INTRODUCTION

Organophosphorus (OP) insecticides still pose a major problem in toxicology. The use of OP compounds for pest control and attempted suicide causes huge numbers of intoxications and several hundreds of thousands of fatalities per year mainly in developing countries (Worek et al., 2004). In the current study, we have chosen dimethylated OP dichlorvos (DDVP) and diethylated OP diazinon (DZN). Both of them are routinely used in veterinary medicine. 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 source of contamination to susceptible hosts. Targeted pest control on 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 intoxication with OP causes a generalized cholinergic crisis due to an irreversible inhibition of acetylcholinesterase (acetylcholine-lyase, acetylcholinesterase (AChE), EC 3.1.1.7) by phosphorylation of their active site serine (Worek et al., 2004) and successive accumulation of the neurotransmitter acetylcholine. 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 enzyme (Morifusa, 1974; Worek et al., 2004).

However, the OP-inhibited AChE changes gradually into a non-reactivatable form on storage. This phenomena is called aging (dealkylation). 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 alkoxyl group of OP residue bound to AChE (Morifusa, 1974; Patocka et al., 2004). Recently, the standard treatment for OP poisoning comprises a combination of atropine, an oxime, e.g. pralidoxime chloride or obidoxime. Oxime compounds that can reactivate OP-inhibited AChE by attaching to the phosphorus atom of the OP compounds forming an oxime-phosphonate, which splits away from the AChE molecule (Worek et al., 2004). The main objectives of this

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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. A further aims to determine the time course of aging of OP-inhibited AChE from liver and muscle of food animals.

**MATERIALS AND METHODS**

**Animals**
Meat from healthy food animals (sheep, cattle, and pigs) from local markets in Plymouth and abattoirs in Cornwall (Callington and Launceston, UK), was used in this study. The samples (liver and muscle) were transported on ice to the laboratory of Biomedical and Biological Sciences at University of Plymouth for immediate processing.

**Sample preparation**
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, 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 10,000 rpm. Homogenisation required between 2 and 5 min depending on the tissue; after every 30 sec or so of homogenisation the mixture was rested for 10 sec to allow cooling. The homogenate was then centrifuged and then decanted into an Eppendorf tube at 9,000 g for 5 min at 4°C (Morizono and Akinaga, 1981; Al-Qarawi and Ali, 2003; Lassiter et al., 2003).

**Enzyme activity measurement**
Acetylcholinesterase activity was determined by the Ellman method (1961), adapted for use with microtitre plates as described by Haigh et al. (2008), and using acetylstiocholine iodide as substrate (1 mM final concentration) for measuring AChE activity, respectively.

**Determination of rate constants for aging ($k_a$) and spontaneous reactivation ($k_s$)**
OP-inhibited AChE was prepared by incubating samples with appropriate OP concentrations for 30 min at 25°C resulting in an inhibition of 85-95% of control activity. OP-treated samples were stored in aliquots at -80°C until use. In order to remove excess OP after affecting inhibition the samples by DDVP and DZN, the samples were filtrated and the absence of inhibitory activity was tested by incubation of OP-treated and control AChE (15 min, 25°C). Aliquots were taken after various time intervals for determination of AChE activity ($k_a$) and of the decrease of oxime-induced reactivation ($k_s$). OP-treated samples were incubated with 500 μM pralidoxime chloride (30 min) (Škrinjaric-Špoljar et al., 1973; Aurbek et al., 2006). All chemicals used in this study were purchased from Sigma Chemical Company (Poole, UK).

**Statistical analysis**
Conventional statistical methods were used to calculate the means and standard errors (S.E.). Differences with $P < 0.05$ were regarded to have statistical significance. The pseudo-first-order rate for constants $k_a$ and $k_s$ were calculated by a non-linear regression analysis (Škrinjaric-Špoljar et al., 1973; Worek et al., 2004). All statistics was carried out using MiniTab statistical software version 15 (MiniTab Ltd., State College, PA, USA).

**RESULTS**
Spontaneous reactivation ($k_s$) and aging ($k_a$) kinetics of AChE inhibited by DDVP and DZN were determined in liver and muscle for sheep and cattle (as well as pig) using the modified Ellman method as described in the Materials and Methods (Tables 1 and 2). Liver $k_a$ and $k_s$ kinetic parameters for reaction between AChE and two OP (DDVP and DZN) are shown in Table 1. The $k_a$ for animals was decreased according to the rank order of sheep > pig > cattle for DDVP and DZN. Nevertheless, $k_s$ values for animals are decreased according to the rank order of cattle > sheep > pig for DDVP while decreased in 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 $k_s$ (0.8). Kinetic $k_a$ and $k_s$ of all tested animals in liver gave no correlation between $k_a$ and $k_s$ ($R^2 < 0.34$) (Table 1).

