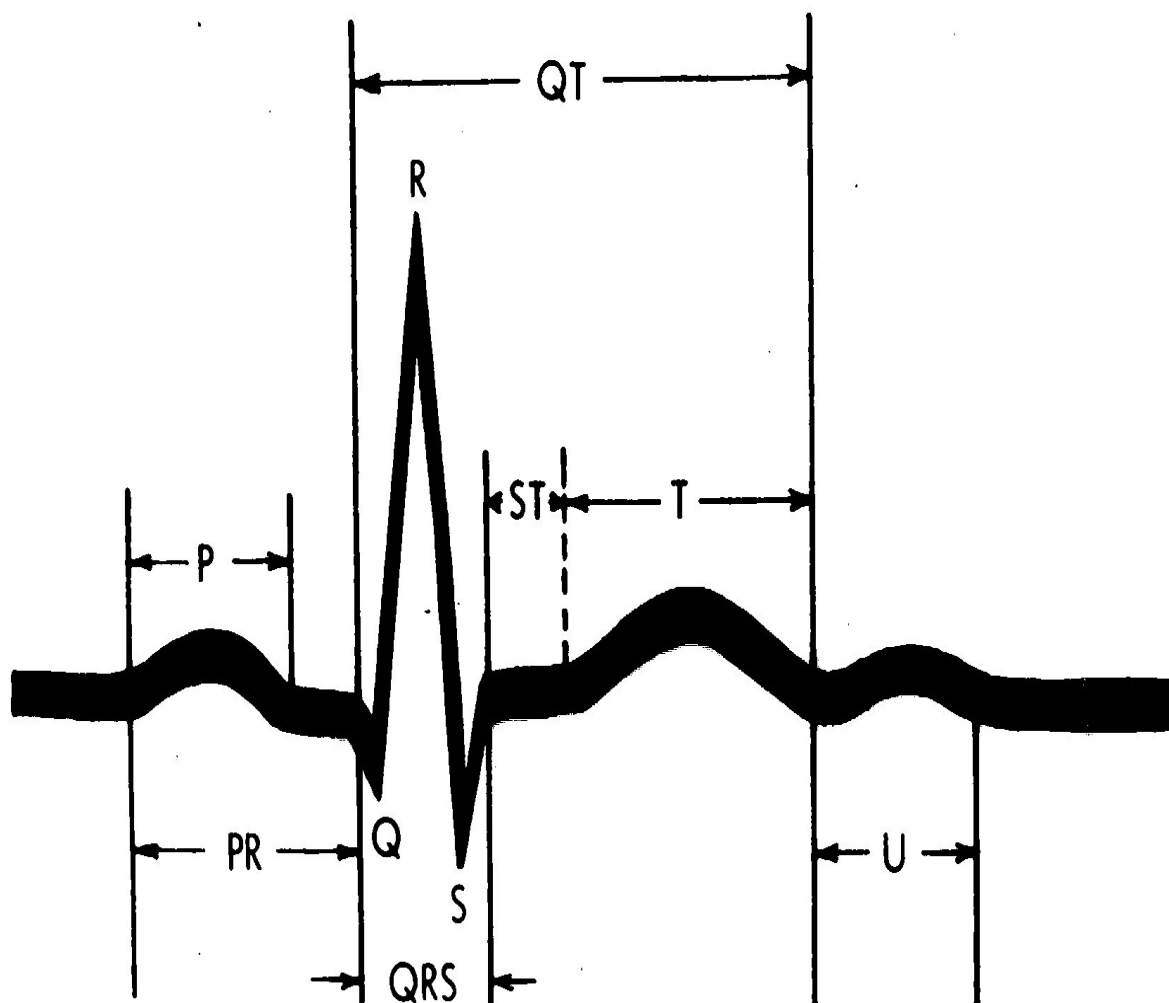


FIG.18: ECG: DIAGRAMMATIC.

The sequence of the electrical impulse leading to the stimulation of the cardiac muscle is essentially the same as that occurring during the stimulation of any cell, viz., a depolarization of the cell membrane during which the cell interior becomes electropositive with respect to its surroundings, followed after a latent period by a repolarization during which it slowly returns to its normal state of electronegativity with respect to its surroundings. The depolarization is the trigger for a series of intracellular events leading to contraction of the cardiac muscle.

This cyclic electrical activity of the heart can be recorded by electrodes placed in spatially strategic points on the body surface. The pattern thus recorded is called the electrocardiograph (ECG).

A normal ECG demonstrates the following waves in a single cardiac cycle (See Fig. 18):

The first deflection from the base line or the isoelectric state is a positive deflection indicating the depolarization of the atria, viz., the P wave. This is followed by a small isoelectric interval during which the impulse is traversing the A-V node, followed by the QRS complex, which represents the successive depolarization of the interventricular septum, the ventricular musculature and the base of the ventricles. This complex is followed by another isoelectric interval during which there is no net

electrical activity since the atria have already repolarised and the ventricles are in a refractory state. The subsequent ventricular repolarization is responsible for the T-wave.

The direction of each deflection is determined by the direction of the net electrical activity with respect to the recording electrode.

The sino-atrial and the atrioventricular nodes of the heart are innervated by the efferent parasympathetic fibres through the vagus nerve.

Stimulation of these cholinergic fibres leads to the release of acetylcholine in the synaptic cleft, thus causing the following modifications of the cardiac activity:

- a) A depression of the S-A node leading to bradycardia,
- b) A decrease in the force of contraction and the refractory period of the atria,
- c) A decrease in the conductivity of the A-V node and the bundle of His, leading to various types and degrees of A-V blockade.

3.2. Effects of Penitrooxon on the ECG

Being an irreversible anticholinesterase agent, Penitrooxon decreases the rate of degradation of acetylcholine at the site of action, thereby producing a high concentration of acetylcholine at the effector site, leading to the

following effects :

1. The decreased refractory period of the atria leading to P wave inversion,
2. depression of A-V node conductivity leading to various degrees of A-V blockade, ranging from a prolongation of the P-R interval to complete atrioventricular dissociation, and
3. depression of the S-A node leading to bradycardia, extrasystoles and arrhythmia.

3.3. Materials and methods

3.3.1. Materials and animals

1. Chemicals:

- a) Fenitrooxon
- b) Urethane (Riedel)
- c) Chloralose (BDH)

Urethane-chloralose solution:

Urethane 250 mg/ml

Chloralose 15 mg/ml

Preparation: Urethane was dissolved in distilled water and chloralose was added to it and the mixture was warmed till chloralose dissolved.

2. Animals :

Twentyone day old male mice of the NIH strain weighing 8 - 10 grams were used.

3.3.2. Methods

3.3.2.1. Determination of the minimum lethal dose of Fenitrooxon with ECG monitoring

An emulsion of Fenitrooxon was prepared in water using Tween 80 as the emulsifying agent.

Mice were anaesthetized by an intraperitoneal injection of the Urethane-chloralose solution, 4 ml/kg. A standard lead 2 ECG was recorded at intervals of 1 minute, by subcutaneous needle electrode on the EKG amplifier model 105 of Instruments and Chemicals private Limited. Continuous monitoring of the ECG was done concurrently on an oscilloscope (Polyvision of Inco, Ambala).

Mice were divided into 6 groups of 6 mice each and anaesthetized as above. After the establishment of anaesthesia fenitrooxon was injected intraperitoneally, 2,4,6,8,10 & 12 mg/kg to the six groups, respectively. Mortality was observed for 6 hours. The doses showing 100 % mortality were noted and a dose for use in protection studies was determined.

3.3.2.2. Effect of antifenitrooxon antibody on experimental poisoning by fenitrooxon

On the basis of the mortality profile observed in the experiment described above, a dose of 11.4 mg/kg was selected as a dose approximating the LD_{100} (minimum lethal dose, MLD) for studying the effect of antifenitrooxon

antibody on experimental poisoning.

Twelve mice were anaesthetized as above and were pretreated alternately with 1 ml of normal saline or 1 ml of antifenitrooxon antibody (21.33 mg/ml) intraperitoneally and then a dose of 11.4 mg/kg of fenitrooxon was administered to them intraperitoneally. A standard lead 2 ECG was recorded at 1 minute intervals, as described in section 3.3.2.1. Mortality in the saline and antibody pretreated groups were recorded.

