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Characterization of cholinesterase activities for pesticide exposure in food animals

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Characterization of cholinesterase activities for pesticide exposure in food animals

by

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Abstract

The primary aim of the work described in this thesis is to establish a foundation for the applicability of a biochemical biomarker, cholinesterase (ChE) activity in food animal species, as an instrument for evaluating exposure to pollutants as well as predicting high-level effects on public health. Secondary aims are to increase the awareness of pesticide users of anti-ChE exposure, to decide whether poisoning episodes involve anti-ChE by measuring residual effects in tissues, and to identify sources of contamination in food animal tissues. The ChE are specialized carboxylic ester hydrolases that break down esters of choline. They are classified as either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE). Both AChE and BChE activities were found to be higher in cattle than in sheep and higher in erythrocytes than in plasma and serum. The anticoagulant heparin significantly affects AChE activity in plasma compared with EDTA. Of the different tissue tested, the mean of ChE activities was found to be highest in tissue from the liver, followed by lung, muscle, kidney and heart for sheep and cattle. In pigs, the ChE activities tested higher in kidney, liver, lung, muscle and heart.

The effect of freezing on ChE activities in liver and muscle tissues was significant inhibition after 6 months at -80 °C, whereas decreased after 3 months at -20 °C. A technique to improve the purification of AChE in sheep tissue was developed. BW284c51 strongly reduced acetylthiocholine iodide (AcTChI) and propionylthiocholine iodide (PrTChI) hydrolysis and slightly affected that of butyrylthiocholine iodide (BuTChI) in the liver, while iso-OMPA had no significant effect for muscle BuTChI of sheep and pigs. Histochemical study of liver tissue found AChE localised mainly in the cytoplasm of the cell lining in the sinusoids. The optimal pH values of AChE and BChE in liver and muscle ranged between 7.8 and 8.5. Both AChE and BChE activities increased when increase the time course and temperature.

The half maximal inhibitory concentration (IC_{50}) was found to be higher for carbaryl than dichlorvos (DDVP) and diazinon (DZN). Very little residual AChE activity was seen in the liver, but more was found in muscles. In general, the

rate constants of inhibition (k_i) values for liver and muscles were increased in different pHs according to the rank order of $8.5 > 7.5 > 6.5$, while in plasma it was decreased in different temperatures as follows: $20\text{ }^\circ\text{C} > 30\text{ }^\circ\text{C} > 40\text{ }^\circ\text{C}$. The final experiments were carried out at the rate of spontaneous reactivation (k_s) of inhibited AChE by DDVP and DZN from liver and muscle was found to be higher in sheep compared to cattle and pig, while the aging of phosphorylated AChE (k_a) was found to be higher in cattle compared to sheep and pig. In addition, this study indicated that the developed bispyridinium symmetric (K048) oxime seems to be promising reactivated to DDVP-inhibited AChE for sheep and pigs while HI-6 was effective in cattle.

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Abbreviations and acronyms

ANOVA	Analysis of variance
anti-ChE	Anticholinesterase
A ₄ , A ₈ and A ₁₂	Asymmetric molecular forms of cholinesterase consisting of 4, 8 and 12 catalytically active subunits, respectively
ACh	Acetylcholine
AChE	Acetylcholinesterase
AcTChI	Acetylthiocholine iodide
BChE	Butyrylcholinesterase
BuTChI	Butyrylthiocholine iodide
BSA	Bovine serum albumin
BW284c51	1: 5-bis (4-trimethylammoniumphenyl) pentan-3-one diiodide
CC	Central canal
CV	Coefficient of variance
ChE	Cholinesterase
DDVP	Dichlorvos
DZN	Diazinon
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EC	Enzyme commission (international union of Biochemistry to standardize enzyme classification and nomenclature)
EDTA	Ethylenediaminetetraacetic acid
HC	Hepatic cell
His	Histidine
IC ₅₀	Half maximal inhibitory concentration
Iso-OMPA	Tetraisopropyl pyrophosphoramidate
IML	Intermediolateral
k _a	Rate of aging
K _{dis}	Dissociation constant of enzyme-reactivator complex
kDa	Kilodalton (unified atomic mass unit)
k _i	Rate constant for inhibition

k_s	Rate of spontaneous reactivation
K_m	Michaelis-Menten constant (molar concentration of substrate at which the velocity of the reaction reaches half the maximum value)
K_f	Dissociation constant
k_f	First-order rate constant for reactivation
k_{f2}	Bimolecular rate constant for reactivation
mM	Millimolar
MN	Motoneurons
OP	Organophosphate
PrTChI	Propionylthiocholine iodide
pH	Potential hydrogen ion
PT	Portal tract
R_f	Relative mobility
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
SD	Standard deviation
SPN	Sacral parasympathetic neurons
$t_{1/2}$	Half time (calculated using equation; $t_{1/2} = \ln 2/k$)
TNB	5-thio-2-nitrobenzoic acid
RPM	Revolutions per minute
μm	Micrometre
μM	Micromolar
V_{max}	Maximum reaction velocity at a given substrate concentration
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

General introduction and literature review

Chapter 1: General introduction and literature review

1.1 Introduction

Enzymes that preferentially catalyse the hydrolysis of ester bonds are classified as esterase EC 3.1. In general, two types of esterases have been identified in mammalian blood and tissues. These are distinguished according to several artificial substrates into two groups "A" and "B" esterases (Nigg and Knaak, 2000). Diisopropyl-fluorophosphate fluorohydrolase (EC 3.1.8.2) and arylesterase/paraoxonase (EC 3.1.1.2) represent subclasses of "A" esterase, while carboxylesterase (EC 3.1.1.1) and cholinesterases (ChE) represent a subclass of "B" esterase, which are characterized by the presence of an anionic site as well as an active (esterase) site. These esterases represent important enzymes in the nervous system, responsible for the hydrolysis of carboxylic esters family and targets for inhibition by pesticides (Fukuto, 1990; Wilson *et al.*, 2001; Wilson, 2010).

In 1914, Dale was the first to propose that the neurotransmitter acetylcholine (ACh) is involved in the chemical transmission at autonomic ganglia. He suggested that enzymatic hydrolysis of the ester linkage would supply a method for the rapid exclusion of ACh from the synaptic cleft (Dale, 1914). Proof for the existence of such ChE came in (1926) when Loewi and Navratil used experiments on frog hearts to demonstrate that ChE could be inhibited by physostigmine (eserine) thus prolonging the effect of administered ACh. In 1932, a crude extract of an ACh-splitting enzyme prepared from horse serum was named "choline-esterase" (Stedman, 1932). Then in 1940, the existence of two major forms of human ChE was discovered; human serum cholinesterase and red blood cell cholinesterase (Alles and Hawes, 1940).

It is now known that ACh is the major neurotransmitter at many central synapses and is used by all parasympathetic and some sympathetic nerves, as well as in innervations of skeletal muscle (Bajgar *et al.*, 2008; Laura *et al.*, 2008; Taylor *et al.*, 2009).

In general, two types of ChE have been identified in mammalian blood and tissues. These are distinguished according to their substrate specificity and sensitivity to selective inhibitors. The first is acetylcholinesterase (AChE, EC 3.1.1.7), which is systematically called acetylcholine acetylhydrolase. Other names include true cholinesterase, specific cholinesterase, red blood cell cholinesterase, erythrocyte cholinesterase and cholinesterase I. The second is butyrylcholinesterase (BChE, EC 3.1.1.8), referred to systematically as acylcholine acylhydrolase. Other names include cholinesterase, pseudocholinesterase, non-specific cholinesterase, plasma cholinesterase, serum cholinesterase, propionylcholinesterase, benzoylcholinesterase and cholinesterase II (Silver, 1974; Chatonnet and Lockridge, 1989; Wilson *et al.*, 2001; Wilson and Philip, 2005).

The preferred substrate for the AChE enzyme is ACh, while the preferred substrates for BChE are butyrylcholine and propionylcholine (Tecles and Ceron, 2001; Wilson *et al.*, 2001; Wilson and Philip, 2005). Both AChE and BChE serve a pivotal role in regulating the transmission of nerve impulses by rapid hydrolysis of the neurotransmitter ACh (Taylor *et al.*, 2009; Wilson, 2010). It appears that AChE is the predominate enzyme performing this function, since AChE catalyses the hydrolysis of ACh much more rapidly than does BChE (Soreq *et al.*, 2005; Taylor *et al.*, 2009; Shang *et al.*, 2012). The ChE are hydrolases which, under optimal conditions, catalyse the hydrolysis of choline

and thiocholine esters at a higher rate than that of other esters. Subsequent studies have shown that the order of the relative rates of hydrolysis by AChE decreases in the series: acetyl > acetyl- β -methyl > propionyl >> butyryl, while in BChE, the rate decreases in the series: butyryl > valeryl > propionyl > acetyl >> acetyl- β -methyl (Moralev and Rozengart, 2007).

In recent times the most common anticholinesterase (anti-ChE) or ChE inhibitor used for validation and evaluation of ChE activity assay methods include organophosphate (OP) compounds, those acting reversibly by the formation of covalent bonds, which are used as nerve agents, flame-retardants and insecticides in veterinary medicine (Pérez *et al.*, 2010; Nurulain, 2011).

Then carbamate compounds, those also acting reversible, forming covalent bonds, which apply as insecticides in veterinary medicine, herbicides in agricultural crops and gardens, as therapeutic drugs in human medicine (Alzheimer's disease, glaucoma and myasthenia gravis). Furthermore, OP and carbamate compounds when animals are exposed to OP and carbamate compounds cause inhibition of ChE with the consequences of over stimulation of muscarinic, nicotinic and central nervous system (Fukuto, 1990; Timothy, 2001; Wilson and Philip, 2005; Jokanovic and Stojiljkovic, 2006; Fodero *et al.*, 2012).

1.2 Physiological functions of cholinesterase

1.2.1 Classical role

AChE serve a pivotal role in regulating nerve impulse transmission by rapid hydrolysis of neurotransmitter ACh (Bajgar *et al.*, 2008; Laura *et al.*, 2008; Taylor *et al.*, 2009; Shi *et al.*, 2012). Likewise, AChE appears to be the predominant enzyme performing this function, since hydrolysis of ACh is much

quicker than does BChE (Soreq *et al.*, 2005; Taylor *et al.*, 2009; Sanchez-Gonzalez *et al.*, 2012). The neurotransmitter ACh is ester of choline and acetic acid, and a specific indicator for monitoring nervous system owing to responsible for transmission of nerve impulses at.

1. autonomic nervous system from preganglionic to postganglionic neurons in both sympathetic and parasympathetic nervous system;
2. between postganglionic parasympathetic nerve fibre and cardiac muscle; smooth muscle and exocrine glands;
3. neuromuscular junction of somatic nervous system; and
4. cholinergic synapse in the central nervous system (Yoshio, 1999; Timothy, 2001; Su *et al.*, 2008).

Yoshio (1999) observed that ACh is formed by the action of choline acetyltransferase (EC 2.3.1.6) from choline and acetyl-CoA at the sites of cholinergic neurotransmission. The choline acetyltransferase is responsible for transporting ACh from the neuronal cytoplasm to the presynaptic vesicle into the synaptic cleft and binds to the postsynaptic cholinergic receptor (muscarinic or nicotinic) producing depolarization of the post synaptic membrane (Bielarczy and Szutowicz, 1989; Sanchez-Gonzalez *et al.*, 2012; Yu and Jiang, 2012). The physiological function of BChE is still unknown (Small *et al.*, 1996; Nese Cokugras, 2003; Jiri *et al.*, 2004; Shi *et al.*, 2012). A decrease of BChE has been found to have no toxicological significance (Mack and Robitzki, 2000).

1.2.2 Non-classical role

The role of AChE in the fetus is a neural development by expression on the stopped stage of neuroepithelial cell migration and start of exertion of adhesive protein stabilizing cell-cell contacts, influences that appear in embryonic brain

(Small *et al.*, 1996; Laura *et al.*, 2008; Su *et al.*, 2008). In addition, AChE is involved in the synthesis of β -amyloid fibril protein which assists in the distribution of β -amyloid plaques by interacting throughout the anionic site of the enzyme in the brain, therefore the pathological characteristic of Alzheimer's disease is β -amyloid plaques with neurofibrillary tangles and a decline in the levels of ACh due to a substantial reduction in the activity of choline acetyltransferase (Small *et al.*, 1996; Stefanova *et al.*, 2003). This is because there is homology among AChE and members of the β -amyloid fold family, including the neurotactins, which are involved in cell-cell adhesion. The preserved domain of neurotactin may be replaced by AChE and still maintain cell-cell interaction (Adamson *et al.*, 1975). In addition, AChE regulates neurite growth through nicotinic or muscarinic receptors (Small *et al.*, 1996; Karun, 2001; Laura *et al.*, 2008).

1.3 Structure of cholinesterase

The ChE is a polymorphic enzyme, whose molecular weights is generally in the range between 70-80 kilodalton (kDa) (Silver, 1974; Massoulie *et al.*, 1993). AChE has a high catalytic activity and each molecule of AChE degrades around 25000 molecule of ACh per second, approaching the limit allowed by diffusion of substrate. The existence of AChE and BChE in different molecular forms is well recognized, and polymorphic enzymes can be classified based on solubility characteristics and sedimentation velocities determined by centrifugation in sucrose density gradients. These forms are divided into two classes.

Firstly, asymmetric or collagen-like tailed forms, the quaternary structure is characterized by the presence of a collagen-like tail, which is formed by the triple helical association of three collagenic subunits (Q subunits); each subunit

may be attached to one catalytic tetramer (G_4). The asymmetric forms consists of one, two or three catalytic tetramers; these forms are called the A_4 , A_8 and A_{12} forms, respectively (Figure 1.1) (Massoulié *et al.*, 1999). Their main characteristics are a large Stokes' radius, and a specific sensitivity to collagenase in low salt conditions in the presence of a polyanion. Localized at the skeletal neuromuscular junction, synapses, brain, heart, muscle and peripheral ganglia, they have glycosylated head, which are joined together by sulfhydryl groups containing the active sites and collagen tails that attach the enzyme to the cell surface (Massoulié *et al.*, 1991; Massoulié *et al.*, 1993; Paul and Elmar, 1995; Small *et al.*, 1996; Jiri *et al.*, 2004).

The ionic interaction is characterized by their capacity to cooperate with polyanionic components, e.g. glycosaminoglycans, at low ionic strength and BChE is less abundant than those of AChE (Massoulié *et al.*, 1993). The similarity between AChE and BChE is emphasized by the continuation of hybrid asymmetric forms, in which both AChE and BChE subunits are joined to the same collagen tail (Chatonnet and Lockridge, 1989; Massoulié *et al.*, 1993). In addition, Chatonnet and Lockridge (1989) observed that the most complex form is A_{12} , which has 12 catalytic subunits and is classified as either hydrophobic water soluble or amphiphilic, linked to a phospholipid membrane or extracellular matrix by sturdy interaction with other molecules.

Secondly, in globular forms the quaternary structure is defined in a negative fashion, by the lack of collagen-like tails. These forms, found in both vertebrates and invertebrates, exist as a monomer (G_1), dimer (G_2) or tetramer (G_4) joined together by disulphide bonds, with each subunit containing an esteratic active site containing the serine hydroxyl group, and an anionic site for the quaternary

ammonium group (Chatonnet and Lockridge, 1989; Massoulie *et al.*, 1993; Rocio Marcos *et al.*, 1998; Wilson and Philip, 2005). The G₁, G₂ and G₄ forms are released into the body fluids or anchored to the cell surface through hydrophobic amino acid sequences or glycopospholipids (Chatonnet and Lockridge, 1989; Paul and Elmar, 1995; Shen, 2004). These forms are localized in the nerves, brain, muscles, serum, erythrocytes, lymphocytes, placenta, liver, urogenital system, digestive tract, exocrine and endocrine glands.

Globular forms may be operationally subdivided into amphiphilic membrane bound and non-membrane bound forms. The former are defined by their ability to bind to micelles of non-denaturing detergents. These interactions are demonstrated by sedimentation changes, increase in Stokes' radius or alterations in electrophoretic migration under non denaturing conditions (Chatonnet and Lockridge, 1989; Massoulie *et al.*, 1991). In addition, (Massoulie *et al.*, 1991) distinguished two types of amphiphilic G₂ forms. Type I corresponds to glycolipid anchored dimers of AChE, occurring not only in nervous tissue, but also in muscles and erythrocytes. Type II dimers are found in soluble form in the plasma but are also clearly amphiphilic, are insensitive to specific phospholipase, and never aggregate in the absence of detergent but only show limited sedimentation (Figure 1.1) (Massoulie *et al.*, 1999).

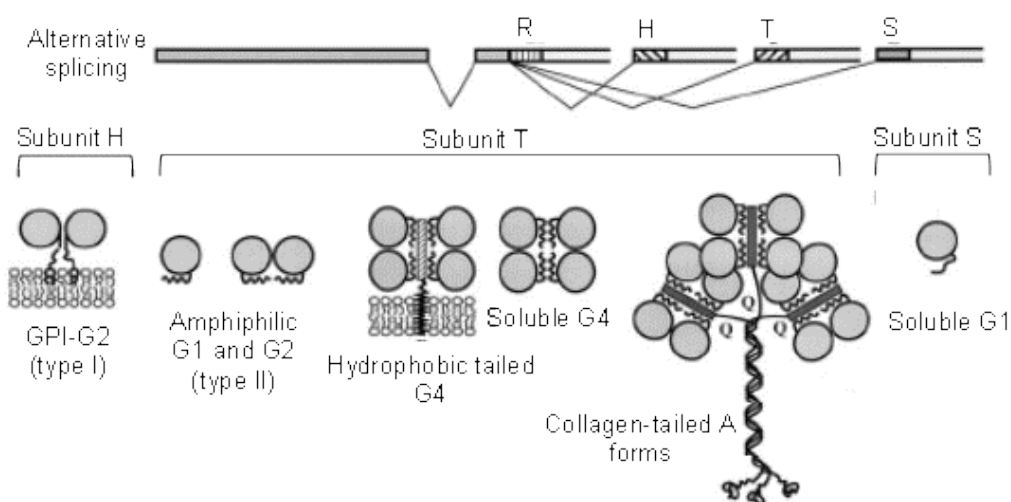


Figure 1.1 Schematic model to demonstrate the molecular polymorphism of AChE and BChE (taken from (Massoulie *et al.*, 1999)).

1.4 Enzymatic mechanism

The primary function of AChE in the tissues is to catalyse the hydrolysis of the neurotransmitter ACh (Bajgar *et al.*, 2008; Taylor *et al.*, 2009; Primožič *et al.*, 2012). The active site of AChE contains two sub-sites, a negatively charged anionic site and an esteratic site containing both an acidic (electrophilic) and a basic (nucleophilic) group.

At the simplest level, AChE action is described as follows. Firstly after the initial formation of the Michaelis complex (Step 1 in Figure 1.2), the acylation pathway starts with nucleophilic attack by the serine hydroxyl residue in the active site on the carbonyl carbon atom of the ACh. The nucleophilicity of the serine hydroxyl is influenced by the proximity of a histidine (His) residue in the active site (general acid/base catalysis; Step 2 in Figure 1.2). This leads to the formation of covalently bound intermediate in which the carbonyl carbon has a tetrahedral arrangement of bonds and protonation of the His residue (Step 3 in Figure 1.2). The final step is the protonation of the choline by the His residue, leading to

cleavage of the ester and release of the choline (Step 4 in Figure 1.2) (Qinmi *et al.*, 1998; Sant' Anna *et al.*, 2006).

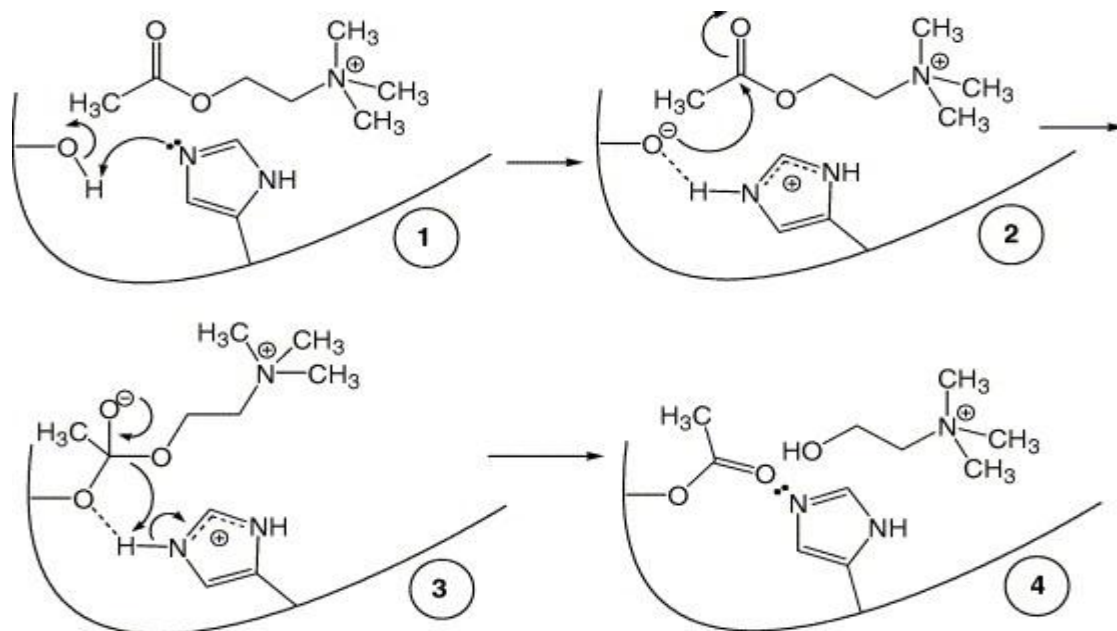


Figure 1.2 Hydrolysis of ACh by acylation (taken from (Sant' Anna *et al.*, 2006)).

The deacylation pathway starts with the nucleophilic attack of a water molecule on the acetyl carbonyl carbon (Step 5 in Figure 1.3). Again, this is promoted by the His residue acting as a base (Step 6 in Figure 1.3). This forms another intermediate in which the carbonyl carbon has a tetrahedral arrangement of bonds (Step 7 in Figure 1.3). In the final step (Step 8 in Figure 1.3) this intermediate rearranges with the resulting release of acetic acid (Sant' Anna *et al.*, 2006). BChE catalyses the hydrolysis of butyrylcholine into choline and butyric acid or propionylcholine into choline and propionic acid using a similar mechanism (Morizono and Akinaga, 1981; Tecles and Ceron, 2001; Wilson *et al.*, 2001; Wilson and Philip, 2005).

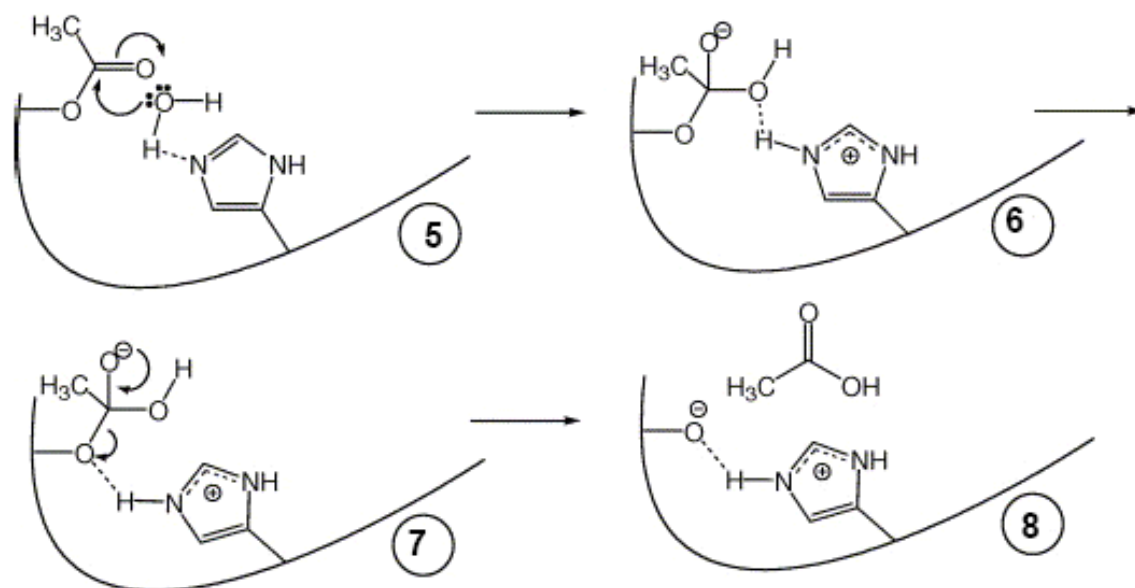


Figure 1.3 Hydrolysis of ACh by deacylation (adapted from (Sant' Anna *et al.*, 2006)).

1.5 Localization of cholinesterase

ChE are widely distributed across different animal species and there is no correlation between the distribution of AChE and BChE in different tissues (Massoulie *et al.*, 1993). Furthermore, AChE has been detected widely in plant species (Sagane *et al.*, 2005; Mukherjee *et al.*, 2007). Generally, AChE and BChE in vertebrates are distributed in different tissues as described below.

1.5.1 Blood

AChE is located on the surface of blood cells (erythrocytes, megakaryocytes, lymphocytes and platelets) (Paulus *et al.*, 1981; Rocio Marcos *et al.*, 1998; Jamshidzadeh *et al.*, 2008; Naik *et al.*, 2008), whereas BChE in blood is located mainly in serum and plasma (Sun *et al.*, 2002; Anglister *et al.*, 2008; Naik *et al.*, 2008; Saxena *et al.*, 2008). Neglecting erythrocyte and plasma ChE activity in animals may result in misinterpretations of the extent of anti-ChE from pesticides, and hence would affect the setting of regulations relating to human exposure and food safety (Wilson, 1996). Also one possibility is that AChE in

the erythrocytes evolved to protect the body from natural anti-ChE (Rodney *et al.*, 1987; Soreq *et al.*, 2005), for example the natural anti-ChE agents chaconine and solanine from tubers and nightshades (Nigg *et al.*, 1996).

BChE in the plasma catalyse the hydrolysis and hence detoxification of many drugs, for example, succinylcholine to succinylmonocholine and choline (Lockridge, 1990), cocaine to ecgonine methyl ester with benzoic acid (Xie *et al.*, 1999; Sun *et al.*, 2002), aspirin (acetylsalicylic acid) is rapidly hydrolysed to salicylate and free acetate (Masson *et al.*, 1998), and heroin rapidly hydrolysed to 6-monoacetylmorphine and more slowly to morphine following an intravenous dose (Asher *et al.*, 1999). Lockridge *et al.* (1980) showed that BChE is the major esterase involved in the hydrolysis of heroin, while Owen and Nakatsu (1983) have identified three further esterases involved in heroin hydrolysis within the blood. Furthermore, BChE rapidly hydrolyses procaine mainly to *p*-aminobenzoic acid and diethylamino ethanol (Dawson and Poretski, 1983). Rapid hydrolysis of procaine to the inactive products results in its relatively short time of action.

1.5.2 Central nervous system

Both AChE and BChE are well distributed in the central nervous system. Almost 80% of brain tissue ChE activity is AChE and the remaining 20% is BChE (Adamson *et al.*, 1975), although BChE activity is greater in the white matter, and where its function appears to be myelin maintenance (Silver, 1974). AChE activity is high in the cerebellum and cerebrospinal fluid of mammals (Silver, 1974; Wilson *et al.*, 2001), and also in other brain areas (pontomedullar, frontal cortex and basal ganglia) (Chatonnet and Lockridge, 1989; Weber *et al.*, 1999; Bajgar *et al.*, 2008). AChE in the central nervous system has important roles in

regulating excitation of nerve cells by destroying the neurotransmitter ACh (Wilson *et al.*, 2001; Su *et al.*, 2008; Taylor *et al.*, 2009).

BChE in the brain is confined to subcortical white matter bundles, for example, corpus callosum, anterior commissure, fornix and the internal capsule fibres traversing the striatum. It is also found in the thalamus and upper brainstem, in regions unrelated to AChE, in capillary endothelial cells of the cerebral cortex (Chatonnet and Lockridge, 1989; Weber *et al.*, 1999; Mesulam *et al.*, 2002; Wilson and Philip, 2005), glial cells and associated structures such as septa in the optic nerve (Silver, 1974), cholinergic neurons and wide-ranging in neuropil reactivity region (between neuronal cell bodies in the grey matter of the brain and spinal cord) (Mesulam *et al.*, 2002). BChE is able to catalyse the hydrolysis of ACh and other choline esters (e.g. butyrylcholine and propionylcholine) (Morizono and Akinaga, 1981; Tecles and Ceron, 2001; Wilson *et al.*, 2001; Wilson and Philip, 2005), but is unrelated to cholinergic neurotransmission (Berman *et al.*, 1987). However, the presence of BChE in the cerebral microcapillaries of the rat may be related to maintenance of the blood brain barrier (Massoulie *et al.*, 1993).

1.5.3 Peripheral nervous system and muscular tissues

Somatic motor nerve fibres, which innervate skeletal muscle, arise from cranial nuclei and the ventral horn of the spinal cord. Both AChE and BChE are present in the neuron cell body, axons, motor end plate and neuromuscular junctions. Most AChE is found post synaptically on the surface of the junction fold in the sarcoplasm (Silver, 1974; Wilson *et al.*, 2001). Also AChE histochemically is well associated with neuron cell bodies in the ventrolateral ventral horn; intermediolateral columns of thoracic, rostral and mid lumbar cord of the

sympathetic preganglionic cells; sacral parasympathetic nucleus; central canal; and partition cells (Figure 1.4) (Anglister *et al.*, 2008).

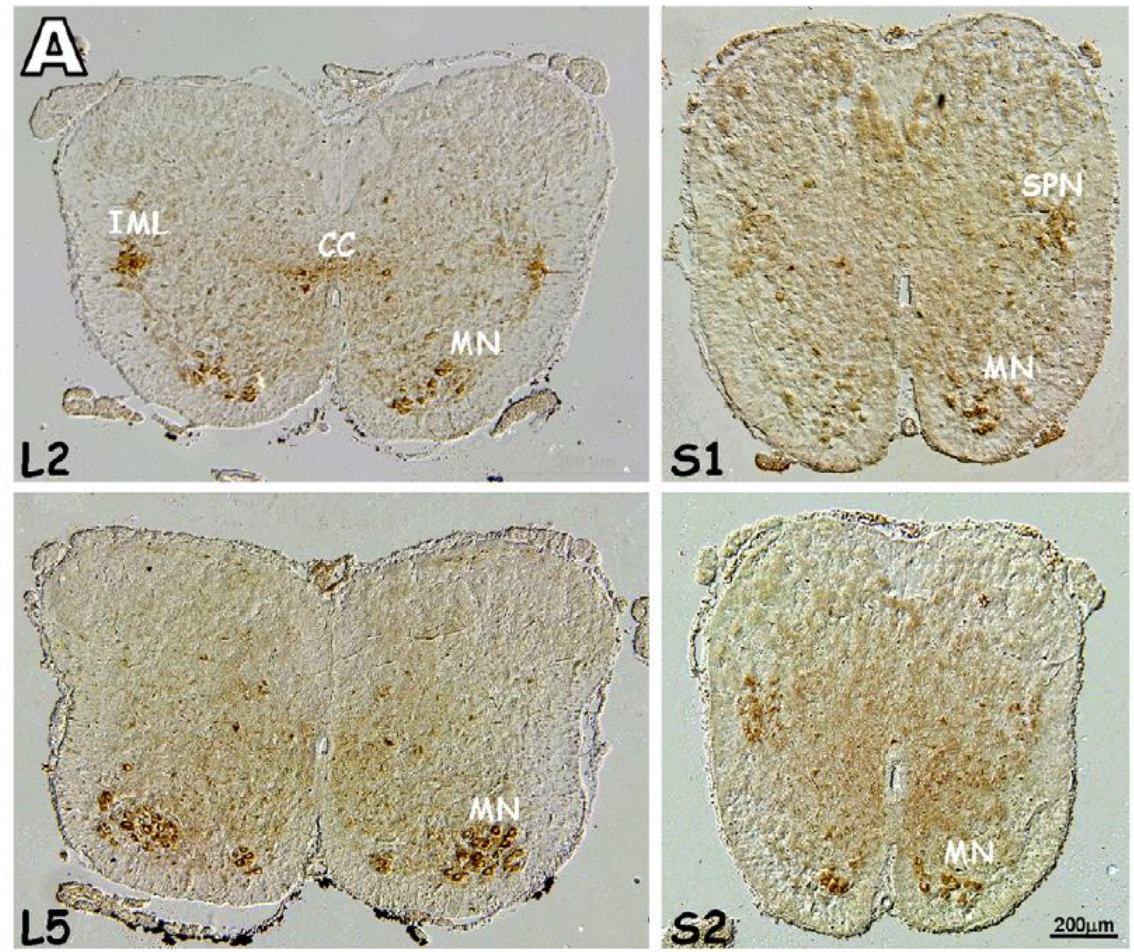


Figure 1.4 Distribution of ChE containing neurons in lumbosacral segments of the spinal cord. Cryosections of L2, L5, S1 and S2 segments of newborn rat spinal cord were histochemically stained for ChE (taken from (Anglister *et al.*, 2008)). ChE containing neurons were concentrated in 4 major groups: motoneurons (MN), central canal (CC) and partition neurons in all segments, intermediolateral (IML) in L2 and sacral parasympathetic neurons (SPN) in S1 segments. Note additional ChE-stained cells in medioventral and intermediate zones.

1.5.4 Vascular tissues

Both AChE and BChE are well distributed in the heart and endothelium of blood vessels (Silver, 1974; Chatonnet and Lockridge, 1989; Mirajkar and Pope,

2008), however BChE levels are higher in the auricles than in the ventricles, and the carotid body contain higher BChE levels than AChE (Silver, 1974).

1.5.5 Respiratory system

ChE activities are predominate in bronchial smooth muscle and in the lungs and AChE co-regulate the degradation of ACh in respiratory tissues (Mann, 1971; Pillai *et al.*, 1993; Mack and Robitzki, 2000). Also AChE and BChE are found in the mucous gland, and some goblet cells and their secretions (Silver, 1974).

1.5.6 Digestive system and associated structures

There are high levels of ChE in the mammalian salivary glands (Khosravani *et al.*, 2007), smooth muscles of the oesophagus, stomach and alimentary tract (Silver, 1974), endoplasmic reticulum membrane in the liver (Weber *et al.*, 1999; Mack and Robitzki, 2000), and spleen (Morizono and Akinaga, 1981; Nieto-Ceron *et al.*, 2004). BChE activity in the pancreas is higher than AChE activity, and distributed mainly in the Islets of Langerhans, acinar cells and pancreatic secretions (Morizono and Akinaga, 1981). The function of ChE in these areas are still unknown (Wilson *et al.*, 2001).

1.5.7 Urogenital system

ChE activity is present in parts of the kidney, bladder, adrenal medulla and in urine samples (Silver, 1974; Yang *et al.*, 2002), however the levels of ChE increase in cases of renal tumours (Yang *et al.*, 2002). The ChE are also distributed on other sites in some endocrine glands including thyroid, parathyroid, pituitary and adrenal glands (Silver, 1974). In addition, ChE are found in the skin (Silver, 1974; Wilson *et al.*, 2006).

1.6 Clinical significance of measurement of cholinesterase activities

There are many reasons for measuring ChE activities in domestic animals:

1. to evaluate of health impact by biomonitoring hazards from anti-ChE exposures in domestic animals (Mohammad, 1997; Wilson *et al.*, 2005; Brown *et al.*, 2006). When anti-ChE overexposure does arise, ChE monitoring assists in clinical management (Brown *et al.*, 2006);
2. to avoid problems from chronic anti-ChE exposure especially in the early stages of poisoning with OP or carbamate compounds when the signs of poisoning are unclear due to both compounds being possible contaminants of water, soil, air, food, dust or environment and when animals consume these compounds (Wilson and Philip, 2005; Angerer *et al.*, 2006; Brown *et al.*, 2006). It may also influence the choice of pesticides to use according to their safety and increase awareness in pesticide user of anti-ChE exposure (Wilson *et al.*, 2001; Wilson and Philip, 2005; Brown *et al.*, 2006; Hernandez *et al.*, 2006). In addition, decide whether poisoning episodes involved ChE inhibiting agents by measuring residues on skin or clothing and identify sources of contamination in animal tissues;
3. to monitor environmental exposure to chemical warfare agents (nerve gases) which cause irreversible ChE inhibition (Dorandeu *et al.*, 2008; Saxena *et al.*, 2008); and
4. to use as a biomarker for different diseases; since ChE is synthesised in the liver the amount of enzyme appearing in the plasma is dependent upon both normal liver function and an adequate delivery of amino acids. Determination of ChE activities may therefore be used as a test of liver function or as an index of protein synthesis. Many forms of liver disease including cirrhosis (Silver, 1974; Hada *et al.*, 1999), chronic hepatitis (Hada *et al.*, 1999), malignant disease with

secondary fatty liver deposits (Vorhaus and Kark, 1953) have been shown to be accompanied by reduction in ChE activity. Likewise, ChE activities are lower in other pathological conditions including chronic renal disease (Ryan, 1977), myocardial infarction (Ceremuzyński *et al.*, 1985), burns (Frolich, 1977), anaemia, malnutrition and chronic debilitating disease (Vorhaus and Kark, 1953), aflatoxin poisoning (Cometa *et al.*, 2005) and Guillaine-Barre syndrome (Dalvie and London, 2006). It is probable that this is due to a secondary reduction in protein synthesis. However, it is also possible that enzyme synthesis is reduced because of derangement of the metabolic processes of the hepatocyte by materials produced in the diseased tissues or toxic materials inhibit that enzyme activity.

With regard to studies that have indicated correlations between exposure of farmers to OP compounds and an increased risk of leukaemia. *In vitro* studies reveal that anti-ChE (e.g. eserine) can induce carcinogenesis and potentiate the action of oestrogen in the epithelium of rat mammary glands (Calaf *et al.*, 2007). Moreover, exposure of rodents to OP (e.g. malathion) can induce breast tumours (Cabello *et al.*, 2003), other than ChE levels shows to vary depending upon the cellular origin of the tumour, so that there is no clear general correlation between increases or decreases in ChE levels and cancer. ChE activity increases in malignant tumours in brain and kidney (Razon *et al.*, 1984; Yang *et al.*, 2002), but decreases in malignant lymph nodes and colon (Francisco *et al.*, 2003; Montenegro *et al.*, 2006). While in lung tumours, enzyme levels vary depending on the type of tumour, they have modified glycosylation and are reduced in activity in squamous cell carcinomas and large cell carcinomas (Martinez-Moreno *et al.*, 2006).

Although the role of ChE enzymes in tumorigenesis is unclear, the fact that AChE and BChE may be involved in the control of cell growth and proliferation during the early stages of development, and the amplification of ChE gene could influence the ability of tumour cells to proliferate more rapidly (Small *et al.*, 1996; Nese Cokugras, 2003). On the other hand, levels of ChE is increased in Alzheimer's disease (Geula and Mesulam, 1995; Greenfield and Vaux, 2002), and in type 2 diabetes mellitus (Rao *et al.*, 2007). A role for the cholinergic system in memory was first suggested in the early 1970s, when cholinergic antagonists were found to damage learning, while anti-ChE could have a positive effect (Silver, 1974). At this time, examination of post mortem Alzheimer's disease in brain tissue also showed reduction in the cholinergic neuronal markers choline acetyltransferase and AChE in the cortex, hippocampus and nucleus basalis (Silver, 1974). These findings led to the formulation of the "cholinergic hypothesis", linking abnormalities in the cholinergic system to functional and pathological changes in Alzheimer's disease. The decreased level of AChE has more recently been localised to the selective loss of the membrane-bound tetrameric form, but upregulation of the monomeric form (Geula and Mesulam, 1995). In addition, ChE is found in senile plaques and neurofibrillary tangles, even at the initial stages of their formation (Morán and Gómez-Ramos, 1992). However, in Alzheimer's disease there is a reduction in ChE secretion from the adrenal gland (Appleyard and McDonald, 1991), and a reduction in salivary ChE, which is being considered as an Alzheimer's disease biomarker (Sayer *et al.*, 2004). This has led to the suggestion by Greenfield and Vaux (2002) that neurodegeneration in Alzheimer's disease, and potentially Parkinson's disease and motor neuron disease, may result from a reversion to an embryonic restoration programme.

However, another possibility is that the AChE associated with the myeloid plaques may be of astroglial origin (Geula and Mesulam, 1995). Recent work suggests the alternative splicing of AChE may also contribute to Parkinson's disease. An increased risk for Parkinson's disease has been found to correlate with mutations in the regulatory region of AChE, which impair AChE-R up-regulation, and mutations in the adjacent paraoxonase gene (Benmoyal-Segal *et al.*, 2005).

1.7 Anticholinesterase

Chemicals may prevent ChE from breaking down ACh, increasing both the level and duration of action of the neurotransmitter ACh (Nigg and Knaak, 2000; Aisa *et al.*, 2012; Cabral *et al.*, 2012). There are natural and synthetic anti-ChE; the natural reversible inhibitors are found in a number of plant toxins, e.g. solanaceous glycoalkaloids and alkaloids are naturally occurring steroids in potatoes and related plants (Rodney *et al.*, 1987; Ercetin *et al.*, 2012), fungal termitremes (Dowd *et al.*, 1992), triazoles, trifluoroacetophenones and fasciculin complex from snake venom (Harel *et al.*, 1995; Radic *et al.*, 2005) and huperzine A from moss (Gordon *et al.*, 2005). Synthetic ChE inhibitors are pesticides that are substances or mixtures intended for use widely within modern agriculture and veterinary medicine to control insect infestation. Increasing concern is being shown towards their indiscriminate use and the long term effects they may cause to the environment, farm animals and human health (Davis *et al.*, 2007). Pesticide contamination causes inhibition of ChE at muscarinic or at nicotinic receptors or in the central nervous system, leading to accumulation of ACh (Timothy, 2001; Ferreira *et al.*, 2008). Generally, pesticides can be divided into two main classes according to their chemical composition and the stability of the enzyme inhibitor complex.

1. Organophosphate (OP) compounds: More than one hundred OP compounds are currently used worldwide; they were developed during World War II and have been used in terrorist attacks in different countries (Table 1.1; (Nurulain, 2011)). They are esters of phosphonic, phosphinic or phosphoric acid. The general structure of OP compounds is shown in Figure 1.5.

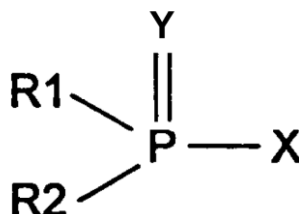


Figure 1.5 Shows the general structure of an OP compounds, where R_1 and R_2 may be almost any group (alkoxy, thioalkyl, alcohol, phenol, mercaptan, amide, alkyl or aryl). Group X can be acyl radical such as fluoride, nitrophenyl, phosphates, thiocyanate, carboxylate, phenoxy or thiophenoxy group. Where Y, the leaving group can be any one of a wide variety of substituted and branched aliphatic, aromatic or heterocyclic groups, generally linked by an oxygen (-oxon) or sulphur (-thion). This distinguishes two groups of OP, P = O compounds, phosphates, are commonly known as oxonates and this can be incorporated into the trivial name, (e.g. paraoxon). P = S compounds, phosphorothioate, are commonly known as thionates, again this can be incorporated into the trivial name (e.g. parathion) (adapted from (Fukuto, 1990; Wilson *et al.*, 2001)).

Table 1.1 A brief history of OP compounds (taken from (Nurulain, 2011)).

Year	Event
1854	The process of synthesis of first OP compounds, tetraethyl pyrophosphate was reported.
1936	The first OP nerve poison, tabun was produced in Germany.
1938	Another nerve agent belongs to OP group was developed and named sarin.
1944	The third neurotoxic compound of the same group was developed in Germany, named soman.
1957	VX, another nerve agent of the same group developed in UK and weaponized by USA.
1983/1984	Iraqi troops used tabun nerve agent against Iranian soldiers during the war.
1988	Sarin was used against Kurdish in Iraq by Iraqi troops.
1991	It is also believed that allied troops have been exposed to sarin during the Gulf war.
1994	In Matsumoto, Japan a terrorist attack was reported with sarin which caused seven deaths and many casualties.
1994/1995	Assassination attempt with VX was made in Japan.
1995	A terrorist attack by sarin was reported in Tokyo subway, Japan.

Intoxication by OP compounds causes a cholinergic crisis due to reversible inhibition of ChE activity in the nervous tissues and skeletal neuromuscular junction. This occurs by a two-step process, the initial formation of an enzyme inhibitor complex by binding to the serine hydroxyl group in the esteratic site (Michaelis complex) (Step 1 in Figure 1.6) followed by phosphorylation of the

tributyl phosphorotrithioate); and (6) laboratory chemicals (e.g. diisopropyl fluorophosphates) (Timothy, 2001; Jiang *et al.*, 2007).

In addition, the high toxicity of some OP compounds has led to their application as chemical warfare agents (e.g. demeton-s-methyl, O-Ethyl-S-[2-(diisopropylamino) ethyl] methylphosphonothionate, tabun, soman, and sarin) (Timothy, 2001; Bajgar *et al.*, 2008). Davis *et al.* (2007) observed that OP compounds are currently commonly used instead of organochlorine pesticides due to their lower persistence in the environment, while still remaining effective. However, these compounds may still find their way into our food and water supplies. Hence the need the use of determination of ChE activity for the reliable detection of pesticides for environmental protection and food safety purposes.

In the current study, I have chosen two types of OP compounds; dichlorvos (DDVP, $C_4H_7O_4Cl_2P$) 2,2-dichlorovinyl dimethyl phosphate is a chlorinated OP insecticide that is extensively used in many countries for controlling insect pests on agricultural, commercial and industrial sites, and diazinon (DZN, $C_{12}H_{21}N_2O_3PS$) O,O-diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate) is a thiophosphoric acid ester first developed in 1952. Both of these compounds are routinely used in veterinary medicine. Compared to other pesticides, these are generally preferred because of their cost-effectiveness and wide range of bioactivity. Their annual worldwide sales in 2003 were about 40 million US dollars (Liu *et al.*, 2009; Sun *et al.*, 2009b). However, there are concerns about the safety of these compounds because of their high acute toxicity.

2. Carbamate compounds: These were originally extracted from the Calabar bean of West Africa. The Calabar bean was known to be toxic and the main toxin, physostigma venenosum, was first isolated in 1865. The structure was identified in 1925 and the compound first synthesized as physostigmine in 1935 (Brufani *et al.*, 2000). These compounds are esters of carbamic acid, unlike OP, and were developed from natural products. The general structure of carbamate compounds is shown in Figure 1.7.

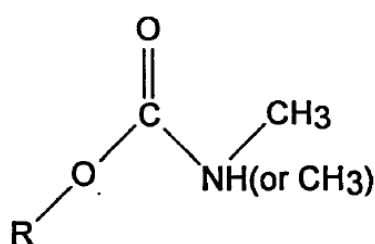


Figure 1.7 Shows the general structure of carbamate compounds, where R is an organic group and usually alkyl or aryl groups and are substituted on the nitrogen or amides, which have one or two methyl groups attached to the nitrogen atom. A range of differing organic groups can be linked to the oxygen atom (adapted from (Timothy, 2001; Wilson and Philip, 2005)).

Carbamate compounds can be divided into main classes according to their use:

1. Treatment of chemical disorder diseases such as myasthenia gravis, glaucoma and paralytic ileus (e.g. physostigmine, neostigmine, pyridostigmine and edrophonium) (Nigg and Knaak, 2000; Corea *et al.*, 2008).
2. Insecticides and herbicides to control external parasites in agriculture and veterinary medicine (e.g. carbaryl, methomyl, carbofuran, formetanate, methiocarb, oxamyl and propoxur) (Gupta *et al.*, 2007; Padilla *et al.*, 2007).

Carbamate compounds also cause a cholinergic crisis due to reversible inhibition of ChE activity in the nervous tissues and skeletal neuromuscular

junctions by the formation of an enzyme inhibitor complex at the serine hydroxyl group in the esteratic site (Michaelis complex) and carbamylation of the enzyme (Fukuto, 1990; Taylor *et al.*, 1995; Gupta *et al.*, 2007). I have chosen in this study carbaryl ($C_{12}H_{11}NO_2$) 1-naphthyl-*N*-methylcarbamate, a broad-spectrum pesticide sold under the trade name Sevin. This is widely used on farms as a contact insecticide because of its effectiveness against numerous insect pests and for the control of pests on food animals, fruit, vegetables, forage, cotton and many other crops (Demirbas, 1998).

However, OP and carbamate compounds bind to ChE and inhibit the enzyme in insects and parasites, resulting in blocked degradation of neurotransmitter ACh accumulation at the synaptic cleft, and prolonged depolarization. Depolarization initially causes overstimulation of the peripheral and central nervous system leading to a range of effects including:

1. Muscarinic effects include anorexia, nausea, vomiting, diarrhoea, abdominal cramps, bronchoconstriction, sneezing, coughing, tightness in chest, dyspnoea, pulmonary oedema, increased bronchial secretions, bradycardia, increases of sweating, salivation and lacrimation (Pope *et al.*, 2005; Jokanovic and Stojiljkovic, 2006).
2. Nicotinic effects, which usually occur after muscarinic effects, that reach moderate severity. Signs include weakness, twitching, cramps, muscle fasciculation, tachycardia, hypertension and mydriasis (Jokanovic and Stojiljkovic, 2006; Ferreira *et al.*, 2008; Sharififar *et al.*, 2012).
3. Central effects include headache, dizziness, convulsion, bronchospasm, seizures, mental confusion, ataxia and coma (Jokanovic and Stojiljkovic, 2006;

Ferreira *et al.*, 2008); death occurs in some cases of anti-ChE poisoning due to central or peripheral respiratory cardiac failure (Bajgar *et al.*, 2008).

1.8 Prophylaxis and treatment of anticholinesterase

Decontamination of the gastrointestinal tract by using activated charcoal, and cutaneous injection of water and alkaline soap is the general treatment of poisoning with OP compounds (Ferreira *et al.*, 2008). Saxena *et al.* (2008) found that BChE could be used as a pre-treatment in cases of OP intoxication leading to the prevention of cardiac abnormalities and seizures. Administration of atropine sulphate, a muscarinic antagonist which binds to the muscarinic ACh receptor, reduces the effectiveness of the excess ACh produced by the inhibition of ChE (Ferreira *et al.*, 2008). Activation of ChE inhibited by OP poisoning using oximes (e.g. pralidoxime, obidoxime, methoxime, HI6 and trimedoxime) eliminates the problem by allowing removal of the excess ACh (Worek *et al.*, 2007; Ferreira *et al.*, 2008; Musilek *et al.*, 2009).

Dimethylated OP structures respond more quickly to reactivation by oximes compared to diethylated OPs, because the methyl group causes less steric hindrance and has greater electronegativity than ethyl group (Timothy, 2001; Ferreira *et al.*, 2008). The reactivity of R groups is in the order of methoxy > ethoxy > propoxy > isopropoxy > amine groups (Wilson *et al.*, 2001). If oximes are administered with suitable rapidity to an animal that has been exposed to OP compounds then they have been shown to effectively reactivate ChE, and as a consequence are the most successful therapeutic agents in the treatment of OP poisoning (Worek *et al.*, 2007). However, in case of carbamate poisoning oxime compounds must be combined with atropine sulphate because if oximes are used alone they cause an increase in the toxicity of carbamates, an

exception being carbaryl where toxicity is reduced to various degrees (Natoff and Reiff, 1973; Ferreira *et al.*, 2008).

Nicotinic antagonists include ganglionic blocking drugs (e.g. hexamethonium or trimetaphan), to alleviate tachycardia and hypertension, followed by skeletal muscle relaxant drugs (e.g. atracurium, tubocurarine or pancuronium) to relieve weakness, muscle twitching and fasciculation (Gibb, 1986). Furthermore, the use of the anticonvulsant diazepam is useful to abolish or reduce convulsion (Ferreira *et al.*, 2008; Musilek *et al.*, 2009).

