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# DI-(2-CHLOROETHYL) ARYL PHOSPHATES

## A STUDY OF THEIR REACTION WITH B-ESTERASES, AND OF THE GENETIC CONTROL OF THEIR HYDROLYSIS IN SHEEP

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**Abstract**—In order to find an explanation of the low acute toxicity of di-(2-chloroethyl) aryl phosphates and their variable ataxic toxicity in sheep, the reactions of these compounds with various esterases found in sheep were studied, and compared with those of other, more toxic compounds.

Di-(2-chloroethyl) aryl phosphates have been shown to form unstable di-(2-chloroethyl) phosphoryl derivatives of various esterases found in sheep. It is suggested that the instability of the cholinesterase derivative is the reason for the low toxicity of these compounds to mammals, when compared with their diethyl analogues. The corresponding ali-esterase derivatives are also unstable, so that inhibition of these enzymes is only temporary.

The rate of hydrolysis of the di-(2-chloroethyl) aryl phosphates by plasma has been shown to be much greater in some sheep than in others. This difference of activity is demonstrated as being genetically determined, and due to the presence or absence of a dominant allele, the sheep of low hydrolytic ability being the homozygous recessives. The rapid hydrolysis of di-(2-chloroethyl) aryl phosphates found in some samples of sheep plasma protects the animals from the ataxia syndrome which sometimes results when large doses (>150 mg/kg) of these compounds are given.

## INTRODUCTION

THE ACTION of many organophosphate compounds on mammalian esterase systems has been fully described and reviewed.[1, 2] The inhibition resulting from organophosphate action is generally non-reversing or slowly reversing, depending upon the type of phosphorylated enzyme formed,[3, 4] those from dimethyl phosphates being generally less stable than higher analogues.

The present work describes the reactions of a new class of organophosphates, di-(2-chloroethyl) aryl phosphates, with the esterases of sheep and other mammals. One of these (Haloxon) is used as an anthelmintic. In an earlier paper[5], Lee and Hodsden described the action of Haloxon on the cholinesterase of *Haemonchus contortus* a parasitic nematode, and compared this with the corresponding reaction on the erythrocyte cholinesterase of the sheep host. The conclusion drawn from this comparison was that the dichloroethyl phosphoryl cholinesterase formed in the reaction was stable in the case of the helminth enzyme, but unstable for sheep cholinesterase.

This difference in stability of the two di-(2-chloroethyl) phosphorylcholinesterases was put forward as the reason for the anthelmintic efficiency combined with low host

toxicity of Haloxon. Further evidence for the formation of an unstable di-(2-chloroethyl) phosphoryl cholinesterase in sheep is given in this paper, and the corresponding B-esterase[6] derivatives have been investigated to determine their stability. The aim of this work was to discover whether any sheep esterase systems were highly susceptible to inhibition by di-(2-chloroethyl) aryl phosphates, and if this inhibition was reversible.

The hydrolysis of di-(2-chloroethyl) aryl phosphates by sheep tissues was also studied to determine which of the complex of A-esterases, found in sheep plasma,[7] was responsible for the breakdown of these compounds, and whether variation in the activity of an A-esterase could be held responsible for the variation in response of individual sheep to doses of Haloxon.

## EXPERIMENTAL

### *Materials*

Blood samples for the work on the genetic inheritance of an A-esterase in sheep were obtained from three sources where the genealogy of the animal was known.

1. The flock of Herdwick sheep maintained at the Agricultural Research Council Institute for Research on Animal Diseases, Compton, Berks.
2. The mixed flock maintained at the Agricultural Research Council Animal Breeding Research Institute, Edinburgh.
3. The pedigree Southdown flock of Mr. Harris, Manor Farm, Bloxworth, Dorset.

Other blood samples and tissues were taken from various breeds of sheep, but most of the experiments on cholinesterase were done with tissues of Welsh Mountain sheep. Blood samples from many other species were used for comparison.

Tissues, other than blood, were obtained by slaughter, homogenized in distilled water in an all glass apparatus and used immediately.