3.4. Results

1. Determination of the minimum lethal dose

Table 1

Percentage mortality with graded doses of fenitrooxon

<u>Dose in mg/kg</u>	<u>% mortality</u>
2	16.66
4	33.33
6	66.66
8	50.00
10	100.00
12	100.00

On the basis of this dose dependent mortality profile, a dose of 11.4 mg/kg was selected as the dose approximating the LD₁₀₀ or the MLD for use in protection studies.

FIG 19 A) NORMAL MOUSE E.C.G.



FIG 19 B) EFFECT OF FENITROOXON ON MOUSE E.C.G.



- 1) Inversion of P wave
- 2) Prolongation of P-R interval
- 3) Increased amplitude of Q-R-S
- 4) Bradycardia

FIG 19 C) EFFECT OF FENITROOXON ON MOUSE E.C.G.-A-V DISSOCIATION



2. Effect of Anti-fenitrooxon antibody on experimental poisoning by Fenitrooxon

i) On intraperitoneal administration of Fenitrooxon to mice pretreated (control) with saline, the following changes in their ECG could be seen (See Fig. 19) :

- a) Prolongation of the P.R. interval and inversion of the P waves,
- b) increased amplitude of the QRS complex, and
- c) varying degrees of atrioventricular blockade leading to complete A-V dissociation, and irreversible bradycardia with extrasystole, leading to death.

As against this, in mice that were given antifenitrooxon antibody, prior to the administration of Fenitrooxon, none of the above effects were seen, saving transient tachycardias and bradycardias, except in the animal which died.

ii) All 6 mice pretreated with saline died on administration of Fenitrooxon. Out of six antibody pretreated mice, only one died on administration of Fenitrooxon, the other survived. The effect was seen for all-or-none activity.

CHAPTER IV : DISCUSSION

Discussion

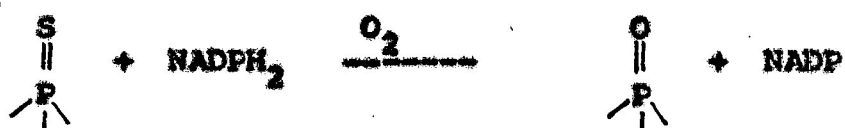
In their search for insecticidal compounds with low toxicity for mammals and a high degree of insecticidal activity, Nishizawa et al., succeeded in obtaining the compound Fenitrothion ("Sumithion") which not only had extremely low toxicity for mammals, but also a superior insecticidal activity, compared to any conventional insecticidal compounds or their isomers, homologues and analogues. It was placed on the market in 1961. The spectrum of activity as a contact and stomach insecticide is comparable to that of Parathion-methyl. Furthermore, it is suitable for controlling flies and mosquitoes which are resistant to chlorohydro-carbons.

Until 1950 it was generally assumed that the administered organophosphate per se was the active antiesterase involved in the poisoning process. In that year observations were inconsistent with that assumption (151). Since then, several experimental approaches have shown that when organophosphate esters are toxic in vivo and yet inefficient as cholinesterase inhibitors in vitro, their metabolic conversion to more active antieserases can be suspected.

Metabolism in vivo may result in both less toxic and more toxic compounds. The conversion of thiophosphates to the more toxic phosphates was shown by Gage (152).

The biochemical existence of a living organism depends upon the effectiveness of internal compensatory mechanisms which protect against external physical and chemical disturbances. The object of degradation of foreign substances is to convert them into a water soluble form which can be excreted. This purpose is served by a relatively small number of basic chemical reactions, for example oxidation, which converts lipophilic thiono compounds into the more soluble oxygen derivatives and thioethers into sulfoxides and sulphones.

Oxidation is one of the most important degradation reactions in the living organism. In case of Fenitrothion and many other insecticides of its class, oxidation however, results not in detoxication but in enhancement of toxic action, thus having an activating effect. In vertebrates, the oxidation reactions take place in the liver microsomes in the presence of the reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH_2) and oxygen (153). In insects this oxidation takes place in the fat body, which may be regarded as the 'liver' of the insect since it not only acts as a depot for metabolic products but also participates very actively in the metabolic processes. The oxidative activation of thiono esters proceeds as follows :



a reaction which would appear to be generally applicable to the thiono esters.

Knack et al., assume that the oxygen is converted by the peroxidase hydrogen donor system into free perhydroxy radicals which form activated, rapidly decomposing intermediates with the P = S grouping (154). This might offer an explanation for the otherwise inexplicable fact that, in an oxidation process, a reducing agent is required.

By oxidation of the thiono group, the water solubility of an ester may, under some circumstances, be markedly increased: for example, the solubility in water at 25°C of Parathion is 24 mg/L, while that of Paraoxon, its oxy-metabolite, is 2400 mg/L, that is a hundred-fold increase in solubility.

The difference between the enzymatic hydrolysis and inhibitory action of organophosphates consists of the fact that, during the detoxification reaction the phosphoryl group is transferred enzymatically to water and in the case of inhibition to the serine alcohol of cholinesterase respectively. The two reactions compete with each other. For a synthesis directly aimed at the production of new insecticides, the molecule must be provided in a hydrolytically insensitive "transport form", from which the "active" form can be obtained in a slow reaction e.g. oxidation. The time required for oxidation permits accumulation of the active form at the site of action. Thus, "Fenitrothion" is the transport form of "Fenitrooxon", and has no inhibitory potency towards cholinesterase. Hence the synthesis of the active and more potent fenitrooxon was undertaken.

The easiest way to prepare Fenitrooxon was by oxidation of Fenitrothion. No literature is available on the conversion of Fenitrothion to Fenitrooxon. However, methods are available for the conversion of Parathion, a member of the Fenitrothion series, to paraoxon. Paraoxon (O,O-diethyl-O-4-nitrophenyl phosphate) is a powerful inhibitor of B-type esterases (E.C.3.1.1.1.) and as such, is much in demand as a biochemical tool to distinguish between A and B type esterase activities (155). In vitro, the oxidation can be effected by several reagents such as N-bromosuccinimide, nitric acid and bromine water (156).

We have followed the method of Giang et al., using nitric acid as the oxidizing agent and we have found a compound which inhibits house blood cholinesterase in a dose dependent manner, and produces the signs of organophosphorus poisoning in mice (141).

In addition to the numerous cases of accidental intoxication resulting from the use and manufacture of organophosphorus compounds as agricultural insecticides, these agents have been employed frequently for homicidal and suicidal purposes, largely on the basis of their ready accessibility.

In cases of poisoning by organophosphates due to endogenous acetylcholine toxification, the following effects are seen:

1. Muscarinic effects:

These include sweating, tears, salivary secretion,

miosis, malaise, vomiting, diarrhoea, increased secretion in the respiratory tract, bronchospasm and an asthma-like condition, cyanosis and oxygen deficiency.

Of these, severe bronchospasm and pulmonary edema may be fatal.

2. Effects on the central nervous system :

These include confusion, ataxia, slurred speech, loss of reflexes, Cheyne-Stokes respiration, generalized convulsions, coma and central respiratory paralysis. Actions on the vasomotor and other cardiovascular centres further complicate the hemodynamic patterns.

Atropine sulphate, which is a competitive blocker of acetylcholine at the muscarinic receptor, produces a reversal of muscarinic, and to a moderate extent, the central effects of Fenitrothion.

However, during the management of a case of poisoning, a delicate balance has to be struck between too low a level of atropine leading to a persistence of the effects of the poison, and too high a level of atropine leading to atropine toxicity viz., dryness of mouth, blurred vision and photophobia, increase in body temperature, tachycardia and palpitations, muscular incoordination, symptoms suggestive of psychosis, convulsions and in severe cases, circulatory collapse and death due to respiratory failure.

Due to the unknown quantity of circulating poison, the achievement of this balance usually necessitates continuous monitoring of the signs and repeated changes in the medication schedule, imposing great stress on the physician and patient alike. Also relapses and atropine toxicity become frequent features.

