An Evaluation of The Effects of DFP (Diisopropyl Phosphorofluoridate) and Atropine on the Disposition, Efficacy and Toxicity of 2-PAM (2-Pyridine aldoxime Methochloride) in Mice

by

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of the degree of Doctor of Philosophy

Department of Chemistry
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JANICE DEBORAH SHILOFF

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The effects of DFP and atropine on the disposition, efficacy and toxicity of 2-PAM were evaluated in mice.

Serum 2-PAM levels were elevated at 3, 7 and 10 mins in mice receiving 2-PAM and atropine (50 and 17.4 mg/kg; i.p. or s.c.) alone or 5 mins after exposure to DFP (3 mg/kg; i.p.). No significant differences in serum 2-PAM levels were seen at the later sampling times.

The serum elimination half-life values for 2-PAM (50 mg/kg; i.p.) administered alone by either the i.m., i.p. or s.c. routes of administration were not significantly different than for 2-PAM administered in combination with atropine (17.4 mg/kg; i.p.). The serum elimination half-life value of 2-PAM administered with atropine 5 mins after DFP (3 mg/kg; i.p.) was significantly ($p < 0.05$) shorter than values obtained for the administration of 2-PAM plus atropine (50 and 17.4 mg/kg; i.p.) alone or 5 mins prior to DFP and 2-PAM (50 mg/kg; i.p.) administered 5 mins after DFP.

Serum and brain 2-PAM concentrations obtained from the administration of increasing doses of 2-PAM (25-125 mg/kg; i.p.) alone or 5 mins prior to or after administration of DFP (3 mg/kg; i.p.) to mice were dose dependent. No significant differences were found in serum and brain concentrations between the treatment groups. Reactivation of serum acetylcholinesterase was found to occur only at the 100 and 125 mg/kg doses of 2-PAM. No significant reactivation of brain

acetylcholinesterase was seen.

Atropine (17.4 mg/kg; i.p.) was demonstrated to increase the toxicity of 2-PAM. The LD50 value was found to increase from 141.8 mg/kg (2-PAM; i.p.) to 86.3 mg/kg (2-PAM plus atropine; i.p.).

A dose dependent hypothermia was demonstrated in mice following 2-PAM (50-125 mg/kg; i.p.) administration alone or 5 minutes prior to or after challenge from DFP (3 mg/kg; i.p.). The administration of 2-PAM after exposure to DFP was not as effective in reversing the DFP-induced hypothermia as was its administration prior to exposure to DFP. A hypothermic response was seen in mice receiving DFP (3 mg/kg; i.p. and s.c.) and atropine (17.4 mg/kg; i.p.) themselves. Administration of 2-PAM and atropine (50 and 17.4 mg/kg; i.p.) exhibited a faster onset and recovery from the hypothermia. The effect of the hypothermia was found to be additive, but not dose dependent.

After 21 days exposure to DFP (1-3 mg/kg; i.p.), the quantity of 2-PAM found in the serum and brains of mice was not significantly different from control values. Serum and brain acetylcholinesterase activities were found to be 74% and 84% inhibited at 21 days.

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GLOSSARY

1. Term / Abbreviation

Term	Abbreviation
Acetylcholine	ACh
Acetylcholinesterase	AChE
Anticholinesterase	antiChE
Association constant	Ka
Bimolecular inhibition rate constant	Ki
Blood brain barrier	BBB
Butyrylcholinesterase	BuChE
Central nervous system	CNS
Cerebrospinal fluid	CSF
Cholinesterase	ChE
Concentration required to inhibit enzyme activity by 50%	IC50
Gastrointestinal tract	GIT
Intramuscular(ly)	i.m.
Intraperitoneal(ly)	i.p.
Intravenous(ly)	i.v.
Mixed function oxidase(s)	MFO
Phosphorylation constant	Kp
Pseudocholinesterase	p-ChE
Subcutaneous(ly)	s.c.
Serum elimination half-life	T1/2

2.

Chemical Name	Common Name
Pinacolyl methylphosphonofluoridate	Soman, GD
Isopropyl methylphosphonofluoridate	Sarin, GB
Ethyl N-dimethylphosphoramidocyanate	Tabun, GA
[[[(4-aminocarbonyl)pyridino]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride	HI-6
1,1'-[oxybis(methylene)]bis[4-(hydroxyimino) methyl]pyridinium dibromide	Obidoxime Toxogonin
2-[(hydroxyimino)methyl]-1-methylpyridinium chloride	2-PAM
1,1'(1,3'-propanediyl)bis[(4-hydroxyimino) methyl]-pyridinium dibromide	TMB-4
Diisopropylphosphorofluoridate	DFP
2-methyl-4-chlorophenoxyacetic acid	MCPA
Diacetyl monoxime	DAM

I. Introduction

Organophosphorus compounds (OP, organophosphates) are denoted by the general formula as indicated in Figure 1 (3,89,99,107).

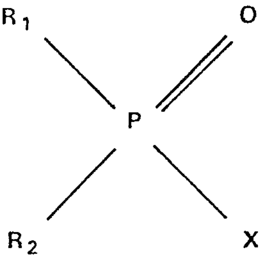

Figure 1

General Structure of Organophosphorus Compounds. R_1 and R_2 represent alcohols, phenols, mercaptans, amides or alkyl or aryl groups. X represents an acyl radical such as fluoride, chloride, paranitrophenol, cyanide, thiocyanate, enol, carboxylate, or almost any phenoxy or thiophenoxy.

Organophosphorus compounds comprise a group of compounds with varying toxicities to different forms of life. The most common use of these compounds is as insecticides. In this usage, organophosphorus compounds of relatively high toxicity to insects and low toxicity to man and other mammals are chosen. In contrast, some organophosphorus compounds have been used medically for the management of myasthenia gravis and

glaucoma. This use, however, has decreased in recent years because of the narrow margin between therapeutic and toxic doses. The most toxic organophosphates, the so-called "nerve agents", have been considered as chemical warfare agents because they are powerful inhibitors of carboxylic esterase enzymes which includes acetylcholinesterase (AChE, true cholinesterase) and pseudocholinesterase (p-ChE, butyrylcholinesterase, BuChE). In man, most cholinesterase (ChE) in the nervous tissue and erythrocytes is AChE whereas ChE in the liver and plasma (or serum) is mostly pseudocholinesterase.

Although not all the answers are presently known regarding the mechanism of biological action of organophosphorus compounds, it is generally agreed upon that the toxic effects of the organophosphates are primarily due to inhibiton of AChE (58,60,71). The resulting accumulation of acetylcholine (ACh) at the synapses initially stimulates and then paralyzes transmissions in cholinergic synapses; adrenergic synapses remain unaffected.

Because of the potential use of organophosphorus compounds as chemical warfare agents, research into finding a prophylaxis and/or therapy universally effective against the toxic effects of the organophosphorus compounds has been an ongoing process since World War II. Of the numerous compounds which have been investigated as possible antidote(s) for

organophosphate intoxication, atropine in combination with an oxime or carbamate have shown the most promise in alleviating the signs and symptoms of organophosphate poisoning (3,89,99,107). The currently recommended emergency therapy against accidental or intentional organophosphate poisoning is the use of atropine in combination with the oxime, pyridine-2-aldoxime methochloride (2-PAM, 2-PAMCl, Pralidoxime, Protopam)(1,51,74,156,158).

A. Normal Cholinergic Neurohumoral Transmission

A review of the actions of the ubiquitous neurohumoral transmitter acetylcholine is necessary in order to understand the complexities involved in the search for effective prophylaxes and/or therapies against organophosphate poisoning.

1. Acetylcholine

Chemically, acetylcholine (Ach) is a quaternary ammonium compound that contains three spatially separated centers which are of importance for its biological activity: the positively charged nitrogen atom, the carboxyl oxygen carrying a relatively negative charge and the relatively electron-poor esteratic oxygen (Figure 2)(25,71). However only the first two of the three reactive sites found in ACh are necessary for the various actions of the compound.

$$H_3C - \overset{\overset{\large CH_3}{|}}{\underset{\underset{\large CH_3}{|}}{N^{\oplus}}} - CH_2 - CH_2 - O - \overset{\overset{\large O}{||}}{C} - CH_3$$

Figure 2

Structure of Acetylcholine (ACh)

2. Role of Acetylcholine in Normal Cholinergic Neurohumoral Transmission

Acetylcholine (ACh) is the major neurohumoral transmitter when both the pre- and postganglion of the parasympathetic nervous system are stimulated (Figure 3). In addition, ACh is the neurotransmitter for preganglionic sympathetic nerve fibers as well as skeletal muscle and sweat glands (71).

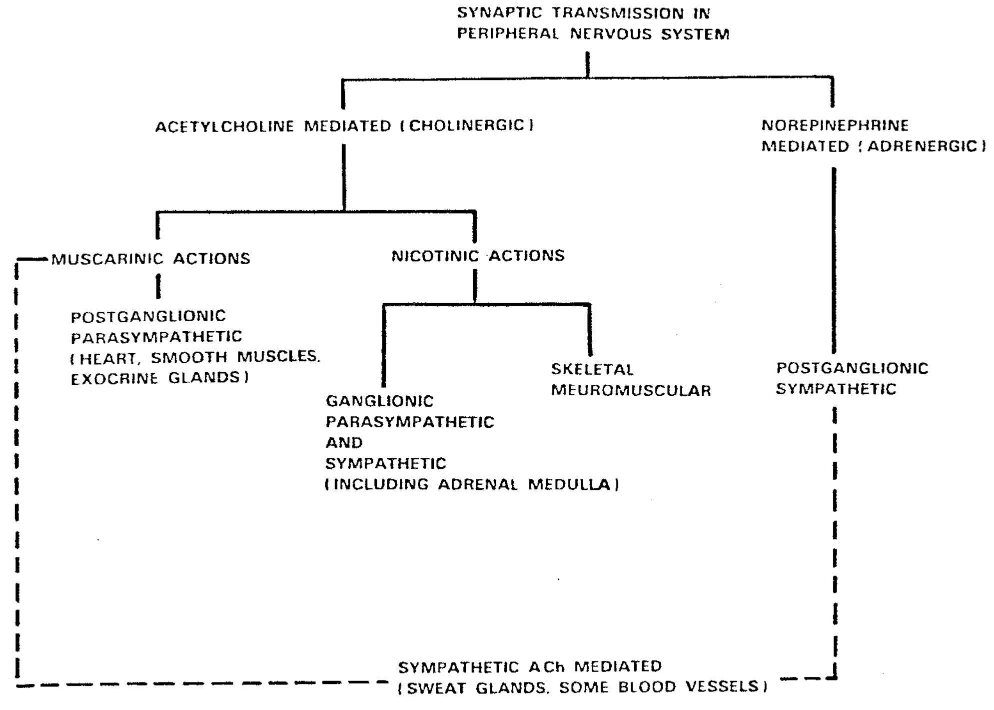

Figure 3

Cholinergic and Adrenergic Neurotransmission

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When ACh binds to a cholinergic receptor, the multitude of effects invoked are dependent upon the type of receptor that is stimulated (Table 1) (208). The two classes of cholinergic receptors are nicotinic and muscarinic. Stimulation of the muscarinic receptors mimics the action of the alkaloid muscarine (Figure 4) and has predominant effects on the secretory glands and smooth muscle (Figure 3). Alternately, stimulation of the nicotinic receptors mimics the action of nicotine (Figure 4) and has predominant effects on the autonomic ganglia and skeletal-neuromuscular junctions (Figure 3).

Figure 4

Principal Cholinomimetics

Table 1

Responses of Effector Organs to Autonomic Nerve Impulses
Reproduced from Gilman, Goodman and Gilman (71)

EFFECTOR ORGANS	ADRENERGIC IMPULSES		CHOLINERGIC IMPULSES
	RECEPTOR TYPE	RESPONSES	RESPONSES
EYE			
Radial muscle iris	α	Contraction (mydriasis)	—
Sphincter muscle iris		—	Contraction (miosis)
Ciliary muscle	β	Relaxation for far vision (slight effect)	Contraction for near vision
HEART			
S-A node	β	Increase in heart rate	Decrease in heart rate; vagal arrest
Atria	β	Increase in contractility and conduction velocity	Decrease in contractility, and (usually) increase in conduction velocity
A-V node and conduction system		Increase in conduction velocity	Decrease in conduction velocity; A-V block
Ventricles	β	Increase in contractility, conduction velocity, automaticity, and rate of idiopathic pacemakers	—
BLOOD VESSELS			
Coronary		Dilatation	Dilatation
Skin and mucosa	α	Constriction	Dilatation
Skeletal muscle	α, β	Constriction; dilatation	Dilatation
Cerebral	α	Constriction (slight)	Dilatation
Pulmonary	α	Constriction	Dilatation
Abdominal viscera	α, β	Constriction; dilatation	—
Salivary glands	α	Constriction	Dilatation
LUNG			
Bronchial muscle	β	Relaxation	Contraction
Bronchial glands		Inhibition (?)	Stimulation
STOMACH			
Motility and tone	β	Decrease (usually)	Increase
Sphincters	α	Contraction (usually)	Relaxation (usually)
Secretion		Inhibition (?)	Stimulation
INTESTINE			
Motility and tone	α, β	Decrease	Increase
Sphincters	α	Contraction (usually)	Relaxation (usually)
Secretion		Inhibition (?)	Stimulation
GALLBLADDER AND DUCTS		Relaxation	Contraction
URINARY BLADDER			
Detrusor	β	Relaxation (usually)	Contraction
Trigone and sphincter	α	Contraction	Relaxation
URETER			
Motility and tone		Increase (usually)	Increase (?)
UTERUS	α, β	Variable	Variable
SEX ORGANS		Ejaculation	Erection
SKIN			
Pilomotor muscles	α	Contraction	—
Sweat glands	α	Slight, localized secretion	Generalized secretion
SPLEEN CAPSULE	α	Contraction	—
ADRENAL MEDULLA		—	Secretion of epinephrine and norepinephrine
LIVER		Glycogenolysis	—
PANCREATIC ACINI		—	Secretion
SALIVARY GLANDS	α	Thick, viscous secretion	Profuse, watery secretion
LACRIMAL GLANDS		—	Secretion
NASOPHARYNGEAL GLANDS		—	Secretion

3. Acetylcholinesterase

Acetylcholinesterase (AChE) is a naturally occurring and widely distributed enzyme present in all animals. Acetyl-cholinesterase is a glycoprotein whose unit is a globular monomer of molecular weight 80,000 Dalton (60,111,197). The active centers of AChE consist of an anionic site which is specific for the quaternary ammonium moiety of ACh and an esteratic site which provides for nucleophilic attack on the acyl carbon of the substrate (Figure 5).

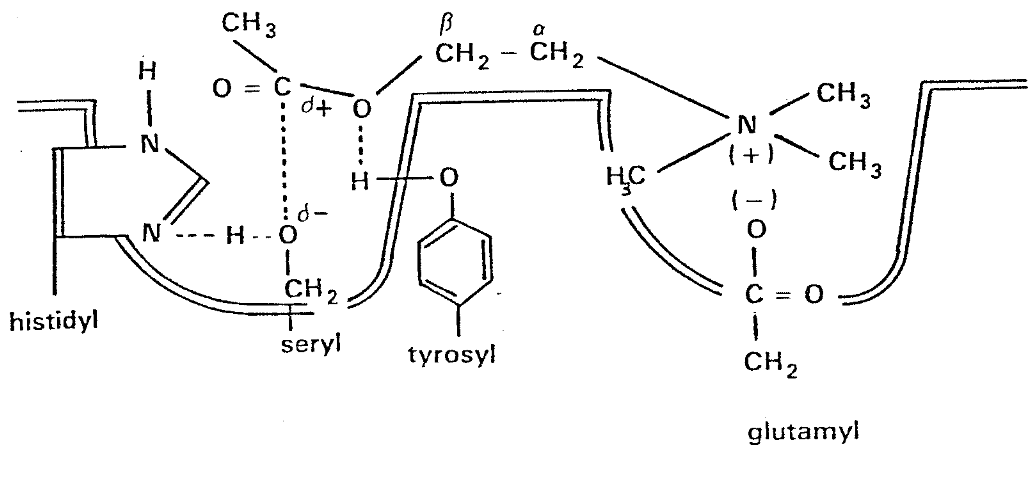

Figure 5

Active Sites of Acetylcholinesterase
Reproduced from Hodgson and Guthrie (95)

If it were not for the enzyme acetylcholinesterase, cholinergic receptor stimulation by acetylcholine would persist indefinitely until death of the organism occurred. The anionic site of acetylcholinesterase is negatively charged and attracts the positively charged nitrogen atom of ACh and binds the attached methyl groups by Van der Waals forces. The esteratic site of AChE, where nucleophilic attack occurs on the acyl carbon of the substrate, is comprised of two basic groups, serine and histidine, and one acid group, the hydroxyl of the amino acid tyrosine. The esteratic site combines with the carbonyl carbon (or phosphoryl phosphorus in the case of organophosphorus compounds) atom of the substrate and forms a covalent bond with the enzyme (95).

The specificities of mammalian acetylcholinesterase towards selected substrates are illustrated in Table 2.

Table 2

Specifications of Mammalian AChE Towards Selected Substrates Relative to ACh = 100. Values are Based on Optimal Substrate Concentrations (95)

SUBSTRATE	AChE	SOURCE OF ENZYME
Acetylcholine	100	Human Serum BuChE
Butrylcholine	2	Human Erythrocyte
Benzoylcholine	1.5	Human Erythrocyte
(D, L) Acetylcholine-β-methylcholine	33	Human Erythrocyte
Propionylcholine	87	Human Erythrocyte
Acetylthiocholine	83	Bovine Erythrocyte
Phenyl Acetate	113	Bovine Erythrocyte
Tributyrin	2	Human Erythrocyte

4. Hydrolysis of Acetylcholine by Acetylcholinesterase

The hydrolysis of acetylcholine (ACh) takes place in three steps and is illustrated in Figure 6 (25,71,89,99). In the case of acetylcholine, the fit of the substrate to the enzyme is so precise that the ACh molecule is split at the ester bond to form acetic acid and choline. Neither of these two compounds has any significant biological activity at the ACh receptor. The net effect of the interaction of ACh with AChE is the cessation of cholinergic receptor stimulation.

ACETYLCHOLINE

ACETYLCHOLINESTERASE

ANIONIC SITE

ESTERATIC SITE
HISTIDINE SERINE

STEP 1

FORMATION OF ENZYME-SUBSTRATE COMPLEX. PRINCIPAL BONDS FORMED WITH THE CATIONIC SITE OF ACh AND THE CARBONYL ATOM OF THE ESTER GROUP

CHOLINE

STEP 2

ACYL TRANSFER THROUGH NUCLEOPHILIC ATTACK ON THE ESTER CARBONYL BY A SERINE RESIDUE IN THE ENZYME

ACETYLATED ENZYME

$+H_2O$

ACETIC ACID

STEP 3

HYDROLYSIS OF ACYL ENZYME (RATE LIMITING STEP). YIELDS ACETATE AND HYDROGEN IONS THUSLY REGENERATING THE ENZYME

REGENERATED ENZYME

Figure 6
Hydrolysis of ACh

B. Effect of Anticholinesterase Compounds on Normal Neurohumoral Transmission

1. Organophosphorus Compounds

Most organophosphorus compounds belong to the irreversible class of cholinesterase inhibitors (135,145,146,159, 185,188,189). Although organophosphorus compounds react with AChE in essentially the same manner as ACh, the enzyme-organophosphate complex that is formed is much more stable than is the ACh-enzyme complex. This in turn causes a delay of the hydrolysis of ACh resulting in its accumulation at cholinergic receptor sites subsequently causing intense and continuous stimulation which can eventually lead to respiratory paralysis and death due to anoxia.

Emphasis will be placed on diisopropylphosphorofluoridate (DFP) (Figure 7) in ensuing discussions because it was chosen as the challenge organophosphorus compound in the present study for its 3 major qualities:

1. slow spontaneous reactivation (irreversible inhibition);
2. good reactivation characteristics when 2-PAM is utilized as the oxime, and;
3. experimental availability for which comparisons to the more potent "nerve agents" can be made.

Figure 7

Structure of Diisopropylphosphorofluoridate (DFP)

a. Absorption and distribution of Organophosphorus Compounds

Absorption, the process whereby a toxicant moves through the body membranes and enters the circulation, and distribution, the process whereby a toxicant is dispersed throughout the body, are dependent upon a number of factors (95). In general, the absorption and distribution of organophosphorus compounds throughout the body occurs very readily.

The biodisposition of DFP is characterized by very rapid uptake into tissues. Martin showed that within 1 minute of an intravenous (i.v.) injection to mice (1 mg/kg; 3H-DFP) the highest levels of radiolabel were in brain, lung, heart and kidney (139). Peak levels of radiolabel in liver, diaphragm and fat occurred within five minutes. During the first hour, tissue levels decreased 20-40%, remained constant for ~6 hours and was cleared in ~3 days. The amount of label extracted from tissues was found to decline with time suggesting irreversible binding or aging. Martin found that 70% of the label was extractable at 1 minute in the brain and 30% in other tissues at the same time interval (139). Only 20% and 10% were extractable from brain and other tissue at 30 minutes. It was concluded that DFP will accumulate in most tissue with chronic exposure. Acute and occasional exposure however, will most likely result in concentration in lung and liver.

In mice, a comparison of routes of administration after injection of 32P-DFP showed that i.p. administration resulted in higher levels of radioactivity in the liver (168). Pretreatment of the animals with 2-PAM and atropine did not influence the amount of tissue bound radiolabel. It appears that DFP interacts with and is absorbed to liver microsomal esterases. There was no correlation between bound 32P in any organ and the onset of death.

A study of the biodisposition of 3H-DFP administered i.v. to guinea pigs (0.1 to 6 mg/kg) showed that at higher doses, the radiolabel was distributed uniformly throughout the body tissue with the highest accumulation in the liver and kidneys (84). DFP was found to be metabolized to diisopropylphosphate with both compounds being excreted primarily through the urine and bile (84,85).

The primary distribution mechanisms for toxicants appears to be in association with plasma proteins (95,97). Plasma proteins are also known to interact with organophosphorus compounds. At 0.1 uM, DFP has been found to bind almost entirely to plasma proteins; the binding being primarily to albumin (178). However, at 0.1 mM less than 2% is bound to serum proteins suggesting that a saturating effect can occur. Means and Wa have shown that DFP reacts with a tyrosine residue of bovine serum albumin and that the binding is reversible with a Ka = 2.8×10^{2} M (147).

It has been shown that administration of 5% of an LD50 of DFP s.c. for 10 consecutive days in rat resulted in decreased AChE activities in erythrocytes (30%), tibialis muscle (30%) and brain (40%)(109). Katkiewicz <u>et al</u> showed that these levels returned to 80, 80 and 50% respectively 7 days after the last exposure (109). Histological examination of the treated animals revealed degeneration of neurons, necrotic changes in the hypothalmus, degeneration of the

myelin sheath in the sciatic nerve and a decrease in nerve succinic dehydrogenase activity. Fonnum and Guttormsen found a 10% loss of AChE activity in the brain 15 minutes after administration of 1.8 mg/kg DFP s.c. to rats with increased ACh levels at 60 minutes (68). They found that AChE levels returned to normal in ~16 hours. In contrast, Menequz et al found that within 3 hours, AChE activity in the cerebral cortex, hippocampus and striatum were decreased 60-80% after administration of 1.1 mg/kg DFP s.c. to rats (154). Recovery to 60% of the baseline AChE levels in that instance required ~48 hours in the cerebral cortex and hippocampus and 12 days in the striatum.

b. The Binding of Organophosphorus Compounds to Acetylcholinesterase

The binding of organophosphorus compounds to acetylcholinesterase (AChE) is a three step process and is illustrated in Figure 8 by the potent irreversible cholinesterase inhibitor diisopropylphosphorofluoridate (DFP) (25,71,89,99). The first step is a binding step which is influenced by the attraction of a portion of the organophosphate to the anionic site of the enzyme. The second step is a phosphorylation step in which the organophosphate is known to phosphorylate the active serine hydroxyl group at the

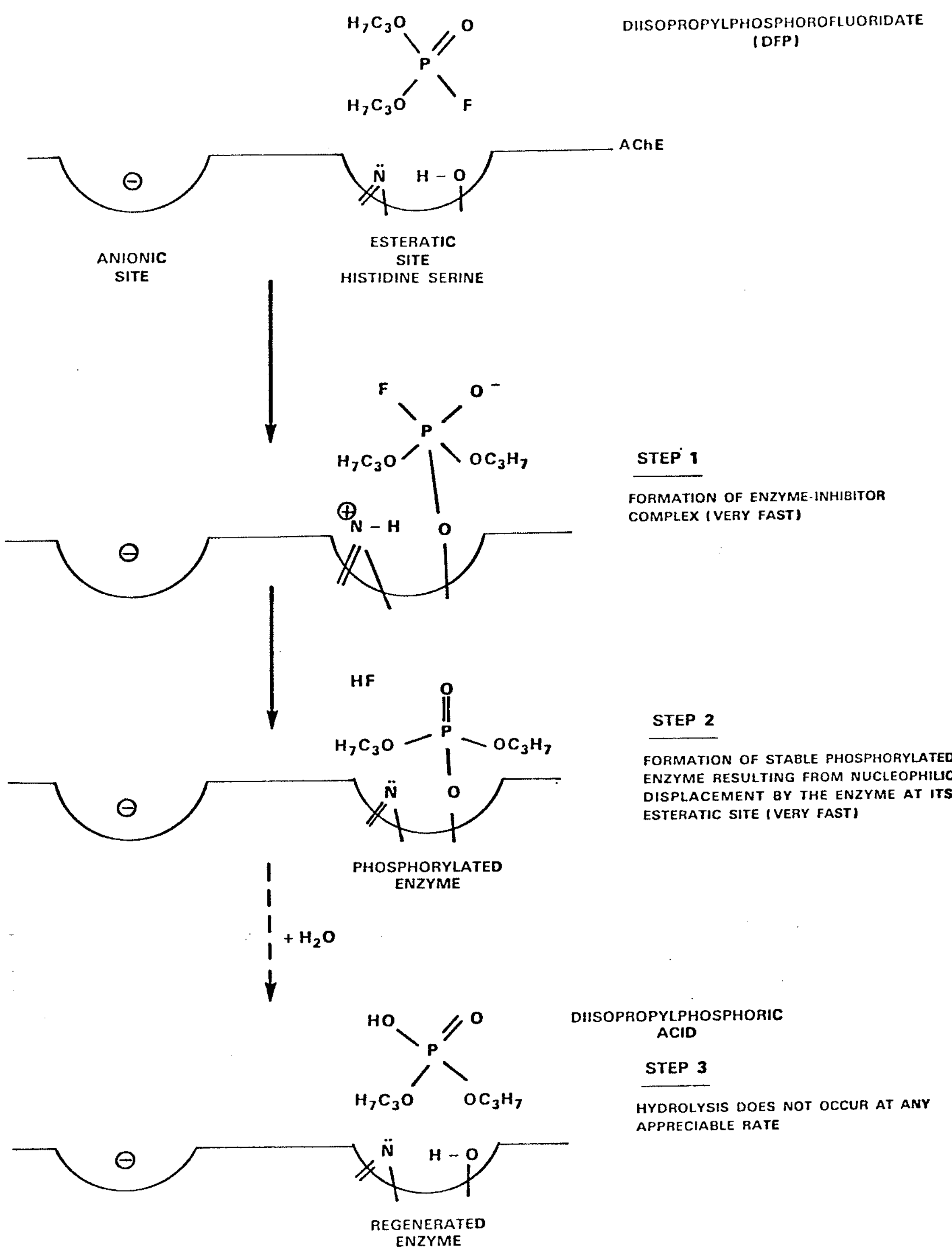

Figure 8
Inhibition of AChE by DFP

esteratic site of the enzyme. If wholly analogous to the binding of acetylcholine to acetylcholinesterase, the third step in the binding of organophosphorus compounds to AChE and related enzymes would be hydrolysis. However, phosphorylated AChE tends to be more stable than either the acetylated or carbamylated AChE. Therefore, unlike the acetylated form of AChE, the phosphorylated AChE is extremely resistant to spontaneous reactivation resulting from hydrolysis by water. Some eventual hydrolysis of the ester bond by water does occur however but this hydrolytic step is extremely slow and unmeasureable for some organophosphate cholinesterase inhibitors.

There is a wide variation in the stability of phosphorylated AChE. The stability of and extent to which phosphorylated AChE can be regenerated is dependent upon a number of factors such as the nature of the groups attached to the phosphate, the ease of carbonium ion formation, temperature, pH, the source of the enzyme and time (70,89, 95,99,107). The order from high to low spontaneous reactivation has been shown to be methyl > ethyl > secondary alkyl groups > tertiary alkyl groups. The phosphorylation (Kp), dissociation (Kd) and rate constants of some organophosphorus compounds from various sources are presented in Table 3.

Table 3

Phosphorylation (k_2), Dissociation (K_d) and Bimolecular Rate Constants (k_i) of Some Organophosphate and Carbamate Inhibitors of Cholinesterases from Various Sources. Reproduced from Hodgson and Guthrie (95)

ENZYME	COMPOUND	k_2 (min^{-1})	K_d (M)	k_i ($M^{-1} min^{-1}$)	TEMP (°C)
AChE (bovine)	Malaoxon	67	2.7×10^{-4}	2.4×10^{5}	5
BuChE (human)	Malaoxon	6.6	6.2×10^{-4}	1.1×10^{4}	25
AChE (bovine)	DFP	40.7	1.2×10^{-3}	3.4×10^{4}	25
BuChE (horse)	DFP	145	2.6×10^{-5}	5.5×10^{6}	25
AChE (bovine)	NO_2-C_6H_4-O-P(=O)-$(OEt)_2$ (ortho)	3	1.44×10^{-3}	2.1×10^{3}	5
	NO_2-C_6H_4-O-P(=O)-$(OEt)_2$ (meta)	0.81	2.2×10^{-4}	3.7×10^{3}	25
	NO_2-C_6H_4-O-P(=O)-$(OEt)_2$ (paraoxon)	43	3.6×10^{-4}	1.2×10^{5}	25
BuChE (human)	$(Et)_2N - CH_2CH_2 - S - P(=O) - (OEt)_2$	90	1.5×10^{-5}	5.8×10^{6}	5
	INSECTICIDAL CARBAMATES				
AChE (bovine)	$CH_3S - C(CH_3)(CH_2) - C(H) = N - O - C(=O) - N(H) - CH_3$ (aldicarb)	146	1×10^{-2}	1.6×10^{4}	—
	PHARMACEUTICAL CARBAMATES				
AChE (bovine)	Eserine	10.8	3.3×10^{-6}	3.3×10^{6}	—
AChE (electrical)	$^{+}N-(CH_3)_3$-C_6H_4-$O - C(=O) - N - (CH_3)_2$ (neostigmine)	46.5	1.2×10^{-6}	4.0×10^{6}	—

Lanks and Seleznic found that after DFP inhibition of electric eel AChE (94% inhibition of activity), the phosphorylated enzyme spontaneously dephosphorylated if excess unbound DFP was removed from the reaction mixture by gel filtration (121,122). Spontaneous reactivation of the DFP-enzyme had a free energy of reactivation of 20.1 Kcal/mol as expected for a hydrolysis of a covalent bond.

c. Aging

The most likely occurrence of the phosphorylated AChE is a process which has been designated as "aging" or more aptly, dealkylation (95). In aging, loss of an alkyl group from the di-alkyl phosphorylated AChE occurs forming a much more stable mono-alkyl phosphorylated AChE (Figure 9).

In the aged form, the AChE cannot be regenerated and the rate of recovery after organophosphate poisoning is dependent upon the rate of synthesis of new AChE. Aging follows first order kinetics under normal physiological conditions and occurs by C-O or P-O fission (16). Various models involving the use of purified extracts of AChE from various species, whole blood, plasma or whole animal models (anesthetized) have been used to study the irreversible inactivation of AChE and aging by dealkylation _in vitro_ and _in vivo_ (10,16,39,47,150,181,190).

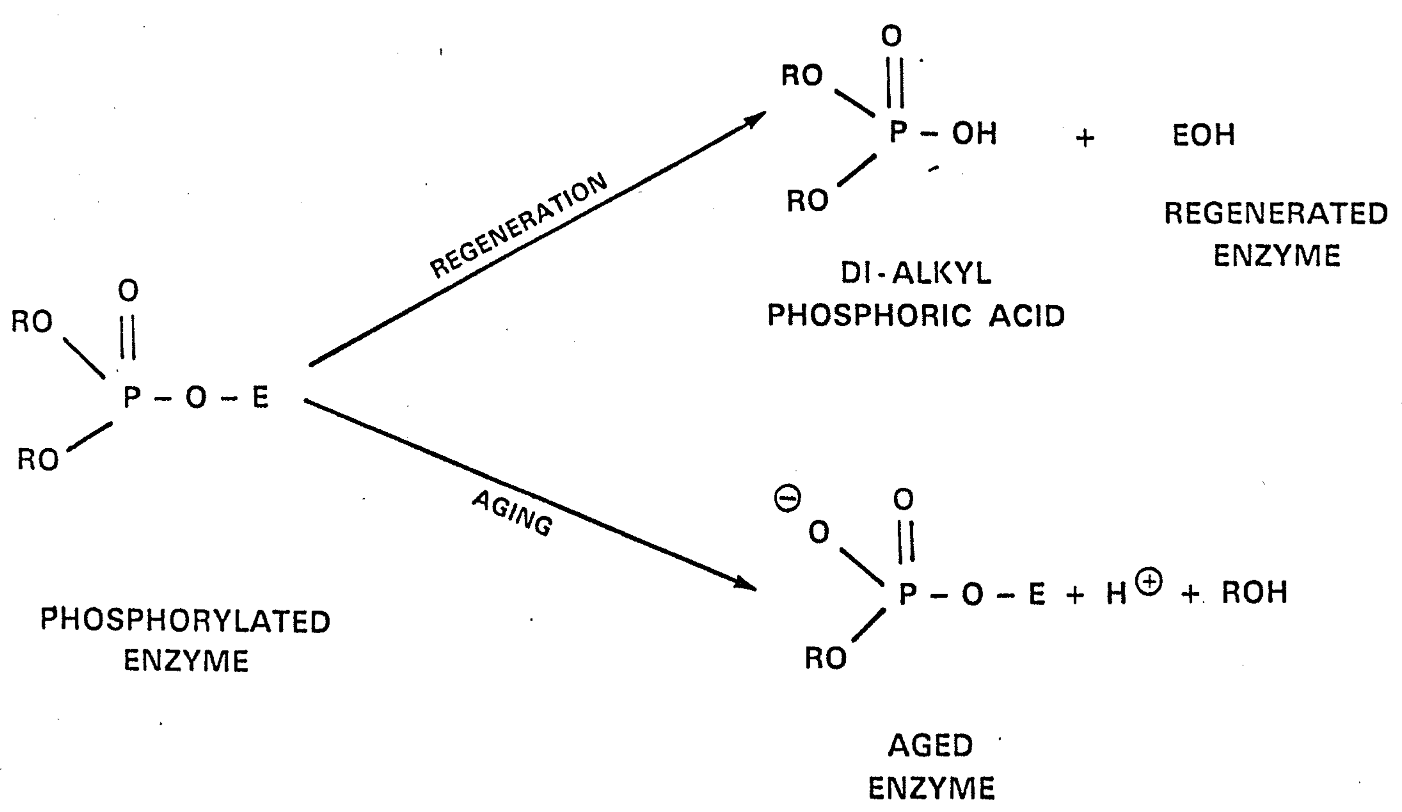

Figure 9

Dealkylation or Aging of Organophosphorus Compounds

Beauregard _et al_ showed that aging of DFP-AChE (bovine erythrocyte) was due to dealkylation (loss of 2-propanol) rather than the β-elimination product diisopropyl-phosphoric acid (16). Modification of the AChE active site histidine with 2 nM diethyl-pyrocarbonate resulted in a slowing of the aging process by 2-3 times. Since diethylpyrocarbonate also inhibits AChE activity, it appears that the active site of histidine in AChE is involved in aging.

Coult _et al_ have shown that the time required for 50% aging of DFP-enzyme complex in bovine erythrocyte AChE was 2140 minutes (47).

Lanks and Seleznic have observed that the aging of DFP-enzyme was dependent upon temperature (122). The percentage of reactivated enzyme was 69, 29 and 3% at 0, 25 and 37 degrees respectively.