Plasma and erythrocyte suspensions were obtained as described previously.[5]

### *Methods*

*Estimation of esterase activity.* Cholinesterase activity was measured colorimetrically by the Fleisher, Pope and Spear[8] modification of the method described by Hestrin,[9] and manometrically in a conventional Warburg apparatus using the technique of Ammon.[10] The medium used for manometric work was 0·031 M $NaHCO_3$, 0·44% gelatin solution saturated with 5% $CO_2$ in $N_2$ (pH 7·7).

Aliphatic esterase activity was also measured manometrically. The evolution of $CO_2$ was followed for sixty min after the addition of substrate.

Aromatic esterase activities were measured colorimetrically whenever possible. The hydrolysis of paraoxon and dichloroethyl 4-nitrophenyl phosphate released 4-nitrophenol, and hydrolysis of dichloroethyl 4-ethoxycarbonylcoumarin-7-yl phosphate produced 4-ethoxycarbonyl-7-hydroxycoumarin. Both these hydrolysis products are yellow, 4-nitrophenol having a maximum absorption at 400 mμ, 4-ethoxycarbonyl-7-hydroxycoumarin at 375 mμ.

The hydrolysis of these organophosphates was carried out in 0·15 M $Na_2HPO_4$, $KH_2PO_4$ buffer (pH 7·2). The organophosphate substrates (2 μmoles) in 0·05 ml of acetone were pipetted into tubes, and the acetone evaporated at 60°C. Buffer solution (3 ml) and enzyme solution (1 ml) were then added, and the tubes incubated at 37°C.

Following incubation, the protein present was precipitated by the addition of an

equal volume of acetone. Filtration through Whatman No. 1 filter paper produced a clear filtrate, the absorption of which was measured at the appropriate wavelength.

Calibration curves were prepared by substituting varying amounts of the appropriate hydrolysis product for the substrates in the above system. In both cases a linear relationship was obtained between quantity of hydrolysis product and the readings from the spectrophotometer (Unicam SP600).

When clear plasma samples were used in a colorimetric estimation of A-esterase activity, each tube was read off at 400 or 375 m$\mu$ before and after incubation, and thus acted as its own blank, removing the need for acetone precipitation. Controls for non-enzymic hydrolysis were treated in similar fashion.

The corresponding hydrolysis product of Coroxon and Haloxon is not coloured, but fluoresces under alkaline conditions. The procedure for measuring the hydrolysis rate of Coroxon and Haloxon was the same as that for the other organophosphates, except that 1 ml of the 50% acetone filtrate was pipetted into 9 ml of N/10 $K_2CO_3$, and the fluorescence, due to the 3-chloro-4-methyl-7-hydroxycoumarin present, was read off using an EIL type 27A fluorimeter. Calibration of the fluorimeter was done in the manner corresponding to that used for the spectrophotometer; linear correlation was found.

The appropriate tissue blanks and non-enzymic hydrolysis controls were run concurrently with each experiment.

*Substrates.*

1. Acetylcholine perchlorate (ACh)—British Drug Houses, Ltd.
2. Glyceryl tributyrate (Tributyrin, TB)—British Drug Houses, Ltd.
3. Ethyl acetate (EtAc)—British Drug Houses, Ltd.
4. Methyl butyrate (MeBu)—British Drug Houses, Ltd.
5. 4-nitrophenyl acetate (NPA)—Cooper Technical Bureau.
6. 4-nitrophenyl butyrate (NPB)—Cooper Technical Bureau.