Apart from such difficulties in medication, atropine is not effective against the nicotinic effects of the poison viz., fibrillations, fasciculations and twitching followed by fatigability, weakness and paralysis due to the accumulation of acetylcholine at the neuromuscular junctions.

Thus, it will be seen that atropine is ineffective against an important component of the respiratory failure that so often leads to death, viz., paralysis of the respiratory muscles.

Due to these inadequacies of atropine therapy, a new class of drugs, viz., cholinesterase reactivators was introduced in therapy. In 1951, Wilson found that hydroxylamine (H_2N-OH) can reactivate the phosphorylated cholinesterase at a significant rate.⁽²¹⁾ Further work showed that a highly effective reactivation should be produced by a molecule containing a quaternary nitrogen and an oxime group spaced at an appropriate distance. This led to the development of pyridine-2-aldoxime (Pralidoxime?) 2-PAM), and certain bis-quaternary oximes like obidoxime chloride, reaction rates with which were a million times faster than that with hydroxyl amine.

Oximes combine with the phosphoryl groups of the phosphorylated esteratic sites, forming a soluble complex. This results in the esteratic site being set free and the enzyme being reactivated. The reactivating action of oximes is most marked at the skeletal neuromuscular junction, where they restore the response to stimulation of a blocked motor nerve within a few minutes. The effects are less striking at autonomic effector sites and insignificant in the CNS, except for non-quaternary compounds such as diacetyl monoxime.

However, the phosphorylated acetylcholinesterase undergoes a fairly rapid process of "aging", so that within the course of minutes or hours it becomes completely resistant to reactivators. This "aging" is due to the splitting-off of one alkyl or alkoxy group, leaving a much more stable monoalkyl or monoalkoxy - AChE. Thus oxime therapy is useful only in the very early stages of poisoning (See Fig. 8).

Various other drugs have been tested as adjuncts to this therapy, in order to control the convulsions which are a side effect of this poisoning, to assist in countering respiratory failure which is the primary cause of death, or because of their antimuscarinic properties (157).

Some of these drugs have been found to enhance the protective effect of atropine against organophosphorus poisoning, e.g. benactysine, chlorpromazine, diazepam, mecamylamine and

representatives of the veratrum alkaloids (157). The anti-convulsant, meprobamate and the alkaloid, galanthamine may well come into this category also. Galanthamine is a potent reversible cholinesterase inhibitor, and as such has been recommended as a prophylactic against nerve gases (157). A similar use of pyridostigmine, a carbamate type of reversible cholinesterase inhibitor, has been proposed (158).

Grudzinska et al., have studied therapeutic effects of phenytoin, pento barbital, diazepam, trimethadione and phensuximide given as adjuvants to atropine and obidoxime in fluostigmine intoxication in the rat (159). Only trimethadione significantly raised the LD_{50} value for fluostigmine. However, it is interesting that all tested anticonvulsants, given as adjuvants to conventional therapy, were prolonging the life of the animals. This was probably due to the cessation of convulsions which play a great part in the mechanism of death.

Sasajima et al., have found that taurine in combination with atropine sulphate showed a tendency to increase the LD_{50} of Dichloroves and Fenitrothion. It also produced a marked increase in survival time and time taken for appearance of convulsions in the poisoning and inhibited the hypothermia and cholinesterase inhibition produced. (160)

A basically new approach to therapy would be the development of an agent which will chemically bind potentially active poison, neutralize its biological activity and remove it from circulation.

Such an agent will prevent conversion of the transport from the poison to its active form. It will prevent the occurrence of relapses. It will bring about effective elimination of the poison from the body, thereby avoiding incidents of chronic neurotoxicity. It will decrease the blockade of cholinesterase and other enzymes. It will decrease the duration of treatment with conventional agents like atropine, since the amount of poison to be contended with, is reduced.

It is well known that low molecular weight compounds when linked with proteins or polypeptide carriers, elicit the production of antibodies capable of specifically binding the hapten used. The concept of using hapten-specific antibodies to treat the toxic effects of the hapten has been advanced recently (25). The application of this concept to poisoning by organophosphorus insecticides was thought to be worthwhile, since treatment by an antibody specific to these poisons would have all the advantages mentioned above. And since the antibody can be used in the form of its fab fragments, the antibody-hapten complex will be amenable to glomerular filtration.

The immunogenicity of a substance depends both on its size and its chemical composition. Both Fenitrothion and Fenitrooxon are small molecules and are not immunogenic as such. Antigenicity was conferred on them by conjugating them to a larger molecule bovine serum albumin. The overall aims of the conjugation reactions were -

1. To have conjugates of high solubility in aqueous media,
2. To couple the molecule in such a way so as to get

maximum exposure of that part of the molecule which is biologically most active, viz., the phosphate moiety.

Antibodies have been known to be directed against that part of the molecule which is the farthest away from the site of coupling to the carrier protein.

Hence, the nitro-group on the phenolic ring in the molecule was selected.

3. To evolve a relatively simple procedure of conjugation for these molecules.

The conjugates so obtained were soluble in normal saline. Phosphorus analysis revealed 6 groups of Fenitrothion and 9 groups of Fenitrooxon in the Fenitrothion-BSA and Fenitrooxon-BSA respectively. Rabbits, when immunized with the conjugates, produced antibodies, as borne out from the results of the agar-gel diffusion tests. This indicates that the conjugation procedures used, the animal species selected, the adjuvant used and the time interval between booster doses were satisfactory for antibody production. Similar methods can be used for the production of antibodies to many other pesticides and drugs.

It has been shown that immunization with hapten-protein conjugate leads to the development of antibodies both for the hapten and the carrier. Hence, to detect the antibodies to the hapten, it is critical to separate the antibodies to the carrier. In the present case, antibodies to the carrier were precipitated out by incubating the antibody solution with the carrier protein-bovine serum albumin in optimal proportions.

The supernatants, when tested on agar-gel showed precipitin lines for the conjugate, but not for the carrier protein. The structural difference between the conjugate and the carrier protein is only the hapten molecule in the conjugate. Further incubation of the supernatants with the requisite hapten resulted in abolition of the precipitin lines against the conjugate, implying thereby that the active sites on the antibodies have been specifically blocked by the hapten, and are thus unavailable for the reaction with the conjugate. Thus, it is evident that antibodies specific to the haptens have been produced.

The biological protection afforded by the antibody was studied on the electrocardiographic pattern and the minimum lethal dose of Fenitrooxon in the anaesthetised mouse. Mice weighing 8-10 grams were used since the amount of antibody available to us was limited.

ECG monitoring was done so as to observe the non-lethal effects of the poison. It was necessary to anaesthetise the animals in order to implant the subcutaneous ECG leads. Urethane-chloralose was chosen as the anaesthetic in order to attain stable anaesthesia for a period of one to two hours.

Of the effects of Fenitrooxon on the ECG, the inversion of the P waves is due to the increase in the conductivity of the atria and other effects like prolongation of the P - R interval, abnoventricular blockade and dissociation are due to the decrease in the conductivity of the A - V node, by the accumulated

acetylcholine. The acetylcholine also causes a depression of the S-A node, leading to bradycardia.

It was observed that not only did five out of the six antibody treated mice survive the minimum lethal dose of Fenitrooxon but also showed no significant alteration of their ECG pattern.

Thus in conclusion, it can be said that a specific antibody can be raised to fenitrooxon and that it affords significant biological protection in poisoning by this agent.

Summary and conclusion

The organophosphorus insecticide Fenitrothion and its active metabolite Fenitrooxon were coupled to bovine serum albumin by diazo coupling reaction. The conjugates were purified by dialysis and were analysed for the hapten-carrier ratio by their phosphorus content. These conjugates were used to immunize rabbits. From the plasma of the immunized rabbits, the globulin fraction was separated and the presence of hapten-specific antibodies was demonstrated using the agar-gel diffusion method. The titre of the anti-Fenitrooxon antibody was determined by the quantitative precipitin reaction.

The biological protection afforded by the anti-Fenitrooxon antibody was tested on mice, and it was found to be significant.

Therefore, we venture to suggest that antibody therapy may prove to be an extremely valuable adjunct in the management of poisoning by organophosphorus insecticides.