The half-life (T1/2) of conversion to the aged form varies from a minute or less to several days. For example, the aging half-life for diisopropylphosphoryl AChE of human erythrocytes is 4.6 hours whereas the diethylphosphoryl aging half-life is 41 hours. The rate of aging is known to increase as pH decreases and temperature increases. In addition, the rate of aging is known to be dependent on the phosphoryl group but not the leaving group (23,47,75-77,95).

d. Binding of Organophosphorus Compounds to Other Enzymes

Binding of organophosphorus compounds to enzymes has been, and still is, of considerable interest from both a pharmacological and toxicological viewpoint. The most cited organophosphate inhibited enzymes are the class known as serine esterases i.e. enzymes catalyzing ester hydrolysis and containing an active serine hydroxyl group (89,99,107). Serine esterases include ChE, chymotrypsin, trypsin, thrombin, urokinase and alkaline phosphatase. Organophosphorus compounds however can bind to and inactivate a variety of other enzymes and proteins. This binding apparently involves phosphorylation at hydroxyl groups similar to serine esterase inactivation or alkylation of hydroxyl or sulfhydryl groups which do not have to reside at the catalytic site. Organophosphate-protein complexing at non-catalytic sites can inhibit enzyme activity by allosteric interaction.

e. Kinetic Parameters used in defining the Interaction of Organophosphorus Compounds with Enzymes

The interaction of organophosphorus compounds with enzymes and the resulting inhibition of the enzyme can best be defined in kinetic parameters by use of the bimolecular (second order) inhibition rate constant (Ki) and the IC50 value which is the concentration of an organophosphorus compound required to inhibit enzyme activity by 50%. These 2 parameters are most frequently reported in the literature and allow for direct comparison of studies involving organophosphate-enzyme interactions. The bimolecular rate constant is based on the presumed 2 stage interaction of organophosphorus compounds with enzymes which consists of an initial binding, defined by its association constant (Ka) and then a phosphorylation, defined by its phosphorylation constant (Kp)(3,52,133,134). In general, it is found that as Ka increases so does the affinity of the organophosphate for the enzyme; as Kp increases so does the enzyme phosphorylation rate and that the higher Ki is, the stronger the inhibition of the enzyme by the organophosphorus compound. Factors such as source and type of enzyme, pH and temperature are known to affect the rate constants. Utilizing DFP as the representative organophosphorus compound, the Ka for AChE isolated from rat

brain synaptosomes is 5.6 x 10^{2} M whereas for BuChE isolated from horse serum it is 2.0 x 10^{6} M (133,134,201,202). The Ki's for the same 2 examples are 1.54 x 10^{4} and 4.0 x 10^{6} M^{-1} min^{-1} respectively (133,134,201,202). The Kp's for AChE isolated from rat and monkey brain synaptosomes have been determined to be 8.7 and 9.0 $minutes^{-1}$ respectively whereas for BuChE isolated from human serum it is 30 $minutes^{-1}$ (133,134,201,202). It has been suggested that when all 3 parameters are reported, binding and phosphorylation are most important because they directly affect the formation of phosphorylated enzyme. The phosphorylation, dissociation and bimolecular rate constants of some organophosphate and carbamate inhibitors of cholinesterases from various other sources are illustrated in Table 3.

The IC50 values, like the rate constants, are found to be dependent on the source, quantity and type of enzyme involved. For instance, the DFP IC50 value for AChE obtained from brain synaptosomal membranes varies from 0.64 uM in monkey to 2.0 uM in rats to 5.3 uM in guinea pigs (202). On the other hand, the DFP IC50 value for BuChE obtained from plasma varies from 0.009 uM in humans to 0.066 uM in male rats to 0.075 uM in guinea pigs (57). Utilizing IC50 values, the most potent inhibitors of the organophosphorus class of irreversible AChE inhibitors are soman (pinacolyl methylphosphonofluoridate, GD), sarin (isopropyl methylphosphonofluoridate, GB) followed by paraoxon and DFP.

f. Detoxification Mechanisms for Organophosphorus Compounds

The primary detoxification mechanism for organophosphorus compounds is hydrolysis which may be enzymatic or non-enzymatic (6,89,99,101,107,167). The general types of biological reactions that are most prominent in the detoxification of organophosphorus compounds involve the mixed function oxidases (MFO); transferases and hydrolases. In certain cases, one or more of these reactions may be involved at the same time on the organophosphate molecule. Hydrolase enzymes include arylesterases, DFP'ase and carboxylesterases. Arylesterases is an A-esterase and preferentially reacts with organophosphates that contain phenolic esters i.e. paraoxonase is an arylesterase which hydrolyses paraoxon to diethylphosphoric acid. Phosphorylphosphatases such as DFP'ase degrades primarily organofluorine compounds to their respective acid i.e. DFP to diisopropylphosphoric acid. Carboxylesterases (or aliesterases) hydrolyze only esters of lower fatty acids like tributyrin but not the higher acids like palmitic or oleic acids. Enzymatic hydrolysis appears to follow first order kinetics under physiological conditions. Athough a number of sources of organophosphate degrading enzymes have been reported, information regarding rates of hydrolysis are in most cases not available.

The principal non-enzymatic process for the detoxification of organophosphorus compounds is hydrolysis. Non-enzymatic hydrolysis has been shown to be influenced by temperature, pH and other ions present in the water (6). In general, DFP is most stable at pH 6-7.5 and as expected, lower temperature increases stability. It can also be seen that phosphate ions accelerate DFP hydrolysis. The oximes 2-PAM, DAM, TMB-4 and toxogonin were found to accelerate hydrolysis by lowering the half-life of DFP by 78, 64, 82 and 87% respectively (167).

g. Consequences of Organophosphate Intoxication

In vivo, the consequences of inhibition of AChE by organophosphorus compounds is an intense and continuous cholinergic stimulation producing generalized peripheral and central cholinergic responses; the degree and appearance of which are dependent on the degree of AChE inhibition and the route of exposure (Table 4) (12). The most pronounced systemic effects are profuse salivation, sweating, miosis, diarrhea, muscular weakness and fasiculations, mental confusion and ataxia, and disturbances of ventilation culminating in respiratory paralysis and death due to anoxia.

Table 4

Signs and Symptoms of Organophosphate Poisoning
Modified from Namba, T., Nolte, C.T., Jackrel, J. and Grob, D. (158)

MUSCARINIC MANIFESTATIONS	NICOTINIC MANIFESTATIONS	CENTRAL NERVOUS SYSTEM MANIFESTATIONS
Bronchoconstriction	Muscular fasciculation	Restlessness
Increased bronchial secretions	Tachycardia	Insomnia
Sweating	Hypertension	Tremors
Salivation		Confusion
Lacrimation		Ataxia
Bradycardia		Convulsions
Hypotension		Respiratory depression
Miosis		Cirulatory collpase
Blurring of vision		
Urinary incontinence		

There are at least four contributing causes that lead to respiratory failure:

1. excessive secretion from salivary and bronchial glands which leads to obstruction of the airway;
2. bronchoconstriction and laryngospasm;
3. neuromuscular blockade of respiratory muscles, and;
4. central respiratory failure.

If the outcome of organophosphate intoxication is not immediately fatal, complete recovery may take several days, weeks or months. For the most part the rate of recovery is then dependent on the rate of synthesis of new AChE.

Although recovery is usually complete, there are long term consequences to surviving an organophosphate exposure. Delayed neurotoxic effects may also occur which could take three or more months to manifest themselves (72). Symptoms of delayed neurotoxicity may include polyneuritis (inflammation of the nerves) with flaccid paralysis of the upper and lower extremities and degeneration of myelin sheaths and neurons in the spinal cord, sciatic nerve and medulla. The mechanism of delayed neurotoxicity due to organophosphate intoxication is not well understood.

2. Carbamates

Although not directly related to the content of this thesis, it is noteworthy to mention the carbamate class of cholinesterase inhibitors.

Most carbamate compounds belong to the reversible class of cholinesterase inhibitors. Carbamates react with AChE in essentially the same manner as ACh to form a carbamylated-enzyme intermediate instead of an acetylated-enzyme intermediate (89,99,107,161). Unlike the acetylated-enzyme, however, the carbamylated enzyme is relatively more stable and free AChE is only slowly regenerated by hydrolysis. This slow regeneration of AChE from the carbamylated intermediate makes the carbamate class of compounds potentially useful as prophylactic agents against organophosphate poisonings.

Because of their reversible interaction with acetylcholinesterase, the carbamate class of cholinesterase inhibitors have been investigated as potential prophylaxes against organophosphate intoxication for they are known to delay the hydrolysis of ACh from 1 to 8 hours (24,25).

C. Therapies Utilized in Organophosphate Intoxication

Since the toxic effects of organophosphates were first realized, there have been major attempts to find therapies that would alleviate the symptoms that result from the excessive accumulation of ACh due to AChE inhibition. The 2 major areas of investigation in this regard have been to:

1. block the active cholinergic receptor sites; and/or;
2. reactivate the phosphorylated enzyme.

Although many compounds have been investigated with respect to their blocking and reactivating capabilities, the 2 compounds of interest to this work are the antimuscarinic, atropine, and the oxime, 2-PAM chloride (Figure 10) because they are the current antidotes of choice in the case of accidental or intentional organophosphate poisoning and because they are included in some Armed Forces chemical defence kits (1,51,74,156,158).

PRALIDOXIME CHLORIDE
(2-PAM, 2-PYRIDINEALDOXIME METHOCHLORIDE)

ATROPINE

Figure 10

Structure of Principal Antidotes Against Organophosphate Intoxication

1. Atropine

Atropine (dl-hyoscyamine) is a naturally occurring alkaloid which is found in a number of Solanaceous plants including _Atropa belladona_ (deadly nightshade) and _Datura stramonium_ (Jimson weed, stinkweed)(71). It is an ester of the complex organic base tropine (tropanol) formed in combination with tropic acid. Atropine is a racemic mixture of equal parts d- and l-hyoscyamine. Although the l-isomer is more potent both centrally and peripherally than is the d-form, the racemic mixture is preferred because it is more stable chemically.

a. Absorption, Distribution and Elimination of Atropine

Atropine is well absorbed by all routes of administration (55,105). Once in the system, atropine disappears rapidly from the blood and is distributed throughout the entire body. Conjugation and enzymatic hydrolysis take place in the liver (73,105). About 85-90% is excreted in urine within 24 hours, 50% excreted unchanged and the rest as tropic acid esters or tropine (71,105).

b. Role of Atropine in Acetylcholinesterase Inhibition

Atropine is a competitive or surmountable antagonist of ACh at muscarinic receptor sites in smooth and cardiac muscle, exocrine glands and the central nervous system (CNS) (25,71). The antagonism can be overcome by increasing the concentration of ACh at the receptor sites, for example, by an anticholinesterase (antiChE). Atropine produces a highly selective antagonism of muscarinic receptors which is greater against the effects of injected cholinergic drugs than for stimulation of cholinergic postganglionic nerves. The difference is most likely due to the release of the chemical mediator in an area in which it has greater proximity and is therefore present in higher concentration than is capable by exogenous administration.

c. Direct Pharmacological Actions of Atropine

Atropine has widespread actions once it has been introduced to a system (25,54,71,106,108,179). It acts at the receptors innervated by cholinergic compounds to antagonize the effects of ACh. In the cardiovascular (CV) system, atropine produces complex effects. Soon after its administration, atropine is known to produce a transient bradycardia which is due to the stimulation of the medullary cardioinhibitory center. The main effect of atropine however

is to produce a tachycardia from blockade of the normal action of the vagus nerve at the postganglionic neuroeffector junction. Atropine can reverse all the cardiac effects of ACh and vagal stimulation i.e. it lengthens the refractory period, shortens the P-R interval, increases cardiac output and oxygen consumption. The effect on cardiac output is generally slight because of compensatory circulatory mechanisms. Atropine has only slight vasoconstricting properties in vascular beds which is not unexpected for parasympathetic innervation and the cholinergic sympathetic vasodilator fibers to vessels supplying skeletal muscle do not appear to be involved in normal regulation of tone.

In the gastrointestinal tract, atropine and related drugs antagonize the increase in smooth muscle tone and activity that follows activation of cholinergic receptors. Generally, they decrease tone and the amplitude and frequency of peristalsis of all segments of the intestinal tract. This inhibition depends on the level of existing cholinergic (vagal) nerve activity since these actions are predominately under parasympathetic control.

Atropine when administered systemically or locally to the eye produces dilation of the pupil (mydriasis), paralysis of accomodation of the lens (cycloplegia) and increased intraocular pressure.

Following administration of atropine, bronchiol

and tracheal smooth muscles relax particularly when parasympathetic tone is high or muscle tone has been increased by muscarinic agents. The bronchodilation produced by atropine results in a larger vital capacity and decreased bronchial resistance. Atropinic drugs also inhibit secretions from the bronchial glands.

In glands receiving cholinergic innervation, primarily sweat and salivary, atropine reduces or abolishes secretions. There is a rise in body temperature seen when large doses of atropine are administered to animals that sweat. Suppression of sweating is doubtless a consideration factor in this regard, especially when environmental temperature is high. Other mechanisms may be important however when large doses are administered. It has been suggested that atropine may exert a central effect on temperature regulation, however, animals that do not sweat, such as dog, do not exhibit increased body temperature after large doses of atropine (71).

Atropine is known to stimulate medulla at higher cerebral centers resulting in a mild increased vagal output. The rate, and occassionally the depth of breathing are increased, but this is probably the result of bronchiolar dilatation. When respiration is seriously depressed, atropine cannot be relied on as a stimulant. In larger or repeated doses of atropine, further depression of respiration may

occur. With toxic doses, central excitation becomes more pronounced leading to restlessness, irritability, disorientation, hallucinations and/or delirium. With still larger doses, stimulation is followed by depression and medullary paralysis causing death. Doses of atropine required to inhibit peripheral responses to choline esters or antiChE agents have themselves almost no detectable central effects. However, atropine directly depresses activation of various types of neurons in the cat spinal cord by acetylcholine and by noncholinergic stimuli, indicating that the drug has central actions other than blocking cholinergic synapses.

2. Oximes

The primary intent in the therapy of intoxication by organophosphorus compounds, past emergency life support, is to reactivate phosphorylated enzymes as rapidly as possible. Early studies on organophosphate-enzyme complexes have shown that nucleophilic compounds such as hydroxylamine (H_2NOH), hydroxamic acids (RCONHOH) and oximes (RCH=NOH) reactivate organophosphate poisoned enzymes with varying degrees of success (22,56,58,89,99,107). The design and synthesis of 2-PAM provided a model for reactivation drugs. Although other mono- and bis-pyridinium oximes are currently under investigation as reactivators, this work will concentrate on 2-PAM for it is widely used as a therapy in combination with atropine in cases of accidental or intentional organophosphate poisoning.

a. Absorption and Distribution of 2-PAM

The reported extent of absorption of an orally administered dose of 2-PAM is low (136,183). Only 27% of 2-PAM was recovered in the urine after oral administration while 5-35% was found in the feces (102). It is possible that 2-PAM is decomposed by intestinal bacteria. Large variations in blood levels of individuals receiving the same dose were also observed. Discrepancies with the amount of unchanged 2-PAM found in the urine are also reported from 20-25% to 31.9%

(102). Large differences between effective oral and i.v. doses have been attributed to incomplete absorption for orally administered quaternary ammonium compounds. It has also been shown that 2-PAM is not transferred by an active transport system and that its diffusion through the intestinal wall is also independent of the water transport which eliminates the solvent-drug effect characteristic of small neutral molecules (48,65). The extent of absorption did not correlate with the chloroform-water partition ratio. The movement of 2-PAM across the intestinal wall was found to be dependent on its concentration and the transmural potential difference. It was assumed that 2-PAM crossed the mucosal membrane by diffusion through the aqueous pores (102).

Duke *et al* found that a greater level of TMB-4 was maintained in the blood of anesthesized rabbits compared to 2-PAM or P2S over a 30 minute period following i.v. or intramuscular (i.m.) administration of equal doses of oxime (55). Half peak values were reached in approximately 1 1/2 to 2 minutes with the peak values for all three oximes administered i.m. occurring at 9-10 minutes. It has been suggested that the longer retention in the blood is a factor as to why TMB-4 is a better antagonist to some antiChE agents than either 2-PAM or P2S. Askew, using dogs and rabbits, and Rutland, using rats, showed that DAM (diacetyl monoxime) reaches maximal concentrations in blood within 10

minutes after i.p. injection (8,175). Lemanowicz *et al* found that 2-PAM was absorbed negligibly from canine stomach or duodenum but that absorption from the small intestine was substantial (123). Less that 20% of drug placed in closed jejunal loops remained after 2 hours. They concluded that the reason for poor plasma levels of 2-PAM after oral administration was not poor absorption but rather slow absorption outpaced by the rapid elimination.

Distribution studies on C14-labeled 2-PAM administered intraperitoneally to mice have shown that after 1 hour 64% of the labeled compound was recovered as follows: 20% of the activity was contained by the liver, duodenum, jejunum, ileum, kidney, caecum and blood while the urine contained the remaining 44% (180). It has also been found that 10 minutes after the completion of i.v. infusion of PAM to rabbits the highest concentration was found in the kidney while the least amount was found in the brain (102). It has been suggested as well that no 2-PAM enters the erythrocytes in either man or dog subjects (58). However, previously it has also been shown that 2-PAM does enter the red blood cells (RBC's) to some extent (59). The whole blood concentration of 2-PAM was found to be between 45-50% of the plasma concentration in human volunteers.

One of the most urgent concerns with AChE reactivation is drug penetration across the blood brain barrier

(BBB) into the brain substance. Oximes like 2-PAM possess a quaternary nitrogen in the pyridinium ring that aids in attaching the molecule to the receptor's anionic site. By the same token being ionized at physiologic pH hampers the drug's passage across the blood brain barrier (2-PAM with a pKa of 8.0 is about 80% ionized at pH 7.4).

Whether or not oximes enter the central nervous system has created much controversy. Much of the controversy of whether or not 2-PAM penetrates the blood brain barrier arises from the poor correlation between reactivating capabilities of 2-PAM centrally and the antidotal value of the oximes (33,41,81,89,132,143,173-175,195). Partial reactivation of AChE in cerebellum and paleocortex has been confirmed by some authors whereas extreme measures such as direct injection of 2-PAM into the carotid artery have demonstrated incomplete restoration of cerebral AChE activity (89,99,107,157, 173-175,195). More recent studies have demonstrated that a bis-oxime such as toxogonin, which is found to have a pKa of 7.8, is only 65% ionized at pH 7.4 and thereby provides for a 92% reactivation of brain AChE versus 49% for 2-PAM under identical conditions (186). It has been suggested that when dissociated in an aqueous media, the non-ionic moiety of toxogonin can assume a nonpolar quinoid configuration that allows transmembrane passage into the central nervous system (186).

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Recently, the use of Pro-PAM, a 1,6-dihydropyridine precursor of 2-PAM which is oxidized to 2-PAM after tissue entry, although exhibiting better central reactivating properties on AChE than 2-PAM, has been shown to have only marginal therapeutic benefits. In fact, Bodor et al and Kenely et al have suggested that the systemic toxicity of these tertiary Pro-oximes outweigh their marginal therapeutic benefits (27-31,110). Clement, although concurring with their marginal therapeutic benefits, found the toxicity of Pro-PAM to be lower than that of 2-PAM in mice (41).

Nonetheless, a review of the literature with respect to 2-PAM by Ellin et al concluded that 2-PAM has some ability to cross the blood brain barrier despite its quaternary character and to induce effects dependent upon the presence of oxime in the brain (58,60). Their conclusions were based largely on direct assays like C14 distribution studies and direct measurement of 2-PAM in brain substances rather than on indirect assays such as central reactivation of poisoned AChE. The mechanism(s) by which 2-PAM enters the central nervous system has not yet been determined (58).

b. Elimination of 2-PAM

Because 2-PAM is a highly polar and water soluble drug it is rapidly eliminated from the body. The primary route of elimination is by tubular secretion which results in a rapid renal excretion, the mechanism of which is not well understood. Enander et al have found that 80-90% of injected 2-PAM is unchanged in the urine of rats within 24 hours (61). Besides the unchanged 2-PAM, numerous other metabolites could be identified in urine.

The main metabolic pathways for 2-PAM are illustrated in Figure 11. When 2-PAM is aerobically incubated with rat liver homogenate it is found to be completely metabolized. Due to its high polarity and the fact that it is excreted largely in its unchanged form indicates that when given i.v. it does not reach its possible sites of metabolism in the liver. Although 2-PAM is slightly more extensively metabolized when given orally to rats, in which case it must pass through the portal system to the liver, it is still mainly eliminated unchanged by the kidney. This suggests that in vitro, 2-PAM can be metabolized by rat liver homogenate. In vivo, however, metabolism by the liver is not a major consideration for it is not a major route of elimination.

Figure 11

Metabolic Transformations of PAM

I: N-methylpyridinium-2-aldoxime. II: N-methylpyridinium-2-nitrile. III: N-methylpyridinium-2-carboxyamide. IV: N-methylpyridinium-2-aldehyde. V: N-methyl-2-pyridone. VI: N-methylpyridinium-2-carboxylic acid. Reproduced from Enander *et al.* (61).

c. Direct Pharmacological Effects of 2-PAM

There are indications that oximes have pharmacological actions which are independent of their reactivating effect on inhibited AChE.

i. Neuromuscular Function

Oximes are known to antagonize the effect of ACh on mammalian and amphibian skeletal and smooth muscle (128). Following exposure to AChE inhibitors, the single stimulation of a motor nerve results if repetitive release of ACh and repetitive discharge of the muscle is continued. It was shown that soman-induced repetitive depolarization of the muscle could be blocked by 2-pyridine aldoxime methyl trichloroacetate without reactivation of the enzyme. The oxime may produce its effect by an action on the motor nerve terminal resulting in direct blockage of the repetitive discharge.

Fleisher et al found that 2-PAM chloride was superior to Toxogonin in reactivation of guinea pig red blood cell AChE which was inhibited by sarin but that Toxogonin was a superior antidote to GB poisoning than 2-PAM chloride (67). An antiAChE action on isolated organs and neuromuscular structures has been postulated for TMB-4, 2-PAM and other oximes (67,92,93,98).

ii. Gastrointestinal Tract

Kuhnen-Clausen demonstrated that 2-PAM, 4-PAM, TMB-4 and Toxogonin antagonized the contractions in isolated guinea pig ileum by acetyl-β-methylcholine chloride (Mecholyl) and furthethonium iodide (119). This indicates that there is a direct parasympathetic action of these oximes.

iii. Cardiovascular and Respiratory Effects

In anesthesized dogs, 2-PAM (i.v.; 10 mg/kg) caused a biphasic response in arterial blood pressure; the first spike being attributed to ganglionic stimulation accompanied by bradycardia, the later sustained pressure plateau to the release of catecholamines (187,209). In those dogs, 80 mg/kg of 2-PAM caused muscle twitching, while a slow infusion of 20 mg/kg caused death by respiratory paralysis. However, evidence to the contrary exists. It has been shown that 2-PAM (i.v.; 5-40 mg/kg) may have a direct effect on the action of the heart at vascular smooth muscle of the dogs (9,34,35).

iv. Ocular Effects

The ocular side effects of 2-PAM such as blurred and double vision, difficulty in near accomodation are well known but have received little research effort (82,88). The possibility of a local effect on intraocular dynamics has been put forth as PAM mesylate (P2S) has been found to enter

the aqueous humor in measurable amounts. However, the oxime does not accumulate in aqueous humor and it appears to be disposed of as rapidly as it enters the eye (15). Since ocular side effects in man are known to become intense and more frequent with repeated dosing, it has been suggested that this possibility represents a threshold curare-like paralysis of the extraocular muscles brought about by cholinomimetic oxime effects on the neuromuscular junction (176). Since disturbances of the visual system are among the earliest manifestations of exposure to organophosphorus compounds (miosis, diplopia which is the perception of 2 images) and since the side effects of the oxime antidote are similar, it has been suggested that further work be carried out in order to be able to discriminate between the two.

v. Central Nervous System Effects

The central effects of oximes as well as organophosphate AChE inhibtors are poorly defined because of the enormous urgency in treating the acute respiratory and cardiovascular cholinergic crisis (36,89,99,107).

Organophosphorus compounds are known to cause a decrease in body temperature (40,46,110,116,117, 127,149,151-153). Some quaternary oximes, which are believed not to cross the blood brain barrier, have been found to reduce the hypothermia induced by organophosphorus compounds

(149,151-153). This phenomenon has been used as an indication that oximes have penetrated the blood brain barrier and induced central action.

d. Toxicity of 2-PAM

Acute toxic doses for some of the various salt forms of 2-PAM in some species are illustrated in Table 5 (7).

Chronic toxicity studies of 2-PAM iodide in dogs and rabbits demonstrated that they could safely tolerate repeated daily doses of 30 mg/kg administered i.v. for as long as 6-8 weeks (49).

e. Role of oximes in Organophosphate Intoxication

The primary role of oximes is the reactivation of phosphorylated enzyme. Although advances have been made, no single oxime has been found that is an effective reactivator against all organophosphate cholinesterase inhibitors. Considering toxicity and solubility factors in the design of new oximes has resulted in 2 major classes of oxime with regard to their reactivating capabilities:

1. those based on a pyridine structure substituted in the 2-position similar to 2-PAM, and;
2. those bis-pyridinium bridge compounds such as HI-6, TMB-4, Toxogonin.

Table 5

Toxicity of 2-PAM Salt Forms in Various Species

OXIME	ROUTE OF ADMINISTRATION	SPECIES	LD_{50} (mg/kg)	REFERENCE
2-PAM I	i.v.	Mouse	117	107
			140 – 178	59
			145	160
2-PAM Cl	i.v.	Mouse	115	59, 160
			90	107
		Rats	96	140
		Rabbits	95	48
2-PAM I	i.p.	Mouse	240	107
			190	55
			136 – 260	59
		Rats	305	59
2-PAM Cl	i.p.	Mouse	205	59
			155	107
2-PAM I	i.m.	Mouse	240	107
2-PAM Cl	i.m.	Mouse	180	107
		Rats	150	65
		Guinea Pigs	168	65

Initially, 2-PAM was designed as the site-directed nucleophile to be utilized in the reactivation of organophosphorus inhibited AChE. Wilson et al concluded that the esteratic site of AChE was occupied by the organophosphorus moiety and that the anionic site was still functional (206,207). They postulated compounds like 2-PAM or 4-PAM have a nucleophilic oxygen moiety which should fall one bond length away from the phosphorus atom. It was suggested that this orientation would result in reactivation of the inhibited, unaged enzyme and that 3-PAM which does not have this orientation would not reactivate the enzyme. These postulates were supported by their experimental results.

In the reactivation of phosphorylated enzyme, the quaternary nitrogen of the pyridine ring interacts with the anionic site of AChE. In doing so, the nucleophilic CH=NOH group is in close proximity to the phosphorus atom which is attracted to the serine residue at the esteratic site. 2-PAM interacts with the electrophilic phosphorus atom leading to the formation of an oxime-phosphonate which no longer fits the enzyme template and thus is released leaving unoccupied, reactivated functional AChE (Figure 12).

A number of reviews have addressed oxime structure and reactivation ability (22,58,60,69,74,89,90,100,107). In its capacity as a reactivator of phosphorylated AChE, 2-PAM has been shown to be most effective in the peripheral nervous system (36).

O
P
H_7C_3O OC_3H_7
N O
DFP PHOSPHORYLATED ENZYME

CH = NOH
N
CH_3
PRALIDOXIME

CH = N – OH
H_3C N
O
P
H_7C_3O OC_3H_7
N O

STEP 1

APPROACH OF PRALIDOXIME TO DFP-INHIBITED AChE

H
C = N — O — P
H_3C N
$O^{\ominus}$
H_7C_3O OC_3H_7
N – H O

STEP 2

FORMATION OF STABLE OXIME-PHOSPHONATE

OXIME-PHOSPHONATE

CH = N — O — P
N
CH_3
O
H_7C_3O OC_3H_7
N H – O

STEP 3

RELEASE OF OXIME-PHOSPHONATE REGENERATION OF AChE

REGENERATED ENZYME

Figure 12

Reactivation of DFP-Inhibited AChE by 2-PAM

There is still however, a great deal of controversy with regard to its effectiveness as a reactivator of central nervous system phosphorylated AChE. Experimentally, some investigators have shown there to be only minimal reactivation of central AChE (30,41,109). Although oxime penetration into the brain may be low and reactivation of cerebral AChE slight, other investigators have demonstrated improved cerebral function following aggressive treatment of organophosphate poisonings by 2-PAM. In a recent case of accidental organophosphate poisoning monitored by continual EEG, increased brain wave activity and arousal occurred after just 5 mg/kg of continuous infusion of 2-PAM (130). Coma is known to occur at a near lethal degree of brain AChE inactivation and any reactivation of brain AChE activity, however small, has been suggested to be sufficient to restore immediate cerebral function. Oximes, such as 2-PAM, are however not effective on aged enzymes.

Because of the voluminous information regarding the effectiveness of oximes against organophosphate poisoning the reader is referred to the literature (8,21,22,32,33,41-45,50,53,56,58,60,62,66,71,74,75,79,87,89,92,93,96,98,99,104,107,113,115,120,124-126,128,130,131,140,143,156-158,162,163,174,184,192,194,195,205-207). In general, it seems that effectiveness of oxime(s) is dependent upon a number of factors including:

1. the nature of the oxime;
2. the particular organophosphorus compound involved;
3. whether or not adjunct therapy is available;
4. whether or not alternate life support were available; and;
5. other physical, chemical and environmental factors.

Suffice it to say that the hydroxamic acids and oximes have been shown to reverse such actions of AChE inhibitors as effects on smooth, skeletal and cardiac muscle and reduce mortality.

3. Non-Oxime Regimens Against Organophosphate Poisoning

Other drugs besides oximes have been evaluated for their prophylactic and therapeutic value in organophosphate poisoning. Most notable of the other compounds under investigation are the carbamate class of antiChE inhibitors and the anticonvulsants. Since this topic is beyond the scope of this work, the reader is referred to the literature regarding non-oxime regimens against organophosphate poisoning (24,71,76,89,107).

D. Aim of the Study

An evaluation of the literature has demonstrated that the question as to whether or not oximes have a significant central action in the therapy of organophosphate intoxication is still debatable. It is not clear to what extent, if any, or by what mechanism(s) oximes penetrate into the central nervous system and whether their occurrence in the central nervous system has any significant contribution to the therapeutic efficacy. As well, there is little clarification as to what extent atropine is involved either in the penetration of oxime(s) into the central nervous system or to its reactivating capabilities once in the central nervous system.

The aim of this study therefore is to contribute to the clarification of this controversy by investigating the biodisposition and evaluation of the effects of 2-PAM alone and administered in a combined solution with atropine prophylactically and therapeutically against the organophosphate cholinesterase inhibitor DFP in mice.

1. Selection of Materials

2-PAM was selected as the oxime and atropine as the cholinolytic because they are the current antidotes of choice in cases of accidental or intentional organophosphate poisoning and because they are included in some Armed Forces chemical defence kits (1,51,74,156,158).

DFP was chosen as the challenge organophosphate because of its slow spontaneous reactivation (i.e. irreversible inhibition) and good reactivation characteristics when 2-PAM is utilized as the oxime. DFP was also the organophosphate available experimentally for which comparisons to the more potent and not readily available nerve agents have been made.

2. Study Design

The experiments in this thesis were designed to investigate the possibility that 2-PAM, administered alone or in combination with atropine, either prophylactically or therapeutically against DFP poisoning, penetrates the central nervous system and exerts centrally reactivating effects on AChE as well as to see if there was any synergism or antagonism between atropine and 2-PAM. This was undertaken using 5 experimental approaches.

a. Acute Studies

i. Routes of Administration/Half-life

This study involved the determination of the serum elimination half-life and serum concentrations for 2-PAM alone and in the presence of atropine by different routes of admininstration as well as for:

1. 2-PAM alone;
2. 2-PAM plus atropine alone;
3. 2-PAM given prophylactically and therapeutically against DFP, and;
4. 2-PAM plus atropine given prophylactically and therapeutically against DFP.

This work was carried out to see if atropine or DFP had any effect on circulating levels of 2-PAM or its serum elimination half-life.

ii. Increasing Dose Study

The aim of this work was to explore the possibility that 2-PAM could be detected in increasing amounts in the brain following administration of increasing doses of 2-PAM. In addition, the experiment was designed to see if challenge with DFP had any effect on the amount of 2-PAM present in the brain and/or the reactivation of central AChE by 2-PAM following administration of 2-PAM prophylactically or therapeutically.

III. Toxicology

LD50 experiments were designed to illustrate what effect atropine had on the toxicity of 2-PAM and/or its usefulness in protection against DFP intoxication. It was also suggested that some comment(s) regarding the central occurrences of 2-PAM and toxicity could be made from these experiments.

IV. Hypothermia

Core body temperature measurements were designed to provide an alternative to HPLC and AChE measurements in determining the central reactivation and central penetration of 2-PAM administered with or without atropine, prophylactically or therapeutically against DFP.

It was suggested that the results from the proposed experiments would allow conclusions to be drawn on the possible central presence of oxime and a mechanism by which it is/is not transported there as well as to its possible central efficacy. In addition, conclusions regarding the relevance of synergism or antagonism of the antidotes in the prophylaxis and therapy against DFP intoxication could be made.

b. Chronic Study

This study was designed to see if chronic administration of DFP increased the amount of 2-PAM in the brain and whether or not 2-PAM had any reactivating properties on AChE.

II. Experimental

A. Materials

1. Chemicals

Atropine sulphate; Triton X-100; scintillation grade 1,4,-dioxane and toluene were purchased from BDH Chemicals. The sodium salt of 1-octanesulfonic acid and 2-PAM methochloride were purchased from Aldrich Chemical Co. Inc.. DFP was purchased from Sigma Chemical Co.. Scintanalyzed bis-MSB; HPLC grade sodium acetate and acetonitrile; 70% perchloric acid and tetramethylammonium chloride were purchased from Fisher. Scintillation grade PPO (2,5-diphenyl oxazole) was purchased from the Amersham Corporation. Pyridostigmine was a gift from the F. Hoffman-LaRoche and Co. Ltd.. All other chemicals were reagent grade quality.

2. Animals

Male mice (CD-1 ; 20-30 g) were obtained from Charles River Canada Ltd., St. Constant, Quebec. The animals were acclimatized in the animal facility at the Faculty of Pharmacy, University of Manitoba for at least one week following their arrival there. The animals were maintained on a 12 hour light/dark cycle starting with lights on at 0700 hours at a temperature of 21 °C for the duration of their stay. Access to food and water before and after drug administration was available _ad libitum_ to all animals utilized in all the studies.

B. Methods

1. Analytical

a. 2-PAM Quantitation by High Pressure Liquid Chromatography

Serum and brain concentrations of 2-PAM were determined using a modified procedure of Benschop *et al* and Ligtenstein (17,124-126). In 6 X 50 mm glass tubes, 100 ul sample (serum or 20% brain homogenate), 5 ul of 20 mg/ml pyridostigmine (internal standard), 45 ul acetonitrile and 5 ul 70% perchloric acid were added in the order specified. Samples were mixed on a vortex mixer and then spun down in a Silencer Model H-103N series centrifuge at 3500 rpm for 15 minutes. The resultant supernatants were transferred by Pasteur pippette to 250 ul capacity polyethylene microcentrifuge tubes and an aliquot injected directly into the HPLC. The HPLC system (Waters Associates, Milford, Mass.) was comprised of a WISP 710B automatic injector, a Model 510 solvent delivery system, a Model 480 variable wavelength UV/Vis spectrophotometric detector set at 295 nM and a Model 730 data module. For all analyses, a Waters 30 X 0.4 cm C18 uBondapak steel column was used.

The mobile phase utilized was 22% acetonitrile in a 78% 0.1 M sodium acetate buffer pH 2.6 containing 0.01 M 1-octanesulfonic acid to form a paired ion and 0.0025 M tetramethylammonium chloride to reduce adsorption. The flow rate was 0.8 ml/min which eluted both 2-PAM and pyridostigmine within 15 minutes (Figure 13).