*Organophosphates*

1. Diethyl 4-nitrophenyl phosphate (Paraoxon), colourless liquid; B.Pt. (1 mm) 169–170° $n_D^{20}$ = 1·5105—Cooper Technical Bureau.
2. Diethyl 3-chloro-4-methylcoumarin-7-yl phosphate (Coroxon), white crystalline solid; M.Pt. 71°—Cooper Technical Bureau.
3. Di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate (Haloxon), white crystalline solid; M.Pt. 92–93°—Cooper Technical Bureau.
4. Di-(2-chloroethyl) 4-nitrophenyl phosphate, pale yellow liquid; $n_D^{20}$ = 1·5315—Cooper Technical Bureau.
5. Di-(2-chloroethyl) 2,4,5-trichlorophenyl phosphate, pale yellow liquid; $n_D^{20}$ = 1·5400—Cooper Technical Bureau.
6. Di-(2-chloroethyl) 4-ethoxycarbonylcoumarin-7-yl phosphate, white crystalline solid; M.Pt. 69–70°—Cooper Technical Bureau.
7. Tri-2-tolyl phosphate (tri-*o*-cresyl phosphate, TOCP)—British Drug Houses, Ltd.

Organophosphates Nos. 3–6 inclusive are commonly referred to in this paper as 'Halons', being di-(2-chloroethyl) aryl phosphates of general formula $(ClC_2H_4O)_2P(O).O.Aryl$.

## RESULTS AND DISCUSSION

1. *The reaction between sheep erythrocyte cholinesterase and dichloroethyl phosphates*

The reaction between Haloxon and sheep erythrocyte cholinesterase has been described previously as reversible,[5] the inhibition being independent of time after the initial 10 min incubation of enzyme with inhibitor, with reactivation occurring in the presence of high concentrations of substrate.

Various other halons, e.g. di-(2-chloroethyl) 4-nitrophenyl phosphate, di-(2-chloroethyl)2,4,5-trichlorophenyl phosphate and di-(2-chloroethyl) 4-ethoxycarbonylcoumarin-7-yl phosphate, were found to have the same pattern of reaction with sheep erythrocyte cholinesterase (Fig. 1.).

The initial reaction between two of these halon compounds and sheep erythrocyte cholinesterase, as measured by the inhibition of ACh hydrolysis, was found to be temperature dependent, with an energy of activation of about 9000 cals/mole. (The

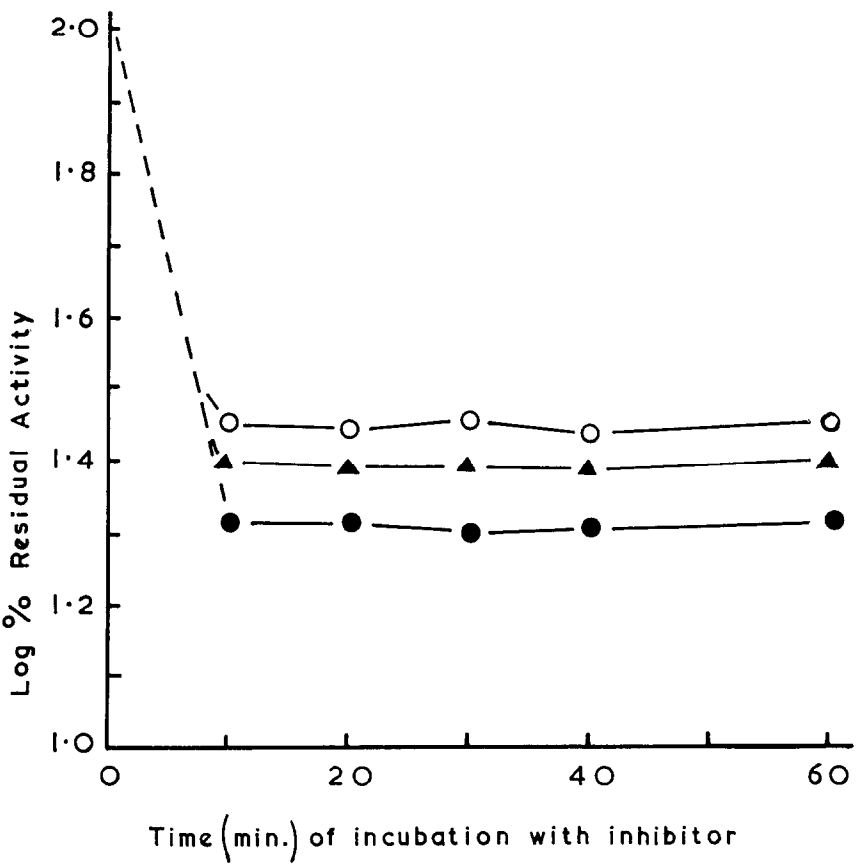


FIG. 1. The inhibition of sheep erythrocyte cholinesterase by three dichloroethyl aryl phosphates at $10^{-6}$ M concentration. (37°).