APPENDIX

BIBLIOGRAPHY

1. Fest C., Schmidt K.- J. eds. : The Chemistry of organophosphorus pesticides. Springer-Verlag Berlin Heidelberg New York 1973 pp. 6.
2. Fest C., Schmidt K.- J. eds. : The Chemistry of organophosphorus pesticides. Springer-Verlag Berlin Heidelberg New York 1973 pp. 11.
3. Hayes W.J., Jr. and Vanglin W.K. : Mortality from pesticides in the United states in 1973 and 1974. Toxicol. Appl. Pharmacol. 42, 235 (1977).
4. "Chemistry and specifications of pesticides", WHO Technical Report series No. 620 pp. 7 (1977).
5. Baker E.L. Jr., Zack M., Miles J.W., Alderman L., Warren M., Dobbin R.D., Miller S., Teeters W.R. : Epidemic malathion poisoning in Pakistan malaria workers ; Lancet, 1, 31 (1978).
6. McKinstry D.N., Koenig E., Koelle W.A. & Koelle G.B. : The release of acetylcholine from a sympathetic ganglion by Carbachol. Can. J. Biochem. Physiol. 41, 2599 (1963).
7. Fest C., Schmidt K.- J. eds. : The Chemistry of organophosphorus pesticides. Springer-Verlag Berlin Heidelberg New York 1973 pp. 165.
8. Report of the commission on enzymes of the International Union of Biochemistry, 1961. Pergamon Press. Oxford. Lond. N.Y. Paris. 1961, pp. 104.
9. Leuzinger W., Baker A.L. : Acetylcholinesterase, 1. Large scale purification, homogeneity and amino acid analysis : Proc. Nat. Acad. Sci. U.S.A., 59, 620 (1966).
10. Wilson I.B., Hatch M.A., Ginsberg S. : Carbamylation of acetylcholinesterase : J. Biol. Chem. 235(8), 2312 (1960).
11. Koelle G.B. : "Anticholinesterase agents" pp. 449 : in Goodman L.S., Gilman A. eds. : "The Pharmacological basis of therapeutics" 5th ed., Macmillan Publishing Co., Inc. N.Y. 1975.
12. Benshop H.P. and Keijer J.H. : On the mechanism of ageing of phosphorylated cholinesterases. Biochem. Biophys. Acta. 128, 586 (1966).
13. Fest C., Schmidt K.- J. eds. : The Chemistry of organophosphorus pesticides. Springer-Verlag Berlin Heidelberg New York 1973 pp. 185.

14. Windholz M., ed. : The Merck Index : Merck and Co., Inc., N.J., U.S.A., 1976, pp. 520.
15. Schrader G.; New insecticides of low toxicity on the basis of phosphoric acid esters. *Angew. Chem.* 73, 331 (1961).
16. Nishizawa Y., Fujii K., Kadota T., Miyamoto J. and Sakamoto H. : Studies on the organophosphorus insecticides Part VII : Chemical and biological properties of new low toxic organophosphorus insecticide, O, O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate. *Agr. Biol. Chem.* 25 (8), 605 (1961).
17. Anand M., Khanna R.N., Misra D. and Sharma H.K. : Changes in brain acetylcholine of rats after dermal application of fenitrothion (Sumithion) : *Ind. J. Physiol. Pharmac.* 21, 121, (1977).
18. Khanna R.N., Misra D., and Hasan M.Z. : Effect of fenitrothion on magnesium level and cholinesterase activity in rats : *Ind. J. Physiol. Pharmacol.* 21, 210 (1977).
19. Koundinya P.R. and Ramamurthy R. : Effect of Sumithion (Fenitrothion) on some selected enzyme systems in fish *Tilapia mossambica* (Peters) *Ind. J. Expt. Biol.* 16 (7), 809 (1978).
20. Vadlamudi V.P., Paul B.S. : Acute toxicity studies on sumithion and its effect on blood and brain cholinesterase activities in mice : *Ind. J. Pharmacol.* 5(4), 423 (1973).
21. Wilson I.B. : Acetylcholinesterase XI, Reversibility of tetraethyl pyrophosphate inhibition : *J. Biol. Chem.* 190, 111 (1951).
22. Wilson I.B. and Ginsberg S. : A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase : *Biochim. Biophys. Acta.* 18, 168 (1955).
23. Koelle G.B. "Anticholinesterase agents" pp. 459, in Goodman L.S., Gilman A. eds. "The pharmacological basis of therapeutics" 5th ed. : Macmillan publishing Co., Inc. N.Y. 1975.
24. Butler V.P. Jr., and Beiser S.M. "Antibodies to small molecules : biological and clinical applications : pp. 255, in Dixon F.J. and Kunkel H.G. eds., : *Advances in Immunology.* Vol. 17, Academic press, N.Y. and London (1973).
25. Smith T.W., Haber E., Yeatman M.D., Butler V.P. : Reversal of advanced digoxin intoxication with Fab fragments of digoxin-specific antibodies : *N. Engl. J. Med.* 294, 797 (1976).

26. Pressman D. and Grossberg A.L. eds. : "The structural basis of antibody specificity" W.A. Benjamin Inc. 1968, pp. 8.
27. Gupta R.M. and Ehida M.B. : "Role of 5-HT in acute inflammation and anaphylaxis : Studies with antibodies to 5-HT. : Aspects of Allergy and Applied Immunology, 11, 141 (1978).
28. Berson S.A., Yalow R.S. : Quantitative aspects of the reaction between insulin and insulin binding antibody. J. Clin. Invest. 38, 1996 (1959).
29. Spector S. : "Radioimmunoassay" Ann. Rev. Pharmacol. 13, 359 (1973).
30. Grota L.J., Brown G.M. : Antibodies to catechol-amines. Endocrinology, 98, 615 (1976).
31. Miwa A., Yoshioka M., Shirahata A., Nakagawa Y., Tamura Z. Preparation of a specific antibody to each one of catecholamines and L-DOPA. Chem. Pharm. Bull. 24, 1422 (1976).
32. Peskar B., Spector S. : Serotonin : Radioimmunoassay. Science, 179, 1340 (1973).
33. Wilkinson M., Arepⁿ J., Bradtke J., de Ziegler D. : Determination of a dark-induced increase of pineal N-acetyl transferase activity and simultaneous radioimmunoassay of melatonin in pineal, serum and pituitary tissue of male rat., J. Endocrinol. 72(2), 243 (1977).
34. Jenkin G., Mitchell M.D., Hopkins P., Matthews C.D., Thorburn G.D. : Concentrations of melatonin in the plasma of the rhesus monkey (*Macaca mulatta*) : J. Endocrinol., 83(3), 489, (1980).
35. Davis T.R.A., Meade K.M. : Biologically active antibodies to Histamine., Nature, 226, 360 (1970).
36. Wilkes M.M., Stewart R.D., Bruni J.F., Quigley M.E., Yen S.S.C., Ling N., Chretien, M. : A specific homologous radioimmunoassay for human β -endorphin; direct measurement in biological fluids : J. Clin. Endocrinol. Metab. 50(2), 309 (1980).
37. Levine L. and Van Vunakis H. : "Antigenic activity of prostaglandins". Biochem. Biophys. Res. Commun. 41, 1171 (1970).
38. Fitzpatrick F.A., Bundy G.L. : Hapten mimic elicits antibodies recognizing prostaglandin E₂ : Proc. Natl. Acad. Sci. U.S.A., 75 (6), 2689 (1978).