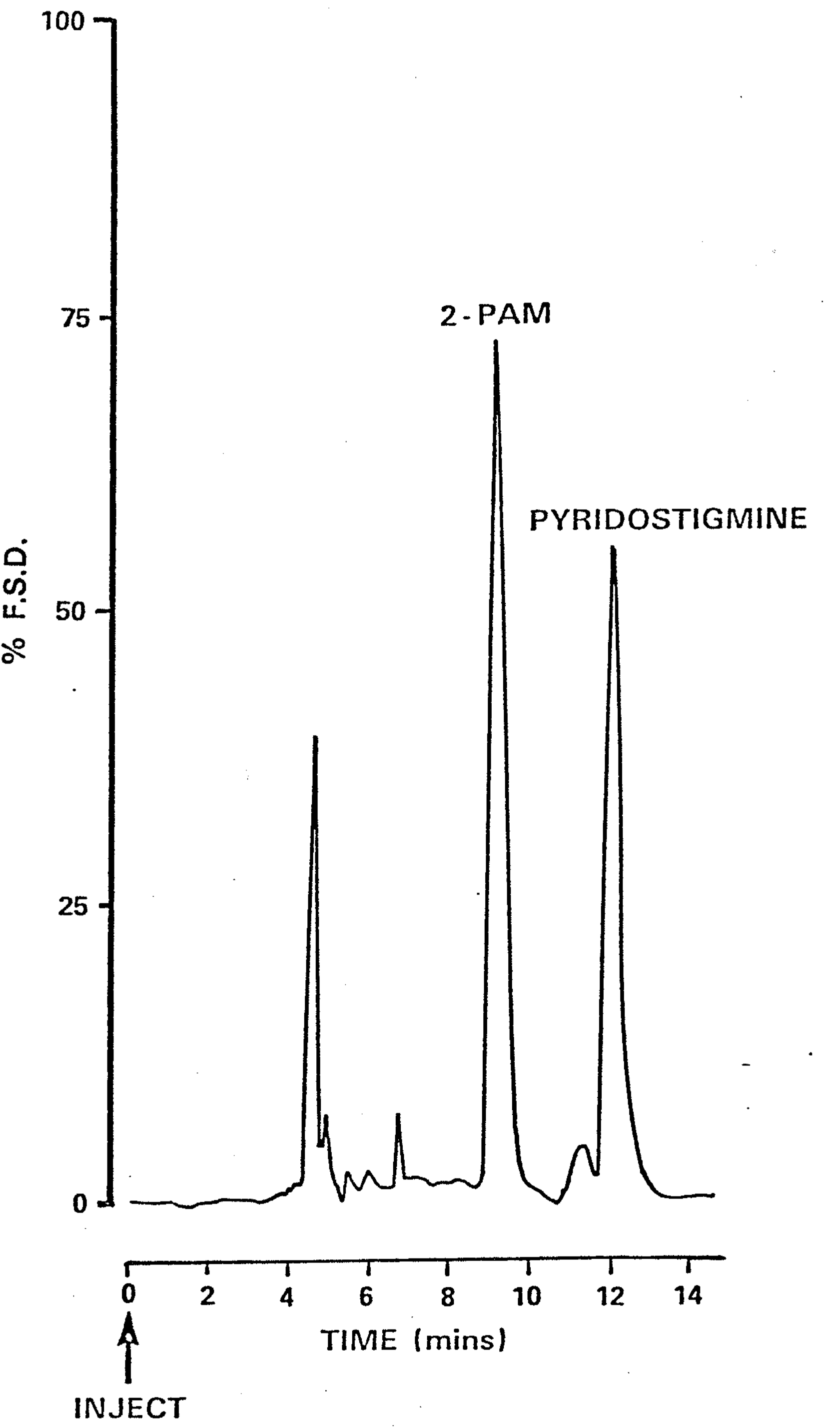

Figure 13

HPLC Chromatograph of 2-PAM and Pyridostigmine

b. Acetylcholinesterase Assay

Acetylcholinesterase activity in brain and serum was determined using the radiometric procedure of Siakotos _et al_ utilizing C14-acetylcholine iodide as the substrate (182). The reaction was carried out at 37 ^{o}C in both cases.

i. Brain

The assay mixture for determining acetylcholinesterase activity in brain homogenates of control and treated mice contained 195 ul of a 0.1 M phosphate buffer (pH 7.4); 5 ul of a 20% (w/v) whole brain homogenate and 100 ul of C14-acetylcholine (3 mM ACh; S.A. = 0.6 x 10 dpm per umol) in a plastic conical tube. The assay mixture was vortexed and incubated for 10 minutes in a constant temperature water bath. Blanks were run concurrently and contained 200 ul of a 0.1 M phosphate buffer (pH 7.4) and 100 ul C14-acetylcholine (3 mM ACh; S.A. = 0.6 x 10 dpm per umol).

The reaction was stopped by the addition of 5 mL of a suspension of resin-dioxane (20 g Amberlite IRP-69 resin in 100 mL 1,4-dioxane). The enzyme-resin-dioxane volume was then brought up to 10 mL with 1,4-dioxane. The tube was capped, mixed by inversion and centrifuged at 500 x g for 4 minutes. A 4 mL aliquot of the resulting supernatant was transferred with a Repipet automatic dilutor to a scintilla-

tion counting vial. The dilutor was rinsed with 10 mL of scintillation cocktail (10 g PPO; 1 g bis-MSB) and 667 mL Triton X-100 to 2000 mL with scintillation grade toluene). The samples were counted in a Beckman LS9000 Liquid Scintillation Spectrometer at an efficiency of 74% for carbon-14. Acetylcholinesterase activity was expressed as nmoles ACh hydrolyzed per mg tissue per minute.

ii. Serum

The assay mixture for determining acetylcholinesterase activity in the serum of control and treated mice contained 190 ul of a 0.1 M phosphate buffer (pH 7.4); 10 ul of serum and 100 ul of C14-acetylcholine (3mM ACh; S.A. = 0.6 x 10 dpm per umol) in a plastic conical tube. The rest of the procedure was as described for brain with the exception that incubation time was 5 minutes and acetylcholinesterase activity expressed as umoles ACh hydrolyzed per mL serum per minute.

2. Animal Studies

a. Acute Studies

i. Route of Administration

Male mice (20-30 g) were given either 50 mg/kg 2-PAM or a combined solution of 50 mg/kg 2-PAM plus 17.4 mg/kg atropine in saline by either i.m., i.p. or s.c. routes of administration. The injection volume was 1/100 of their body weight. One complete serum blood level curve was generated on one day. The sampling time intervals after injection were 3, 7, 10, 15 and 20 minutes. At the appropriate intervals mice were sacrificed by decapitation. Whole blood and brain samples were collected for later workup and analyses.

The results of multiple runs were combined to obtain serum half-lives for the various routes of administration. Tukey's and Bonferroni's methods of analysis were applied to determine any significant differences.

ii. Serum Half-life Determinations in the Presence of DFP and atropine

Groups of 5 mice (20-30 g) were given DFP (3 mg/kg; i.p.) either 5 minutes prior to or 5 minutes after the administration of either 2-PAM (50 mg/kg; i.p.) or a combined solution of 2-PAM and atropine (50 mg/kg and 17.4 mg/kg respectively; i.p.). Injection volume was 1/100 of the animal's body weight.

Control animals received only 2-PAM (50 mg/kg; i.p.) or a combined solution of 2-PAM and atropine (50 mg/kg and 17.4 mg/kg respectively; i.p.). At 7, 10, 15, 20, 25 and 30 minutes total exposure to 2-PAM or a combined solution of 2-PAM and atropine, the mice were decapitated. Whole blood and brain samples were collected for later workup and analyses.

Tukey's and Bonferroni's methods of analyses were applied to the results in order to determine any significant differences.

III. Increasing Dose Study

Groups of 10 mice (20-30 g) were treated with 5 different doses of 2-PAM (25-125 mg/kg; i.p.) either alone or 5 minutes before (prophylactically) or 5 minutes after (therapeutically) treatment with DFP (3 mg/kg; i.p.). At 15 minutes after the 2-PAM injection, the mice were decapitated. Whole blood and brain samples were collected for later workup and analyses.

A Student Newman-Keul's (SNK) analysis of the results determined any significant differences.

iv. Toxicology (LD50 Determinations)

Ten animals per dose and 4-6 different doses were used to construct LD50 curves. DFP (8-13 mg/kg); atropine (210-330 mg/kg); 2-PAM (90-200 mg/kg) and a combined solution of 2-PAM (50-125 mg/kg) plus a constant dose of atropine (17.4 mg/kg) or a combined solution of atropine (1.1-17.4 mg/kg) plus a constant dose of 2-PAM (130 mg/kg) were administered by i.p. injection. DFP (4-12 mg/kg; i.p.) was administered 5 minutes prior to either 2-PAM (100 mg/kg; i.p.) or a combined solution of 2-PAM (100 mg/kg) and atropine (17.4 mg/kg) given by i.p. injection.

In all cases, the mortality at 24 hours was recorded and the LD50 values calculated by probit analysis according to the method of Finney (63).

v. Hypothermia Studies

Groups of 10 mice (20-30 g) were treated with 5 different doses of 2-PAM (25-125 mg/kg; i.p.) either alone or 5 minutes before (prophylactically) or 5 minutes after (therapeutically) treatment with DFP (3 mg/kg; i.p.). In addition, groups of 10 mice were treated with DFP (3 mg/kg; i.p.) and atropine (17.4 mg/kg; i.p.). A group of 10 control animals receiving only a saline injection was also included in the study.

Initially, the core temperature of each animal in the group was taken. This involved insertion of a rectal thermistor to an approximate distance of 3-4 cm. Once stabilized, usually 10-15 seconds, the core temperature was read on a YSI Model 73ATA Indicating Controller. After all control temperatures were taken, the mice were treated as indicated above. Core temperatures were taken starting at 10 minutes after the last injection and continued to be taken over a 24 hour period. The room temperature was maintained between 21-23 °C over the experimental period.

Core temperatures for each time interval were averaged and plotted. An SNK analysis was performed to identify any significant differences.

b. Chronic Study

On day 1 of the study a total population of 50 mice (20-30 g) received a dose of DFP (1 mg/kg; i.p.). On day 3 and subsequent alternating days to day 21, 5 mice from the total population were set aside and received an i.p. injection of a combined solution of 2-PAM (50 mg/kg) and atropine (17.4 mg/kg). The rest of the population continued to receive a daily injection of DFP (1, 2 & 3 mg/kg; i.p.) until the end of the study. On day 7 and day 14 the dose of DFP was increased to 2 and 3 mg/kg respectively. At 15 minutes after the 2-PAM and atropine injection, mice were decapitated. Whole blood and brain samples were collected for later workup and analyses.

Tukey's and Bonferroni's methods of analyses were applied to the results and yielded significant differences.

3. Sample Preparation

a. Serum Samples

Whole blood was collected directly into Microtainer[R] capillary serum separators and spun at 5000 X g in a Fisher Model 59 centrifuge for 2 minutes. The resultant serum was kept in an ice-bath until AChE and HPLC measurements were performed.

b. Homogenate Preparation

Whole brain was excised immediately after decapitation of the animal and was placed in an ice-cold 0.9% (w/v) saline solution. The brain (minus cerebellum) was then blotted dry on filter paper to remove microblood vessels/trace residual blood and weighed. A 20% (w/v) homogenate was made utilizing a 0.9% saline solution. The homogenate was kept on ice until AChE and HPLC measurements were performed.

III. Results

A. Analytical

1. HPLC Quantitation of 2-PAM

Standard curves were obtained from peak height determinations of known quantities of 2-PAM in water, in water plus the cleanup procedure of perchloric acid and acetonitrile as well as from serum and brain spikes utilizing the perchloric and acetonitrile cleanup procedure (Figures 14 to 17). The standard curves obtained for each evaluation were found to be linear and reproducible from day to day (correlation coefficient=0.99). Determinations of 2-PAM quantities in serum and brain were subsequently made by direct calculation from peak height ratio values of unknowns to the standard curves.

2. Acetylcholinesterase Assay

The results of the AChE activities in brain homogenates and serum are included with their respective animal study group.

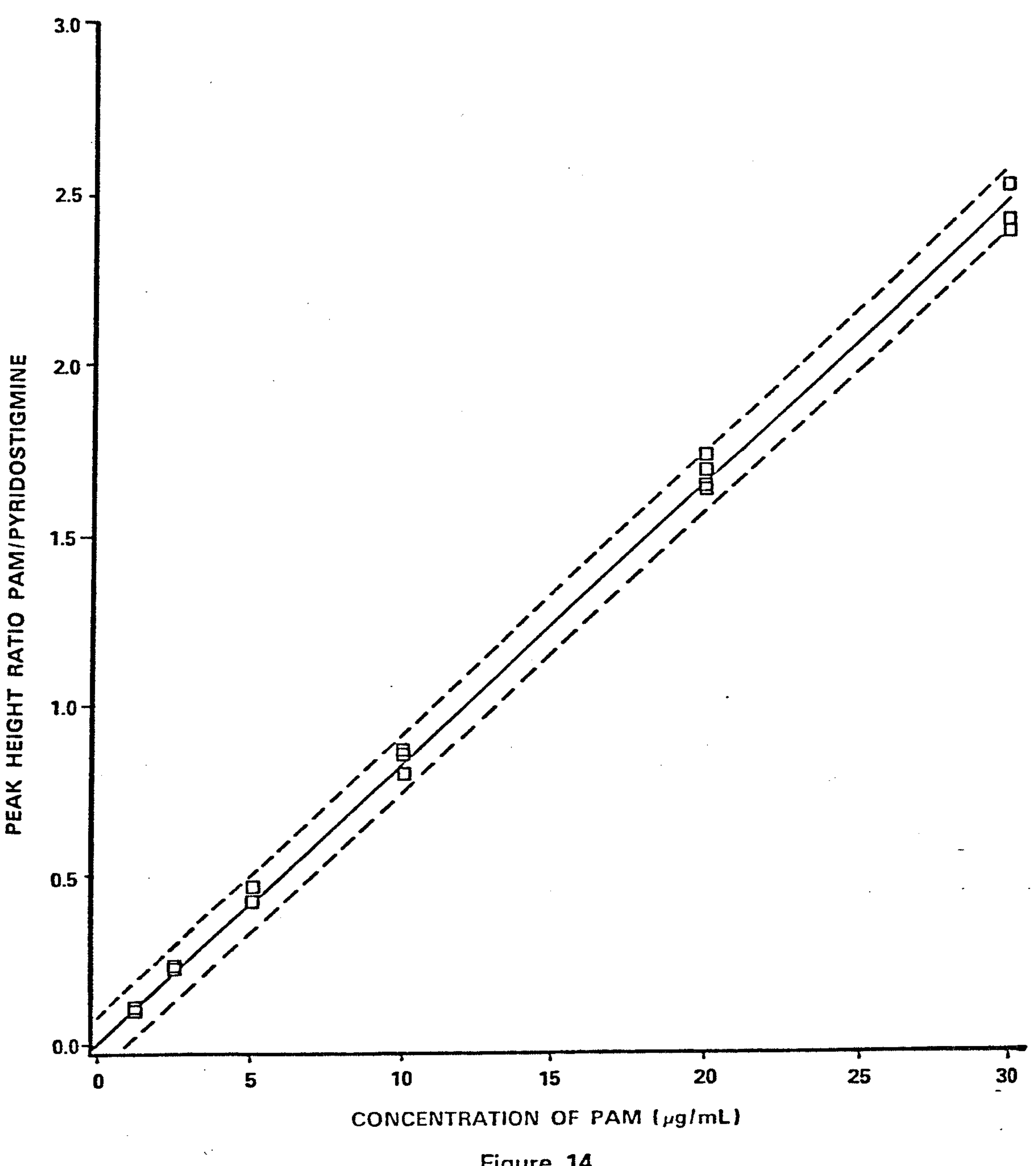

Figure 14

HPLC Determination of the Standard Curve of 2-PAM in Water with 95% Confidence Limits

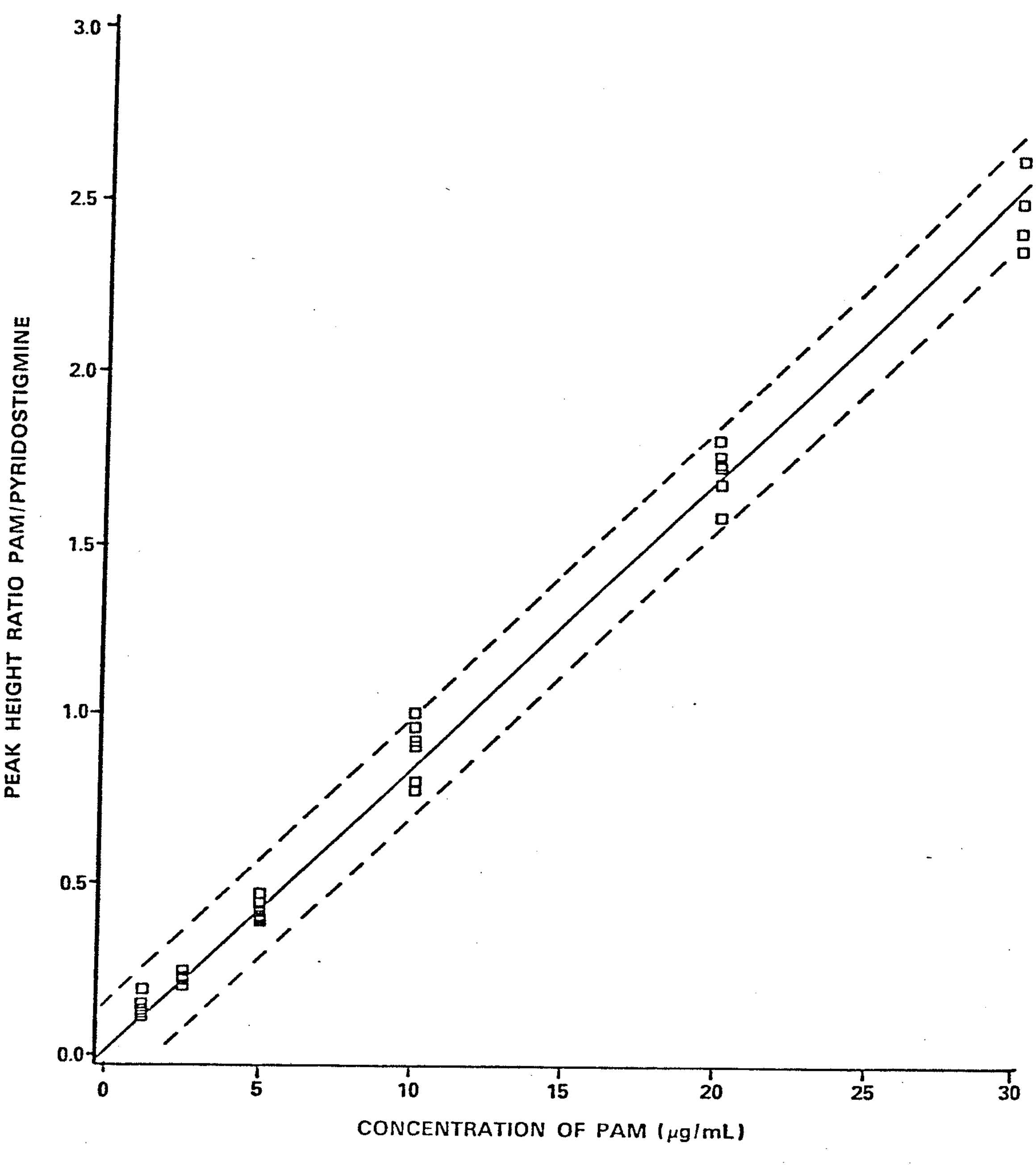

Figure 15

HPLC Determination of the Standard Curve of 2-PAM in Water Utilizing Cleanup Procedures with 95% Confidence Limits

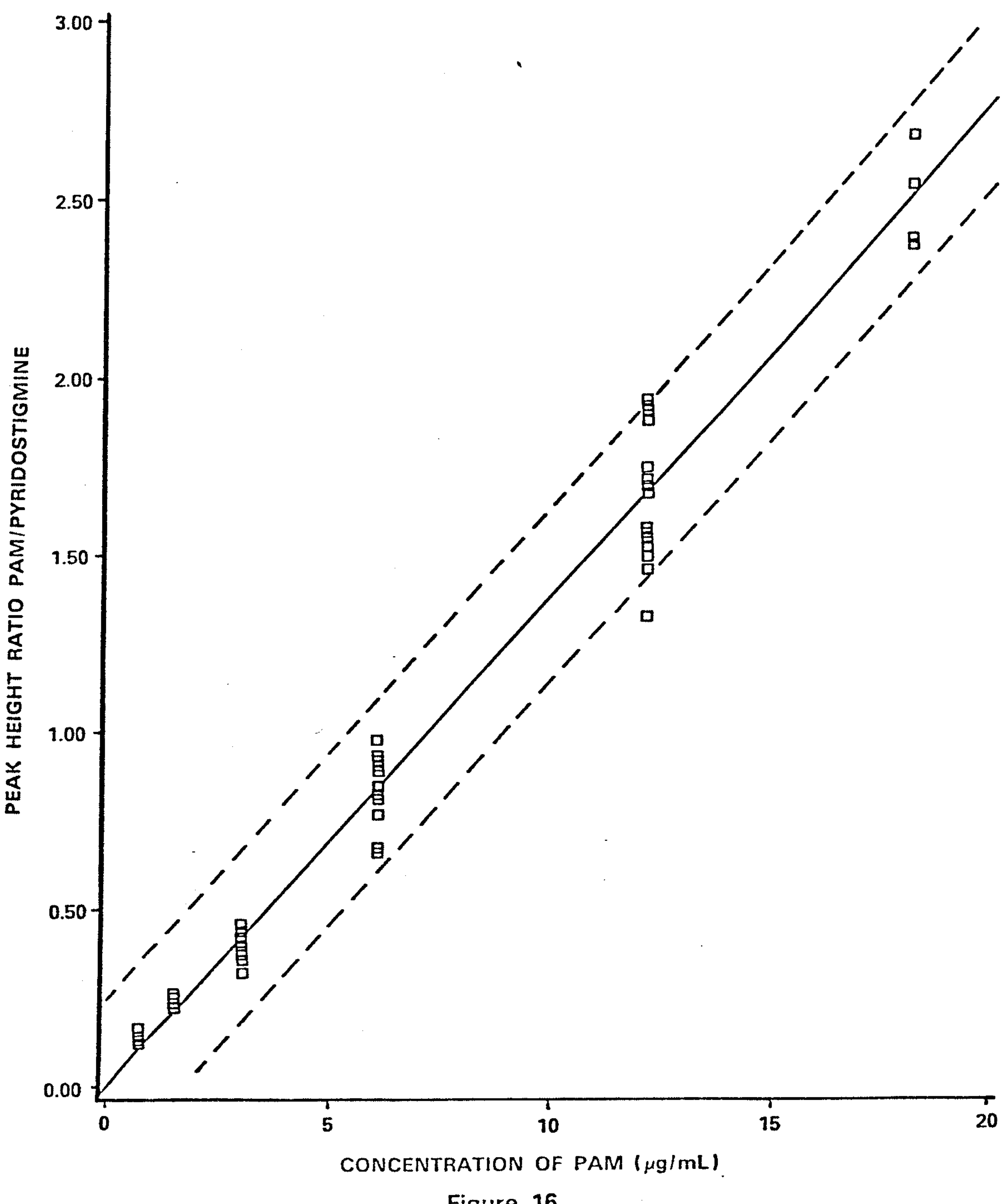

Figure 16

HPLC Determination of the Standard Curve of 2-PAM; Serum Spike

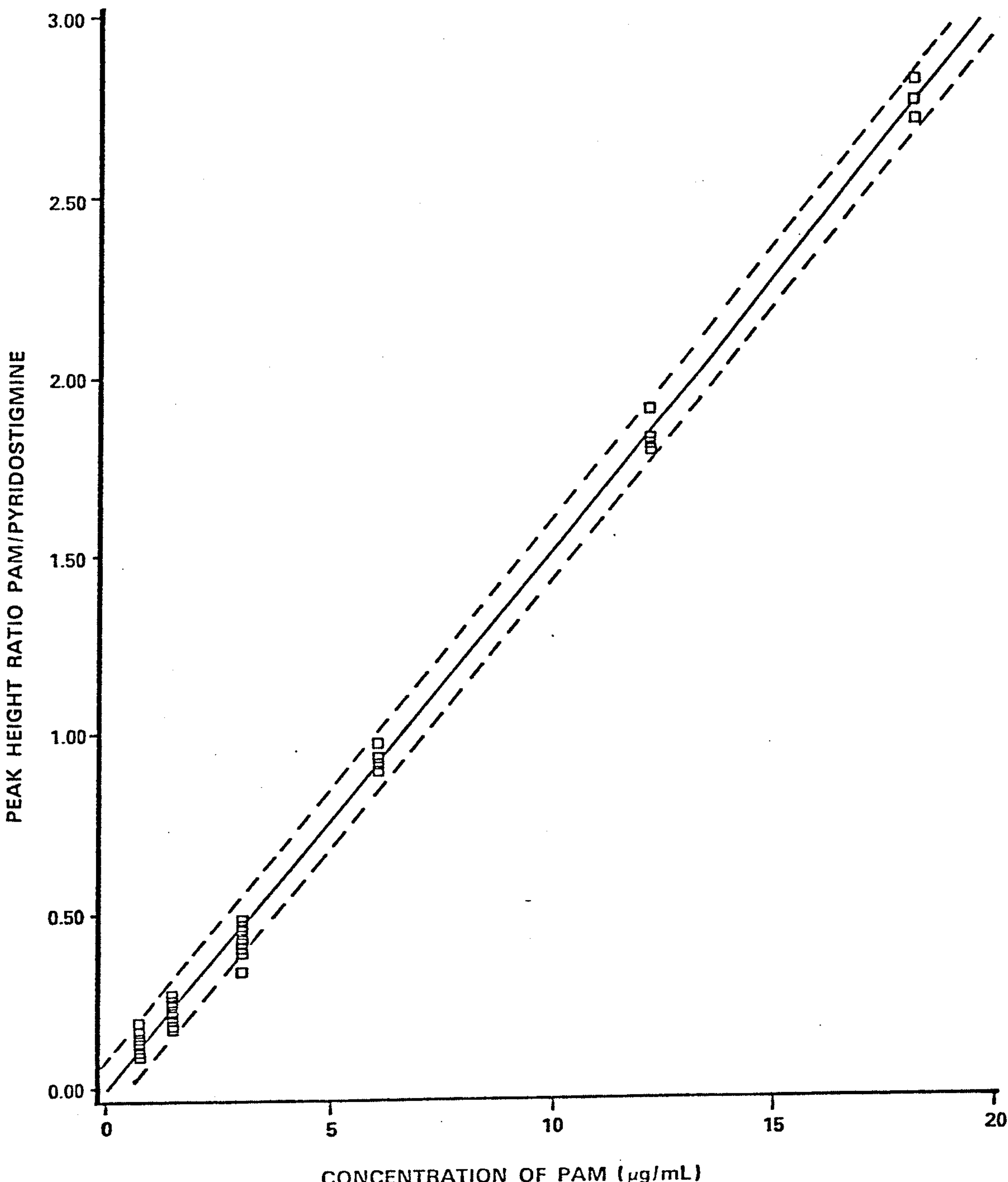

Figure 17

HPLC Determination of the Standard Curve 2-PAM; Brain Homogenate Spike

B. Animal Study

1. Acute Study

a. Route of administration

Figure 18 represents the serum level curves obtained from the administration of 2-PAM or 2-PAM and atropine by either i.m., i.p. or s.c. routes of administration. At 3 minutes, there was no significant difference in serum levels attained by the i.m., i.p. or s.c. administration of 2-PAM (Table 6). In addition, at 3 minutes there was no significant difference in the blood levels attained by the i.p. or s.c. administration of a combined solution of 2-PAM and atropine. However at 3 minutes, 2-PAM administered in combination with atropine by either i.p. or s.c route was significantly different from ($p < 0.05$) the 2-PAM administered by either route alone. At the later time intervals (7-20 minutes), the quantity of 2-PAM in the serum which had been administered i.p. was found to be significantly different ($p < 0.05$) from the other routes of administration as well as when given in combination with atropine.

It was found however that the serum elimination half-life for 2-PAM administered alone by either the i.m, i.p. or s.c. routes of administration was no different than 2-PAM administered in combination with atropine either i.p. or s.c. (Table 7; Figure 18). Error bars were omitted in Figure 18 for clarity.

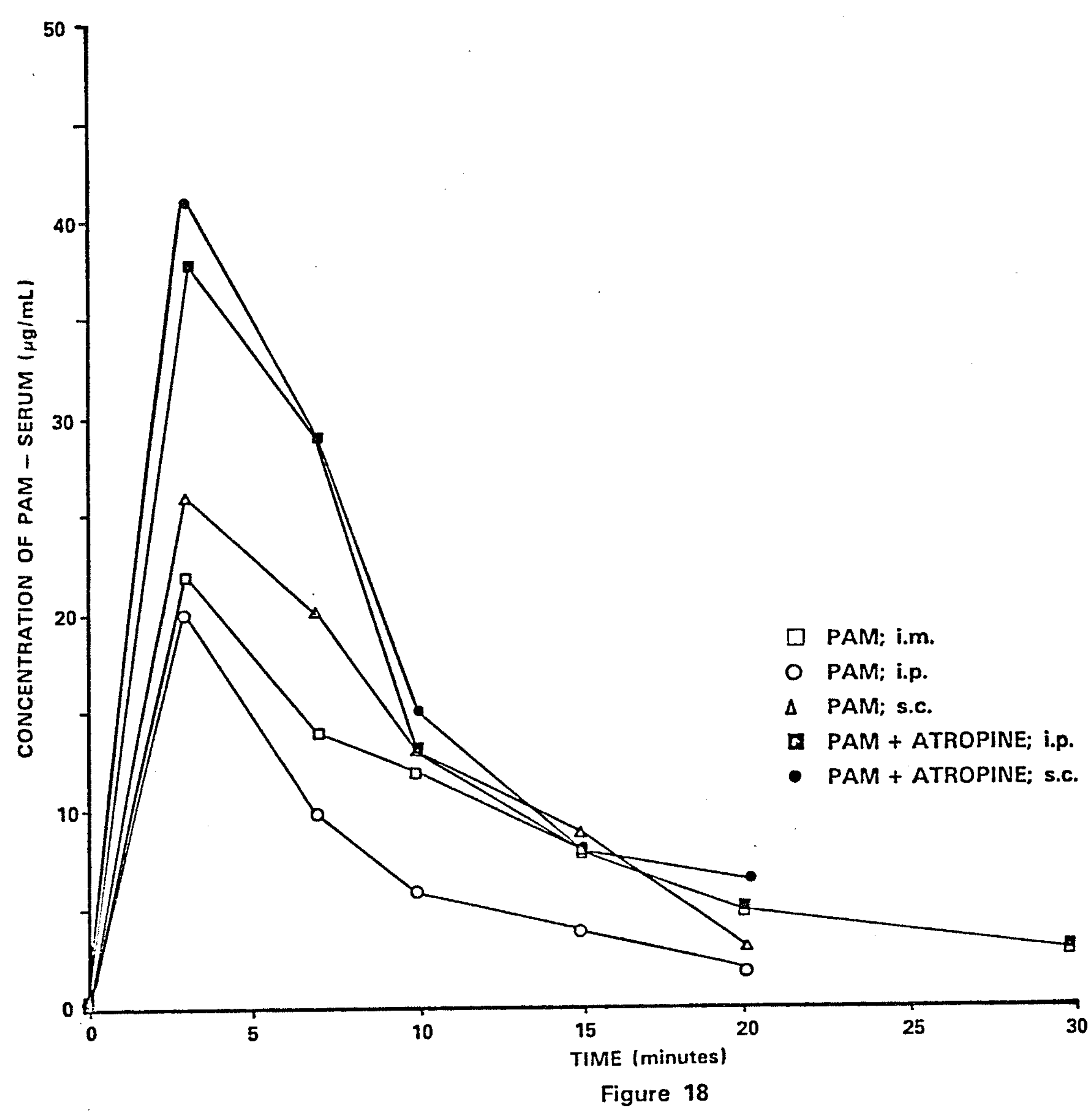

Figure 18

Serum Level Curves for 2-PAM; Different Routes of Administration
Dose of 2-PAM and atropine (50 mg/kg and 17.4 mg/kg, respectively) was administered as a combined solution. Each data point represents the mean from 3 – 5 mice. Error bars are omitted for clarity.

Table 6

Serum 2-PAM Concentrations ($\mu g/mL$) from Mice Given 50 mg/kg 2-PAM or 50 mg/kg 2-PAM Plus 17.4 mg/kg Atropine

ROUTE OF ADMINISTRATION	TIME (minutes)					
	3	7	10	15	20	30
i.m.	22.30 ± 1.35[1] (3)[2]	13.73 ± 1.43 (3)	12.17 ± 1.54 (3)	5.21 ± 0.68 (3)	4.47 ± 0.76 (3)	2.67 (1)
i.p.	20.12 ± 1.17 (3)	9.61 ± 0.86 (3)	5.88 ± 0.42 (3)	4.11 ± 0.32 (3)	2.24 ± 0.53 (3)	—
s.c.	25.69 ± 1.23 (4)	19.89 ± 1.07 (4)	12.68 ± 1.24 (4)	5.57 ± 0.95 (4)	2.95 ± 1.13 (4)	—
i.p. Combo	38.16 ± 1.43 (3)	29.06 ± 1.25 (3)	12.49 ± 0.55 (3)	7.99 ± 0.52 (3)	4.99 ± 0.02 (3)	—
s.c. Combo	40.76 ± 1.21 (2)	29.35 ± 1.32 (2)	14.46 ± 1.02 (2)	8.06 ± 0.37 (2)	6.47 ± 0.44 (2)	—

1 Mean ± SEM

2 Number of Animals

Table 7

Serum Elimination $T_{1/2}$ Values (minutes) for 2-PAM in Mice Following Different Routes of Administration

ROUTE OF ADMINISTRATION	DOSE (mg/kg)	SERUM ELIMINATION $T_{1/2}$ (minutes)
i.m.	50 PAM	7.86 ± 1.66[1] (3)[2]
i.p.	50 PAM	6.96 ± 2.15 (3)
s.c.	50 PAM	6.39 ± 1.49 (4)
i.p.	50 PAM + 17.4 Atropine	7.59 ± 0.46 (3)
s.c.	50 PAM + 17.4 Atropine	8.61 ± 0.03 (2)

1 Mean ± SEM

2 Number of Trials

The AChE levels in brain and serum were determined. There were no significant differences found between control brain and serum AChE activities and those determined at the sampling times after the administration of 2-PAM or 2-PAM and atropine (Tables 8 and 9).

b. Serum Half-life Determinations

Table 10 represents the concentration of 2-PAM determined by HPLC analysis of serum as a function of time for 2-PAM and 2-PAM plus atropine given i.p. either alone or prophylactically or therapeutically against DFP (3 mg/kg; i.p.). Significant differences ($p < 0.05$) were found between the 2-PAM and 2-PAM plus atropine groups at time intervals from 3 to 15 minutes; no differences were seen for the later times.

Results in Table 11 demonstrate the serum elimination half-life for 2-PAM or a combined solution of 2-PAM plus atropine given i.p. either alone or 5 minutes prior to or 5 minutes after an i.p. challenge of DFP which were determined from the data presented in Table 10.