1.9 Aims and objectives

The primary aim of the study described in this thesis is to start a foundation for the applicability of a biochemical biomarker, ChE activity in food animal tissues, as an instrument for evaluating exposure to pollutants, in addition to predicting high-level effects on public health. Secondary aims are to increase the awareness of pesticide users, to decide whether poisoning episodes involve anti-ChE by measuring residual effects in tissues and blood, and to recognise sources of contamination in food animal species of anti-ChE exposure.

The specific objectives of this study were:

1. to implement and validate simple methods for determining of AChE and BChE activities for food animals (Chapter 2);
2. to evaluate basal levels and characterize AChE and BChE activities in food animals, in blood (erythrocyte, serum and plasma) and tissue (liver, kidney, muscle, heart and lung), to investigate the most accurate and precise method for determination of ChE activities for food animals, and to determine the most sensitive (target) tissues for measurement of ChE activity (Chapter 3);

3. to investigate the effects of storage at -20 °C and -80 °C on AChE and BChE activities, in addition, to determining the best method for storage of samples for the determination of AChE and BChE activities in food animals (Chapter 4);
4. to develop a protocol for the purification of AChE and to extend this method for further enzyme characterization (Chapter 5);
5. to characterize ChE activities, e.g. with respect to the effects of dilution, selective inhibitors, pH, temperature and histochemical localisation (Chapter 6);
6. to investigate the kinetic properties of ChE, and also to establish a foundation to determine the effects of pH and temperature on the rate of the constants of inhibition with OP and carbamate compounds (Chapter 7); and
7. to investigate the rates of spontaneous reactivation of AChE inhibited by OP compounds, and moreover, to determine the time course of aging of OP-inhibited AChE. Finally, to find suitable conditions to reactivate of OP-inhibited AChE and to determine the most efficacious oxime compounds as antidotes for intoxication by OP compounds (Chapter 8).

The final chapter of the thesis (Chapter 9) discusses the importance of applications of biochemical biomarker responses in AChE and BChE activities posed by the OP and carbamate compounds for the food animal species.

Chapter 2

General materials and methods

Chapter 2: General materials and methods

2.1 General chemicals

Cholinesterases (ChE) substrates (acetylthiocholine iodide, AcTChI, 98% purity; S-butyrylthiocholine iodide, BuTChI, 98% purity; propionylthiocholine iodide, PrTChI; acetylcholine iodide, 97% purity; and butyrylcholine iodide, 99% purity); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB); dichlorvos (DDVP), 98.1% purity; carbaryl; tetraisopropyl pyrophosphoramidate, iso-OMPA; 1: 5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide, BW284c51; epoxy-activated Sepharose; edrophonium chloride; α -methyl-D-mannoside; Sephacryl S-200; polyvinyl alcohol; obidoxime; pralidoxime 99% purity; heparin; and ethylenediaminetetraacetic acid (EDTA) were supplied by the Sigma Chemical Company (Poole, Dorset, UK). Diazinon (DZN) was obtained from Chem Services, Riedel de Haen, Seelze-Hannover, Germany. All other reagents and solvents used in this thesis were of analytical grade and were supplied by Fisher (Loughborough, Leicestershire, UK).

2.2 Animal tissue

Fresh meat from healthy food animals (sheep, *Ovis aries*; cattle, *Bos taurus*; and pig, *Sus domesticus*) were obtained from local markets in Plymouth and abattoirs in Cornwall (Callington and Launceston), UK (Figure 2.1). These had been killed by a blow to the head after which the brain was pithed in the manner approved for use in abattoirs. The samples were transported on ice to the laboratory at the University of Plymouth for immediate processing. The time between death of the animal and the start of processing was about an hour. During sample collection from the animal it was ensured that there was no possibility of introduction of anti-ChE compounds from the skin of the animals.

As noted by Fairbrother *et al.* (1991) this can be a source of contamination by anti-ChE compounds.

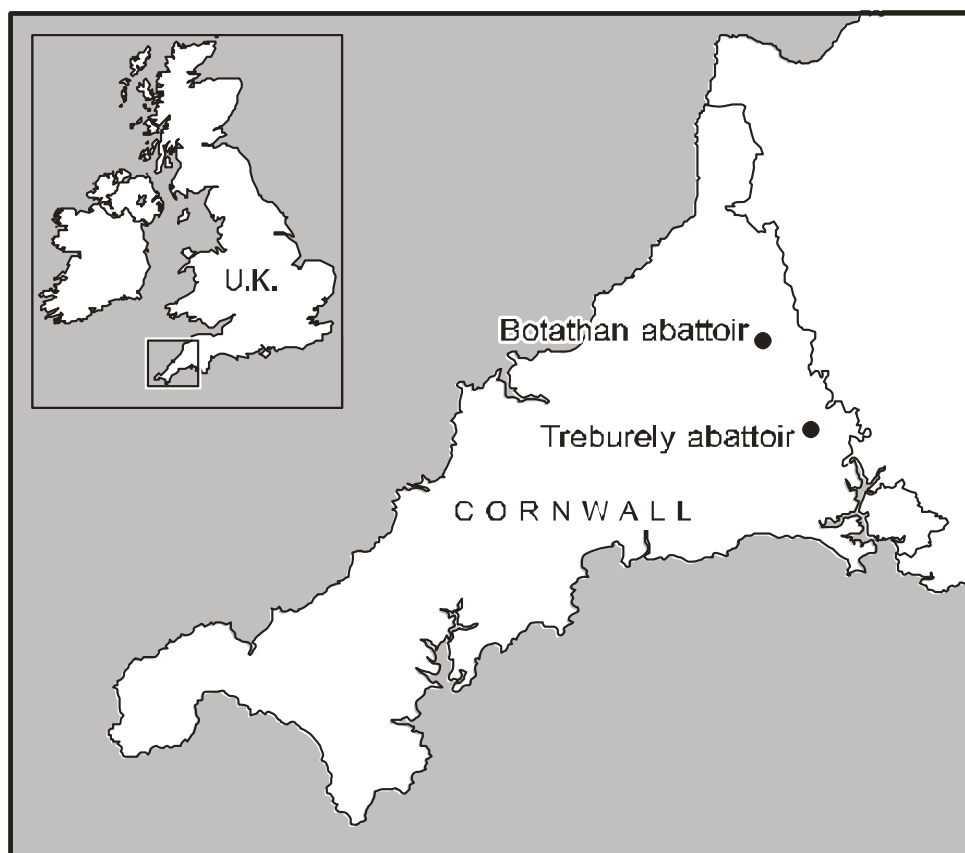


Figure 2.1 Map of south-west UK showing sample collection sites in Cornwall.

2.3 Sample preparation

2.3.1 Blood samples

To obtain serum, blood samples were allowed to clot for at least 1 h at 20 °C, after which they were centrifuged at 3000 g for 10 min in a Biofuge Pico micro-centrifuge (Heraeus Instrument, Osterode, Germany). To obtain plasma, 4 ml blood samples were added to anticoagulant (either EDTA, 7.2 mg, final concentration 0.18%, or heparin, 3.7 mg, final concentration 0.1%) in 12 ml centrifuge tubes. Plasma was separated by centrifugation at 3000 g for 10 min. The erythrocytes were washed three times with two volumes of 0.1 M sodium

phosphate buffer, pH 7.4, centrifuging as described above between washes. Next, the packed erythrocytes were diluted in 20 volumes of hypotonic sodium phosphate buffer (6.7 mM, pH 7.4) to facilitate haemolysis followed by centrifugation at 3000 *g* for 10 min. The supernatant was removed and the pellet re-suspended in hypotonic phosphate buffer. Aliquots of the erythrocyte ghosts were stored at -80 °C until use (Tecles *et al.*, 2000; Aurbek *et al.*, 2006).

2.3.2 Tissue samples

One gram of each tissue was removed using a scalpel, cut into small pieces (3-4 mm³), and rinsed until the blood was fully removed. The tissue was then placed on ice in 12 ml tubes (7.5 mm internal diameter) and homogenized using a mechanically-driven homogenizer (Model X520-D, T6 probe, Bennett and Company, Weston Super Mare, North Somerset, England, UK) with sodium phosphate buffer (0.1 M, pH 8) at a ratio of 1 part of tissue to 9 parts of buffer, and a speed of 10000 rpm. Homogenisation required between 2 and 5 min depending on the tissue; after every 30 s or so of homogenisation the mixture was rested for 10 s to allow cooling. The homogenate was then centrifuged in Eppendorf tubes at 9000 *g* for 5 min at 4 °C (Morizono and Akinaga, 1981; Lassiter *et al.*, 2003). It was important during homogenization to ensure that (a) samples were fully homogeneous and that aliquots taken reflected the homogenate as a whole, and (b) that ChE activities were not altered in the process (e.g. through heat-induced denaturation) (Fairbrother *et al.*, 1991). Overall, each experiment in this thesis was designed as described separately in each Chapter.

2.4 Enzyme activity measurement

Two methods were used to analysis ChE as described below.

2.4.1 Ellman method

2.4.1.1 Introduction

At present, the most widely used method for the determination of ChE activity is the colorimetric method of Ellman *et al.* (1961). This is a simple, accurate, fast and direct method of measuring ChE activity in blood and tissues. It is based on the reaction between thiocholine, which is one of the products of the enzymatic hydrolysis of the synthetic substrates AcTChI, BuTChI or PrTChI with the sulfhydryl group of a chromogen such as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The formation of the yellow product of this reaction, 5-thio-2-nitrobenzoic acid (TNB) is measured by monitoring absorbance at 410 nm in addition mixed disulphide (Figure 2.2).

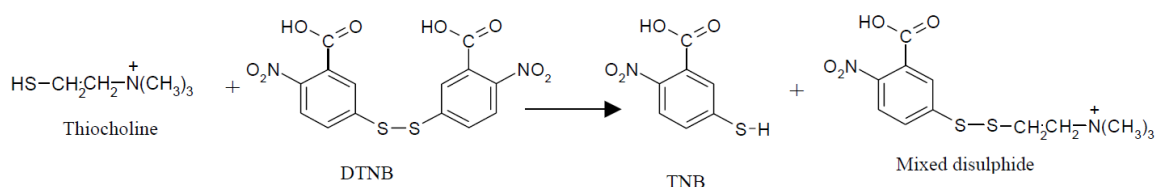


Figure 2.2 Ellman colorimetric reaction (taken from (Frasco *et al.*, 2005)).

Each mole of anion produced represents the hydrolysis of one mole of substrate (Morizono and Akinaga, 1981; Tecles and Ceron, 2001; Frasco *et al.*, 2005; Sinko *et al.*, 2007). The advantages of DTNB are that it is water-soluble, it can be used at neutral pH with few side reactions, and its reaction with thiocholine is fast and sensitive due to the high molar absorption coefficient of TNB (Ellman *et al.*, 1961; Tecles and Ceron, 2001; Timothy, 2001).

2.4.1.2 Assay

Cholinesterases (ChE) activity was determined by the Ellman method (1961), adapted for use with microtitre plates as described by Haigh *et al.* (2008), and using either AcTChI or BuTChI as the substrate (1 mM final concentration of each) for measuring AChE and BChE activities, respectively. Incubation of the sample with 4 mM final concentration of iso-OMPA, a potent inhibitor of BChE but not for AChE in case measuring of AChE. In preparing solutions during measuring, it is essential that the substances have been stored properly and have not exceeded their shelf life, however substrates and reagents in solution have a much shorter duration of stability and should not to be used on experiment extending over one day, and kept on ice during use. Briefly, 0.02 ml of sample and 0.24 ml of assay mixture [9.75 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA, and 0.25 ml of 0.2 mM final concentration of DTNB] were mixed, allowed to stand for 5 min, and then 0.04 ml of substrate solution were added. The assay temperature in each case was 25 °C.

The absorbance increase was monitored for 5 min at 410 nm in a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA) (Haigh *et al.*, 2008). There may be some non-enzymic (endogenous) reaction between the sample and the DTNB which may interfere with the analysis. To control for this, a pre-incubation of DTNB and sample is performed prior to the addition of substrate. In each case the rate of absorbance increase was corrected by subtracting, the rate observed for a reagent blank (i.e. without sample). ChE activities were calculated using an extinction coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ for TNB (Worek *et al.*, 1999), and are expressed as units (1 U \equiv 1 $\mu\text{mol} \equiv$ 1000 nmol of substrate

hydrolysed per min) per g wet weight of tissue. All measurements were carried out in triplicate.

2.4.2 Michel method

2.4.2.1 Introduction

Another method for measuring ChE activities is the electrometric method of Michel (1949), that has been used to detect ChE activity in human plasma and erythrocytes. In this method the change in pH arising from the production of H⁺ in the hydrolysis of cholinester substrates (ACh, butyrylcholine and propionylcholine) to form choline and a carboxylic acid is directly determined using a pH meter (Michel, 1949; Wilson *et al.*, 2001; Wilson and Philip, 2005). The acids produced immediately dissociate liberating H⁺ to form acetate, butyrate and propionate, respectively, resulting in a decrease in pH in the reaction mixture (Fedosseeva *et al.*, 2000; Wilson *et al.*, 2001; Ahmed and Mohammad, 2005). More recently various modifications to the Michel method have been developed including (a) increase in sample volume; (b) decrease in incubation time; (c) increase in incubation temperature; and (d) use of a range of different buffers (Mohammad, 1997; Hamm, 1998; Ahmed and Mohammad, 2005).

2.4.2.2 Assay

Cholinesterases (ChE) activity was also determined by the Michel method (1949), further adapted by Mohammad (2007). In brief, 3 ml of distilled water was placed in a 10 ml beaker. Three millilitres of barbital phosphate buffer [6 mM sodium barbitone, 4 mM potassium dihydrogen phosphate and 600 mM sodium chloride, and adjusted to pH 8.1 with a few drops of 1 mM HCl by (Hanna GLP Bench-top pH/mV/ISE/°C meter)] was added, followed by a 0.2 ml

sample of blood or homogenised tissue. The pH of the mixture was measured just after addition of the substrate using a glass electrode connected to a pH meter (Hydrus 500^R, Thermo Orion, USA). Substrate (0.1 ml of either 27.5 mM acetylcholine iodide or butyrylcholine iodide, for measurement of AChE or BChE activities, respectively) was added and the mixture incubated in a water bath at 37 °C for 20 min, after which the pH was again measured (Hawkins and Knittle, 1972; Domenech *et al.*, 2007). The activity was calculated as the change in pH over the 20 min (Δ pH/20 min) minus Δ pH of the blank, i.e. without added sample (Mohammad, 2007; Jamshidzadeh *et al.*, 2008). All measurements were carried out in duplicate. Again, substrate solutions were prepared and used on the same day and kept on ice during use.

2.5 Statistical analysis

Conventional statistical methods were used to calculate the means, coefficient of variance (CV), standard deviation (SD) and standard error (SE). Pearson's correlation coefficient, regression analysis, two sample *t-test* and one-way analysis of variance (ANOVA) were applied to test for any significant differences (associated probability < 0.05). All statistics was carried out using MiniTab statistical software version 15 (MiniTab Ltd., Coventry, UK).

Chapter 3

Comparative analysis of cholinesterase activities in food animals using modified Ellman and Michel assays

The results from this Chapter have been presented at the 1st Annual Meeting of the Combined Universities in Cornwall Environmental Research Forum with the European Centre for Environment and Human Health presents: Environmental Research, Business and Innovation in South West England. September 2010, Cornwall, UK (poster presentation). The results have also been published in *Canadian Journal of Veterinary Research* 75(4):261-270 (Abass Askar *et al.*, 2011).

Chapter 3: Comparative analysis of cholinesterase activities in food animals using modified Ellman and Michel assays

3.1 Introduction

Cholinesterases (ChE) are specialized carboxylic ester hydrolases that catalyse the hydrolysis of choline esters. They are classified as either acetylcholinesterase (AChE, EC 3.1.1.7) or butyrylcholinesterase (BChE, EC 3.1.1.8) (Chatonnet and Lockridge, 1989; Rao *et al.*, 2007; Wilson, 2010). The most widely used method for the determination of AChE and BChE activities is the colorimetric method of Ellman *et al.* (1961). It is based on the reaction between thiocholine, which is one of the products of the enzymatic hydrolysis of the synthetic substrates AcTChI, BuTChI or PrTChI with the DTNB. The formation of the yellow product of this reaction, TNB is measured by monitoring absorbance at 410 nm. Each mole of anion produced represents the hydrolysis of one mole of substrate (Morizono and Akinaga, 1981; Tecles and Ceron, 2001; Frasco *et al.*, 2005; Sinko *et al.*, 2007). Another method for determination AChE and BChE activities is the electrometric method of Michel (1949), that has been used to detect ChE activity in human plasma and erythrocytes (Michel, 1949; Wilson *et al.*, 2001; Wilson and Philip, 2005). In this method the change in pH arising from the production of H⁺ in the hydrolysis of cholinester substrates (ACh, butyrylcholine and propionylcholine) to form choline and a carboxylic acid is directly determined using a pH meter (Michel, 1949; Wilson *et al.*, 2001; Wilson and Philip, 2005). The acids produced immediately dissociate liberating H⁺ to form acetate, butyrate and propionate, respectively, resulting in a decrease in pH in the reaction mixture (Fedosseeva *et al.*, 2000; Wilson *et al.*, 2001; Ahmed and Mohammad, 2005). Methods governing ChE activities of tissues from food animals are still poorly understood. Our objective were (a) to

investigate correlations between the modified Ellman and Michel methods, and (b) to establish a foundation for the applicability of AChE and BChE activities in food animal species as biochemical biomarkers for evaluating of exposure to OP and carbamate compounds. Also part of the study is to characterize the level of AChE and BChE activities in the selected organs/tissues and determined the best organ/tissue in which to measure ChE activity. Biomarkers of exposure can be further divided into three groups: (a) potential dose or external dose, (b) internal or absorbed dose, and (c) biologically effective dose.

3.2 Materials and methods

3.2.1 Sample collection and preparation

The sample collection and preparation from the sheep, cattle and pig were done as described in Sections 2.2 and 2.3. Following this it was then measured the enzyme activity as described in Section 2.4. All chemicals were of the highest analytical grade obtained from Sigma Chemical Company (Poole, Dorset, UK), unless stated otherwise.

3.2.2 Statistical analysis

All enzyme measurements were conducted in ten individual samples from each animal with results expressed as mean values \pm SE. Pearson's correlation coefficient, percentage coefficient of variance (%CV), standard deviation (SD), regression analysis and the two-sample *t*-test were applied to test for any significant differences ($P < 0.05$). The Bland-Altman method was also used to compare between two methods as described in Dewitte *et al.* (2002). All statistics was carried out using MiniTab statistical software version 15.

3.3 Results

3.3.1 Determination of cholinesterase activity in blood

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were determined in blood and blood derivatives (plasma with either heparin or EDTA, serum and erythrocytes for sheep and cattle) using the modified Ellman and Michel method as described in Section 2.4 (Figure 3.1). There was significantly higher AChE activity in plasma when heparin was used as the anticoagulant compared to when EDTA was used, using both assay methods (Figure 3.1A and 3.1B). In all cases (cattle and sheep, using both assay methods) AChE activity was higher than BChE activity in plasma (Figure 3.1A and 3.1B). It was found that BChE activity in plasma was higher in cattle than in sheep, using both assay methods (Figure 3.1A and 3.1B). Using the modified Ellman method AChE activity in both in serum and erythrocytes was found to be significantly higher in sheep than in cattle (Figure 3.1C and 3.1D, respectively). In contrast, BChE activity was significantly higher in both serum and erythrocytes from cattle than sheep (Figure 3.1C). Similar to plasma, there was no significant correlation between AChE and BChE activities in either serum or erythrocytes from sheep or cattle. Taking the overall data set there was a significant correlation between both AChE and BChE activities measured by the modified Ellman and modified Michel methods (Pearson's correlation coefficient = 0.90, $P < 0.0001$; Figure 3.2A). However, the percentage coefficient of variance (%CV) values for each tissue were generally higher (13 out of 16 sets of data) using the Michel method than the Ellman method (Figure 3.3A-D). The Bland and Altman plot of the ratio of two methods Ellman and Michel was showed the mean differences between two methods to be 0.57 and SD was 1.57 and -0.45 for upper and lower limits, respectively (Figure 3.4A).

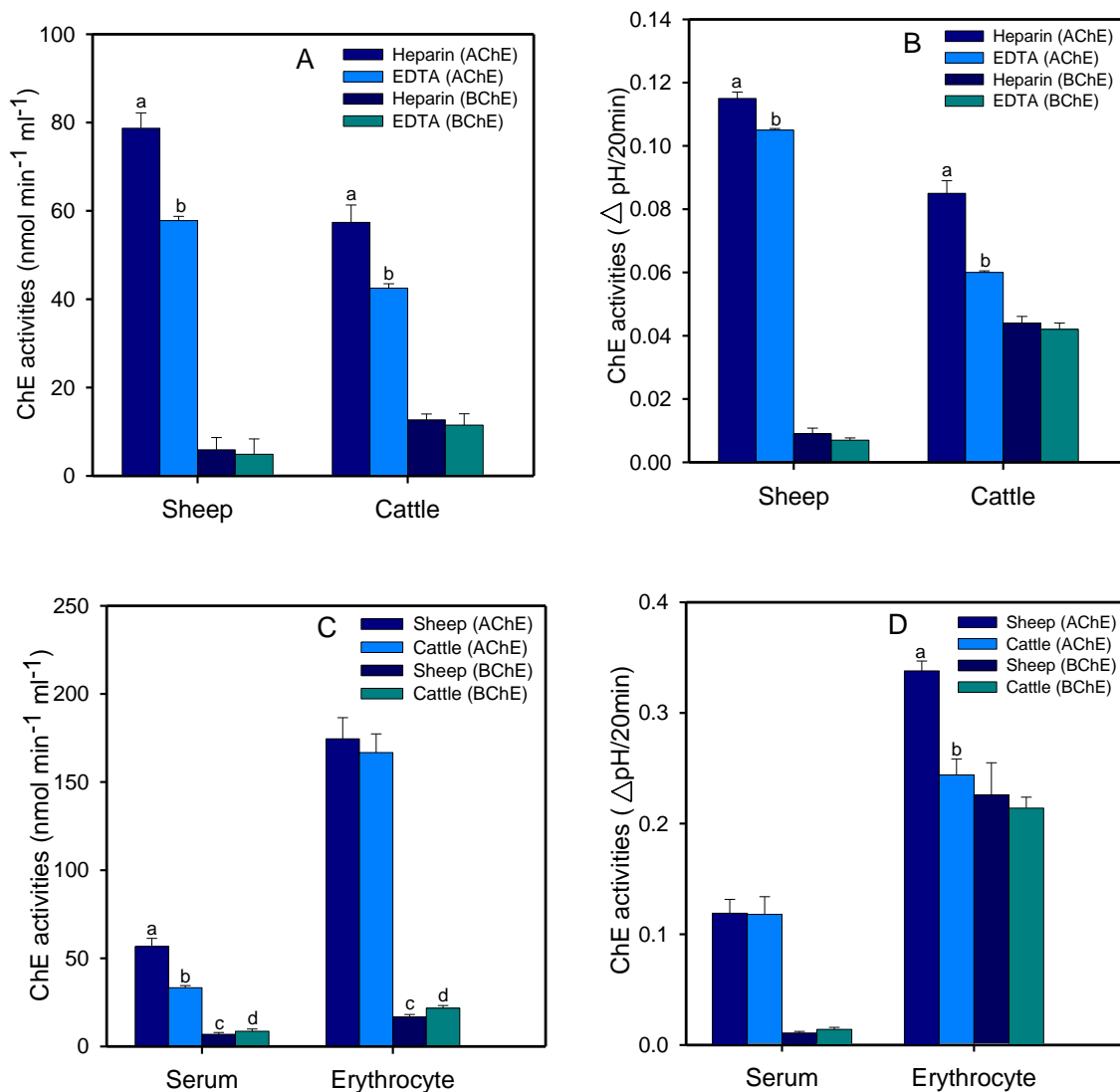


Figure 3.1 AChE and BChE activities in plasma (with heparin and EDTA), serum and erythrocyte for sheep and cattle using (A and C) modified Ellman method and (B and D) modified Michel method. Data are expressed as the mean \pm SE, ($n = 10$ in each animal). The different letters are significantly different (t -test, $P < 0.05$).

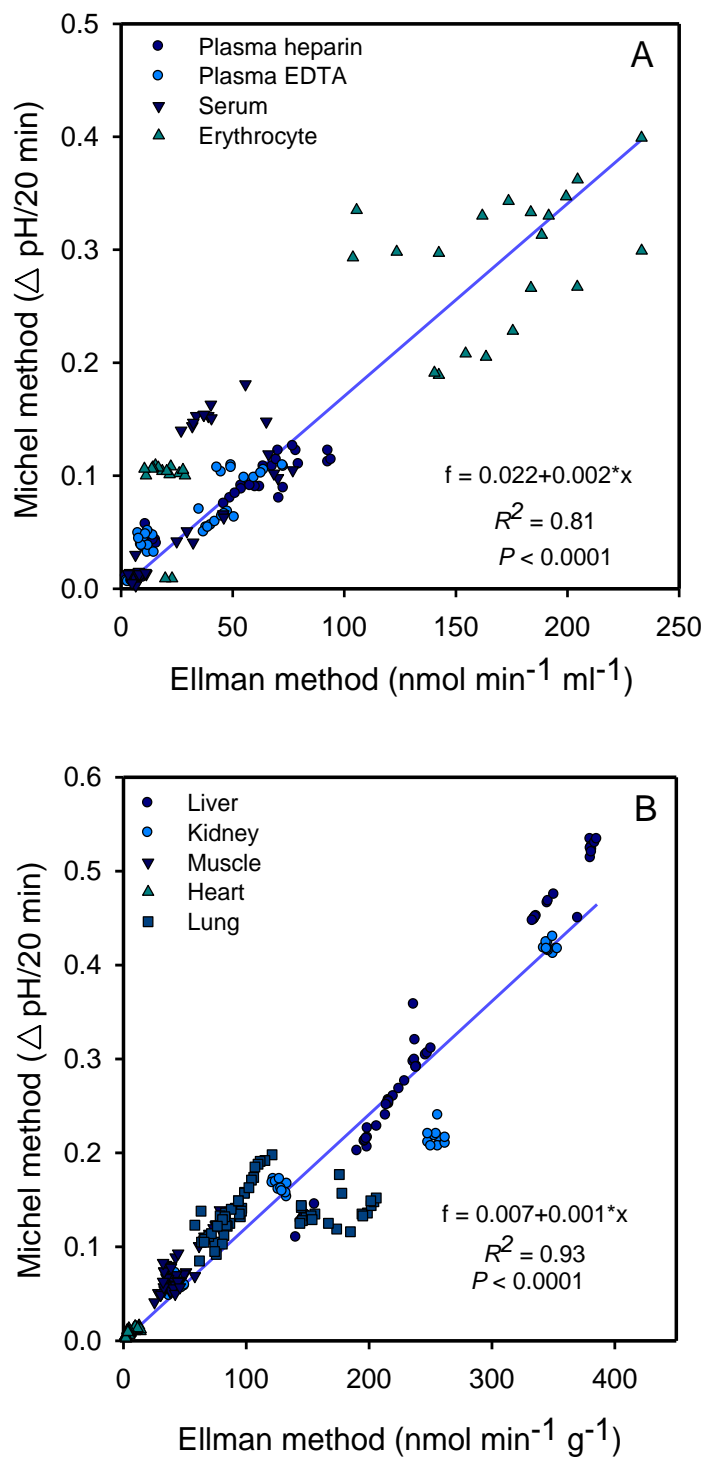


Figure 3.2 Regression analysis of the individual AChE and BChE activities using modified Ellman and Michel assays from blood derivatives (A) and tissues (B) of food animals.

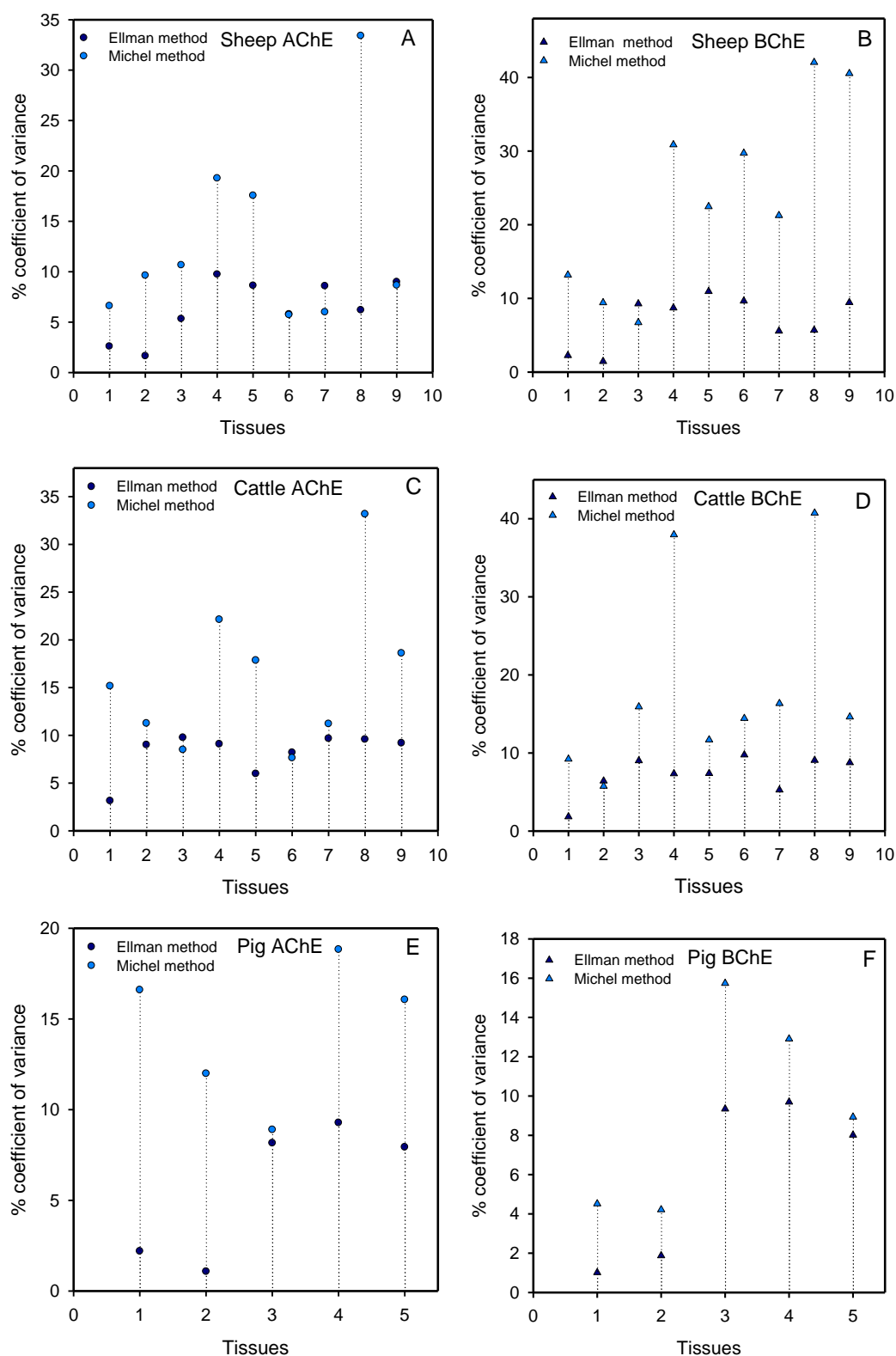


Figure 3.3 Percentage coefficient of variance (%CV) between modified Ellman and modified Michel methods for ChE activities of sheep, cattle and pigs. The x-axis from tissues 1 = liver, 2 = kidney, 3 = muscle, 4 = heart, 5 = lung, 6 = plasma with heparin, 7 = plasma with EDTA, 8 = serum and 9 = erythrocyte.

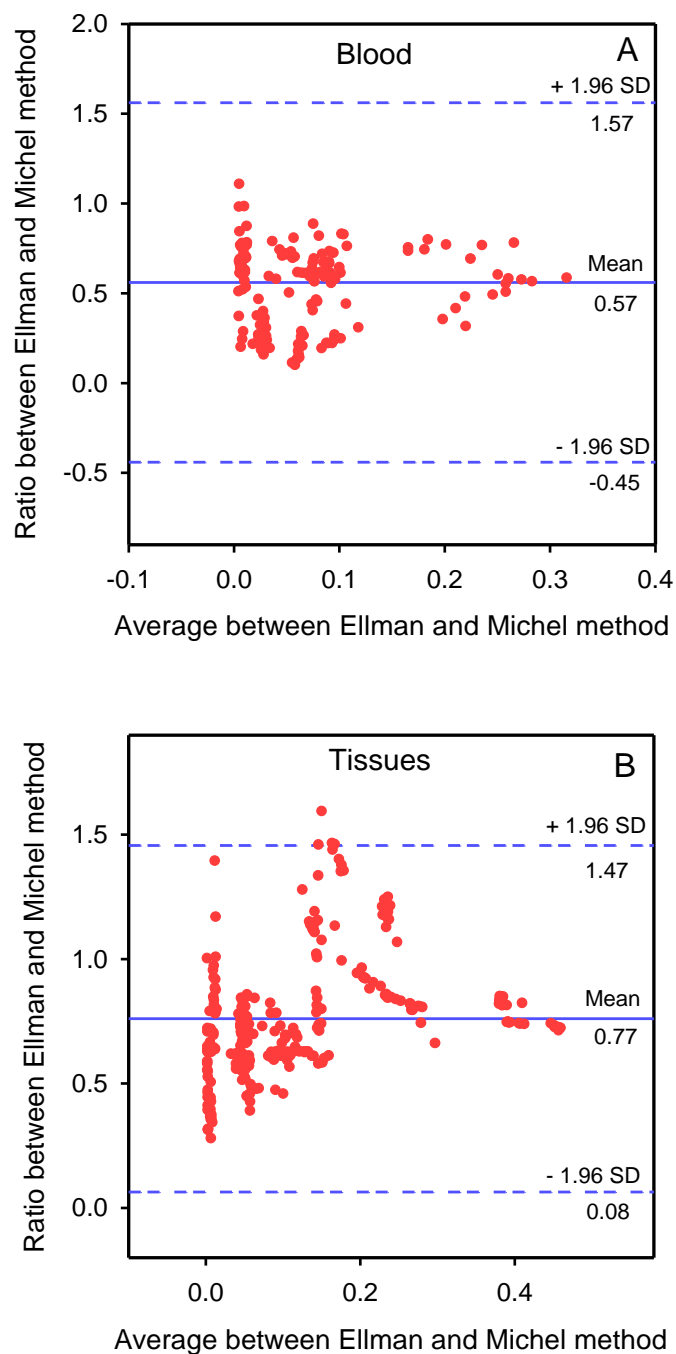


Figure 3.4 Bland and Altman plot of the ratio of the Ellman and Michel (Δ pH) assay (plotted on the y-axis) versus the average of the two methods (x-axis) for AChE and BChE activities in food animal. Horizontal lines are drawn at the mean difference, and at the mean difference \pm 1.96 SD of the differences (dashed line). If the differences within mean \pm 1.96 SD are clinically not important, the two methods cannot be used interchangeably.

3.3.2 Determination of cholinesterase activities in animal tissues

Both AChE and BChE activities were also determined in a range of other tissues (liver, kidney, muscle, heart and lungs from sheep, cattle and pigs) using the modified Ellman and Michel method as described in Section 2.4 (Figure 3.5). Tissue from the heart had the lowest activities for both AChE and BChE in all three animals compared with other tissues using both assay methods (Figure 3.5A-D).

In general both enzyme activities were highest in liver, with the exception of tissue from pigs in which AChE activity was higher in kidney than that seen in liver and much higher (8 times) than that seen in kidney from sheep and cattle (Figure 3.5A and 3.5B). Both enzyme activities tended to be highest in pig, with the exception of lung, in which activities were highest in cattle (Figure 3.5A-D). Using both assay methods, in the case of lung tissue from sheep, there was a significant positive correlation (Pearson's correlation coefficient, $r = 0.79$, $P = 0.003$ and $r = 0.78$, $P = 0.006$ for the Ellman and Michel methods, respectively; Figure 3.6A-B).

Again, taking the overall data set there was a significant correlation between AChE and BChE activities measured by the modified Ellman and modified Michel methods (Pearson's correlation coefficient = 0.96, $P < 0.0001$; Figure 3.2B), and again, the %CV values for each tissue were generally higher (27 out of 30 sets of data) using the Michel method than the Ellman method (Figure 3.3A-F). The Bland and Altman plot of the ratio of the Ellman and Michel two methods showed the mean differences between the two methods to be 0.77 and SD was 1.47 and -0.08 for upper and lower limits, respectively (Figure 3.4B).

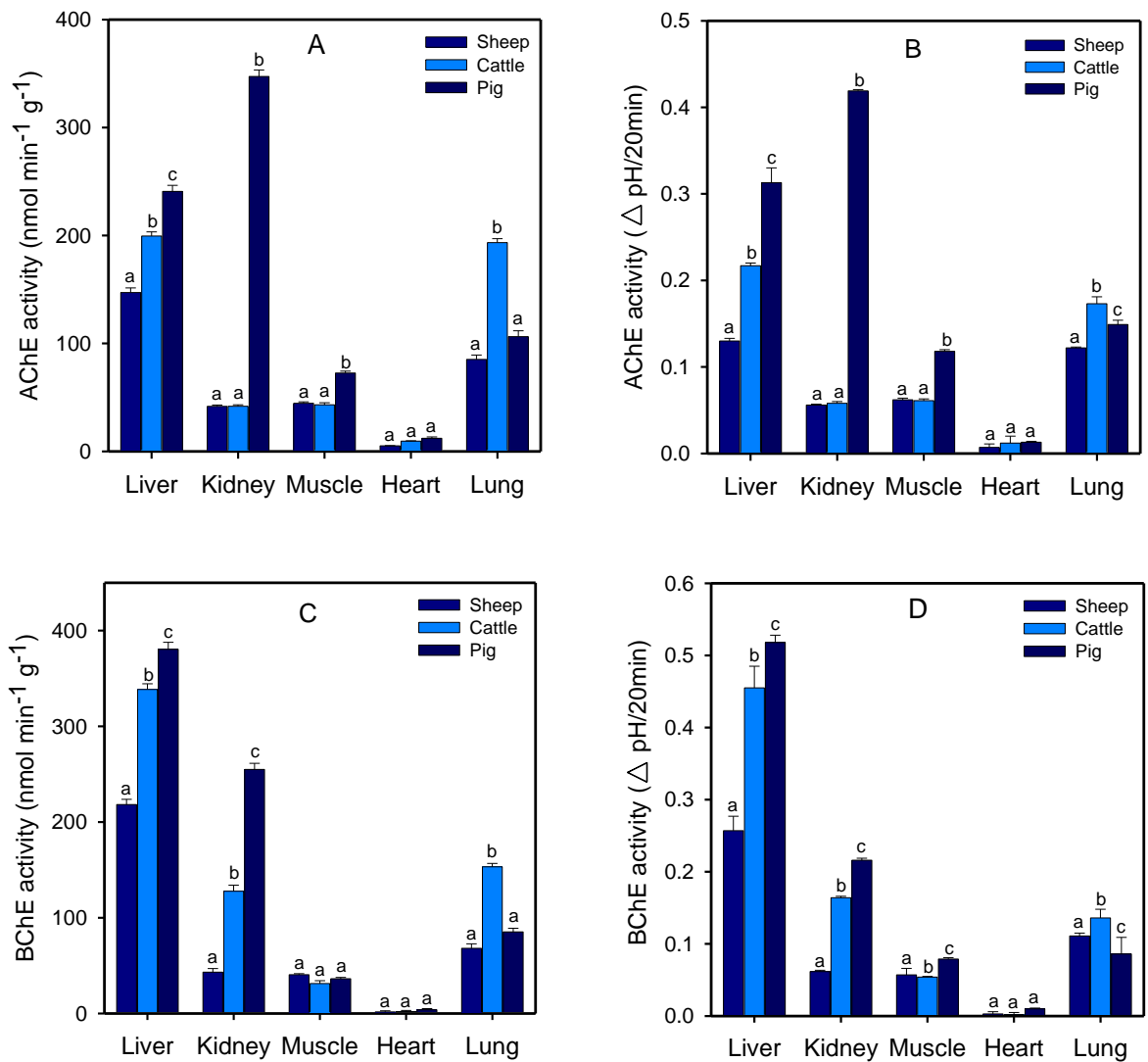


Figure 3.5 AChE and BChE activities from tissues of sheep, cattle and pigs using (A and C) modified Ellman method and (B and D) modified Michel method. Data are expressed as the mean \pm SE, ($n = 10$ in each animal). The different letters between the animals are significantly different [analysis of variance (ANOVA), $P < 0.05$].

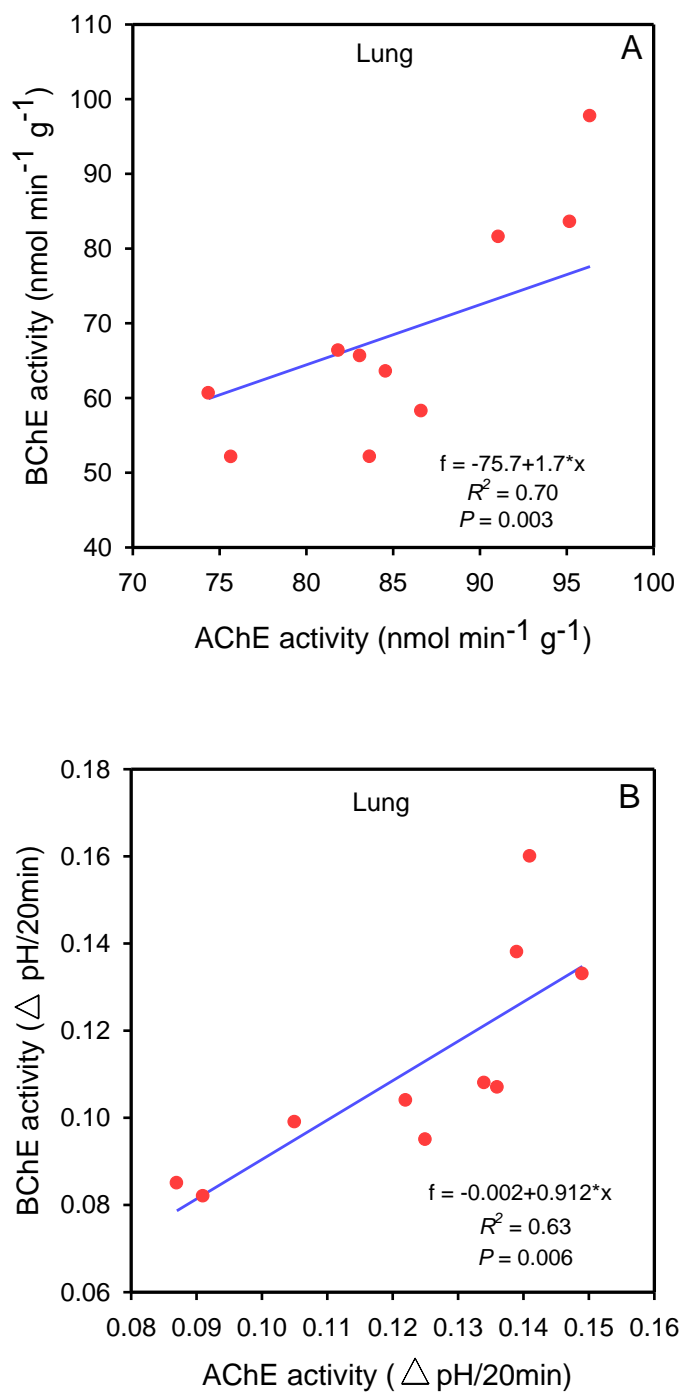


Figure 3.6 Pearson's correlation coefficient values between AChE and BChE activities from the lungs of sheep using (A) modified Ellman method and (B) modified Michel method.

3.4 Discussion

3.4.1 Cholinesterase activities in blood

The widespread use of OP and carbamate compounds and the dangers associated with their application have resulted in ChE activities being used as biomarkers for evaluating both exposure to and the effect of these pesticides (Wilson *et al.*, 2001). As noted by Wilson *et al.* (2001) determining ChE activities may form the basis for the establishing of safe levels of such pesticides in food and the environment. There are two methods widely used to measure of ChE activities, are the modified Ellman method (Haigh *et al.*, 2008) and the modified Michel method (Mohammad, 1997). These methods have been used successfully to measure ChE activities in blood from goats (Guhathakurta and Bhattacharya, 1989; Al-Jobory and Mohammad, 2004). However, neither method has been validated for use either in blood from other food animals or in other tissues. The present study is the first attempt to standardize and validate methods for ChE activity determining in tissues, including blood, from sheep and cattle.

Plasma separation from erythrocytes requires a suitable anticoagulant. Some anticoagulants, e.g. oxalate and citrate cannot be used for blood samples intended for ChE measurement because they bind Ca^{2+} and Mg^{2+} which are necessary for the enzyme activity (Whitter, 1963). Both anticoagulants used in the present study, heparin and Ca^{2+} -EDTA, have been recommended for this type of enzyme study (Fairbrother *et al.*, 1991), and apparently did not cause major interference with the measurement of either AChE or BChE activities. I found significantly higher activities when heparin was used as the anticoagulant, than when EDTA was used (Figure 3.1A and 3.1B). This is in agreement with previous research by Mohri and Rezapoor (2009), in which they found generally

higher levels of analytes (metabolites, ions and enzyme activities, not including ChE) in heparinised plasma from sheep compared to EDTA plasma. They also found little difference in the measurement obtained for heparinised plasma compared to serum. In contrast, when ChE activities were measured in this study, activities were higher in plasma than in serum.

The BChE activities I obtained using the Ellman method for sheep and cattle plasma (Figure 3.1A) are lower with those found by Al-Qarawi and Ali (2003). However, these authors used a modified Hestrin method (Lee and Livett, 1967) in which butyrylcholine is used as the substrate instead of BuTChI which is used in the Ellman method. They also used a ten-fold higher concentration of substrate. Both of these factors may explain the apparent difference in activity.

In all cases, both assay methods showed high levels of ChE activity in erythrocytes, about 2-3 times higher than that of serum and plasma for AChE activity, and about 35 times higher than that of serum and plasma for BChE activity (Figure 3.1A-D). This is in agreement with the findings of Al-Jobory and Mohammad (2004) reported AChE activity to be about 2-times higher in erythrocytes than in plasma in goats. Furthermore, both Ahmed and Mohammad (2005) and Silvestri (1977) observed higher ChE activities in erythrocytes from humans and horses than for plasma, using the Michel method. The high ChE enzyme activities seen in erythrocytes are due to the large amount of ChE enzyme located on their surface (Suhail and Rizvi, 1989). In general, the level of AChE activity in blood derivatives in sheep was higher than in cattle (Figure 3.1A-D). This is in agreement with an earlier report by Crookshank and Palmer (1978) observed higher ChE activities in erythrocytes, plasma and serum from sheep than cattle using the Michel method, but

contrasts with Wilson *et al.* (2001) observed that activity in erythrocyte from cattle was much higher than those by the other food animals (e.g. horses and rabbits) by using the manometric method according to Zajicek (1957).

In all cases, ChE activities measured by both the Michel and Ellman methods were directly proportional (Figure 3.2A). This is in agreement with the findings of other authors for these activities in human plasma and erythrocytes (Groff *et al.*, 1976; Crookshank and Palmer, 1978). For example, Groff *et al.* (1976) found an excellent positive correlation found between results for the Ellman and Michel methods in human erythrocytes and plasma. Chuiko *et al.* (2003) found a positive correlation between AChE and BChE activities in citrate plasma across a range of sixteen teleost fish species. Of the two species studied here, sheep and cattle, a correlation between AChE and BChE levels was only seen in EDTA plasma from sheep ($R^2 = 0.61$ and $P = 0.008$). A similar relationship was not seen in heparin plasma, nor was it seen in EDTA or heparin plasma from cattle, making it likely that this is a spurious observation (Hawkins and Knittle, 1972).

Higher coefficient of variance (%CV) values were obtained using the Michel method than with the Ellman method (Figure 3.3A-F). The modified Ellman method is therefore more precise than the modified Michel method in determining contents of blood for sheep and cattle. These interesting results differ with these of Lewis *et al.* (1981) who observed that a good precision for both Ellman and Michel assays for measuring ChE activities in human plasma and erythrocyte. The modified Ellman ChE assay method can give a more reliable result than the laboratory based modified Michel assay. Hence, either AChE or BChE would be used to determine ChE activities in the blood tissues.

Finally, these results supported the idea that AChE and BChE activity in erythrocyte, plasma and serum for sheep and cattle may be a suitable biomarker for anti-ChE compounds. The mean differences in the blood derivatives between two methods are plotted by Bland and Altman plot and showed clinically important, due to the mean was found lesser than ± 1.96 (Figure 3.4A).

3.4.2 Tissue cholinesterase activities

Both AChE and BChE activities were also determined in a range of other tissues. The results for sheep and cattle in the present study show that the AChE activity was far higher in tissue from the liver than from that of kidney, muscle, heart or lungs (Figure 3.5A). This is in agreement with the work of Morizono and Akinaga (1981), in which they found AChE activity to be higher in liver than in kidney, muscle, lung and heart tissue in cattle. In contrast, AChE activity in kidney tissue from pigs was found to be higher than those by the other tissues (i.e. lung, muscle and heart) (Figure 3.5A). This is again in agreement with previous research by Morizono and Akinaga (1981) found that AChE activity was generally higher in tissue from kidney than in that from the heart and lung of pigs.

The AChE activities obtained using the Ellman method for muscle tissue from sheep, cattle and pigs (Figure 3.5A) are lower than those found by Sharma *et al.* (1994). However, these authors used a high temperature (30 °C) to dilute samples and also filtered the homogenised samples through muslin cloth. In our study lower temperature (25 °C) was used, and the homogenates were not filtered. Both of these factors may explain the apparent difference in activity.

The BChE activities I obtained using the Ellman method for tissue from sheep and cattle liver (Figure 3.5C) are lower than those found by Al-Qarawi and Ali (2003). However, these authors used a modified Hestrin method (Lee and Livett, 1967) and used butyrylcholine as the substrate instead of BuTChI which is used in the Ellman method. They also used a ten-fold higher concentration of substrate. Both of these factors may explain the apparent difference in activity.

In general, the total BChE activity in tissue from liver was higher than in the tissue from kidney, muscle, lungs and about 51-88 times higher than tissue from the heart for sheep, cattle and pig (Figure 3.5D). This is in agreement with an earlier report by Mohammad (2007) reported ChE activities to be higher in liver than in muscle in rock dove, quail and chickens. Indeed, the liver is considered to be a vital organ and effective in detoxification and hence involved in creating of BChE (Ogunkeye and Roluga, 2006).

The lowest AChE and BChE activities were found in tissue from the heart compared with other (e.g. liver, kidney, muscle and lungs in all three animal species). This is in agreement with the findings of Abdelsalam and Ford (1985) observed that ChE activities were lower in tissue from the heart than that of lung, liver and kidney in cattle. But in contrast with Chemnitus *et al.* (1992) BChE activity in tissue from pig heart was observed to be $216 \text{ nmol min}^{-1} \text{ g}^{-1}$ which is higher compared to that found in our study (Figure 3.5C). These authors used a thirty-fold higher concentration of substrate, however, which may explain the apparent difference in activity.

In general, ChE activities were highest in tissue from pigs followed by tissues from cattle and sheep (Figure 3.5A-D). These results disagree with those of Al-Qarawi and Ali (2003) who observed that ChE in tissue liver from sheep was

higher than in tissue from cattle. The biological significance of these differences may be related to a varying ability of the ChE of different animals to metabolize xenobiotics (e.g. anti-ChE compounds) (Al-Qarawi and Ali, 2003).