39. Fitzpatrick F.A., Gorman R.R., Bundy G.L. : An antiserum against 9, 11- α -15-hydroxy-prosta-5,13 dienoic acid recognizes and binds prostaglandin endoperoxides : *Nature*, 273(5660), 302 (1978).
40. Haber E., Page L.B., Jacoby G.A. : Synthesis of antigenic branch-chain copolymers of angiotensin and Poly-L-lysine. *Biochemistry*, 4, 693 (1965).
41. Goodfriend T.L., Levine L., Fasman G.D. : Antibodies to bradykinin and angiotensin : a use of carbodiimides in Immunology. *Science*, 144, 1344 (1964).
42. Verma P.S., Lorenz P.E., Sander G.E. : Simplified radio-immunoassay of bradykinin in human plasma. : *Clin. Chem.* 26 (3), 439 (1980).
43. Bruynzeel P.L.B., Hamelink M.L., Van den Bogaard W. : Interference of EDTA in determinations of cAMP and cGMP in plasma by radioimmunoassay : *Clin. Chem.* 26(5), 668 (1980).
44. Skomedal T., Gryne E., Osnes J.B., Sjetnen A.E., Deye I. : A radioimmunoassay for cyclic AMP obtained by acetylation of both unlabelled and labelled ligand or of unlabelled ligand only : *Acta. Pharmacol. Toxicol.* 46(3), 200 (1980).
45. Cuello A.C., Galfre G., Milstein C. : Detection of substance P in the central nervous system by a monoclonal antibody : *Proc. Natl. Acad. Sci. U.S.A.*, 76(7), 3532 (1979).
46. De Weck A.L. : Immunological effects of aspirin anhydride, a contaminant of commercial acetylsalicylic acid preparations. : *Int. Arch. Allergy*. 41, 393 (1971).
47. Spector S., Parker C.W. : Morphine : radioimmunoassay. *Science*, 168, 1347 (1970).
48. Henderson G.L., Frincke J., Leung C.Y., Torten M. and Benjamini E. : Antibodies to fentanyl., *J. Pharmac. Exp. Ther.* 192, 489 (1975).
49. Liu C.T. and Adler F.L. : Immunologic studies on drug addiction : I. Antibodies reactive with methadone and their use for detection of the drug. *J. Immunol.* 111, 472 (1973).
50. Spector S. : Quantitative determination of morphine in serum by radioimmunoassay. *J. Pharmac. Exp. Ther.* 178, 259 (1971).
51. Takatori T., Yamaoka A. : "Production of anti-antipyrine antibody by immunization with 4- α -antipyrin-conjugated bovine serum albumin". *Forensic Sci.* 12(2), 151 (1978).
52. Van Vunakis H., Langone J.J., Riceberg L.J., Levine L. : Radioimmunoassays for adriamycin and daunomycin., *Cancer. Res.* 34, 2546 (1974).

53. Lewis J.E., Nelson J.C., Elder H.A. : Amikacin : Rapid and susceptible radioimmunoassay., *Antimicrob. Agents Chemother.*, 7, 42 (1975).
54. Hamburger R.N. : Chloramphenicol-specific antibody., *Science*, 152, 203 (1966).
55. Berk L.S., Lewis J.L., Nelson J.C. : One-hour radioimmunoassay of serum drug concentrations as exemplified by digoxin and gentamycin., *Clin. Chem.* 20, 1159 (1974).
56. Schwenk R., Kelly K., Tse K.S., Sehon A.H. : A radioimmunoassay for isoniazid., *Clin. Chem.* 21, 1059 (1975).
57. Gleich G.J., Stankievic R.R. : Measurement of penicilloyl antibodies by radioimmuno precipitation., *Immunochemistry*, 6, 85 (1969).
58. Wadum A.G. : Immunological specificity of sulfonamide axoproteins., *J. Infect. Dis.* 70, 173 (1943).
59. Queng J.T., Dukes C.D., McGovern J.P. : Antigenicity of the tetracyclins., *J. Allergy*. 36, 565 (1965).
60. Tigelaar R.E., Rapport R.L., Inman J.K., Kupferberg H.J. : Radioimmunoassay for diphenylhydantoin., *Clin. Chim. Acta.* 43, 231 (1973).
61. Stanley P.E., Peikert M.R. : Determination of phenytoin in plasma : A comparison of procedures using a new radioimmunoassay, gas chromatography and enzyme immunoassay : *Epilepsia* (N.Y.) 19(3), 265 (1978).
62. Robinson J.D., Morris B.A., Aherne G., Wynne and Marks V. : Development of radioimmunoassays for anticonvulsant drugs., *J. Endocrinology*, 64, 7p (1975).
63. Pettiger W.H., Keeton K., Tanaka K. : Radioimmunoassay and pharmacokinetics of saralasin in the rat and hypertensive patients., *Clin. Pharmac. Ther.* 17, 146 (1975).
64. Jaton J.C. and Ungar-Waron H. : Antibodies to folic acid and methotrexate obtained with conjugates of synthetic polypeptides. : *Arch. Biochem. Biophys.* 122, 157 (1967).
65. Kamal R., Landon J., Forrest G.C. : A fully automated, continuous flow radioimmunoassay for methotrexate : *Clin. Chem.* 26(1), 97 (1980).
66. Spector S., Berkowitz B., Flynn E.J. and Peskar B. : Antibodies to morphine, barbiturates and serotonin., *Pharmac. Rev.* 25, 281 (1973).
67. Flynn E.J. and Spector S. : Determination of barbiturate derivatives by radioimmunoassay., *J. Pharmac. Exp. Ther.* 181, 47 (1972).

68. Chung A., Kim S.Y., Cheng C.T., Castro A., : Phenobarbital specific antisera and radioimmunoassay., *Experientia*, 29, 820 (1973).
69. Kovalov K.E. : Covalent bonding of phenobarbital with proteins to produce antibodies which neutralize the toxic action of phenobarbital., *Khim. Farm. Zh.* 10(10), 7 (1976).
70. Oliver G.C., Parker B.M., Brasfield D.L. and Parker W.P. : The measurement of digitoxin in human serum by radioimmunoassay., *J. Clin. Invest.* 47, 1035 (1968)
71. Smith T.W., Butler V.P., Haber E. : Determination of therapeutic and toxic serum digoxin concentration by radioimmunoassay., *New Engl. J. Med.* 261, 1212 (1969).
72. Lesne M. : Dosage radioimmunologique de la Gitaloxine et de la Gitoxine., *Arch. Int. Pharmacodyn. Ther.* 199, 206 (1972).
73. Smith T.W. : Ouabain-specific antibodies : Immunochemical properties and reversal of Na^+ , K^+ -activated adenosine triphosphatase inhibition., *J. Clin. Invest.* 51, 1583 (1972).
74. Teale J.D., Forman E.L., King L.J., Marks V. : Radioimmunoassay of cannabinoids in blood and urine. *Lancet* II, 593 (1974).
75. Van Vunakis H., Farrow J.T., Gjika H.B. and Levine L. : Specificity of the antibody receptor site to D-lysergamide: Model of a physiological receptor for lysergic acid diethylamide., *Proc. Natl. Acad. Sci. U.S.A.*, 68 (7), 1483 (1971).
76. Van Vunakis H., Bradvika H., Benda P., Levine L. : Production and specificity of antibodies directed towards 3,4,5-trimethoxy-phenyl-ethylamine, 3,4-dimethoxyphenyl ethylamine and 2,5-dimethoxy-4-methylamphetamine., *Biochem. Pharmacol.* 18, 393 (1969).
77. Centeno E.R., Johnson W.J. and Schon A.H. : Antibodies to two common pesticides, DDT and malathion., *Int. Arch. Allergy Appl. Immun.* 37, 1 (1970).
78. Fatori D., Hunter W.M. : Radioimmunoassay for serum paraquat : *Clin. Chim. Acta.* 100(2), 81 (1980).
79. Ercegovitch C.D. : Pesticide analysis by radioimmunoassay presented at the 174th meeting of the American Chemical Society (Paper No.2, Pesticide Chemistry Division) 1977.
80. Vijay H.M., Mendoza C.E., Lavergne G. : Production of a homocytotropic antibody to malathion in the rat., *Toxicol. Appl. Pharmacol.* 44(1), 137 (1978).