The serum elimination T1/2 = 9.33 minutes from 2-PAM and atropine administered i.p. 5 minutes after DFP i.p. was found to be significantly different ($p < 0.05$) from the i.p. administration of 2-PAM and atropine administered alone (T1/2 = 11.98) or from the same regimen administered 5 minutes

Table 8

Acetylcholinesterase Levels (μmol/mL/min) in Serum from Mice Given 50 mg/kg 2-PAM or 50 mg/kg 2-PAM Plus 17.4 mg/kg Atropine

GROUP	TIME (minutes)					
	0	3	7	10	15	20
Control	2.61 ± 0.07[1] (49)[2]	—	—	—	—	—
i.m.		2.75 ± 0.03 (2)	2.52 ± 0.02 (2)	2.48 ± 0.25 (2)	2.29 ± 0.18 (2)	1.78 ± 0.28 (2)
i.p.	—	2.81 ± 0.16 (4)	2.81 ± 0.17 (6)	2.94 ± 0.09 (10)	2.61 ± 0.11 (10)	2.68 ± 0.13 (5)
s.c.	—	2.72 ± 0.24 (5)	2.86 ± 0.12 (10)	2.82 ± 0.11 (10)	2.89 ± 0.18 (5)	3.05 ± 0.19 (5)
i.p. Combo	—	2.85 ± 0.27 (3)	2.76 ± 0.20 (3)	2.31 ± 0.26 (6)	2.47 ± 0.27 (7)	2.64 ± 0.41 (3)
s.c. Combo	—	2.49 ± 0.20 (2)	1.73 ± 0.15 (2)	2.37 ± 0.18 (2)	2.38 ± 0.17 (2)	2.77 ± 0.18 (2)

1 Mean ± SEM

2 Number of Animals

Table 9

Acetylcholinesterase Levels (nmol/mg/min) in Brain from Mice Given 50 mg/kg 2-PAM or 50 mg/kg 2-PAM Plus 17.4 mg/kg Atropine

GROUP	TIME (minutes)					
	0	3	7	10	15	20
Control	10.27 ± 0.28[1] (49)[2]	—	—	—	—	—
i.m.		11.28 ± 0.55 (2)	11.52 ± 0.48 (2)	10.75 ± 0.38 (2)	11.25 ± 0.46 (2)	11.89 ± 0.53 (2)
i.p.	—	9.27 ± 0.62 (5)	9.43 ± 1.06 (6)	10.11 ± 0.32 (10)	9.60 ± 0.46 (10)	9.31 ± 0.79 (5)
s.c.	—	11.06 ± 0.49 (5)	10.42 ± 0.29 (10)	10.87 ± 0.21 (9)	10.50 ± 0.39 (4)	11.35 ± 0.38 (4)
i.p. Combo	—	10.91 ± 0.68 (3)	10.67 ± 0.25 (3)	9.43 ± 0.72 (6)	9.89 ± 0.66 (7)	10.83 ± 0.75 (3)
s.c. Combo	—	9.27 ± 0.45 (2)	10.32 ± 0.60 (2)	9.99 ± 0.40 (2)	10.06 ± 0.35 (2)	12.35 ± 0.80 (2)

1 Mean ± SEM

2 Number of Animals

Table 10

Serum 2-PAM Concentrations (μg/mL) in Mice

TREATMENT GROUP	TIME (minutes)					
	7	10	15	20	25	30
PAM[3]	11.75 ± 0.24[1] (5)[2]	9.02 ± 1.12 (3)	6.47 ± 0.23 (13)	4.34 ± 0.05 (3)	3.71 ± 0.11 (4)	—
PAM + Atropine[4]	18.53 ± 0.91* (5)	13.13 ± 0.35* (5)	7.50 ± 0.25 (14)	5.47 ± 0.12 (3)	—	—
PAM after DFP[5]	—	8.76 ± 0.25 (5)	5.75 ± 0.20 (6)	4.68 ± 0.20 (5)	3.55 ± 0.13 (3)	—
PAM + Atropine after DFP[5]	—	14.41 ± 0.80* (4)	9.49 ± 0.33 (9)	6.57 ± 0.41 (5)	4.45 ± 0.19 (4)	3.28 ± 0.12 (3)
PAM prior to DFP[6]	—	8.92 ± 0.40 (5)	5.91 ± 0.24 (6)	—	3.46 ± 0.01 (2)	2.58 ± 0.11 (2)
PAM + Atropine prior to DFP[6]	—	—	7.64 ± 0.35 (9)	5.86 ± 0.55 (5)	4.27 ± 0.41 (3)	3.48 ± 0.13 (2)

1 Mean ± SEM

2 Number of Animals

3 Dose = 50 mg/kg 2-PAM; i.p.

4 Dose = 50 mg/kg 2-PAM + 17.4 mg/kg Atropine i.p. as a combined solution

5 Dose of 2-PAM or 2-PAM + Atropine administered 5 min after 3 mg/kg DFP i.p.

6 Dose of 2-PAM or 2-PAM + Atropine administered 5 min prior to 3 mg/kg DFP i.p.

* $p < 0.05$

Table 11

Serum Elimination $T_{1/2}$ Values (minutes) for 2-PAM

TREATMENT GROUP[1]	SERUM ELIMINATION $T_{1/2}$ (minutes)	95% CONFIDENCE LIMITS
PAM[2]	10.60	9.38 – 11.99
PAM + Atropine[3]	11.98	9.76 – 15.54
PAM after DFP[4]	11.78	10.24 – 13.85
PAM + Atropine after DFP	9.33[5]	8.56 – 10.26
PAM prior to DFP	11.40	10.06 – 13.16
PAM + Atropine prior to DFP	12.78	10.03 – 17.63

1 Number of animals used in the determination of $T_{1/2}$ was at least 5

2 Dose PAM = 50 mg/kg; i.p.

3 Dose PAM + Atropine = 50 + 17.4 mg/kg, respectively; i.p.

4 Dose DFP = 3 mg/kg

5 PAM + Atropine after DFP was found to be significantly different from PAM + Atropine; PAM after DFP and PAM + Atropine prior to DFP; $p < 0.05$. No other significant differences were found.

prior to DFP (T1/2 = 12.78). Additionally, the T1/2 = 9.33 minutes from 2-PAM and atropine administered therapeutically 5 minutes after DFP was found to be significantly different ($p < 0.05$) from a T1/2 = 11.78 minutes determined for 2-PAM administered therapeutically 5 minutes after DFP. Figures 19 to 24 represent the linear regression analysis of the mean data of the various treatment groups from which determinations of serum elimination half-lives were calculated. Statistical analyses of all other treatment group comparisons were not significantly different from one another.

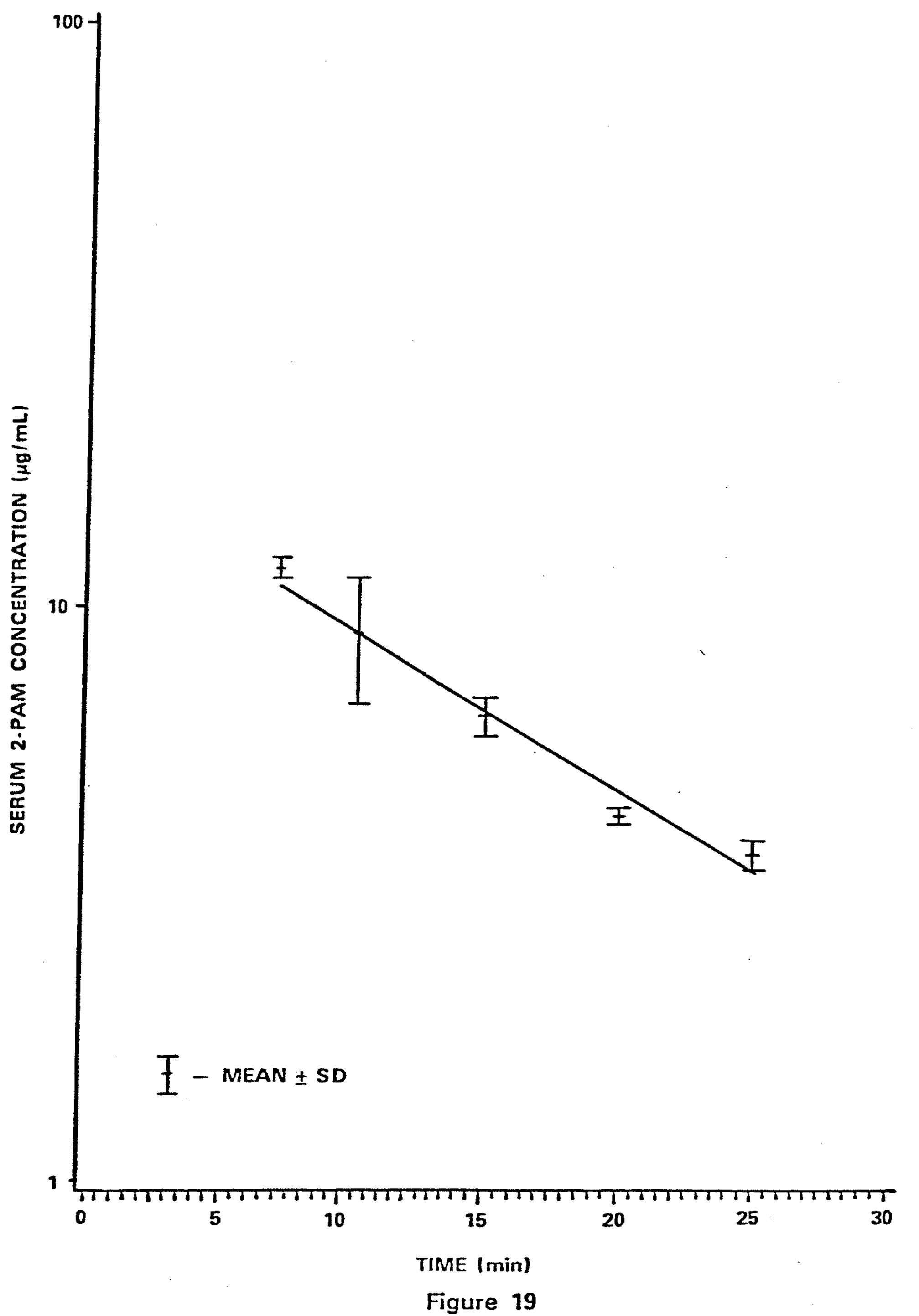

Figure 19

Serum 2-PAM Concentration *Versus* Time Plot
Linear regression analysis of data from mice given 2-PAM (50 mg/kg; i.p.)

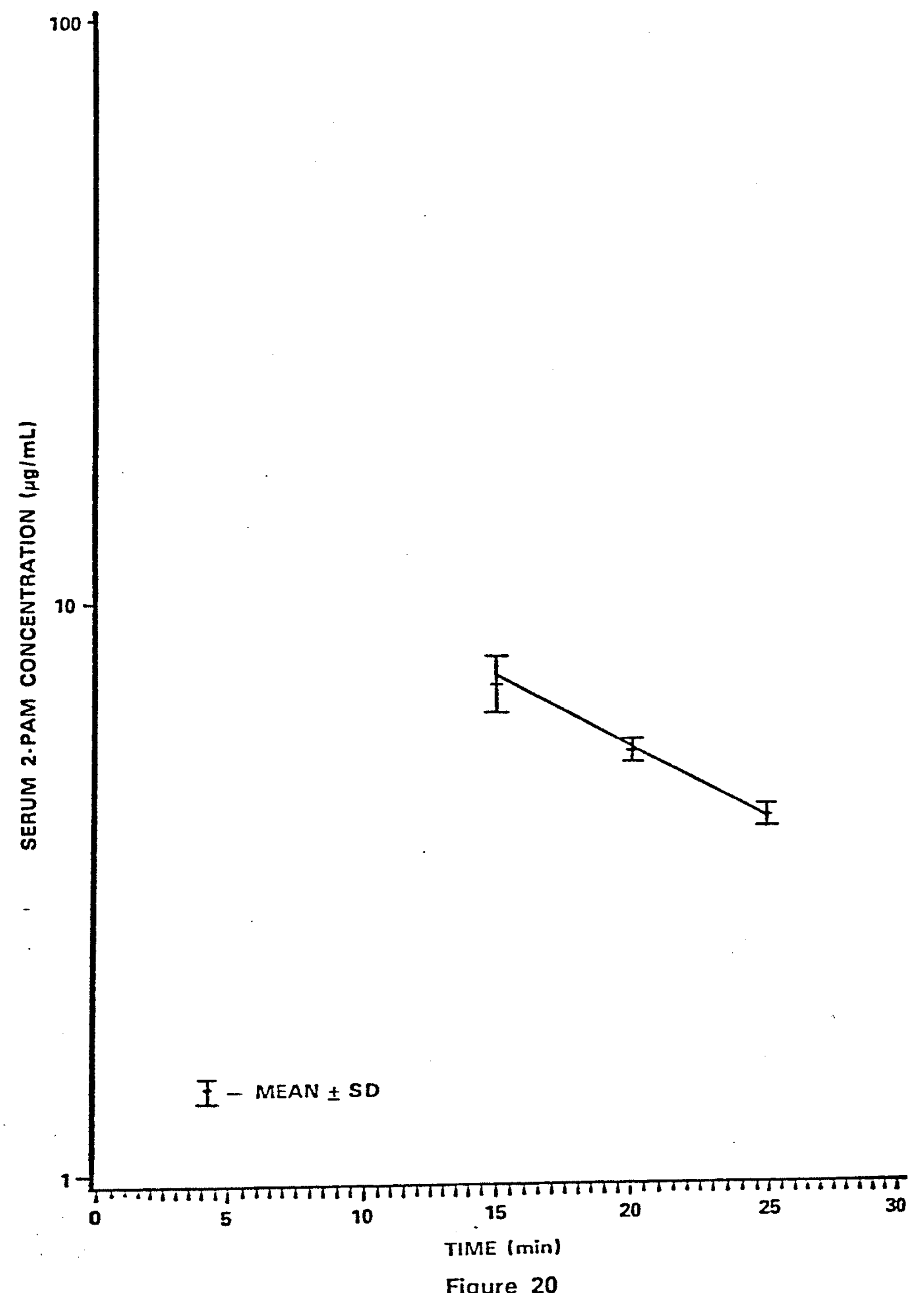

Figure 20

Serum 2-PAM Concentration *Versus* Time Plot
Linear regression analysis of data from mice given 2-PAM plus atropine (50 and 17.4 mg/kg; respectively; i.p.)

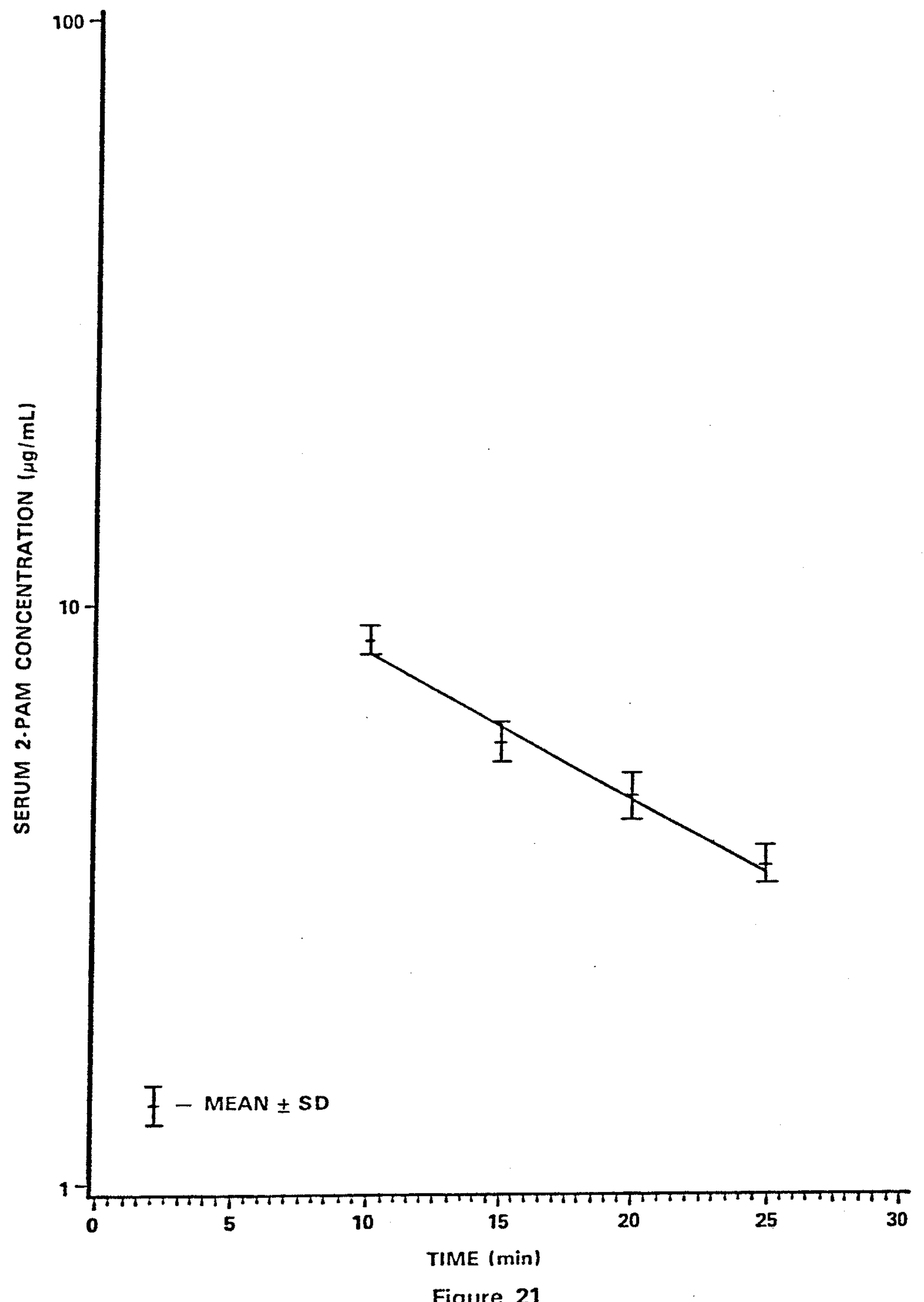

Figure 21

Serum 2-PAM Concentration *Versus* Time Plot
Linear regression analysis of data from mice given 2-PAM (50 mg/kg; i.p.) 5 minutes after DFP (3 mg/kg; i.p.)

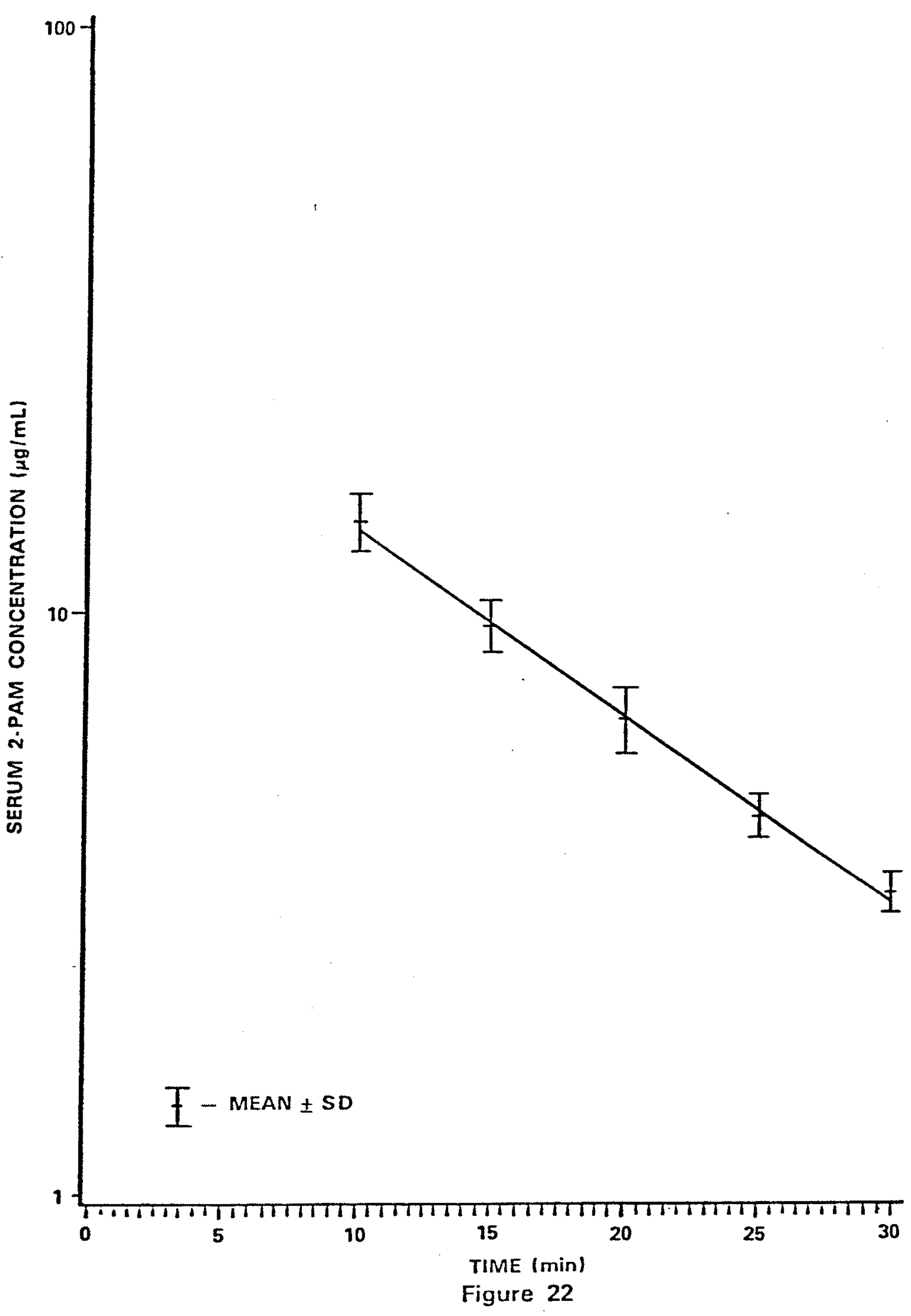

Figure 22

Serum 2-PAM Concentration *Versus* Time Plot
Linear regression analysis of data from mice given 2-PAM plus atropine (50 and 17.4 mg/kg; respectively; i.p.) 5 minutes after DFP (3 mg/kg; i.p.)

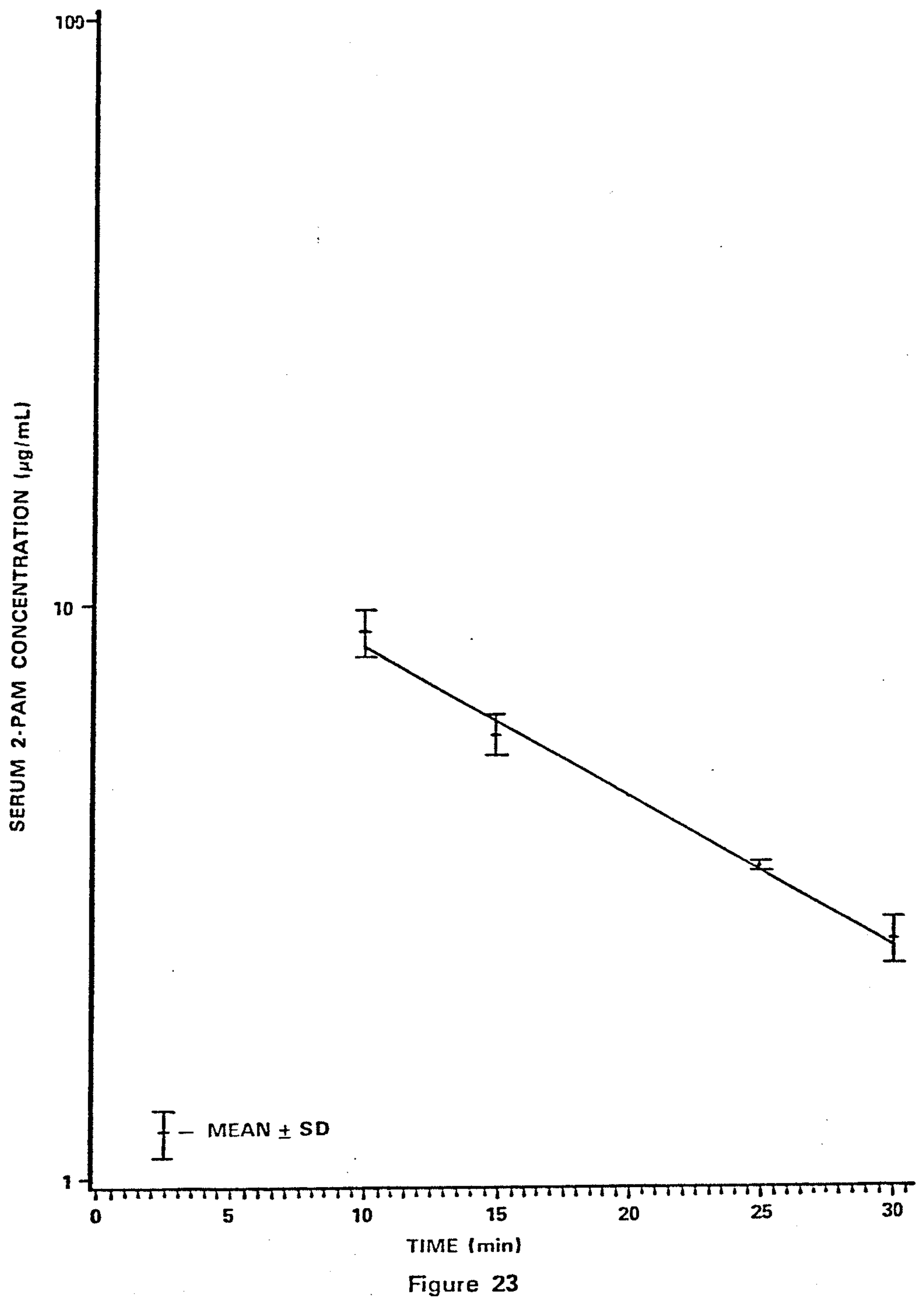

Figure 23

Serum 2-PAM Concentration *Versus* Time Plot
Linear regression analysis of data from mice given 2-PAM (50 mg/kg; i.p.) 5 minutes prior to DFP (3 mg/kg; i.p.)

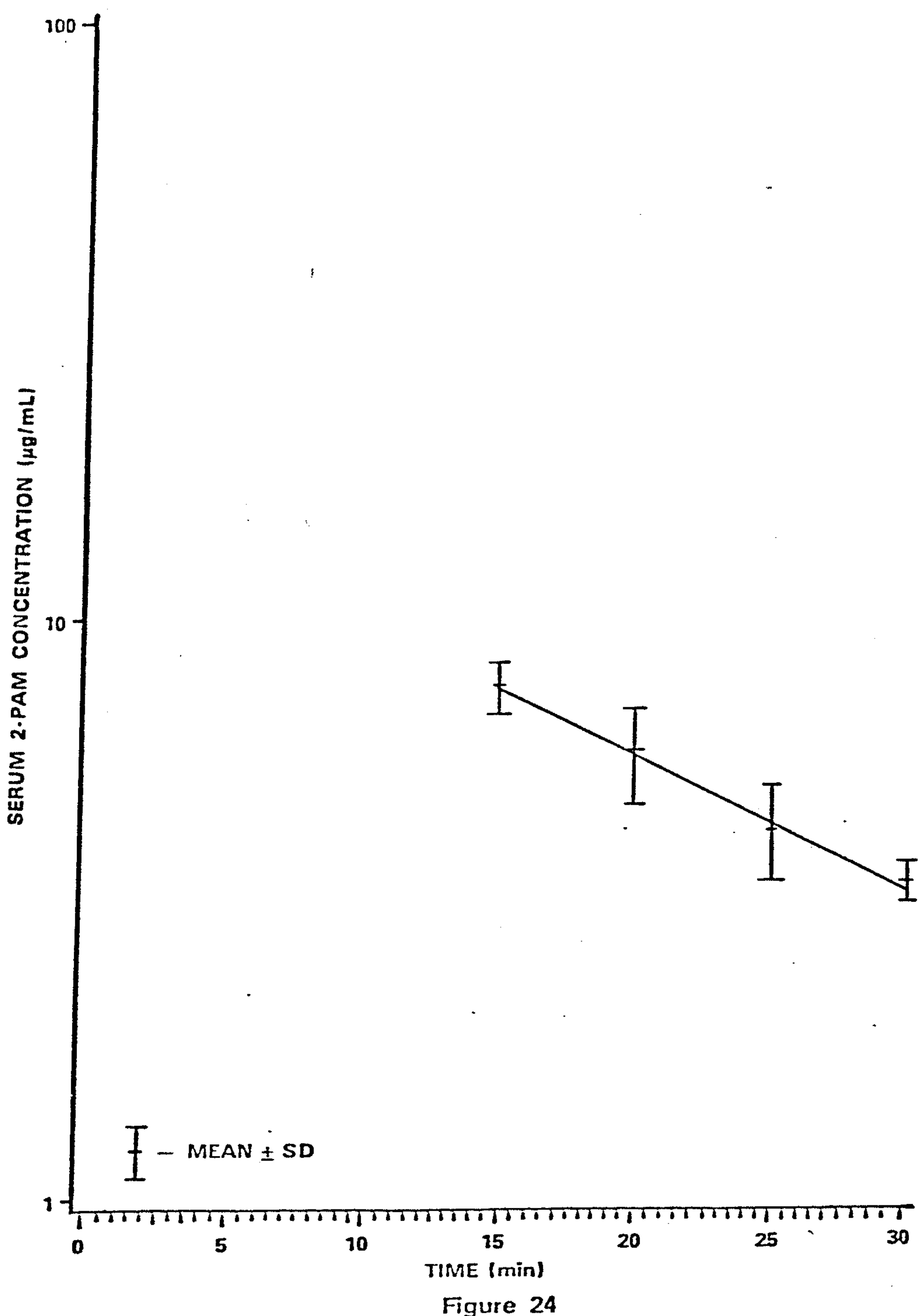

Figure 24

Serum 2-PAM Concentration *Versus* Time Plot
Linear regression analysis of data from mice given 2-PAM plus atropine (50 and 17.4 mg/kg; respectively; i.p.) 5 minutes prior to DFP (3 mg/kg; i.p.)

c. Increasing Dose Study

The serum and brain concentrations of 2-PAM obtained after the i.p. administration of varying doses of 2-PAM either alone or 5 minutes prior to or after the i.p. administration of DFP are presented in Tables 12 and 13 and Figures 25 to 27 respectively.

A serum concentration of 14.38 ug/ml of 2-PAM was found to be significantly different ($p < 0.05$) for the 100 mg/kg 2-PAM dose administered alone when compared to the serum concentration for that same dose of 2-PAM administered either prophylactically (11.50 ug/ml) or therapeutically (11.15 ug/ml) after DFP (Table 12). All other comparisons among the 3 treatment groups were not significantly different from one another. In addition, no significant differences were found when comparing the amounts of 2-PAM present in brain homogenates from the 3 different treatment groups (Table 13).

AChE activities, determined from the same samples as utilized for HPLC analyses of 2-PAM, demonstrated that increasing doses of 2-PAM prophylactically or therapeutically had no reactivating effect on serum AChE. No significant differences other than for the 100 and 125 mg/kg doses of 2-PAM administered 5 minutes prior to DFP and the 125 mg/kg dose of 2-PAM administered 5 minutes after DFP were found between the AChE activities present in serum obtained from administration of 2-PAM prophylactically or

Table 12

Serum Concentrations (μg/mL) Obtained from Increasing Doses of 2-PAM

DOSE OF PAM[1] (mg/kg)	TREATMENT GROUP		
	PAM	PAM PRIOR TO DFP	PAM AFTER DFP
25	2.42 ± 0.23[2] (8)[3]	2.47 ± 0.22 (9)	2.52 ± 0.17 (9)
50	3.64 ± 0.31 (8)	3.42 ± 0.27 (7)	4.23 ± 0.33 (7)
75	8.89 ± 0.46 (7)	7.49 ± 0.30 (9)	8.09 ± 0.44 (8)
100	14.38 ± 1.21[4] (5)	11.50 ± 0.52 (4)	11.15 ± 0.75 (4)
125	17.32 ± 0.89 (8)	18.35 ± 2.50 (3)	15.68 ± 0.85 (7)

1 Dose of PAM was administered i.p. alone or 5 min prior to or after the i.p. administration of 3 mg/kg DFP to CD-1 male mice. Animals were sacrificed at 15 min exposure to PAM.

2 Mean ± SEM

3 Number of Animals

4 Significantly different $p < 0.05$ than the other 100 mg/kg doses

Table 13

Brain Concentrations (ng/mL) Obtained from Increasing Doses of 2-PAM

DOSE OF PAM[1] (mg/kg)	TREATMENT GROUP		
	PAM	PAM PRIOR TO DFP	PAM AFTER DFP
25	58.89 ± 6.11[2] (9)[3]	65.00 ± 5.67 (8)	68.75 ± 5.49 (8)
50	148.00 ± 17.72 (5)	188.00 ± 16.55 (5)	190.00 ± 16.43 (5)
75	204.29 ± 37.15 (7)	237.14 ± 29.70 (7)	247.50 ± 28.52 (8)
100	232.50 ± 31.46 (4)	272.50 ± 28.39 (4)	—
125	412.50 ± 18.49 (8)	402.30 ± 16.40 (3)	407.00 ± 25.95 (10)

1 Dose of PAM was administered i.p. alone or 5 min prior to or after the i.p. administration of 3 mg/kg DFP to CD-1 male mice. Animals were sacrificed at 15 min exposure to PAM.

2 Mean ± SEM

3 Number of Animals

4 No significant differences were found between the treatment groups

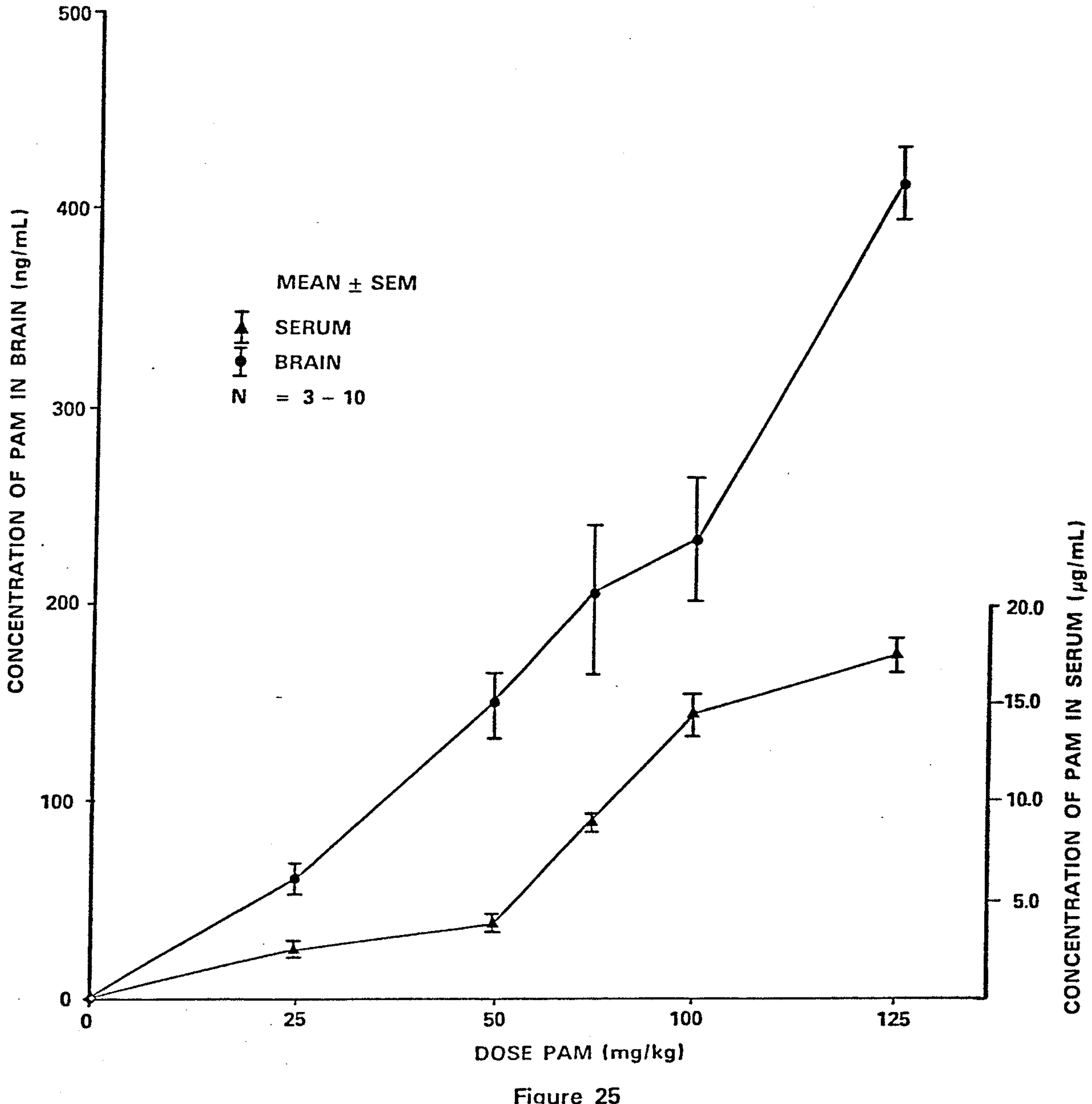

Figure 25

Concentration of 2-PAM in Serum and Brain as Determined by HPLC Analysis after the i.p. Administration of Varying Doses of 2-PAM to Mice

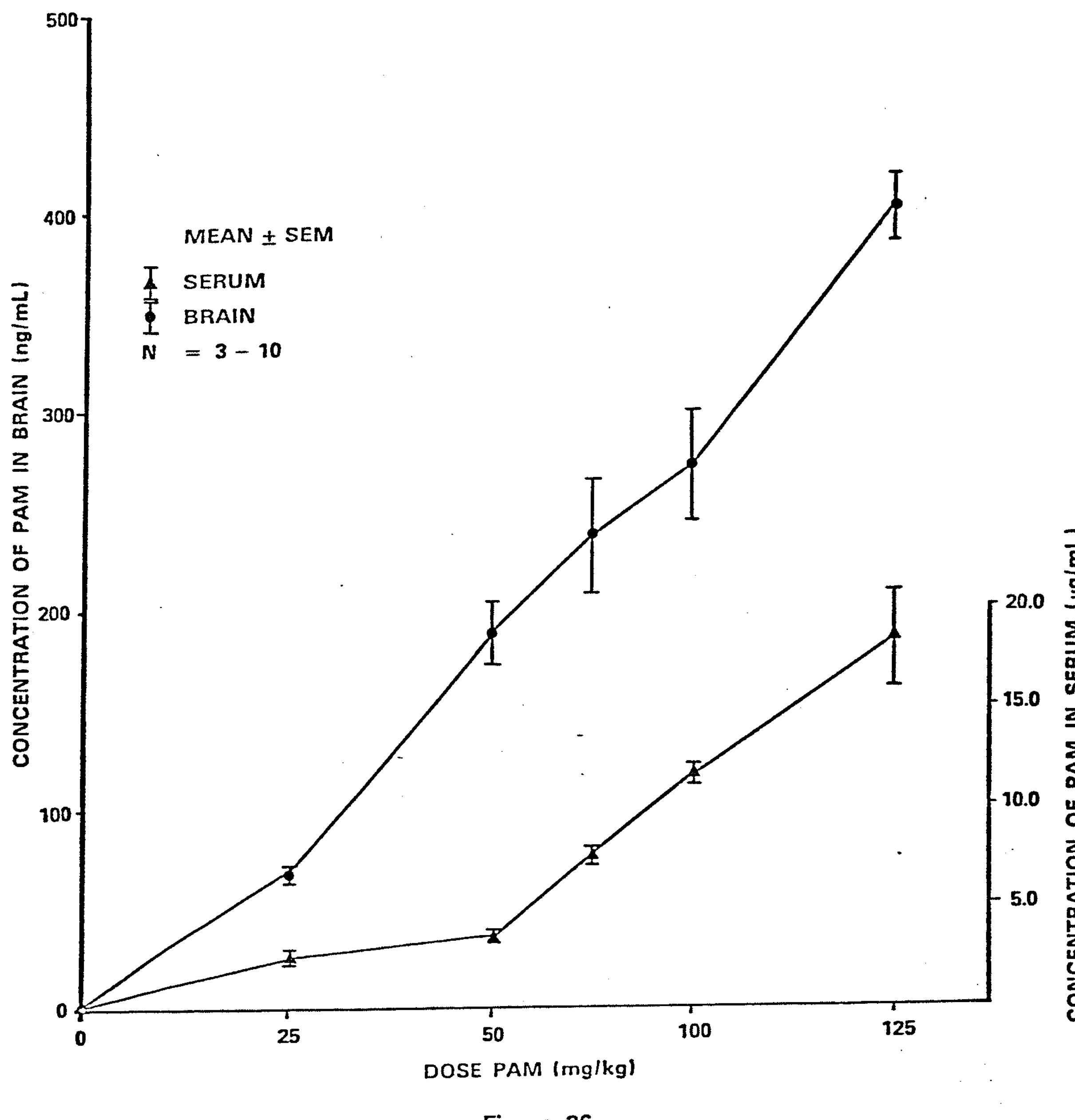

Figure 26

Concentration of 2-PAM in Serum and Brain as Determined by HPLC Analysis after the i.p. Administration of Varying Doses of 2-PAM to Mice 5 Minutes Prior to Exposure to DFP (3 mg/kg; i.p.)