In all cases, I found that ChE activities measured by both the Michel and Ellman methods were directly proportional (Figure 3.2B). This is in agreement with the findings of Hawkins and Knittle (1972) observed that the regression lines were directly proportional to each other when both the Ellman and Michel methods were compared in the brain of birds. This differs from the results of Padilla *et al.* (2007) who observed that the regression line was not proportional in the brain of rats. These authors compared the Ellman method to the radiometric method, however rather than to the Michel method.

Once again higher %CV values were obtained using the Michel method in comparison with the Ellman method (Figure 3.3A-F). The modified Ellman method is therefore more precise than the Michel method in the tissues of sheep, cattle and pigs. These results differ from with the work of Hawkins and Knittle (1972) found that %CV values obtained using the Ellman method were higher than with the Michel method. However, these authors compared tissue from brain of birds rather than from food animals tissues, which may explain the apparent difference in activity. Finally, of all the cases, I found that there was a significantly positive correlation between AChE and BChE activities in the tissues from the lungs of sheep (Figure 3.6A-B). Again, the mean differences in the tissues over all animals between the two methods are plotted by Bland and Altman plot and was seen clinically important (Figure 3.4B).

3.5 Conclusions

This study shows for the first time that the level of AChE and BChE activities by two methods (modified Ellman and Michel) from tissues of sheep, cattle and pigs. To summarise the main points of this Chapter, the following conclusions can be drawn.

1. Erythrocytes are the main source for AChE and BChE activities found in the blood of sheep and cattle, while the liver and kidney were the main sources of ChE activities found in the tissues of sheep, cattle and pigs. In addition, heparin was a more sensitive anticoagulant than EDTA for measuring AChE activity. AChE and BChE activities in the blood contents were higher in cattle than in the sheep.
2. Both AChE and BChE activities were found in blood contents for sheep and cattle, the erythrocyte higher than those by the plasma and serum. Both AChE and BChE activities were found highest in the liver, followed by lung, muscle, kidney and heart for sheep and cattle, whereas in pigs the AChE and BChE activities tested higher in kidney, liver, lung, muscle and heart.
4. Ellman modified method was more precise than the Michel method in determining AChE and BChE activities.

Chapter 4

Comparison of two storage methods for the analysis of cholinesterase activities in food animals

The results from this Chapter have been presented at the Universities Federation for Animal Welfare (UFAW) International Animal Welfare Symposium, June 2011, Portsmouth, UK (poster presentation). The results have also been published in *Enzyme Research* 2010:1-11 (Abass Askar *et al.*, 2010).

Chapter 4: Comparison of two storage methods for the analysis of cholinesterase activities in food animals

4.1 Introduction

One of the most useful tools in diagnosing exposure of food animals to OP and carbamate compounds is measurement of tissue cholinesterases (ChE) activity. ChE is specialized carboxylic ester hydrolases that catalyse the hydrolysis of choline esters. Two types of ChE activity have been identified in mammalian blood and tissues; these are distinguished according to their substrate specificity and sensitivity to the selective inhibitors. The first is acetylcholinesterase (AChE, EC 3.1.1.7), which is systematically called acetylcholine acetylhydrolase. The second is butyrylcholinesterase (BChE, EC 3.1.1.8), referred to systematically as acylcholine acylhydrolase (Silver, 1974; Chatonnet and Lockridge, 1989; Wilson *et al.*, 2001; Wilson and Philip, 2005). In recent years, two freezing methods for keeping ChE activity have predominated in diagnostic situations (Fairbrother *et al.*, 1991).

There have been different studies investigating the stability of AChE and BChE activities over time at different freezing temperatures, yet there is disagreement between authors and many unanswered questions still remain. AChE and BChE enzymes are sensitive to different freezing temperatures. Methods governing effects of freezing of ChE from tissues of food animals are not completely understood. Our objective was to investigate the effects of storage at -20 °C and -80 °C on AChE and BChE activities, in addition to determining the best method for storage of samples for the determination of AChE and BChE activities in food animals, and to establish a foundation for the applicability of

AChE and BChE activities in food animal species as biochemical biomarkers for evaluating both exposures to and the effects OP and carbamate compounds.

4.2 Materials and methods

4.2.1 Sample collection and preparation

The sample collection and preparation were done as explained in Sections 2.2 and 2.3. However, the tissue homogenates were thoroughly mixed and distributed into 16 equal portions in all animals (sheep, cattle and pigs) and tissues (liver and muscle), representing freezing temperatures (-80 °C and -20 °C) for immediate processing one-month intervals, over a period of 8 months. Both AChE and BChE activities were measured by Ellman method as explained in Section 2.4.1. All the chemicals used in this research were purchased from analytical grade.

4.2.2 Statistical analysis

All enzyme measurements were conducted in ten individuals from each animal with results expressed as mean values \pm SE. Pearson's correlation coefficient, coefficient of variance (CV), standard deviation (SD), regression analysis and the one-way analysis of variance (ANOVA) were applied to test for any significant differences ($P < 0.05$). The Bland-Altman method was also used to compare between two freezing methods as described in Dewitte *et al.* (2002). All statistics was carried out using MiniTab statistical software version 15.

4.3 Results

4.3.1 Liver freezing

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities were determined in liver for sheep, cattle and pigs of each of the 8 freezing times rates at -80 °C and -20 °C as described in Section 2.4.1 (Figure 4.1A-D). There

was significantly higher AChE and BChE activities in pigs compared to when cattle and sheep used, using both effects of freezing (Figure 4.1A-D). In all cases (sheep, cattle and pigs using both freezing methods), BChE activity was higher in liver than does AChE activity (Figure 4.1A-D). Freezing for cases (sheep, cattle and pigs using both freezing methods) at -80 °C was a significant decrease after 6 months (Figure 4.1A and 4.1B). In general, at -20 °C AChE and BChE activities were significant decreased after 3 months with exception in case of sheep AChE was significant after 1 month (Figure 4.1C).

The linear regression of means ChE activities of liver in 8 months of freezing is shown in (Figure 4.2A-D). The R^2 values was tended to be very high in case of BChE activity at -20 °C ($R^2 = 0.98$, $P = 0.0001$; Figure 4.2D). Taking the overall data set there was a significant correlation between both AChE and BChE activities measured by -80 °C and -20 °C freezing (Pearson's correlation coefficient = 0.70, $P < 0.0001$; Figure 4.3A).

However, the percentage coefficient of variance (%CV) values for each month were generally higher (35 out of 48 sets of data) using the -20 °C than that of -80 °C freezing (Figure 4.4A-F). The Bland and Altman plot of the ratio of two freezing -80 °C and -20 °C was shown the mean differences between two freezing methods to be 8.8 and SD was 144.7 and -127.6 for upper and lower limits, respectively (Figure 4.5A).

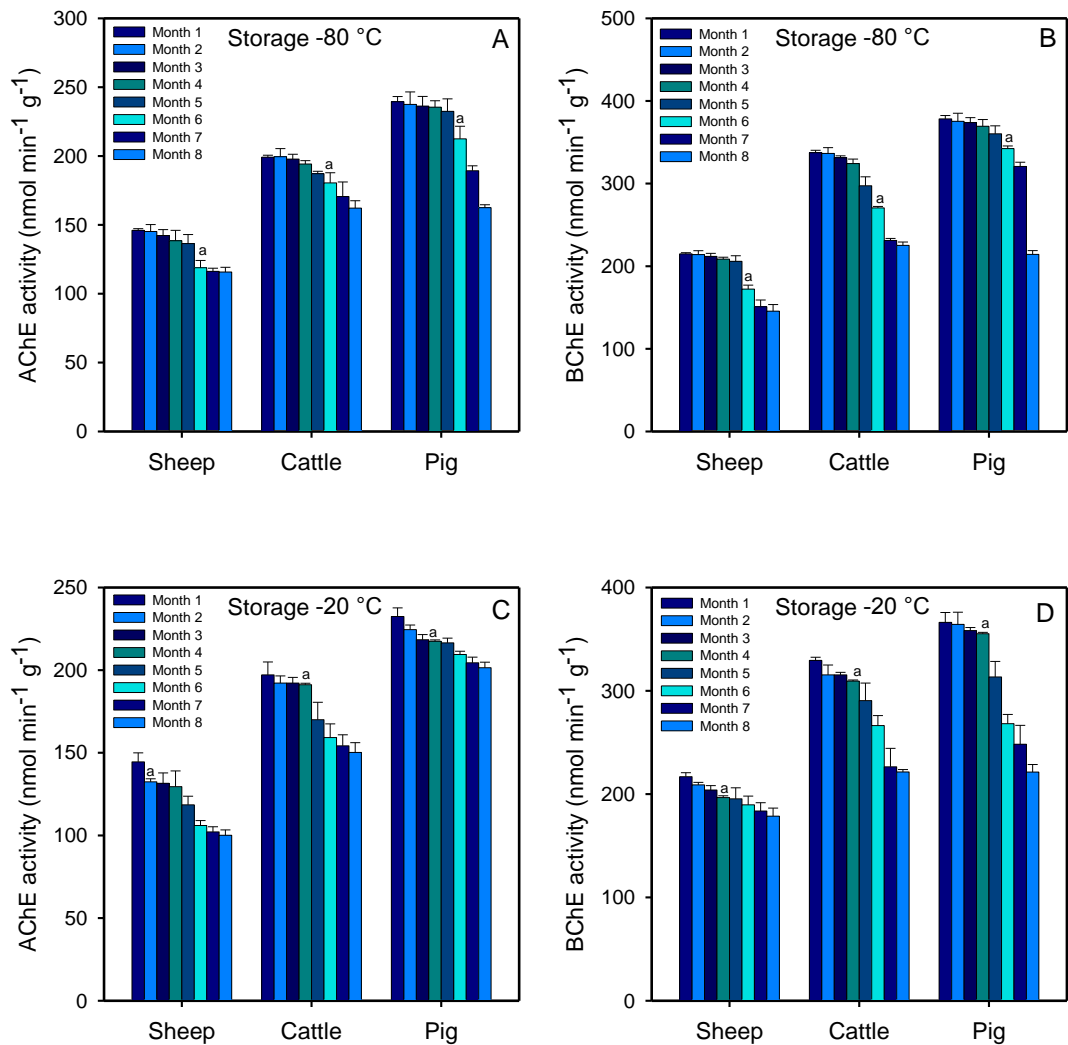


Figure 4.1 AChE and BChE activities in liver for sheep, cattle and pigs using (A and C) freezing at -80 °C and (B and D) -20 °C. Data are expressed as the mean \pm SE, (n = 10 in each animal). The letter in the column is significantly different [analysis of variance (ANOVA), $P < 0.05$].

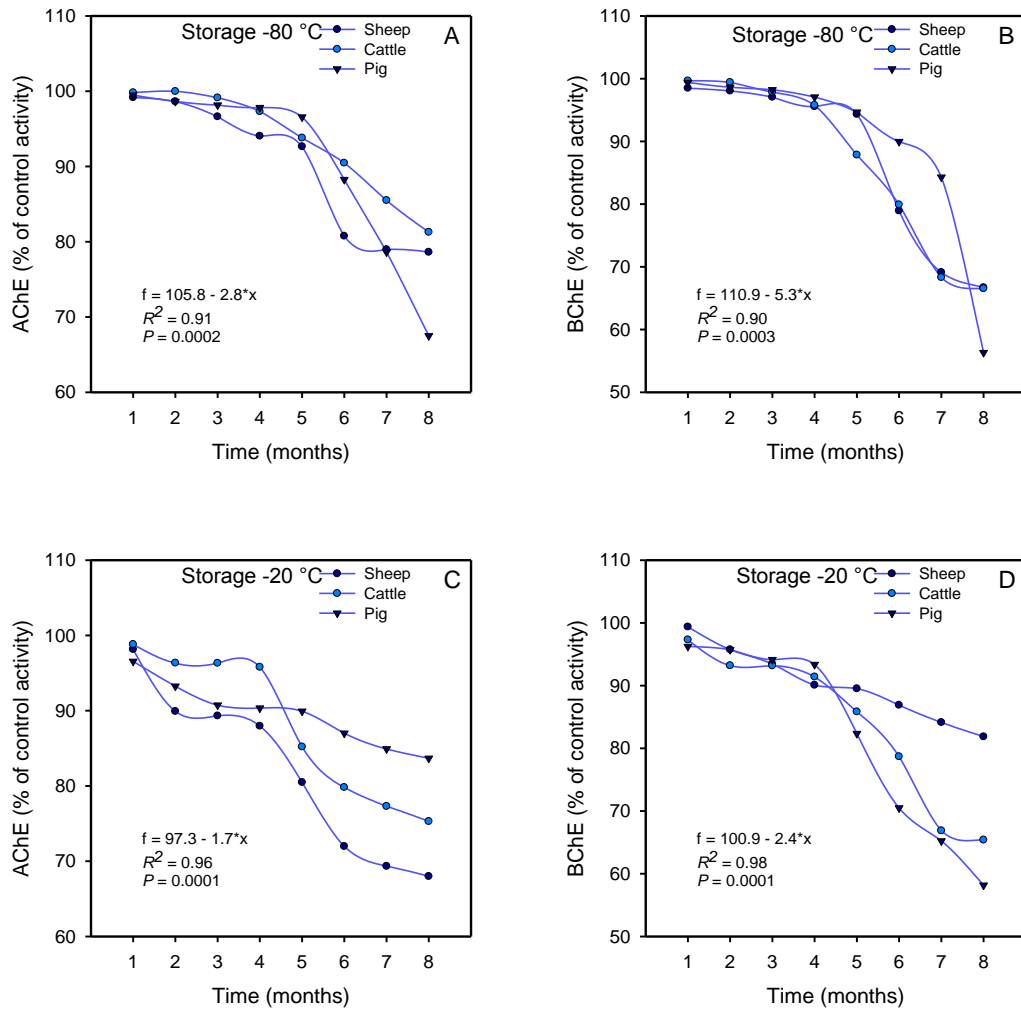


Figure 4.2 AChE and BChE (% of control) over time in liver freezing at -80 °C and -20 °C for sheep, cattle and pigs. A linear regression obtained between three species of animals (sheep, cattle and pig) and the equation usually written: $f = a + bx$, where f is the predicated mean AChE and BChE activities, a is the intercept of the regression line with f -axis, b is the slop or regression coefficient and x was any month of storage. These equations indeed could be used for predication of ChE activities in different sites for any month of freezing.

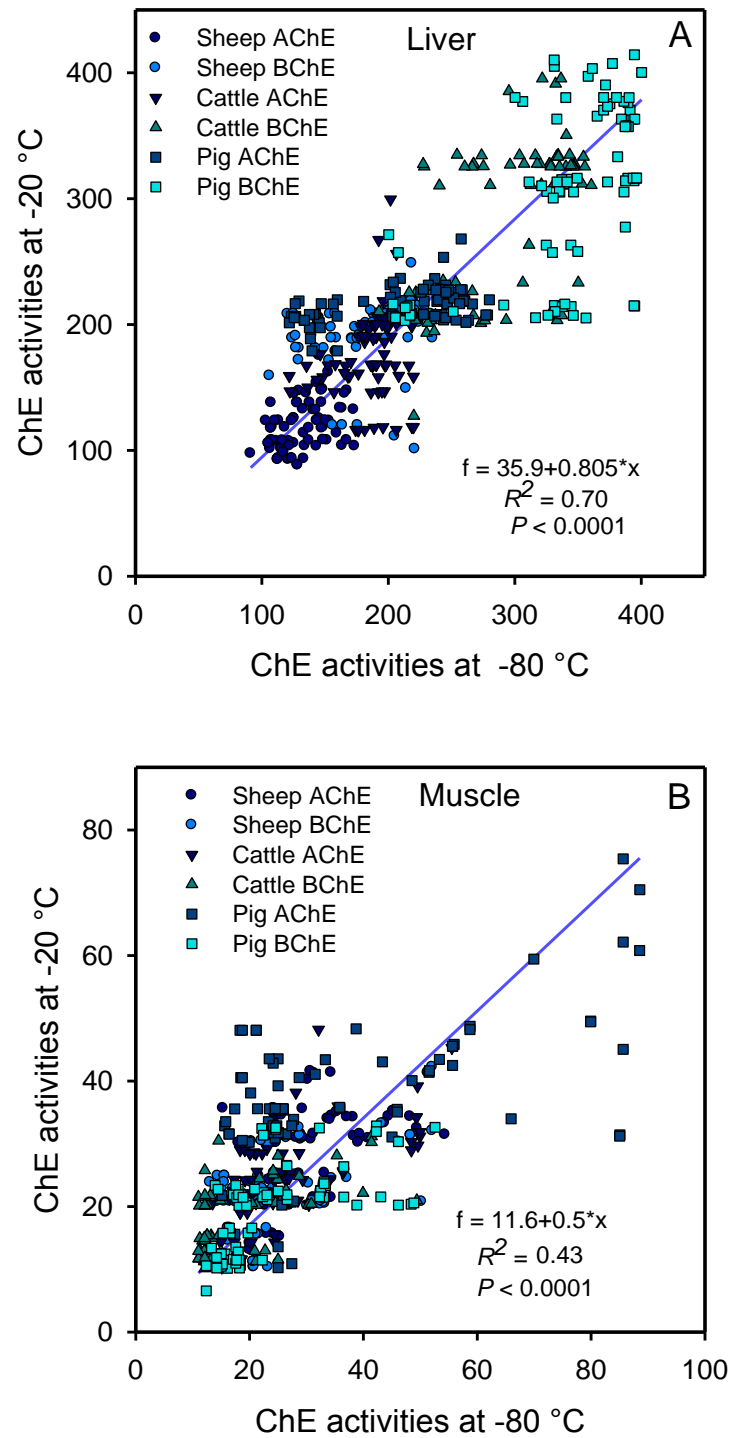


Figure 4.3 Regression analysis of individual activity of AChE and BChE activities using storage -80 °C and -20 °C from liver (A) and muscle (B) of sheep, cattle and pig.

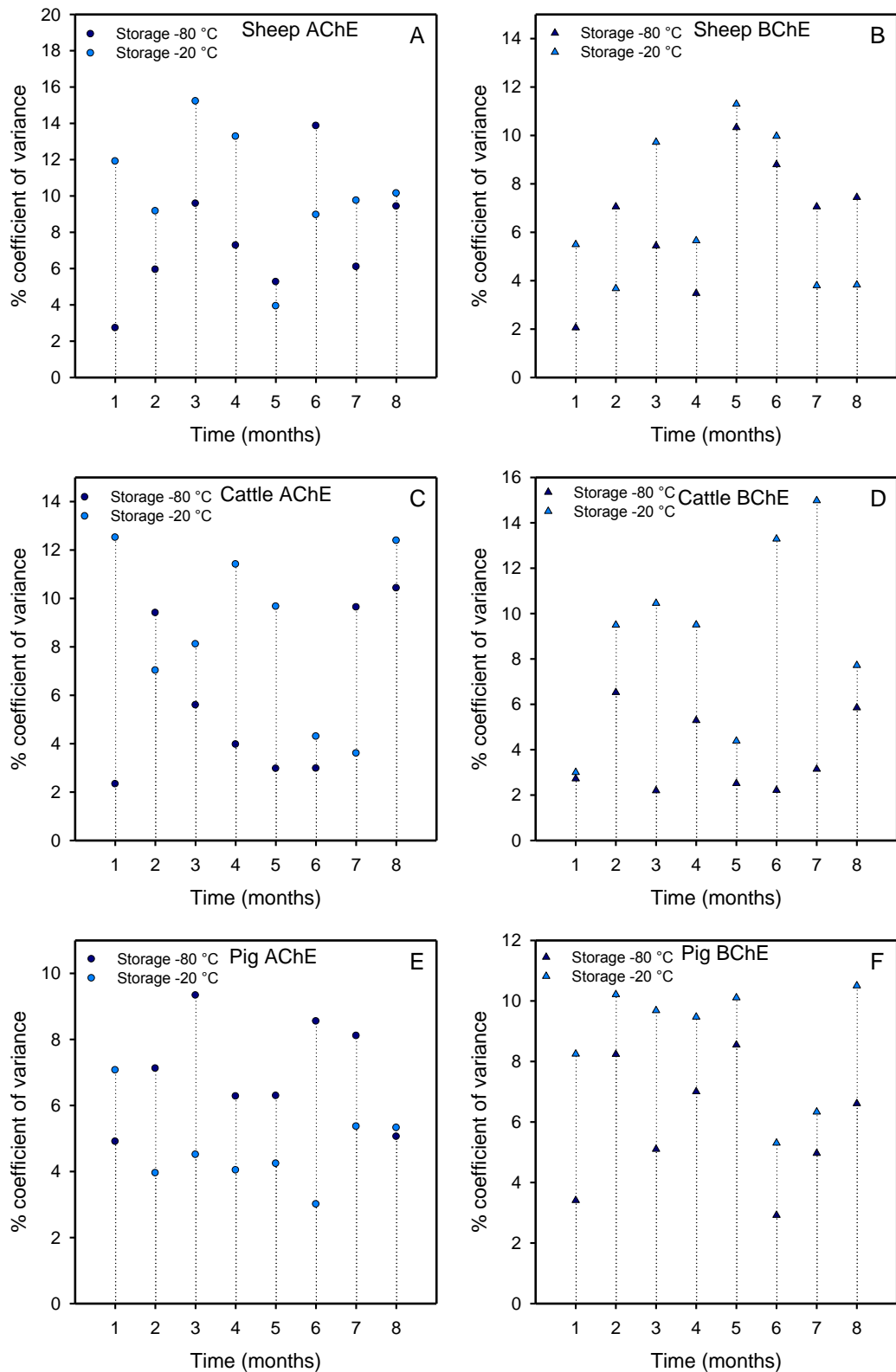


Figure 4.4 Percentage coefficient of variance between freezing at -80 °C and -20 °C for AChE and BChE activities in liver for sheep, cattle and pigs in 8 months.

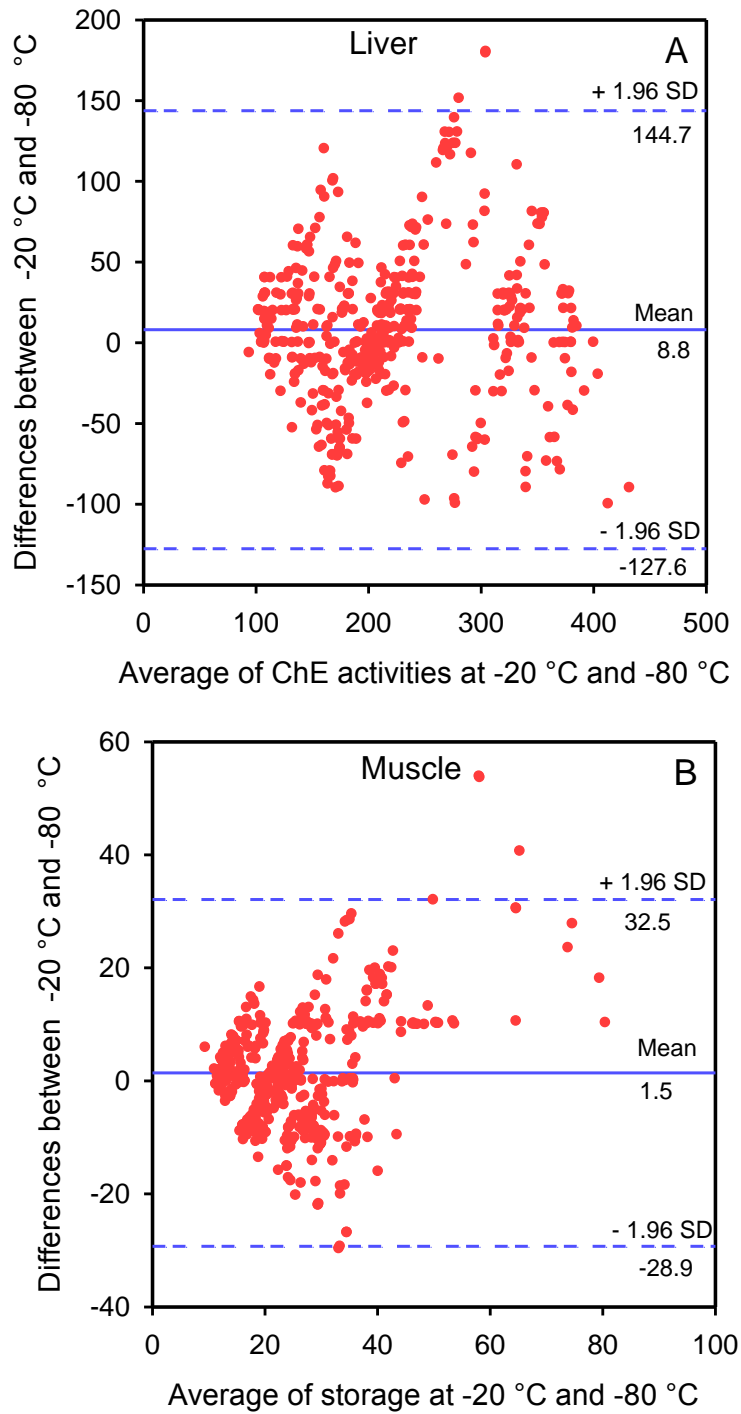


Figure 4.5 Bland and Altman plot of the ratio of the storage -80 °C and -20 °C (plotted on the y-axis) versus the average of the storages (x-axis) for food animal AChE and BChE activities. Key to the figures are listed under the figure 3.4.

4.3.2 Muscle freezing

Effect of freezing for AChE and BChE activities were also determined in muscle for sheep, cattle and pigs using of each of the 8 freezing times rates at -80 °C and -20 °C as described in Section 2.4.1 (Figure 4.6A-D). In all cases (sheep, cattle and pigs using both freezing methods), AChE activity was higher in muscle than does BChE activity (Figure 4.6A-D). In all cases (sheep, cattle and pigs using both freezing methods), the freezing at -80 °C was found a significant decrease after 3 months for AChE and BChE activities (Figure 4.6A and 4.6B). Freezing in all cases (sheep, cattle and pigs using both freezing methods) at -20 °C, there are a significant decreases after 1 month for BChE activity (Figure 4.6D), while for AChE there are a significant after 2 months for cattle and pigs with exception for sheep after 3 months (Figure 4.6C).

Once, again linear regression of means ChE activities was seen in muscle in 8 months of freezing (Figure 4.7A-D). The R^2 values was tended to be very high in case of AChE activity at -20 °C ($R^2 = 0.98$, $P = 0.0001$; Figure 4.7C). Again taking the overall data set there was a significant correlation between both AChE and BChE activities measured at -80 °C and -20 °C freezing (Pearson's correlation coefficient = 0.43, $P < 0.0001$; Figure 4.3B), and again, the %CV values for each month were generally higher (35 out of 48 sets of data) using the -20 °C than that of -80 °C freezing (Figure 4.8A-F). The Bland and Altman plot of the ratio of freezing -80 °C and -20 °C was shown the mean differences between two freezing methods to be 1.5 and SD was 32.5 and -28.9 for upper and lower limits, respectively (Figure 4.5B).

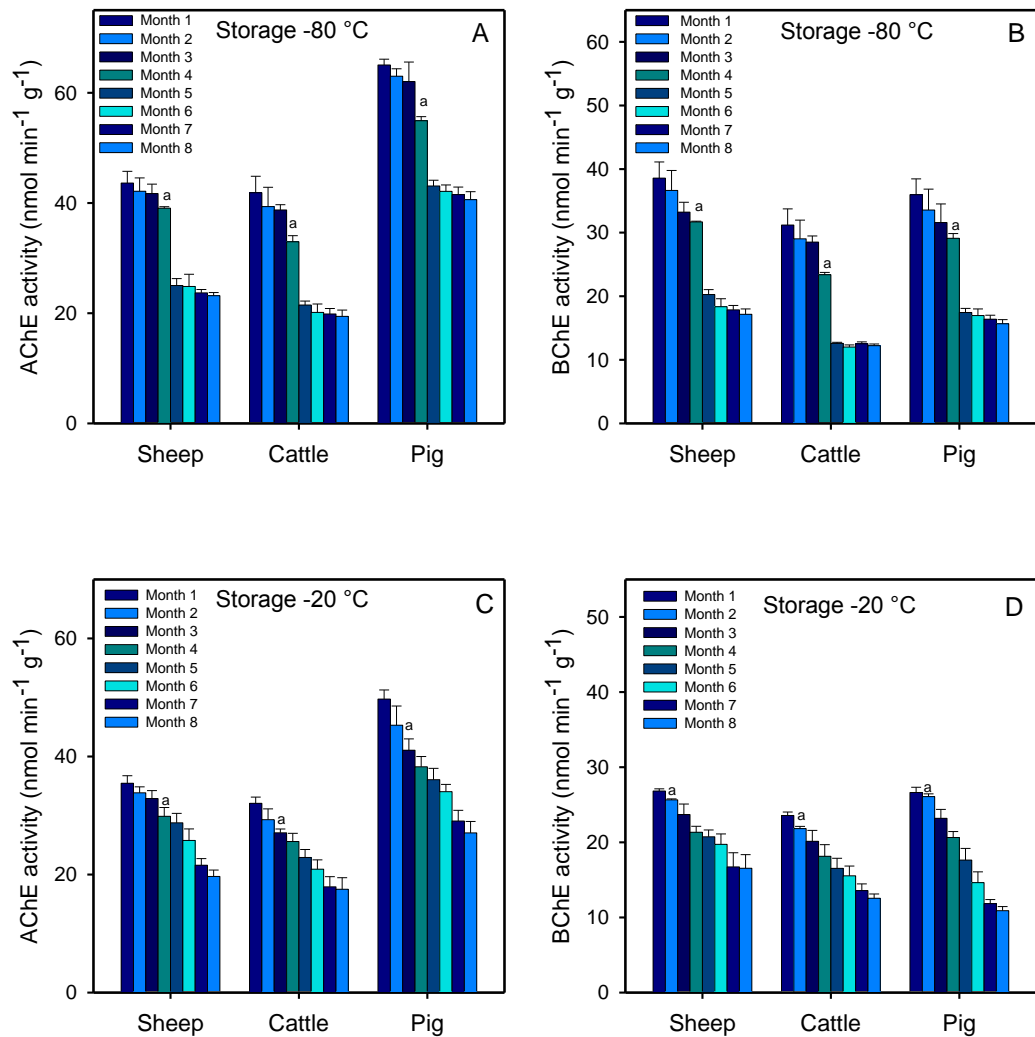


Figure 4.6 AChE and BChE activities in muscle for sheep, cattle and pigs using (A and C) freezing at -80 °C and (B and D) -20 °C. Data are expressed as the mean \pm SE, ($n = 10$ in each animal). The letter in the column is significantly different [analysis of variance (ANOVA), $P < 0.05$].

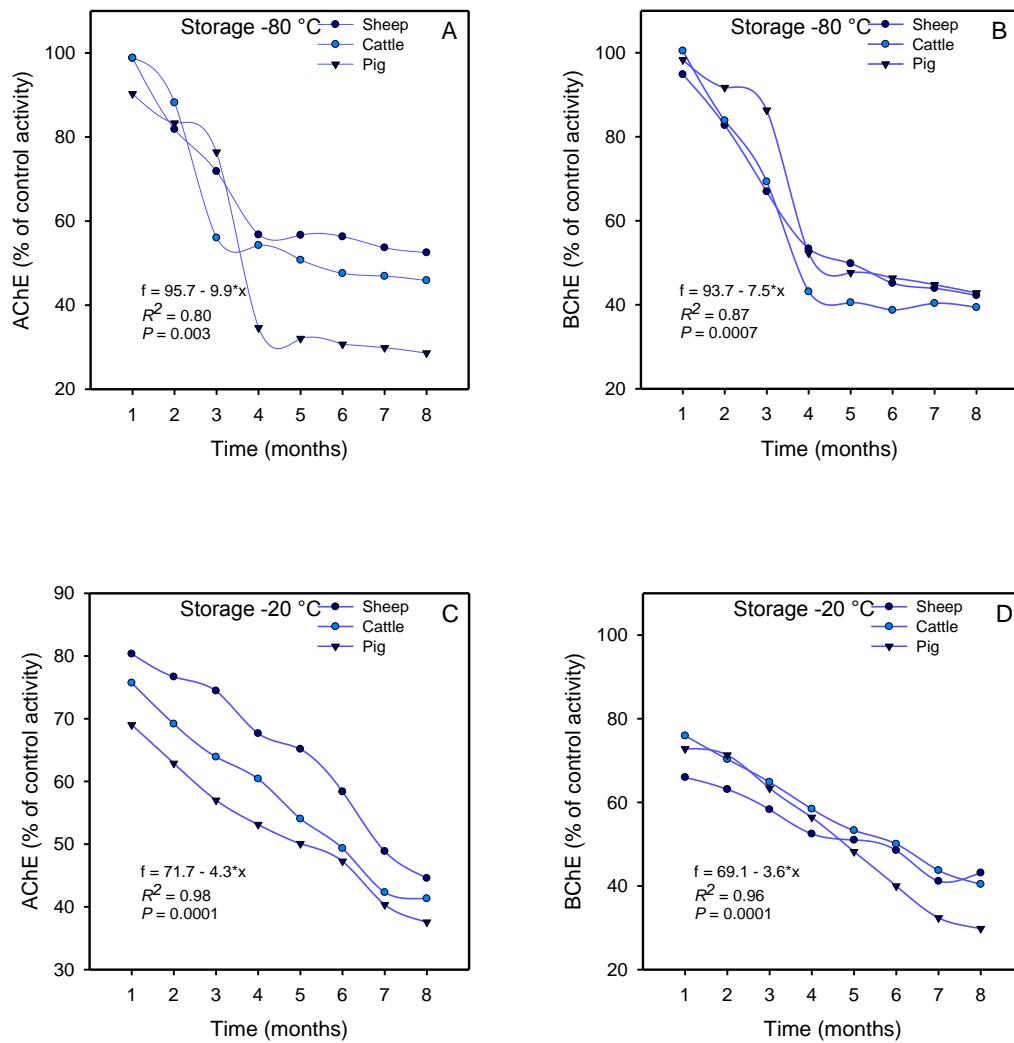


Figure 4.7 AChE and BChE activities (% of control) over time in muscle freezing at -80 °C for sheep, cattle and pigs. Key for the figures are listed under the figure 4.2.

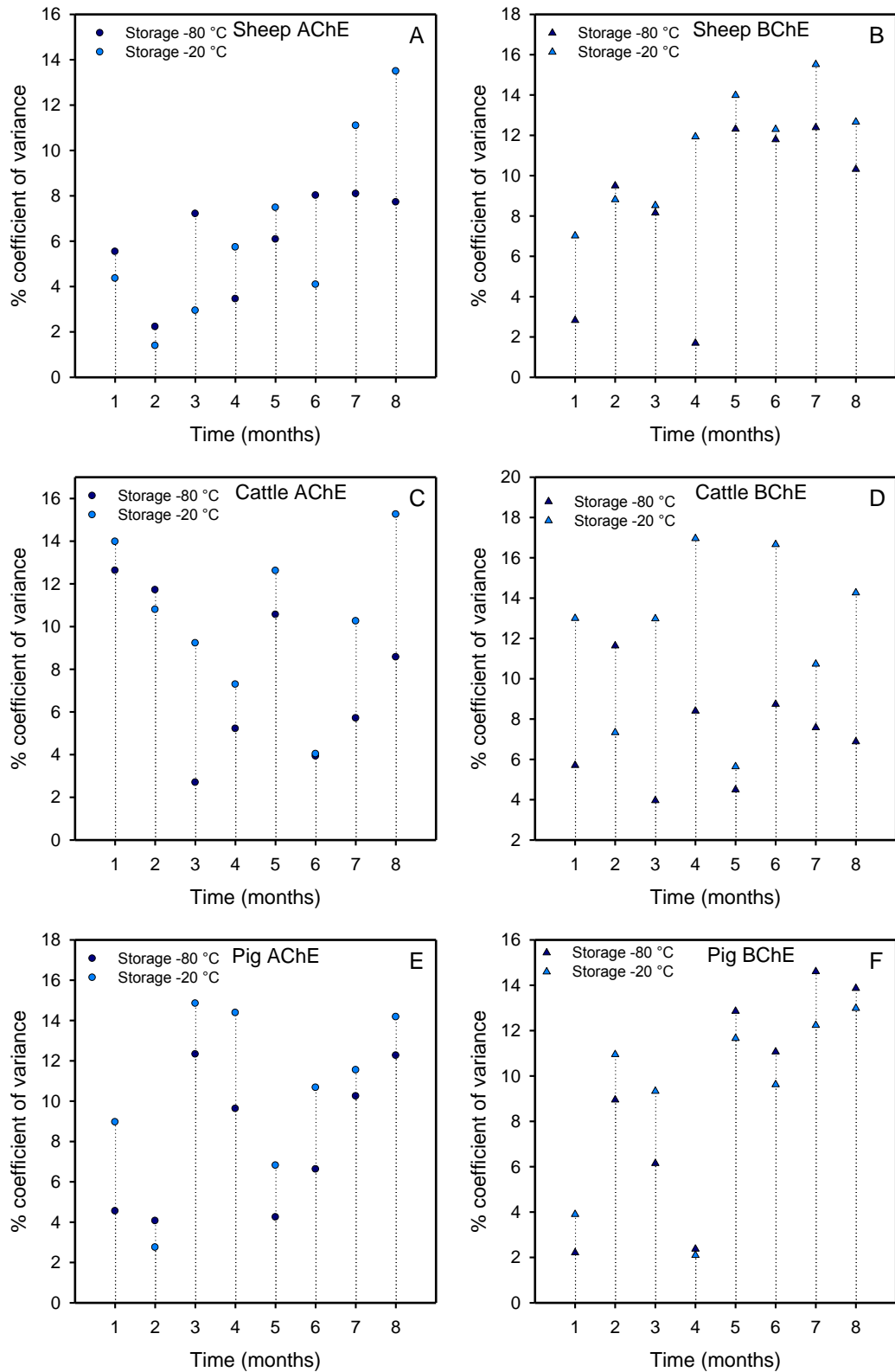


Figure 4.8 Percentage coefficient of variance between freezing -80 °C and -20 °C for AChE and BChE activities in muscle sheep, cattle and pigs in 8 months.

4.4 Discussion

There are two effects of freezing currently described for the measurement of ChE activities, the -20 °C and -80 °C. However, neither effect of freezing has been validated for use either in tissues from other food animal or in other tissues. The present study was to investigate the effect of freezing (8 months) on activity of AChE and BChE for sheep, cattle and pigs using modified Ellman method in liver and muscles as described in Section 2.4.1 (Figures 4.1 and 4.6). In all cases the results from our study are shown a significant decrease of AChE and BChE activities at -80 °C after 6 months in liver (Figure 4.1A and 4.1B). In contrast, with muscle I found significant after 3 months (Figure 4.6A and 4.6B). This is in agreement with the work of Kirby *et al.* (2000) found no changes or loss in ChE activities for 4 months in freezing at -80 °C for flounder muscle tissue. Nigg and Knaak (2000) who observed a little change in human plasma BChE activity when freezing at -70 °C after 10 times of frozen and thawing. In addition, ChE activities of fish brain tissue freezing at -68 °C and -70 °C for up 55 days and 5 months, respectively did not differ significantly (Fairbrother *et al.*, 1991; Nigg and Knaak, 2000).

In general freezing at -20 °C were significant decreases in all cases (sheep, cattle and pigs for liver and muscle) after 1-3 months (Figures 4.1 and 4.6). This is in agreement with the work of Crane *et al.* (1970) observed plasma and erythrocyte ChE activities freezing at -20 °C is remain stable after 6 weeks. This is in contrast with the work of Nigg and Knaak (2000) stated using freezing at -20 °C for 14 month without significance loss of plasma BChE activity. Panteghini *et al.* (1986), observed human plasma ChE activities to be stable for several months and years using freezing at -20 °C. These authors used a human blood plasma measuring rather than from food animals. This factor may

explain the apparent difference in activity. There is a 30% loss in BChE activity using freezing at -20 °C in human serum, while there is no loss in AChE activity at storage for one year (Turner *et al.*, 1984; Huizenga *et al.*, 1985). And there is a 23% decrease in up to 6 months in sheep AChE activity using freezing at -20 °C in whole blood and a 9% decrease of whole blood from dog using freezing at -20 °C (Tecles *et al.*, 2002a). Moran and Gomez-Ramos (1992) explained that some loss of AChE activity is due to particularly of G₄ molecular form of the enzyme, which has been described in unfixed human brain tissue, stored frozen -20 °C for 4 weeks.

In addition, there is a great variety of freezing degrees that can be found among different laboratories, for example, there were no changes in ChE activities when stored more than ten years at lower than 4 °C (Holmstedt, 1971), and a 10% decrease after 2 months in bovine erythrocyte ChE, in addition, a 95% decrease at 37 °C for 4 days (Stefan *et al.*, 1977). Furthermore, Balland *et al.* (1992) found that ChE loses 15% of its activity after 240 days of storage at room temperature; additionally who reported that freezing for 1 h at -40 °C and -196 °C did not affect ChE activities in plasma and stored samples. High correlation coefficient was seen at between 8 months of freezing -80 °C and -20 °C in the liver and muscle (Figures 4.2 and 4.7). One objective of the present study was to investigate whether the frozen animal product had effect on activity of ChE (Balland *et al.*, 1992).

Linear regression of mean ChE activities was observed in all individual samples on months of freezing at -80 °C and -20 °C (Figure 4.3A and 4.3B). The regression is used in present study to find the line that best predicts y (% control ChE activities) from x (months). The mean differences between two freezing

methods are plotted by Bland and Altman plot and were seen only in muscle storage clinically important the mean less than ± 1.96 (Figure 4.5B). With regards to precision of the both freezing methods, they showed higher coefficient of variance (%CV) values in -20 °C freezing compared with freezing at -80 °C (less than 13% and less than 15.2% for freezing -80 °C and -20 °C, respectively for liver) and (less than 12.8% and less than 16.9% for freezing -80 °C and -20 °C, respectively for muscle) (Figures 4.4 and 4.8); therefore freezing at -80 °C provided more precise than freezing at -20 °C in muscle and liver for sheep and cattle. Finally, it was noticed that the decreases of ChE inhibition levels after freezing were broadly similar to those found in the original analysis and, therefore, long-term freezing could still be used as an option during monitoring programmes, especially where samples are not allowed to thaw during storage.

4.5 Conclusions

This is the first study that provided the original data concerning the effect of freezing for AChE and BChE activities for sheep, cattle and pig. The AChE and BChE activities were freezing by two methods (-20 °C and -80 °C) from liver and muscle for sheep, cattle and pigs for 8 months. Summarizing the results of this Chapter, the following conclusions can be drawn.

1. Freezing at -80 °C in all three animal species (sheep, cattle and pigs), there are found a significant inhibition of AChE and BChE activities after 6 months in the liver, while after 3 months in muscle.
2. Liver extracts using freezing at -20 °C from all three animal species (sheep, cattle and pigs) showed a significant decrease in AChE activity after 3 months with the exception of sheep.

3. Muscle extracts using freezing at -20 °C from all three animal species (sheep, cattle and pigs) showed a significantly decreased in BChE activity after 1 month, while significant decreased after 2 months for AChE activity, with the exception in sheep AChE activity after 3 months.

4. Freezing at -80 °C was more precise than the freezing at -20 °C in determining AChE and BChE activities.

5. Finally, despite this further studies are necessary under different laboratories, in order to improve and strengthen these results and to increase in our knowledge about this very interesting enzyme as a potential biochemical marker for pesticide intoxication.

Chapter 5

Purification of soluble acetylcholinesterase from sheep liver by affinity chromatography

The results from this Chapter have been presented as poster presentations at the following conferences in the UK: Winter Meeting of the British Pharmacological Society, December 2010, Queen Elizabeth II Conference Centre, London; 23rd Annual Symposium of the Biochemical Society of Recent Advances in Membrane Biochemistry, January 2011, University of Cambridge; and 2nd European Conference on Process Analytics and Control Technology, April 2011, Glasgow. The results have also been published in *Applied Biochemistry and Biotechnology* 165(1):336-346 (Abass Askar *et al.*, 2011).

Chapter 5: Purification of soluble acetylcholinesterase from sheep liver by affinity chromatography

5.1 Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is specialized carboxylic ester hydrolases that catalyse the hydrolysis of choline esters, which is systematically called acetylcholine acetylhydrolase. Other names include true cholinesterase, specific cholinesterase, red blood cell cholinesterase, erythrocyte cholinesterase and cholinesterase I. The preferred substrate for AChE is ACh (Silver, 1974; Wilson *et al.*, 2001; Wilson and Philip, 2005; Wilson, 2010). AChE serves a pivotal role in regulating nerve impulse transmission by rapid hydrolysis of the neurotransmitter ACh.

Most of the information about the properties of purified AChE has been obtained from studies of the AChE from the electric tissue of the eel *Electrophorus electricus* (Lawler, 1961). Lord in (1961) indicated a partially purified AChE from the German cockroach *Blattella germanica* by grinding insects in sodium taurochlorate, dialysing against di-sodium hydrogen phosphate, precipitating with ammonium sulphate, dialysing against sodium citrate, incubating in protamine sulphate, dialysing against water and precipitating in acetone with the final sample being dissolved in sodium citrate buffer. Leuzinger and Baker in (1967) purified AChE as a crystalline and electrophoretically homogenous form by chromatographic procedures.

Efficient purification of AChE has since become possible using affinity chromatography, which has been described by Cuatrecasas *et al.* (1968) as permitting a given enzyme to be readily separated from a mixture of proteins by its selective and reversible adsorption on a resin to which a specific competitive

inhibitor of that enzyme has been covalently attached. Early affinity techniques for AChE purification were investigated by Reavill and Plummer (1978) who compared the efficiency of three affinity columns.

Recently, a variety of affinity resins such as tacrine (Carroll *et al.*, 1995), procainamide (Philipp, 1994), edrophonium (Son *et al.*, 2002), and *m*-tri-methylaminophenylamine (Pascale *et al.*, 1996) have been developed for the purification of AChE from various organisms. Most of these ligands are specific inhibitors of AChE. I attempted to purify the soluble AChE from sheep liver using the two-step affinity chromatography such as Concanavalin A-Sepharose 4B column and edrophonium-Sepharose 6B column that turned out to be very rapid and sensitive. The first goal of this study was to develop a protocol for the purification of AChE and to extend this method for further enzyme characterization. A further aim was to study whether the edrophonium pharmacologic action is due primarily to the inhibition or inactivation of AChE at sites of cholinergic transmission.

5.2 Materials and methods

5.2.1 Sample preparation

Ten grams of liver tissue was removed using a scalpel, cut into small pieces (3-4 mm³), and rinsed until the blood was fully removed. The tissue was then placed on ice in 50 ml tubes (10 mm internal diameter) and homogenized using a mechanically driven homogenizer with sodium phosphate buffer (0.1 M, pH 8) containing 0.5 M NaCl at a ratio of 1 part of tissue to 9 parts of buffer, and a speed of 10000 rpm. Homogenisation required 2 min; after every 30 s or so of homogenisation the mixture was rested for 10 s to allow cooling. The homogenate was then centrifuged in 50 ml tubes by using (MES, T8 probe,

Europa 284) at 30,000 g for 1 h, at 4 °C (Son *et al.*, 2002). The AChE activity was measured by Ellman method as described in Section 2.4.1. All chemicals used in this research were analytical grade.

5.2.2 Determination of protein

The protein content was quantified either by measuring the absorbance at 280 nm (Berg *et al.*, 2002) or by Bradford method (colorimetric protein assay at 595 nm) (Bradford, 1976) based on the binding of coomassie brilliant blue dye to proteins. Bovine serum albumin (BSA) is unique, which plays an important role in stabilizing protein structure. The BSA standards were made at concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1 and 1.2 mg/ml BSA in distilled water (Figure 5.1).

Samples (50 µl) were placed in dry clean tubes and the volume was made up to 2.55 ml by the addition of the Bradford buffer (100 mg coomassie brilliant blue in 50 ml 95% ethanol, add 100 ml 85% phosphoric acid), volume was made up to 1 litre by distilled water and when the dye has completely dissolved, and filter through Whatman filter paper. Then sample with Bradford buffer was mixed briefly by vortex machine (MS1 Minishaker, IKA Works, Inc.) and incubated for 5 min at room temperature 20 °C, and analysed spectrophotometrically using a Helios Beta UV-Vis spectrophotometer (UK). The protein concentration of the test samples could be determined from the standard curve (Figure 5.1).

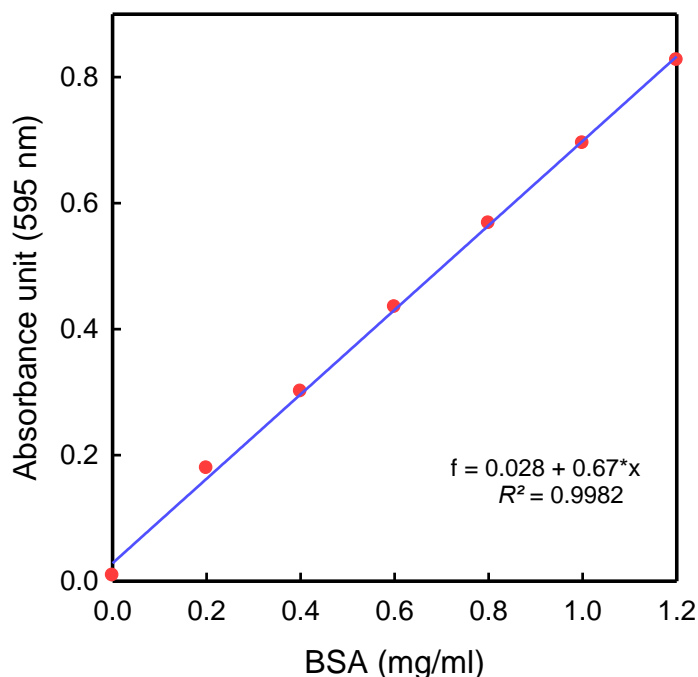


Figure 5.1 Standard calibration curve of BSA for Bradford assay. The data was showed as mean for triplicate.

5.2.3 Synthesis of edrophonium-Sepharose affinity gel

Preparation of affinity gel was followed by the process of Anthony and Ian (1983) with minor modification of Son *et al.* (2002). Epoxy-activated Sepharose was hydrated and washed with deionised distilled water on a sintered glass filter as recommended by the producer by the Sigma Chemical Company (Poole, Dorset, UK). Before use, the gel could be washed in sequence with 10 volumes each of 100 mM sodium acetate buffer (pH 4.5); 12 mM sodium borate buffer (pH 10); and deionised distilled water. The gel slurry was dried on a Buchner funnel and transferred into a solution (12 mM borate buffer, pH 11) containing 20 mM edrophonium chloride (1 part gel/2 parts mixture solution). The pH of aliquots was then adjusted to 12 by the addition of 0.1 M sodium hydroxide. The mixture was shaken for 48 h at 50 °C on an incubator (LEEC, UK). The efficiency of edrophonium coupled to Sepharose 6B was measured based on

the variation at 280 nm. All purification steps were performed at 4 °C, according to Anthony and Ian (1983).

5.2.4 Isolation and purification of acetylcholinesterase

Essentially, this followed the method of Son *et al.* (2002), by which AChE retained on an affinity Concanavalin A-Sepharose 4B column (GE Healthcare, UK Ltd.) was equilibrated with 50 mM sodium phosphate buffer (pH 7.4) and was followed by 0.5 M NaCl at 4 °C previous to packing liver extracts onto the column. The column was washed with the sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl until the protein content of the eluate was under the detection limit at 280 nm. AChE was then eluted with the sodium phosphate buffer (pH 7.4) containing 0.5 M methyl α -D-mannopyranoside at a flow rate of 30 ml/h. Active fractions monitored as a sole peak were collected and pooled, and then concentrated by using Amicon Ultra Centrifugal Filter Devices (Millipore, Carrigtwohill, Ireland).