81. Wing K.D., Hammock B.D., and Klustner D.A. : Development of an S-Bioallethrin specific antibody : J. Agric. Food Chem. 26(6), 1328 (1978).
82. Wing K.D., Hammock B.D. : Stereoselectivity of a radio-immunoassay for the insecticide S-Bioallethrin : Experientia, 35(12), 1619 (1979).
83. Horowitz P.E. and Spector S. : Determination of serum d-tubocurarine concentration by radioimmunoassay. J. Pharmac. Exp. Ther. 185, 94 (1973).
84. Al-Dujaili E.A.S., Forrest G.C., Edwards C.R.W. : Development and application of an automated direct radio-immunoassay for plasma aldosterone : J. Endocrinol. 81 (2), 111p (1979).
85. Morris H.G., De Roche G., Caro C.M. : Detection of synthetic corticosteroid analogs by the competitive protein-binding radioassay. Steroids 22, 445 (1973).
86. Nathanielsz P.W., Lowe K.C., Buster J.E. : Comparison of two rapid cortisol radioimmunoassays for use in the fetal sheep : Steroids 34(1), 111 (1979).
87. Meikle A.W., Lagerquist L.G., Tyler F.H. : Plasma dexamethasone radioimmunoassay. Steroids 22, 193 (1973).
88. Werawatgocumpa S., Pongpradit T., Leepipatpaiboon S., Sukanthanak A. : A radioimmunoassay for serum methoxyprogesterone acetate in Thai women receiving injectable DMPA contraceptive. Contraception, 20 (4) 319 (1979).
89. Kundu N. : Radioimmunoassay of contraceptive steroids. Steroids, 23, 155 (1974).
90. Tateishi K., Kato A., Yamamoto H., Hayashi C., Kitagawa M. : Anti-testosterone antisera produced in mice of different strains and sexes : Steroids, 32(2), 233 (1978).
91. Gross S.J., Grant J.D., Bennett R., Wong S.L.R., Lomax P. : Estriol-specific antibody for steroid assays. Steroids, 18, 555 (1971).
92. Nambara T., Hasoda H., Tadano K., Yamashita K. : Preparation and antigenic properties of 5- α -dihydrotestosterone-15 α -bovine serum albumin conjugate : Chem. Pharm. Bull. 26(12), 3851 (1978).
93. Colburn W.A. : Radioimmunoassay for flutxymesterone (Halotestin), Steroids, 25, 43 (1975).
94. Pang S., Shine S., Levine L.S., New M. : Practical advances in cortisol and dehydroepiandrosterone sulphate radio-immunoassay using the microfilter paper method. J. Lab. Clin. Med. 95(4), 515 (1980).

95. Bedolla-Tovar N., Rahman S.A., Cekan S.Z., Diczfalusy E. : Assessment of the specificity of norethisterone radio-immunoassays. *J. Steroid. Biochem.* 9(6), 561 (1978).
96. Gross S.J., Campbell D.H. and Westall H.H. : Production of antisera to steroids coupled to proteins directly through the phenolic A ring. *Immunochemistry* 5, 55 (1968).
97. Warren R.J. and Fotherby K. : Radioimmunoassay of synthetic progestogens, norethisterone and norgestrel. *J. Endocrinol.* 62, 605 (1974).
98. Colburn W.A. : Radioimmunoassay for prednisone. *Steroids*, 24, 95 (1974).
99. Pek B., Holynes A.L. : A radioimmunoassay for plasma androstenedione using an iodine-125 tracer. *Clin. Chim. Acta.* 103(2), 165 (1980).
100. Mule S.J., Whitlock E. and Jukofsky D. : Radioimmunoassay of drugs subject to abuse : Critical evaluation of urinary morphine - barbiturate, morphine, barbiturate and amphetamine assays. *Clin. Chem.* 21, 81 (1975).
101. Hooker S.B., Boyd W.C. : Antibodies to strychnine. *J. Immunol.* 38, 479 (1940).
102. Arens H., Zenk M.H. : Radioimmunoassays for the determination of the peptide alkaloids ergotamine and ergocristine in *claviceps purpurea* : *Planta Med.* 38(3), 214 (1980).
103. Shigeru M., Sakamoto N., Seino Y., Tamada T., Matsuyama H., Muranaka H. : Cotinine excretion and daily cigaret smoking in habituated smokers. *Clin. Pharmacol. Ther.* 25 (5, Pt.1) 555 (1979).
104. Haines C.F., Mahajan D.K., Miljkovic D., Miljkovic M., Vessell E.S. : Radioimmunoassay of plasma nicotine in habituated and naive smokers. *Clin. Pharm. Ther.* 16, 1083 (1974).
105. Castonguay A., Van Vunakis H. : Radioimmunoassay of N'-nitrosonornicotine. *Anal. Biochem.* 95(2), 387 (1979).
106. Dixon W.R., Earley J., Postma E. : Radioimmunoassay of chlordiazepoxide in plasma. *J. Pharm. Sci.* 64, 937 (1975).
107. Midha K.K., Loo J.C.K., Hubbard J.W., Rowe M.L., McGilveray I.J. : Radioimmunoassay for chlorpromazine in plasma. *Clin. Chem.* 25(1), 166 (1979).
108. Peskar B. and Spector S. : Quantitative determination of diazepam in blood by radioimmunoassay. *J. Pharmac. Exp. Ther.* 186, 67 (1973).
109. Inayama S., Tokunaga Y., Hosoya E., Nakadate T., Niwaguchi T., Aoki K., Saito S. : "Preparation of a specific antibody to mephentermine", *Chem. Pharm. Bull. (Tokyo)* 25 (4), 838 (1977).

110. Ullman F.E., Yoshida R.A., Blakemore J.I., Maggio E.,
Leute R. : "Mechanism of inhibition of malate dehydrogenase
by thyroxine derivatives and reactivation by antibodies".
Biochim. Biophys. Acta. 567(1), 66 (1979).
111. Walsh P.R., Wang M., Gittermann M.L. : Development and
validation of a choriogonadotropin beta-subunit radio-
immunoassay for serum choriogonadotropin using commercially
available components. *Clin. Biochem.* 13(2), 69 (1980).
112. Horn H., Berger S., Bar-Ilan A., Jonas Z. : Polyethylene
glycol and second antibody in radioimmunoassay of human
choriogonadotropin : *Clin. Chem.* 26(5), 666 (1980).
113. Verhoff F.H., Lisi P.J., Fischer C.D., Teipel J.W.,
Goldstein G., Schiffman M.B. : Graphical determination
of specific activity, binding constants and antibody-site
concentrations for radioimmunoassays with application to
thymopoietin. : *Clin. Chem.* 26(6), 718 (1980).
114. Hammond J.E., Philips J.C., Straight C.B., Hammon M.G. :
Problems in radioimmunoassay of human lutropin with
commercially available reagents. : *Clin. Chem.* 26(6),
772 (1980).
115. Dermody W.C., Ananthaswamy R., Rosen M.A., Perini F.,
Levy A.G. : Immunological, biochemical and enzymatic
validation of radioimmunoassays specific to the amino
and carboxy terminal of human calcitonin. : *Clin. Chem.*
26(2), 235 (1980).
116. Langone J.J. : Immunoassay of 5-methyltetrahydrofolate :
Use of iodine - 125 - labelled protein A as the tracer
molecule for specific antibody. : *Anal. Biochem.*
104(2), 347 (1980).
117. Shinomiya T., Funayama-Machida C., Uchida T. :
Characterization of Anti-ApApA antibodies. : *J. Biochem.*
84(6), 1447 (1978).
118. Koopman W.J., Schroten Holer R.E. : A sensitive radio-
immunoassay for quantitation of IgM rheumatoid factor. :
Arthritis Rheum. 23(3), 302 (1980).
119. Riceberg L.J., Van Vunakis H. : Determination of N,N-di-
methylindolealkylamines in plasma, blood, urine extracts
by radioimmunoassay and high pressure liquid chromatography.
J. Pharmacol. Exp. Ther. 206(1), 158 (1978).
120. Clemens T.L., Hendy G.N., Graham R.F., Baggiolini E.G.,
Uskokovic M.R., O'Riordan J.L.H. : Production of antibodies
to 1 α ,25-dihydroxycholecalciferol-25-hemisuccinate. :
development of a sensitive radioimmunoassay for 1 α ,25-
dihydroxycholecalciferol. : *J. Endocrinol.* 77(2), p.49
(1978).