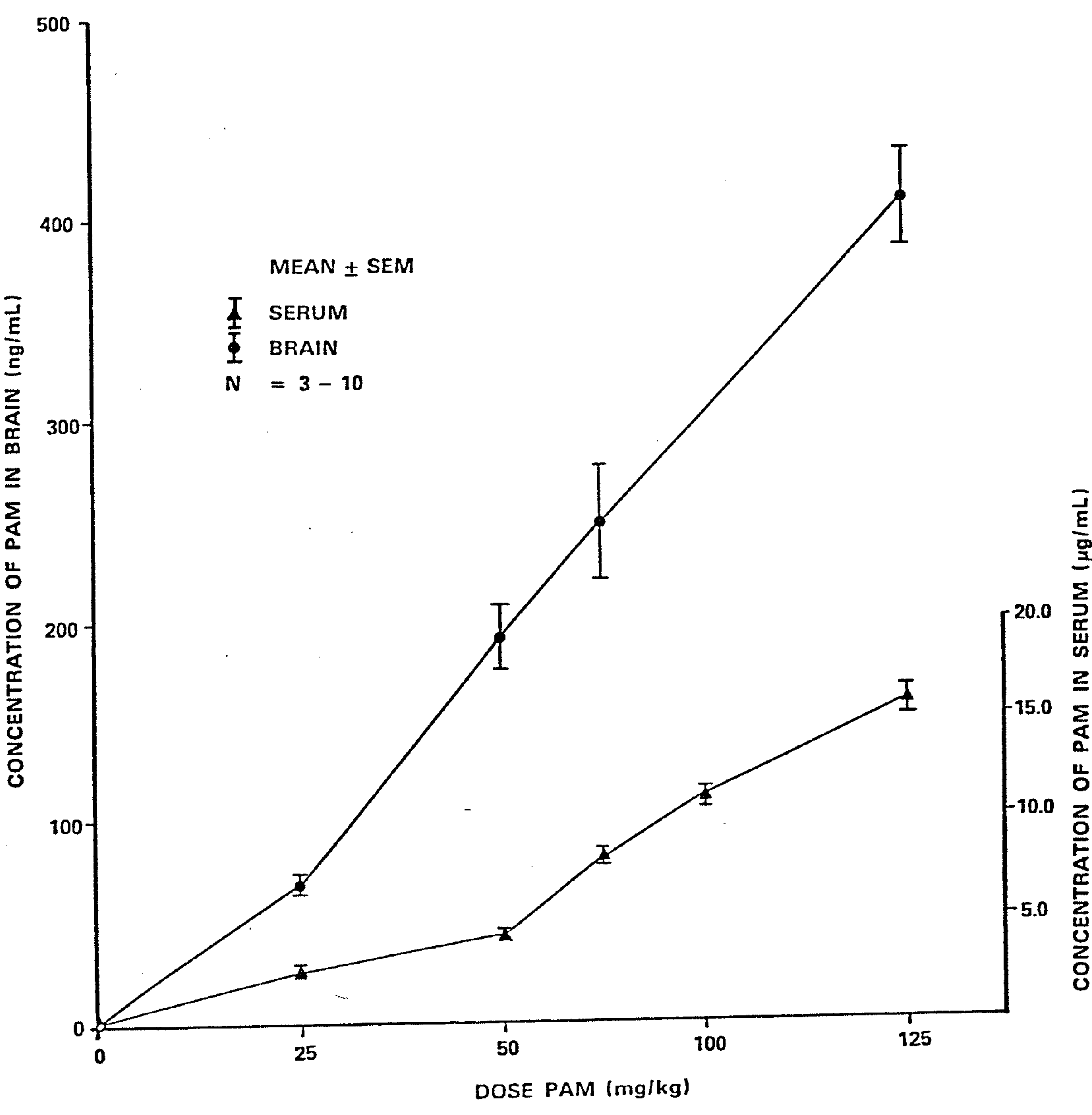

Figure 27

Concentration of 2-PAM in Serum and Brain as Determined by HPLC Analysis after the i.p. Administration of Varying Doses of 2-PAM to Mice 5 Minutes After Exposure to DFP (3 mg/kg; i.p.)

therapeutically than in serum from administration of DFP alone (Tables 14 & 15). The 125 mg/kg 2-PAM dose exhibited a good protection against the challenge from DFP when administered prophylactically however administered therapeutically, that same dose had a minimal effect in increasing the serum AChE activity (Figure 28). No significant reactivation of brain AChE was found (Figure 29).

Table 14

Serum Acetylcholinesterase Activities (μg/mL/min) from Increasing Doses of 2-PAM

DOSE OF PAM[1] (mg/kg)	TREATMENT GROUP		
	PAM	PAM PRIOR TO DFP	PAM AFTER DFP
0	—	0.39 ± 0.03[2] (5)	0.37 ± 0.02[2] (5)
25	2.40 ± 0.11[3] (9)[4]	0.17 ± 0.02 (9)	0.24 ± 0.05 (9)
50	2.50 ± 0.11 (9)	0.45 ± 0.07 (9)	0.51 ± 0.05 (8)
75	1.57 ± 0.10 (9)	0.44 ± 0.03 (9)	0.49 ± 0.05 (9)
100	1.72 ± 0.05 (5)	0.65 ± 0.07[5] (5)	0.56 ± 0.08 (5)
125	2.15 ± 0.10 (10)	1.24 ± 0.08[5] (10)	0.69 ± 0.07[5] (10)

1. Dose of PAM was administered i.p. alone or 5 min prior to or after the i.p. administration of 3 mg/kg DFP to CD-1 male mice. Animals were sacrificed at 15 min exposure to PAM.
2. Control values; administration of DFP (3 mg/kg; i.p.) only
3. Mean ± SEM
4. Number of Animals
5. Significantly different ($p < 0.05$) from controls

Table 15

Brain Acetylcholinesterase Activities (nm/mg/min) from Increasing Doses of 2-PAM

DOSE OF PAM[1] (mg/kg)	TREATMENT GROUP		
	PAM	PAM PRIOR TO DFP	PAM AFTER DFP
0	—	5.33 ± 0.63[2] (5)	5.39 ± 0.28[2] (5)
25	11.03 ± 0.32[3] (6)[4]	5.41 ± 0.42 (6)	4.27 ± 0.32 (6)
50	10.31 ± 0.59 (8)	5.56 ± 0.42 (7)	4.43 ± 0.30 (9)
75	7.71 ± 0.44 (9)	3.58 ± 0.38 (9)	2.86 ± 0.36 (9)
100	7.41 ± 0.12 (4)	3.08 ± 0.71 (5)	3.64 ± 0.67 (4)
125	6.71 ± 0.29 (10)	4.41 ± 0.50 (9)	3.96 ± 0.32 (10)

1 Dose of PAM was administered i.p. alone or 5 min prior to or after the i.p. administration of 3 mg/kg DFP to male CD-1 mice. Animals were sacrificed at 15 min exposure to PAM.

2 Control values; administration of DFP (3 mg/kg; i.p.) only

3 Mean ± SEM

4 Number of Animals

5 No significant differences were found when compared to control values

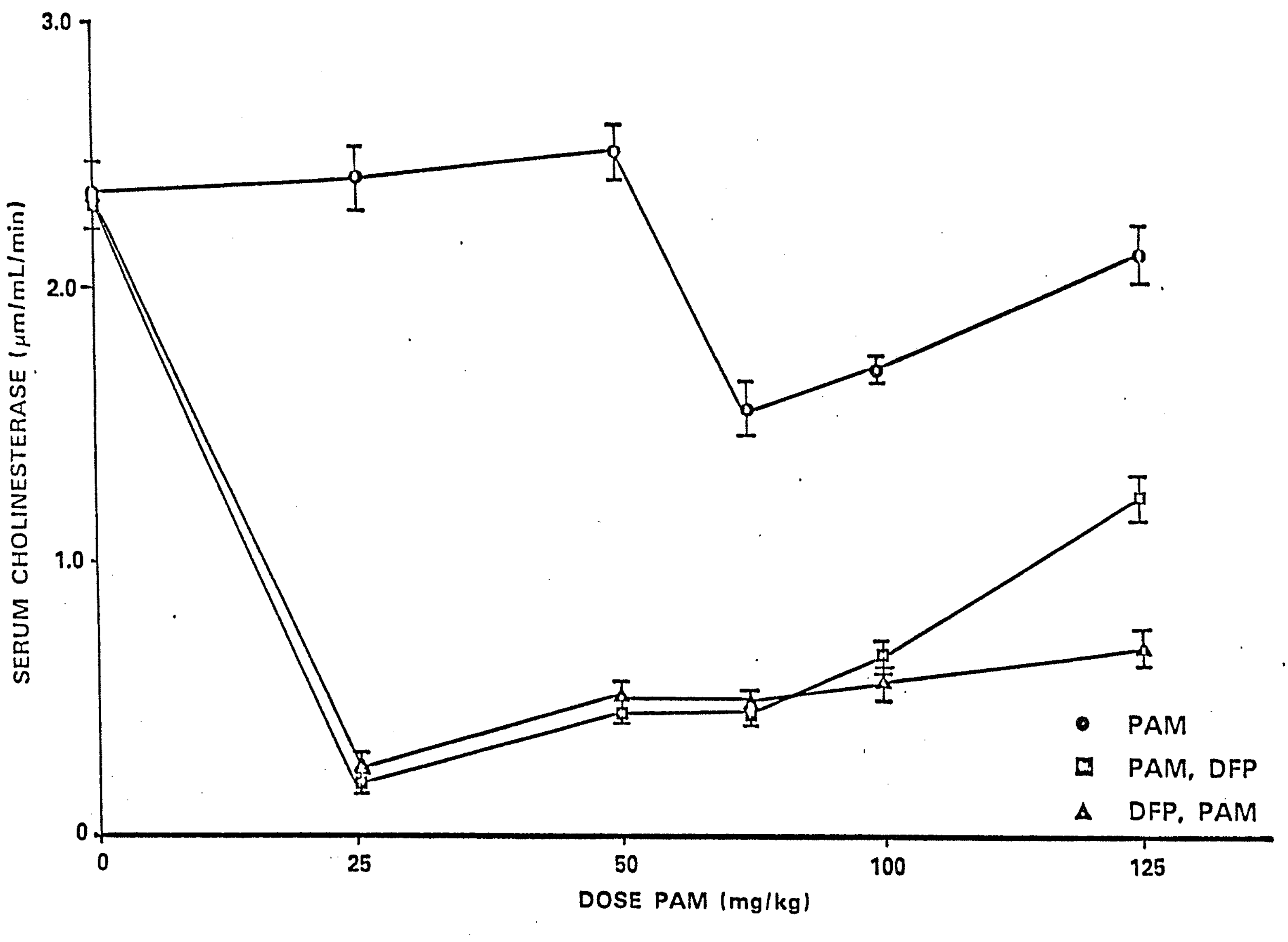

Figure 28

Serum Cholinesterase Activity (μm/mL/min) *Versus* Doses of 2-PAM Administered i.p. Alone or Prophylactically or Therapeutically Against DFP (3 mg/kg; i.p.)

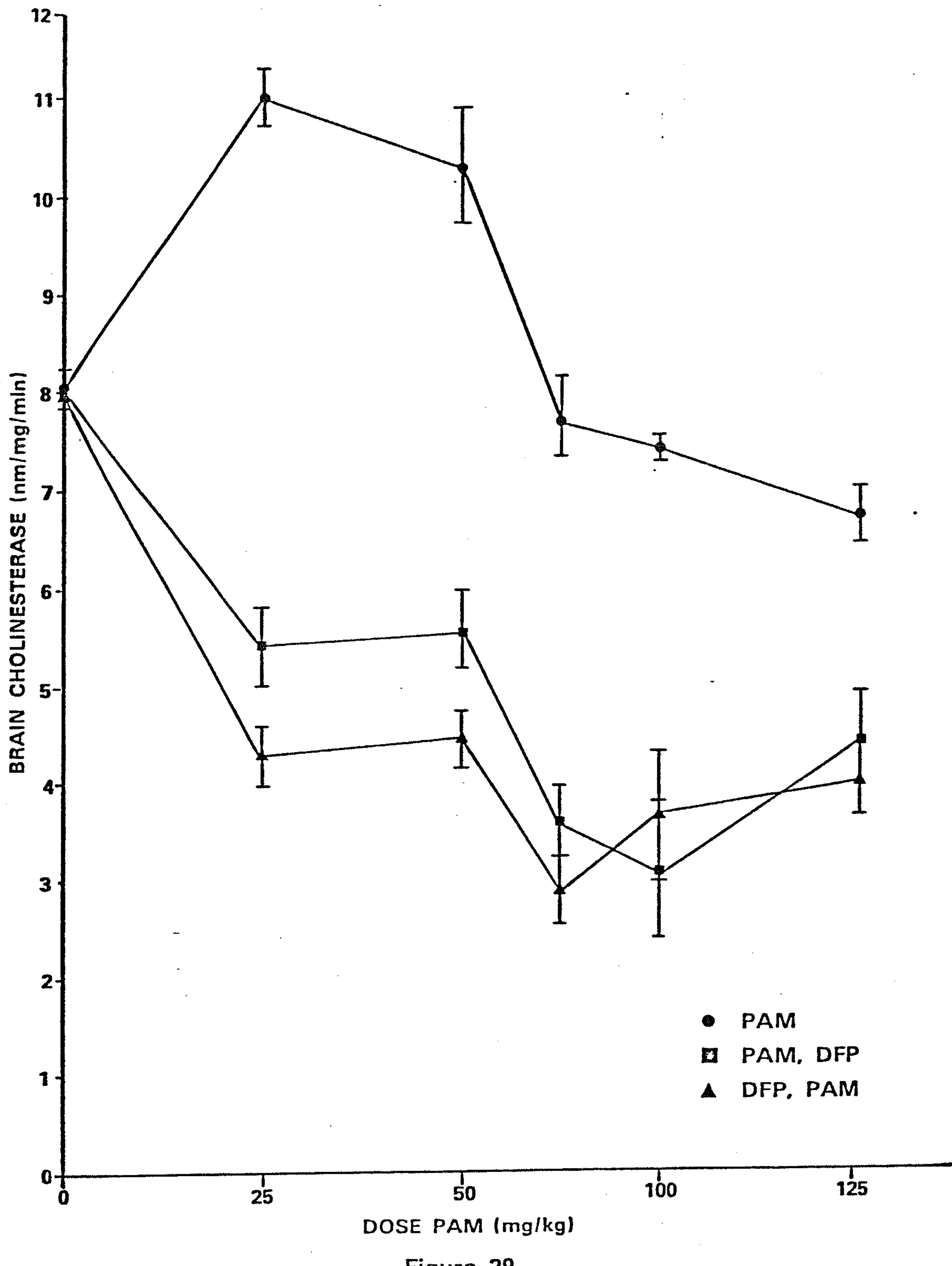

Figure 29

Brain Cholinesterase Activity (nm/mg/min) *Versus* Doses of 2-PAM Administered i.p. Alone or Prophylactically or Therapeutically Against DFP (3 mg/kg; i.p.)

d. Toxicology (LD50) Determinations

The LD50 values and 95% confidence limits for DFP, 2-PAM, atropine and combinations of the aforementioned compounds are given in Table 16.

Table 16

LD_{50} Values and 95% Confidence Limits for DFP, Atropine and 2-PAM in Mice

COMPOUND	LD_{50} (mg/kg)	95% CONFIDENCE LIMITS
DFP	11.6	10.7 – 12.7
Atropine	226.7	166.8 – 252.7
PAM	141.8	128.9 – 157.6
PAM + Atropine[3]	86.3	74.0 – 96.9
Atropine + PAM[4]	2.0	1.0 – 3.1

1 Route of administration for all compounds was i.p.
2 Number of animals per group = 10; 4 – 6 dosage groups
3 Compounds were injected as combined solutions. Atropine, 17.4 mg/kg
4 Compounds were injected as combined solutions. PAM, 130 mg/kg

Administered as a combined injection with a constant dose of 17.4 mg/kg atropine, the LD50 value of 2-PAM was found to drop significantly from 141.8 to 86.3 mg/kg. However, the reverse schedule of a constant dose of 130 mg/kg 2-PAM and varying doses of atropine suggested that if a lower concentration of atropine (2 mg/kg) was administered the dosage of 2-PAM could be increased to a value approaching the LD50 value for 2-PAM itself (Figure 30).

The mortality curves for DFP and DFP followed 5 minutes later by 100 mg/kg 2-PAM or a combined solution of 100 mg/kg 2-PAM and 17.4 mg/kg atropine are illustrated in Figure 31.

An expected mortality of 0-1 deaths due to the 100 mg/kg dose of 2-PAM was seen when challenged with up to 8 mg/kg DFP. At the 10 mg/kg dose of DFP, 2-PAM administration was found to offer some protection against the toxicity of DFP. In the presence of 2-PAM, no deaths were seen whereas for a 10 mg/kg dose of DFP alone 2 deaths were seen.

In comparison, when challenged with up to 8 mg/kg DFP, an increased number of deaths (3 out of 10 mice) were seen in the animals which had been given 100 mg/kg 2-PAM and 17.4 mg/kg atropine as a combined solution therapeutically whereas zero out of 10 mice were dead from administration of DFP alone.

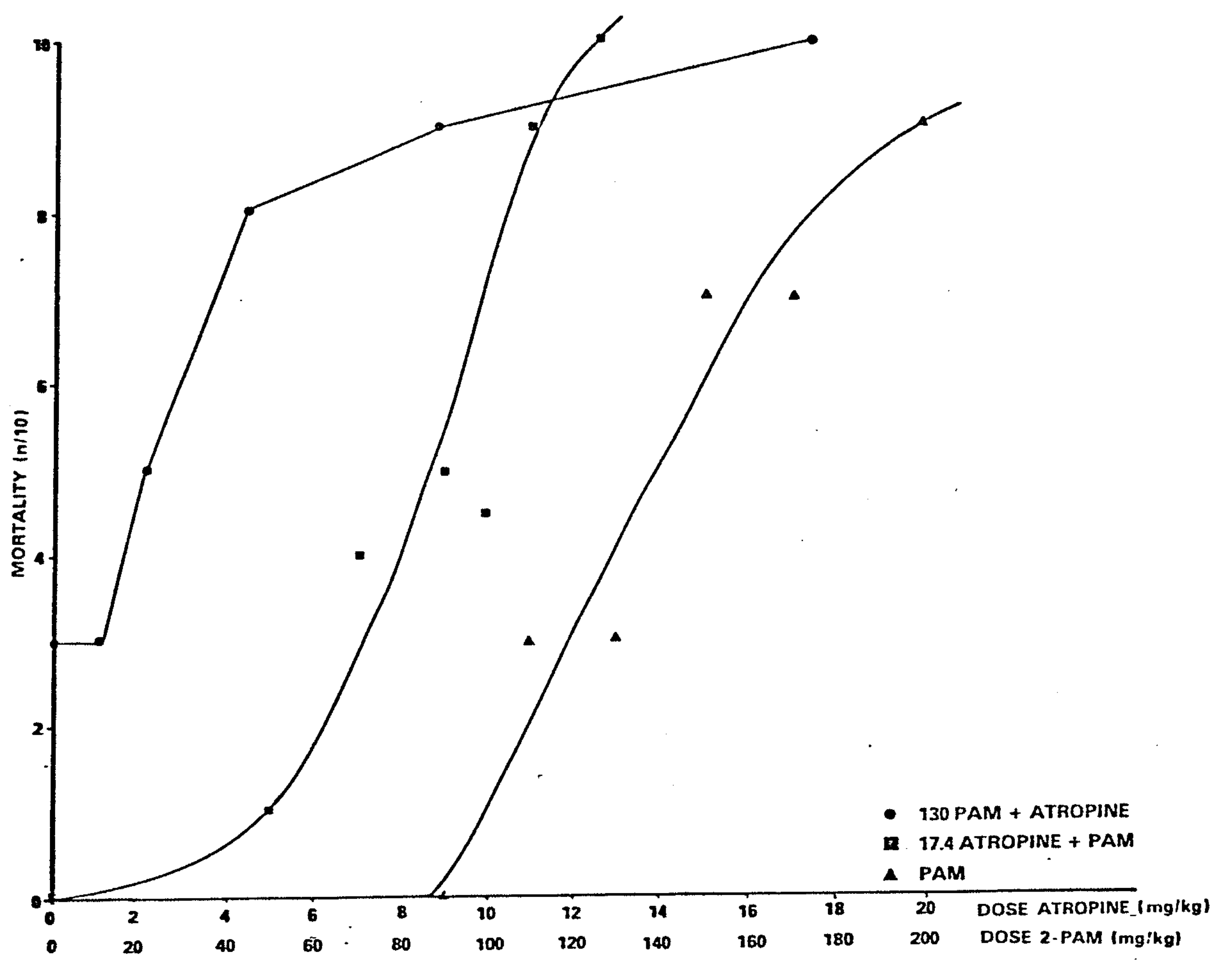

Figure 30

LD_{50} Curves (i.p.) for 2-PAM Alone and Combined Solutions of Atropine and 2-PAM. The concentration of atropine was held constant at 17.4 mg/kg and 2-PAM at 130 mg/kg in the combined solutions.

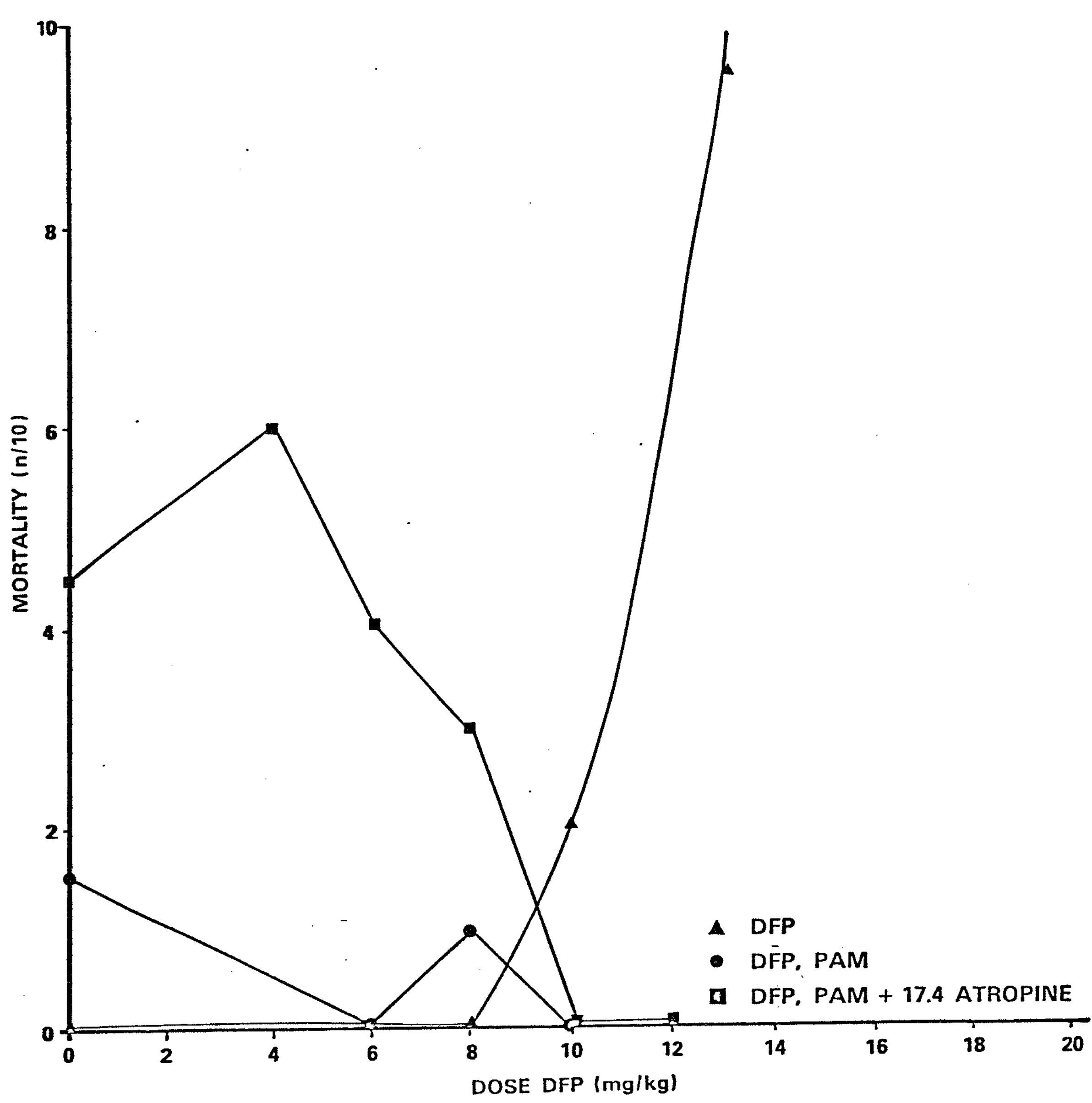

Figure 31

Mortality Curves (i.p.) for DFP and 2-PAM (100 mg/kg) or 2-PAM and Atropine (100 and 17.4 mg/kg; Respectively) Administered as a Combined Solution 5 Minutes After Exposure to DFP

e. Hypothermia Study

The core temperatures of control and treated mice are presented in Table 17. Control mice, those receiving a sham injection of saline, exhibited a slight but insignificant decrease in core temperature over 6 hours.

A dose dependent hypothermia following administration of 50-125 mg/kg doses of 2-PAM is illustrated in Figure 32. Error bars have been left out in all of the core temperature figures for clarity. The reader is referred to Table 17 for presentation of experimental errors. Although significant differences ($p<0.05$) were found among the temperatures at the various sampling times and treatment groups, the general trend, as indicated by evaluating the differences at the various sampling times, suggests that the curves generated for the 50, 75 and 100 mg/kg doses of 2-PAM might be significantly different from the curve generated for the 125 mg/kg dose of 2-PAM. The same trend was seen for the hypothermia data generated for the 50-125 mg/kg doses of 2-PAM administered prophylactically against DFP (Figure 33). The 50, 75 and 100 mg/kg doses of 2-PAM appear to be different from the 125 mg/kg dose of 2-PAM. The hypothermia data however, seem to indicate that the hypothermia observed after the 125 mg/kg dose of 2-PAM is not different from that obtained by the administration of DFP alone.

Table 17

Core Temperatures (°C) Obtained for DFP (3 mg/kg), Atropine (17.4 mg/kg) and Increasing Doses of 2-PAM (50 – 125 mg/kg) in Mice

CORE TEMPERATURE (°C)[1]

GROUP \ TIME (MINUTES)	0	10	20	30	45	60	90	120	150	180	240	300	360	1440
CONTROL MICE	37.3 ± 0.1[2] (10)[3]	37.3 ± 0.1 (10)	37.2 ± 0.1 (10)	37.2 ± 0.1 (10)	37.3 ± 0.1 (10)	36.9 ± 0.1 (10)	37.0 ± 0.1 (10)	37.1 ± 0.1 (10)	36.8 ± 0.1 (10)	36.9 ± 0.1 (10)	36.8 ± 0.1 (10)	36.9 ± 0.1 (10)	36.7 ± 0.1 (10)	
3 DFP[4]	37.7 ± 0.2 (10)	36.5 ± 0.2 (10)	35.5 ± 0.2 (10)	35.2 ± 0.2 (10)	35.0 ± 0.2 (10)	34.7 ± 0.2 (10)	34.5 ± 0.2 (10)	35.0 ± 0.2 (10)	35.5 ± 0.2 (10)	35.4 ± 0.2 (10)	35.7 ± 0.2 (10)	35.6 ± 0.2 (10)	35.7 ± 0.2 (10)	36.6 ± 0.2 (10)
3 DFP[5]	38.1 ± 0.1 (10)	36.3 ± 0.1 (10)	34.5 ± 0.1 (10)	33.5 ± 0.2 (9)	32.7 ± 0.2 (9)	31.6 ± 0.2 (9)	31.3 ± 0.2 (9)	31.0 ± 0.2 (9)	30.8 ± 0.3 (9)	30.6 ± 0.1 (9)	30.3 ± 0.4 (9)	30.2 ± 0.3 (9)	30.7 ± 0.2 (9)	33.1 ± 0.9 (9)
17.4 ATROPINE[4]	37.6 ± 0.1 (10)	37.2 ± 0.1 (10)	36.5 ± 0.1 (10)	36.6 ± 0.1 (10)	36.6 ± 0.1 (9)	36.6 ± 0.1 (9)	36.7 ± 0.2 (9)	36.7 ± 0.2 (9)	36.7 ± 0.2 (9)	36.7 ± 0.2 (9)	36.6 ± 0.2 (9)	36.4 ± 0.3 (9)	36.6 ± 0.2 (9)	35.9 ± 0.3 (9)
50 PAM[4]	37.2 ± 0.1 (10)	36.7 ± 0.1 (10)	36.8 ± 0.1 (10)	36.8 ± 0.1 (10)	36.6 ± 0.1 (10)	36.9 ± 0.1 (10)	36.7 ± 0.1 (10)	36.6 ± 0.1 (10)	36.5 ± 0.1 (10)	36.5 ± 0.1 (10)	36.4 ± 0.1 (10)	36.4 ± 0.1 (10)	36.3 ± 0.1 (10)	36.3 ± 0.1 (10)
50 PAM 5 MIN PRIOR TO 3 DFP	37.3 ± 0.1 (10)	36.5 ± 0.1 (10)	36.2 ± 0.1 (10)	36.0 ± 0.2 (10)	36.0 ± 0.2 (10)	36.0 ± 0.2 (10)	36.0 ± 0.2 (10)	36.1 ± 0.2 (10)	36.1 ± 0.2 (10)	36.0 ± 0.2 (10)	36.2 ± 0.1 (10)	36.3 ± 0.1 (10)	36.2 ± 0.1 (10)	36.1 ± 0.1 (10)
50 PAM 5 MIN AFTER 3 DFP	37.4 ± 0.1 (10)	36.5 ± 0.1 (10)	36.2 ± 0.1 (10)	36.6 ± 0.2 (10)	36.6 ± 0.2 (10)	36.6 ± 0.2 (10)	36.7 ± 0.1 (10)	36.7 ± 0.1 (10)	36.7 ± 0.1 (10)	36.6 ± 0.1 (10)	36.7 ± 0.1 (10)	36.6 ± 0.1 (10)	36.6 ± 0.1 (10)	36.4 ± 0.1 (10)
50 PAM + 17.4 ATROPINE[6]	37.8 ± 0.1 (10)	36.5 ± 0.1 (8)	35.3 ± 0.2 (8)	35.3 ± 0.1 (8)	35.6 ± 0.1 (8)	35.7 ± 0.1 (8)	35.8 ± 0.1 (8)	35.8 ± 0.1 (8)	35.9 ± 0.1 (8)	36.1 ± 0.1 (8)	36.2 ± 0.1 (8)	36.0 ± 0.0 (8)	36.0 ± 0.0 (8)	36.1 ± 0.1 (8)
50 PAM + 17.4 ATROPINE 5 MIN PRIOR TO 3 DFP	37.7 ± 0.1 (10)	35.4 ± 0.2 (9)	35.4 ± 0.2 (9)	35.7 ± 0.2 (9)	36.1 ± 0.1 (9)	36.0 ± 0.1 (9)	36.1 ± 0.1 (9)	36.2 ± 0.1 (9)	36.3 ± 0.1 (9)	36.4 ± 0.1 (9)	36.3 ± 0.1 (9)	36.3 ± 0.1 (9)	36.3 ± 0.1 (9)	36.3 ± 0.1 (9)
50 PAM + 17.4 ATROPINE 5 MIN AFTER 3 DFP	37.7 ± 0.1 (10)	36.5 ± 0.1 (10)	35.6 ± 0.1 (10)	35.7 ± 0.1 (10)	36.2 ± 0.1 (10)	36.3 ± 0.1 (10)	36.3 ± 0.1 (10)	36.2 ± 0.1 (10)	36.3 ± 0.1 (10)	36.4 ± 0.2 (10)	36.4 ± 0.1 (10)	36.4 ± 0.1 (10)	36.4 ± 0.1 (10)	36.5 ± 0.1 (10)
75 PAM	37.6 ± 0.1 (20)	36.8 ± 0.1 (20)	36.8 ± 0.1 (20)	36.9 ± 0.1 (20)	37.0 ± 0.1 (20)	37.1 ± 0.1 (20)	36.6 ± 0.1 (10)	36.4 ± 0.2 (10)	36.2 ± 0.1 (10)	36.2 ± 0.1 (10)	35.9 ± 0.1 (10)	36.0 ± 0.1 (10)		
75 PAM 5 MIN PRIOR TO 3 DFP	37.4 ± 0.1 (10)	36.0 ± 0.2 (10)	35.7 ± 0.2 (10)	35.8 ± 0.3 (10)	35.8 ± 0.3 (10)	35.8 ± 0.4 (10)	35.6 ± 0.3 (10)	35.6 ± 0.3 (10)	35.8 ± 0.3 (10)	35.8 ± 0.3 (10)	35.9 ± 0.2 (10)	36.0 ± 0.2 (10)	36.2 ± 0.1 (10)	36.5 ± 0.1 (10)
75 PAM 5 MIN AFTER 3 DFP	37.6 ± 0.1 (9)	35.4 ± 0.1 (9)	34.7 ± 0.2 (9)	34.1 ± 0.4 (9)	34.0 ± 0.5 (9)	33.9 ± 0.5 (9)	33.7 ± 0.8 (9)	34.4 ± 0.6 (8)	34.6 ± 0.6 (8)	34.6 ± 0.6 (8)	35.0 ± 0.4 (8)	35.5 ± 0.4 (8)	35.4 ± 0.4 (8)	36.0 ± 0.2 (7)
75 PAM + 17.4 ATROPINE COMBINED	37.4 ± 0.1 (10)	35.7 ± 0.3 (6)	35.2 ± 0.1 (6)	35.6 ± 0.2 (6)	36.1 ± 0.2 (6)	36.3 ± 0.1 (6)	36.2 ± 0.1 (6)	36.3 ± 0.1 (6)	36.3 ± 0.1 (6)	36.2 ± 0.1 (6)	36.2 ± 0.1 (6)	36.2 ± 0.1 (6)	36.2 ± 0.1 (6)	36.3 ± 0.1 (6)
75 PAM + 17.4 ATROPINE 5 MIN AFTER 3 DFP	37.7 ± 0.1 (10)	36.7 ± 0.1 (10)	35.7 ± 0.3 (9)	35.3 ± 0.2 (9)	35.6 ± 0.2 (9)	35.6 ± 0.2 (9)	36.0 ± 0.1 (9)	36.0 ± 0.2 (9)	36.0 ± 0.2 (9)	36.0 ± 0.2 (9)	36.2 ± 0.1 (9)	36.2 ± 0.1 (9)	36.3 ± 0.1 (9)	36.3 ± 0.1 (9)
100 PAM	37.3 ± 0.1 (10)	36.2 ± 0.2 (9)	36.1 ± 0.2 (9)	36.3 ± 0.1 (9)	36.1 ± 0.2 (9)	36.0 ± 0.2 (9)	36.0 ± 0.2 (9)	36.0 ± 0.1 (9)	36.2 ± 0.1 (9)	36.2 ± 0.1 (9)	36.2 ± 0.1 (9)	36.2 ± 0.1 (9)	36.2 ± 0.2 (9)	36.1 ± 0.2 (9)
100 PAM 5 MIN PRIOR TO 3 DFP	37.4 ± 0.1 (10)	35.9 ± 0.2 (8)	35.6 ± 0.2 (8)	35.8 ± 0.2 (8)	35.8 ± 0.2 (8)	35.6 ± 0.2 (8)	35.7 ± 0.2 (8)	35.8 ± 0.2 (8)	35.9 ± 0.2 (8)	36.1 ± 0.1 (8)	36.1 ± 0.1 (8)	36.3 ± 0.1 (8)	35.9 ± 0.1 (8)	36.2 ± 0.1 (8)
100 PAM 5 MIN AFTER 3 DFP	37.3 ± 0.1 (10)	36.4 ± 0.1 (10)	35.6 ± 0.1 (10)	35.4 ± 0.1 (10)	35.7 ± 0.1 (10)	35.5 ± 0.1 (10)	35.4 ± 0.1 (10)	35.3 ± 0.2 (10)	35.3 ± 0.1 (10)	35.6 ± 0.2 (10)	35.6 ± 0.1 (10)	35.9 ± 0.1 (10)	36.1 ± 0.2 (10)	36.3 ± 0.1 (9)
125 PAM	37.7 ± 0.1 (10)	36.6 ± 0.1 (10)	36.0 ± 0.2 (6)	35.5 ± 0.3 (6)	35.8 ± 0.3 (6)	35.8 ± 0.2 (6)	35.0 ± 0.2 (6)	34.7 ± 0.3 (6)	34.7 ± 0.4 (6)	34.8 ± 0.4 (6)	35.3 ± 0.3 (6)	35.8 ± 0.3 (6)	35.8 ± 0.1 (6)	36.3 ± 0.1 (6)
125 PAM 5 MIN PRIOR TO 3 DFP	37.6 ± 0.1 (20)	35.5 ± 0.5 (7)	35.2 ± 0.5 (6)	34.9 ± 0.4 (6)	34.8 ± 0.3 (6)	34.7 ± 0.4 (6)	34.7 ± 0.6 (6)	34.3 ± 0.7 (6)	34.5 ± 0.7 (6)	34.6 ± 0.6 (6)	35.2 ± 0.3 (6)	35.7 ± 0.2 (6)	35.8 ± 0.1 (6)	36.2 ± 0.2 (6)
125 PAM 5 MIN AFTER 3 DFP	37.5 ± 0.1 (10)	36.6 ± 0.1 (10)	34.7 ± 0.2 (10)	34.6 ± 0.2 (10)	34.6 ± 0.3 (10)	34.6 ± 0.3 (10)	34.4 ± 0.4 (10)	34.3 ± 0.5 (10)	34.7 ± 0.5 (10)	34.6 ± 0.5 (10)	35.3 ± 0.3 (10)	35.6 ± 0.2 (10)	35.9 ± 0.1 (10)	35.9 ± 0.1 (10)