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

Muscle $k_a$ and $k_s$ kinetics for reaction among AChE and two OP (DDVP and DZN) are seen in Table 2. The values of $k_a$ 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, first-order rate constants for $k_a$ values was decreased according to the rank order of cattle > sheep > pig for DDVP where-
Spontaneous reactivation and aging kinetics of acetylcholinesterase

Table 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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Sheep</th>
<th>Cattle</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_s$ (hr$^{-1}$)</td>
<td></td>
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</tr>
<tr>
<td>DDVP</td>
<td>0.323 ± 0.05</td>
<td>0.218 ± 0.09</td>
<td>0.245 ± 0.03</td>
</tr>
<tr>
<td>DZN</td>
<td>0.088 ± 0.04</td>
<td>0.061 ± 0.001</td>
<td>0.070 ± 0.003</td>
</tr>
<tr>
<td>Ratio$^a$</td>
<td>3.7</td>
<td>3.6</td>
<td>3.5</td>
</tr>
<tr>
<td>$k_a$ (hr$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDVP</td>
<td>0.019 ± 0.003</td>
<td>0.021 ± 0.003</td>
<td>0.013 ± 0.002</td>
</tr>
<tr>
<td>DZN</td>
<td>0.014 ± 0.002</td>
<td>0.018 ± 0.001</td>
<td>0.017 ± 0.006</td>
</tr>
<tr>
<td>Ratio$^a$</td>
<td>1.4</td>
<td>1.7</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Values are mean ± S.E. obtained from nonlinear regression analysis, each performed in triplicate (n = 3 in each animals). Different letters in column is significantly different (ANOVA, $P < 0.05$). $^a$ Ratio (DDVP vs. DZN).

Fig. 1. 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 is significantly different (ANOVA, $P < 0.05$).

as 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 $k_s$ and $k_a$ ($R^2 < 0.26$) (Table 2).

Half time ($t_{1/2}$) from muscle for $k_s$ and $k_a$ kinetic parameters for reaction among AChE and two OP (DDVP and DZN) are shown in Fig. 2. 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 (Fig. 2A). Other than $t_{1/2}$ for aging was nearly similar in DZN than DDVP in pig (Fig. 2B).

DISCUSSION

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 of these food animals. Phosphorylated AChE is susceptible to spontaneous hydrolysis of an alkyl-ester bond, resulting in a negatively charged residue which is resistant towards nucleophilic attack.
The rate of \( k_s \) to inhibit erythrocyte AChE by DDVP has been observed to be 0.92 \( \text{min}^{-1} \) for cattle (Skrinjaric-Spoljar et al., 1973), 0.347 \( \text{hr}^{-1} \) for rat (WHO, 2007), while \( k_s \) values to DZN was 0.012 \( \text{hr}^{-1} \) for human (WHO, 2007), but was 0.408 and 0.019 \( \text{hr}^{-1} \) 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 erythrocyte, which ranged between 0.133 and 0.323 \( \text{hr}^{-1} \), but lower than DZN, and ranged between 0.021 to 0.088 \( \text{hr}^{-1} \). These differences may be due to AChE activity in the erythrocyte 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 1 and 2).

Literature values of the half time (t\(_{1/2}\)) of \( k_s \) for AChE are about 2 and 58 hr for DDVP and DZN, respectively in human erythrocyte (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\(_{1/2}\) (Fig. 1A).

The recovery of 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

### Table 2.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Sheep</th>
<th>Cattle</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_s ) (hr(^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDVP</td>
<td>0.161 ± 0.016</td>
<td>0.133 ± 0.03(^a)</td>
<td>0.139 ± 0.032</td>
</tr>
<tr>
<td>DZN</td>
<td>0.062 ± 0.039</td>
<td>0.026 ± 0.06(^b)</td>
<td>0.021 ± 0.005</td>
</tr>
<tr>
<td>Ratio(^a)</td>
<td>3</td>
<td>5.1</td>
<td>6.6</td>
</tr>
<tr>
<td>( k_s ) (hr(^{-1}))</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDVP</td>
<td>0.017 ± 0.001</td>
<td>0.018 ± 0.001(^a)</td>
<td>0.013 ± 0.003</td>
</tr>
<tr>
<td>DZN</td>
<td>0.010 ± 0.003</td>
<td>0.009 ± 0.001(^b)</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td>Ratio(^a)</td>
<td>1.7</td>
<td>2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Key to the table is listed under the Table 1.
Spontaneous reactivation and aging kinetics of acetylcholinesterase
dZN 0.017 hr⁻¹ for human (Wilson et al., 2005). In agreement with our result with DZN that ranged between 0.009 to 0.018 hr⁻¹, while lower than reported with DDVP. Except for muscle DZN-inhibited AChE ka proceeded markedly later than kₐ of cattle and pigs. In addition, t₁/₂ of kₐ for human erythrocyte AChE is about 41 hr for DZN (WHO, 2007), concurring with our results which ranged between 39.1 to 85.3 hr (Fig. 1B). This indicates that the reduction of DDVP t₁/₂ in the liver and muscle might alter the use of oximes. In clinical research, the level and time course of kₐ 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; Aubek et al., 2006). Finally, this study found that values of kₐ and/or kₐ could also play a role on the administration of oximes in the liver and muscles of food animals. In conclusions, this study that provide original data concerning an enzymological characterization of these inhibitors in these food animals. As well as in this research, we surveyed recent developments in our understanding of the kinetic properties of kₐ and kₐ for sheep, cattle, and pig AChE is 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 either OP. This was also true in muscle with DDVP and DZN, although the difference was less. Hence, in the case of t₁/₂ AChE inhibited with DZN the enzyme will tends to become much higher than 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 inhibition as well as for spontaneous reactivation but not for dealkylation and oxime-induced reactivation.

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REFERENCES