121. Landon J., Moffat A.C. : The radioimmunoassay of drugs : a review : *The Analyst*, 101(1201), 225 (1976).
122. Sela M. : Immunological studies with synthetic polypeptides pp. 29 in Dixon F.J. Jr. and Humphrey J.H., eds. : *Advances in immunology*, Acad. Press. N.Y. and Lond., 1966.
123. Bomford R. : The comparative selectivity of adjuvants for humoral and cell-mediated immunity. : *Clin. Exp. Immunol.* 39, 426 (1980).
124. Lader S., Burn B.A.L., Court G. : A comparative assessment of immunization procedures for radioimmunoassay pp. 31 in *Radioimmunoassay and related procedures in medicine* : Vol. 1, Int. Atomic energy press, Vienna (1974).
125. Dawson E.C., Denissen A.E.H.C., Van Weeman B.K. : A simple and efficient method for raising steroid antibodies in rabbits. : *Steroids*, 31(3), 357 (1978).
126. Rodke L.S., Farnsworth V., Goodfliesch R., Hood L. : Rabbit kappa chain immunoglobulin allotypes : evidence for a polymorphic control mechanism regulating closely-linked duplicated genes. : *Fed. Proc.* 35, 639 (1976).
127. Green I., Paul W.E., Benacerraf B. : Genetic control of immunological responsiveness in guinea pigs to 2,4-dinitrophenyl conjugates of poly-L-arginine, protamine and poly-L-ornithine. : *Proc. Natl. Acad. Sci. U.S.A.* 64, 1095 (1969).
128. Dressman G.R. and Benedict A.A. : Reductive dissociation of chicken γ G immunoglobulin in neutral solvents without a dispersing agent. : *Proc. Natl. Acad. Sci. U.S.A.* 54, 822 (1968).
129. Farr R.S. : A quantitative immunochemical measure of the primary interaction between I^{*}-BSA and antibody. : *J. Infect. Dis.* 103, 238 (1958)
130. Grant G.H. and Butt W.R. : Immunochemical methods in clinical chemistry pp. 383. in Bodansky O. and Steward C.P. eds. : *Advances in clinical chemistry*. Vol. 13, Acad. Press. N.Y. and Lond. (1970).
131. Kwapinski J.B. : In "Methods of Serological Research". : John Wiley and Sons, New York (1965), pp. 131.
132. Mayer M.N. : In "Experimental Immunochemistry", Eds. : Kabat S.A. and Mayer M.M., Charles G. Thomas U.S.A., (1961), p. 133.
133. Cohn E.J., Strong L.E., Hughes W.L., Mullford D.T., Ashworth J.N., Melin M., Taylor H.L. : Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. : *J. Am. Chem. Soc.* 68, 459 (1946).

134. Deutsch H.F.: "Preparation of immunoglobulin concentrates", pp. 316, in Williams C.A. and Chase M.W. eds. : "Methods in immunology and immunochemistry", Vol. 1, Acad. Press. N.Y. and Lond. (1967).
135. Horejsi J. and Smetana R. : : Isolation of gamma-globulin from blood serum by Rivanol. : Acta. Med. Scand. 155, 65 (1956).
136. Deutsch H.F. : "Preparation of immunoglobulin concentrates", pp. 321 ; in William C.A. and Chase M.W. eds. : "Methods in immunology and immunochemistry", Vol.1, Acad. Press. N.Y. and Lond. (1967).
137. Landsteiner K.; The specificity of serological reactions., Harvard University Press, Boston (1945).
138. Pauling L., Pressman D., Campbell D.H., Ikeda C., Ikawa M.: The serological properties of simple substances. I. precipitation reactions between antibodies and substances containing two or more haptenic groups. : J. Am. Chem. Soc. 64, 2994 (1942).
139. Howard A.N. and Wild F. : The reactions of diazonium compounds with amino acids and proteins. : Biochem. J. 65, 651 (1957).
140. Specifications for pesticides used in public health : 5th edition (1979), World Health Organization, Geneva, pp. 158.
141. Giang P.A. and Hall S.A. : "Enzymatic determination of organic phosphorus insecticides". : Analyt. Chem. 23, 1830 (1951).
142. Ellman G.L., Courtney D.K., Andres V. and Featherstone R.M. : A new and rapid colourimetric determination of acetylcholinesterase activity. : Biochem. Pharmacol., 7, 88 (1961).
143. Horwitz W. ed., : "Official methods of Analysis of the association of Official Analytical Chemists, published by Association of official analytical chemists, Washington, 1975, pp. 11.
144. Fiske C.H. and Subbarow Y. : "The colourimetric determination of phosphorus". J. Biol. Chem. 66, 375 (1925).
145. Gitlin D. : Use of ultraviolet absorption spectroscopy in the quantitative precipitin reaction., J. Immunol. 62, 437 (1949).
146. Burn E.A.L. : in Radioimmunoassay methods., eds.: Kirkham K.E., Hunter W.M., E and S., Livingstone, Edinburgh (1970), pp. 31.

147. Chase M.W. : "Collection and handling of serum"., pp. 237, in Williams C.A. and Chase M.W., eds. : *Methods in immunology and immunochemistry*, Vol. 1, Acad. Press, N.Y. and Lond. 1967.
148. Bartholini G., Pletscher A. and Gey K.F. : Diminution of 5-hydroxytryptamine in thrombocytes in vitro by chlorpromazine and related compounds. : *Experientia* 17, 541 (1961).
149. Ouchterlony O. : Antigen-antibody reaction in gels. *Acta. Pathol. Microbiol. Scand.* 26, 507 (1949).
150. Mayer M.M. : in "Experimental Immunochemistry", Eds. Kabat S.A. and Mayer M.M., Charles G. Thomas, U.S.A. (1961), p. 133.
151. Holmsted, B. : Pharmacology of organophosphorus cholinesterase inhibitors. : *Pharmac. Rev.* 11, 567 (1959).
152. Gage J.C. : A cholinesterase inhibitor derived from O,O-diethyl O-p-nitrophenylthiophosphate in vivo. : *Biochem. J.* 54, 426 (1953).
153. Brodie B.B., Gillette J.R. and LaDu B. : Enzymatic metabolism of drugs and other foreign compounds. : pp. 427 in Luck J.M., Allen F.W., Mackinney G. eds. : *Ann. Rev. Biochem. Annual reviews*, Inc. Palo Alto, Calif. U.S.A. (1958)
154. Knaak J.B., Stahmann M.A., Casida J.E. : Peroxidase and ethylenediamine-tetra acetic acid-ferrous iron- catalyzed oxidation and hydrolysis of parathion. : *J. Agr. Food. Chem.* 10, 154 (1962).
155. Aldridge W.N. : Serum Esterases : 1 : Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate and a method for their determination. : *Biochem. J.* 53, 110 (1953).
156. Ramakrishna N. and Ramachandran B.V. : Preparation and monitoring the extent of conversion of parathion to paraoxon by bromine water : *Indian J. Biochem. Biophys.* 13, 190 (1976).
157. Dawson R.M., Bladen M.P. : Some adjuncts to oxime-atropine therapy for organophosphate intoxication- their effects on acetylcholinesterase : *Biochem. Pharmacol.* 28, 2211 (1979)
158. Dirhuber P., French M.C., Green D.M., Leadbeater L., Stratton J.A. : The protection of primates against soman poisoning by pretreatment with pyridostigmine. *J. Pharm. Pharmacol.* 31(5), 295 (1979).
159. Grudzinska E., Gidynska T. and Rump S. : Therapeutic value of anti-convulsant drugs in poisonings with an organophosphate : *Arch. Int. Pharmacodyn.* 238, 344 (1979).
160. Sasajima M., Nakane S., Aihara H., Tarunto Y., Ohshima T., Akiyama F., Tsuchida K., Abe S., Nagata H., Kurachi M., Noda K., Yoshizawa H., Tanaka I. : Antagonistic effects of taurine to organophosphorous insecticides : *Pharmacometrics.* 16(3), 473 (1978).

SYNOPSIS

S Y N O P S I S

STUDIES WITH ANTIBODIES TO PENITROTHION AND ITS METABOLITES

It is well known that low molecular weight compounds, when linked to proteins or polypeptide carriers, elicit the production of antibodies capable of reacting specifically with the hapten used (1). The development of sensitive methods of detecting and quantitating reactions between hapten and antibody and the perfection of techniques for obtaining highly purified preparations of antihapten antibodies have played a significant role in the development of modern immunological concepts. Antihapten antibodies have proved particularly useful in the study of antigenic determinants, structural requirements for immunogenicity, the nature of antigen-antibody reactions and the chemical, physical and biological properties of antibody (2).