1 Core temperature was taken 10 minutes after the last injection by use of a rectal thermistor

2 Mean ± SEM

3 Number of animals

4 Doses were administered i.p. and are represented as mg/kg values

5 DFP was administered s.c. in this study

6 PAM and Atropine were administered as a combined solution

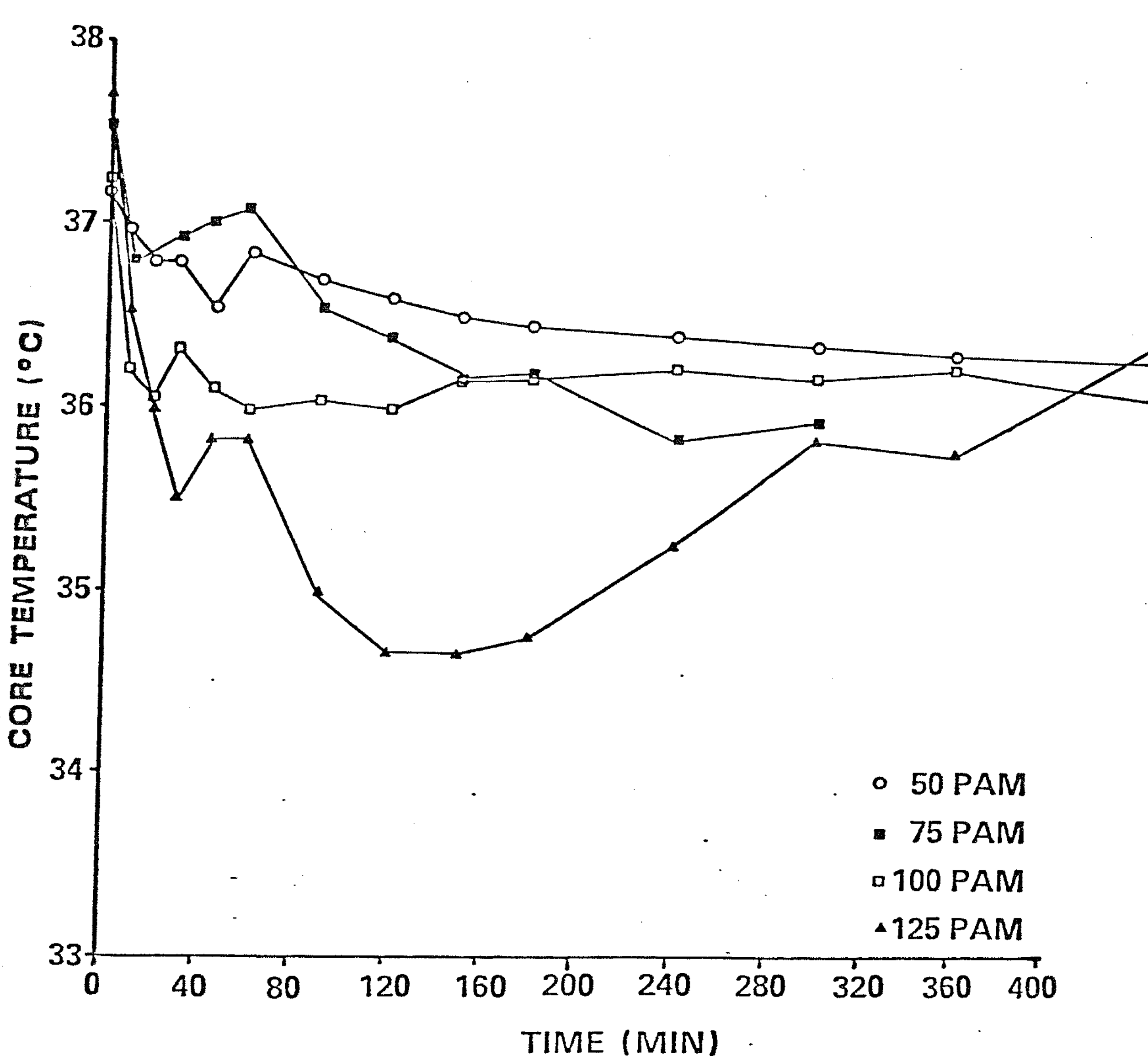

Figure 32

Core Temperatures (°C) Obtained for 2-PAM (50 – 125 mg/kg; i.p.) in Mice

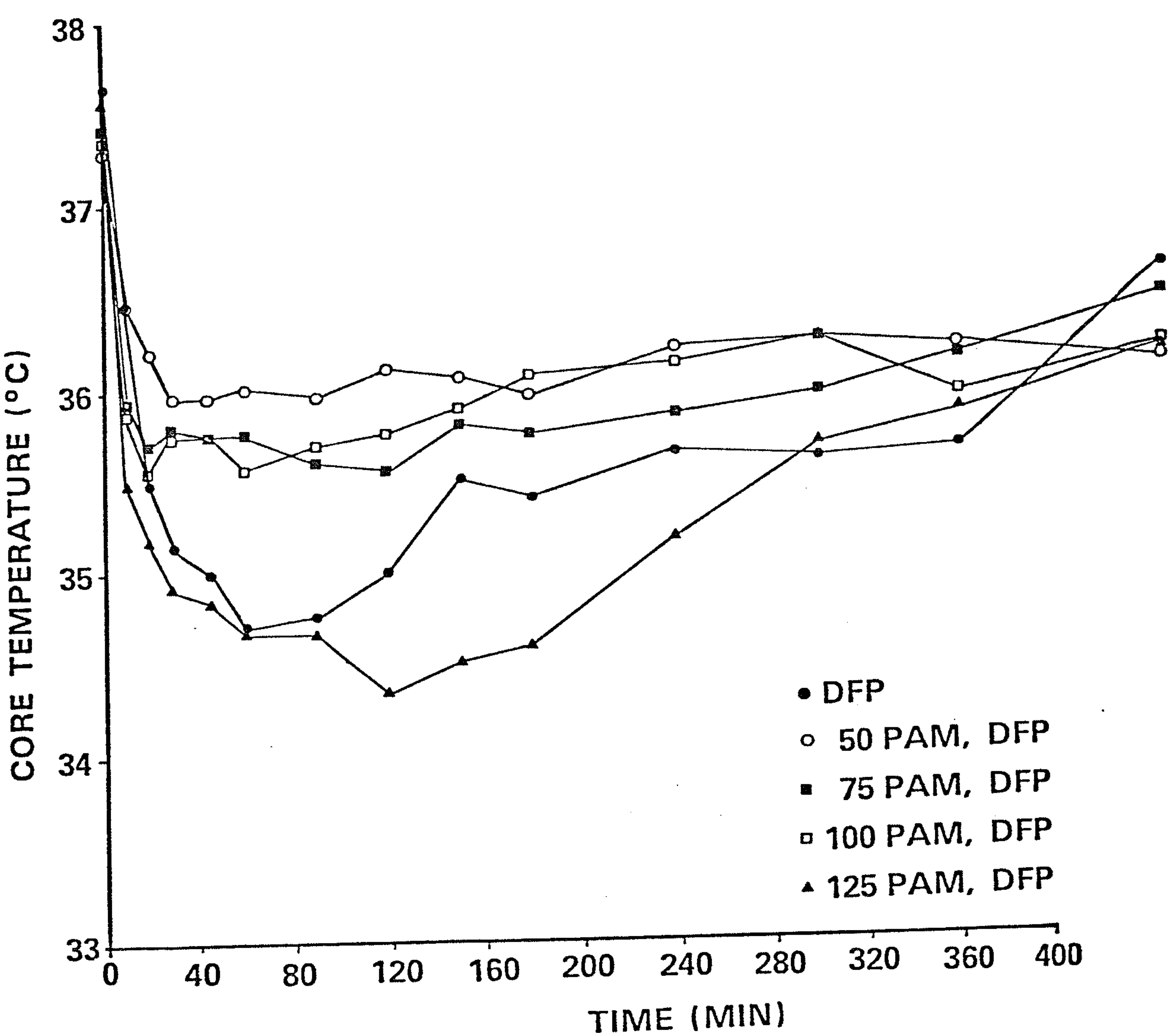

Figure 33

Core Temperatures (°C) Obtained for 2-PAM (50 – 125 mg/kg; i.p.) Administered Prophylactically 5 Minutes Prior to Exposure with DFP (3 mg/kg; i.p.) in Mice

The hypothermia data obtained from the therapeutic administration of 50-125 mg/kg doses of 2-PAM was found to illustrate the reverse trend from that seen for 2-PAM administered alone or prophylactically (Figure 34). The data for the 50 mg/kg dose 2-PAM appeared to be different from the 75, 100 and 125 mg/kg doses of 2-PAM as well as from the data obtained from the administration of DFP alone.

Figures 35 to 38 illustrate the hypothermia data for a single dose of 2-PAM over the range of 50-125 mg/kg administered alone, prophylactically or therapeutically 5 minutes before or after DFP as well as for DFP alone. The data for the 50 mg/kg dose of 2-PAM administered before or after DFP illustrates differences from the hypothermia induced by DFP alone (Figure 35). The hypothermia for the 75 and 100 mg/kg doses of 2-PAM administered prophylactically indicates differences from the data obtained for DFP alone. No differences could be observed when those doses of 2-PAM were given therapeutically against DFP. The hypothermia data for 125 mg/kg 2-PAM given before or after DFP indicate no differences when compared to the data for DFP alone.

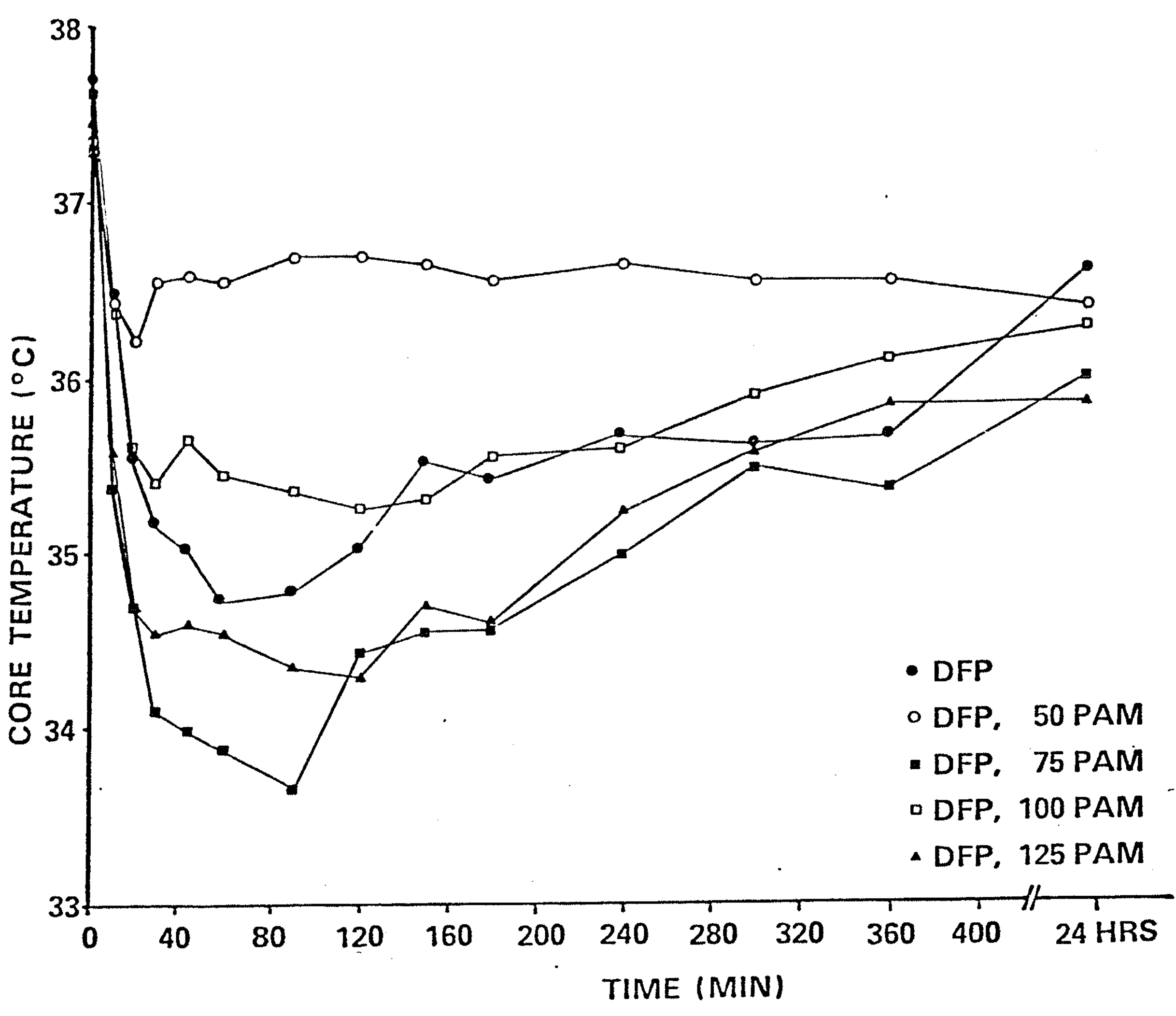

Figure 34

Core Temperatures (°C) Obtained for 2-PAM (50 – 125 mg/kg; i.p.) Administered Therapeutically 5 Minutes Ater Exposure with DFP (3 mg/kg; i.p.) in Mice

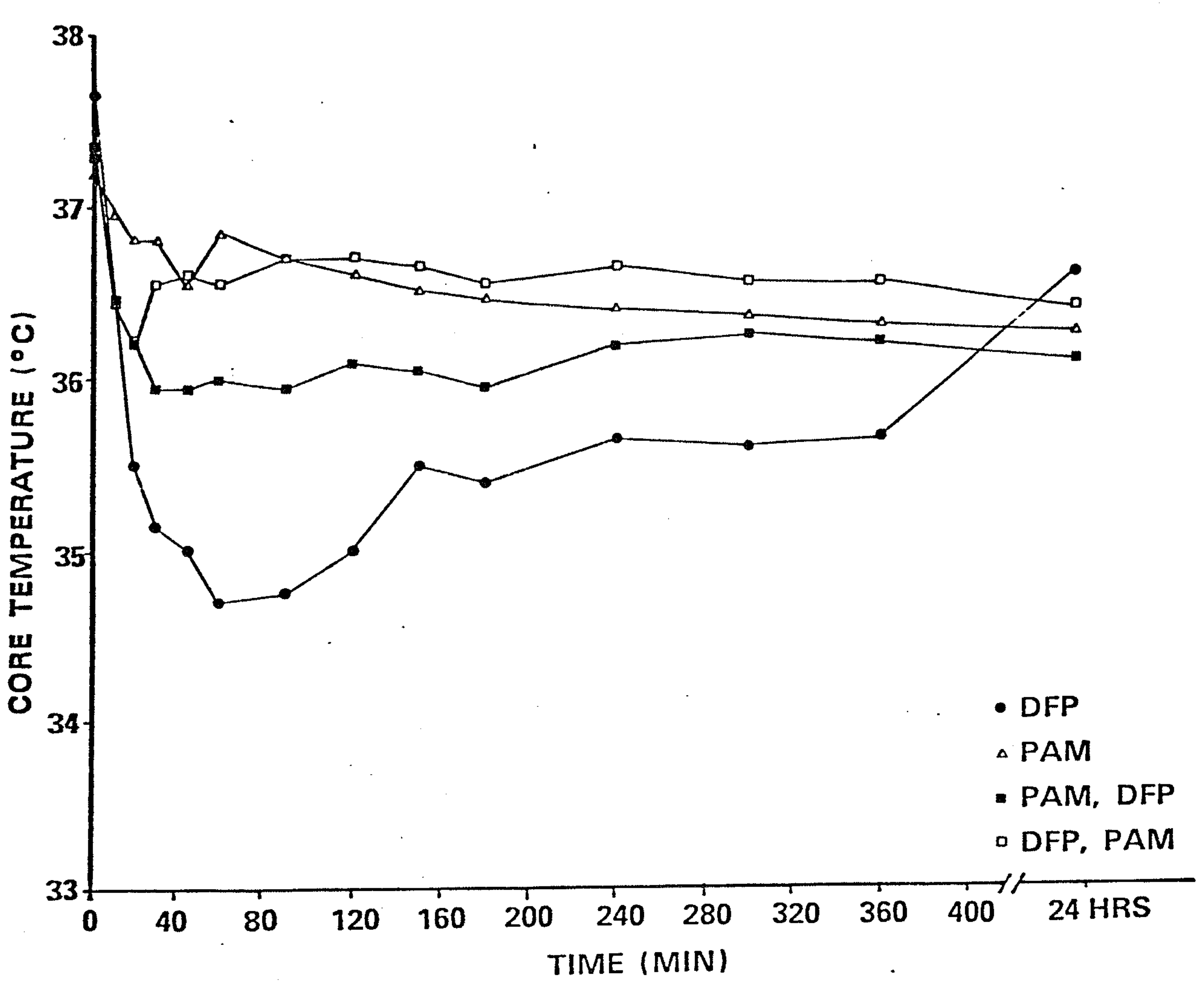

Figure 35

Core Temperatures (°C) Obtained for 2-PAM (50 mg/kg; i.p.) Administered Alone or 5 Minutes Before or After Exposure to DFP (3 mg/kg; i.p.) in Mice

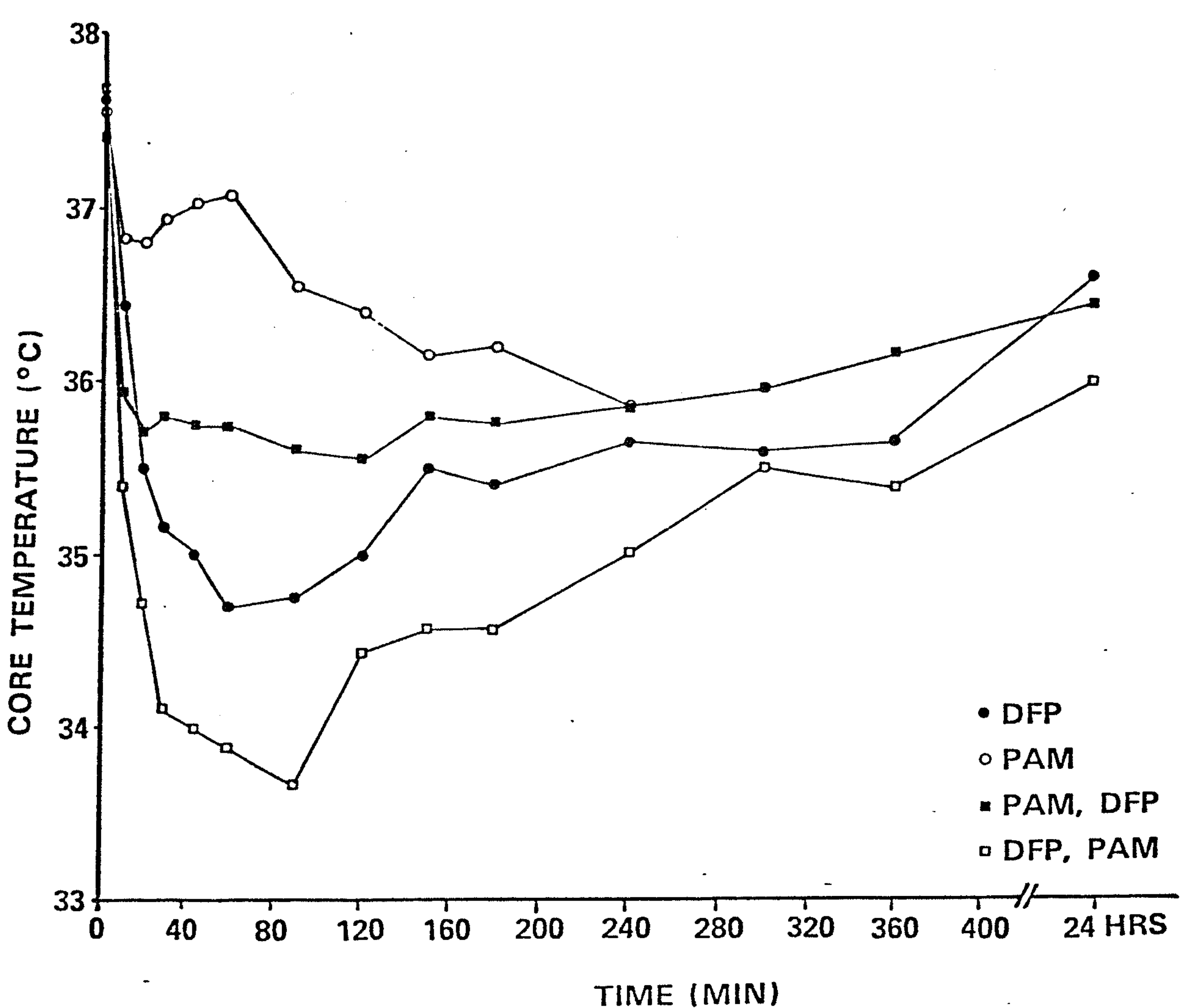

Figure 36

Core Temperatures (°C) Obtained for 2-PAM (75 mg/kg; i.p.) Administered Alone or 5 Minutes Before or After Exposure to DFP (3 mg/kg; i.p.) in Mice

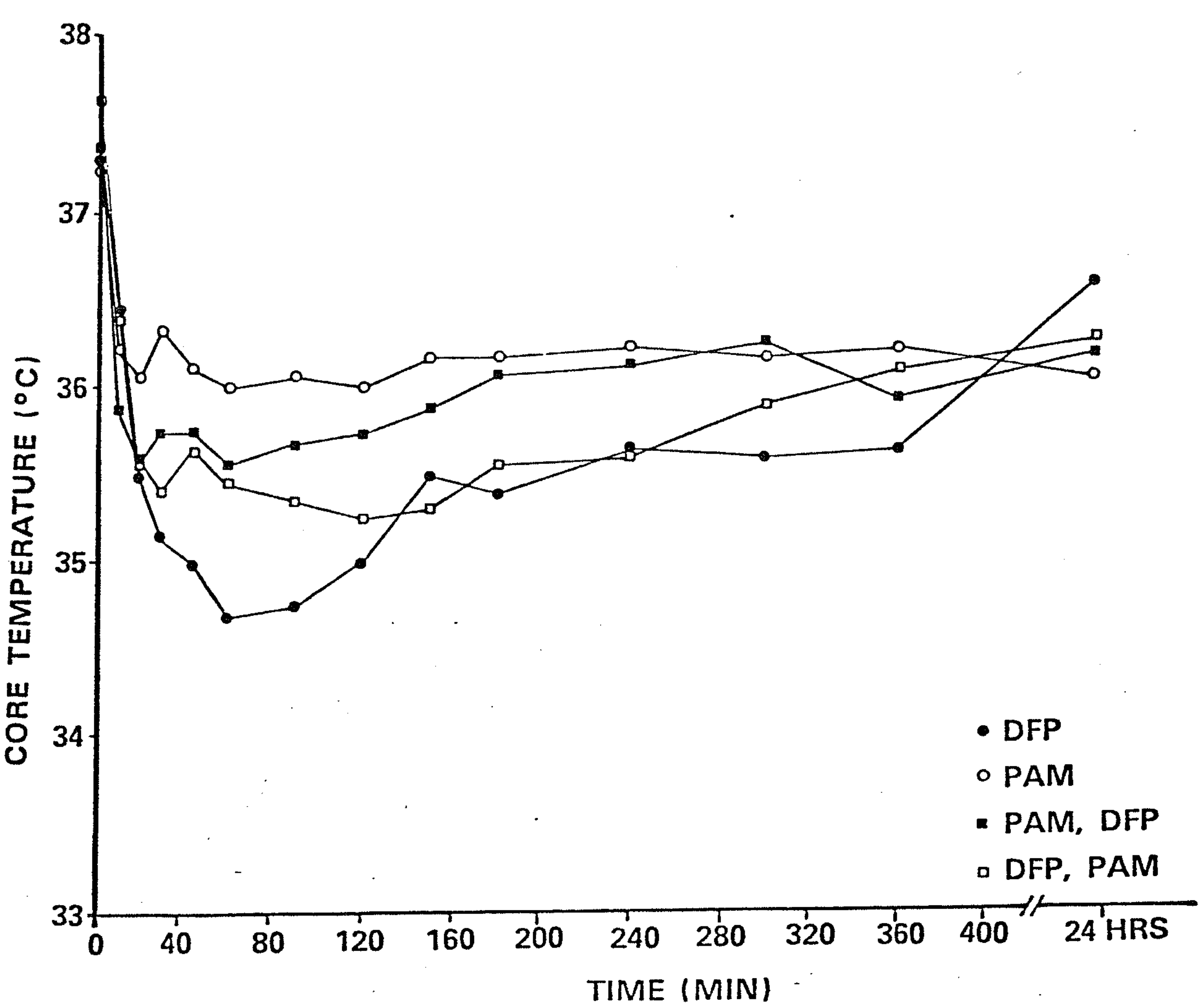

Figure 37

Core Temperatures (°C) Obtained for 2-PAM (100 mg/kg; i.p.) Administered Alone or 5 Minutes Before or After Exposure to DFP (3 mg/kg; i.p.) in Mice

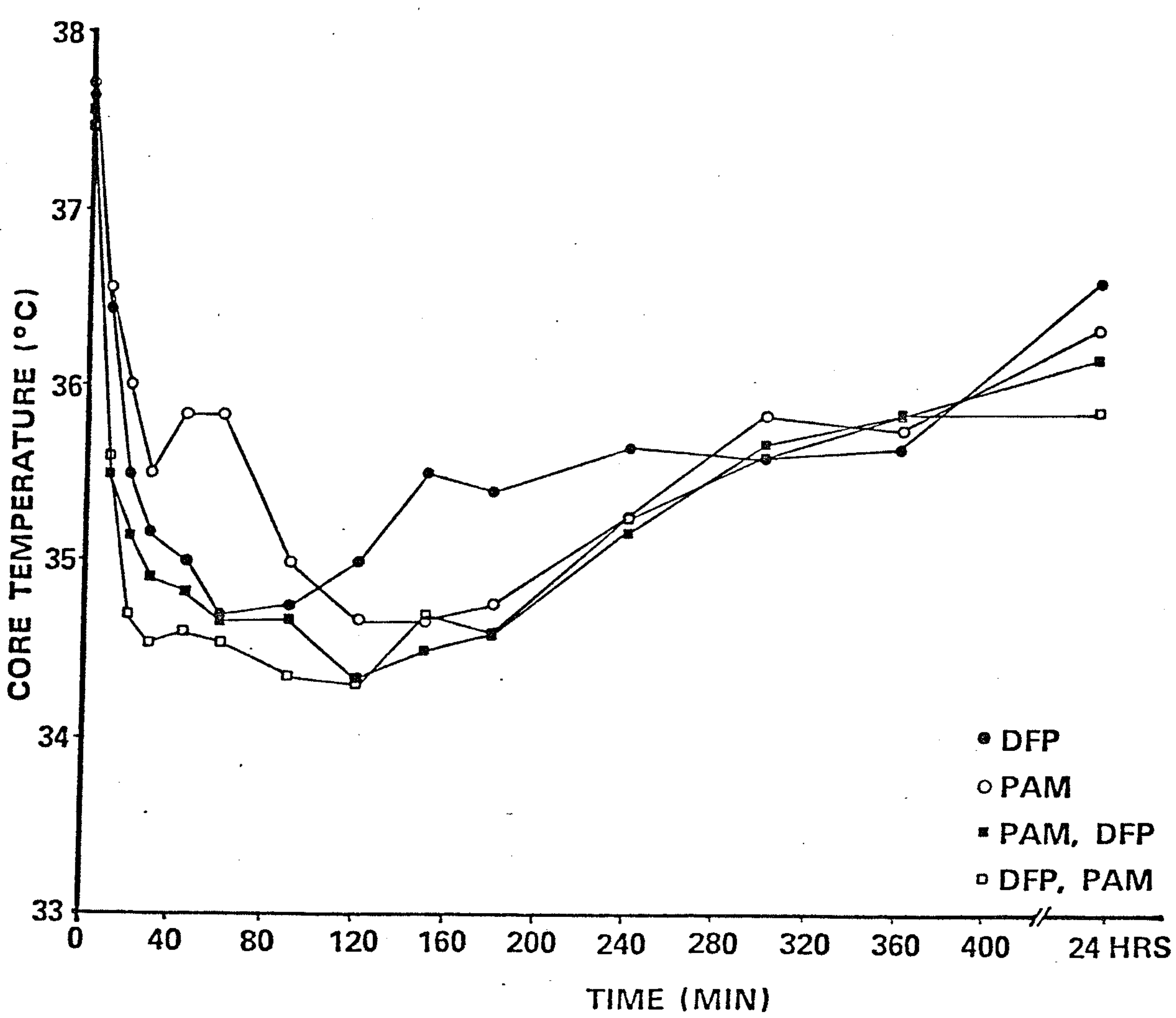

Figure 38

Core Temperatures (°C) Obtained for 2-PAM (125 mg/kg; i.p.) Administered Alone or 5 Minutes Before or Ater Exposure to DFP (3 mg/kg; i.p.) in Mice

Figures 39 and 40 illustrate what happened to the core temperature when atropine was administered with 2-PAM in a combined solution. As presented, both 2-PAM and atropine were found to induce a hypothermia in mice on an individual basis producing a core temperature drop of between 1-1.5 °C. This hypothermic effect was found to be additive when they were injected as a combined solution. Within 30 minutes the core temperature was found to have dropped 2-2.5 °C. Unlike the dose dependent hypothermia seen with 50-125 mg/kg doses of 2-PAM on its own, no dose dependent hypothermia was seen when 50 and 75 mg/kg 2-PAM were administered as a combined solution with 17.4 mg/kg atropine (Figure 41).

In addition, when administered as a combined solution, 2-PAM (50 and 75 mg/kg; i.p.) and atropine (17.4 mg/kg) given alone, prophylactically, or therapeutically against DFP were similar to one another (Figures 42 and 43). When compared to the hypothermia data obtained from the administration of DFP alone however, those doses appeared to be different from the DFP data. This was unlike what was found to occur for the administration of 2-PAM alone. In addition it is noteworthy to mention that when administered as a combined solution, 2-PAM and atropine seemed to have a faster onset to hypothermia (10 minutes) than that seen with 2-PAM alone (20-30 minutes). The recovery from the hypothermia as well seemed to be more rapid for the combined solution than for 2-PAM alone (Figures 44 to 46).