The eluant was then applied onto a column of edrophonium-Sepharose 6B previously equilibrated with 50 mM sodium phosphate buffer, pH 7.4 containing 0.5 M NaCl. Then, the column was washed with the 50 mM sodium phosphate buffer, pH 7.4 containing 0.5 M NaCl until the protein content was below 0.01 at 280 nm. The enzyme was then specifically eluted with 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM edrophonium chloride. Each fraction of 0.5 ml was collected by a peristaltic pump (Miniplus 3, Gilson, UK) connected to a fraction collector (FC 2112 Fraction Collector, Redirac, UK), and the active fractions were pooled and dialyzed (see dialysis section) overnight against 50 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl, with three changes of the buffer (Anthony and Ian, 1983; Son *et al.*, 2002).

5.2.5 Dialysis

Dialysis is a purification method which allows proteins to be separated from small molecules by use of a semipermeable membrane, usually made from cellulose (Berg *et al.*, 2002). This membrane contains pores of a defined size so that there is a molecular weight cut-off. This ensures that small molecules will be free to pass through the membrane into the surrounding medium, whilst keeping larger macromolecules within (Berg *et al.*, 2002). Dialysis as a method can be used to exchange buffers or remove salts or other small molecules from the sample of proteins. The dialysis membrane containing protein and small molecules are immersed in an aqueous solution into which the latter can diffuse via the pores. Once equilibrium is reached, the dialysate is replaced until most of the small molecules have been removed from the environment of the protein, and the concentration is at an acceptable level (Figure 5.2).

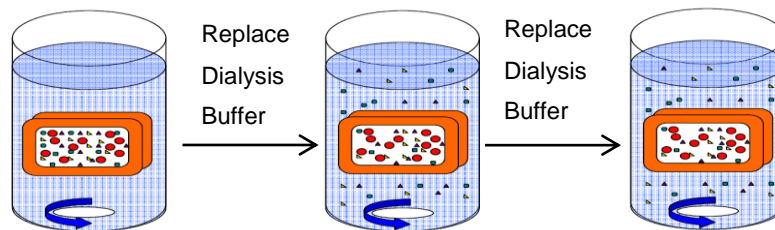


Figure 5.2 Gradual removal of small molecules from protein sample.

5.2.6 Sephacryl S-200 HR

Molecular weight was estimated under non-denaturing conditions by gel filtration technique (molecular exclusion) using Sephacryl S-200. High molecular weight proteins will go down through a column quickly, while lower molecular weight proteins will go down through a column slowly (Son *et al.*, 2002). This is because the structure of the gel beads within the column excludes molecules that are too big to pass through the bead pores. A standard protein markers

mixture (1 ml) containing (in milligram): Carbonic anhydrase 1.5, β -amylase 2, alcohol dehydrogenase 2.5, cytochrome C 4, BSA 5 and apoferritin 5 were applied to a Sephacryl S-200 column, followed by 100 ml of 0.15 M sodium phosphate buffer (pH 7.2), flow rate (0.5 ml/min) and volume of each fraction collected, a sample was detecting at 595 nm after loading to the column. The column (2.4 × 24 cm) was calibrated with standard molecular weight: Carbonic anhydrase (29,000), BSA (66,000), alcohol dehydrogenase (150,000), β -amylase (200,000) and apoferritin (443,000).

5.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) gels were prepared according to the method of Laemmli (1970) (Table 5.1). Polymerisation was achieved by the addition of ammonium persulphate and *N, N, N', N'*-tetramethylethylenediamine. Protein samples were dissolved with three times concentrated sample buffer at a ratio 3:1. 15 μ l was mixed with 5 μ l of sample buffer containing [0.2 M Tris buffer (pH 6.8), 10% (w/v) SDS, 20% (w/v) glycerol, 0.05% (w/v) bromphenolblue]. Next, the prepared samples were completely loaded on the gel, and electrophoresis was carried out in a Mini Protean 3 system (Bio-Rad, UK) at a constant current of about 60 volt until the dye front reached the resolving (separating) gel and then at 120 volt until the dye front had reached the bottom of the gel.

After removal of the staining gel, the resolving gel was placed in stainer [0.125% Coomassie blue G-250 in 10% (v/v) phosphoric acid, 10% (w/v) ammonium sulphate, 20% (v/v) methanol for 2 h at room temperature 20 °C] and left over night at room temperature with constants shaking. Gels were then destained by placing in destainer. The destainer [10% (v/v) methanol and 10% (v/v) acetic

acid] was changed regularly until the gel was fully destained. All chemicals and protein markers used in this study were of analytical grade.

Table 5.1 Chemical composition of 10% SDS-PAGE gels (adapted from (Laemmli, 1970)).

Chemical	Separating layer 10% (ml)	Stacking layer 4% (ml)
Distilled water	12.3	3.075
1.5 M Tris buffer (pH 8.8)	7.5	-
Acrylamide/bisacrylamide (30%/0.8% w/v)	9.9	0.67
0.5 M Tris buffer (pH 6.8)	-	1.25
The above reagents were mixed and degassed and the following reagents were then added:-		
20% (w/v) SDS	0.15	0.025
<i>N, N, N', N'</i> -tetramethylethylenediamine	0.02	0.005
10% (w/v) ammonium persulphate	0.15	0.025

5.2.8 Estimation of the relative mobility

The relative mobility (*R_f*) refers to the mobility of the protein of interest measured with reference to a tracking dye. Standard molecular weight markers were run on every SDS gel conducted. After destaining, the *R_f* of each marker was determined according to Garfin (2009) as follows;

$$R_f = \frac{\text{Distance of protein migration from top of resolving gel}}{\text{Distance of tracking dye migration from top of gel}}$$

The *R_f* values was then plotted versus the log of known molecular weights. This allowed the construction of a calibration curve from which the *R_f* values of the

unknown protein could be read off to give the molecular weight of the protein (Figure 5.7).

5.3 Results and discussion

5.3.1 Purification of acetylcholinesterase

The present approach to purification of sheep liver AChE entailing affinity chromatography was based on the approach of Anthony and Ian (1983) and Son *et al.* (2002). AChE is primarily involved in cholinergic synaptic transmission and found in a variety of neuronal and non-neuronal tissues (Wilson, 2010). The purification of soluble AChE from sheep liver is summarized in Table 5.2. Total protein activity was detected to be 183 mg in 50 ml of homogenate, with a specific AChE activity of 0.025 U/mg of protein. After affinity 1, the purified AChE contained 3.9 mg of protein in 41 ml of supernatant, with a specific activity of 0.289 U/mg of protein. The supernatant obtained by ultracentrifuge has about 66% of the total AChE activity and about 46% of the total protein recovery. When the supernatant was applied onto Concanavalin A-Sepharose 4B column, AChE was quantitatively adsorbed by Concanavalin A-Sepharose 4B column (Figure 5.3). About 25% of the enzyme and 2% of protein were eluted from the column with 0.5 M methyl α -D-mannopyranoside and purification fold was nearly 12. The edrophonium-Sepharose 6B affinity chromatography resulted in an AChE activity of 0.382 (U) and a purification fold of 842.

Table 5.2 Purification of AChE from sheep liver.

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Protein recovery (%)	Activity recovery (%)	Purification factor
Homogenate	50	183.0	4.6	0.025	100	100	1
Supernatant	41	83.3	2.9	0.036	45.5	65.7	1.5
Affinity 1: Concanavalin A-Sepharose 4B column	22	3.9	1.1	0.289	2.1	24.7	11.6
Affinity 2: Edrophonium- Sepharose 6B column	10	0.018	0.382	20.9	0.009	8.4	841.9

The specific activity of AChE, expressed as micromole hydrolysed per minute per milligram of protein. The recovery (%) of protein and activity was based on the total protein and AChE activity, respectively.

Although there were some tailings at the end of a peak shoulder in the adsorption of enzyme from the column, a reasonable amount of enzyme was recovered. It has been known that the globular form of AChE, predominant in the mammalian liver and muscle, is a glycoprotein (Massoulie *et al.*, 1993). The chromatographic behaviour of sheep liver AChE on Concanavalin A-Sepharose 6B column resin indicates that soluble AChE from sheep liver may be of glycoprotein nature (Michizo *et al.*, 1985). The enzyme eluted with 0.5 M methyl α -D-mannopyranoside showed a high protein with very sharp peak with AChE activity (Figure 5.3).

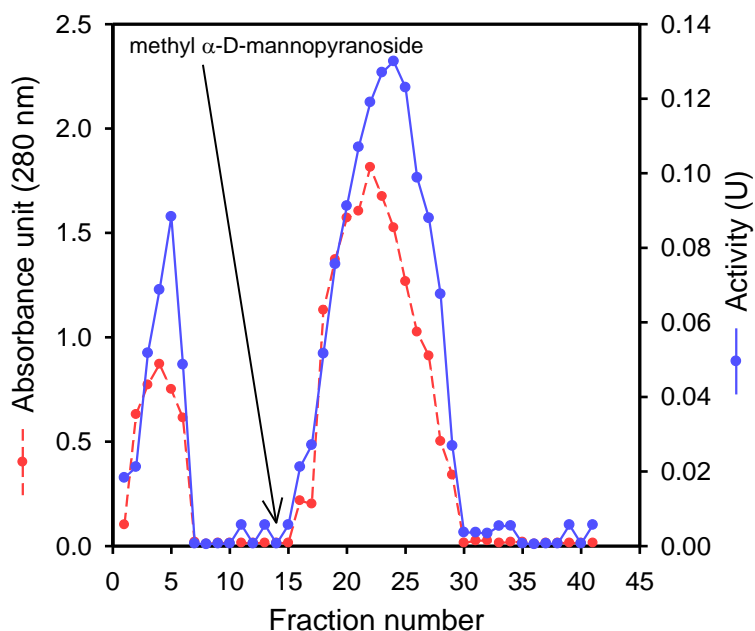


Figure 5.3 A typical elution profile for the chromatography of sheep liver AChE on Concanavalin A-Sepharose 4B column (1.5 × 5 cm) previously equilibrated with 0.05 M sodium phosphate buffer containing 0.5 M methyl α -D-mannopyranoside at a flow rate of 0.5 ml/min.

Then, the pooled sample concentrated and loaded on the edrophonium-Sepharose 6B column. After the column was washed with 50 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl, AChE was eluted with edrophonium chloride. They demonstrated a very sharp peak with high AChE activity but with very low protein. However, this enzyme did not show any enzyme activity because of the inhibitory action of edrophonium bound to the active site of AChE. Most of the non-specifically bound proteins were removed in 50 mM sodium phosphate buffer and 0.5 M NaCl washing (Figure 5.4).

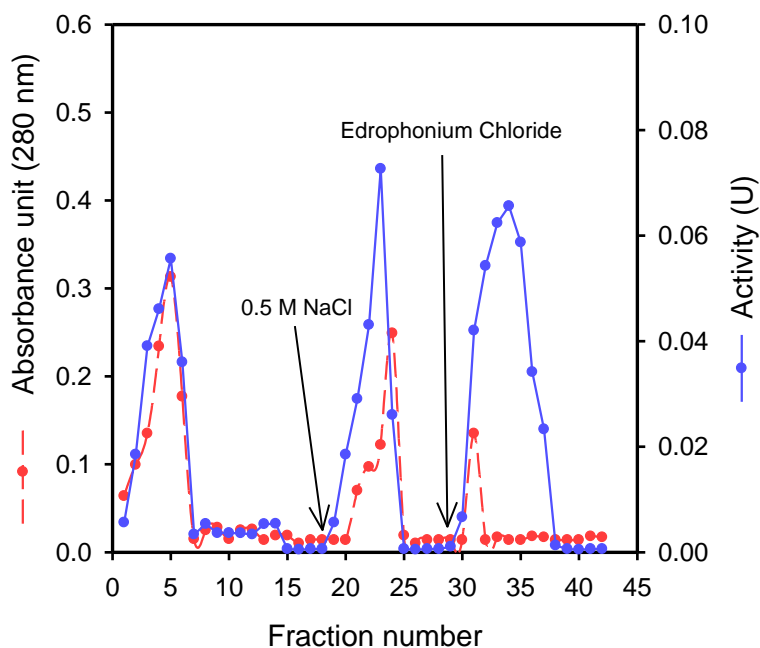


Figure 5.4 A typical elution profile for the chromatography of sheep liver AChE on edrophonium-Sepharose 6B column (1 × 15 cm) previously equilibrated with 50 mM sodium phosphate buffer containing 20 mM edrophonium chloride at a flow rate of 0.5 ml/min.

The purity of the purified AChE is quite compatible with that of AChE from other sources such as 600-fold for rat liver (Leuzinger, 1971), cattle erythrocyte (930-fold) (Schmidt-Dannert *et al.*, 1994), cattle serum (44,000-fold) (Ralston *et al.*, 1985), Housefly (400-fold) (Im *et al.*, 2004). The specific activity of the AChE I obtained from liver of sheep (Table 5.2) is much lower with those found by Eileen (1977), Son *et al.* (2002) and Im *et al.* (2004). These authors use brain tissues instead of liver which I used. In addition, very high AChE activity found in the tissue brain than other tissues. This factor may explain the apparent difference in specific activity.

5.3.2 Molecular weight determination

The molecular weight by gel filtration analysis of sheep liver AChE as calculated from the calibration curve and estimated to be 201.5 kDa as tetrameric using Sephacryl S-200 (Figure 5.5). Our results (Figure 5.5) are lower when compared with other food animals, e.g. cattle (Ralston *et al.*, 1985). However, this author used serum instead of liver tissue. This factor may explain the apparent difference in tetrameric molecular weight.

Monomeric subunit molecular weight of AChE was calculated from the calibration curve and estimated to be 67.1 kDa as single monomeric subunits using 10% SDS-polyacrylamide gel electrophoresis (coomassie blue stained) (Figures 5.6 and 5.7). It was higher than those in quail brain (62.5 kDa) (Son *et al.*, 2002), human brain (66 kDa) (Zhu *et al.*, 1993), human serum (65 kDa) (Lockridge *et al.*, 1987), electric organ of the electric eel (25-59 kDa) (Dudai and Silman, 1974), while lower than in studied *Torpedo californica* (71-82 kDa) (Taylor and Lappi, 1975; Lwebuga-Mukasa *et al.*, 1976), cattle serum (83 kDa) (Ralston *et al.*, 1985), and cattle superior cervical ganglia (75 kDa) (Marc *et al.*, 1979). The above results differ with our results due to these authors using different tissues instead of liver tissue. Finally, I found that in this case, purification was most successful on a column containing edrophonium chloride covalently linked to epoxy-activated Sepharose 6B and eluted with methyl α -D-mannopyranoside.

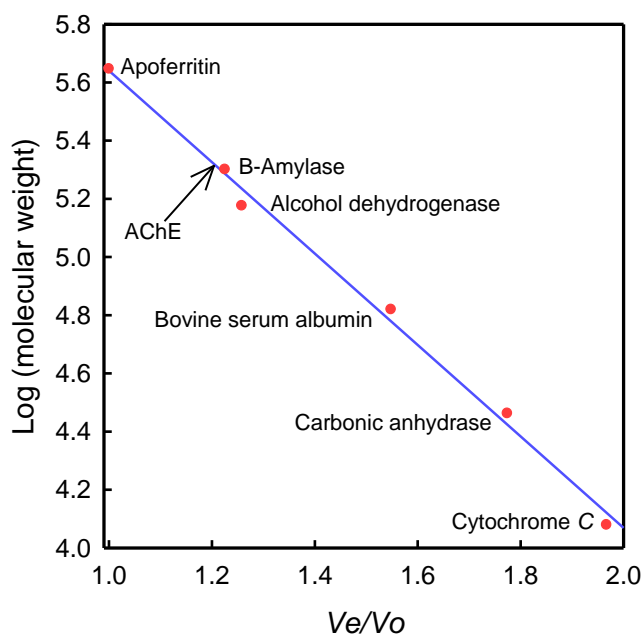


Figure 5.5 Calibration curve for gel permeation determination of the molecular weight of purified AChE by Sephacryl S-200 HR chromatography. The protein markers used in order of increasing molecular weight: Cytochrome C (12,000), carbonic anhydrase (29,000), BSA (66,000), alcohol dehydrogenase (150,000), *b*-amylase (200,000) and apoferritin (443,000). Dextran blue (2,000,000) was used to determine the void volume (V_0), while (V_e) is the elution volume.

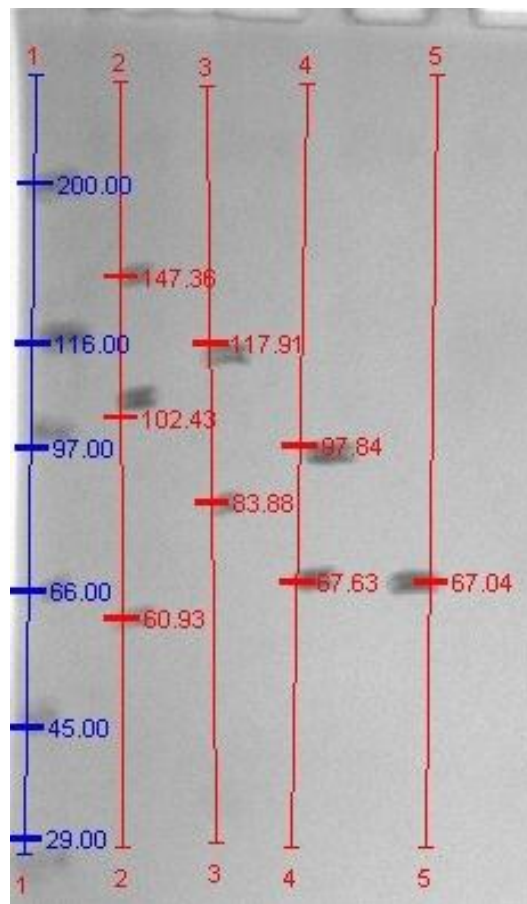


Figure 5.6 SDS-polyacrylamide gel electrophoresis pattern of AChE from sheep liver. SDS-PAGE was conducted in gel and the protein stained with coomassie blue. *Lane 1:* Standard proteins (molecular weights insert) are carbonic anhydrase (29,000), egg albumin (45,000), BSA (66,000), phosphorylase *b* (97,000), β -galactosidase (116,000) and myosin (200,000). *Lane 2:* Supernatant AChE. *Lane 3:* 50 mM sodium phosphate buffer fraction on Concanavalin A-Sepharose. *Lane 4:* 0.5 M methyl α -D-mannopyranoside fraction on Concanavalin A-Sepharose. *Lane 5:* 20 mM edrophonium fraction on edrophonium-Sepharose.

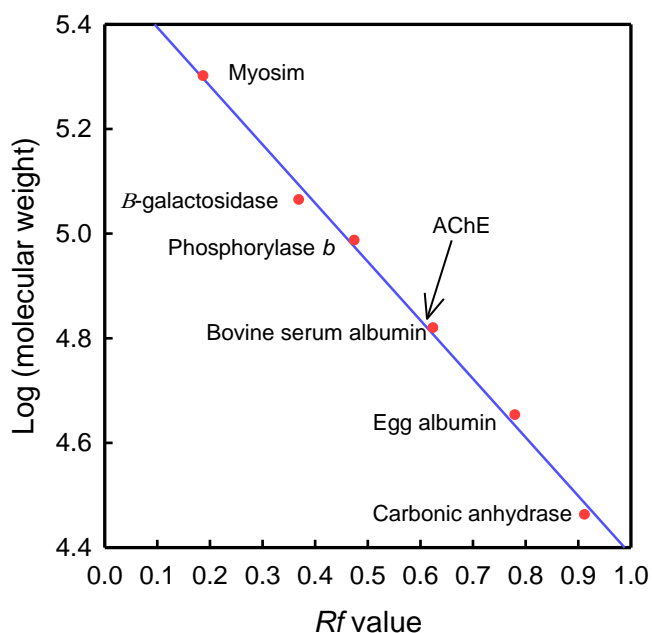


Figure 5.7 A typical standard calibration curve for a SDS gel. The protein markers used in order of increasing molecular weight: Carbonic anhydrase (29,000), egg albumin (45,000), BSA (66,000), phosphorylase *b* (97,000), β -galactosidase (116,000) and myosin (200,000).

5.4 Conclusions

A new method for the purification of acetylcholinesterase (AChE, acetylcholine acetylhydrolase, EC 3.1.1.7) enzyme and to extend a purification method for further enzyme characterization was developed. A research question that could be developed from the foregoing fact are as follows.

1. I succeeded in establishing a gentle solubilization technique that provided a favourable detergent during further purification procedure by stabilizing the native form of this fragile protein?
2. The purify AChE by a two-step separation on Concanavalin A-Sepharose 4B column followed by edrophonium-Sepharose 6B column. The monomeric molecular weight was detected 67.04 kDa by using SDS page, while tetrameric form detected to be 201.5 kDa by using the Sephacryl S-200 column?

3. Lastly, this protocol, in our opinion, (combined use of Concanavalin A-Sepharose 4B and edrophonium affinity 6B chromatography) could be a useful resource for purifying soluble AChE from sheep liver, which is readily applicable to the purification of soluble AChE from other sources?

Understanding these questions might provide vital clues on strategies to improve efficiency in sheep AChE and food safety.

Chapter 6

Characterization of cholinesterase

The results from this Chapter have been presented as poster presentations at the following conferences: Annual Meeting of European Federation of Food Science Technology (EFFoST)-Food and Health, November 2010, Dublin, Ireland; 23rd Annual Symposium of the European Society of Veterinary Neurology-European College of Veterinary Neurology, September 2010, University of Cambridge; Winter Meeting of the British Pharmacological Society, December 2010, Queen Elizabeth II Conference Centre, London; 32nd Annual Congress of the British Toxicology Society, March 2011, University of Durham; and Annual Conference of British Society of Animal Science, April 2011, University of Nottingham.

These results have also been published in:

Kasim Abass Askar, A. Caleb Kudi and A. John Moody (2010). The use of selective inhibitors to estimation of cholinesterase activity of liver and muscle for food animals. *Journal of British Pharmacological Society* 8:165-165.

Kasim Abass Askar, A. Caleb Kudi and A. John Moody (2011). Histochemical localization of acetylcholinesterase. *Toxicology* 290(2-3):104-104.

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Chapter 6: Characterization of cholinesterase activities

6.1 Introduction

Cholinesterases (ChE) prefers to hydrolyse the natural substrate ACh, which is the predominant choline ester, therefore ACh cannot be used as the sole substrate in the laboratory determination of AChE activity in blood or tissues containing AChE and BChE enzymes (Wilson *et al.*, 2001). To overcome this problem, specific substrates are used e.g. acetyl- β -methylcholine, commonly employed to measure AChE, while synthetic butyrylcholine or benzoylcholine are used to measure BChE (Morizono and Akinaga, 1981; Tecles and Ceron, 2001; Wilson *et al.*, 2001). Whereas variability in substrate affinity in tissues of cattle and pigs yielded highest BuTChI in pancreas, liver, kidney and lungs (except AcTChI in the heart and spleen) and PrTChI is poorly hydrolysed, while horse, dog and cats are yielded highest AcTChI in pancreas, liver, kidney, heart and lungs (except BuTChI in spleen) and PrTChI poorly hydrolysed (Morizono and Akinaga, 1981). This variability is due to the different biological function of the tissues. The variability in substrate affinity of ChE indicates the need for characterisation and selection of the appropriate substrate before determining ChE activity in animal species (Tecles and Ceron, 2001).

The two enzymes may also be distinguished by their affinity for or reactivity with various selective inhibitors, e.g. 1: 5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (BW284c51) (Anglister *et al.*, 2008; Naik *et al.*, 2008), 1: 5-bis (4-trimethylammoniumphenyl) pentan-3-one diiodide (BW284c50) (Silver, 1974), or methanesulfonyl fluoride (Borlongan *et al.*, 2005), all of these are specific selective to AChE. While tetraisopropyl pyrophosphoramidate (iso-OMPA) (Anglister *et al.*, 2008; Naik *et al.*, 2008), quinidine sulphate (Wilson *et*

al., 2001), bambuterol (Massoulié *et al.*, 1993), or ethopropazine [(10-2-dimethylaminopropyl) phenothiazine hydrochloride] (Cometa *et al.*, 2005), all of these compounds are a reversible and selective inhibitor to BChE. An important distinction between AChE and BChE in response to substrate specificity and selective inhibitor (Wilson *et al.*, 2001).

It is well known that pH and temperature depend on the charge of the enzyme and/or of the mixture. Protonation of the active site amino acids of the ChE is influenced by the pH of the surrounding medium (Silver, 1974; Fairbrother *et al.*, 1991). The imidazole nitrogen of the His residue attracts serine hydroxyl hydrogen to the active site of the ChE, reporting a nucleophilic character to the serine oxygen. Serine hydroxyl His imidazol charge transmits structure has a three possible forms; one active and other inactive. The active forms contain a protonated serine residue in the active site. ChE can assume deactivated forms if the serine decreases a proton (at high pHs) or imidazole nitrogen increases a proton (at low pHs), forming a quaternary nitrogen incapable of attracting the serine hydrogen (Fairbrother *et al.*, 1991; Tecles *et al.*, 2002b).

ChE can also be demonstrated histochemically at the optical microscope level. In brief, the enzyme in the tissue hydrolyses substrate present in the incubation medium and the hydrolysed product then reacts with some other component of the medium to form an insoluble precipitate at the site of enzyme activity. Localisation of AChE activity histochemically has been done for over 50 years. At present, the most widely used methods for the histochemical localisation of ChE activities is the direct method of Gomori (1948). This is a simple and direct method of localisation ChE activities in the tissues. It is based on the reaction between thiocholine, which is one of the products of the enzymatic hydrolysis of

the synthetic substrates AcTChI or BuTChI, with the medium containing; copper sulphate, glycine, maleic acid, magnesium chloride, sodium hydroxide and sodium sulphate. The formation of the shadow yellow colour in the tissues is indicated enzyme activity. Another method for histochemical localisation of ChE activities is the thiocholine method of Kugler (1987). It is based on the reaction between polyvinyl alcohol with the medium containing; sodium citrate, copper sulphate, potassium ferricyanide and AcTChI. The formation of the shadow brown colour in the tissues of this reaction indicates enzyme activity. The histochemical localisations of AChE in the tissues have been important in supplying supporting evidence for central cholinergic transmission. Table 6.1 shows specific and general characteristics of AChE and BChE.

However, the fundamental characterization of ChE in food animals is still not completely understood. This study aims to confirm and extend our current knowledge towards (a) to investigate the effect of dilution on ChE activities in the tissues from food animals used for human consumption; (b) to investigate the presence of different ChE by using two selective inhibitors for AChE or BChE; (c) to investigate the optimum pH and temperature for AChE and BChE; and (d) to determine the histochemical localisation of AChE in the liver of sheep used for human consumption. It also aims to identify the values of enzyme activity as biomarkers for evaluating exposure to OP and carbamate compounds and increase awareness in pesticide user to anti-ChE compounds. These objectives are interesting. In fact, it has been known for some time that one of the most common causes for human illness is toxic intake, and that associated with food is one of the most serious.

Studies by researchers worldwide have revealed that even in industrialised nations the food contains an ever-increasing quantity of pesticide residues (Timothy, 2001). Therefore, the aim of the work in this Chapter was also develop the assessment of pesticides residues in meat products and to build of a model to assess the residue concentration is meat products in veterinary medicine. In practice, supplemental security is a given by the fact that the real residue level and the eventual exposure to animal health is largely under the established acceptable daily intake (Harris *et al.*, 2001). Consequently, the user of animal treatment products applying good veterinary practices to meat products with a concentration of substance that does not exceed the commercially legal tolerable value. However, the presence of residue does not by itself explain the toxicity of a meat product. Hence, maximum concentration of pesticide residue (expressed as milligrams of residue per kilogram of food/animal feeding stuff) likely to occur in or on food and feeding stuffs after the use of pesticides according to Good Agricultural Practice (i.e., when the pesticide has been applied in line with the product label recommendations and in keeping with local environmental and other conditions) (MAF260/90).

It is also necessary to take into account the exposure and the toxicological effect to effectively minimise the risk of toxicity. Advantage should be use from the high variation of animal treatment products and the various pesticides compounds. Quantitative distinction should be put in evidence between meat products, such as insecticides applied early at the begin of intoxication period and substances applied late to protect the meat product, between old products requiring a high dose to be effective and recent substances with high bioactivity at low rate (FAO/WHO, 1970).

Hence, the Food and Agriculture Organization/ World Health organization in (1970) found that when cattle and sheep sprayed with 0.025% DDVP, three times within two weeks intervals producing no residues detectable at the limits of sensitivity in tissues (muscle, liver and kidney) of cattle (<0.25 ppm) or sheep (<0.05 ppm). As well as, Noetzel (1964) observed that when cattle received dermal applications with 0.5% DDVP, twice daily for 28 day, also causing no residues were detectable at the limits of sensitivity in milk (<0.02 ppm) (FAO/WHO, 1970).

Sheep plunge dipped with 250 mg DZN which indicated that residues at low levels in liver (<0.01-0.01 mg/kg) and in kidney (0.01-0.04 mg/kg) from one day after application of the dipping. Other study for sheep dipping in a DZN solution containing 250 mg. The animals were slaughtered in pairs at 1, 3, 7, 14 or 21 days after treatment and liver and muscle analysed for DZN content (Diazinon, 2002). Cattle fed in the diet 100 ppm of carbaryl had 1 ppm in kidney, 0.4 ppm in liver and 0.1 ppm in muscle. It is therefore recommended that the tolerance for meat of cattle reduced from 1 ppm to 0.2 ppm. Other study for pigs when receiving 200 mg/kg by gavage or 300 mg/kg in the diet administered during gestation or organogenesis showed no effect on reproduction (Weil *et al.*, 1972).

Table 6.1 Properties of two different classes of ChE (adapted from (Silver, 1974; Wilson *et al.*, 2001)).

Characteristics		AChE	BChE
Preferred substrate		ACh	Butyrylcholine or Propionylcholine
Activity towards	Acetyl- β - methylcholine	+	-
	Benzoylcholine	-	+
	Acetylcarnitylcholine	+	-
Specific inhibitors	BW284c51	Susceptible to 10^{-5} M	Resistant to 10^{-5} M
	Iso-OMPA or Ethopropazine	Resistant to 10^{-5} M	Susceptible to 10^{-5} M
Effect of ions	Ni and Zn	Strong inhibition	Weak or no inhibition
	Mn and Mg	Mg ⁺⁺ the more effective activator	Mn ⁺⁺ the more effective activator
Optimum substrate concentration		3 mM	10 mM
Reaction catalyzed		ACh + H ₂ O ↓ Choline + Acetate	Acylcholine + H ₂ O ↓ Choline + Corresponding acid
Inhibition by excess substrate		Yes. Most active towards substrate in low concentration	No. Most active towards substrate in high concentration
Localization		Membrane bound	Soluble

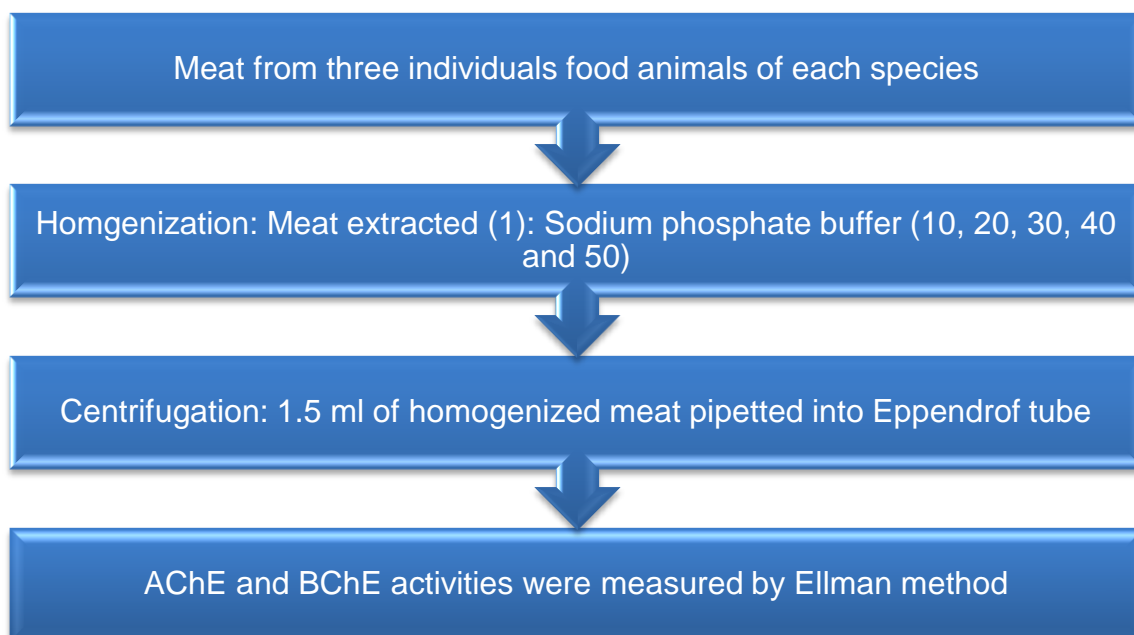
6.2 Materials and methods

6.2.1 Sample collection and preparation

The sample collection and preparation were done as explained in Sections 2.2 and 2.3. Following this it was then assayed the enzyme activity as described in Section 2.4.1. All chemicals were of the highest analytical grade obtained from Sigma Chemical Company (Poole, Dorset, UK), unless stated otherwise.

6.2.2 Determination the effect of dilution

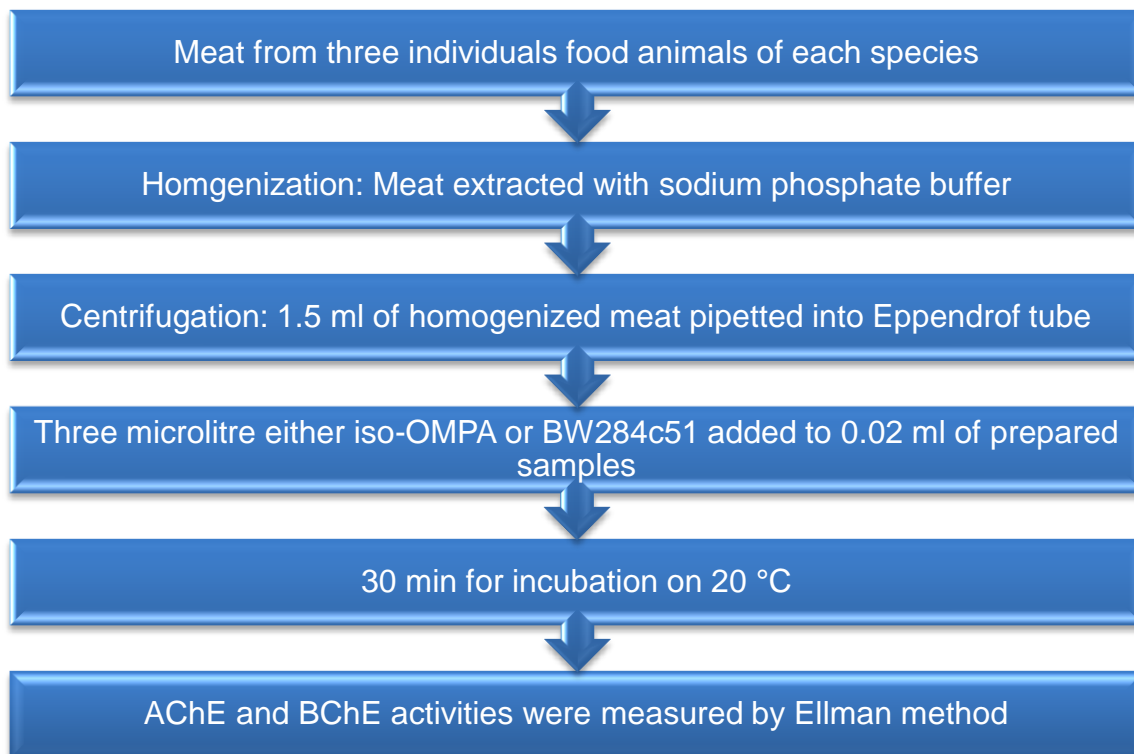
Summarize the steps for measuring AChE and BChE activities in the different dilutions for the tissues of food animals by using Ellman method can be drawn in below;



The tissues (liver, muscle and kidney) from sheep, cattle and pigs were diluted at a ratio of 1:10, 20, 30, 40 and 50 with sodium phosphate buffer (0.1 M, pH 8). Blanks were also run at each selected dilution for each based on absorbance tested. Then the AChE and BChE activities were determined as described in Section 2.4.1.

6.2.3 Determination the effect of specific inhibitor

Determination of AChE and BChE activities by using specific inhibitors in the tissues of food animals can be drawn in below;



Iso-OMPA and BW284c51 were used as specific inhibitors of BChE and AChE, respectively. Stock solutions of iso-OMPA were prepared in ethanol and BW284c51 was dissolved in distilled water (Massoulie *et al.*, 1993; Varo *et al.*, 2002). From these stock solutions, five dilutions were prepared for each inhibitor. Samples (liver and muscle) were individually homogenized and centrifuged. Then homogenates (0.02 ml) were incubated at room temperature 20 °C for 30 min with 3 µl of each inhibitor dilution solution. Controls were incubated with 3 µl of sodium phosphate buffer pH 8.0 were included when appropriated. A blank without inhibitor and with either distilled water for BW284c51 or ethanol (a solvent used for iso-OMPA) was also performed. Final inhibitor concentrations ranging from 0.5 to 16 x 10⁻³ M for iso-OMPA and from

0.25 to 8×10^{-6} M for BW284c51 (Figure 6.1). The effect of inhibitors of AChE and BChE activities was measured by using (AcTChI, BuTChI and PrTChI as substrates) as explained in Section 2.4.1.

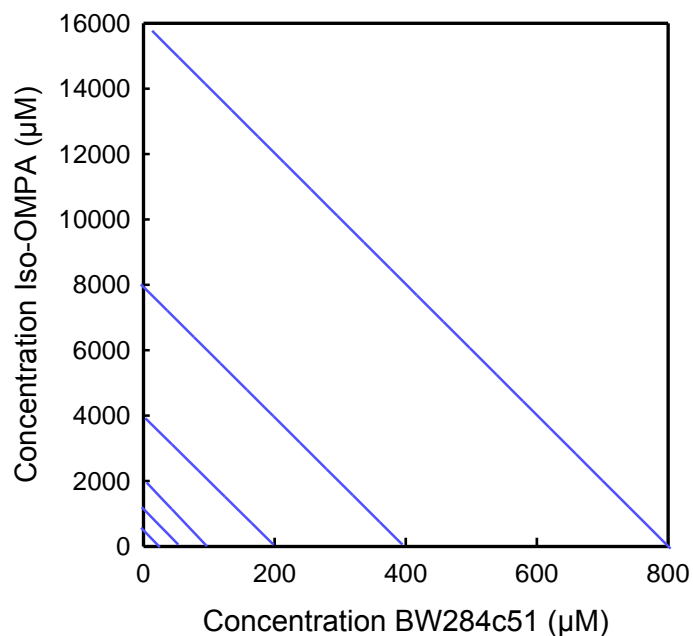
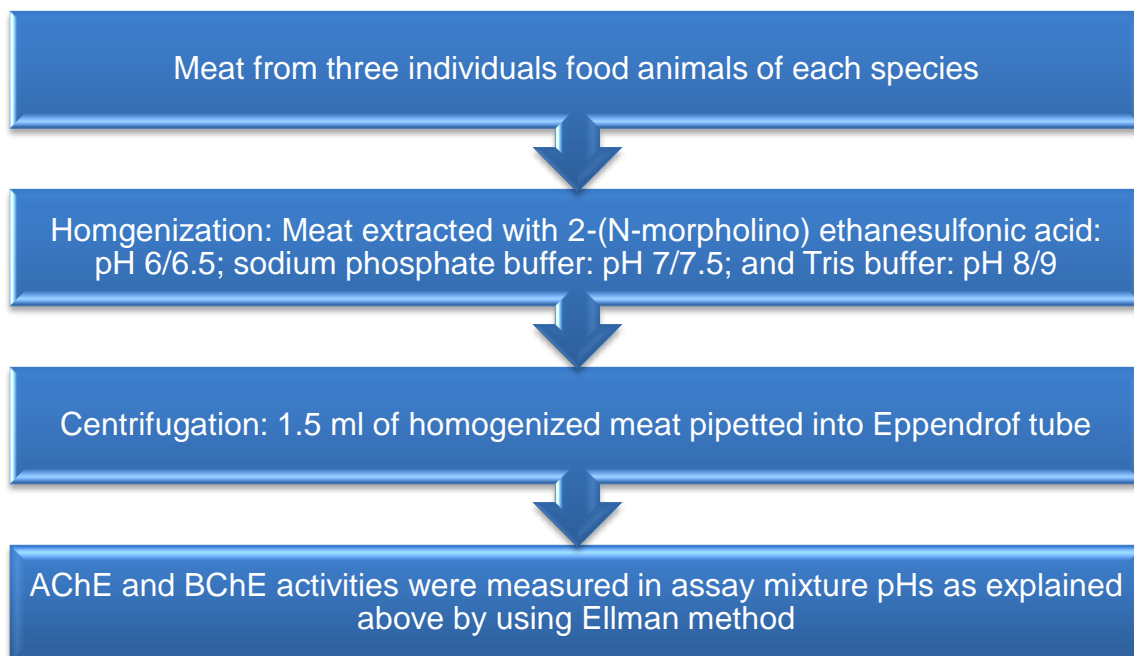


Figure 6.1 Comparison of the two ChE specific inhibitor concentrations. The dashed lines represent the relationship between the concentrations of BW284c51 and iso-OMPA, respectively (μM).

6.2.4 Determination of optimum pH

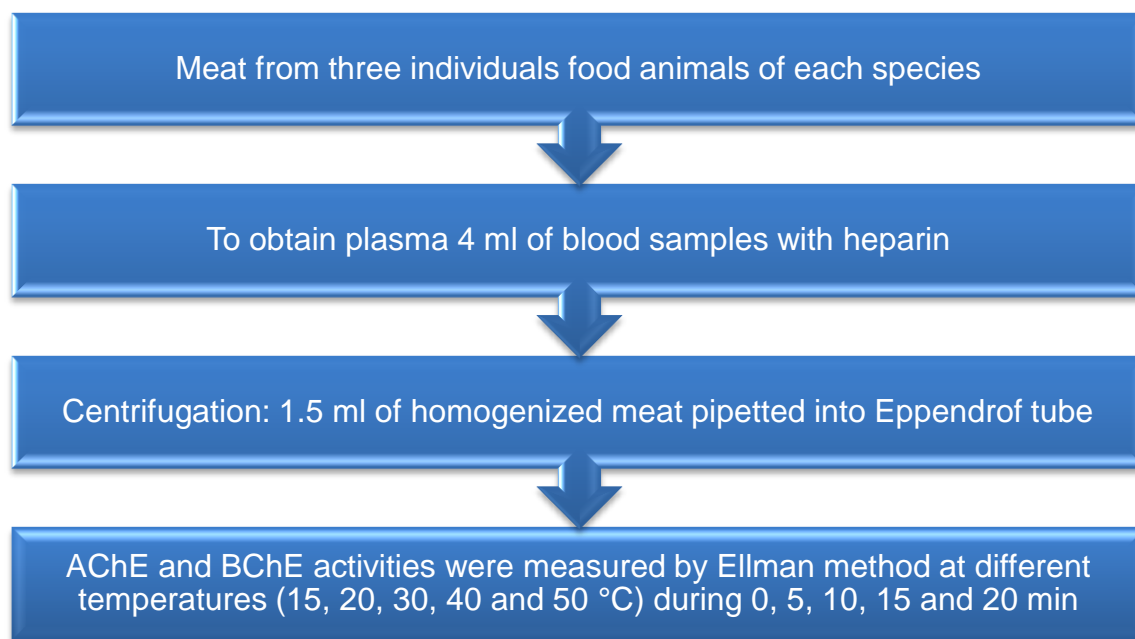
For detection of optimal AChE and BChE activities by using different pHs in the tissues of food animals can be drawn below;



For the measurement of optimum pHs, ChE was measured for different pHs (6-9). Hence, different pHs were 0.1 M buffer concentrations of 2-(N-morpholino) ethanesulfonic acid: pH 6/6.5; sodium phosphate buffer: pH 7/7.5; and Tris buffer: pH 8/9. Often, different buffer components are present due to the variety of pH used. Blanks were also run at each selected pH for each based on absorbance tested. Then the enzyme activity was determined as described in Section 2.4.1. The increase and decrease in ChE activity over different pHs was then monitored. The data were fitted with a Gaussian peak using SigmaPlot version 11 (Systat software, Inc.).

6.2.5 Determination of optimum temperature

Experimental design for detection of optimal AChE and BChE activities by using different temperatures in the blood contents from food animals can be drawn below;



For detection effect of temperature at 5 different reaction temperatures (15, 20, 30, 40 and 50 °C) during 0-20 min of measuring ChE activities. For these studies, the 96-well microtitre plate reader also used. Blanks were also run at each selected temperature for each based on absorbance tested. Following, this the enzyme activity was determined as described in Section 2.4.1. The data were fitted with an exponential rise to maximum over different times using SigmaPlot 11.

6.2.6 Enzyme histochemical localization

6.2.6.1 Tissue sections

The unfixed cryostat sections as described, according to Cornelis *et al.* (1992), as follows.

1. one piece of sheep liver tissue was required that measures at least 0.5 cm³, separate using a scalpel blade and rinsed until the blood was fully removed;
2. the piece of liver was then wrapped in aluminium foil and carefully immersed into the liquid nitrogen (-170 °C to -210 °C) at least for 5 min;
3. the liver was placed in block to metal chunk in the cryostat cabinet (Portable Bench-top Cryostat for Mobile Diagnostics Leica CM1100) using cryoprotectant at an ambient temperature between -20 °C and -30 °C, without allowing the liver block to be warmed up by the liquid cryoprotectant;
4. sectioning started when the block was trimmed to the desired level in the tissue block; and
5. cut into blocks up to 12-µm thick sections and placed on a polysine slide. Since the section is cut correctly, it remains flat on the microtome knife under the anti-roll plate. Following this it was then placed on the slides. It was important during sectioning to ensure that (a) tissue block adjusts to the cryostat cabinet temperature; (b) knife and knife holder is firmly fixed; (c) anti-roll plate is properly adjusted; and (d) the speed of sectioning should be constant (Cornelis *et al.*, 1992).

6.2.6.2 Histochemical detection of acetylcholinesterase

Two methods were used to determine the histochemical localisation of AChE as described below.

1. Gomori method: AChE activity was determined histochemically by the Gomori method (1948), with minor modification by staining with haematoxylin-eosin and mounted with glycerin. Outlines of the procedure of preparing of medium and sample sections are shown below.

- unfixed cryostat sections were fixed in formaldehyde vapour for 10 min, to preventing diffusion of some enzymes and other constituent;
- the medium was prepared as follows: Copper sulphate 0.3 g, glycine 0.375 g, maleic acid 1.75 g, magnesium chloride 1 g, 4% sodium hydroxide (30 ml), 40% sodium sulphate (170 ml), adjusted pH 6.0 stored at the 38 °C (LEEC, UK). Following this it was then added 20 mg of AcTChI to 10 ml of preparing medium; and
- the prepared section was incubated for at least an hour at 38 °C in the media and rinse 3 times with saturated sodium sulphate. Following this it was then treated with dilute yellow ammonium sulphide for 2 min. Lastly, it was washed seven times for 1 min each in distilled water, and equal amount of glycerine jelly. The sections were then rinsed, slightly counter stained with haematoxylin-eosin and mounted with glycerine.

2. Kugler method: AChE activity was also determined histochemically by the Kugler method (1987). An outline of the procedure of preparing of medium and sample sections is shown below.

- unfixed cryostat sections were used;
- dissolve 18 g polyvinyl alcohol in 100 ml 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.0) (1.192 g/100 ml);

- to 1 ml polyvinyl alcohol-containing medium, the following was added (strictly in the following order) 10 μ l sodium citrate (882 mg/ml distilled water; final concentration 30 mM), 10 μ l copper sulphate (450 mg/ml of medium; final concentration 18 mM), 10 μ l potassium ferricyanide (127 mg/ml of medium; final concentration 3 mM), and 10 μ l AcTChI (88 mg/ml of medium; final concentration 3 mM), adjust the pH of the medium to pH 6.0; and
- sections were incubated in the media for at least 1 h at 37 °C, and rinsed at least 3 times with distilled water and mounted with glycerine.

6.2.7 Statistical analysis

All enzyme measurements were conducted in three individuals from each animal with results expressed as mean values \pm SE. Data were analysed by one-way analysis of variance using MiniTab statistical software version 15. Probability values < 0.05 were considered statistically significant.

6.3 Results

6.3.1 Effect of dilution on cholinesterase activities

The effect of dilutions on ChE activities were determined from kidney, liver and muscle of sheep, cattle and pigs using the Ellman method as described in Section 6.2.2 (Figures 6.2 and 6.3). The level of AChE and BChE activities in dilution 1:10, observed highest activity in the liver, muscle and kidney for sheep, cattle and pigs (Figures 6.2 and 6.3). It was found that AChE activity in tissue from the liver significantly different ($P < 0.05$) between dilution 1:10 and dilution 1:50 for cattle, and between dilution 1:50 among other dilutions and between dilution 1:20 with dilutions 1:30, 1:40 and 1:50 for pigs. Tissue from the liver BChE, was significant ($P < 0.05$) between dilution 1:20 and dilution 1:20 with dilutions 1:40 and 1:50 for cattle, and the level of AChE and BChE ranged between 40.4 and 138.4 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for sheep, and 100.7 and 254.6 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for cattle, and 284.4 and 383.9 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for pig samples across different dilutions (Figure 6.2A-C).

Tissue from muscle AChE was significantly different ($P < 0.05$) between dilution 1:30 among other dilutions for sheep, cattle, while in pig was seen significant ($P < 0.05$) between dilution 1:30 and dilutions 1:10, 1:20, BChE was significant ($P < 0.05$) between dilution 1:30 and among other dilutions for cattle, and between dilutions 1:40, 1:50 with other dilutions for sheep, in addition a significant ($P < 0.05$) differences occurs within dilution 1:20 and dilution 1:40 among other dilutions used for pig, and the level of AChE and BChE ranged between 34.8 and 63.8 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for sheep and 30.1 and 50.8 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for cattle and 20.9 and 84.4 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for pig across different dilutions (Figure 6.2D-F).

There was significant ($P < 0.05$) in kidney AChE within dilution 1:30 among other dilutions used for sheep, cattle and pig, while kidney BChE was significant ($P < 0.05$) between dilution 1:30 among other dilutions used for sheep, in addition a significantly different ($P < 0.05$) occurs within dilution 1:10 and dilution 1:20 among other dilutions used for cattle, while in pig was seen significant ($P < 0.05$) between dilution 1:10 and dilutions 1:30, 1:40 and 1:50. Level of AChE and BChE ranged between 44.7 and 75.6 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for sheep, and 40.6 and 145.6 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for cattle, and 259.7 and 367.3 $\text{nmol min}^{-1} \text{g}^{-1}$ respectively for pig samples across different dilutions (Figure 6.3A-C). The enzyme activity in the tissues at the higher dilution ratio declined faster than that of samples at lower dilution ratio. Overall, the dilution 1:10 gave a higher enzymatic activity, and this was selected for the rest of the work.