However, the concept of use of hapten specific antibodies to reverse the toxic effects of the hapten has been advanced only recently (3). Smith et al., have successfully reversed advanced digoxin intoxication with digoxin-specific antibodies in a human patient (4). They state, "Our previous experience with digitalis intoxication of this magnitude, along with that of others leads us to believe that the present case would have had a fatal outcome without this intervention".

Chemical crop protection has become one of the major concerns of mankind. In this respect the phosphoric acid esters present

a very favourable class of compounds, for, by combining four different substituents on the central phosphorus atom, it is possible to fit the properties of an active agent to the special requirements.

Due to their ester nature, these compounds are metabolised in the living organism to inorganic phosphates. This property is advantageous toxicologically.

However, these agents act by inhibition of the enzyme acetylcholinesterase and thus the toxicity for mammals and that for insects cannot basically be separated.

Fenitrothion is an organophosphorus compound with wide use today as insecticide. Unfortunately, poisoning due to this agent is also quite common. The poisoning may be accidental or with suicidal intent. At present, the poisoning due to these agents is treated with atropin and oximes. However, the latter is not available in the country and still the treatment is inadequate.

The aim of this work was to prepare antibodies specific to its active metabolite fenitrooxon and to study whether they could be used in the treatment of poisoning by this agent.

Production of antibodies to fenitrooxon :

To induce antibodies to fenitrooxon, the approach was to couple it with a carrier molecule such as a protein and to use this conjugate as an antigen. Fenitrothion was converted to its active metabolite, fenitrooxon, by the nitric acid oxidation method (5).

The aromatic nitro group on fenitrooxon was reduced by using Raney Nickel as a catalyst and hydrogen under pressure. The amino compound was then diazotized by sodium nitrite and hydrochloric acid. This diazonium salt was then added to a solution of bovine serum albumin in borate buffer, pH 9.2, which was cooled in an ice-salt bath. pH was maintained between 9 and 9.5 by using either 0.1 N sodium hydroxide or hydrochloric acid. A golden red conjugate of bovine serum albumin with fenitrooxon was obtained. It was dialyzed extensively against normal saline, and then lyophilized and stored.

From U.V. absorption data it was concluded that the coupling of fenitrooxon to albumin had occurred.

The molar ratio of the hapten to the carrier protein was determined by calculating the phosphorus content of the conjugate. The conjugate was ashed at 580°C in a muffle furnace and the phosphorus content of the ash was determined by Fiske and Subbarow's method (7).

Rabbits were immunized using Freund's complete adjuvant. A solution of the conjugate in normal saline was emulsified in the adjuvant and inoculated at multiple intradermal sites. Following the primary injections, booster injections were given at monthly intervals. Animals were bled 10-14 days following the last injection. Blood was collected by cardiac puncture, using heparinized glassware and syringes. Plasma was separated by centrifugation. The immunoglobulin fraction of the plasma

proteins was isolated by 33 % saturation with ammonium sulphate and centrifugation of the resultant precipitates of immunoglobulins. The precipitate was then dissolved in normal saline and stored at -20°C .

Antibodies were detected by the double diffusion test in agar (7). The precipitin bands were formed both for conjugate and the carrier protein. Adsorption of the antisera with the carrier protein resulted in total removal of the antibodies to the carrier. This plasma exhibited specificity for the conjugate alone.

Further, the precipitin band obtained by the reaction of antibody with the conjugate could be inhibited by the pure hapten. An alcoholic solution of the hapten was added to the antibody solution, incubated for 30 minutes at 30°C , followed by 15 minutes at 4°C , and then reacted against the conjugate in the agar-gel diffusion test.

From these results, it was interpreted that antibodies are produced to Fenitrooxon.

Absolute amount of the antibodies in the plasma was calculated by quantitative precipitin test (8).

Biological studies :

The effect of anti-fenitrooxon antibodies was studied on the isolated frog rectus abdominis muscle. The muscle was dissected out and mounted in an organ bath in frog Ringer's solution, at room temperature, aerated with oxygen.

Responses of the tissue to acetylcholine were recorded on a moving drum using a gimbal lever, and a dose response curve was established. A standard dose of acetylcholine was selected for further work.

Two strips of the rectus abdominis muscle from the same frog served as experimental and control strips respectively. When the control strip was incubated with fenitrooxon, a potentiation of the response to the standard dose of acetylcholine was obtained. Pretreatment of the experimental strip with the antibody did not appreciably block this potentiation.

The effect of fenitrooxon was next studied in vivo on the electrocardiographic pattern of the mouse.

On intraperitoneal administration of fenitrooxon to anaesthetized mice, the following changes could be seen :

- a) Prolongation of the P-R interval and inversion of the P waves.
- b) Increased amplitude of the QRS complex.
- c) Varying degrees of atrioventricular blockade leading to complete A-V dissociation and irreversible bradycardia with extrasystoles, leading almost invariably to death.

As against this in mice that were given anti-fenitrooxon antibodies intraperitoneally prior to administration of fenitrooxon as above, none of the above effects were seen, saving transient tachycardias and bradycardias.

Summary

Fenitrothion, an organophosphorus insecticide in routine use today, was converted chemically to its active metabolite fenitrooxon. This active metabolite was conjugated to bovine serum albumin. Rabbits immunized with these conjugates, showed the presence of a specific antibody to fenitrooxon, using agar-gel diffusion test. The antibodies thus prepared were evaluated for their binding capacity and neutralisation of the biological effects of fenitrooxon. On the isolated frog rectus abdominis muscle, no protection from the effects of fenitrooxon by the antibodies could be demonstrated. However, in anaesthetized mice, pretreatment with the antibody afforded significant protection against the cardiotoxic effects of fenitrooxon.

Thus, it is demonstrated that a specific antibody to fenitrooxon can be produced and that it may prove to be of value in poisoning by this agent.

Bibliography

1. Landsteiner, K. (1945).
"The specificity of serological reactions".
Harvard University Press, Boston.
2. Beiser, S.M., Butter, V.P., Jr., Erlanger, B.F. (1968).
In "Text book of Immunopathology" (Eds. P.A. Meischer and H.J. Muller-Eberhard), pp. 15-24, Grune and Stratton, New York.
3. Butter, V.P., Jr., and Chen, J.P. (1967).
"Digoxin-specific antibodies",
Proc. Natl. Acad. Sci. USA, 57 : 71-78

4. Smith T.W., Haber, E., Yeatman, L. and Butler, V.P. Jr. (1976). "Reversal of advanced digoxin intoxication with fragments of digoxin-specific antibodies", N. Engl. J. Med., 294 : 797-800.
5. Giang, P.A. and Hall, S.A. (1951). "Enzymatic determination of organic phosphorus insecticides". Analyt. Chem., 23 : 1830
6. Fiske, C.H. and Subbarow, Y. (1925). "The colorimetric determination of phosphorus", J. Biol. Chem., 66 : 375-400.
7. Gregor, G.H. and Bidt, W.R. (1970). In "Advances in Clinical Chemistry", Vol. 13 (Eds., Bodansky, O. and Stewart, C.P.), Academic Press, N.Y., p. 383.
8. Maurer, P.H. (1971). In "Methods in Immunology and Immunochemistry", Vol. III (Eds. Williams, C.A. and Chase, M.W.), Academic Press, N.Y. & London.

.....

MMPadki

(M. M. PADKI)

Signature of the Candidate.

M.B. Bhida

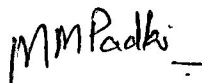
(Dr. M.B. Bhida)

Signature of the Research Guide.

Statement Under University Rule Nos. O.412 & O.413

As required by the University Rule No. O.412 and O.413, I state that the work presented in this thesis is of original in nature and so far to the best of my knowledge has not been submitted for any degree of this or any other University.

Whenever reference has been made to the work of others, it has been clearly indicated along with the source of information in the "References".



(M. M. Padki)

signature of the candidate

Forwarded through the Research Guide



(Dr M.B. Ehide)

Signature of the Research Guide