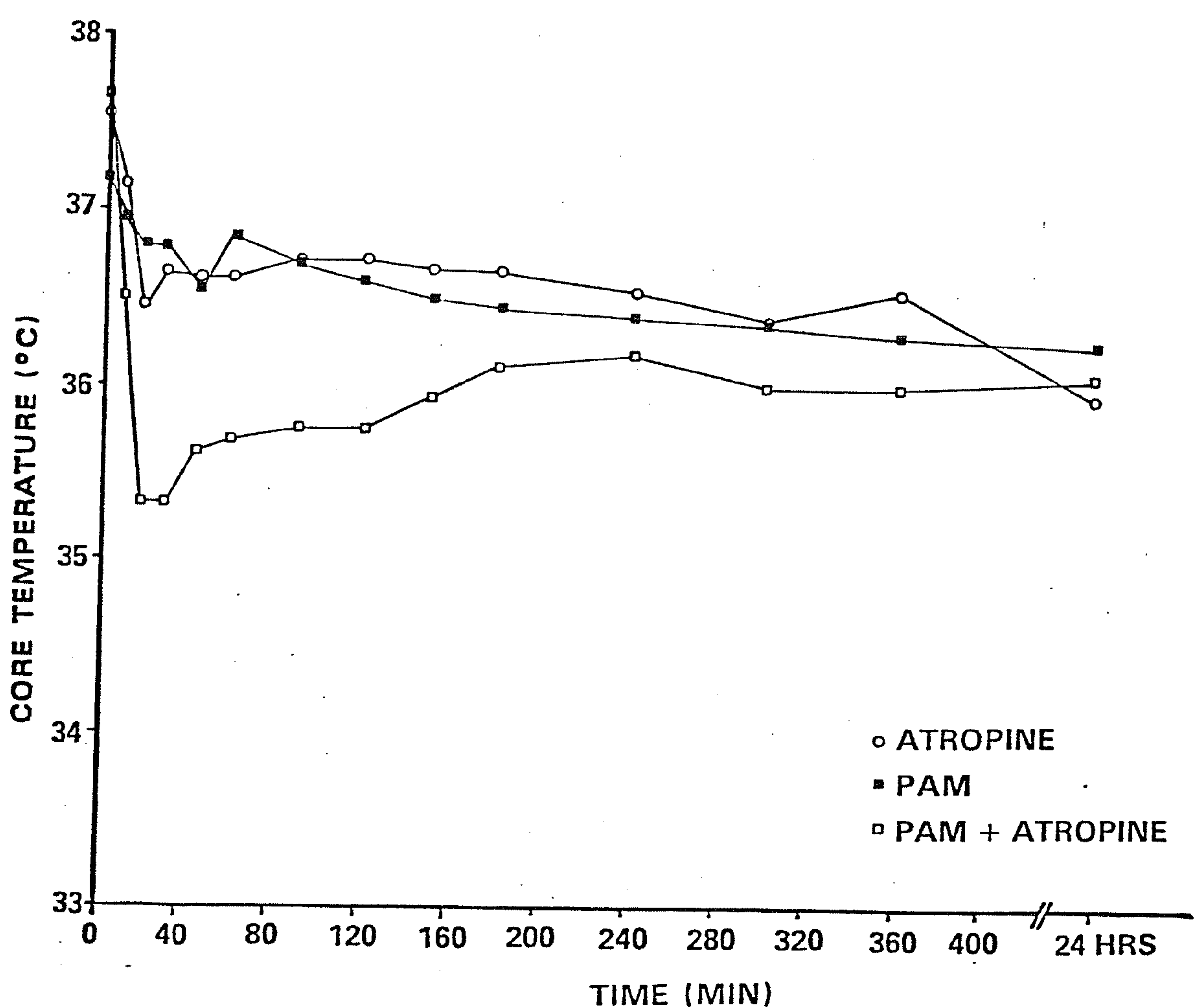

Figure 39

Core Temperatures (°C) Obtained for the Combined Administration of 2-PAM and Atropine (50 and 17.4 mg/kg, Respectively; i.p.) in Mice

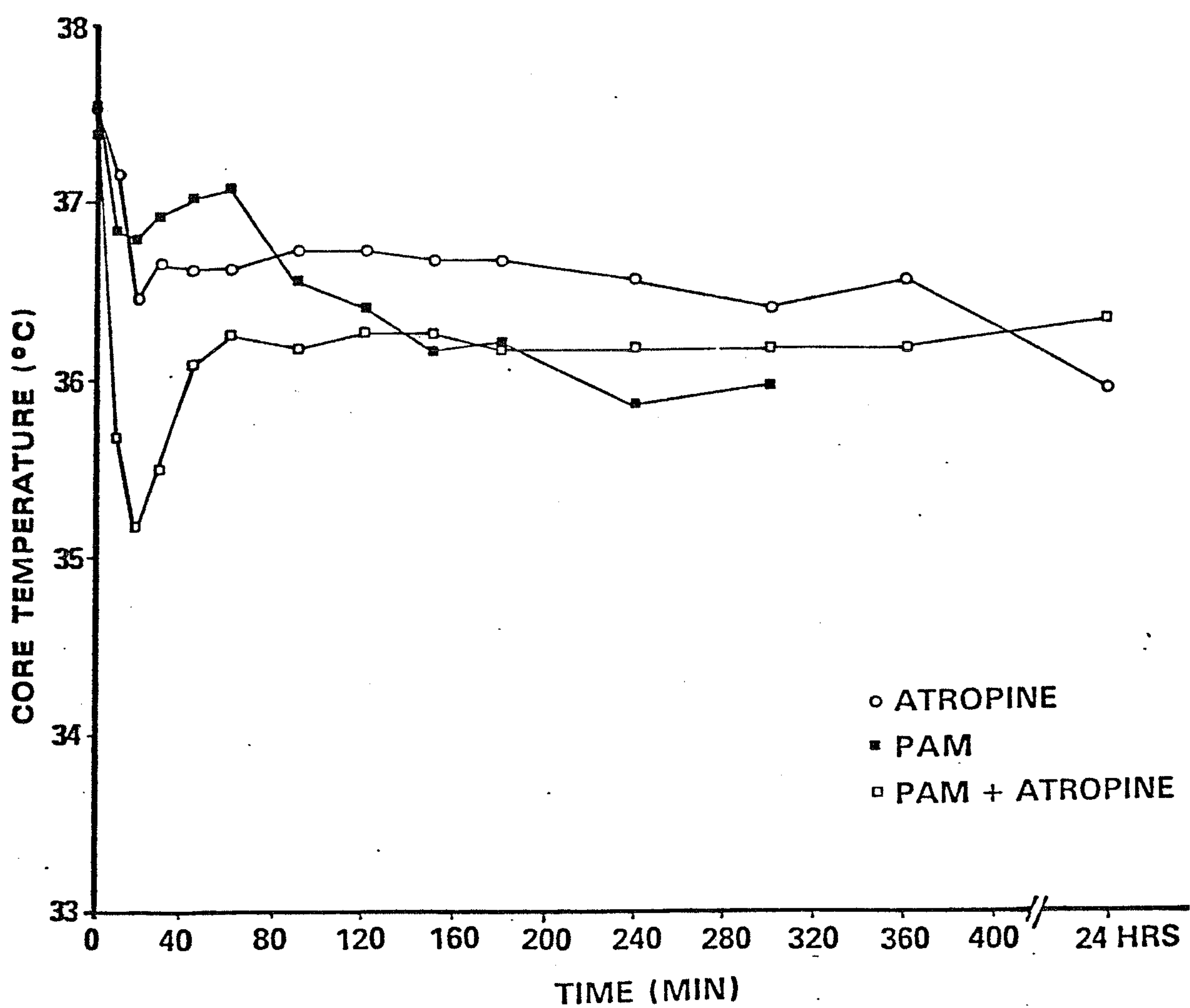

Figure 40

Core Temperatures (°C) Obtained for the Combined Administration of 2-PAM and Atropine (75 and 17.4 mg/kg, Respectively; i.p.) in Mice

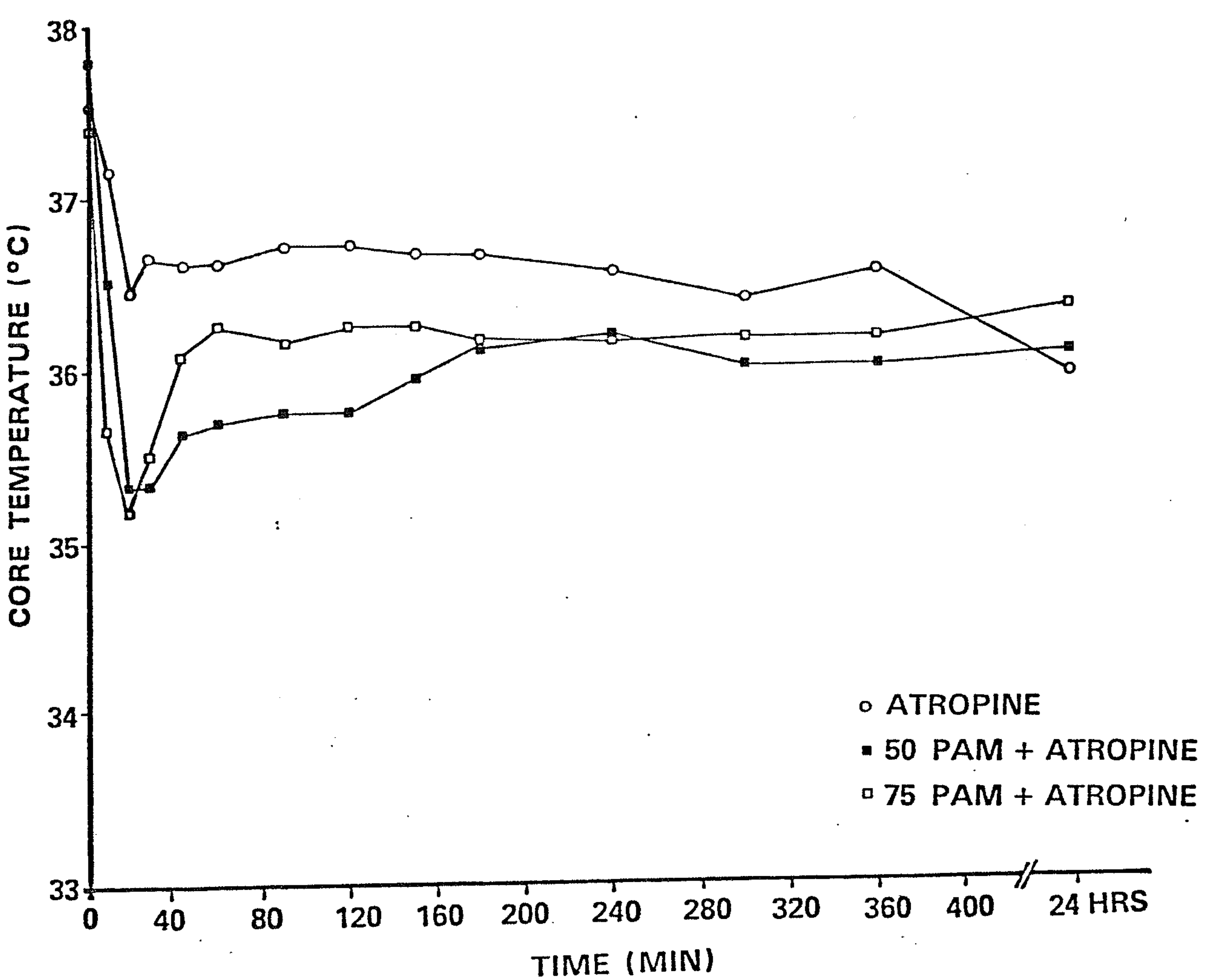

Figure 41

Core Temperatures (°C) Obtained for 2-PAM (50 and 75 mg/kg; i.p.) Administered as a Combined Solution with Atropine (17.4 mg/kg; i.p.) in Mice

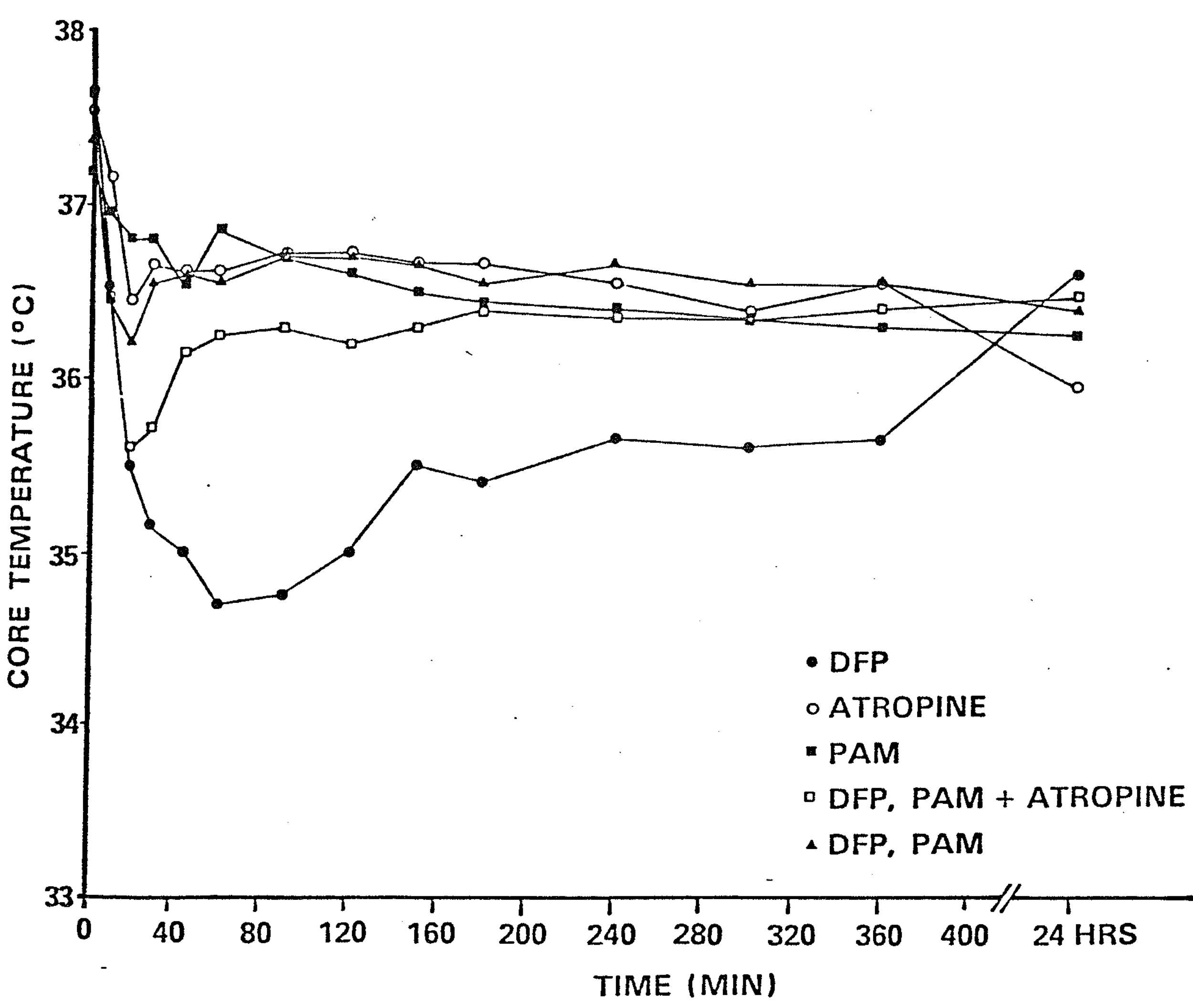

Figure 42

Core Temperatures (°C) Obtained for 2-PAM (50 mg/kg; i.p.) Administered Alone or in Combination with Atropine (17.4 mg/kg; i.p.) 5 Minutes After Exposure to DFP (3 mg/kg; i.p.) in Mice

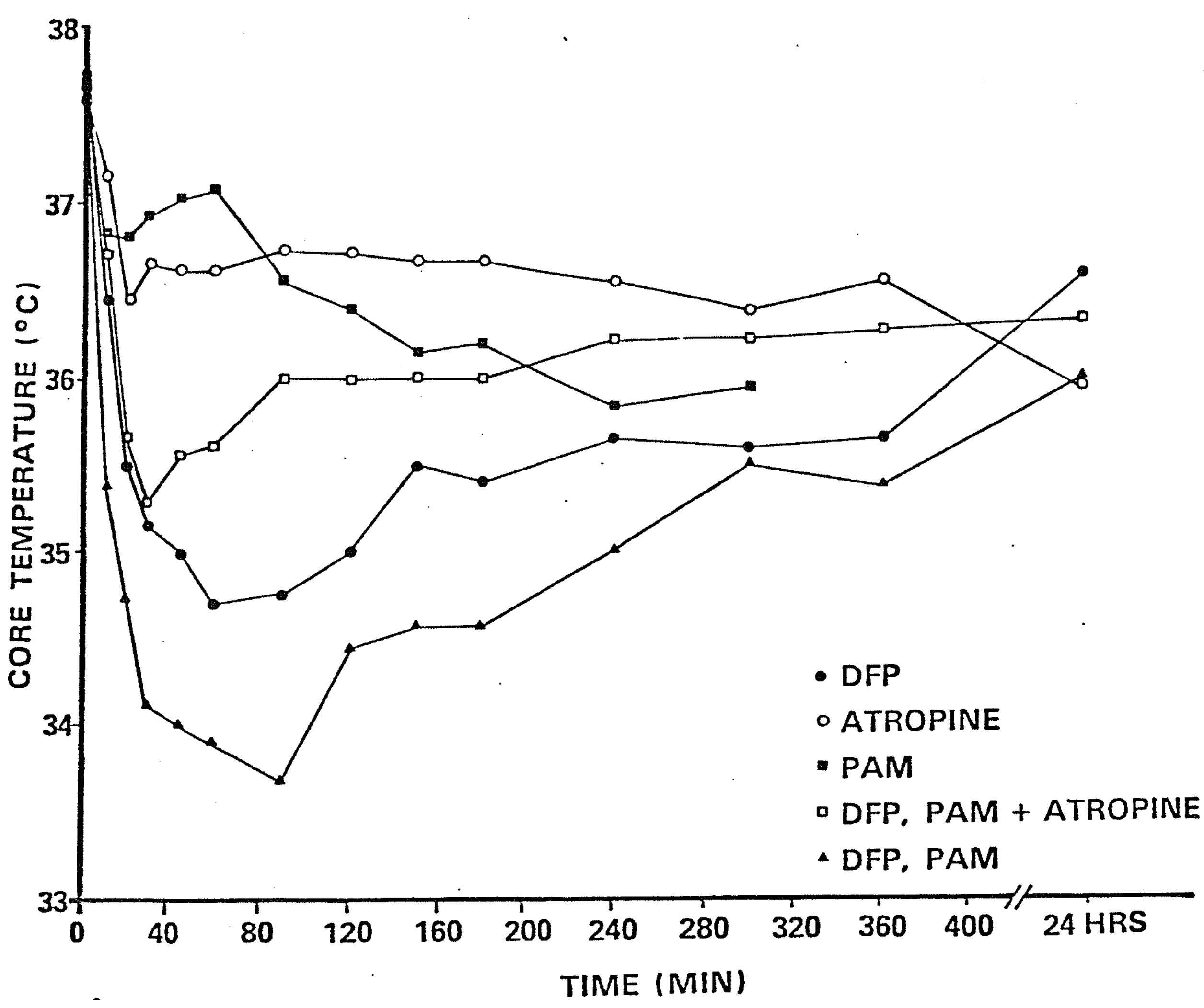

Figure 43

Core Temperatures (°C) Obtained for 2-PAM (75 mg/kg; i.p.) Administered Alone or in Combination with Atropine (17.4 mg/kg; i.p.) 5 Minutes Ater Exposure to DFP (3 mg/kg; i.p.) in Mice

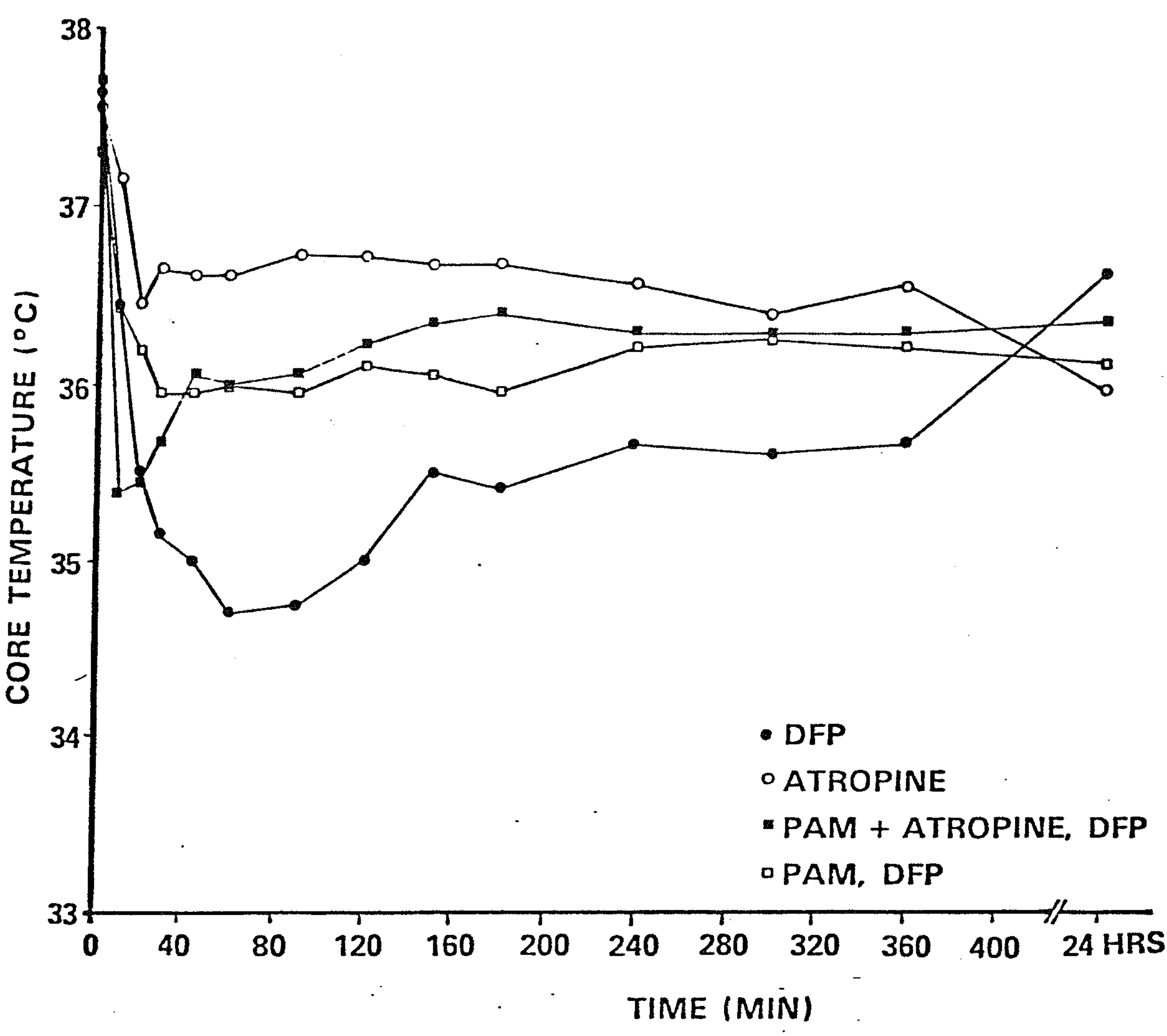

Figure 44

Core Temperatures (°C) Obtained for 2-PAM (50 mg/kg; i.p.) Administered Alone or in Combination with Atropine (17.4 mg/kg; i.p.) 5 Minutes Prior to Exposure to DFP (3 mg/kg; i.p.) in Mice

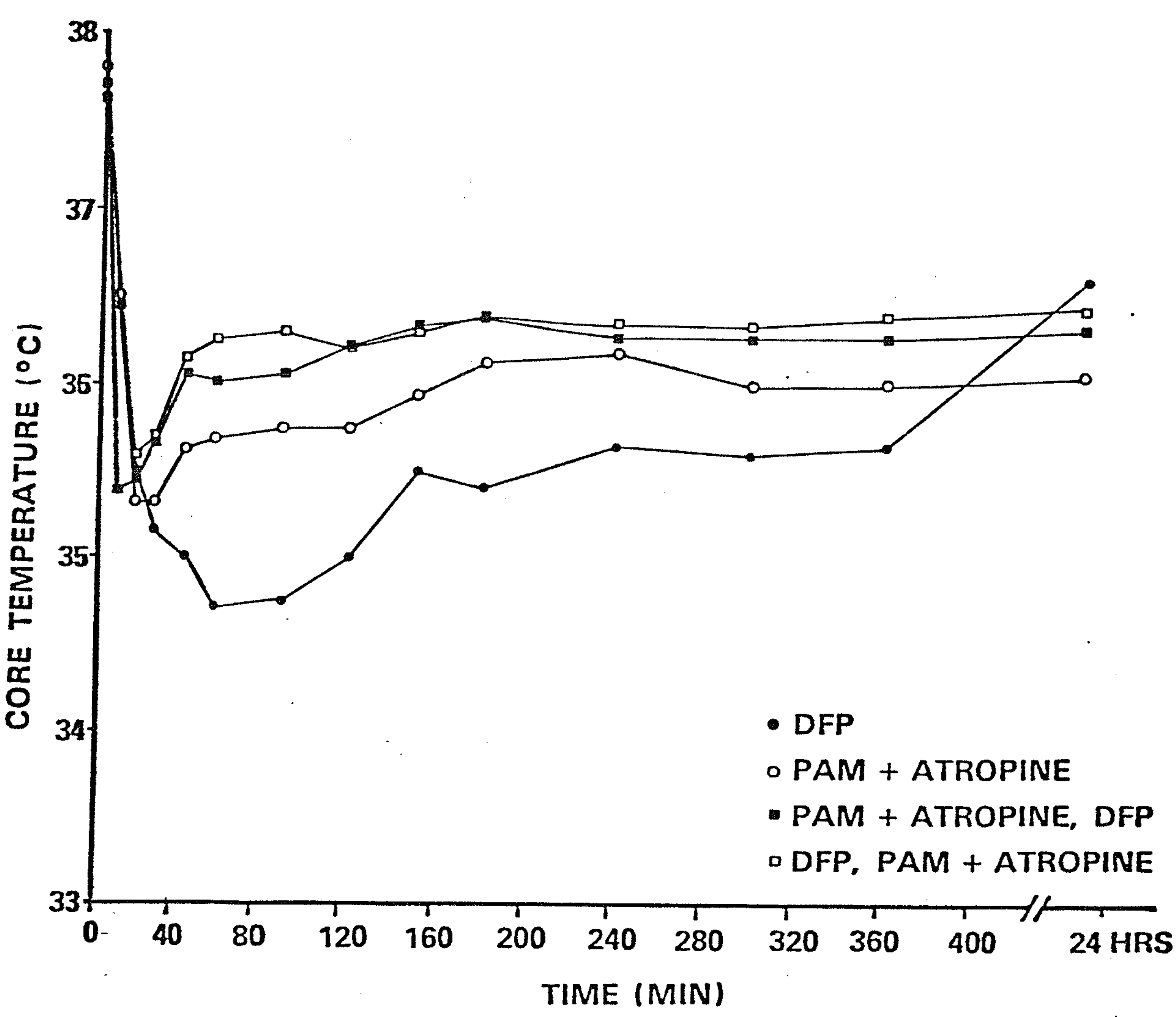

Figure 45

Core Temperatures (°C) Obtained from the Administration of a Combined Solution of 2-PAM and Atropine (50 and 17.4 mg/kg, Respectively; i.p.) Alone or 5 Minutes Before or After Exposure to DFP (3 mg/kg; i.p.) in Mice

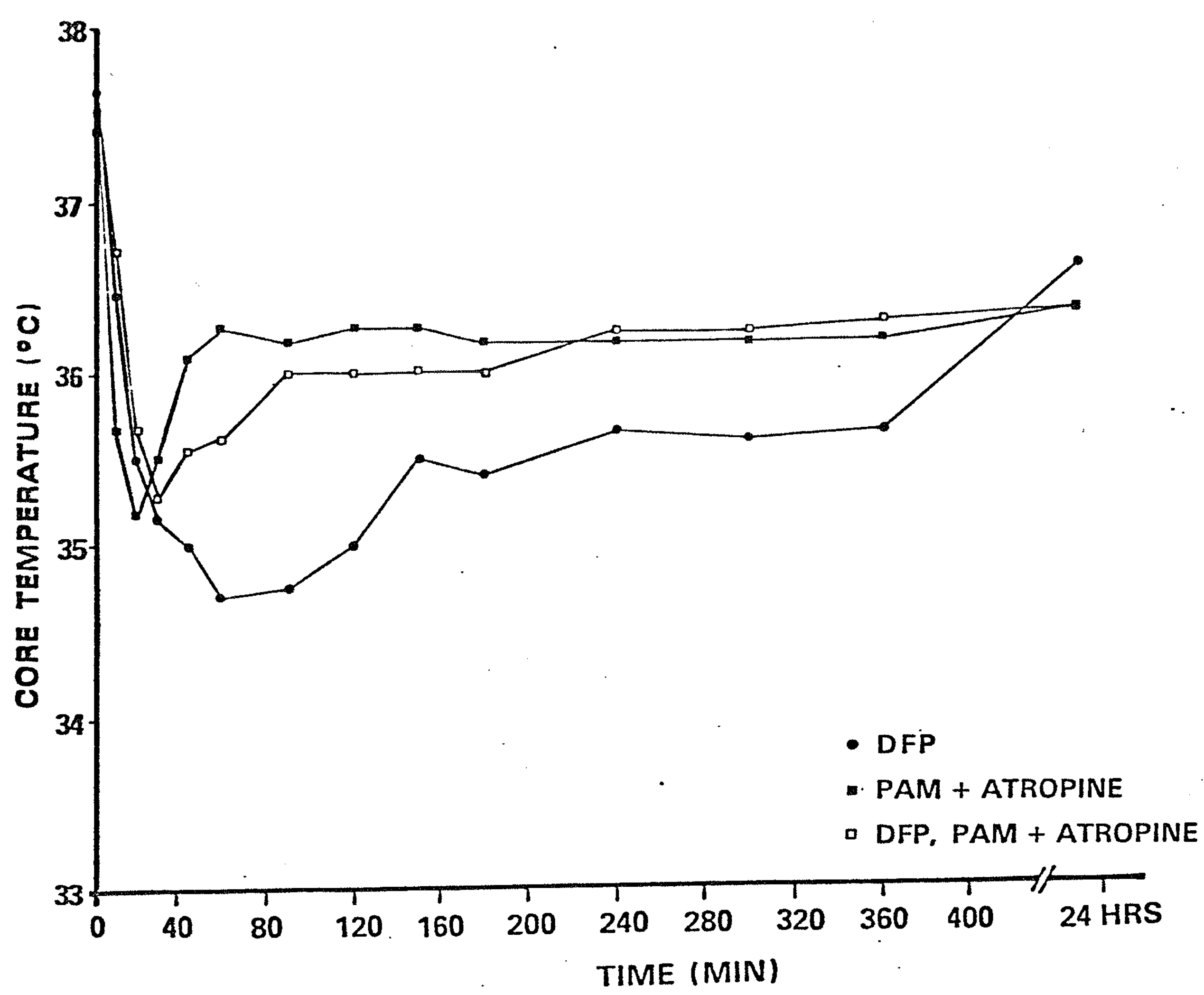

Figure 46

Core Temperatures (°C) Obtained from the Administration of a Combined Solution of 2-PAM and Atropine (75 and 17.4 mg/kg, Respectively; i.p.) Alone or 5 Minutes After Exposure to DFP (3 mg/kg; i.p.) in Mice

2. Chronic Study

The concentration of 2-PAM in the serum and brain of mice chronically exposed to DFP for 21 days are given in Table 18. Bonferonni's method of analysis applied to the experimentally determined 2-PAM serum and brain concentrations suggested that there were no significant differences ($p < 0.05$) over the 21 day study period.

AChE activities in both serum and brain of mice chronically exposed to DFP for 21 days are given in Table 18. Significant differences were seen in both serum and brain AChE activities however these were expected because increasing doses of DFP (1-3 mg/kg; i.p.) were given over the 21 day study period. Figures 47 and 48 show the relationship between concentration of 2-PAM and AChE activity.

Table 18

Brain and Serum Acetylcholinesterase Activities and 2-PAM Concentrations in Mice During Chronic Exposure to DFP

DAY	AChE ACTIVITY		CONCENTRATION OF 2-PAM	
	BRAIN (nm/mg/min)	SERUM (μm/mL/min)	BRAIN (ng/mL)	SERUM (μg/mL)
0	12.7 ± 0.4[1] (4)[2]	2.5 ± 0.1 (4)	167.9 ± 68.6 (7)	9.5 ± 0.3 (9)
1	8.3 ± 0.4 (5)	1.5 ± 0.1 (5)	185.8 ± 17.2 (5)	11.4 ± 1.2 (5)
3	5.2 ± 0.5 (5)	1.3 ± 0.1 (5)	202.7 ± 31.1 (5)	11.5 ± 1.1 (5)
5	4.8 ± 0.5 (5)	1.2 ± 0.1 (5)	191.0 ± 16.5 (5)	10.4 ± 0.8 (5)
7	3.6 ± 0.4 (5)	1.1 ± 0.0 (5)	146.6 ± 8.4 (5)	9.3 ± 1.1 (5)
9	2.7 ± 0.2 (5)	1.0 ± 0.0 (5)	185.9 ± 8.5 (5)	11.0 ± 0.8 (5)
11	2.1 ± 0.1 (5)	1.1 ± 0.1 (5)	184.9 ± 10.7 (5)	12.4 ± 1.3 (5)
15	1.8 ± 0.0 (2)	0.7 ± 0.1 (2)	159.3 ± 0.7 (2)	9.4 ± 1.5 (2)
17	1.5 ± 0.1 (4)	0.7 ± 0.1 (4)	154.2 ± 10.9 (4)	9.5 ± 1.0 (4)
19	1.6 ± 0.1 (4)	0.7 ± 0.0 (4)	106.9 ± 22.9 (4)	9.6 ± 1.4 (4)
21	1.9 ± 0.5 (2)	0.7 ± 0.0 (2)	173.8 ± 31.6 (2)	11.3 ± 3.4 (2)

1 Mean ± SEM

2 Number of Animals

3 DFP was administered daily by i.p. injection. Initial dose was 1 mg/kg. Dose was increased to 2 and 3 mg/kg on days 7 and 14 respectively.

4 A combined solution of 50 mg/kg PAM + 17.4 mg/kg Atropine was administered i.p. on alternating days starting at day 0 and continuing to day 21 to a group of 5 animals. Animals were sacrificed after 15 min exposure to PAM.

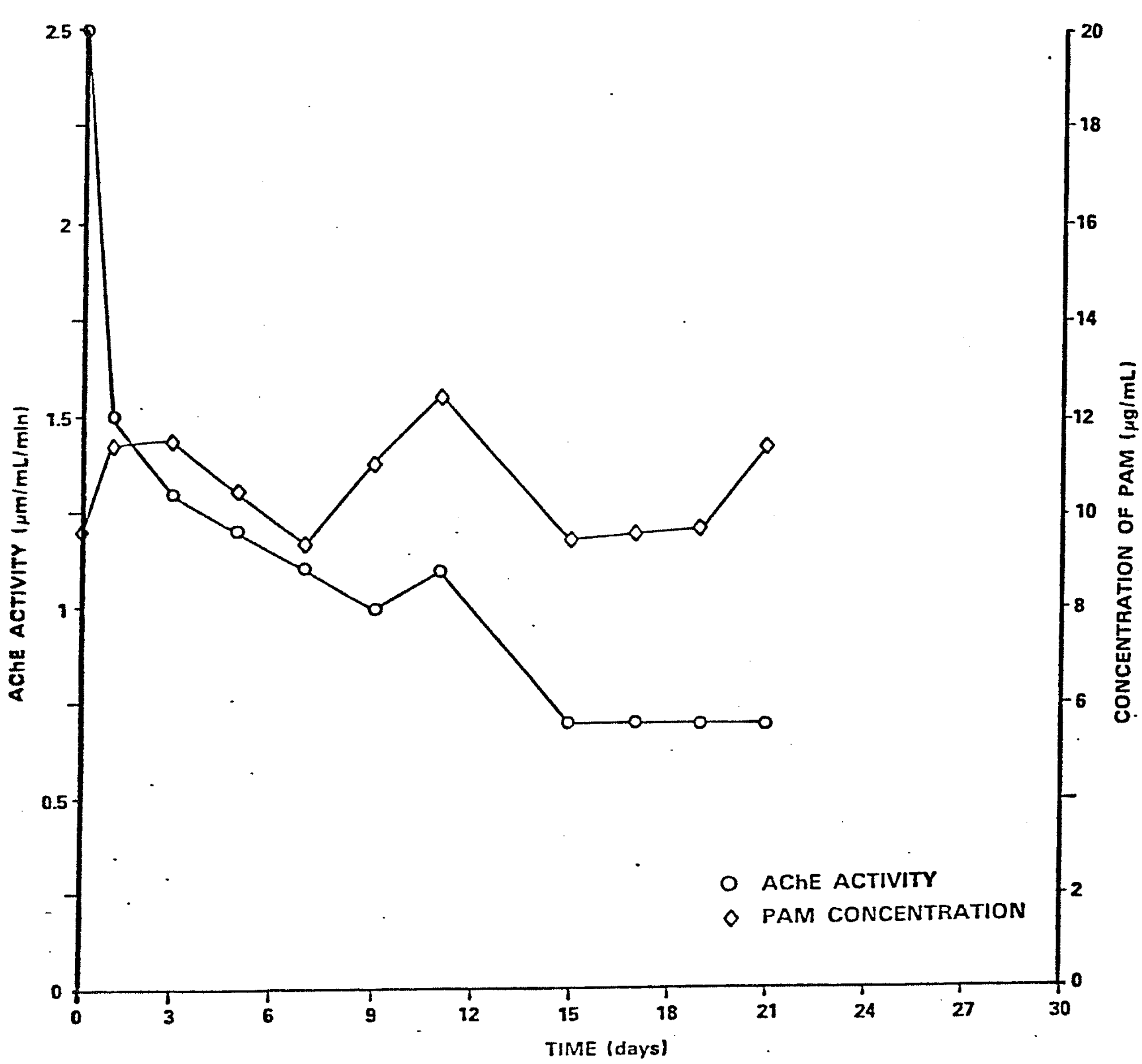

Figure 47

Serum AChE Activity (µm/mL/min) and 2-PAM Concentration (µg/mL) *Versus* Time (days) in Mice

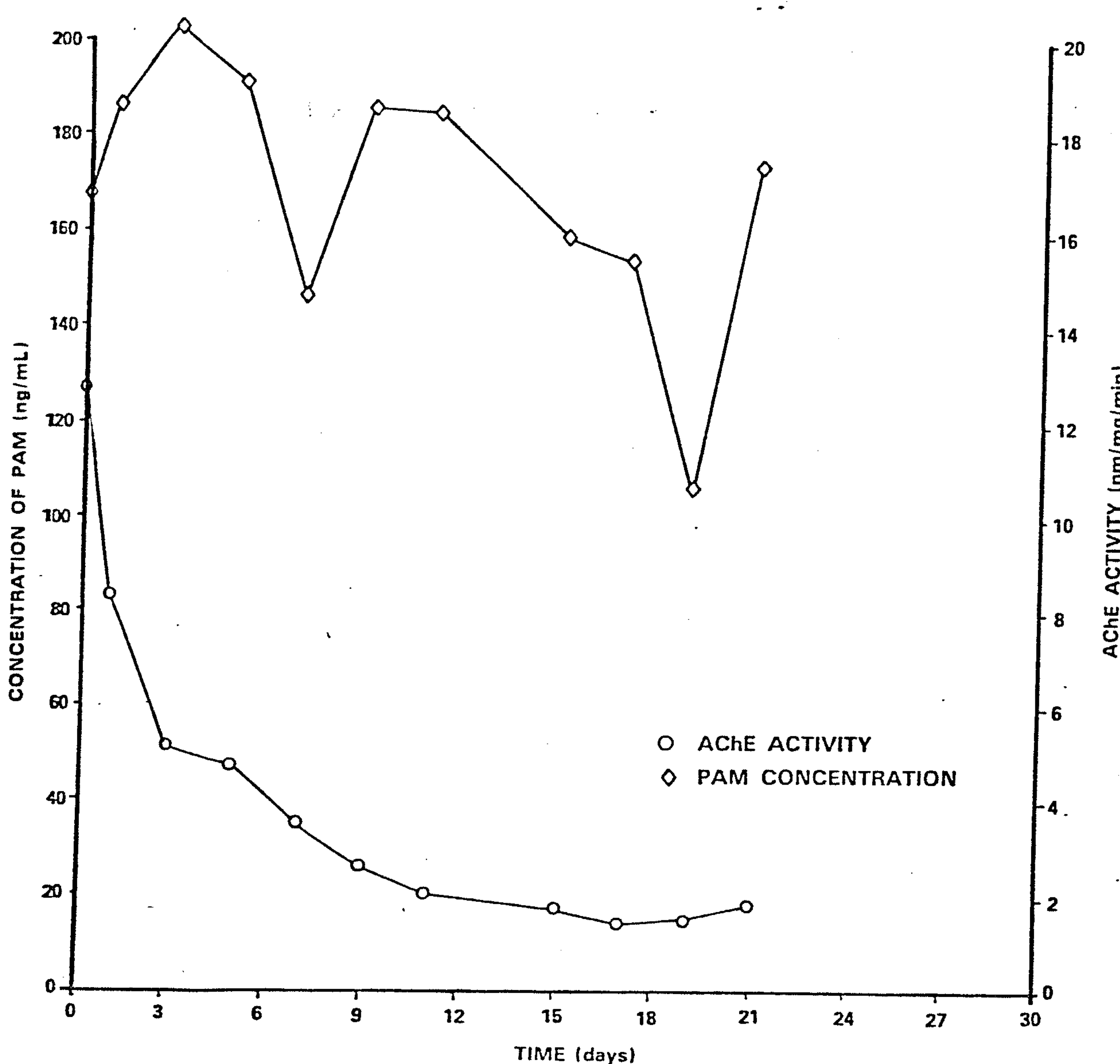

Figure 48

Brain AChE Activity (nm/mg/min) and 2-PAM Concentration (ng/mL) *Versus* Time (days) in Mice

IV. Discussion

A. Analytical

1. HPLC Quantitation of 2-PAM

The use of HPLC to determine quantities of oxime in biological specimens is not a novel concept (17,38,124,126, 196). In this work, the standard curves obtained for each preliminary evaluation of the procedure were found to be reproducible and linear throughout the study. Additionally, they were not significantly different from one another even though the sensitivity of the HPLC method was challenged. Because the standard curves for the preliminary trials in water and water plus the cleanup procedure of perchloric acid and acetonitrile as compared to brain and serum spiked samples were found to be reproducible, linear and not significantly different from one another over time, subsequent determinations of 2-PAM quantities in serum and brain were made by direct comparisons of peak height ratio values of unknowns to the standard curves.