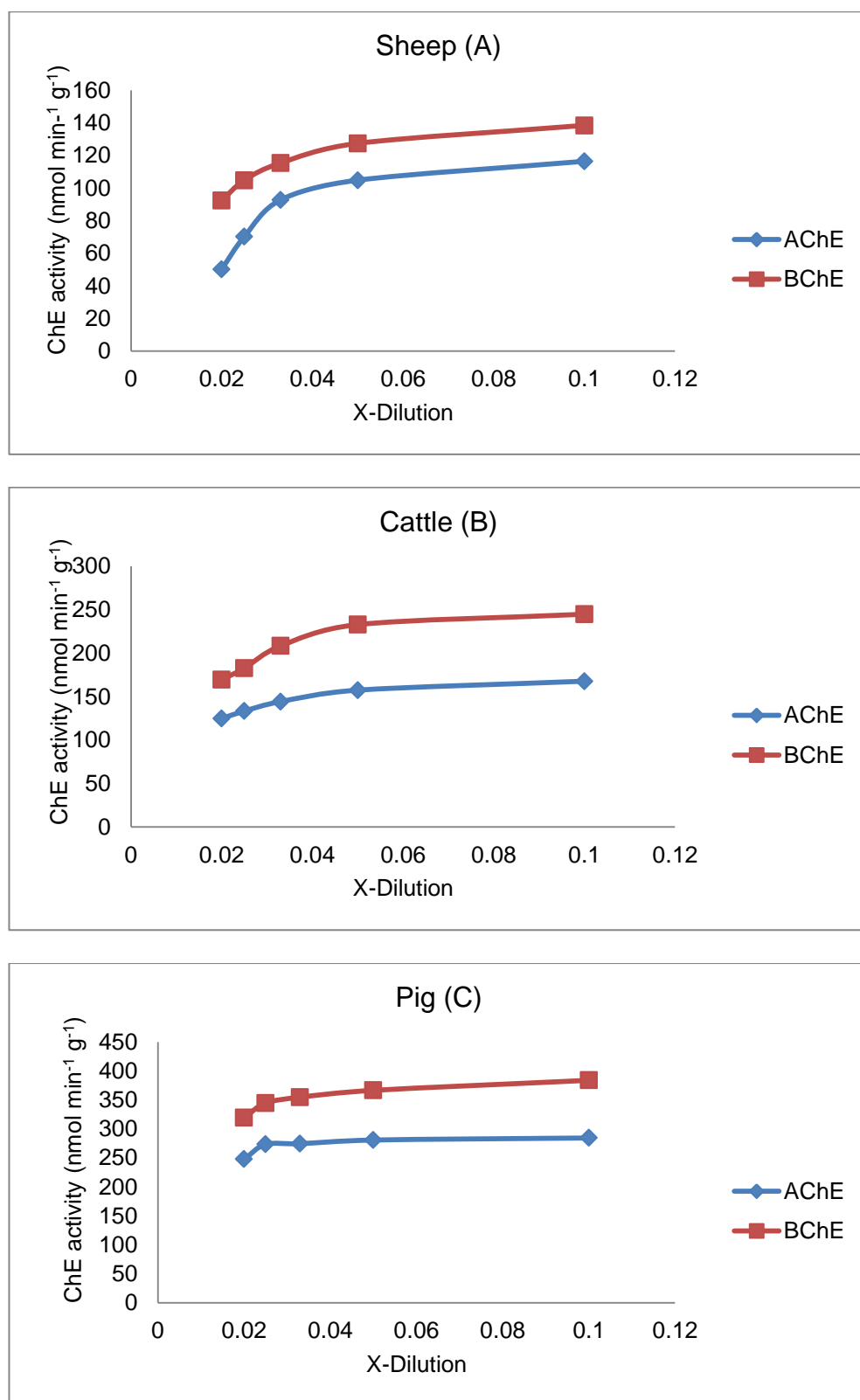


Figure 6.2 (A-C) Mean of specific AChE and BChE activities in diluted liver from sheep, cattle and pigs.

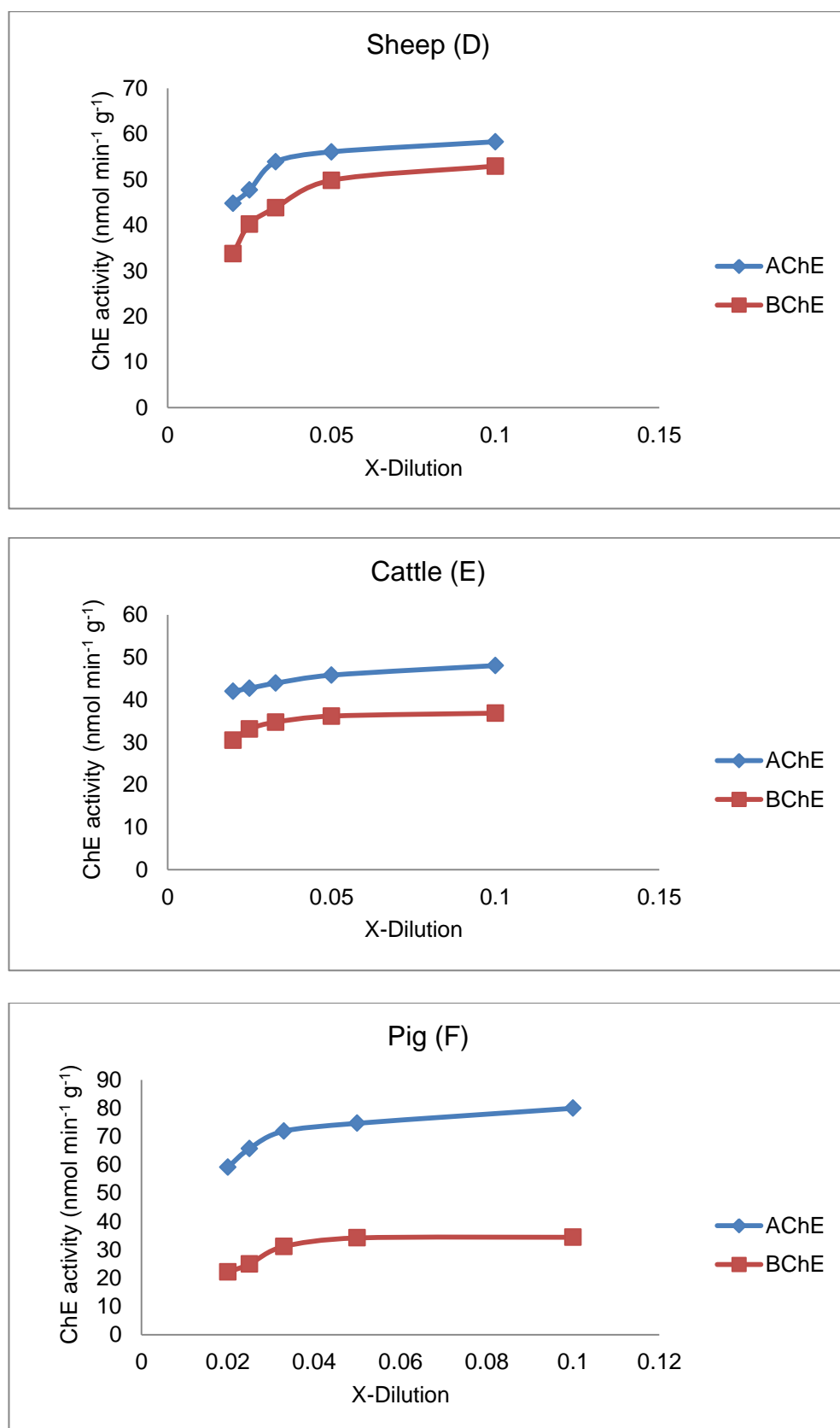


Figure 6.2 (D-F) Mean of specific AChE and BChE activities in diluted muscle from sheep, cattle and pigs.

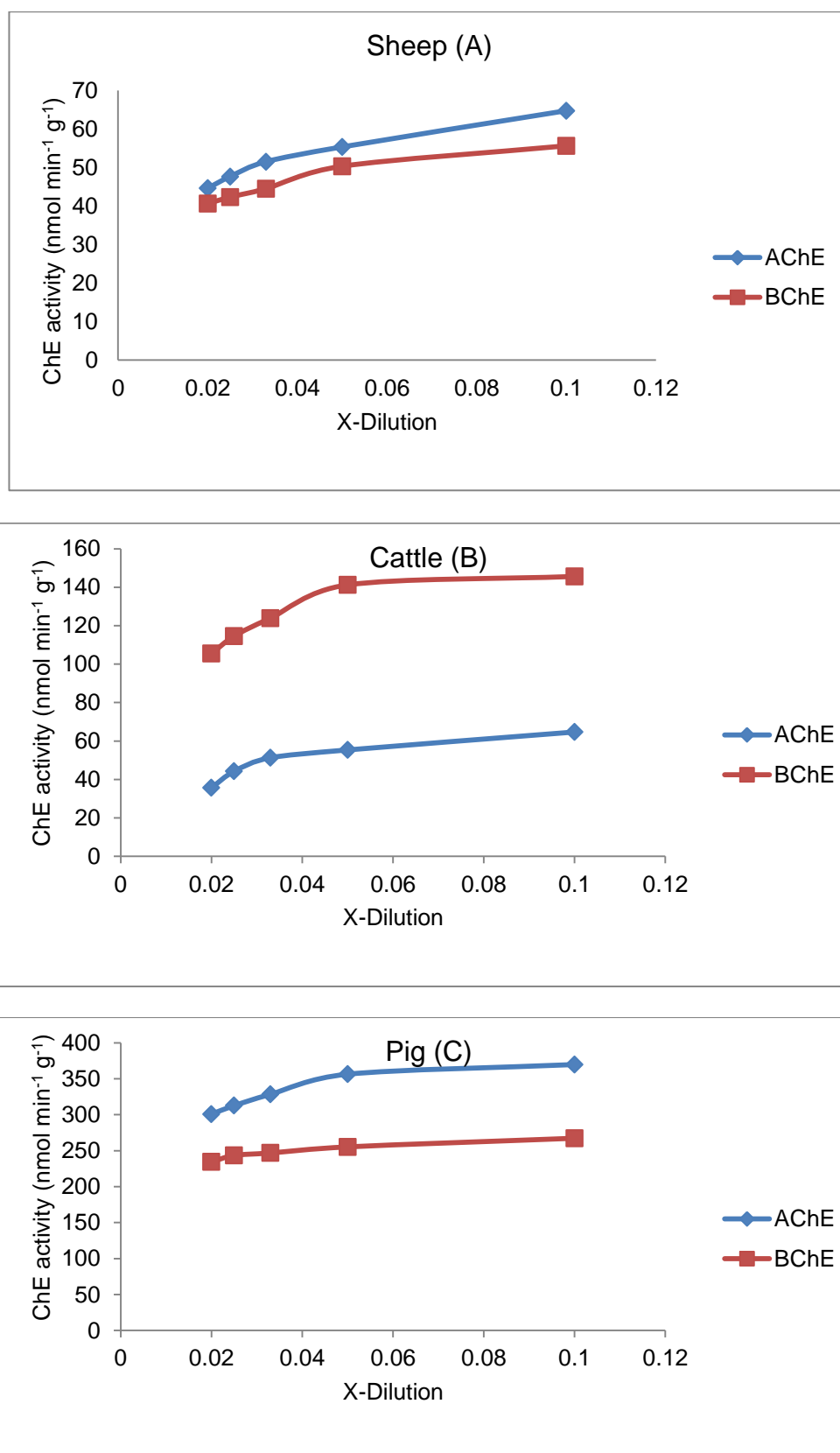


Figure 6.3 (A-C) Mean of specific AChE and BChE activities in diluted kidney from sheep, cattle and pigs.

6.3.2 Use of selective inhibitor to estimate cholinesterase activities

The effect of BW284c51 and iso-OMPA on ChE was determined from issue of liver for sheep, cattle and pigs using the Ellman method as described in Section 6.2.3 (Figure 6.4A-F). Sheep liver BW284c51 had significantly different ($P < 0.05$) between control and different inhibitor concentrations for AcTChI and BuTChI (Figure 6.4A). It found that significantly different ($P < 0.05$) effect for cattle and pig between control and different inhibitor concentrations for AcTChI and at concentration 50 μ M for BuTChI and PrTChI (Figure 6.4C and 6.4E). Iso-OMPA in sheep had ($P < 0.05$) effect between control and different inhibitor concentrations for BuTChI and at concentration 16 mM for AcTChI (Figure 6.4B). Cattle iso-OMPA had ($P < 0.05$) effective between control and different inhibitor concentrations for AcTChI and at concentration 1 mM for BuTChI (Figure 6.4D). It was ($P < 0.05$) for pig between control and concentration 1 mM for AcTChI, BuTChI and PrTChI (Figure 6.4F).

In general, BW284c51 effect was highest in tissues of pigs liver followed by cattle and sheep for AcTChI and PrTChI, while it increased in rank of cattle > sheep > pig for BuTChI. Iso-OMPA increased according rank order of cattle > pig > sheep for AcTChI and cattle > sheep > pig for BuTChI, while it increased: pig > cattle > sheep for PrTChI (Figure 6.4A-F). Liver percentage inhibition of BW284c51 was almost ranged AcTChI (40.7-94%), BuTChI (8.5-79.7%) and PrTChI (50.2-80.7%), while iso-OMPA ranged AcTChI (23.8-75.3%), BuTChI (41.1-63.8%) and PrTChI (13.9-48.2%) (Figure 6.4A-F).

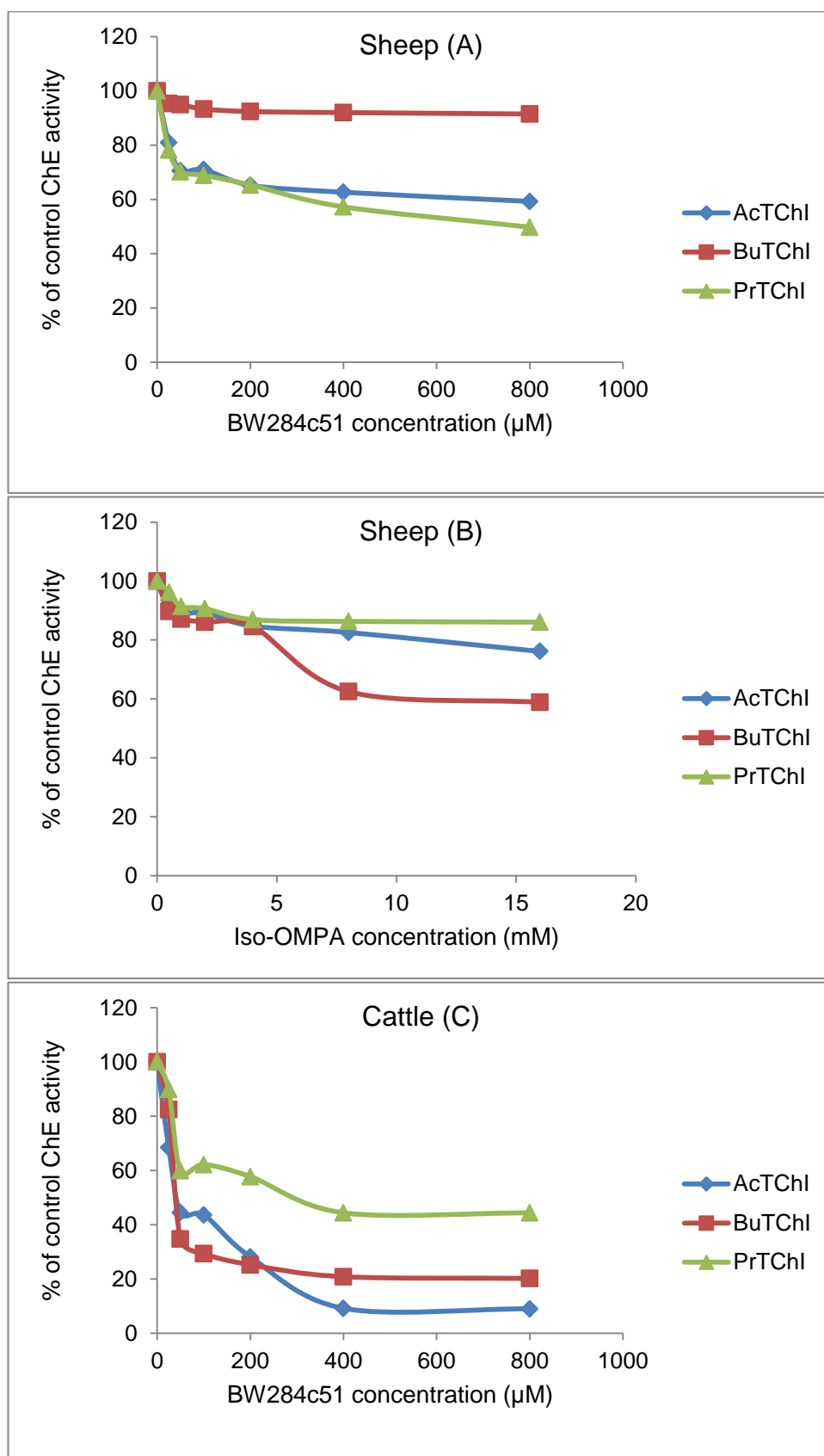


Figure 6.4 A-C Specific inhibitors were measured of liver with 1 mM of AcTChI, BuTChI and PrTChI hydrolysis. Data are expressed as the mean. Each experiment performed in triplicate ($n = 3$ in each animal).

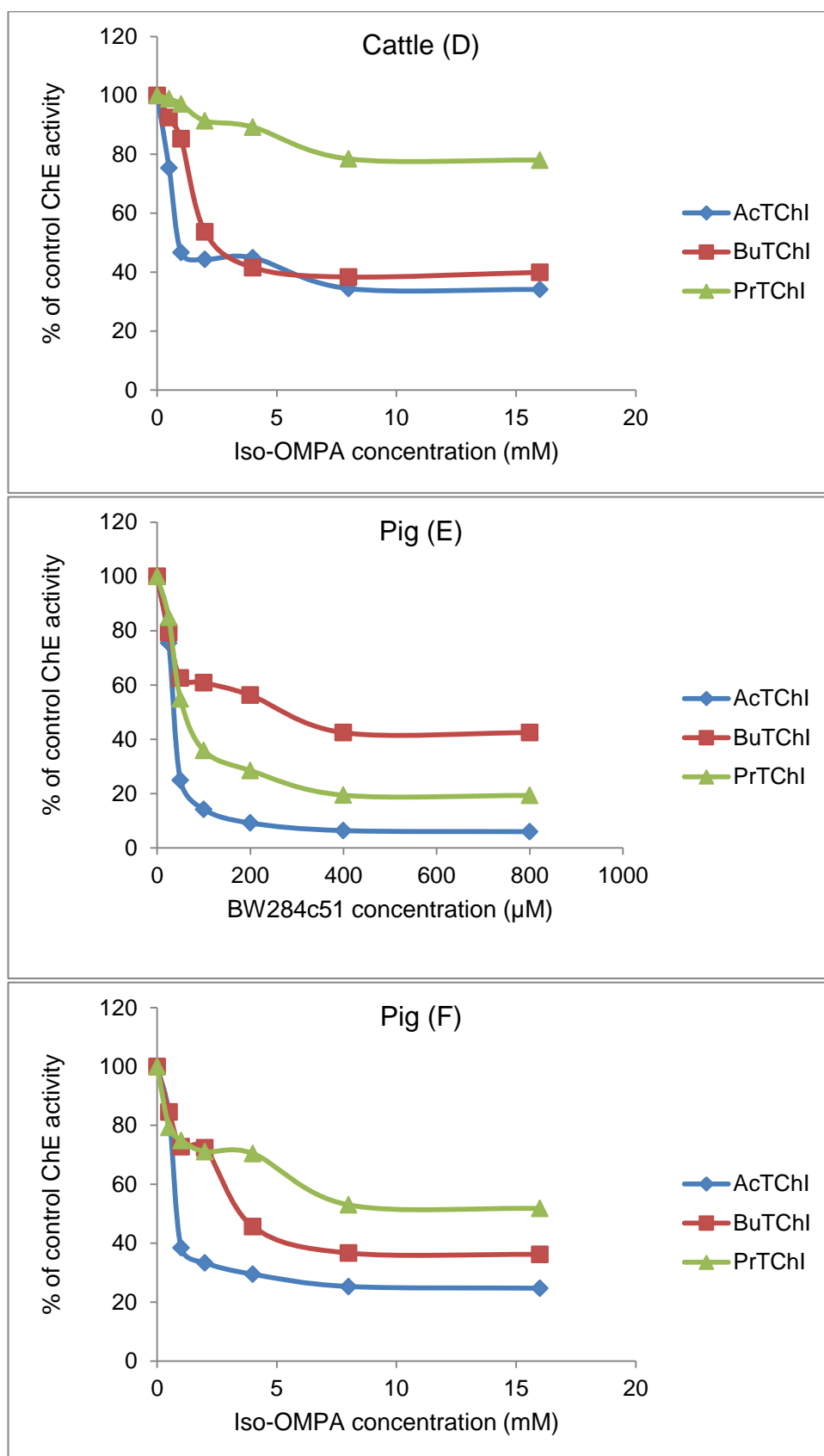


Figure 6.4 D-F Specific inhibitors were measured of liver with 1 mM of AcTChI, BuTChI and PrTChI hydrolysis. Data are expressed as the mean. Each experiment performed in triplicate ($n = 3$ in each animal).

Once, again the effects of BW284c51 and iso-OMPA on ChE were also determined from the muscle of sheep, cattle and pigs using the Ellman method as described in Section 2.4.1 (Figure 6.5A-F). BW284c51 had ($P < 0.05$) effect between control and different inhibitor concentrations for AcTChI and PrTChI for sheep and cattle (Figure 6.5A and 6.5C), whereas pig had ($P < 0.05$) effect between control and different inhibitor concentrations for three substrates used (Figure 6.5E). Iso-OMPA had ($P < 0.05$) effect between control and different inhibitor concentrations with PrTChI and 4 mM concentration for AcTChI and BuTChI for cattle (Figure 6.5D) and at concentration 2 mM for AcTChI and PrTChI in pig (Figure 6.5F). Rates of inhibition for the three animals, BW284c51 were increased as follows: pig > sheep > cattle for AcTChI and PrTChI, while it increased: cattle > pig > sheep for BuTChI. Iso-OMPA in animals was increased in this rank: pig > cattle > sheep for AcTChI and pig > cattle > sheep for BuTChI, while it increased cattle > pig > sheep for PrTChI (Figure 6.5A-F). Percentage muscle inhibition of BW284c51 almost ranged AcTChI (51.9-81.5%), BuTChI (9.3-30%) and PrTChI (32.7-87.5%), whereas iso-OMPA AcTChI (21.6-27.2%), BuTChI (10.5-29.5%) and PrTChI (18-65.8%) (Figure 6.5A-F).

Pearson correlation coefficient (r) calculated to measure the degree of relationship between iso-OMPA and BW284c51 in the liver and muscles for testing animals was observed significant ($P < 0.05$ and $r > 0.84$), with the exception of muscle where BuTChI activity was poor correlation and insignificant ($P = 0.087$; $r = 0.69$ and $P = 0.137$, $r = 0.62$) for sheep and cattle, respectively (Table 6.2).

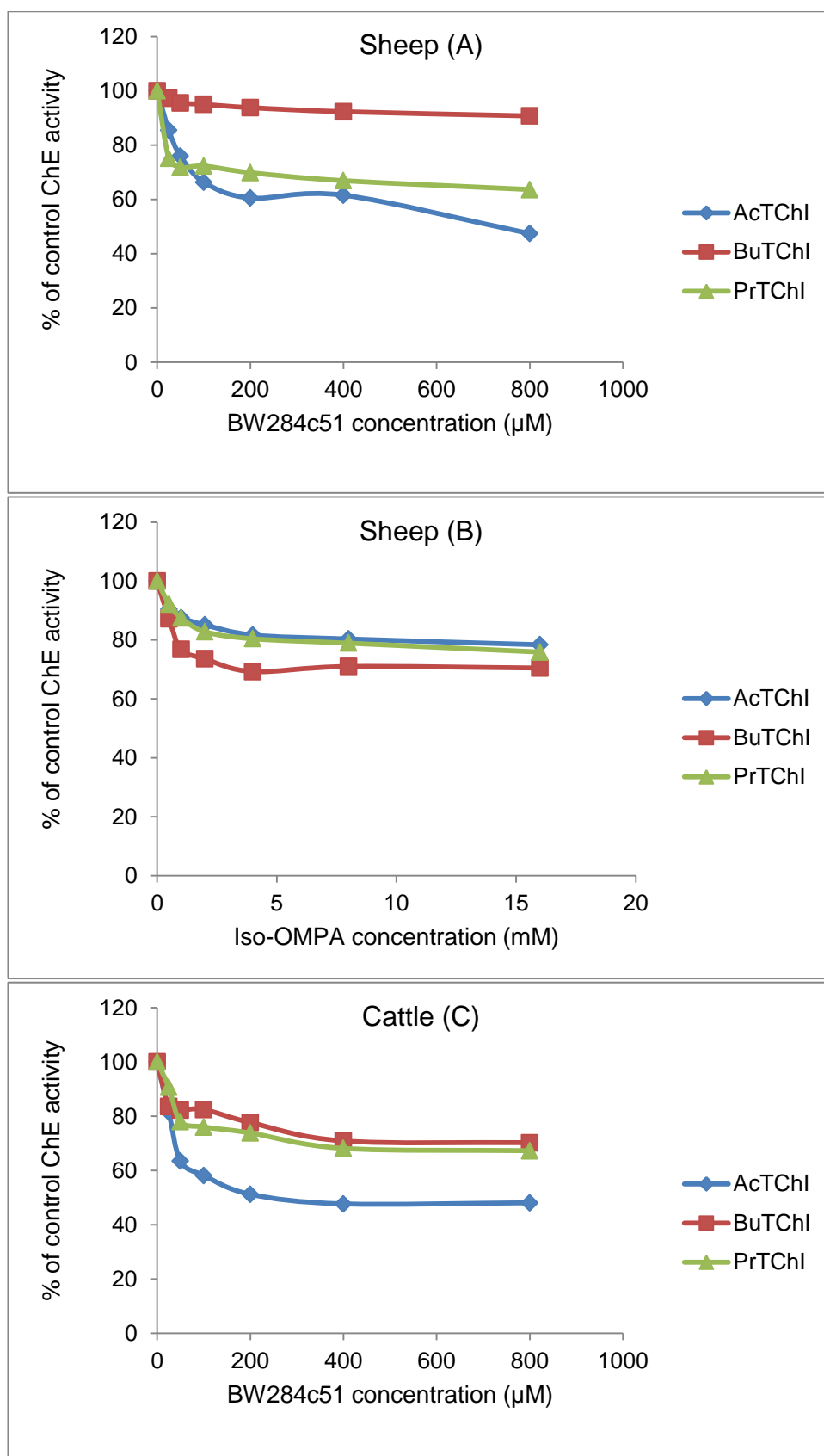


Figure 6.5 A-C Specific inhibitors were measured of muscle with 1 mM of AcTChI, BuTChI and PrTChI hydrolysis. Data are expressed as the mean. Each experiment performed in triplicate ($n = 3$ in each animal).

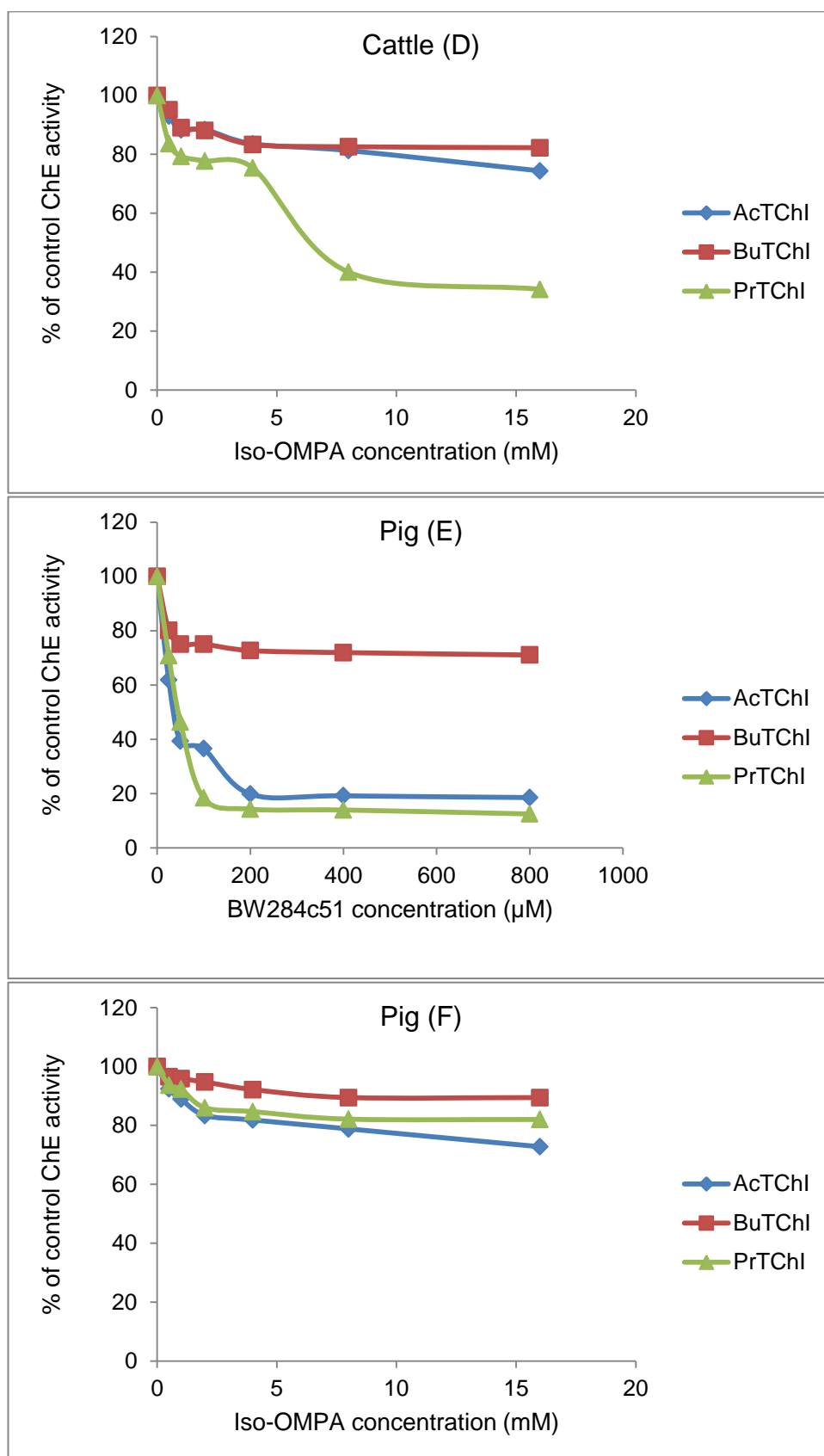


Figure 6.5 D-F Specific inhibitors were measured of muscle with 1 mM of AcTChI, BuTChI and PrTChI hydrolysis. Data are expressed as the mean. Each experiment performed in triplicate ($n = 3$ in each animal).

Table 6. 2 Pearson correlation coefficient and (*P*) values between BW284c51 and iso-OMPA in the liver and muscle for sheep, cattle and pigs.

Substrates	Sheep		Cattle		Pig	
	Liver	Muscle	Liver	Muscle	Liver	Muscle
AcTChI	0.92 (0.003)	0.88 (0.010)	0.85 (0.016)	0.95 (0.001)	0.98 (0.000)	0.97 (0.000)
BuTChI	0.96 (0.000)	0.69 (0.087)	0.84 (0.019)	0.62 (0.137)	0.92 (0.004)	0.97 (0.000)
PrTChI	0.89 (0.007)	0.90 (0.006)	0.98 (0.000)	0.94 (0.002)	0.96 (0.001)	0.91 (0.000)

Values in the table are the *r* (*P*-value).

6.3.3 Determination of optimal pH for cholinesterase activities

Optimal pH for AChE and BChE activities was determined for three different buffers [2-(N-morpholino) ethanesulfonic acid, sodium phosphate buffer, and Tris buffer] at levels ranging between 6 and 9 as described in Section 6.2.4 (Figure 6.6A-F). In all cases, there was significantly increased AChE activity from pH 6.0 to 7.5 and remained at a high level between pH 7.0 and 8.0 and enzyme activity peaked (100%) at 99.69 nmol min⁻¹ g⁻¹ for sheep, 177.12 nmol min⁻¹ g⁻¹ for cattle, and 288.13 nmol min⁻¹ g⁻¹ for pig liver AChE, while 75.23 nmol min⁻¹ g⁻¹ for sheep, 83.31 nmol min⁻¹ g⁻¹ for cattle, and 180.09 nmol min⁻¹ g⁻¹ for pig muscle AChE at pH 8.0, and became severe as pH increased (Figure 6.6A-F). In general, the optimal pHs in both enzyme activities were ranged from pH 7.9 to 8.1. Pig muscle BChE had the lowest optimum pH effect compared with sheep and cattle (Figure 6.5F). AChE was highest in liver for cattle (Figure 6.6C). BChE in liver was increased from pH 6.0 to 8.0 and remained at a high

level from pH 8.0 to 8.5. While the enzyme activity peaked (100%) at 262.54 nmol min⁻¹ g⁻¹ for sheep, 375.54 nmol min⁻¹ g⁻¹ for cattle, and 482.54 nmol min⁻¹ g⁻¹ for pig liver BChE at pH 8.5. The optimal pH for the enzymatic reaction is pH 8.3 to 8.5, but muscle BChE increased from pH 6.0 to 7.0 and remained at a high level from pH 7.0 to 7.5, whereas the activity peaked (100%) at 74.45 nmol min⁻¹ g⁻¹ for sheep, 84.42 nmol min⁻¹ g⁻¹ for cattle, and 83.54 nmol min⁻¹ g⁻¹ for pig muscle BChE at pH 7.5, and became severe as pH increased.

The optimal pH for the enzymatic reaction is 7.7 to 7.9 (Figure 6.6A-F). Both AChE and BChE enzymes in the liver, there was a significant positive correlation (Pearson's correlation coefficient, $r = 0.83$, $P = 0.0191$; $r = 0.82$, $P = 0.024$; $r = 0.96$, $P = 0.001$ for sheep, cattle and pig, respectively). Once again the AChE and BChE enzymes in muscle, there was a significant positive correlation (Pearson's correlation coefficient, $r = 0.94$, $P = 0.002$; $r = 0.88$, $P = 0.009$; $r = 0.85$, $P = 0.017$ for sheep, cattle and pig, respectively). In all cases (sheep, cattle and pigs used different pHs), the relationship was seen between liver and muscle ChE activities (% of control at peak) to be ($R^2 = 0.64$; $P < 0.001$) (Figure 6.7).

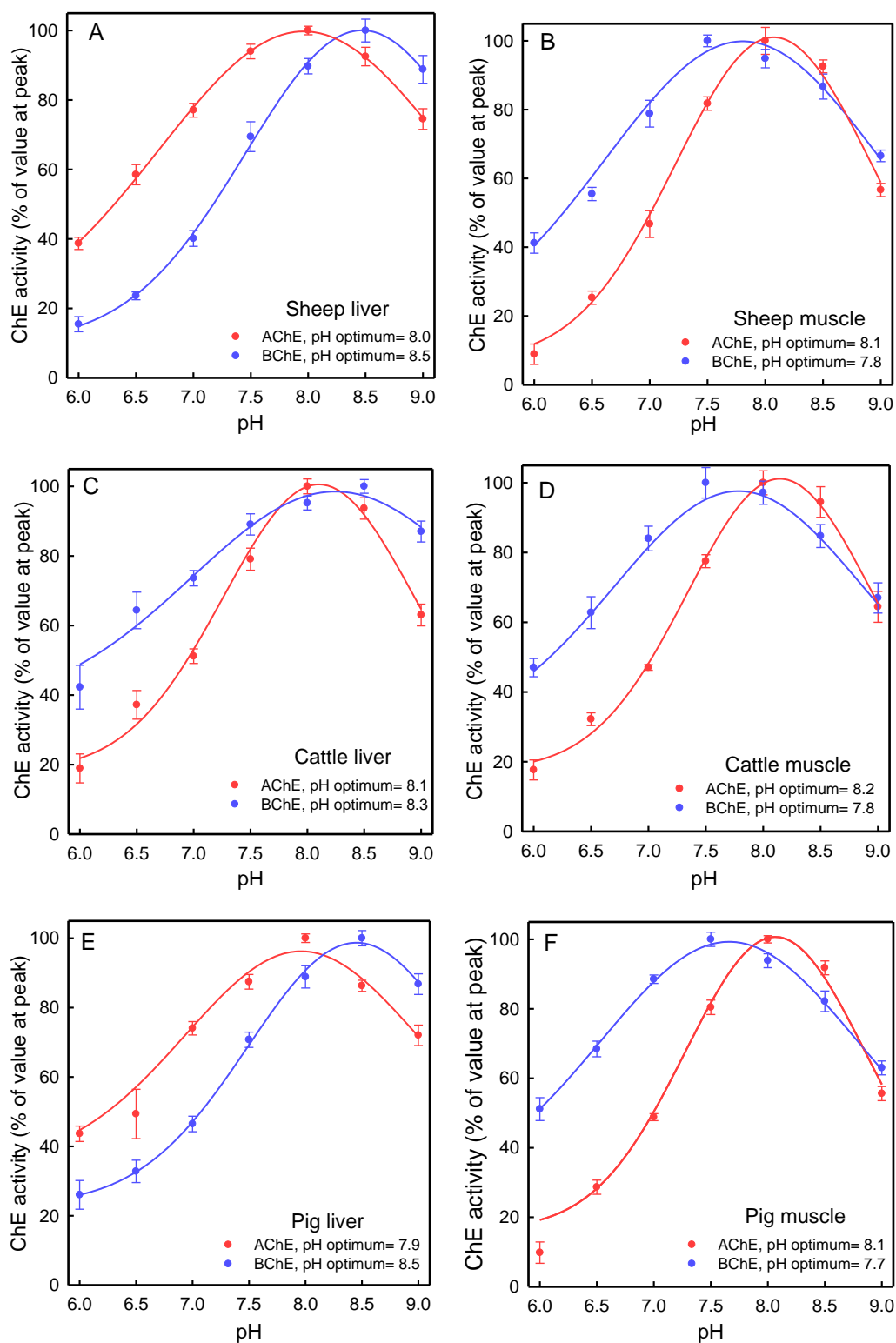


Figure 6.6 Effect of pH on AChE and BChE activity extracts from food animals. Using 0.1 M buffers of 2-(N-morpholino) ethanesulfonic acid (pH 6/6.5), sodium phosphate buffer (pH 7/7.5), and Tris buffer (pH 8/9). Each measurement is expressed as the mean \pm SE of three separate determinations ($R^2 > 0.82$; $P < 0.05$).

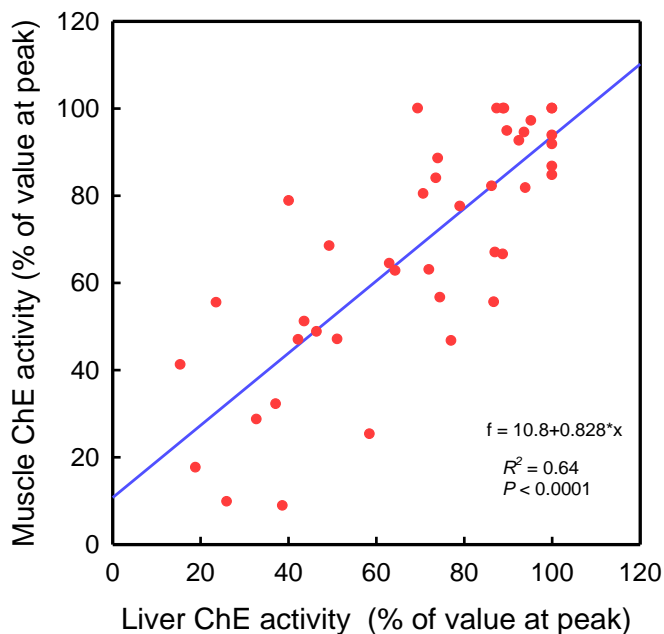


Figure 6.7 Comparison analysis between liver and muscle ChE activities (% of value at peak) of sheep, cattle and pigs within a range of pHs (6/9).

6.3.4 Effect of temperature on cholinesterase activities

AChE and BChE activities were determined in plasma with heparin for sheep and cattle using a temperature range of 15-50 °C from 0-20 min of reaction time of measuring as described in Section 6.2.5 (Figures 6.8 and 6.9). In all effects (e.g. temperature 15-50 °C and time 0-20 min) AChE and BChE activities increased with increasing temperature and reaction time of measuring and optimised in temperature 50 °C, 20 min than other temperature and time used (Figures 6.8 and 6.9). Lower reaction temperature resulted in decreased in enzymatic reaction. It was found that AChE activity from temperature 15-50 °C, 0-20 min was ranged between 0.765 and 166.9 nmol min⁻¹ ml⁻¹ for sheep and from 0.471 to 84.5 nmol min⁻¹ ml⁻¹ for cattle (Figures 6.8A and 6.9A), while BChE activity was ranged between 0.089 and 55.1 nmol min⁻¹ ml⁻¹ for sheep and from 0.166 to 36.5 nmol min⁻¹ ml⁻¹ for cattle (Figures 6.8B and 6.9B).

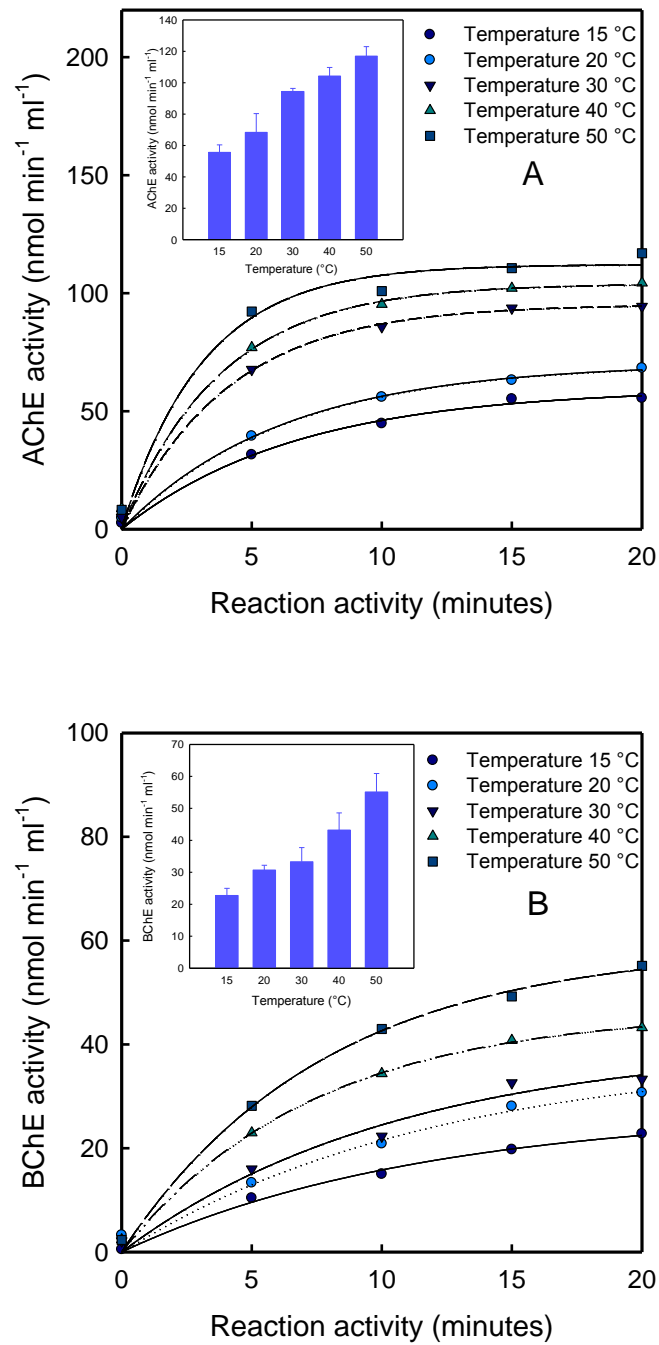


Figure 6.8 Effect of temperature and reaction activity on AChE and BChE activities in plasma of sheep. The maximum level of each temperature in each case at the same time was then plotted (insert). Data are expressed as the mean \pm SE of three separate determinations ($n = 3$).

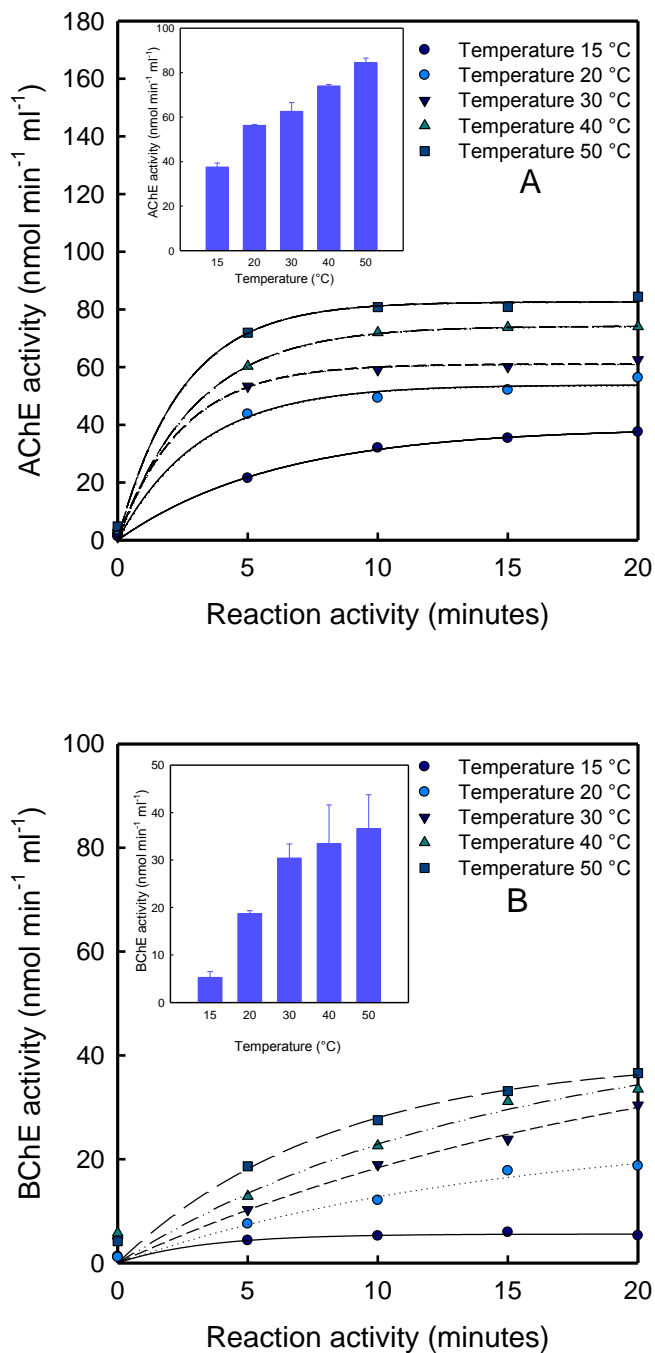


Figure 6.9 Effect of temperature and reaction activity on AChE and BChE activities in plasma of cattle. The maximum level of each temperature in each case at the same time was then plotted (insert). Data are expressed as the mean \pm SE of three separate determinations ($n = 3$).

6.3.5 Histochemical localization of acetylcholinesterase

The various methods available for the demonstration of AChE under the microscope have been outlined in Section 6.2.6 (Figures 6.10 and 6.11), indicates the discrepancies in the presence of histochemical localization by using modified Gomori (Figure 6.10) and Kugler method (Figure 6.11). It showed in both methods the most AChE located in the cytoplasm of the cell lining in the sinusoids, with a decreasing concentration gradient from the central vein to the periphery of the lobule for liver.

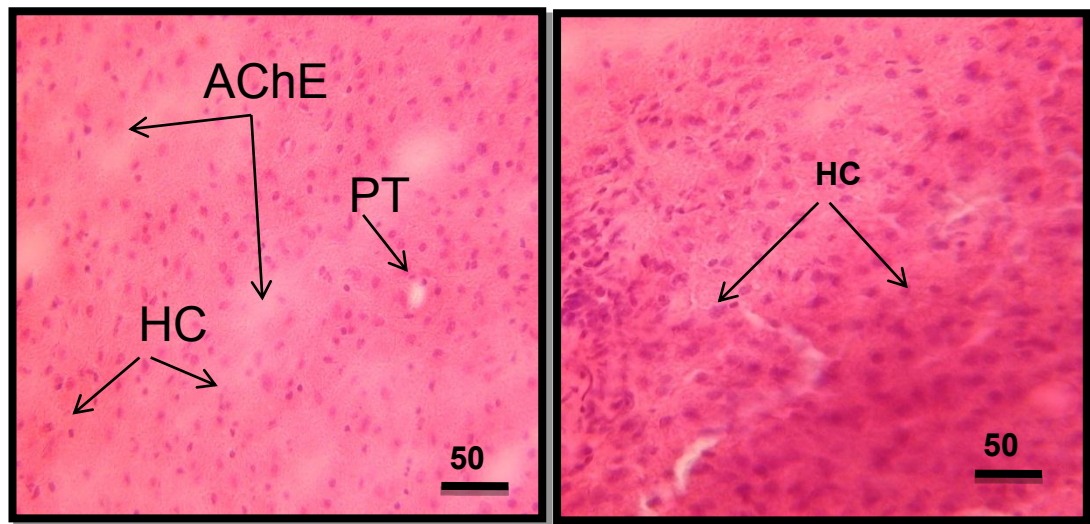


Figure 6.10 The figures above show histochemical Gomori method used to characterize the location of AChE in liver sections from sheep showing in the cytoplasm and in the sinusoids of the liver (left) and normal liver without substrate AcTChI (right) (scale bar = x 40). PT = portal tract and HC = hepatic cells.

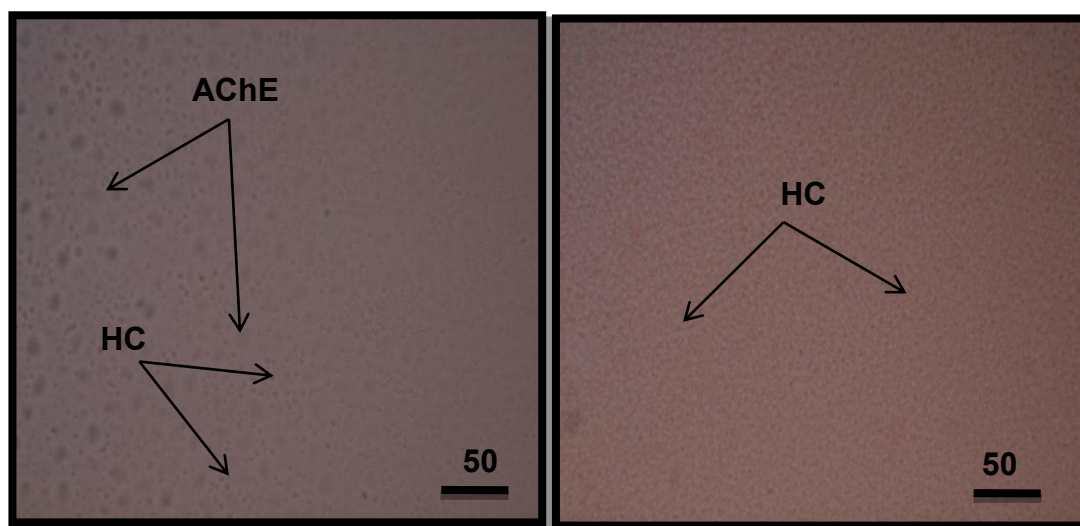


Figure 6.11 The figures above show histochemical Kugler method used to characterize the location of AChE in liver sections from sheep showing in the cytoplasm of liver (left) and normal liver without substrate AcTChI (right) (scale bar = x 40). HC = hepatic cells.

6.4 Discussion

6.4.1 Effect of dilution on tissue cholinesterase activities

Effects of dilution for AChE and BChE activities in liver, kidney and muscle for sheep, cattle and pig was studied to reduce the turbidity of the samples; to ensure that the optical signal falls within the linear range of detection throughout the measurement, and the enzyme dilution slows down the rate of spending of substrate, so providing an extended time window for observation of steady-state enzyme kinetics (Kao and Gratzl, 2009). The results from this study show that dilution 1:10 had high enzyme activity over different dilutions used and ranged between 116.4 and 383.9 $\text{nmol min}^{-1} \text{g}^{-1}$ for liver and between 34.4 and 69.9 $\text{nmol min}^{-1} \text{g}^{-1}$ for muscle, and between 44.7 and 359.7 $\text{nmol min}^{-1} \text{g}^{-1}$ for kidney (Figures 6.2 and 6.3). A similar observation has been made by Lassiter *et al.* (2003), reported that dilution 1:10 for liver and muscle had higher activity than dilution 1:50. The reason for this is probably due to adsorption changes occurring faster at dilution 1:10 (Soreq, 2001). Enzyme activity in the tissues

was at the higher dilution ratio declined faster than that of samples at lower dilution ratio.

6.4.2 Effect of selective inhibitors on tissue cholinesterase activities

Most test systems for AChE activity (EC 3.1.1.7) are using toxic inhibitors (BW284c51 and iso-OMPA) to distinguish the enzyme from BChE (EC 3.1.1.8), which occurs simultaneously in the tissues. They are supplied as early warnings and also offer essential support to chemical analysis when the habitat suffers for a complex mixture of pesticides (Massoulie *et al.*, 1993; Caselli *et al.*, 2006). The inhibitors BW284c51 was used to inhibit AChE, thus BChE activity alone was measured in the presence of these inhibitors while iso-OMPA inhibit BChE, therefore AChE activity only determined in the presence of this inhibitor. Although ChE cannot classified neither as AChE nor like BChE since they explain the characteristics of each form. In fact, AChE prefers AcTChI as substrate (at low concentrations) but at high substrate concentrations, it hydrolyses PrTChI at a higher rate. Similarly to BChE, prefer BuTChI as substrate (at low concentrations) (Tecles and Ceron, 2001; Wilson and Philip, 2005).

BW284c51, are able to infiltrate the deep and narrow catalytic gorge of enzyme and in doing so can attach to both the active and peripheral sites, therefore resulting inhibition of AChE activity (Talesa *et al.*, 2001). A differential sensitivity to BW284c51 had strong inhibition of AChE activity, up to 81% inhibition in the liver and muscles (Figures 6.4 and 6.5). It was found that the AChE activity is not fully inhibited by BW284c51 up to 80 μM . These results suggest that both liver and muscle may be present ChE as Mohammad (2007) reported that high level of ChE in the liver and muscles for sheep and cattle. These results are

also in agreement with an earlier study carried out on the goat and camel (Al-Qarawi and Ali, 2003).

Tissue from the muscle ChE enzyme behaviour towards iso-OMPA did not clearly inhibit BChE activity in a concentration-related manner and the remaining activity, despite for several concentration incubations, was still between 11 and 30% of inhibition. Because the contribution of BChE activity to the total enzyme activity is varied between animals studied. When the percentage of activity on BuTChI sensitive to 16 mM iso-OMPA (Figure 6.5) is taken as an estimate of the quantity of BChE relative to total enzyme in muscle, the following order of animals is obtained sheep (30%) > pig (22%) > cattle (18%). Although, it has a strong inhibitory (up to 64% inhibition) effect on liver compared controls. This is in agreement an earlier report by Rodriguez-Fuentes and Gold-Bouchot (2004) who shown that iso-OMPA in the vertebrate produced potentiation inhibition due to binding iso-OMPA of the active sites BChE. But in contrast with Varo *et al.* (2002) found that no inhibition was observed in invertebrates with iso-OMPA or with excess substrate, which is characteristic properties of BChE.

This interpretation appears to be only explanation consistent with the well-established views that there are only two types of ChE in the liver and muscles of food animals, AChE and BChE activities (Massoulie *et al.*, 1993; Wilson, 2010). A good correlation was obtained in liver and muscle assessed with two inhibitors (Table 6.2). An important result of this work is the finding that AChE and BChE activities were predominantly distributed in the liver, while in the muscle is only AChE activity. Hence based on the present investigation it can

be differentiated liver and meat between sheep, cattle and pigs depending on the level of ChE enzyme.

6.4.3 pH optimum of tissue cholinesterase activities

The effect of pH on the optimum activity of acetylcholinesterase (AChE, EC 3.1.1.7) or butyrylcholinesterase (BChE, EC 3.1.1.8) from liver and muscle of sheep, cattle and pigs have been investigated. In the last decade, the use of AChE and BChE enzymes as biomarkers for ecological monitoring has largely been supported because of their exclusive contribution to determine the toxicity of a mixture of the pesticides, although each contaminant may be found in the habitat below the law threshold. They are supplied as early warnings and also offer essential support to chemical analysis when the habitat suffers for a complex mixture of pesticides (Massoulie *et al.*, 1993; Caselli *et al.*, 2006). The main purpose of this study was to investigate the optimum pH for AChE and BChE enzymes from liver and muscle of sheep, cattle and pigs. The pH change is useful in accepting the relationship between the structure and functional group of the enzyme. Therefore, AChE and BChE compared as a function of pH (Akkaya *et al.*, 2009). A bell shaped curve when enzyme activity in the pH range of 6.9 at 25 °C, presented towards substrates is plotted against pH has been taken (Figure 6.5A-F). Optimal pH values found to be pH 7.9-8.1 and pH 7.7-8.5 for AChE and BChE respectively, slightly more acidic than the optimal pH value for AChE (about 8.5) (Zou *et al.*, 2006).

On the other hand, as Bergmann *et al.* (1958) found the optimum pH for AChE from human plasma to be 7.4-8.0 while the pH for BChE from human serum 7.5-8.1. This is in excellent agreement with the literature data on optimal pH in the liver obtained for AChE (from pH 7.9-8.0) found in the literature (Detra and

Collins, 1986; Tecles *et al.*, 2002b). In addition, to concurring with the works of Cohen and Oosterbaan (1963) found that the optimum pH varies with the source of ChE but for most preparations, the range is 8.0-8.5. But in contrast with the works of Kok *et al.* (2001), found higher pH optimum 9.0 for AChE. Bui and Ochillo (1987) observed that the lower pH optimum 7.4 from muscle for invertebrates. These authors used a higher temperature 38 °C rather than 25 °C. This factor may explain the apparent difference in optimum pH. Good correlation and significant obtained between AChE and BChE to different pHs ($r > 0.82$, $P < 0.05$). While poor correlation obtained between liver and muscle ChE activities within different pHs (6/9) ($r = 0.64$; $P < 0.0001$) (Figure 6.7) (Wackerly *et al.*, 1996).

Moreover, there is a great variety of pHs that can be found among different laboratories, for example, 7 (Li *et al.*, 2008; Mirajkar and Pope, 2008), 7.4 (Imai *et al.*, 2006; Dorandeu *et al.*, 2008), 7.5 (Martinez-Moreno *et al.*, 2006; Zhu *et al.*, 2007), 7.6 (Bajgar *et al.*, 2008), 8.0 (Padilla *et al.*, 2007; Yucel *et al.*, 2008; Bosgra *et al.*, 2009), 8.1 (Mohammad, 2007), 8.2 (Haigh *et al.*, 2008) and 9 (Frasco *et al.*, 2005).

6.4.4 Effect of temperature on cholinesterase activities

The effect of temperature on the optimum activity on AChE or BChE from plasma of sheep and cattle has been also investigated. The catalytic activity of AChE and BChE enzymes are likes most enzymes are temperature dependent. Optimal rates of temperature for mammalian ChE activity generally occur a temperature between 37 °C and 40 °C (Fairbrother *et al.*, 1991). Sahin *et al.* recommended that the optimal temperature being 30 °C (Sahin *et al.*, 2005). But in contrast with Bui and Ochillo (1987) who observed that the optimum

temperature to be 38 °C. In general, the typical temperature range used in the determination of AChE and BChE activities is between 20 °C and 40 °C (Worek *et al.*, 2004; Borlongan *et al.*, 2005; Aurbek *et al.*, 2006; Martinez-Moreno *et al.*, 2006; Al-Badrany and Mohammad, 2007; Oliveira *et al.*, 2007; Padilla *et al.*, 2007; Mirajkar and Pope, 2008). The temperature difference between the AChE and BChE could be attributed to conformational limitation on the enzyme movement as a consequence of ionic interactions between the ChE and the supports or restriction in the diffusion of the substrate at high temperature (Sahin *et al.*, 2005). Authors also observed that AChE do not usually chemically bond with polymeric matrices. Regarding AChE and BChE activities were decreased at lower temperatures and increases gradually with increasing temperature and incubation time. These results in agreement with the literature data on temperature values of canine whole blood ChE activities obtained for AChE and BChE in a range of temperatures 25-40 °C for (Tecles *et al.*, 2002b). In addition, in agreement with the work of Reiner *et al.* (1974), observed that of human plasma AChE and BChE increased with increasing temperature in a range 10-40 °C. But in contrast with the work of Bui and Ochillo (1987) who observed that the temperature effect on ChE activities gradual increases from temperature 20 °C and much decreases of enzyme activity after 37 °C. However, these authors used a muscularis muscle from *Bufo marinus* as sample instead of plasma sample. This factor may explain the apparent difference in enzyme activity. To our knowledge the when temperature increases beyond 55 °C, loss of activity of the protein is likely and above 60-70 °C, total loss of activity due to protein denaturation is common (Alles and Hawes, 1940; Fairbrother *et al.*, 1991).

6.4.5 Histochemical localization of acetylcholinesterase

The histochemical studies of AChE demonstrate that the modified Gomori and Kugler histochemical methods described in Section 6.2.6, can effectively indicated the distribution of AChE in the liver for sheep. The controls included in the procedure provides a check against errors in the identification of AChE types, at least as important a consideration in any histochemical method for AChE as the elimination of diffusion artefacts (Figures 6.10 and 6.11). However, neither method has been validated for use either in liver from other food animals or in other tissues. The present study is the first attempt to standardize and validate Gomori and Kugler histochemical methods for AChE activity localisation in the liver of sheep.

Zajicek *et al.* (1954) and Malmgren and Sylven (1955) they observed a needle-shaped crystals of copper thiocholine were deposited at the site of AChE activity, which gradually increased in size during the reaction and eventually projected outside the site of AChE activity, especially after prolonged incubation; therefore incubation time has been kept as short as possible. In addition, they suggested, that the conversion of copper thiocholine to copper sulphate increased the risk of artefacts in Gomori method (Figure 6.10). In both histochemical methods (Gomori and Kugler methods), I show AChE localised in the cytoplasm (Figures 6.10 and 6.11).

Since AcTChI is a common substrate for AChE, histochemical reactions observed using this substrate and hence it can localise only AChE. In this tissue (liver), staining in the sinusoids of the liver of the sections, incubated in the medium containing AcTChI, indicated that the liver did contain AChE. Hence, the enzyme activity, which was visualised in the liver using AcTChI.

Accordingly, the present research has shown that the cells of liver possess AChE enzymes. The present finding of AChE activity in the cells of the liver could indicate that AChE enzyme is primarily involved in the function of the liver. This observation is in line with the findings of similar studies, which have reported the localisation of AChE from the liver of cat (Koelle, 1951).

6.5 Conclusions

This study provided the original data concerning an AChE and BChE characterization in the tissues of sheep, cattle and pigs. Some of the principal results of this Chapter could be concluded as follows.

1. Substrate AcTChI indicate that, preferential for detecting enzymes selective inhibition of muscle and liver. Hence, in all three animal species (sheep, cattle and pigs) the liver and muscle tissues were more sensitive to inhibition with BW284c51 than iso-OMPA when used three substrates (AcTChI, BuTChI and PrTChI), with exception for BuTChI in sheep and pig iso-OMPA were more sensitive to inhibition than BW284c51 in the liver. In addition, in muscle BuTChI for sheep and PrTChI for cattle were stronger inhibition in iso-OMPA than in BW284c51.
2. In all animals (sheep, cattle and pigs) and tissues (liver and muscle), the optimum pH values for liver in BChE were higher than does AChE, while in muscle optimum pH values in AChE higher than does BChE.
3. The significant effect of time course and temperatures on AChE and BChE activities was increased with increase of temperature in plasma for sheep and cattle.
4. Histochemical localisation of AChE in tissue from liver had provided strong evidence to suggest that AChE is involved in the cells of liver. In addition, the histochemical procedure for showing the presence of AChE is used widely in

diagnosing neurodegenerative disease, and in most laboratories dealing with ChE tissues are therefore already familiar with its application (Silver, 1974).

Chapter 7

Investigation of kinetic properties of cholinesterase

The results from this Chapter have been presented as poster presentations at the following conferences in the UK: 23rd Annual Symposium of the European Society of Veterinary Neurology-European College of Veterinary Neurology, September 2010, University of Cambridge; Annual Meeting of the Society of Environmental Toxicology and Chemistry, September 2010, London; 32nd Annual Congress of the British Toxicology Society, March 2011, University of Durham; and Annual Conference of British Society of Animal Science, April 2011, University of Nottingham.

These results have also been published in:

Kasim Abass Askar, A. John Moody and A. Caleb Kudi (2011). A kinetic characterization of acetylcholinesterase and butyrylcholinesterase in the tissues of food animals. *Advances in Animal Biosciences* 2:185-185.

Kasim Abass Askar, A. Caleb Kudi and A. John Moody (2011). Effect of temperature on rate constants of inhibition of organophosphate and carbamate compounds. *Toxicology* 290(2-3):103-104.

Kasim Abass Askar and A. Caleb Kudi (2012). *In vitro* kinetic characterization of inhibition of acetylcholinesterase by organophosphate and carbamate. *Toxicological and Environmental Chemistry* 94(3):596-604.

Kasim Abass Askar, A. John Moody and A. Caleb Kudi (2012). Effects of different pH on cholinesterase activity and bimolecular rate constant of dichlorvos in the tissues of food animals. *Journal of Veterinary Internal Medicine* 26(1):221-221.

Chapter 7: Investigation of kinetic properties of cholinesterase

7.1 Introduction

Cholinesterases (ChE) are specialized carboxylic ester hydrolases that break down esters of choline. In general, two types of ChE activity have been identified in mammalian tissues; these are distinguished according to their substrate specificity and sensitivity to the selective inhibitors. The first is AChE, which is systematically called acetylcholine acetylhydrolase. The second is BChE, referred to systematically as acylcholine acylhydrolase (Musilek *et al.*, 2009; Gholivand *et al.*, 2010). The preferred substrate for AChE enzymes is ACh; BChE enzymes prefer butyrylcholine or propionylcholine, depending on the species (Wilson, 2010).

Numerous chemical compounds, routinely used in agriculture and chemical industry, can form persistent toxic residues in air, soil, water and foods. Among pesticides, organophosphate (OP) and carbamate pesticides that are mainly used in agriculture show low environmental persistence but display high acute toxicity. The mechanism of toxicity of OP and carbamate compounds is acknowledged as anti-ChE agents, represent the main classes concerned in cases from mild to severe poisoning. The anti-ChE have in common the same mechanism of action but they arise from two different chemical classes. The derivatives of phosphoric, phosphorothioic, phosphorodithioic and phosphonic acids (OP) and those of carbamic acid (carbamate) (Ferreira *et al.*, 2008; Wilson, 2010). Due to ChE sensitivity to these pesticides (several are considered to be effective inhibitors of ChE), its inhibition has been used as a biomarker of exposure and effects of these pesticides in selected species (Frasco *et al.*, 2006). However, it is estimated that of one to five million cases of

pesticide intoxication occur every year, resulting in several thousands of fatalities chiefly among agricultural workers. Most of these poisonings happen in expanding countries where the lack of hygiene, information or sufficient control has created unsafe working conditions (Aldridge and Reiner, 1973).

The intoxication with OP and carbamate compounds cause a generalized cholinergic crisis due to an irreversible and reversible inhibition of ChE by phosphorylation or carbamylation of their active site serine, respectively (Worek *et al.*, 2004; Musilek *et al.*, 2009) and successive accumulation of the neurotransmitter ACh. OP compounds are a large class of chemicals. Since World War II, an estimated several thousand OP has been synthesized for various purposes. Furthermore, ChE enzyme group that is inhibited by OP pesticides based on time and pH-dependent reaction (Aldridge and Reiner, 1973).

However, the fundamental investigation of kinetic properties of ChE for food animals is poorly understood. The aim of the present work were (a) to investigate the kinetics of characterization of ChE activities in the tissues from food animals used for human consumption; (b) to investigate the kinetics of inhibition of AChE activity by these OP and carbamate compounds in the tissues from animals used for human consumption; (c) to establish a foundation for the more sensitive pH effect to the rate of constants (k_i) of DDVP-inhibited AChE and BChE from tissues from food animal species as biochemical biomarkers for evaluating of exposure to OP pesticides; and (d) to study the more sensitive temperature effect to the rate of k_i of OP as well as carbamate-inhibited AChE and BChE in food animals.

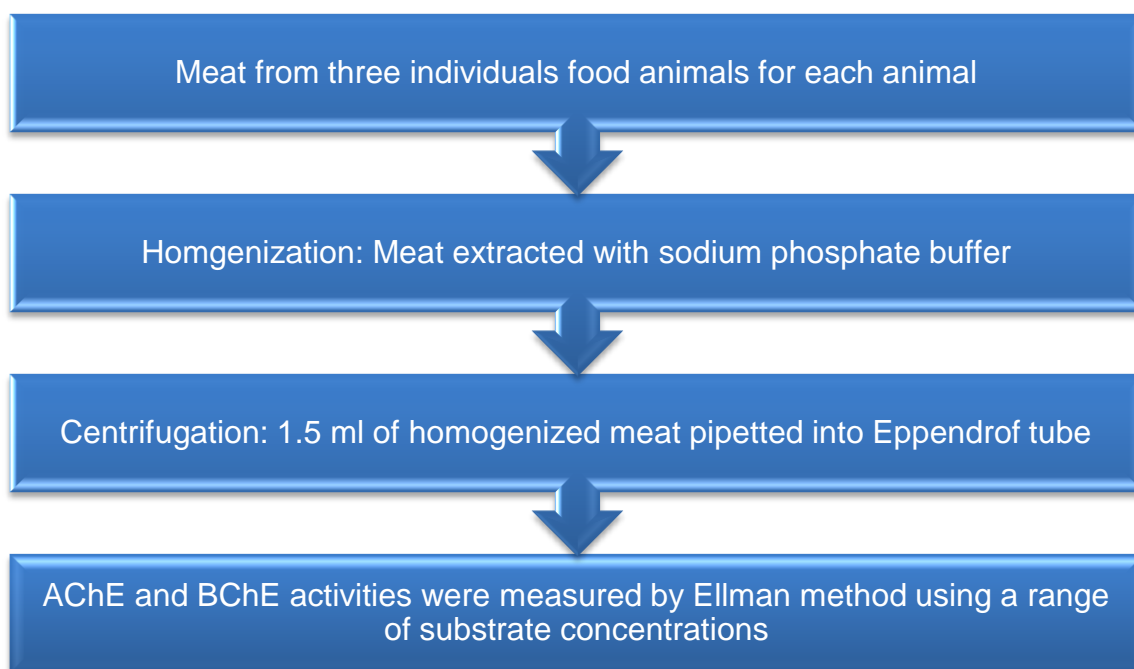
7.2 Materials and methods

7.2.1 Sample collection and preparation

The sample collection and preparation were done as explained in Sections 2.2 and 2.3. Following this it was then measured the enzyme activity as described in Section 2.4.1. All the chemicals used were of analytical grade.

7.2.2 Determination of maximum reaction velocity and Michaelis-Menten constant

Experimental design for detection ChE kinetics in the tissues of food animals by using Ellman can be drawn in below;



For the measurement of maximum reaction velocity (V_{max}), the substrate AcTChI as prepared at concentrations ranging 0.05, 0.1, 0.2, 0.5, 1, 2 and 3 mM in distilled water, while BuTChI concentrations ranging 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 mM in distilled water for measuring AChE and BChE, respectively. Blanks were also run at each selected substrate for each based on absorbance tested. The changes of absorbance were measured at 410 nm for 5 min, 25 °C.

The enzyme becomes saturated with substrate and the rate reaches V_{\max} , the enzyme maximum rate (e.g. Figure 7.1). The Michaelis constant (K_m) is experimentally defined as the concentration at which the rate of the enzyme reaction is half V_{\max} .

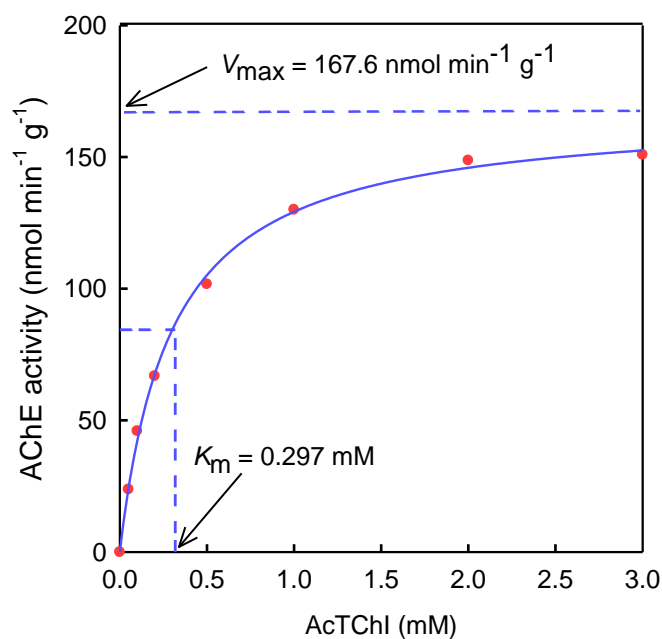
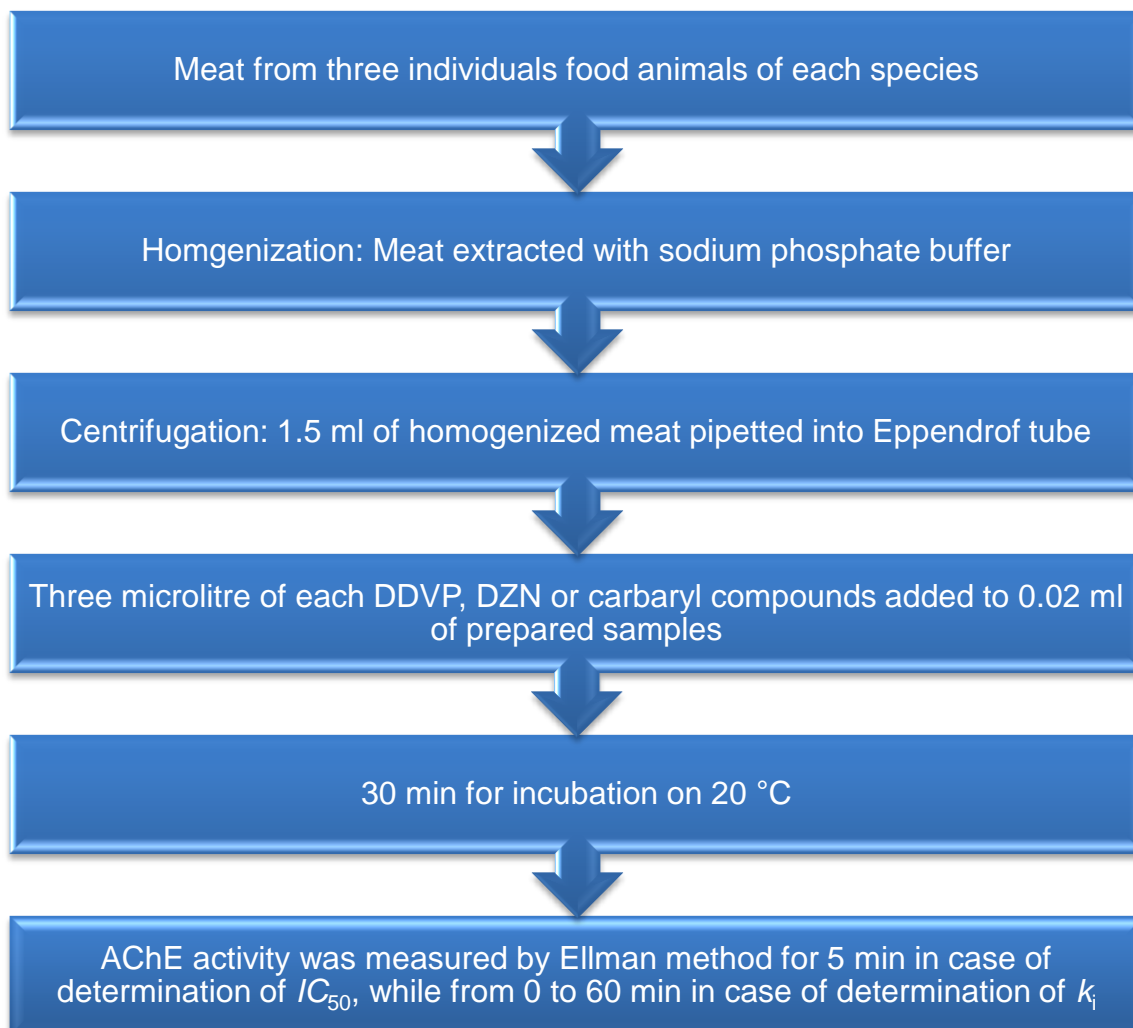


Figure 7.1 The illustrate figure is the data analysis used to obtain maximum reaction velocity (V_{\max}). The data are for sheep liver AChE. In each case data were fitted with non-linear regression analysis using a single rectangular hyperbola by SigmaPlot 11.

7.2.3 Determination half maximal inhibitory concentration and rate constants for inhibition

Experimental design for detection kinetics effect of OP and carbamate compounds for food animals can be drawn below;



For the measurement of half maximal inhibitory concentrations (IC_{50}), AChE was inhibited for 30 min at room temperature 20 °C with either 1-8 μM OP compounds or 5-40 μM carbamate compounds, depending on preliminary range finding tests. Controls were incubated with sodium phosphate buffer pH 8.0 were included when appropriated. Blanks were also run at each selected inhibitor for each based on absorbance tested. Then the enzyme activity was determined as described in Section 2.4.1. The decrease in AChE activity with increasing concentrations of compounds was then plotted (e.g. Figure 7.2A).

Stock solutions of inhibitors were prepared weekly, stored at 4 °C and appropriately diluted in distilled water just before the experiments. The effect of inhibitors of AChE in different concentrations was assayed as explained in enzyme activity measurement.

For the measurement of rate constants of inhibition (k_i), AChE was inhibited as above with 8 μ M DDVP or DZN, or 40 μ M carbaryl resulting in an inhibition of 83-99% of control activity. Controls were also incubated with sodium phosphate buffer pH 8.0 were included when appropriated. Blanks were also run at each selected inhibitor for each based on absorbance tested. Then the enzyme activity was determined as described in Section 2.4.1. The decrease in AChE activity over different times (0-60 min) at room temperature 20 °C was then plotted (e.g. Figure 7.2B). Half time to inhibition, ($t_{1/2}$, expressed as min), was calculated using the equation, $t_{1/2} = \ln 2/k_i$. The remaining AChE activity after OP and carbamate compounds inhibition represents residual AChE activity. However, the percentage inhibition was calculated from the ratio between the activity of an exposed sample and unexposed controls by the formula:

$$\% \text{ Inhibition} = \frac{\text{AChE without inhibitor} - \text{AChE with inhibitor}}{\text{AChE without inhibitor}} \times 100$$

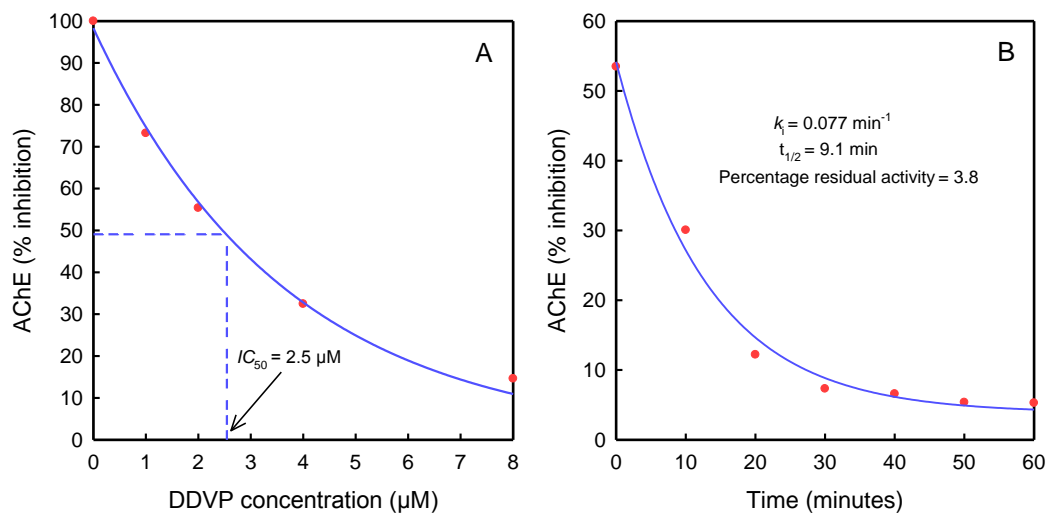
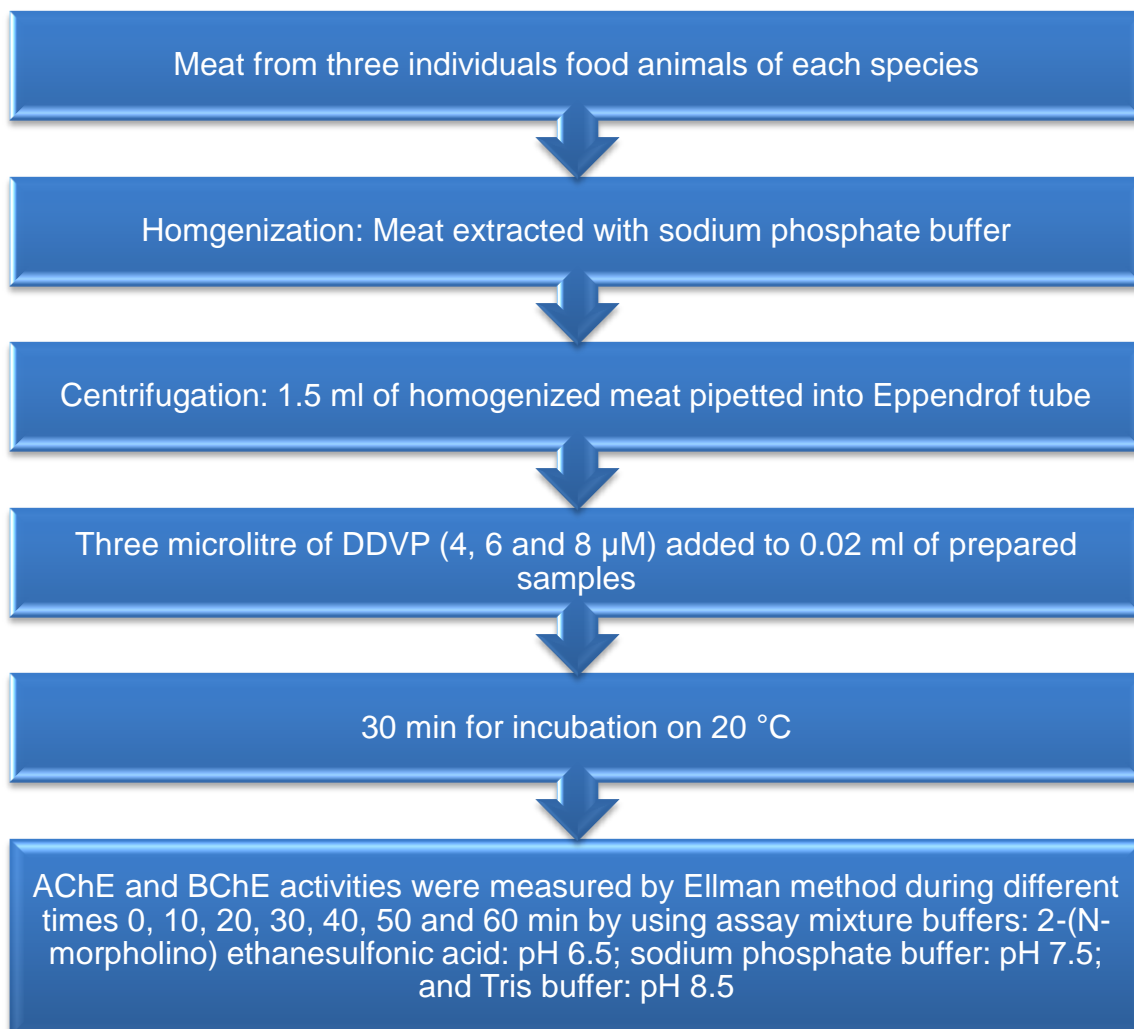


Figure 7.2 The figures above illustrate the data analysis used to obtain half maximal inhibitory concentrations (IC_{50} ; A) and rate constants of inhibition (k_i ; B). The data are for pig liver AChE inhibited with DDVP. In each case, data were fitted with non-linear regression analysis using a single exponential decay by SigmaPlot 11.

7.2.4 Effect of pHs on rate constants of inhibition

Experimental design for detection of rate constants of inhibition in different pHs for food animals can be drawn below;



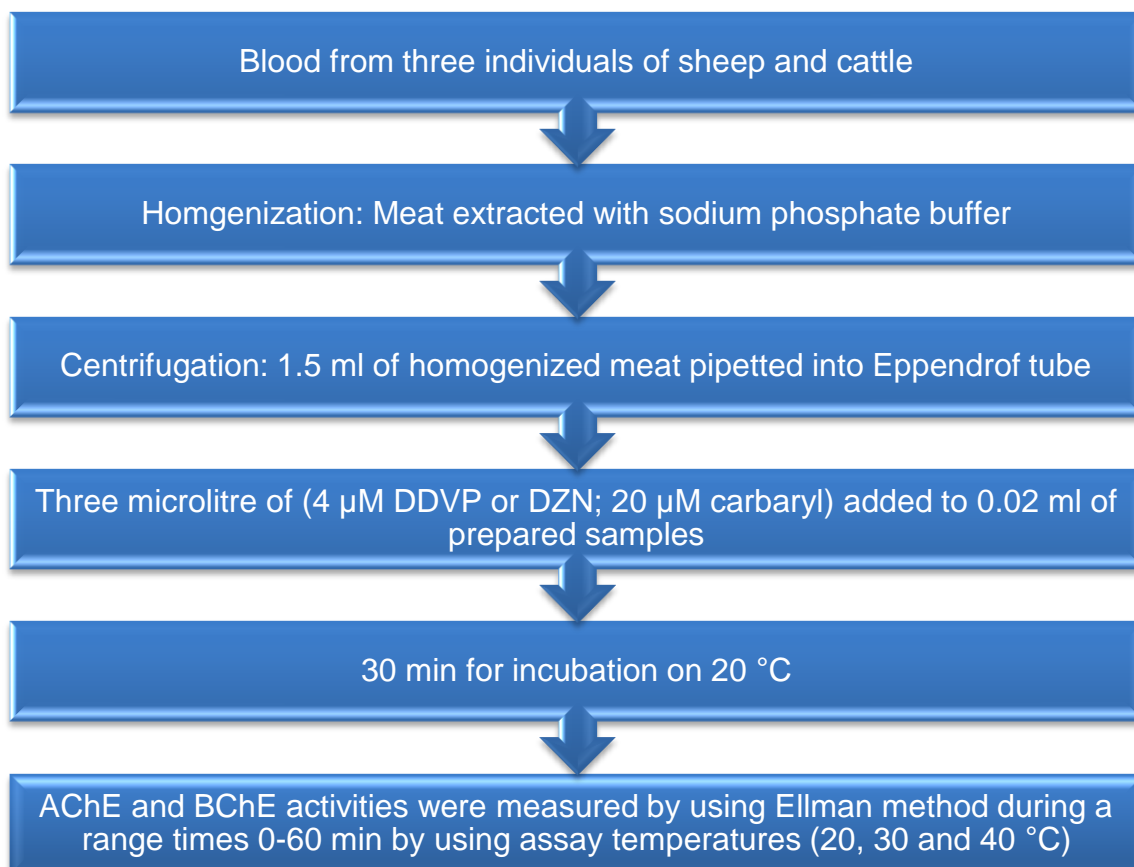
For the measurement of rate constants of inhibition (k_i) in different pHs (6.5, 7.5 and 8.5), ChE was inhibited as above with 4, 6 and 8 μM DDVP concentrations and enzyme was measured in the assay mixture as above pHs, respectively. In preparing pHs solutions during measuring, it is essential that the substances have been stored properly and have not exceeded their shelf life, and materials used in pHs solution should not to be used to experiment extending over one month, and kept on ice during use. Hence, three different pHs were 0.1 M

concentrations buffers of 2-(N-morpholino) ethanesulfonic acid: pH 6.5; sodium phosphate buffer: pH 7.5; Tris buffer: pH 8.5. Controls were incubated with distilled water were included when appropriated. Blanks were also run at each selected pH for each based on absorbance tested.

Then the enzyme activity was determined as described in Section 2.4.1. The decrease in enzyme activity over different times (0-60 min) at room temperature 20 °C, was then plotted. Then the data of inhibition time courses at different times after inhibition was fitted with a single exponential decay using SigmaPlot 11.

7.2.5 Effect of temperature on rate constants of inhibition

Experimental design for detection of rate constants of inhibition in different temperatures for sheep and cattle can be drawn below;



For the measurement of rate constants of inhibition (k_i) in different temperatures (20 °C, 30 °C and 40 °C). The AChE and BChE activities were inhibited with (4 μ M DDVP or DZN; 20 μ M carbaryl). Controls were incubated with sodium phosphate buffer pH 8.0 were included when appropriated. Blanks were also run at each selected inhibitor for each based on absorbance tested. Then the enzyme activity was determined as described in Section 2.4.1. The decrease in enzyme activity over different times (0-60 min) at different temperatures (20 °C, 30 °C and 40 °C) was then plotted as above (Section 7.2.3). Then the data of inhibition time courses at different times after inhibition was fitted with a single exponential decay using SigmaPlot 11.

7.2.6 Statistical analysis

All enzyme measurements were conducted in three individuals from each animal with results expressed as mean values \pm SE. The one-way analysis of variance (*ANOVA*) was applied to test for any significant differences ($P < 0.05$). All analyses were done using Minitab statistical software version 15.

7.3 Results

7.3.1 Determination of maximum reaction velocity and Michaelis-Menten constant

Maximum reaction velocity (V_{max}) and Michaelis-Menten constant (K_m) were determined in liver, muscle and kidney for sheep, cattle and pig using the modified Ellman method as described in Section 7.2.2 (Table 7.1). There was significantly higher V_{max} in liver and kidney was used as the BuTChI substrate compared to when AcTChI substrate in all cases with the exception of sheep where kidney AcTChI substrate activity was higher than that seen in BuTChI substrate (Table 7.1). The V_{max} AChE in animals was decreased according to

the rank order of pig > cattle > sheep for liver, muscle and kidney (Table 7.1). Nevertheless, V_{\max} for BChE was decreased according to the rank order of pig > cattle > sheep for liver and pig > sheep > cattle for muscle, while decreasing pig > cattle > sheep for kidney (Table 7.1). In all cases (sheep, cattle and pig, using both substrate concentrations) AcTChI activity was higher in muscle than BuTChI activity (Table 7.1). Michaelis-Menten constant (K_m) values present the substrate concentrations (mM) necessary to obtain half of V_{\max} of the final reaction velocity ($\text{nmol min}^{-1} \text{g}^{-1}$ tissue). K_m value for sheep, cattle and pig using AcTChI substrate were higher than BuTChI substrate, with the exception of cattle liver and pig kidney where BuTChI activity was higher than that seen in AcTChI, and much higher (Table 7.1). The K_m AChE in animals was decreased as follows: sheep > pig > cattle for liver and cattle > sheep > pig for muscle and kidney (Table 7.1). However, the K_m for BChE was decreased as follows: pig > sheep > cattle for liver and sheep > cattle > pig for muscle, while decreasing according to the rank order of sheep > pig > cattle for kidney (Table 7.1). In this sense, it can be expected that ChE with lower substrate affinity should have lower sensitivity to anticholinergic agents.

Table 7.1 Substrate affinity constant (K_m , expressed in mM) and maximum velocity (V_{max} , expressed in $\text{nmol min}^{-1} \text{g}^{-1}$).

Animal	Substrate	Kinetics	Liver	Muscle	Kidney
Sheep	AcTChI	V_{max}	167.6 ± 2.43	49.2 ± 2.03	48.1 ± 1.91
		K_m	0.297 ± 0.016	0.317 ± 0.047	0.309 ± 0.044
	BuTChI	V_{max}	234.8 ± 3.49	43.1 ± 2.54	46.6 ± 1.34
		K_m	0.178 ± 0.015	0.254 ± 0.077	0.271 ± 0.041
Cattle	AcTChI	V_{max}	218.1 ± 11.17	50.5 ± 2.02	49.2 ± 2.59
		K_m	0.166 ± 0.035	0.348 ± 0.049	0.334 ± 0.062
	BuTChI	V_{max}	352.6 ± 21.3	33.6 ± 2.57	132.1 ± 9.44
		K_m	0.176 ± 0.058	0.188 ± 0.078	0.144 ± 0.047
Pig	AcTChI	V_{max}	270.7 ± 14.4	79.3 ± 2.14	360.2 ± 0.179
		K_m	0.261 ± 0.052	0.159 ± 0.018	0.018 ± 8.92
	BuTChI	V_{max}	386.5 ± 14.48	36.6 ± 2.087	261.4 ± 18.45
		K_m	0.218 ± 0.043	0.069 ± 0.024	0.514 ± 0.157

Values in the table are the mean \pm SE obtained from nonlinear regression analysis, each performed in triplicate ($n = 3$ in each animal).

7.3.2 *In vitro* kinetic characterization of acetylcholinesterase inhibited by organophosphate and carbamate compounds

The liver kinetic parameters for reaction among AChE and two OP (DDVP and DZN) and one carbamate (carbaryl) inhibitors were determined for sheep, cattle and pigs as described in Section 7.2.3 (Tables 7.2 and 7.3). The IC_{50} values were significantly different ($P < 0.05$) between all animals within each inhibitor. The order of potency of inhibition decreased according to the rank order of DDVP > DZN > carbaryl, the carbaryl ranged between (4.8-8.4 μM) higher

about 3 to 4-times than the DDVP and DZN (Table 7.2). Significant differences ($P < 0.05$) were seen in $t_{1/2}$ between sheep and cattle and between sheep and pig with inhibitors. It was higher in pig than cattle (1.8 to 3.3-times) and then sheep (1.2 to 1.3-times). Always, with OP and carbamate compounds, the $t_{1/2}$ was higher in pigs than in sheep and in cattle. A rate constants inhibition (k_i) value was $P < 0.05$ between sheep and cattle and between sheep and pig within inhibitors. It ranged between 43.1×10^{-3} - 140.9×10^{-3} , 44.5×10^{-3} - 159×10^{-3} and 51.4×10^{-3} - $167.5 \times 10^{-3} \text{ min}^{-1}$ for DDVP, DZN and carbaryl, respectively. Percentage residual AChE activity in extracts from liver had $P < 0.05$ between sheep and cattle and between sheep and pig for DDVP and carbaryl, while $P < 0.05$ between animals for DZN, and ranged from 1.6-3.5%, 1.4-6.1% and 1.8-4.8% for DDVP, DZN and carbaryl, respectively, corresponding to 93.9-98.6% of inhibition (Table 7.2).

Table 7.2 *In vitro* kinetic characterization for AChE from liver of sheep, cattle and pig inhibited by DDVP, DZN and carbaryl.

Inhibitor	Animal	IC_{50} (μM)	$t_{1/2}$ (min)	$k_i \times 10^{-3}$ (min^{-1})	%Residual AChE activity
DDVP	Sheep	1.0 ± 0.217	4.9 ± 0.301	140.9 ± 9.14	1.6 ± 0.292
	Cattle	1.6 ± 0.017^a	13.5 ± 0.159^a	51.3 ± 0.599^a	3.5 ± 0.25^a
	Pig	2.5 ± 0.402^{bc}	16.2 ± 0.955^{bc}	43.1 ± 2.67^b	3.5 ± 0.867^b
DZN	Sheep	1.4 ± 0.245	4.6 ± 0.295	152 ± 9.17	1.4 ± 0.057
	Cattle	2.8 ± 0.049^a	10.8 ± 0.079^a	63.9 ± 0.467^a	4.6 ± 0.077^a
	Pig	2.6 ± 0.202^{bc}	14.5 ± 2.078^{bc}	44.5 ± 10.7^b	6.1 ± 0.394^{bc}
Carbaryl	Sheep	4.8 ± 0.31	4.2 ± 0.25	167.5 ± 9.5	1.8 ± 0.445
	Cattle	7.0 ± 0.217^a	9.9 ± 0.014^a	68.2 ± 1.44^a	4.8 ± 0.799^a
	Pig	8.4 ± 0.304^{bc}	13.5 ± 0.323^{bc}	51.4 ± 1.21^{bc}	4.7 ± 0.337^b

Values in the table are the mean \pm SE obtained from nonlinear regression analysis, , each experiment performed in triplicate (n = 3 in each animal).

^aSignificant differences [analysis of variance (ANOVA), $P < 0.05$] between cattle and sheep within same inhibitor are in the same column.

^bSignificant differences [analysis of variance (ANOVA), $P < 0.05$] between pig and sheep within same inhibitor are in the same column.

^cSignificant differences [analysis of variance (ANOVA), $P < 0.05$] between pig and cattle within same inhibitor are in the same column.

The muscle kinetic parameters for reaction among AChE and two OP (DDVP and DZN) and one carbamate (carbaryl) inhibitors were also determined for sheep, cattle and pigs as described in Section 7.2.3 (Table 7.3). IC_{50} values was $P < 0.05$ between DDVP and carbaryl for all animals by inhibitors, while in DZN $P < 0.05$ was seen between sheep and cattle and between sheep and pig. Carbaryl ranged between 7.1-9.9 μM higher about 4-6 fold than DDVP and

DZN. The order of potency of inhibition decreased according to the rank order of DDVP > DZN > carbaryl; in addition, a significant ($P < 0.05$) $t_{1/2}$ was seen in between sheep and cattle and between sheep and pig by inhibitors, and cattle was higher than sheep (2.5 to 2.6-times) and pig (1.2 to 2.4-times) for DDVP and carbaryl; while pig DZN was higher than sheep (2.4-times) and cattle (1.3-times). In all cases, the $t_{1/2}$ was higher in cattle than sheep and pig, with exception with DZN where $t_{1/2}$ was higher than sheep and cattle. Rate constant of inhibition (k_i) values are ($P < 0.05$) between sheep and cattle and between sheep and pig by inhibitors, and were in range 45.5×10^{-3} - $115.3 \times 10^{-3} \text{ min}^{-1}$. Percentage residual AChE activity in extracts from muscle was $P < 0.05$ between sheep and cattle and between pig and cattle for DDVP, while $P < 0.05$ between all tested animals for DZN and carbaryl, and ranged from 6.6-17, 4.2-9.6 and 5.6-12.2 for DDVP, DZN and carbaryl, respectively, about 83-95.8% of inhibition (Table 7.3).

Table 7.3 *In vitro* kinetic characterization for AChE from muscle of sheep, cattle and pig inhibited by DDVP, DZN and carbaryl.

Inhibitor	Animal	IC_{50} (μ M)	$t_{1/2}$ (min)	$k_i \times 10^{-3}$ (min^{-1})	%Residual AChE activity
DDVP	Sheep	1.1 ± 0.038	6.0 ± 0.169	115.3 ± 3.34	7.1 ± 0.371
	Cattle	2.0 ± 0.064^a	15.6 ± 7.39^a	46.1 ± 11.1^a	17.0 ± 0.011^a
	Pig	2.6 ± 0.031^{bc}	13.3 ± 4.11^b	60.9 ± 8.34^b	6.6 ± 0.203^c
DZN	Sheep	1.4 ± 0.136	6.6 ± 0.01	105.7 ± 1.64	4.2 ± 0.103
	Cattle	2.2 ± 0.229^a	12.6 ± 0.814^a	55.2 ± 3.34^a	9.6 ± 0.253^a
	Pig	2.3 ± 0.177^b	15.8 ± 2.21^b	45.5 ± 5.85^b	7.2 ± 0.136^{bc}
Carbaryl	Sheep	7.1 ± 0.209	6.3 ± 0.296	110.7 ± 5.09	5.6 ± 0.096
	Cattle	8.1 ± 0.153^a	15.3 ± 1.589^a	46.4 ± 5.37^a	12.2 ± 0.499^a
	Pig	9.9 ± 0.336^{bc}	12.3 ± 1.86^b	58.7 ± 7.7^b	8.5 ± 0.116^{bc}

Values in the table are the mean \pm SE. Key to the table is listed under the table 7.2.

7.3.3 Determination the effect of pH on rate constants of inhibition

The effect of enzyme activity in three different pHs inhibited by DDVP from the liver of sheep, cattle and pigs as described in Section 7.2.4 (Figure 7.3). There are nearly similar to k_i values for sheep liver AChE and cattle BChE between pH 7.5 and 8.5 (Figure 7.3A and 7.3D). The k_i values are significantly increased with increasing pHs in the reaction mixture and reaches a maximum at pH 8.5. The activity of BChE in different pHs was higher than AChE activity about 1.8 to 3.1-times for sheep, 3.5 to 4-times for cattle, and 2.5 to 3-times for pigs, respectively.

Percentage AChE inhibition was seen at the end of the study highest in sheep and ranged between 92.1 to 96.7%, while in cattle between 90.6 to 93.8%. In the pig was 90.4 to 94.7%, in addition a BChE activity elevated for sheep 85.9 to 89.9%, while lowest in cattle and pig and ranged between 66.8 to 83.8%. Non-linear regression was observed between AChE and BChE and three different of pHs revealed highly significant ($R^2 = 0.90$; $P < 0.001$) relationship with sheep, cattle and pigs (Figure 7.3A-F). In general, the residues (ChE activity) of the DDVP from liver for three different pHs was seen to be higher in BChE than in AChE (Figure 7.4A-B). In all cases (sheep, cattle and pigs used different three pHs), the relationship between AChE and BChE was seen to be ($R^2 = 0.93$; $P < 0.0001$) (Figure 7.5).