2. Acetylcholinesterase Assay

The use of the radiometric procedure of Slakotos *et al* to determine acetylcholinesterase activities is a standard procedure utilized for investigational purposes in the area of organophosphate intoxication (182). Acetylcholinesterase activities are determined in the linear portion of the enzyme

activity versus enzyme concentration curve. The inclusion of blank and control samples provides the necessary information with respect to the efficiency of the assay. In addition, all determinations of significance are made directly to control samples which are included in each run of the assay and which provide for any shortcomings the assay may have.

B. Animal Study

The value of oximes for treating organophosphate poisoning was suggested initially by experiments involving hydroxylamine (8,58,60). In conjunction with atropine, oximes have been demonstrated to be the most efficacious antidotes for the protection against several doses of most organophosphorus compounds. Whereas the mode of action of oximes as reactivators has been attributed to their ability to displace the phosphorus-containing moiety from the inhibited AChE, their effect as antidotes is not wholly ascribed to this mechanism (58,94).

It has been shown that oximes themselves are poor antidotes. However, when given in combination with atropine, which by itself is also a weak antidote for organophosphate poisoning, they are able to protect an animal against an organophosphate poison (8,58,60,94). This potentiating effect is not well understood but it has been suggested that the

central antimuscarinic effects of atropine act to prolong life as well as provide more time for the oxime to exert its effects (58,60,94).

Oximes, because of their ionized nature, do not appear to be able to penetrate the blood brain barrier to any appreciable extent and consequently their effects have been ascribed to be predominantly peripheral (36,112,128). This in itself has lead to a degree of discrepancy. Central reactivation of AChE, in past, has been used as a measure of oxime in the brain. It has been demonstrated that there is poor correlation between central reactivating characteristics of the oxime with the amount of oxime shown to be present centrally and the degree of recovery of the animal models (27-31,41,90, 132,143,173).

The use of oximes as therapeutic agents does presuppose the phosphorylation of AChE following exposure to organophosphorus compounds. Oximes, however, have also been shown to interact directly with organophosphorus compounds thereby reducing their half-lives from several hours to a few minutes (138). In addition, oximes and organophosphorus compounds are known to exert direct pharmacological effects on their own. As esterase content may be limited, it is not clear how an animal can survive a quantity of organophosphate which is more than sufficient to inhibit and saturate all the esterases in the body. There is difficulty in determining how much these "other" actions contribute to the recovery of the animal.

The mechanism by which oximes protect against the toxic effects of organophosphate esters is complex and may be the net result of at least 3 factors:

1. reactivation of phosphorylated AChE;
2. direct chemical reaction between the oxime and OP, and/or;
3. certain pharmacological actions which the oximes exert directly on tissues other than by reactivation of ACh.

Factor 1 is considered to be the predominant mechanism of protection against organophosphate intoxication while factors 2 and 3 are considered to be subsidiary in this regard.

1. Acute Study

a. Route of administration and Half-life

Duke and deCandole have shown that TMB-4 was maintained at a higher blood level when compared to similar doses of PAM chloride or P2S by either the i.v. or i.m. routes of administration (55). In addition, they found that there was no difference in the speeds of absorption from the muscle following i.m. injection. They did find however, that after i.v. injection, blood concentration fell rapidly for approximately 3 minutes and slowly thereafter. It was suggested that there was an establishment of an equilibrium between

intravascular and extravascular oxime and that the slow fall was due to excretion of the oxime. In addition, they suggested that TMB-4 was a more superior reactivator than PAM chloride and P2S in antiChE poisoning because of its longer retention in blood. They found a T1/2 = 35 minutes for PAM chloride after an 18 mg/kg dose in rabbits. Jager et al found a T1/2 = 54 minutes for PAM after a 15 mg/kg i.v. injection in man (102).

Green et al have shown that in guinea pigs, the plasma levels of 2-PAM were elevated in a dose dependent manner following increasing doses of sarin (78). They demonstrated that at 2 minutes after i.m. injection of 2-PAM and atropine that maximal plasma concentration of PAM occurred. Green et al also suggested that a reasonable explanation for the elevated 2-PAM levels was that following AChE inhibiton by sarin, tissue and blood levels of ACh increased which caused profound changes in blood flow to various organs (78). They have also suggested that there is a decreased volume of distribution (VD) of 2-PAM which is commensurate with changes in the pattern and rate of blood flow to the various organs (78). They concluded that 2-PAM plasma levels increased as blood flow to the sites of distribution and excretion decreased.

Since 2-PAM is primarily excreted from the body by renal activity, alterations in blood flow or active

transport mechanisms in the kidney would yield increased levels of oxime in the plasma (18,193,200). There have also been suggestions which have indicated an interaction between ACh and renal function producing effects as just described. The direct inhibitory effect of sarin on renal enzymes or alteration in tissue distribution of 2-PAM by endogenous compounds can not be precluded as well. In relation to the level of exposure to OP compounds, it must be taken into consideration that any interaction which influences the plasma level of 2-PAM (which is dependent upon the toxin and antidote) will be contributing to and complicating any effects which may be seen experimentally.

DFP is known to penetrate all types of tissues freely. The direct chemical reaction between oximes and DFP cannot completely account for the high protecting effect of oximes. Although the exact reaction between oxime and DFP is not known, it is assumed that oximes themselves are decomposed in the reaction (141,166). Ramachandran has shown that 100 umol/kg of oxime is able to protect mice against several LD50 values of DFP (168). He suggested that even if the whole amount of injected oxime takes part in the chemical reaction, not more that 18.4 mg/kg (100 umoles) of DFP can be decomposed when 2-PAM is the oxime. Doses of DFP very much above these levels have been demonstrated to be "safe" to mice which have been administered atropine either prophylactically or

therapeutically.

It is often hard to visualize a situation in which an animal continues to live even after complete inhibition of all AChE in its system which is assumed to happen in the presence of DFP. Some authors have expressed doubt about reactivation of AChE being the sole mechanism of oxime-induced antidotal action (93,94,168). Oxime antidotes are known to shield or protect AChE and/or other vital receptors. Ramachandran has suggested that functional AChE is external and present on cell membranes (168). DFP is known to be freely permeable to those membranes but quaternary oximes are not as freely permeable and atropine makes them even less so. Consequently, there is much more oxime available externally to protect or reactivate the functional AChE while rapid enzyme degradation of DFP is occurring within the cell. Ramachandran found that the capacity of the cell to bind DFP is limited and that the amount bound to microsomes is fixed irrespective of the dose of DFP injected, the excess being found in other fractions (168). He showed that the rate of elimination of 32P from microsomes was slow but in the toxogonin treated group that excretion of 32P was extremely rapid. He suggested that higher elimination of organophosphate in this group was not a result of any reactivating process since that DFP had not taken part in enzyme phosphorylation but had been hydrolyzed by natural detoxifying mechanisms (DFPase) in the

animal. It was also suggested that if the intracellular distribution of DFP in the brain cells was analogous to the liver cells it was likely that small amounts of oximes would be sufficient to protect the vital centers from the lethal effects of DFP (Figure 48).

Berndt et al have shown that an increased urine flow associated with decreased inulin clearance and increased renal blood flow was a direct effect of DFP on renal tubular function which did not appear to be related to inhibition of ChE (19,20). They suggested that DFP may react with renal tissue sites which were involved with normal homeostatic renal mechanisms and through this interaction could alter or produce a modest, self-limiting, acute renal damage. This may exert an effect on the tissue levels of 2-PAM for as has already been mentioned 2-PAM is primarily eliminated by tubular secretion.

Vetterlein et al have shown that 150 ug/kg paraoxon infusion into rats decreased the perfusion rate of the kidney, skeletal muscle, skin and spleen within 15 minutes (199). No changes were seen in the heart, brain, stomach, intestine and liver. When atropine or obidoxime (toxogonin) were given during infusion of paraoxon, all perfusion rates were higher and some flow rates even exceeded control values. The known effects of ACh at vascular smooth muscle sites and indirectly the increase of glandular secretion of the intes-

tinal organs favor a rise in blood flow. On the other hand, paraoxon is known to increase sympathetic activity and thus would counteract vasodilation (199). Increased blood pressure and decreased flow rate to skin, skeletal muscle, spleen and kidney were suggested to be indicative of increased sympathetic activity. Adverse influences of increased sympathetic and parasympathetic activation were suggested to be responsible for the lack of changes in myocardial and splanchnic blood flow during paraoxon infusion (199). Atropine was found to counteract the decreases in perfusion rates induced by paraoxon. In heart and intestine increased blood flow was found to increase 2-fold.

Ramachandran has shown that DFP depresses blood pressure and slows the heart rate (164,165). He suggested that accumulation of DF32P may result from a decreased glomerular filtration of DF32P as well as the cardiovascular effects produced by DF32P. Atropine was found to correct this situation. Ramachandran suggested that animals with depressed circulatory rates may show oxime concentrations in blood higher than normal but they may not be circulating rapidly enough to effect a reactivation of AChE.

Animals given atropine in conjunction with an oxime prophylactically against challenge from organophosphorus compounds have a prolonged life but ultimately were found to die from doses not much more above normal LD50's (165).

Potentiating effects of atropine on oximes may be due to:

1. protection against organophosphate in initial crucial hours;
2. restoration of the circulatory rate thereby effecting a higher turnover of oxime at greater pressure, and/or;
3. maintaining a higher concentration of oxime in the blood.

The results of this present work found 2-PAM levels to be elevated at 3 and 7 minutes in mice receiving a 50 mg/kg dose of 2-PAM and 17.4 mg/kg dose of atropine administered as a combined solution over those just receiving 2-PAM by either the i.m., i.p. or s.c. routes of administration (Table 6). The same was found to be true for 50 mg/kg 2-PAM or 50 mg/kg 2-PAM and 17.4 mg/kg atropine administered i.p. alone or prophylactically against DFP (Table 10). Additionally, in the present study, serum 2-PAM elimination half-lives were determined not to be different from one another (Tables 7 and 11). Distribution was apparently unchanged but an increase in absorption was seen when DFP and/or atropine was administered. The elevated levels of 2-PAM seen in this work could possibly be due to the combined effects of DFP and atropine on factors such as blood flow, renal transport and cardiovascular circulation in addition to

effects normally found to be occurring with the oximes themselves.

b. Increasing Doses of 2-PAM

The effectiveness or noneffectiveness of 2-PAM in reversing the effects of organophosphate antiChE compounds has been a long ongoing controversial discussion which has given rise to a major secondary question of whether or not 2-PAM penetrates into the brain. Conflicting opinions and inconsistent evidence has arisen in the literature regarding these issues (27-31, 41,64,112,129,132,143,172). Earlier studies have indicated that there is no reactivation of brain AChE in animals which have been poisoned by various organophosphorus compounds while other studies have demonstrated some reactivation of brain AChE by 2-PAM in rabbits, rats and mice (92,93,112,113,158,175,177). Reactivation of AChE activity has also been found to be greater when the activity was measured in intact or sliced brain tissues rather than in brain homogenates (109,114). Some studies have indicated a low concentration of 2-PAM in the brain of experimental animals after systemic administration while others have indicated greater concentrations in brain similar to those concentrations found in skeletal muscle (158). In rabbits, EEG changes due to sarin or physostigmine have been prevented by

administration of 2-PAM, trimedoxime and obidoxime. In addition, convulsions due to TEPP have been reduced by administration of 2-PAM (158).

The effectiveness of 2-PAM in the management of organophosphate poisoning in man has been successfully demonstrated for a wide variety of organophosphorus compounds (156,158). It has also been found that 2-PAM is effective in the management of intoxication by quaternary ammonium antiChE compounds such as neostigmine, pyridostigmine and ambenonium which are used in the management of myasthenia gravis (158). Another use of 2-PAM has been to reverse the systemic effects of DFP and the local intraocular effect of DFP and echothiophate when excessive amounts are used in the management of glaucoma (158). The most striking support however for the action of 2-PAM on the central nervous system comes from clinical observations which demonstrate prompt recovery from coma and convulsions due to parathion poisoning where 2-PAM therapy was administered and no improvement of respiration or blood pressure was seen which could be attributed to 2-PAM (140,158). On the other hand, 2-PAM has also been shown to be ineffective against poisoning from a different range of organophosphorus compounds.

The discrepancies in the literature seem to be due to the small number of poisonings involving various organophosphorus compounds as well as differences in dose and

the time of administration of 2-PAM. In addition, a major contributor towards the discrepancies reported in the literature has been the correlation of 2-PAM penetration into the brain with reactivating effectiveness as determined by AChE activity in serum and brain. Certainly many results reported in the literature indicate that when given in large doses at the proper time 2-PAM antagonizes the central nervous system effects of organophosphorus compounds. One is therefore forced to conclude that 2-PAM has some ability to penetrate the blood brain barrier despite its quaternary, ionic and polar characteristics as well as to exert some effect both at a central and peripheral location. The C14-radiolabelled distribution studies of 2-PAM which have been carried out have shown the presence of 2-PAM in the brain, albeit in very small quantities (64,102,129,180,181).

The results of this present study show a dose dependent appearance of 2-PAM in serum and brain which appears to indicate a partitioning or concentration gradient between serum and the brain (Tables 12 and 13; Figures 25 to 27). This partitioning was found not to be disturbed even though 2-PAM was utilized prophylactically and therapeutically against challenge from DFP. Little or no reactivation of brain or serum AChE was demonstrated above levels attained with just DFP up to a 75 mg/kg i.p. dose of 2-PAM (Tables 14 and 15; Figures 28 and 29). The 100 and 125 mg/kg doses of 2-PAM

administered i.p. were found to reactivate serum AChE by 50% while no appreciable reactivation over administration of DFP alone was seen in the brain. In fact 2-PAM itself was found to have an inhibiting effect on AChE in the brain at those doses. Certainly this work is consistent with the work of Mayer and Bain which emphasized the importance of a concentration gradient between the blood and brain in the penetration of quaternary compounds across the blood brain barrier and Ligtenstein's study which demonstrated that HI-6 was found in brain in a dose dependent manner (124,144).

The concept of a blood brain barrier has been consolidated through approximately a century of research by demonstration of impeded access to the brain, when compared to other organs, of various substances circulating in the blood. The pharmacological and physiological significance of this barrier is generally accepted although there is some question as to the exact nature, permeability and location(s) of the blood brain barrier (2,4,5,86,137,170). Certain areas of the brain such as pineal body, tuber cinereum, hypophysis, area postrema, have been claimed to be more accessible to circulating substances such as dyestuffs, silver salts, inorganic 32P-phosphates, quaternary nitrogen compounds and proteins than the brain generally (2,4,27,28,31,36,169,170). There are indications of local areas of increased permeability for instance the tuber cinereum, supraoptic crest and area postrema.

Recently, evidence obtained using horse radish peroxidase (HRP) as a tracer suggests that a blood brain barrier exists at the level of capillaries and arterioles in the brain but not in the median eminence of the hypothalamus (37). However, open junctions between median eminence ependymal cells have been shown to permit a bidirectional exchange of other substances between the median eminence capillaries and the third ventricle cerebrospinal fluid (CSF) (170).

Apart from areas of the brain which have a questionable existence of a blood brain barrier, other factors are known to enhance the permeability of the blood brain barrier (80,103,169). The blood brain barrier at cerebral blood vessels is due to a continuous layer of endothelial cells that are connected by tight junctions (169,170). Rapoport has suggested that these junctions deform and increase their permeability when subjected to tensile stresses (169,170). Hypertonic solutions have been shown to reversibly open the blood brain barrier by shrinking the endothelial cells and widening the tight junctions. Convulsions, hypercapnia (excess carbon dioxide in the blood) and acute hypertension have also been shown to open the blood brain barrier by associated cerebral vasodilation, increased blood pressure in small cerebral vessels and increased blood flow. It has been speculated that endothelial cells are stretched by these changes and that tight junctions widen thereby increasing

permeability of the blood brain barrier. Many noxious stimuli are also known to induce alterations in the blood brain barrier functioning. Damage of the cerebrovascular endothelium can also occur from trauma, disease tumors or cytotoxic agents in experimental and pathological conditions. These insults can irreversibly destroy the cellular integrity of the blood brain barrier and thereby open the blood brain barrier.

Hervonen et al have recently shown that 2-methyl-4-chlorophenoxyacetic acid (MCPA), a herbicide, causes reversible blood brain barrier damage which leads to the abnormal entry of serum proteins and the herbicide itself into the brain (91). At the outset of this present work, it was postulated that damaging of the blood brain barrier occurs in the presence of organophosphorus compounds and that this is responsible for allowing quaternary oxime compounds like 2-PAM into the brain. This present study does not support that hypothesis but does support the hypothesis that 2-PAM enters the brain due to a partitioning from blood to brain. Other factors may also have an influence on the amount of 2-PAM crossing the blood brain barrier.

Ligtenstein in his doctoral thesis investigating the synergism of the cholinesterase reactivating bispyridinium oxime HI-6 and atropine in the treatment of organophosphate poisoning found that the concentration of HI-6 in the brain was dose dependent but concluded that simple

increases in HI-6 concentration in the blood was not the only cause of the increased amount of HI-6 in the central nervous system (125). He suggested that damage to the blood brain barrier provoked by seizures and by mere AChE inhibition in the central nervous system contributed to the entry of HI-6 into the brain as well. In the present work, a definite dose dependent partitioning of 2-PAM between the serum and brain, which was maintained in the presence or absence of DFP, was seen and this partitioning was of primary importance for transport 2-PAM into the brain. There was an indication that the same might be occurring for a combined solution of 2-PAM and atropine, however only 2 doses (50 & 75 mg/kg) were obtained because of enhanced toxicity of 2-PAM by atropine i.e. significant deaths occurred at the 75 mg/kg dose of 2-PAM in the absence of DFP.

Certainly other factors, such as increased AChE inhibition resulting in accumulation of ACh in the central nervous system, hypercapnia, blood pressure, seizures and temperature are known to increase or decrease the permeability of the blood brain barrier. These factors are secondary effects of oximes and organophosphates and should be considered as secondary importance in the passage of 2-PAM from serum to brain. Although all the parameters will be hard to maintain, certainly further work considering their involvement with 2-PAM and its passage into the brain from serum will be

necessary in order to completely understand how all are interrelated.

A review of the literature does give rise to a few explanations of why some of the discrepancies regarding the presence or absence of 2-PAM in the brain have arisen as well as provides some suggestions as to what may possibly be occurring. Some of the controversies found in the literature with respect to the central effectiveness or noneffectiveness of oximes can be attributed to the dose and time of administration of the oximes. In some instances low doses given well after the aging of the enzyme AChE has occurred have yielded poor reactivation results. Other variables such as the nature of the organophosphorus compound, presence or absence of other drug therapies and/or life supporting treatment(s) have all aided in the confusion regarding the effectiveness or noneffectiveness of 2-PAM.

The work of Sundwall, which concluded plasma levels of 4 ug/ml P2S counteracted the neuromuscular blockade, bradycardia, hypotension and respiratory distress of sarin poisoned animals, placed a lot of emphasis on obtaining that level of oxime for protection (191). Sundwall did say that lower concentrations of P2S may be utilized if atropine was to be used as a supporting therapy. This suggestion has recently been supported by Jovanovic _et al_ who found that a serum concentration of 2.7 ug/ml HI-6 obtained by an osmotic minipump

implant in the presence of atropine and diazepam was approximately 3-fold more protective (LD50 = 62.2 mg/kg) than atropine and diazepam alone (LD50 = 162.5 mg/kg) when challenged with sarin (104). In addition, a plasma level of 2.5 ug/ml 2-PAM in the presence of atropine and diazepam supporting prophylaxis was found to be 2-fold (LD50=270 mg/kg) more protective. They found that there was good peripheral reactivation of sarin-inhibited AChE and that reactivation of the CNS AChE was increasing with time. With the level that Jovanovic <u>et al</u> described and the level found in this study one could extrapolate from this work and suggest that approximately 60 ng/ml 2-PAM would be found in mouse brain. This seemingly unimportant quantity of 2-PAM found in the brain might be of importance in the brain afterall. Green has suggested that a "minimal" level of AChE activity is necessary for the life or death of an organism and that this level be between 0.1-10% of control values (75,76,77). Kewitz and Nachmansohn determined this minimal level to be approximately 2.5% of normal AChE activity of the whole brain homogenate (112). Bajgar <u>et al</u> have carried the minimal level suggestion a little further in that they have suggested that the minimal level is different for different parts of the brain with the pontomedullar area being most important (13,14). From their results, the minimal level of AChE activity in the pontomedullar area was proposed to be 2% of normal actvity. The factors

effecting blood brain barrier penetration as discussed previously coupled with the difference in concentration of homogenates on a weight per volume basis could have also affected central reactivation results. This may have been the case in the present investigation for no central reactviation of AChE was found even though a level of approximately 400 ng/ml 2-PAM was found in the brain. However, it is possible that a some central reactivation did occur in localized area(s) of the brain but since whole brain homogenates were used, this could not be evaluated in this study. Further work examining discrete areas of the brain with regard to the amount of 2-PAM present versus reactivation properties needs to be carried out to answer that question. In addition, the 2-3 minute peak serum level of 2-PAM obtained after injection and discrete brain area amounts of 2-PAM and the reactivating capabilities of the central 2-PAM need to be investigated to determine whether or not the presence of nanogram quantities of 2-PAM centrally offer that small protection in a discrete area of the brain which is necessary to maintain life.

Consideration of residual blood content in the brain was also taken into account. Care was taken to remove any traces of blood due to microvessels on the surface of the whole brain as well as any clots which may have formed after excision of the whole brain. In a recent report by Ligtenstein looking at the concentration of HI-6 in rat brain, he

employed a corrective measure for residual blood in rat brain by considering the blood content of the brain as measured by a hemoglobin assay (126). For rat brain, the blood content in the brain of exsanguinated animals ranged from 16.8 to 24.2 ul/g w/w and was not dependent upon the HI-6 concentration. He concluded that brain tissue levels after correction for HI-6 content of residual blood in brain are measurable but was unable to conclude whether or not the concentration of HI-6 found in brain was contributing to the therapeutic effect in the case of organophosphate poisoning. It was found that applying the correction factor Ligtenstein obtained for the residual blood in rat brain to the mouse brains utilized in the present work that 2-PAM would still be present in measurable quantities in the mouse brain. As has already been mentioned, although no prophylactic or therapeutic effect, as measured by AChE reactivation, was demonstrated in the present study, it does not mean that a small reactivation occurring in a localized part of the brain, which would have been masked by measurement of whole brain AChE activity, has not occurred. Some reactivation of serum AChE activity was found but only at the higher doses of 2-PAM (Tables 14 and 15). This result is consistent with the theory that peripheral effects of 2-PAM may be more important than its central effects. It must be kept in mind however that 2-PAM has been shown to enter the central nervous system and its action there must be clarified

in order to determine its significance in the overall efficacy against organophosphate poisonings.

Ligtenstein also noted that loading of the central nervous system with HI-6 occurred rapidly (within 2 minutes) when the concentration gradient over the blood brain barrier was large enough to provoke passage into the central nervous system (126). Several authors have shown the existence of a rapidly equilibrating compartment in the brain behind the blood brain barrier and link the measured compartment to a physically existing rapidly equilibrating space (126). Ligtenstein also found that after loading of the equilibrating spaces, HI-6 was further distributed over large areas of the central nervous system and suggested that most of it will be located in the cerebrospinal fluid as has been documented for C14-obidoxime. The mean residence time of a molecule of HI-6 in the central nervous system was found to be 3.5 times longer than in the blood. In the present work, a peak level of 2-PAM which was enhanced by atropine was obtained at 3 minutes (Tables 6 and 10). As Ligtenstein has suggested, that peak level may be sufficient to provoke passage into the central nervous system on its own.

c. Toxicology

Initially, in order to explain why an increased number of animals were expiring from injection of a combined solution of atropine and increased doses of 2-PAM, toxicity studies were undertaken. The results of the studies have since been determined to be consistent with the small amount of literature available on this subject (Table 16). In a paper by Sanderson and Edson, it was found that simultaneous administration of 17.4 mg/kg atropine i.p. reduced the i.p. LD50 of 2-PAM in female rats from 400 to 150 mg/kg (177). Although they excluded this "anomalous" result to the chemicals which had been used, all deaths were not attributable to the quality of the chemicals. In their discussion, Sanderson and Edson referred to the work of Fournel who had also indicated a slight mortality increase of atropinized mice when the total PAM dose was increased from 50 to 100 mg/kg. Davies <u>et al</u> have shown that the i.m. administration of a combined solution of P2S and 17.4 mg/kg atropine increased the LD50 in mice to 218 mg/kg from 231 mg/kg found for P2S administered alone (50). A similar increase in toxicity for P2S and atropine was demonstrated in guinea pigs but was not found to occur in the rat. They have suggested that since the probit/log dose regression lines were found to be essentially parallel for a given species in the presence and absence of atropine and provided it is known in which direction atropine influences

toxicity in a given species, an appropriate adjustment in such estimates of "safe" doses could be made. Boskovic et al have obtained similar results in rat, mouse and guinea pigs for the administration of PAM and 14.4 umol/kg atropine (32). They found that atropine affected the acute toxicity of oximes in two opposing ways, i.e. increased toxicity in mouse and guinea pig but decreased toxicity in rats.

As has been illustrated there is a paucity of information regarding the acute toxicity of oximes in the presence or absence of atropine. Results in this work which utilized the mouse as the animal model are consistent with the available references. The LD50 for 2-PAM i.p. in mice was found to increase substantially to 86.3 mg/kg from 141.8 mg/kg when coadministered with atropine (Table 16). This occurrence may possibly be connected to the belief that low concentrations of 2-PAM potentiate the actions of ACh at nerve endings and so possibly increase the toxic effects produced by ACh accumulation (32). This effect may also occur for atropine which does have central muscarinic effects and a short duration of action. PAM, although acting primarily at peripheral nicotinic receptors, may also be exerting some effects centrally for this work, as well as others, have demonstrated the presence of 2-PAM in the brain. Overall, both the central and peripheral effects of 2-PAM and/or the accumulation of ACh may be involved in the increased toxic effects seen in the

presence of atropine. Although this work does not dispute the efficacy of the combined use of 2-PAM and atropine in the treatment of accidental or intentional poisonings by organophosphorus compounds, it does suggest that care be taken when administering large doses of oxime, in particular 2-PAM, in the presence of atropine for the acute toxicity of 2-PAM in dogs, LD50 = 75 mg/kg, and in mice, LD50 = 86.3 mg/kg (32; this study). These results point to the need of careful administration to humans who have been exposed to organophosphorus compounds because the highest recommended dose of 2-PAM in that instance has been suggested to be 50 mg/kg (1,74,156,158).

d. Hypothermia

The coordination of sympathetic and parasympathetic responses is shown in the regulation of temperature. This complex function involving widespread physical and chemical processes is mediated by 2 hypothalamic mechanisms, one concerned with the dissipation of heat and the other with its conservation. Both mechanisms are antagonistic to each other but do function independently with continual interrelationship and counterbalancing. The anterior hypothalamus is concerned with mechanisms for dissipation of heat while the posterior hypothalamus is concerned with control mechanisms for conservation and increased heat production.

It has been shown that cholinomimetics injected into the anterior hypothalamus induce a short lasting hypothermia in the rat which could be prevented by atropine (149,151-153). Zeitler has observed hypothermia in mice following administration of centrally acting cholinergic stimulating drugs and concluded that it was the result of central muscarinic stimulation because hypothermia was found to appear in peripherally protected animals and was antagonized by atropine(210). Meeter and Wolthuis found that sublethal doses of ChE inhibitors evoked a hypothermia in the rat (151). Subsequent investigations by Meeter and Wolthuis regarding the mechanism of hypothermia concluded that it was the result of cholinergic excitation of synapses in the

anterior hypothalamus which leads to a lowering of the set point for heat release (SPHR) of the hypothalamic thermostat so that heat is lost by vasodilatation and a reduced heat production probably caused by a decrease of metabolism in the liver (149). In further investigations, Meeter and Wolthuis concluded that antiChE hypothermia could be used in the evaluation of central effectiveness of antidotes (149). They found that after 1 hour there were hypothermia-reducing effects of P2S (150 mg/kg; i.p.) in rats that had been pretreated with DFP (1.2 mg/kg; i.p.). Also when introduced in to the subarachnoid space, hypothermia-reducing abilities of P2S, trimedoxime and obidoxime were seen with varying abilities. This suggested that the differences in efficacy known for the 3 oximes following i.p. injection may be due to their hypothermia-reducing abilities. Kenely *et al* have shown a dose dependent hypothermia for the i.p. administration of DFP to rats which was found to be antagonized by a combined s.c. injection of atropine and 2-PAM for a 10 mg/kg dose of DFP i.p. (110). No antagonism of hypothermia however was seen with a 2 mg/kg dose of DFP administered by i.p. injection when 2-PAM was administered by a s.c. injection. They also found that a slight hypothermia was present for the s.c. administration of atropine and 2-PAM alone.

There are a number of studies which have suggested that an artificially induced hypothermia as opposed to

a voluntary hypothermia (in hibernators) is accompanied by an increase in blood brain barrier permeability (117). The results of those investigations are far from consistent. For example the temperature at which the barrier becomes permeable appears to be different between studies and between species. In addition, the duration of hypothermia insult required to "open" the barrier remains unclear and may reflect variability in the conditions of inducing and maintaining a hypothermic state and in the measurement techniques employed.

In the present study, inclusion of appropriate controls and consistency of methodology were carefully maintained. A dose dependent hypothermia was found for the i.p. administration of 2-PAM (50-125 mg/kg; Table 17; Figure 32). This dose dependent hypothermia correlated well to the dose dependent nature seen when the concentration of 2-PAM in serum and brain were determined by HPLC analysis. In addition some hypothermia-reducing effects were seen when 2-PAM or 2-PAM plus atropine were administered prophylactically or therapeutically in mice challenged with DFP (Table 17; Figures 33 to 46). Utilizing the suggestion of Meeter and Wolthuis that hypothermia can be used to evaluate the central effectiveness of oximes, the results of this work correlate well (151). In addition the possibility of the barrier insult due to hypothermia increasing the permeability of the blood brain barrier cannot be ignored for certainly a low dose of DFP

and/or increasing doses of 2-PAM can induce a substantial hypothermia themselves.

Consideration of the effects of hypothermia of normal metabolic occurrence must also be taken into consideration for they may account for 2-PAM's presence in the brain as well as the enhanced toxicity of 2-PAM in the presence of atropine and the increased levels of 2-PAM in the presence of atropine. Atropine was found to induce a slight hypothermia by itself that was found to reach a maximal effect slightly faster than 2-PAM or DFP. When combined with 2-PAM, the effect of the individual hypothermias from 2-PAM and atropine was found to be additive. Kaiser et al have found using C14-atropine that hypothermia primarily reduces the biological transformation of atropine and secondarily decreases the rate of its excretion (105). The prolonged presence of atropine may account for its efficacy, when administered alone, as well as the enhanced protection when administered in combination with an oxime. The rapid onset of hypothermia and faster recovery from the hypothermia may also afford the greater protection seen when both an oxime and atropine are administered therapeutically against organophosphate poisoning.

2. Chronic Study

The data obtained from the chronic studies supports the data obtained from the dose dependent partitioning of 2-PAM from serum to brain and the hypothermia studies. 2-PAM was detected in the brain in nanogram quantities but no significant differences were found when comparisons from day 21 to control (Table 18). This suggests that 2-PAM was not entering the brain by an altered permeability of the blood brain barrier due to a presence of DFP. Rather, since serum concentrations of 2-PAM were consistent over 21 days, one would expect the brain concentrations to be consistent in view of the fact that partitioning from the serum to the brain (as has already been discussed) was occurring.

Since the DFP dosage was increased weekly to guard against development of tolerance, reactivation of AChE could not be determined. It can be suggested that a minimal AChE activity is necessary to maintain life. However the lower limit was not challenged because the study was terminated at 21 days. At 21 days, 74% inhibition of the serum AChE and 84% inhibition of brain AChE was found.

V. Conclusions

Regardless of the parenteral route of administration, the amount of 2-PAM in the serum and its serum elimination half-life were not significantly different. The administration of 2-PAM and atropine as a combined solution was found to elevate the peak levels of 2-PAM which were obtained in the serum at 3 minutes but did not effect its serum elimination half-life. This data suggests the importance of obtaining a peak serum level within the first few minutes after therapeutic administration of oxime. It appears that the rapid absorption of 2-PAM may be affording the initial protection necessary both centrally and peripherally to maintain life which can then be subsidized by other therapeutic agents and life supporting techniques. Experiments examining the value of the peak concentration versus maintaining a constant serum level need to be carried out to determine the importance of the experimental findings.

The mechanism for the increased toxicity of 2-PAM in the presence of atropine, the increase in serum level of 2-PAM when atropine is administered cojointly and its significance in the role of therapy have incomplete explanations at present. It is paramount however that further work addressing those areas be carried out for when humans are to be the recipients of the available oxime-atropine therapies care must be taken not to induce further toxic events which may be

occurring from the antidotes themselves.

The presence of 2-PAM in the brain has long been questioned in the literature. It was determined that 2-PAM does penetrate the blood brain barrier because of a concentration gradient which is established between the serum and the brain. There is some indication from the hypothermia studies carried out in this thesis that there is some central effectiveness of the 2-PAM that is present. Questions regarding what level is necessary to cause reactivation, what specific brain area is most vital to be reactivated and/or what overall effect of the presence of low level quantities of 2-PAM in the brain have to be addressed. The effect of the hypothermia induced by organophosphorus compounds and oximes themselves as well as the determination of the peripheral versus central effectiveness in combination with other therapies and life support techniques remain to be carried out. Certainly the pharmacokinetic mechanism(s) regarding the functioning of oxime and atropine in a hypothermic model would be helpful in determining how they may be functioning in an organophosphate poisoned system. As most authors now agree, reactivation may not be the only mechanism by which oximes exert their antidotal action. A carefully controlled study examining most of the variables mentioned such as dose of oxime, particular oximes in response to particular organophosphorus compounds, presence or absence of supporting therapy(s), mechanistic

responses of therapy(s) to hypothermic conditions as well as knowledge of the various organs affected and in what way they have been affected would prove beneficial to the understanding of the mode of action of oximes in the protection against organophosphate poisoning.

It seems most evident that one universal antidote which would be effective against all organophosphorus compounds is not forthcoming. It is this author's feeling that a full investigation of therapies including particular oxime function with regard to particular organophosphates at various times after exposure and doses be carried out so that a listing of the best possible treatments are at hand should one be confronted with the possibility of having to treat a case of accidental or intentional organophosphate poisoning. It is hoped however that future investigations would provide a more universal emergency treatment, if not prophylaxis, which would be available for routine use against organophosphate intoxication.

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