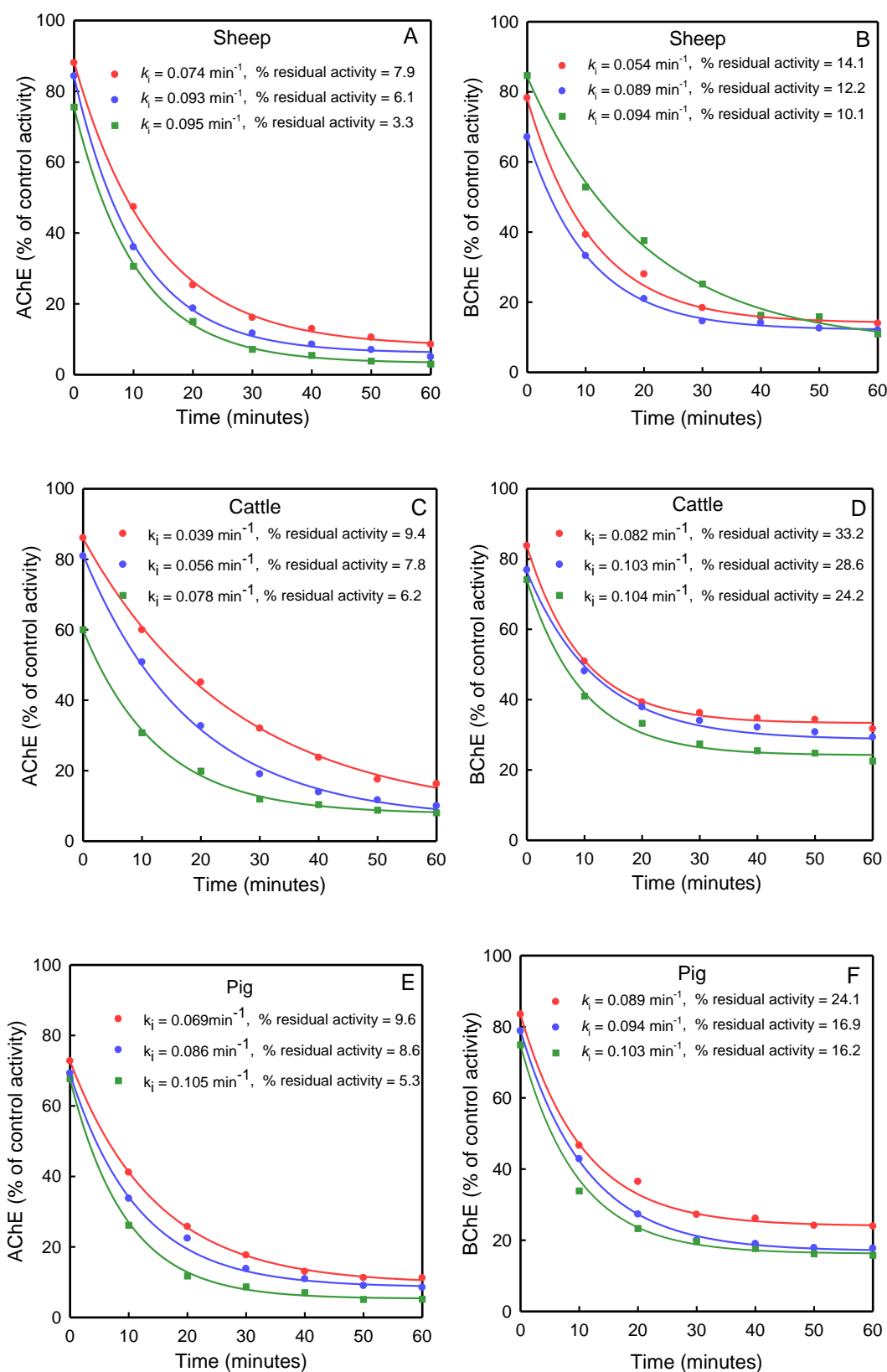


Figure 7.3 The figures above illustrate the data analysis used to obtain the effect of the pH on k_i from the liver of sheep, cattle and pig following *in vitro* exposure to DDVP (● pH 6.5, ○ pH 7.5 and ■ pH 8.5, respectively) ($R^2 > 0.90$; $P < 0.001$).

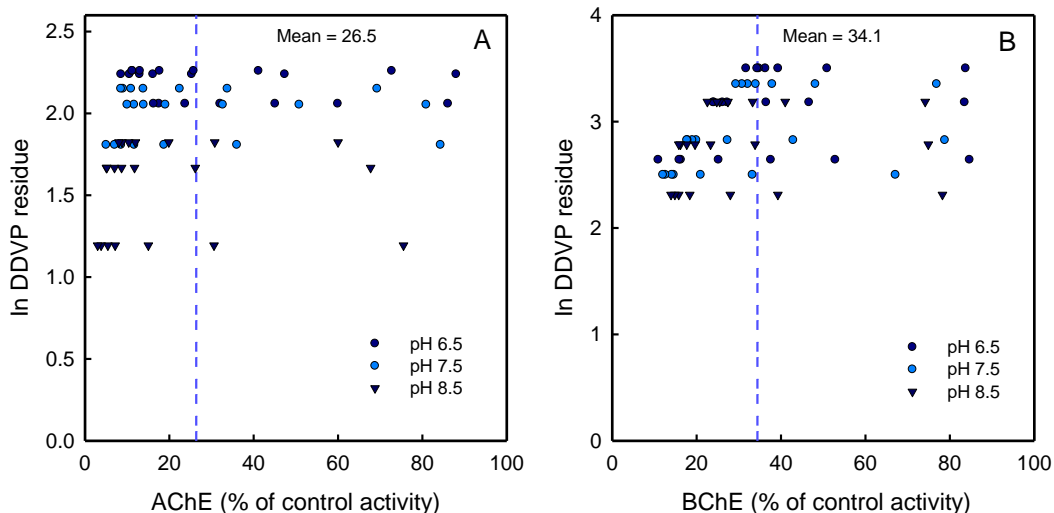


Figure 7.4 Residues of the DDVP for remaining ChE activity after inhibition (plotted on the y-axis) versus the percentage liver ChE activities (x-axis) for food animal at different pHs (6.5/8.5). Vertical dashed lines are drawn at the mean percentage control of ChE activities. The In DDVP residue was calculated as $\ln 2/k_i$.

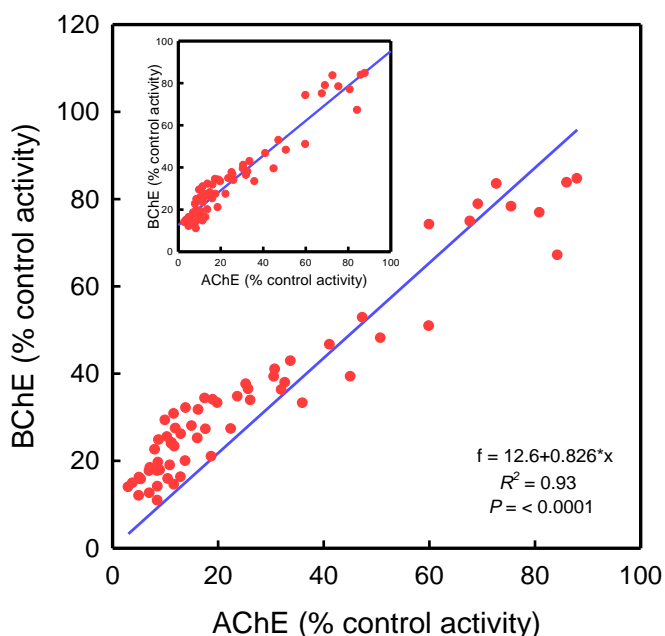


Figure 7.5 Comparison analysis between AChE and BChE activities (percentage control) in liver from sheep, cattle and pigs inhibited with DDVP within three different pHs (6.5/8.5).

7.3.4 Effect of temperature on rate constants of inhibition

The effect of enzyme activity in three different temperatures inhibited by DZN from plasma of sheep and cattle as described in Section 7.2.5 (Table 7.4). Significant differences ($P < 0.05$) were seen between temperature 20 °C and 30 °C for sheep AChE and BChE inhibited with DDVP, and cattle BChE inhibited with DZN. In addition, a significant ($P < 0.05$) between temperature 20 °C and 40 °C for cattle AChE and BChE inhibited with DDVP and sheep and cattle AChE and BChE inhibited with DZN, and sheep AChE and BChE inhibited with carbaryl and cattle AChE inhibited with carbaryl (Table 7.4). In addition ($P < 0.05$) between temperature 30 °C and 40 °C for sheep AChE and BChE inhibited with DDVP, and cattle BChE inhibited with DDVP, in addition a significant ($P < 0.05$) sheep AChE and BChE inhibited with DZN and cattle AChE inhibited with DZN, sheep and cattle AChE inhibited with carbaryl (Table 7.4).

There are nearly comparable k_i value for plasma BChE between 30 °C and 40 °C for sheep inhibited with carbaryl and DDVP. In addition, they were similar k_i value between 20 °C and 30 °C for sheep plasma AChE inhibited by DZN and carbaryl. In general, the k_i values were increases according to the rank order of 20 °C > 30 °C > 40 °C. In all cases the k_i values for BChE was higher than AChE with the exception of sheep plasma DDVP at 40 °C and cattle plasma inhibited with carbaryl at 20 °C, there was AChE higher than does BChE (Table 7.4).

Table 7.4 Effect of different temperatures on rate constants inhibition ($k_i \times 10^{-3} \text{ (min}^{-1}\text{)}$) for DDVP, DZN and carbaryl from plasma AChE and BChE of sheep and cattle.

Inhibitor	Temp. (°C)	Sheep		Cattle	
		AChE	BChE	AChE	BChE
DDVP	20	22.7 ± 0.3	53.8 ± 1.6	27.4 ± 2.0	52.4 ± 10.4
	30	18.6 ± 2.5 ^a	41.1 ± 2.9 ^a	21.0 ± 0.7	39.4 ± 0.9
	40	7.1 ± 2.2 ^c	25.1 ± 1.7 ^c	14.0 ± 4.2 ^b	34.0 ± 2.0 ^{bc}
DZN	20	25.8 ± 0.1	40.1 ± 0.8	29.0 ± 1.2	103.6 ± 2.2
	30	23.2 ± 0.6	33.3 ± 1.1 ^a	23.8 ± 4.0	64.0 ± 1.7
	40	16.4 ± 1.0 ^{bc}	28.8 ± 1 ^{bc}	13.0 ± 3 ^{bc}	37.5 ± 1.9 ^b
Carbaryl	20	37.9 ± 6.2	54.1 ± 4.0	43.0 ± 1.1	273.1 ± 9.6
	30	34.3 ± 1.6	43.8 ± 1.2	38.0 ± 0.6	81.4 ± 1.5
	40	19.3 ± 2.0 ^{bc}	40.1 ± 0.5 ^b	294 ± 5.9 ^{bc}	73.4 ± 2.4

Values in the table are mean ± SE obtained from nonlinear regression analysis, each experiment performed in triplicate (n = 3 in each animal).

^aSignificant differences [analysis of variance (ANOVA), $P < 0.05$] between temperature 30 °C and 20 °C within same inhibitor in the same column.

^bSignificant differences [analysis of variance (ANOVA), $P < 0.05$] between temperature 40 °C and 20 °C within same inhibitor in the same column.

^cSignificant differences [analysis of variance (ANOVA), $P < 0.05$] between temperature 40 °C and 30 °C within same inhibitor in the same column.

7.4 Discussion

7.4.1 Determination maximum reaction velocity and Michaelis-Menten constant

Maximum reaction velocity (V_{\max}) and Michaelis-Menten constant (K_m) were determined in a range of tissues. The results of the present study of sheep, cattle and pigs showed that the V_{\max} was far higher in liver than in the kidney and in muscle, with the exception of the pig where kidney AcTChI substrate activity was higher than that seen in BuTChI substrate (Table 7.1). This is in agreement with the works of Chatonnet and Lockridge (1989) found AcTChI and BuTChI substrates were observed to have higher activity for liver and kidney. The high activity of choline substrate activity in the liver and kidney occurs due to synthesis of enzyme from liver and kidney (Massoulie *et al.*, 1993; Wilson *et al.*, 2001; Wilson and Philip, 2005). Other studies have shown that ChE specific activity, its kinetic parameters and their sensitivity to anti-ChE compounds vary either within tissues or within species (Chuiko *et al.*, 2003). Oliveira *et al.* (2007) suggested that such interspecific variation in ChE kinetic characteristics is phylogenetically based, or due to stress and pollutants.

In all cases, AcTChI substrate gave a higher K_m value than BuTChI substrate affinity, with the exception of the pig where kidney BuTChI activity was higher than that seen in AcTChI (Table 7.1). In this sense, it can be expected that ChE with lower substrate affinity should have lower sensitivity to anticholinergic agents (Chuiko *et al.*, 2003). In general, the results are similar to that of Morizono and Akinaga (1981) observed that K_m values in AcTChI higher than BuTChI in liver and kidney. Results from the present study indicate the need for estimating enzyme kinetic parameters prior to using ChE activity as a biomarker as supported by Chuik *et al.* (2003).

7.4.2 *In vitro* kinetic characterization of acetylcholinesterase

This is the first research that compares the different kinetic parameters for insecticide inhibitor in the tissues of food animals. The kinetic determination of AChE is the basic method for the diagnosis of OP and carbamate poisoning, some preferring the clinical signs of poisoning as a leading tool for insecticide poisoning diagnosis and monitoring, therefore the decrease of AChE activities is the most important manner for the confirmation of intoxication (Hamm, 1998; Wilson *et al.*, 2005; Dalvie and London, 2006; Gupta *et al.*, 2007; Ferreira *et al.*, 2008). For the survey of possible interaction between insecticides and AChE from food animals, Mohammad (2007) found significant inhibition of the enzyme by several OP and carbamate pesticides. Regarding distinguishing inhibitor efficiency, determination of IC_{50} is accepted. DDVP showed higher susceptibility than DZN and carbaryl (Tables 7.2 and 7.3). This reason may be due the constitution and sensitivity AChE in liver and muscle that may preferentially bind DDVP, thereby protecting AChE, resulting in greater tolerance to DDVP. However, carbaryl inhibited the AChE at a concentration five-fold higher than that of DDVP and DZN. This agrees with the findings of Anguiano *et al.* (2010) reported that DDVP in invertebrates was more potent than carbaryl. In contrast with that of Laguerre *et al.* (2009) they observed that carbaryl in the snail was more potent than DDVP (Table 7.5).

Table 7.5 Comparison of IC_{50} of inhibitors, DDVP, DZN and carbaryl, for some vertebrates and invertebrates.

Inhibitor	Species	Tissue	IC_{50} (μ M)	Reference
DDVP	Human	Erythrocytes	0.23	(Quistad <i>et al.</i> , 2005)
DDVP	Mouse	Brain	0.56	(Quistad <i>et al.</i> , 2005)
DDVP	Oysters	Gills	1.08	(Anguiano <i>et al.</i> , 2010)
DDVP	Snail	Shell and soft tissues	13.5	(Laguerre <i>et al.</i> , 2009)
DDVP	Earthworm	Crop/gizzard	0.005	(Sanchez-Hernandez <i>et al.</i> , 2009)
DDVP	Earthworm	Foregut	0.006	(Sanchez-Hernandez <i>et al.</i> , 2009)
DDVP	Rat	Liver	0.2	(Jokanović <i>et al.</i> , 1996)
DZN	Fish	Auricle	164	(Tryfonos <i>et al.</i> , 2009)
Carbaryl	Earthworm	Whole worm	0.005	(Caselli <i>et al.</i> , 2006)
Carbaryl	Oysters	Gills	1.37	(Valbonesi <i>et al.</i> , 2003)
Carbaryl	Mussels	Gills	0.614	(Valbonesi <i>et al.</i> , 2003)
Carbaryl	Snail	Shell and soft tissues	0.057	(Laguerre <i>et al.</i> , 2009)

The k_i values of liver and muscle AChE determined in this study range from 43.1×10^{-3} - $167.5 \times 10^{-3} \text{ min}^{-1}$, and DDVP more sensitive than other compounds (Tables 7.2 and 7.3). This is in agreement with previous research by Frasco *et al.* (2006) where they found that the DDVP was generally more sensitive than carbaryl for the Arthropod and Chordata. In general, DZN was less sensitive in

muscle than carbaryl. This is in agreement with the findings of Mohammad *et al.* (2000) where they found that DZN relatively less sensitive to cattle retinal AChE compared to carbaryl.

But in contrast with Herzprung *et al.* (1989) they found k_i for cattle erythrocytes AChE about 2.3×10^4 , 3.4×10^4 and $1.8 \times 10^4 \text{ min}^{-1}$ for DDVP, DZN and carbaryl, respectively, i.e. 51.3×10^7 to 68.2×10^7 and 46.1×10^7 to 55.2×10^7 fold higher than liver and muscle, respectively, than our studied results. However, these authors used methanol to dilute the OP and carbamate compounds and a potassium phosphate buffer (pH 7.0, 0.05 M) during measuring enzyme activity instead of distilled water for dissolving these compounds and sodium phosphate buffer (pH 8.0, 0.1 M) in our study. They also used a four-fold higher concentration of substrate and unmodified Ellman method. All these factors may explain the apparent difference in rate constant of inhibition.

Sturm *et al.* (1999) observed that k_i values of AChE for DDVP in fish are about 7.3×10^3 to $8.9 \times 10^4 \text{ min}^{-1}$ (Sturm *et al.*, 1999), i.e. 46.1×10^6 to 167.5×10^7 fold greater than that calculated in our study. Since food animals AChE is much more sensitive to DDVP than fish (Herzprung *et al.*, 1989), I suggest a higher sensitivity of AChE-inhibited with OP and carbamate compounds from food animals in comparison with AChE-inhibited with these compounds for invertebrates. These interesting results should be taken into account since food animals are considered good sentinel species. Additionally, the use of k_i for further validation study of $t_{1/2}$ for inhibition revealed higher $t_{1/2}$ in the muscle than liver.

The half time ($t_{1/2}$) of inhibition (8 μ M DDVP or DZN; 40 μ M carbaryl) (Tables 7.2 and 7.3) are lower than those found by Herzprung *et al.* (1989). However, these authors used methanol to dilute the OP and carbamate compounds and a potassium phosphate buffer (pH 7.0, 0.05 M) during measuring enzyme activity instead of distilled water for dissolving these compounds and sodium phosphate buffer (pH 8.0, 0.1 M) in our study. They also used a four-fold higher concentration of substrate. All these factors may explain the apparent difference in the rate of inhibition.

Percentage residual AChE activity in extracts from tissues is also monitored, by simply comparing the activity of the non-inhibited enzyme with the inhibited one. It was significantly lowered in all inhibitors and mostly more than 83-98.6% inhibition detected. This is in agreement with previous studies carried out with AChE inhibition in the range 70-100%, after exposure to OP compounds (Bocquené and Galgani, 1991; Mcloughlin *et al.*, 2000). It is well accepted that a 20% or greater inhibition in AChE in birds, fish and invertebrates indicates exposure to pesticides (Varo *et al.*, 2002). Some animals are able to survive with more than a 50% AChE inhibition but this is an indication of a life-threatening situation (Ludke *et al.*, 1975). On the other hand, as Escartin and Porte (1996) described, the inhibition of AChE at 40% or below caused fatal effects, whereas Lundebye *et al.* (1997) had reported that a 30% of reduction in AChE activity is not lethal.

7.4.3 Effect of pH on rate constants of inhibition

To the best of our knowledge, this is the first work that compares the k_i in different pHs from liver of food animals. The k_i values increased with increasing pH in the reaction mixture, since the pH 6.5 has a more sensitive to inspection

AChE and BChE inhibition with DDVP than other pHs (Figure 7.3A-F). They are, in general, in good concord with the literature data on k_i values obtained for rat brain AChE more sensitive in pH higher than 6.5 (Chiappa *et al.*, 1995). Based on our result I suggest that the DDVP is more sensitive to inhibition AChE than BChE within different pHs from liver of food animals (Figure 7.4). This may be due to AChE is loaded ChE and primarily found in the liver. This is in agreement with the findings of (Bajgar *et al.*, 2008; Laura *et al.*, 2008) they stated that AChE is higher and more sensitive effect to OP compounds than does BChE. Hence, in the all cases, I found that ChE activities by both the AChE and BChE were directly proportional to each other's (Figure 7.5).

7.4.4 Effect of temperature on rate constants of inhibition

The k_i values were decreased with increasing temperature, since the temperature 40 °C had a more sensitive to inspection AChE and BChE inhibition with OP and carbamate compounds than other temperatures (Table 7.4). This is in agreement with previous research by Boon *et al.* (2000) found generally higher levels of k_i value (oligosaccharides and enzyme activities, not including ChE) in temperature 20 °C from *Bacillus circulans* compared to 40 °C. But in contrast with the works of Sun *et al.* (2009a), they observed that k_i value in temperature 30 °C > 20 °C \equiv 40 °C. These authors used orange G in aqueous solution instead plasma from sheep and cattle, however this factor may explain the apparent difference in k_i values.

7.5 Conclusions

The work described in this Chapter focuses on distinguishing an animal species at the degree by the inhibition of ChE activity organizing liver, kidney, muscle and plasma. Furthermore, these results are pointed at the importance of

estimating kinetic parameters for insecticide inhibitor prior to using in animals as biomarker tools of environmental exposure to anti-ChE pesticides. This is clearly a very complex issue but on the strength of the evidence I have seen I would say.

1. There were differences between the three species (sheep, cattle and pig) with high individual variability were detected. The V_{\max} for substrate AcTChI in animals are decreased according to the rank order of pig > cattle > sheep for liver, muscle and kidney. Whereas, V_{\max} for substrate BuTChI increased as follows: pig > cattle > sheep for liver and pig > sheep > cattle for muscle, while it increased in rank order of pig > cattle > sheep for kidney.
2. The IC_{50} values in pig were higher than in sheep and cattle for AChE inhibited with OP and carbamate compounds, except for liver from cattle with DZN where the IC_{50} was higher than for pig and sheep. AChE activity was found to be more sensitive to inhibition by DDVP than by DZN or carbaryl, and activity in liver was slightly more sensitive to these pesticides than that in muscle tissue. In both liver and muscle, k_i values for the inhibition of AChE by OP and carbamate compounds were higher in sheep than in cattle and pigs. However, in general for the same tissue and same animal k_i values were similar for all three inhibitors. In all animals, residual AChE activity after inhibition of all three compounds (DDVP, DZN and carbaryl) was lower in liver than in muscle.
3. The effect of pHs on k_i values from the liver of sheep, cattle and pig inhibited by DDVP compounds found to be a higher in pH 6.5 than the other (e.g. pH 7.5 and pH 8.5). The percentage residual ChE activities in liver extracts after inhibition of DDVP compounds were lower in AChE activity than in BChE activity. In addition, the effect of k_i for different pHs were decreased according rank order of 8.5 > 7.5 > 6.5. Therefore, this data pointed that pH 6.5 is more

recommended for measuring DDVP-AChE and BChE inhibition than other pHs used and AChE were more sensitive than does BChE to inhibition with DDVP.

4. Effect of different temperatures (20 °C, 30 °C and 40 °C) from plasma for sheep and cattle, the k_i values for the inhibition by DDVP, DZN and carbaryl compounds were higher in BChE than AChE with the exception of sheep plasma DDVP at 40 °C and cattle plasma inhibited with carbaryl at 20 °C, there was AChE higher than does BChE. In addition, the effect of k_i for different temperatures were decreased according rank order of 20 °C > 30 °C > 40 °C. So, this data pointed that temperature 40 °C is more recommended for measuring DDVP, DZN, carbaryl-AChE and BChE inhibition in plasma than other temperatures used.

Chapter 8

Reactivation of acetylcholinesterase

The results from this Chapter have been presented as oral presentations at the following conferences: 13th Associations of Institutions for Tropical Veterinary Medicine, August 2010, Bangkok, Thailand and First Joint Meeting between the Society for Experimental Biology, British Ecological Society, Biochemical Society, January 2011, London, UK.

These results have also been published in:

Kasim Abass Askar, A. Caleb Kudi and A. John Moody (2011). Spontaneous reactivation and aging kinetics of acetylcholinesterase inhibited by dichlorvos and diazinon. *Journal of Toxicological Sciences* 36(2):237-241.

Chapter 8: Reactivation of acetylcholinesterase

8.1 Introduction

Although there are many natural and synthetic compounds that inhibit the enzyme AChE, the organophosphate (OP) insecticides (e.g. DDVP and DZN) remain one of the most dangerous and deleterious series of the compounds to animals. The necessary treatment after OP exposure involves the use of parasympatholytic (e.g. atropine), oxime reactivators (e.g. pralidoxime) and anticonvulsant drugs (e.g. diazepam). Therefore, the reactivation of AChE is essential compounds in the treatment of OP intoxications (Musilek *et al.*, 2009). The use of OP compounds for pest control and attempted suicide cause huge numbers of intoxications and several hundreds of thousands of fatalities per year mainly in developing countries (Worek *et al.*, 2004). OP products are prevalent in animals destined for human consumption in the world with serious public health implications. Animal handlers are at risk of contamination and can serve as a source of contamination to susceptible hosts. Targeted pest control of animals, concerted veterinary/medical efforts, professional health instruction, active attachment of animal careers and good health care systems are necessary for effective control (Marrs, 1993; Worek *et al.*, 2004).

The mechanism of action of OP compounds is a progressive inhibition of AChE by phosphorylation (both phosphorylation and phosphonylation) of the esteratic site of the serine hydroxyl group of the enzyme leading to an inhibition ChE (Timothy, 2001; Worek *et al.*, 2004; Ferreira *et al.*, 2008), and successive accumulation of the neurotransmitter ACh. AChE primary function is to catalyse hydrolysis of released ACh and thus maintain homeostasis of this neurotransmitter ACh in the central and peripheral nervous systems. After

inhibition, AChE is not able to serve its physiological function and poisoning animals (Timothy, 2001; Saxena *et al.*, 2008).

Phosphorylated AChE is spontaneously hydrolysed, liberating phosphoric acid and the original active enzyme. This phenomena spontaneous reactivation (dephosphorylation) proceeds very slowly and depends on the leaving group of the original OP inhibitor, but on the remaining substituted groups on the phosphorus atom and the source of the enzyme (Figure 8.1B) (Morifusa, 1974; Worek *et al.*, 2004). However, the OP-inhibited AChE changes gradually into a non-reactivable form on storage. This phenomena is called aging (dealkylation) (Figure 8.1A) (Worek *et al.*, 2004). It was assumed that the aging might be caused by a migration of the phosphoryl group from an initial position to form more stable bond or by the elimination of serine phosphate to lose serine hydroxyl group. It is generally accepted that spontaneous reactivation and aging mechanism for alkoxy group of OP residue bound to AChE (Morifusa, 1974; Patocka *et al.*, 2004).

Oxime compounds can reactivate phosphorylated AChE by displacing the phosphoryl group from the enzyme by virtue of their high affinity for the enzyme and their powerful nucleophilicity. This is characterized by the presence of several structural features: Functional oxime group, quaternary nitrogen group and a different optimal length of linking chain between two pyridinium rings in the case of bispyridinium reactivator (Figure 8.1C) (Kassa, 2002; Worek *et al.*, 2004).

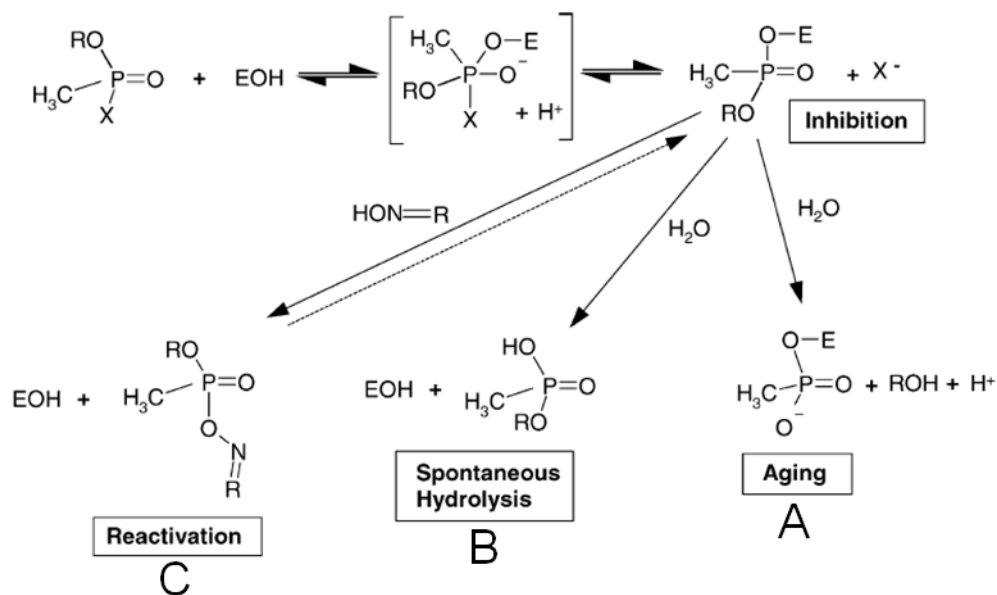


Figure 8.1 Reaction scheme for the reactivation of OP-inhibited ChE by oximes (adapted from (Worek *et al.*, 2004)).

Unfortunately, none of the currently used oximes are sufficiently effective against all OP compounds, for example pralidoxime (2-PAM, Pyridine-2-aldoxime methiodide); obidoxime (1,1'-(Oxydimethylene)bis(pyridinium-4-carbaldoxime) dichloride bis(4-formylpyridiniummethyl) ether dioxime); and HI-6 1-(4-carbamoyl pyridinium)-3-hydroxyiminomethylpyridinium)-2-oxapropane dichloride (Figure 8.2) (Worek *et al.*, 2007; Ferreira *et al.*, 2008). Furthermore, the toxic effect of these compounds may cause death due to respiratory paralysis (Sevelová *et al.*, 2005). There has been some concern about the hepatotoxicity of obidoxime (Marrs, 1993). Likewise, side effects of pralidoxime when administered intravenously to human in the absence of nerve agent poisoning, are only of transient type-like dizziness and blurred vision. Transient diplopia is observed when high doses of pralidoxime (10 mg/kg) are applied and occasionally, nausea and vomiting may occur. Furthermore, the most serious side effect of pralidoxime is hypertension and tachycardia (Sidell, 1992). To overcome these problems, I used the newly synthesized oximes,

bisquaternary symmetric (K005 and K033) or asymmetric (K027 and K048) pyridinium aldoximes with the functional aldoxime group at position two (K005 and K033) or four (K027 and K048) at the pyridine rings (Figure 8.2) (Kuca and Cabal, 2004).

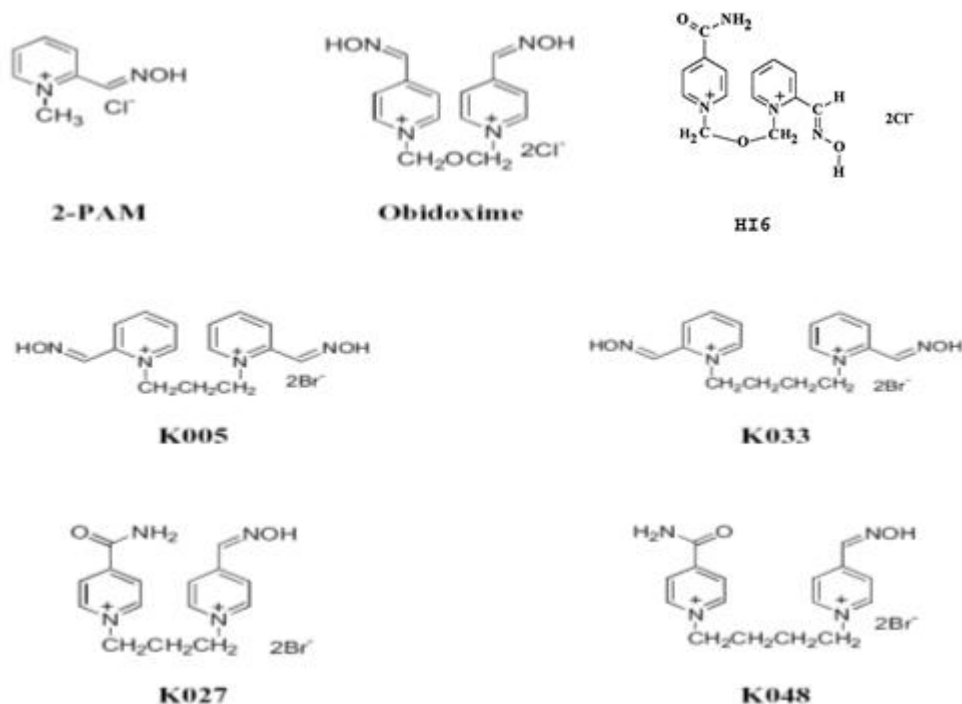


Figure 8.2 Structure of currently used oximes and their new analogues (adapted from (Kuca and Kassa, 2003)).

The main objectives of this study were to investigate the rate of spontaneous reactivation of AChE from liver and muscle of food animals inhibited by the OP, DDVP and DZN. It also aims to identify the time course of aging of OP-inhibited AChE from liver and muscle of food animals. In addition, to find suitable reactivator of AChE and to recommend the most efficacious oxime compounds for the next evaluation as antidotes for intoxication by DDVP. The results of this study confirm that the reactivation effect depends on (a) number of pyridinium rings, (b) number of oxime groups and their position, and (c) length and the shape of the linkage bridge between the pyridinium rings.

8.2 Materials and methods

8.2.1 Chemicals

Obidoxime and pralidoxime were supplied by the Sigma Chemical Company (Poole, Dorset, UK). Oximes K005, 1,3-bis(2-hydroxyiminomethylpyridinium) propane dibromide; K027, 1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium) propane dibromide; K033, 1,4-bis(2-hydroxyiminomethylpyridinium) butane dibromide; K048, 1-(4-hydroxyiminomethylpyridinium)-4-(4-carbamoylpyridinium) butane dibromide; and HI-6 were synthesized and supplied at the Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Czech Republic (Kuca and Kassa, 2003). All other chemicals used in this study were of analytical grade.

8.2.2 Sample collection and preparation

The sample collection and preparation were done as described in Sections 2.2 and 2.3. Following this it was then measured the AChE enzyme activity as described in Section 2.4.1.

8.2.3 Determination of rate constants for aging and spontaneous reactivation

For the detection of rate constants for spontaneous reactivation, AChE was inhibited with either 8 μ M DDVP or DZN for 30 min at room temperature 20 °C resulting in an inhibition of 85-95% of control activity. The excess inhibitor was removed by gel filtration using Sephadex G-25 column (1 x12 cm) to separate enzyme from un-reacted inhibitors, with 0.1 M sodium phosphate buffer, pH 8.0 (Figure 8.3). Hence, the column used was designed to give a good separation of proteins present in small samples of relatively impure homogenates. Then equilibrated 16 fraction times (0.2 ml each) with sodium phosphate buffer (0.1

M, pH 8.0). Eluted with potassium ferricyanide salt (5 mM) $[K_3Fe(CN)_6]$ and measured at 420 nm, 25 °C on endpoint (0.06 ml of three coloured fractions was added to 0.24 ml sodium phosphate buffer for 96-well microtitre plates and measuring by using a plate reader.

In a single stage, sample is desalted, exchanged into a new buffer and low molecular mass materials, such as unwanted salts, and is removed. The high speed and high volume capacity for this separation allows even large sample volumes to be processed rapidly and efficiently. Active fractions from monitored samples as a sole peak were collected. AChE activity measured as described in Section 2.4.1. The increase in AChE activity over time was then monitored (e.g. Figure 8.4A). OP-treated samples were stored in aliquots at -80 °C until use. For detection of aging, AChE was inhibited as above. Excess inhibitor was also removed in the same way. Then at different times 500 μ M pralidoxime concentrations for 30 min at room temperature 20 °C was added to reactivate the enzyme (Figure 8.4B). The decrease of oxime (pralidoxime)-induced reactivation in AChE activity over time was then monitored (Škrinjarić-Špoljar *et al.*, 1973; Aurbek *et al.*, 2006; Worek *et al.*, 2008). The pseudo-first-order rate for constants k_s and k_a were calculated by a non-linear regression analysis (Škrinjarić-Špoljar *et al.*, 1973; Worek *et al.*, 2004).

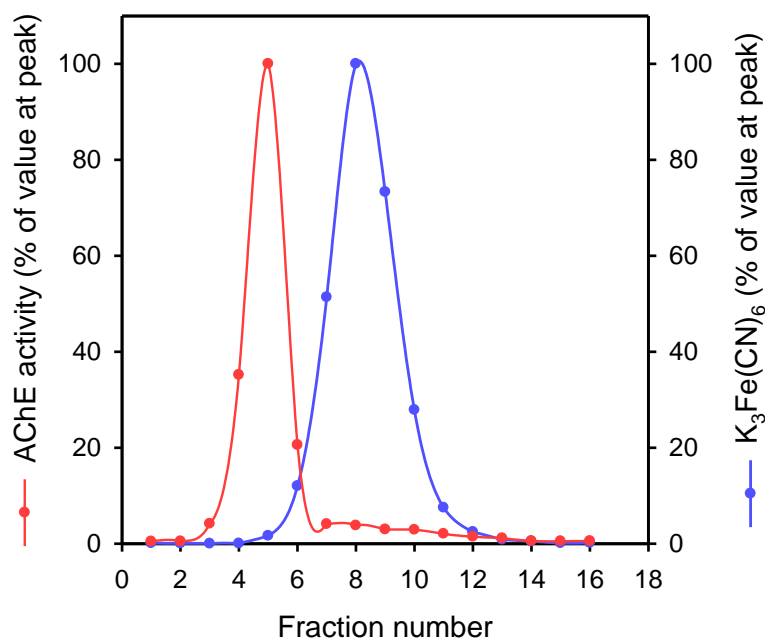


Figure 8.3 The fractionation of AChE on sodium phosphate buffer (0.1 M, pH 8.0). The flow rate was 1ml/20 min⁻¹. Data are for sheep liver.

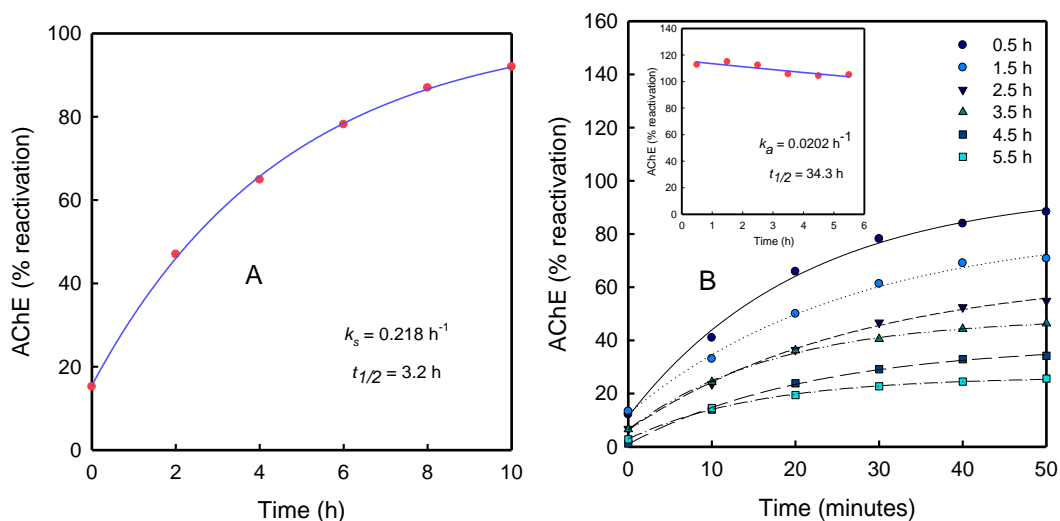


Figure 8.4 This illustrates the data analysis used to obtain rate constants for spontaneous reactivation (k_s , expressed as h⁻¹; A) and for aging (k_a , expressed as h⁻¹; B). The data are for cattle liver AChE inhibited with DDVP. Spontaneous reactivation data were fitted with a single exponential rise to maximum using SigmaPlot 11. For the aging data the reactivation time courses at different times after inhibition was fitted in the same way, and the maximum level of oxime-induced reactivation in each case plus the level of spontaneous reactivation at the same time was then plotted (B, insert). Data were then fitted with a single exponential decay. Half times were calculated using equation; $t_{1/2} = \ln 2/k$.

8.2.4 Preparation of organophosphate-inhibited samples

Samples of liver extract were incubated with a small volume ($\leq 1\%$ v/v) of $8 \mu\text{M}$ DDVP for 30 min at 20°C to achieve $>90\%$ inhibition of AChE activity in the sample. Then, the excess inhibitor was removed by gel filtration using Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 8. Stock solutions of DDVP were prepared weekly, stored at 4°C and appropriately diluted in distilled water just before the experiments. The OP-inhibited samples were stored at -80°C until use.

8.2.5 Reactivation of organophosphate-inhibited sample

The reactivation rate constants of AChE treated with various oximes were determined by a discontinuous procedure (Worek *et al.*, 2010). Six parts of OP-inhibited sample incubated with one part of oxime solution (10^{-2} - 10^{-5} M final concentration) were transferred to a microtitre plate after specified time intervals (0-40 min), after which the AChE activity was measured as explained in the Section 2.4.1. AChE activities of OP-inhibited samples after time-dependent reactivation are expressed relative to maximum reactivation, i.e. the maximum AChE activity at the end of the observation period, which was close to the control AChE activity in all cases.

8.2.6 Determination of dissociation constant of enzyme-reactivator complex (K_{dis})

For the measurement of K_{dis} , samples were incubated with oximes (concentrations ranging between 10^{-2} and 10^{-6} M), and then AChE activity was measured over different times at 20°C as explained in the Section 2.4.1. Then monitored AChE activity within different concentrations over times (0-50 min). The data were fitted with a single exponential rise to maximum using SigmaPlot

11 (Figure 8.5). Stock solutions of oximes were prepared weekly, stored at 4 °C and appropriately diluted in distilled water just before the experiments. Controls were incubated with sodium phosphate buffer pH 8.0 were included when appropriated. Blanks were also run at each selected oxime for each based on absorbance tested.

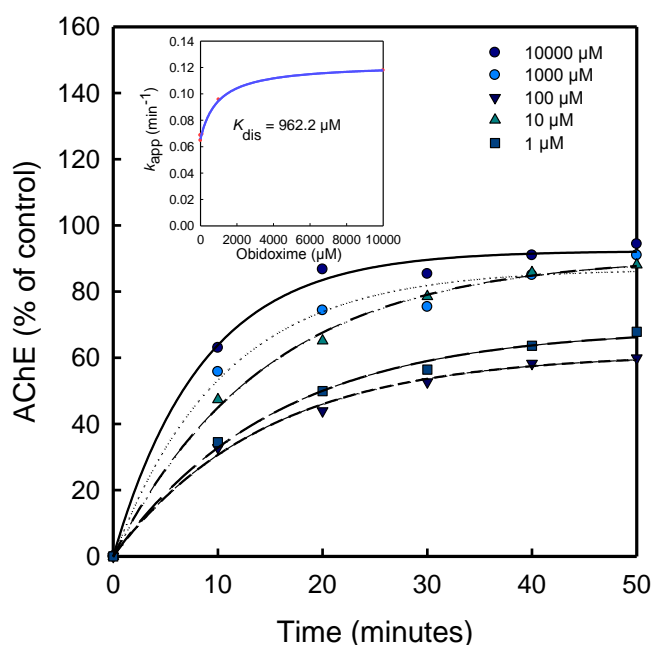


Figure 8.5 The figure above illustrates the data analysis used to obtain the rate constant of oxime (k_{app}). The data are for sheep liver AChE with obidoxime. Then plotted the k_{app} values against different oxime concentrations with a single rectangular hyperbola (insert) by using SigmaPlot 11 and the slope of the curve is representing the K_{dis} .

8.2.7 Determination of reactivation constants

Reactivation constants separated into the enzyme-inhibitor-reactivator complex (dissociation constant, K_R) and rate constant (first-order rate constant for reactivation, k_r) describing the displacement of the OP compound from the active-site serine were determined as previously described (Worek *et al.*, 2007; Artursson *et al.*, 2009). The measurements were made in 0.1 M sodium phosphate buffer (pH 8.0) at 20 °C and 4-5 different oxime concentrations were

used in each experiment. The observed first-order rate constant at a given oxime concentration (k_{oi}) was determined by non-linear regression (SigmaPlot 11).

In brief, for the measurement of K_R , different oxime concentrations ranging between 10^{-2} and 10^{-5} M were added to samples of AChE inhibited with DDVP (prepared as described above). After this AChE activity was measured over different times at 20 °C as described above. The level of reactivation of AChE activity over different times (0-40 min) was then plotted (e.g. Figure 8.6), and from this the rate constant (k_{obs}) was obtained by non-linear regression (SigmaPlot 11). After this the k_{obs} values obtained with different oxime concentrations were plotted against oxime concentration and fitted with a single rectangular hyperbola (SigmaPlot 11). From this the maximum rate of reactivation (k_r) and the concentration of oxime that gives a rate of reactivation equal to 50% of k_r , i.e. K_R , were obtained. The % of reactivation potency was calculated as the % of increase in the activity of a reactivated enzyme in the reaction mixture as follows;

$$\% \text{ Reactivation} = \frac{\text{AChE activity with (DDVP and oxime)}}{\text{AChE activity without (DDVP and oxime)}} \times 100$$

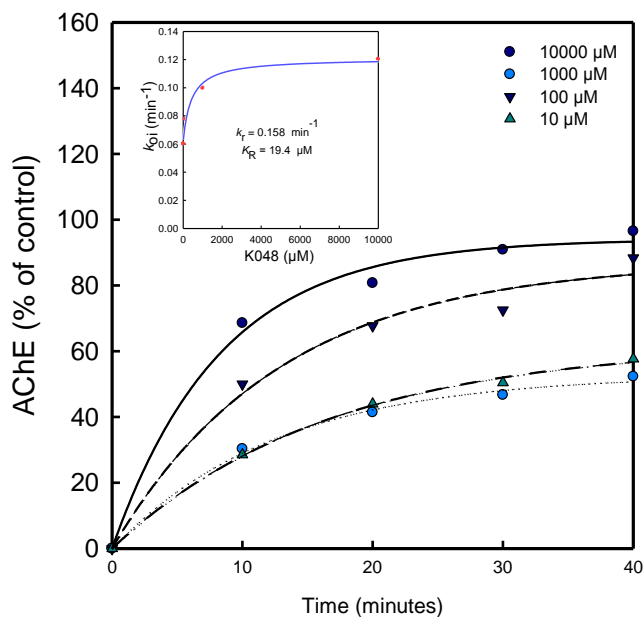


Figure 8.6 The figures above illustrate the data analysis used to obtain rate constant of reactivation in different time and dissociation constant of enzyme-inhibitor-reactivator complex were calculated after plotted slope of reactivation after inhibition against oxime concentrations. The data are for sheep liver AChE inhibited with DDVP and reactivated with K048.

8.2.8 Bimolecular constants of reactivation (k_{r2})

It represents the overall reactivation ability and values of k_{r2} were calculated by dividing k_r with K_R as described in (Kuca and Kassa, 2003; Artursson et al., 2009).

8.2.9 Preparation of the isolated perfusate liver

Sheep liver from a local abattoir in Launceston, Cornwall (Figure 2.1), UK, was killed by a blow to the head; the brain was pithed (ethically approved by humanely sacrificed) and transported to the laboratory at the University of Plymouth for immediate processing. The time between death of the animal and the start of perfusion was about an hour. The decision to use sheep model in this research was made as the metabolic rate is more rapid in small animal than larger one (relative to body size) (Couture and Hulbert, 1995). The second

reason was economic, because all sheep liver samples were obtained at a low cost.

Appropriate captures loosely placed around both the aorta and vena cava, posterior to the liver. A snare was also loosely placed around the portal vein and caudal lobe of the liver removed quickly. A tube (cannula) connected to the perfusion apparatus inserted via the portal vein into the branch leading to the caudal lobe, and secured with a purse string suture. The caudal lobe was immediately perfused into 100 ml of the Krebs Henseleit bicarbonate buffer which contained (in g L⁻¹) according to Ali *et al.* (2000): Glucose, 2; magnesium sulphate, 0.141; potassium dihydrogen phosphate, 0.16; potassium chloride, 0.35; and sodium chloride, 6.9. In addition, add to this buffer 2.1 mM lactate, 0.3 mM pyruvate, and 10 U heparin ml⁻¹ at body temperature (37 °C), gassed with 95% O₂: 5%CO₂ (normal CO₂).

The liver started perfused [1 ml min⁻¹ via an peristaltic pump (Miniplus 3, Gilson, UK)] with another volume of the same Krebs-Henseleit bicarbonate buffer (described above) for up to 1 h, the control (no added DDVP or K048) and treatment groups will be run separately after adding the amount of 16 µM DDVP alone or with 1 mM K048, that wanted to use in the experiment because the high bimolecular rate constant than other oximes used in our previous work. At the end of the experiment, the liver carefully washed in deionised water, and extracted as described in Section 2.3.2. Following this it was then measured the enzyme activity as described in Section 2.4.1.

8.2.10 Statistical analysis

Processing of experimental data for the determination of mean values ± SE. Differences with $P < 0.05$ was regarded to have statistical significance. The

kinetic constants were performed by using the curve-fitting programs provided by SigmaPlot 11.

8.3 Results

8.3.1 Spontaneous reactivation and aging of acetylcholinesterase

Spontaneous reactivation (k_s) and aging (k_a) kinetics of AChE inhibited by DDVP and DZN were determined in liver and muscle for sheep, cattle and pig using the modified Ellman method as described in Section 8.2.3 (Tables 8.1 and 8.2). Liver k_s and k_a kinetic parameters for insecticide inhibitor for reaction between AChE and two OP (DDVP and DZN) are showed in Table 8.1. The k_s for animals was decreased according to the rank order of sheep > pig > cattle for DDVP and DZN. k_a values for animals were decreased according to the rank order of cattle > sheep > pig for DDVP, while decreased in the range cattle > pig > sheep for DZN. The relative activity (ratio of mean) between DDVP and DZN was found highest in sheep k_s (3.7) lowest in pig k_a (0.8). Kinetic k_s and k_a of all tested animals in liver gave no correlation between k_a and k_s ($R^2 < 0.34$) (Table 8.1).

Half time ($t_{1/2}$) from liver for k_s and k_a kinetic parameters for insecticide inhibitor for reaction among AChE and (DDVP and DZN) are showed in Figure 8.7. All cases (e.g. sheep, cattle and pigs), the DZN was higher than DDVP for k_s and k_a and much higher (5-times) than that seen in sheep k_s (Figure 8.7A). In addition, $t_{1/2}$ for aging was higher in DZN than DDVP in sheep (1.5-times), whereas cattle and pig (1.2-times) (Figure 8.7B).

Table 8.1 Rate constants for the spontaneous reactivation (k_s) and aging (k_a) of AChE inhibited by DDVP and DZN from liver of sheep, cattle and pig.

Inhibitor	Sheep	Cattle	Pig
k_s (h^{-1})			
DDVP	0.323 ± 0.05 ^a	0.218 ± 0.09 ^a	0.245 ± 0.03 ^a
DZN	0.088 ± 0.04 ^b	0.061 ± 0.001 ^b	0.070 ± 0.003 ^b
Ratio ^a	3.7	3.6	3.5
k_a (h^{-1})			
DDVP	0.019 ± 0.003	0.021 ± 0.003	0.013 ± 0.002
DZN	0.014 ± 0.002	0.018 ± 0.001	0.017 ± 0.006
Ratio ^a	1.4	1.7	0.8

Values in the table are the mean ± SE obtained from nonlinear regression analysis, each experiment performed in triplicate ($n = 3$ in each animal). Different letters in column are significantly different [analysis of variance (ANOVA), $P < 0.05$]. ^a Ratio (DDVP versus DZN).

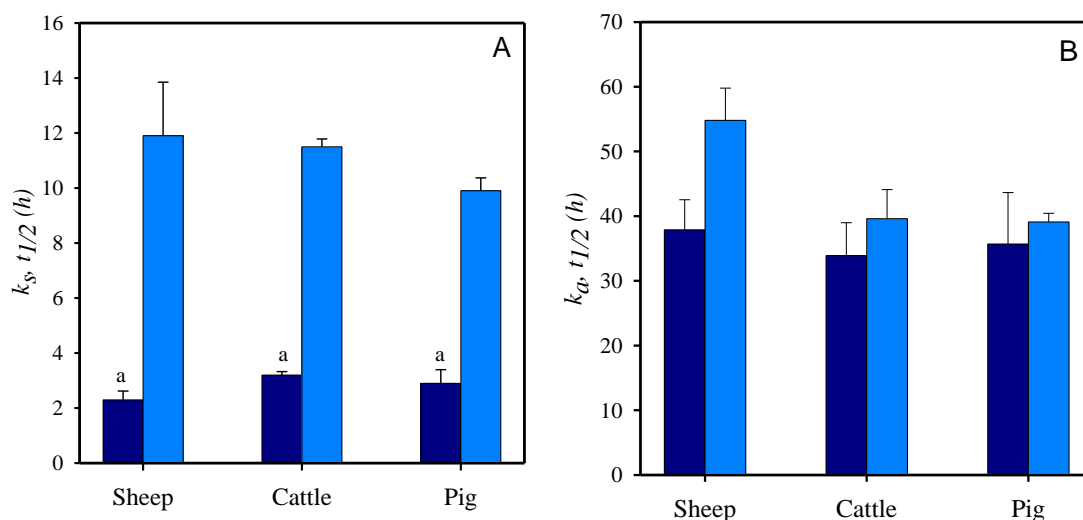


Figure 8.7 Half times for the spontaneous reactivation (k_s) and aging (k_a) of AChE inhibited by ■ DDVP and ■ DZN from liver of sheep, cattle and pig. The letters between the DDVP and DZN are significantly different [analysis of variance (ANOVA), $P < 0.05$].

Muscle k_s and k_a kinetics for reaction among AChE and two OP (DDVP and DZN) are seen in Table 8.2. The values of k_s for animals were decreased

according to the rank order of sheep > pig > cattle for DDVP, while in range sheep > cattle > pig for DZN. On the other hand, the first-order rate constants for k_a values was decreased according to the rank order of cattle > sheep > pig for DDVP whereas decreased: sheep > pig > cattle for DZN. DDVP/DZN was 3, 5.1 and 6.6, respectively, for k_s and 1.7, 2 and 0.9, respectively, for k_a . The comparison between the k_s and k_a kinetics in the muscles of all tested animals gave also poor correlation between k_a and k_s ($R^2 < 0.26$) (Table 8.2).

Half time ($t_{1/2}$) from muscle for k_s and k_a kinetic parameters for insecticide inhibitor for reaction among AChE and two OP (DDVP and DZN) are showed in Figure 8.8. Again in all cases (e.g. sheep, cattle and pigs), the DZN was higher than DDVP for k_s and k_a and much higher (6-times) than that seen in pig spontaneous reactivation (Figure 8.8A). In addition, $t_{1/2}$ for aging was nearly similar in DZN than DDVP in pig (Figure 8.8B).

Table 8.2 Rate constants for the spontaneous reactivation (k_s) and aging (k_a) of AChE inhibited by DDVP and DZN from muscle of sheep, cattle and pig.

Inhibitor	Sheep	Cattle	Pig
k_s (h^{-1})			
DDVP	0.161 ± 0.016	0.133 ± 0.03 ^a	0.139 ± 0.032
DZN	0.062 ± 0.039	0.026 ± 0.06 ^b	0.021 ± 0.005
Ratio ^a	3	5.1	6.6
k_a (h^{-1})			
DDVP	0.017 ± 0.001	0.018 ± 0.001 ^a	0.013 ± 0.003
DZN	0.010 ± 0.003	0.009 ± 0.001 ^b	0.014 ± 0.001
Ratio ^a	1.7	2	0.9

Values in the table are the mean ± SE. Key to the table is listed under the table 8.1.

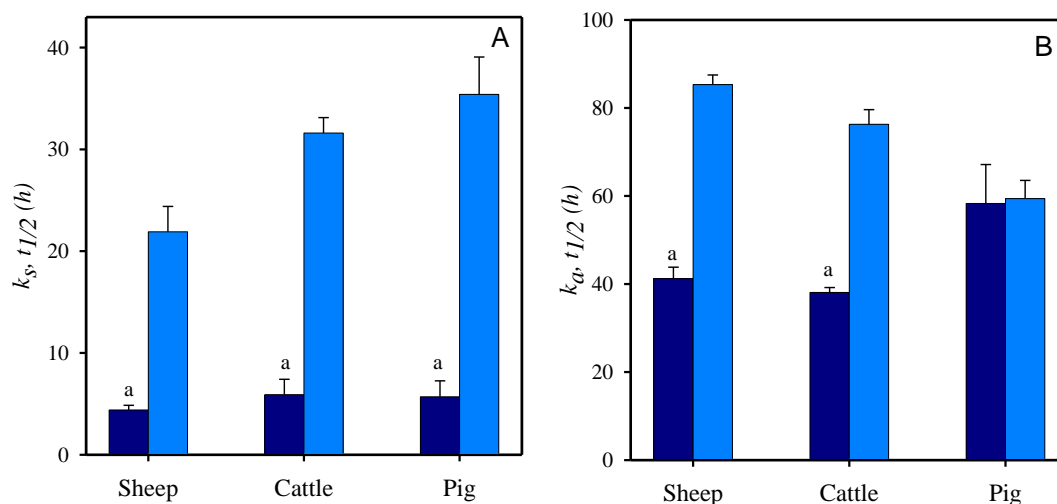


Figure 8.8 Half times for the spontaneous reactivation (k_s) and aging (k_a) of AChE inhibited by ■ DDVP and ■ DZN from muscle of sheep, cattle and pig. The letters between the DDVP and DZN are significantly different [analysis of variance (ANOVA), $P < 0.05$].

8.3.2 Affinity towards the whole acetylcholinesterase and reactivation of dichlorvos-inhibited acetylcholinesterase

8.3.2.1 Affinity towards the whole acetylcholinesterase

The dissociation affinity constants (K_{dis}) of currently available (pralidoxime, obidoxime and HI-6) and newly synthesized oximes (K005, K027, K033 and K048) to intact AChE was investigated as described in Section 8.2.6 (Table 8.3). It expressed the creation of the AChE-reactivator complex decreased of this constant indicated a higher affinity to reactivate of AChE. The values of K_{dis} obtained in this study had high affinities for the AChE from sheep, while HI-6, pralidoxime, K005, obidoxime, K027, K048 and K033 had higher affinity for cattle AChE. K027 had lower affinity for the AChE of pigs compared with the other oximes (K005, K033, K048, HI-6, obidoxime and pralidoxime). Affinity between K033 and K048 oximes for cattle and between K005 and HI-6 for pigs was comparable. The values of K_{dis} for the three animals studied increased

according to the rank order of cattle > pig > sheep for K005, K027 and HI-6. It was increased as follows: cattle > sheep > pig for K033 and obidoxime, while for K048 the rank order was sheep > cattle > pig. However, pralidoxime affinity in the animals was increased as follows: pig > cattle > sheep (Table 8.3). The relative activity (ratio of mean) between highest and lowest oximes affinity was found maximum in sheep (808) and minimum in cattle (22) (Figure 8.9).

Table 8.3 Dissociation constant (K_{dis} , μM) of the tested oximes towards AChE.

Oximes	Sheep	Cattle	Pig
K005	20.7 \pm 2.01	465.3 \pm 18.5	30.5 \pm 2.11
K027	47.4 \pm 5.36	2,836 \pm 27.1	670.6 \pm 52.5
K033	1,962 \pm 22.7	3,036 \pm 42.4	415.2 \pm 23.9
K048	6,062 \pm 63.1	3,000 \pm 23.9	149.2 \pm 14.7
HI-6	7.5 \pm 2.23	139.9 \pm 12.5	24.3 \pm 6.78
Obidoxime	962.2 \pm 25.5	1,956 \pm 10.1	64.5 \pm 10.2
Pralidoxime	100.3 \pm 27.8	294.4 \pm 13.1	304.5 \pm 24.9

Values in the table are the mean \pm SE obtained from nonlinear regression analysis, each performed in triplicate ($n = 3$ in each animal).

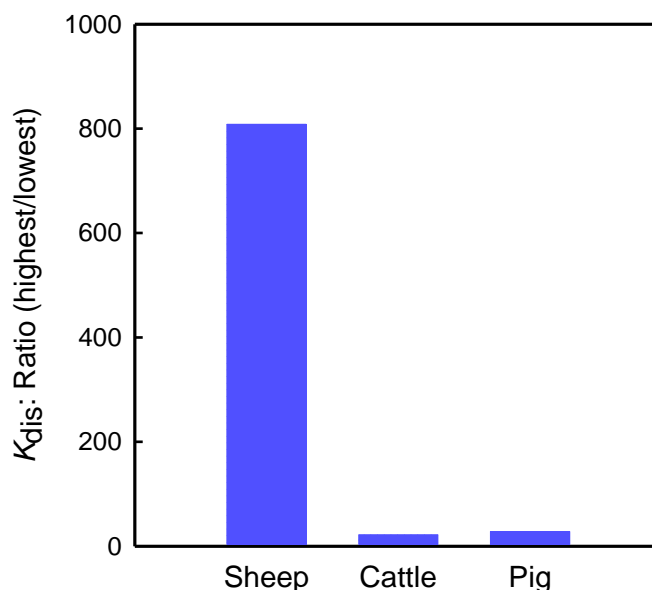


Figure 8.9 Ratio of dissociation constants (K_{dis}) from all tested oximes (K005, K027, K033, K048, HI-6, obidoxime and pralidoxime). The ratio of K_{dis} was formed between highest and lowest from table 8.3.

8.3.2.2 Reactivation of dichlorvos-inhibited acetylcholinesterase

The ability of all studied oximes kinetic parameters (K_R , k_f and k_{f2}) to reactivate DDVP-inhibited AChE *in vitro* were also investigated as described in Sections 8.2.7 and 8.2.8 (Tables 8.4-8.6). The value of K_R constant characterized the affinity of all new oximes to DDVP-inhibited AChE indicated that the K033 for sheep, cattle and pigs. But the rates of K_R for currently used oximes was as follows: HI-6 > obidoxime > pralidoxime for sheep and obidoxime > HI-6 > pralidoxime for cattle, while pralidoxime > obidoxime > HI-6 for cattle. The ratio of mean between the highest and lowest of K_R constant was found largest in cattle (568) and smallest in pig (9) (Figure 8.10A).

The values of K_R for the three animals studied increased according to the rank order of cattle > sheep > pig for K005, K027 and pralidoxime. It was increased: in order of cattle > pig > sheep for K033 and K048, while for obidoxime the

order was sheep > pig > cattle. However, the rank order for HI-6 was increased as follows: pig > sheep > cattle (Tables 8.4-8.6).

Higher k_r values were obtained for K048 followed by K033, K027 and K005 for sheep. So, highest to K033 followed by K005, K048 and K027 for cattle. Whereas in pigs the constant of k_r ranged as follows: K005, K048, K027 and K033. The rates of k_r for currently used oximes were as follows: pralidoxime > obidoxime > HI-6 for sheep, pralidoxime > HI-6 > obidoxime for cattle, and HI-6 > pralidoxime > obidoxime for pig. The relative ratio of k_r between highest and lowest was found utmost in cattle (88) and least for pig (11) (Figure 8.10B).

k_r for the three animals studied increased according to rank order of cattle > sheep > pig for K033 and HI-6. In addition, it was increased as follows: pig > cattle > sheep for K005 and cattle > pig > sheep for K027. The k_r value of K048 increased in rank order of pig > sheep > cattle and sheep > pig > cattle for obidoxime, while it was sheep > cattle > pig for pralidoxime (Tables 8.4-8.6).

K048 has the highest k_{r2} representing the overall reactivation ability, followed by K027 (520-times), K033 (629-times) and K005 (1230-times) for sheep (Table 8.4), as well as K048 have had highest for pig followed by K005 (2-times), K027 (6-times) and K033 (49-times), respectively, (Table 8.6), while K005 was highest in cattle (Table 8.5). The k_{r2} values for K033 and K048 was comparable in cattle (Table 8.5). On the other hand, pralidoxime had the highest k_{r2} followed by HI-6 and obidoxime for sheep, while HI-6 in cattle had highest k_{r2} followed by pralidoxime and obidoxime. Obidoxime had the highest k_{r2} followed by HI-6 and pralidoxime for pigs. k_{r2} between K027 and K033 for sheep and between K033 and K048 were comparable in cattle.

The values of k_{r2} for the three animals studied increased as follows: pig > cattle > sheep for K005 and K027. It was increased according to the rank order of cattle > sheep > pig for K033 and HI-6, while for pralidoxime and obidoxime, the rank order was sheep > pig > cattle. However, k_{r2} of K048 was increased as follows: sheep > cattle > pig (Tables 8.4-8.6). The ratio of highest/lowest of reactivation constants was found the biggest in sheep (1229) and smallest for cattle (15) (Figure 8.10C). The percentage reactivation potency of the oximes at concentration 10^{-3} M was higher than that seen 10^{-2} M in the case of K005 and K033 for sheep (Figure 8.9) as well as in the case of cattle with obidoxime (Figure 8.10), but for K005 and obidoxime in pigs (Figure 8.11). On the other hand, the perfusion of sheep liver treated by oxime K048 alone or K048 with DDVP was seen significantly different between control and treated groups (Figure 8.14).

Table 8.4 Kinetic parameters of reactivation of DDVP-inhibited AChE from sheep.

Oximes	K_R (μ M)	k_r (min^{-1})	k_{r2} ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
K005	1,020 \pm 33.2	0.007 \pm 0.0006	7
K027	1,283 \pm 54.4	0.020 \pm 0.0041	16
K033	3,179 \pm 41.6	0.041 \pm 0.002	13
K048	19.4 \pm 4.14	0.158 \pm 0.005	8
HI-6	1,896 \pm 12.7	0.234 \pm 0.045	123
Obidoxime	4,392 \pm 69.7	0.279 \pm 0.036	64
Pralidoxime	2,093 \pm 16.9	0.321 \pm 0.0741	153

Values in the table are the mean \pm SE obtained from nonlinear regression analysis, each experiment performed in triplicate ($n = 3$).

Table 8.5 Kinetic parameters of reactivation of DDVP-inhibited AChE from cattle.

Oximes	K_R (μM)	k_r (min^{-1})	k_{r2} ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
K005	3,016 \pm 103.02	0.185 \pm 0.002	62
K027	1,968 \pm 10.46	0.085 \pm 0.0854	43
K033	4,381 \pm 17.074	0.245 \pm 0.0225	26
K048	3,606 \pm 41.18	0.090 \pm 0.0127	25
HI-6	713.6 \pm 9.2	0.265 \pm 0.0085	371
Obidoxime	7.7 \pm 2.62	0.003 \pm 0.001	39
Pralidoxime	2,245 \pm 11.57	0.132 \pm 0.0205	59

Values in the table are the mean \pm SE obtained from nonlinear regression analysis, each experiment performed in triplicate (n = 3).

Table 8.6 Kinetic parameters of reactivation of DDVP-inhibited AChE from pig.

Oximes	K_R (μM)	k_r (min^{-1})	k_{r2} ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
K005	426.1 \pm 31.1	0.0593 \pm 0.009	139
K027	516 \pm 37.3	0.025 \pm 0.004	48
K033	3,321 \pm 47.4	0.020 \pm 0.0002	6
K048	624.1 \pm 36.7	0.184 \pm 0.026	296
HI-6	3,934 \pm 120.5	0.215 \pm 0.002	55
Obidoxime	2,204 \pm 45.4	0.133 \pm 0.008	60
Pralidoxime	329.1 \pm 42.8	0.023 \pm 0.006	69

Values in the table are the mean \pm SE obtained from nonlinear regression analysis, each experiment performed in triplicate (n = 3).

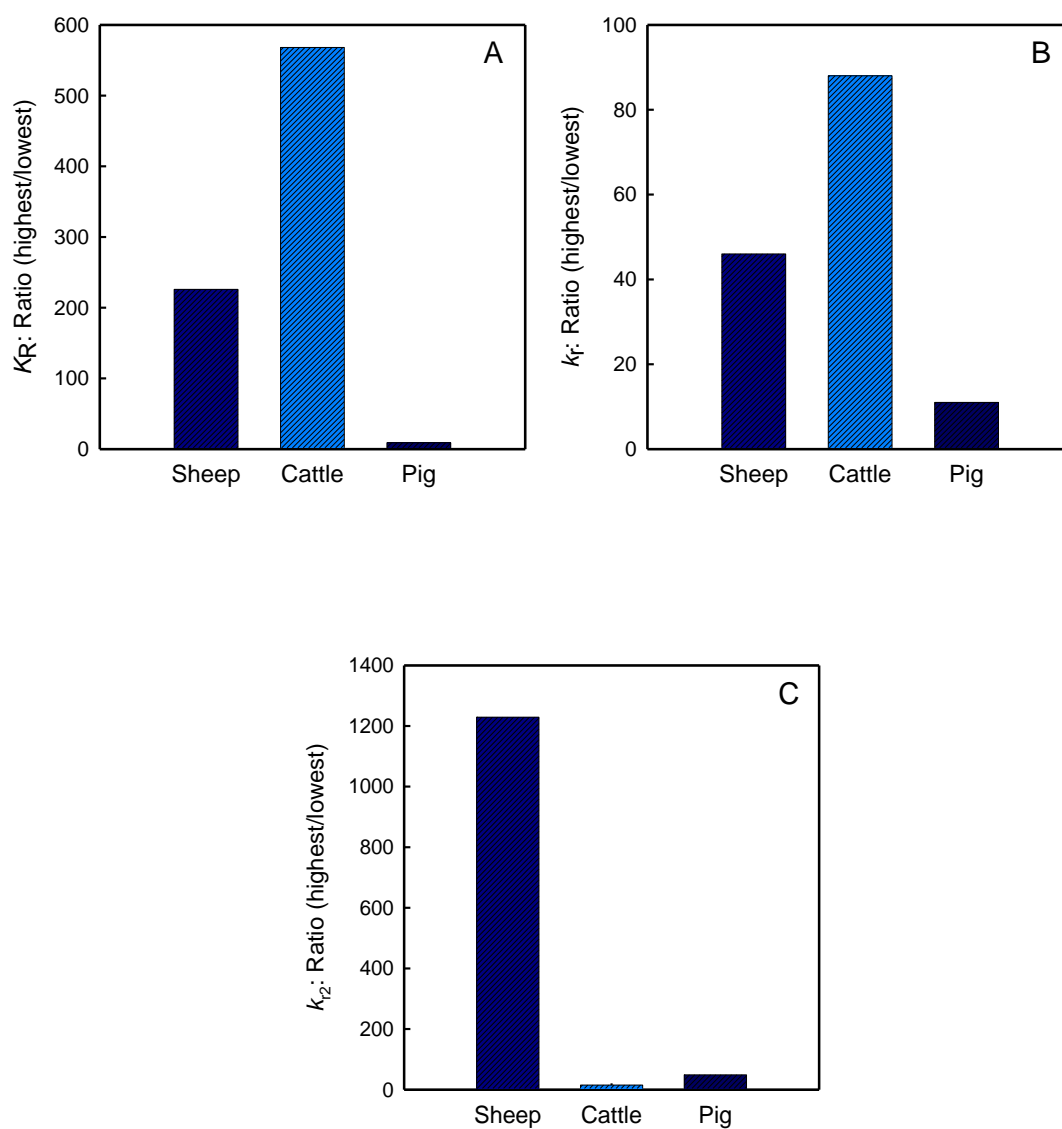


Figure 8.10 Ratio of reactivation constants from all tested oximes (K005, K027, K033, K048, HI-6, obidoxime and pralidoxime). The ratio of K_R (A), k_r (B) and k_2 (C) was formed between highest and lowest from tables 8.4-8.6.

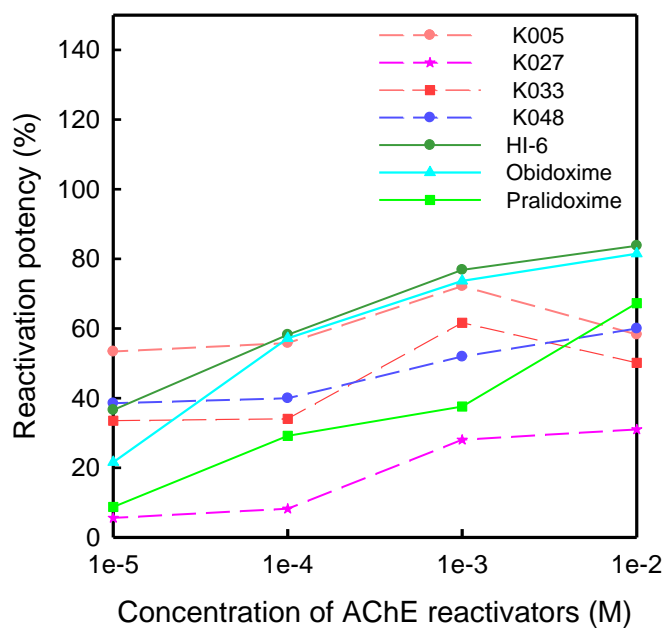


Figure 8.11 Effect of oximes to reactivate sheep liver AChE inhibited by DDVP. Homogenated liver of sheep treated with DDVP (8 μM final concentration) for 30 min at 20 $^{\circ}\text{C}$ to achieve a sample inhibition of >90%. Oximes (10^{-2} - 10^{-5} M final concentration) were then added and incubated for 0-40 min under the same conditions. Then measured the enzyme activity as explained in the section on ChE determination.

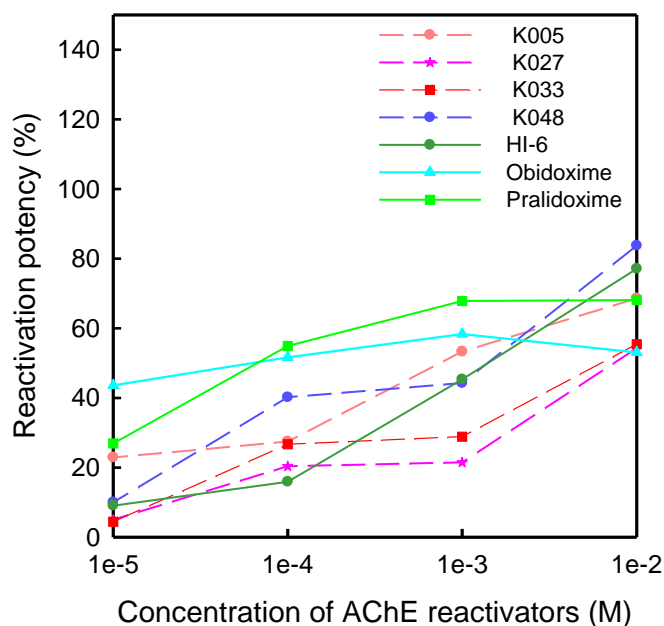


Figure 8.12 Effect of oximes to reactivate cattle liver AChE inhibited by DDVP. Homogenated liver of cattle was treated with DDVP (8 μ M final concentration) for 30 min at 20 °C to achieve a sample inhibition of >90%. Oximes (10^{-2} - 10^{-5} M final concentration) were then added and incubated for 0-40 min under the same conditions. Then measured the enzyme activity as explained in section on ChE determination.

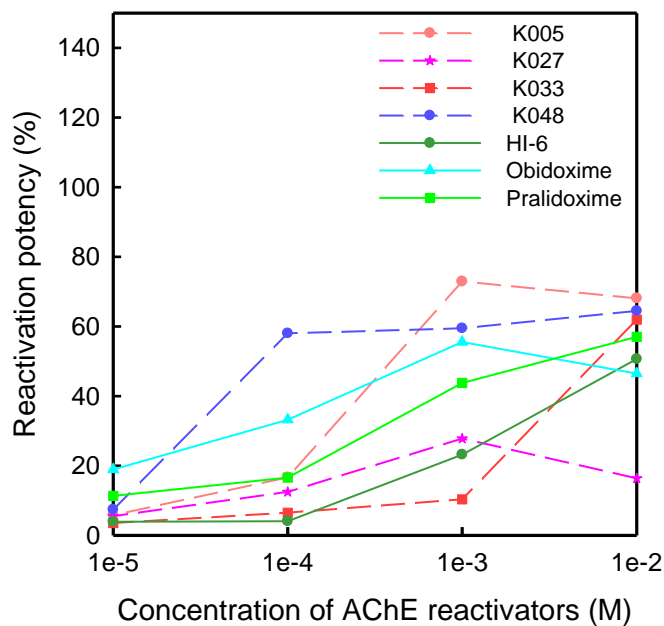


Figure 8.13 Effect of oximes to reactivate pig liver AChE inhibited by DDVP. Homogenated liver of pig was treated with DDVP (8 μ M final concentration) for 30 min at 20 °C to achieve a sample inhibition of >90%. Oximes (10^{-2} - 10^{-5} M final concentration) were then added and incubated for 0-40 min under the same conditions. Then measured the enzyme activity as explained in the section on ChE determination.

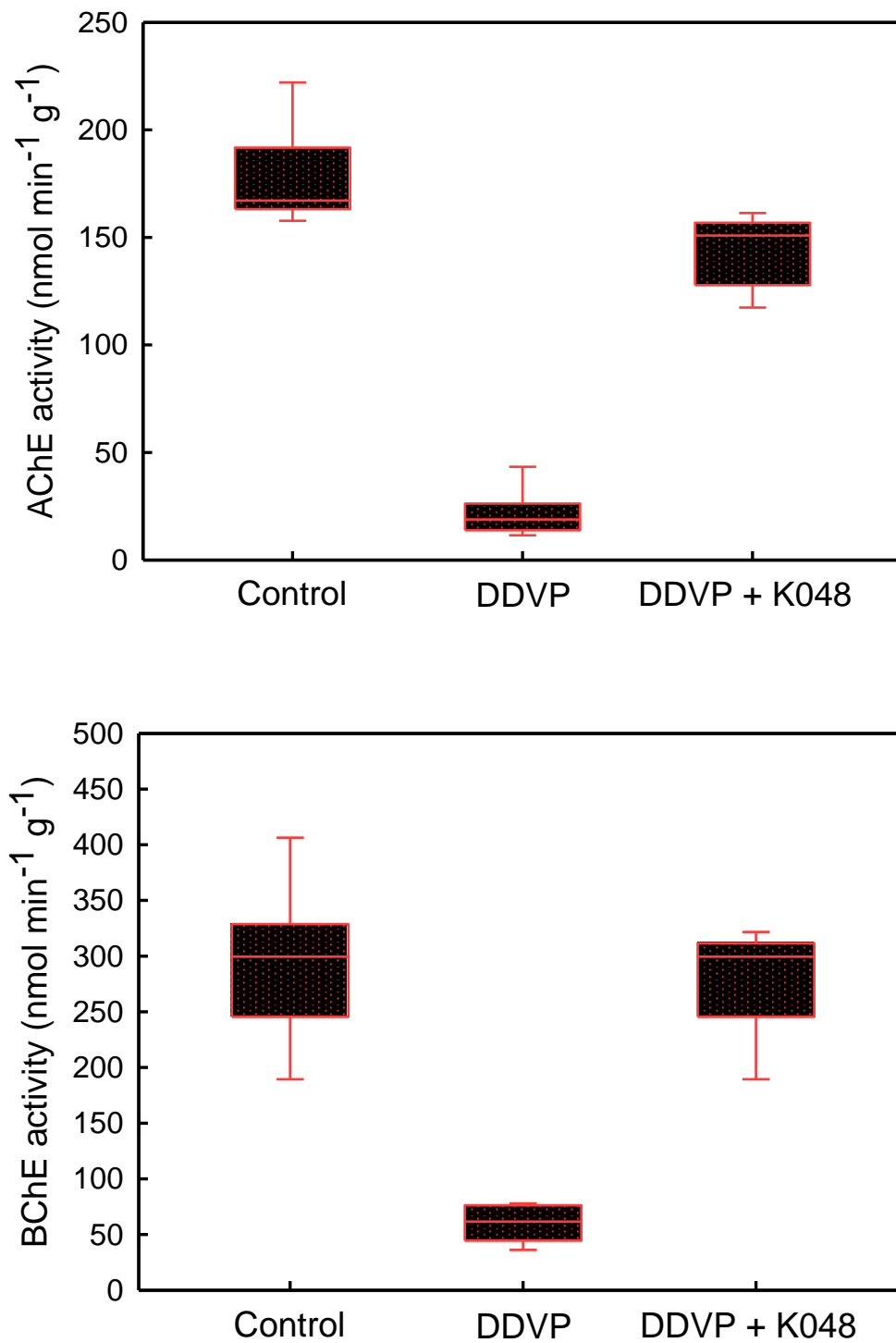


Figure 8.14 Box plots of the range AChE and BChE activities reported to be taking DDVP and DDVP with K048 of liver perfusion.

8.4 Discussion

8.4.1 Spontaneous reactivation of acetylcholinesterase

To the best of our knowledge, this is the first study, which compares the k_s , and k_a of OP inhibited AChE in the liver and muscles for sheep, cattle and pigs. Phosphorylated AChE is susceptible to spontaneous hydrolysis of an alkyl-ester bond, resulting in a negatively charged residue which is resistant towards nucleophilic attack (Aldridge and Reiner, 1973). The rate of k_s to inhibit erythrocytes AChE by DDVP has been observed to be 0.92 min^{-1} for cattle (Škrinjarić-Špoljar *et al.*, 1973), 0.347 h^{-1} for rat (WHO, 2007), while k_s values to DZN was 0.012 h^{-1} for human (WHO, 2007), but was 0.408 and 0.019 h^{-1} for DDVP and DZN, respectively in ethanol (Morifusa, 1974). Comparable to our work DDVP was higher in the liver and muscle of cattle than in erythrocytes, which ranged between 0.133 and 0.323 h^{-1} , but lower than DZN, and ranged between 0.021 to 0.088 h^{-1} . These differences may be due to AChE activity in the erythrocytes being more sensitive to DDVP than in the liver and muscle cells, unlikely with DZN. However, k_s in the liver proceeded substantially faster with cattle DDVP and DZN compared with the other animals. While muscle k_s was faster with cattle exposed to DDVP and pig to DZN compared with other animals (Tables 8.1 and 8.2).

Literature values of the half time ($t_{1/2}$) of k_s for AChE is about 2 and 58 h for DDVP and DZN, respectively in human erythrocytes (Wilson and Philip, 2005; WHO, 2007). These findings are in agreement with our results, where observed DDVP $t_{1/2}$ of k_s was lower than DZN $t_{1/2}$ (Figure 8.7A). The recovery of the rate k_a of reserved erythrocyte AChE by DDVP was $2.62 \times 10^4 \text{ min}^{-1}$ and $7.77 \times 10^4 \text{ min}^{-1}$ for cattle and horse, respectively (Aurbek *et al.*, 2006), and DZN 0.017 h^{-1} for human (Wilson *et al.*, 2005). In agreement with our result with DZN that

ranged between 0.009 to 0.018 h⁻¹, while lower than reported with DDVP. Except for muscle DZN-inhibited AChE k_a proceeded markedly later than k_a of cattle and pigs. In addition, $t_{1/2}$ of k_a for human erythrocytes AChE is about 41 h for DZN (WHO, 2007), concurring with our results which ranged between 39.1 to 85.3 h (Figure 8.7B). This indicates that the reduction of DDVP $t_{1/2}$ in the liver and muscle might alter the use of oximes. In clinical research, the level and time course of k_a is important, because it is the factor that limits the period for useful oximes administration after affecting food animals with OP pesticides (Fairbrother *et al.*, 1991; Worek *et al.*, 2004; Aurbek *et al.*, 2006). Furthermore, this study found that values of k_a and k_s could also play a role on the administration of oximes for food animals.

8.4.2 Affinity towards the whole acetylcholinesterase and reactivation of dichlorvos-inhibited acetylcholinesterase

The our work has presented *in vitro* experiments for several oximes because a previous study showed that *in vitro* and *in vivo* results were similar (Kassa and Cabal, 1999). DDVP has been pointed in this work due to the main contaminants in the cases of OP intoxication in the world. However, the antidotal treatment for OP poisonings are not sufficiently effective (Timothy, 2001). DDVP is an OP compounds and poisoning with this compound has been treated with the cholinergic receptor antagonist atropine and with oximes (mainly pralidoxime) in an attempt to reactivate the OP-inhibited AChE. It has been proposed that early death from severe poisoning with DDVP is mediated through the central, but not peripheral, nervous system actions (Steven *et al.*, 2003). However, pralidoxime has not been efficient to reactivate DDVP-inhibited AChE and its routine use has been questioned. One of the objectives of this study was evaluated the *in vitro* potency of standards and newly developed

oximes in reactivating DDVP-inhibited AChE derived from food animals liver supernatants.

Steric compatibility, electrostatic effects and hydrophobic interaction effect of the affinity constants K_{dis} and K_R . Oxime HI-6 has a highest affinity for AChE among all oximes used for sheep, cattle and pigs followed by K005 for sheep and pigs, while pralidoxime has the highest affinity in cattle (Table 8.3). This is in agreement with the finding of Kuca and Cabal (2004) stated that the oxime HI-6 had higher affinity than other oximes such as pralidoxime, obidoxime, K005, K027, K033 and K048).

Dissociation constants (K_R) to reactivate DDVP-inhibited AChE by K005 for sheep and pigs as well as obidoxime for cattle (Tables 8.4 and 8.6) are lower with those found by Kuca *et al.* (2005). These authors used a rat brain and OP (VX)-inhibited instead liver and OP (DDVP)-inhibited. Both of these factors may explain the apparent difference in the value of K_R .

K_R constant affinity to DDVP showed the highest K048 for sheep, obidoxime for cattle, and K005 for pigs. Thus, have a high velocity of reactivation of DDVP-inhibited AChE, and are able to extensively reactivate DDVP-inhibited AChE at relatively low concentrations. Oxime obidoxime for sheep and HI-6 for pigs were lower affinity compared to K-series compounds (Tables 8.4 and 8.6). This is in agreement with an earlier report by Kuca *et al.* (2005) who observed that tabun-inhibited AChE is lowest affinity to obidoxime compared K027, K033 and K048 (Kuca *et al.*, 2005). It also in agrees with the work of Petroianu *et al.* (2007) who mentioned that the K-series compounds had higher affinity than the other currently used oxime pralidoxime.

Oxime K048 for sheep and pig, while oxime HI-6 for cattle was found to be higher efficacious reactivator AChE inhibited with DDVP than the other oximes used in this study. This is in agreement with the work of Aurbek *et al.* (2006) found that oxime HI-6 for pig was found to be higher efficacious reactivator AChE inhibited with VR and CVX than other (e.g. pralidoxime and obidoxime). These interesting results differ with those of (Aurbek *et al.*, 2006; Herkert *et al.*, 2010) they stated that oxime HI-6 for pig AChE inhibited to be lowered efficacious reactivator than other oximes (e.g. obidoxime). Oxime K005 was lowered efficacious reactivator for sheep (Table 8.4). This is in agreement with the finding of Kuca and Kassa (2003) who mentioned that K005 was less efficacious than the other K-series compounds and currently used oximes in the case of sarin inhibited AChE. Oximes K033 seems to be lowered efficacious reactivator than other oximes for cattle and pig (Tables 8.5 and 8.6). This differs from the results of Kuca *et al.* (2005) who found that K033 was more effective for reactivating AChE than the other (e.g. pralidoxime, obidoxime, HI-6, K005 and K027). These authors used OP-VX-inhibited AChE instead DDVP, however, may explain the apparent difference in efficacious reactivator.

The percentage of AChE reactivates to DDVP-inhibited AChE was higher in HI-6 for sheep (Figure 8.11), K005 for cattle (Figure 8.12), and K048 for pig (Figure 8.13). This is in agreement with the work of Kuca *et al.* (2005) who found that the percentage AChE reactivation by HI-6 was higher than those by other oximes in AChE inhibited with cyclosarin. Kassa (1995) found that HI-6 was more efficacious reactivation than obidoxime to reactivate soman inhibited AChE. While differ with the work of oxime inhibited with sarin and tabun (Kuca *et al.*, 2005). Likewise, the reactivation potency in case of K027 at concentration M^{-3} was 21-27% and at concentration M^{-5} was 4-6%. This is in agreement with

the work of Kuca *et al.* (2010) where they found that K027 in case of DDVP inhibition was ranged between 5-26% at for concentrations M^{-3} and M^{-5} , respectively.

I confirmed these interesting results by perfusion of liver sheep by K048 in Figure 8.14. This was done by reasons of (a) to build a simple *in vitro* liver perfusion model for investigating the clinical effectiveness of OP poisoning, (b) to determine the effect of perfusion on the OP compounds of newly synthesized oximes, and (c) to evaluate the effect of liver perfusion model of anti-ChE effects. It is our aim also to develop a portable liver perfusion system that allows reactivating of inhibited AChE and BChE activities by DDVP compound to longer preservation than with *in vivo* while maintaining liver viability. The requirements were at 37 °C, gassed with 95% O₂: 5%CO₂ (normal CO₂) system has been indicated in several experiments (Ali *et al.*, 2000). I examined the changes in liver perfusion at two phases in this study. I found liver perfusion with K048 detects in higher levels of AChE and BChE activities than liver inhibited with DDVP compound (Figure 8.14). These results suggest that in the sheep liver, the toxicity of DDVP is mediated by hepatic and extra-hepatic activation. This is agreement with work of Sultatos *et al.* (1985) found in the mouse liver, the acute toxicity of chlorpyrifos is mediated by extra-hepatic production of oxon, whereas that of parathion is likely mediated by both hepatic and extra-hepatic activation.

8.5 Conclusions

In conclusions, this study shows for the first time that original data concerning an AChE activity reactivation after inhibiting with OP compounds for sheep, cattle and pigs. Furthermore, these results are pointed at the importance of

identifying the suitable oximes for treatment of exposed animals with anti-ChE pesticides.

In view of the evidence I have studied I think it is fair to say.

1. In this research, we designed recent developments in our understanding of the kinetic properties of k_s and k_a for sheep, cattle and pig AChE are comparable in view of interactions with DDVP and DZN. In liver from all three animals studied, the rate of the aging process is much slower than spontaneous reactivation of AChE inhibited with DDVP and DZN. This was also true in muscle with DDVP and DZN, although the difference was less. Hence, in the case of $t_{1/2}$ AChE inhibited with DZN the enzyme will tend to become much higher than those by the DDVP. The determination of reactivation and aging constants of dimethylated and diethylated OP pesticides with food animals AChE indicates that a structure activity relationship can be derived for the inhibition as well as for spontaneous reactivation but not for dealkylation and oxime-induced reactivation.

2. This study shows also the first time that no single, broad-spectrum oxime is suitable for the antidote treatment of poisonings with OP compounds for food animals. In addition, indicated that the developed bispyridinium symmetric (K048) oxime seems to be promising reactivated to DDVP-inhibited AChE for sheep and pigs while HI-6 was effective in cattle. However, obidoxime, ineffective against DDVP compared to pralidoxime. Furthermore, little change in the structure of the AChE reactivator can greatly affect its affinity to intact or inhibited enzyme. Oxime-induced reactivation of phosphorylated AChE activity is becoming a complementary methodology for assessing field exposure to OP pesticides (Worek *et al.*, 2007). Finally, the present data is very important because they indicated that asymmetric bispyridinium oximes of the K-series

compounds are promising antidotes for DDVP poisoning and it was more efficacious for sheep and pig compared to cattle.

Chapter 9

General discussion and recommended future work

Chapter 9: General discussion and recommended future work

9.1 General discussion

This thesis focuses on establishing a foundation for the applicability of a biochemical biomarker, ChE activity, in food animal species, as an instrument for evaluating exposure to pollutants or pesticides as well as predicting high-level effects to public health (Wilson *et al.*, 2001; Wilson and Philip, 2005). Measurements of ChE activity have been made for over 60 years. The techniques employed to determine of ChE activities include titrimetry (Stedman, 1932), manometry (Ammon, 1933), the Hestrin method (Hestrin, 1949), Δ pH method (Michel, 1949), spectrophotometry (Ellman *et al.*, 1961), and the radiometric method (Johnson and Russell, 1975). Most of these methods suffer problems of reproducibility or complexity (Wilson *et al.*, 2001; Wilson *et al.*, 2002).

The first step for the thesis was to investigate a good quality method to determine ChE activities in the tissues of slaughtered food animals. Hence, two methods extensively used for the measurement of ChE activities were tested. The first, the Ellman spectrophotometric method (Ellman *et al.*, 1961) does not use the natural substrate, however it was accurate and sensitive. When this method was used manually, it was not rapid enough to analyse a sufficient number of samples to achieve a reliable estimation of the kinetic parameters. This method was then modified for use in a microtitre plate reader system (Haigh *et al.*, 2008; Leticia and Gerardo, 2008). This modified system was capable of testing the effect of anti-ChE (inhibitor or reactivator) rapidly (up to 28 samples in triplicate in 5 min). The sensitivity of the method was good and

low amounts of reaction mixture (0.3 ml of 1 mM substrate per measurement) were used.

The second method, a modified Michel method, was used for the initial stages of this study as it had the advantage of using the natural substrate ACh (Mohammad, 1997). This method was fairly reliable for high enzyme activity determinations but was unreliable for the measurement of low enzyme activity samples due to atmospheric interference and fluctuations in the pH meter. Therefore, the work described in Chapter 3 was designed to compare these two most widely used methods (Figures 3.1 and 3.5) to confirm the most precise method and to establish a foundation for the most applicable method in food animal species. I found that the Ellman modified method provided better precision than the modified Michel method in determining ChE activities because the %CV values were lower in the Ellman method (Figure 3.3). In general, an increase of %CV values has been demonstrated to be associated with a reduction in the precision of methods (Lewis *et al.*, 1981), whereas other studies have demonstrated that a lower %CV value in the tissues is associated with increased precision of the method (Hawkins and Knittle, 1972; Lewis *et al.*, 1981).

Few studies have investigated the stability of AChE and BChE activities. The aim of the work described in Chapter 4 was to determine the stability of AChE and BChE activities during long-term freezing. There are two methods extensively used for the storage of enzymes, freezing at -80 °C (Kirby *et al.*, 2000) and at -20 °C (Crane *et al.*, 1970). Therefore, the work described in Chapter 4 was designed to compare the effects of the two most widely storage methods on AChE and BChE activities. In Chapter 4 a significant loss of both

AChE and BChE activities was found after 6 months of freezing at -80 °C and after 3 months of freezing at -20 °C. Furthermore, a linear relationship was found between mean AChE and BChE activities and storage time. These results could be important to freezing of meat in semiarid tropical regions of the world. This especially so in Asia, where intensification of food animal production is rapidly increasing (Al-Jobory and Mohammad, 2004).

The fourth objective of this study was to develop a protocol for the purification of AChE from sheep liver and to extend the purification method for further enzyme characterization. Currently, affinity chromatography has been extensively used for the purification of AChE in Japanese quail (Son *et al.*, 2002) and in humans (Philipp, 1994). Therefore, the aim of the work in Chapter 5 was to develop a protocol for the purification of AChE from sheep liver based on methods previously applied to other animal tissues (Table 5.2). The outcome of this work was that AChE was purified 842-fold with a specific activity of 21 U/mg protein. The purified protein had the expected monomeric molecular mass (SDS-PAGE), whereas gel filtration chromatography with Sephacryl S-200 under nondenaturing conditions showed the protein to have the expected tetrameric molecular mass.

In order to achieve the lowest detection limit for pesticide measurements, it was first necessary to determine the effects of dilution on the enzyme, the relative levels of AChE and BChE activities by using a specific inhibitors, optimal pH, temperature effects and histochemical localisation of the enzyme. All of these factors gave optimal results for AChE and BChE in tissues of food animals which had not had any exposure to pesticide compounds. Many studies have already characterized ChE activities in different species, e.g. *Bufo marinus* (Bui

and Ochillo, 1987) and earth worm (Caselli *et al.*, 2006; Rault *et al.*, 2007). The work in Chapter 6 was therefore designed to characterize AChE and BChE and to investigate samples for assessment of risk to meat products entering the human food market. The practical implications of the findings are:

1. BW284c51 were strongly reduced AcTChI and PrTChI hydrolysis and slightly affected that of BuTChI in the liver, and the iso-OMPA had no significant effect for muscle BuTChI of sheep and pig;
2. the optimal pH values ranging between 7.8 and 8.1 for liver and muscle AChE, while in BChE pH values were ranged between 8.3 and 8.5 for liver and between 7.7 and 7.9 for muscle;
3. significant effects of the time course (5 to 20 min) and temperatures effects (15 to 50 °C) on AChE and BChE activities were increased with an increase in temperature; and
4. histochemical localisation of AChE in sheep liver by using two methods (Gomori and Kugler) showed the most AChE was located in the cytoplasm of the cell lining in the sinusoids, with a decreasing concentration gradient from the central vein to the periphery of the lobule.

The significance of these results has been discussed within Chapter 6, but it is important to state that the reason why AcTChI is higher in the liver and muscle samples is not known until now.

The sixth objective of this thesis was to investigate the kinetic properties of ChE activities. In addition, to establishing the effects of pH and temperature with the objective of diagnosing of OP and carbamate poisoning and to monitor the recovery of intoxicated animals. The use of AChE or BChE activities for the

inhibition based for determination of pesticides shown great promise for environmental screening analysis. AChE or BChE are irreversibly and reversibly inhibited by pesticides (e.g. OP and carbamate compounds, respectively). OP and carbamate insecticides are still represent important pesticides, which are used worldwide in agriculture to protect plants and animals and to prevent crop damages due to insects. Hence, a comparison of activity of AChE or BChE activities before and after exposure to tissue samples can provide an evaluation of the pesticide level. Several studies have already demonstrated the kinetic properties of ChE activities in different species, e.g. auricle (Tryfonos *et al.*, 2009), rat (Jokanović *et al.*, 1996), earth worm (Sanchez-Hernandez *et al.*, 2009) and snail (Laguerre *et al.*, 2009). Therefore, the aim of the work in Chapter 7 was dealt with the *in vitro* kinetic affects of OP and carbamate compounds in tissue samples by using modified Ellman method. In addition, it assessed the risk of contaminated meat products entering the human food market. A new method for the detection of pesticides using in food animals by the inhibition of ChE was developed. In addition, Ellman modified method seemed very promising as a biosensing test for screening pesticides in environmental samples. The practical implications of the findings are:

1. V_{\max} AcTChI in animals decreased in rank order of pig > cattle > sheep for liver, muscle and kidney. Whereas, V_{\max} for BuTChI increased as follows: pig > cattle > sheep for liver and pig > sheep > cattle for muscle, while it increased from pig > cattle > sheep for kidney;
2. IC_{50} was found to be higher in carbaryl than in DDVP and DZN, about 3-4 times higher in liver and about 4-6 times higher in muscle; and

3. in general, the k_i values increased in different pHs according to the rank order of $8.5 > 7.5 > 6.5$, while they decreased in different temperatures as follows: $20\text{ }^\circ\text{C} > 30\text{ }^\circ\text{C} > 40\text{ }^\circ\text{C}$.

The last objective of this study was to investigate the rate of spontaneous reactivation of AChE inhibited by OP compounds, and to determine the time course of aging of OP-inhibited AChE. In addition, to find a suitable reactivator for AChE and to recommend the most efficacious oxime compounds for the next evaluation as antidotes for intoxication by OP compounds. Other studies have already found oximes that reactivate AChE in other species (Kuca *et al.*, 2005; Worek *et al.*, 2010). Therefore, the aim of the work in Chapter 8 was to investigate *in vitro* reactivation of AChE activity. In Chapter 8, also the implication of oxime compounds as a treatment for food animals was investigated. It was designed with the aim of understanding the relationship between standard and new synthesis oxime as a treatment used in optimising animal health through their ability to resist intoxication by OP compounds. In addition, to consider the most suitable oximes (Tables 8.3-8.6) for treatment of intoxicated food animals by pesticide compounds. It was hypothesized to confirm that the reactivation effects depend on:

1. number of pyridinium rings;
2. number of oxime groups and their position; and
3. length and the shape of the linkage bridge between the pyridinium rings.

The results demonstrated that:

1. first-order rate constants obtained for k_s were higher in sheep than in cattle and pig;
2. the aging (k_a) was higher in cattle than in sheep and pig;

3. K_{dis} for oximes were obtained a highest affinity for intact AChE to HI-6 for sheep, cattle and pigs;
4. K_R affinity of new oximes to DDVP-inhibited AChE indicates that the high affinity to K048 in sheep and obidoxime in cattle, while K005 was highest in pig. First-order rate constant for reactivation (k_r) was obtained highest for pralidoxime in sheep and HI-6 in cattle and pig; and
5. oxime K048 have had the highest k_{r2} in sheep and pig, while HI-6 was highest in cattle. However, this aspect was beyond the scope of this study and further research on this area is recommended. Therefore, it will be of economic importance to undertake more studies in this area to make this process more efficient.

9.2 Recommended future work

This study has identified areas for future research both generally for OP and carbamate compounds and more specifically with regard to the AChE and BChE activities in food animals. Regarding the former, it is important that future studies monitor and check for drifts in reaction-controlling variable like temperature and pH especially in replicated batch type tissues of the kind undertaken here. Studies should also investigate and quantify the effect of these factors for other food animals (e.g. rabbit, goat, horse and deer) and for improved estimates of AChE and BChE activities.

Other areas for future work include conducting parallel experiments in the different organs (e.g. brain, pancreas, intestine, stomach and spleen). Regarding the kinetic effects of ChE, a better understanding of the kinetic inhibition of OP and carbamate compounds used in this study for food animals requires a better understanding of their speciation. Thus, the kinetic

characterization for these compounds requires an improvement and expansion. In particular, little is known about their kinetics with AChE and BChE. Among the OP, therefore, soman and sarin should perhaps be considered more urgently for future studies. Further studies are suggested based on present results to determine possible differences of AChE and BChE activities between male and female and to make further comparisons with this study.

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Appendix: Training courses and taught sessions attended

1. Courses and workshops

- English Language Summer School (Intensive course)-Academic writing (June 2008 to September 2008), University of Plymouth.
- Postgraduate Research Skills and Methods in Biology (October 2008 to January 2009), University of Plymouth.
- Supporting English Language Classes (October 2008 to January 2009), University of Plymouth.
- Further Supporting English Language Classes (October 2009), University of Plymouth.
- General Teaching Associates Course (8th October 2009 to 12th November 2009), University of Plymouth.
- Laboratory Based Teaching and Methods Practice (Env 5101) (October 2009 to December 2009), University of Plymouth.
- Continuing Professional Development Course for Clinical Pharmacologists (14th-16th December 2010), Royal College of Physicians of London.
- Continuing Professional Development Course for Clinical Trials and Regulatory Affairs (10th-11th January 2011), University of Plymouth.
- Continuing Professional Development Course for Continuing Education Programme Clinical Biomarkers (27th-30th March 2011), University of Durham.
- Student Associate Scheme Training, organized by the Faculty of Education (6th-24th June 2011), Prince Rock Primary School, Plymouth.
- Postgraduate English Language Summer Classes (June 2011 to July 2011), University of Plymouth.
- Real-time PCR course Exeter (November 2011), Dartington Hall Conference Centre, Totnes.
- General Teaching Associates Course (9th-13th July 2012), University of Plymouth.

2. Taught sessions

- Impact factors (19th September 2008).
- Endnote for beginners (7th November 2008).
- LaTeX part 1 (11th November 2008).
- Free sessions on learning academic vocabulary (14th November 2008).
- Introduction to electronic resource (18th November 2008).
- Word 2007 creating forms (19th November 2008).
- Planning a carrier in research (19th November 2008).
- Introduction of Microsoft excel 2007 (20th November 2008).
- Dr in three years (21st November 2008).

- Introduction to R (26th November 2008).
- Career shaping planning for a career in a non-academic sector (26th November 2008).
- Overview of the University of Plymouth intranet for PGR's (3rd December 2008).
- Giving and receiving feedback (4th December 2008).
- LaTeX Part 2 (20th January 2009).
- Writing and developing CV's for academic purposes (21st January 2009).
- Excel 2007: further features (27th January 2009).
- C.V. writing for non-academic roles and the creative job search (28th January 2009).
- Risk management for research student (29th January 2009).
- Managing tough application (4th February 2009).
- Presentation skills part 1 (9th February 2009).
- Career Planning-How your personality can impact on career decisions (11th February 2009).
- The transfer process (18th February 2009).
- Winning at job interviews (4th March 2009).
- Preparing for the viva (5th March 2009).
- Plagiarism, your word or others (10th March 2009).
- Preparing effective poster presentation (12th October 2009).
- New features of office 2007 (13th October 2009).
- Creating web pages using Microsoft office share point designer 2007 (20th October 2009).
- Microsoft office 2007: Structuring your thesis (23rd October 2009).
- Introduction to E-Portfolio with pebble pad (28th October 2009).
- Creating graphics using paint shop pro X2 (4th November 2009).
- Overview of the University of Plymouth intranet for pgr's (10th November 2009).
- Presenting to an audience part 1 (10th November 2009).
- Word 2007 proofing and tracking changes (24th November 2009).
- Presenting to an audience-part 2 (25th November 2009).
- Analyses that-stats clinic (9th December 2009).
- Postgraduate society short conference 2011 (29th June 2011).

3. Awards

- British Pharmacological Society, Travel grant for British Pharmacological Society-(BPS Winter Meeting), London-UK, December 2010, £75.
- Biochemical Society, Travel grant for Stress Responses-Molecules Organisms and Environments conference, London-UK, January 2011, £200.
- EuroPACT 2011, Travel grant for 2nd European Conference on Process Analytics and Control Technology, Glasgow-UK, April 2011, €500.