○ = dichloroethyl 4-ethoxycarbonylcoumarin-7-yl phosphate.

△ = dichloroethyl 2,4,5-trichlorophenyl phosphate.

● = dichloroethyl-4-nitrophenyl phosphate.

Substrate-ACh $2 \times 10^{-3}$ M, hydrolysis measured colorimetrically.

short time available for measuring that part of the inhibition reaction which is linearly time-dependent precludes the accurate estimation of the activation energy). (Fig. 2).

Reactivation of sheep erythrocyte cholinesterase after inhibition by halon compounds, was achieved by adding the plasma (0·5 ml) of certain sheep (see Section 3), which had previously been found to have a high hydrolytic activity against halons, and which would prevent inhibition of cholinesterase by the concentration of inhibitors used in the experiments.

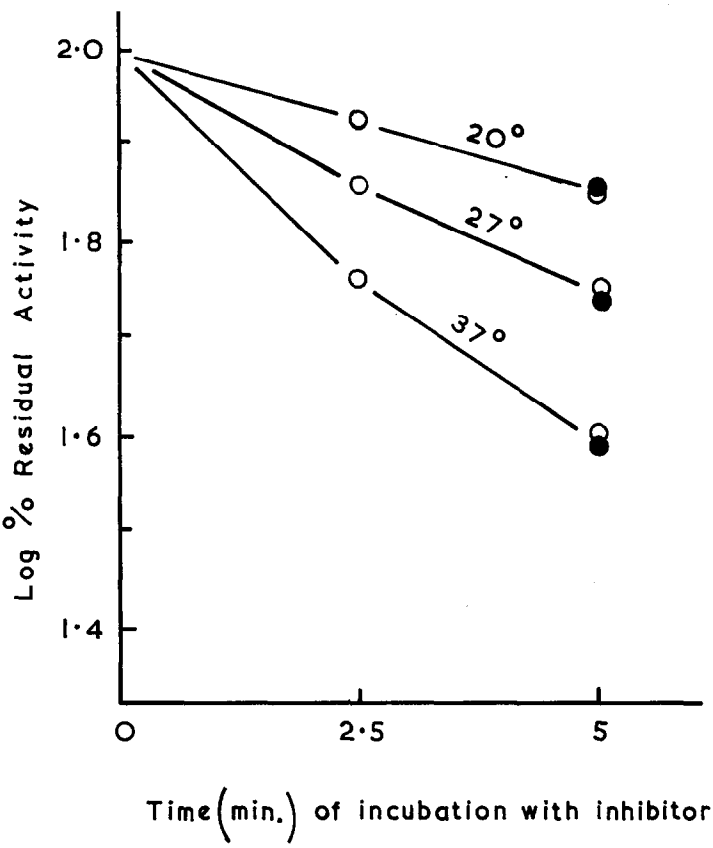


FIG. 2. The inhibition of sheep erythrocyte cholinesterase by Haloxon (⊙) and dichloroethyl-4-nitrophenyl phosphate (●) at various temperatures.
Substrate-ACh $2 \times 10^{-3}$ M, hydrolysis measured colorimetrically.

The rates of reactivation at 37°C of sheep erythrocyte cholinesterase, after inhibition by four halon compounds, are shown in Fig. 3. No difference in reactivation rate was found when one halon was substituted for another.

The unimolecular rate constants for recovery of activity at various temperatures of sheep erythrocyte cholinesterase after inhibition by Haloxon and di-(2- chloroethyl) 4-nitrophenyl phosphate are given in Table 1. The energy of activation of the recovery reaction was calculated from these rate constants, and found to be 11,000 cals/mole. (See also Fig. 4).

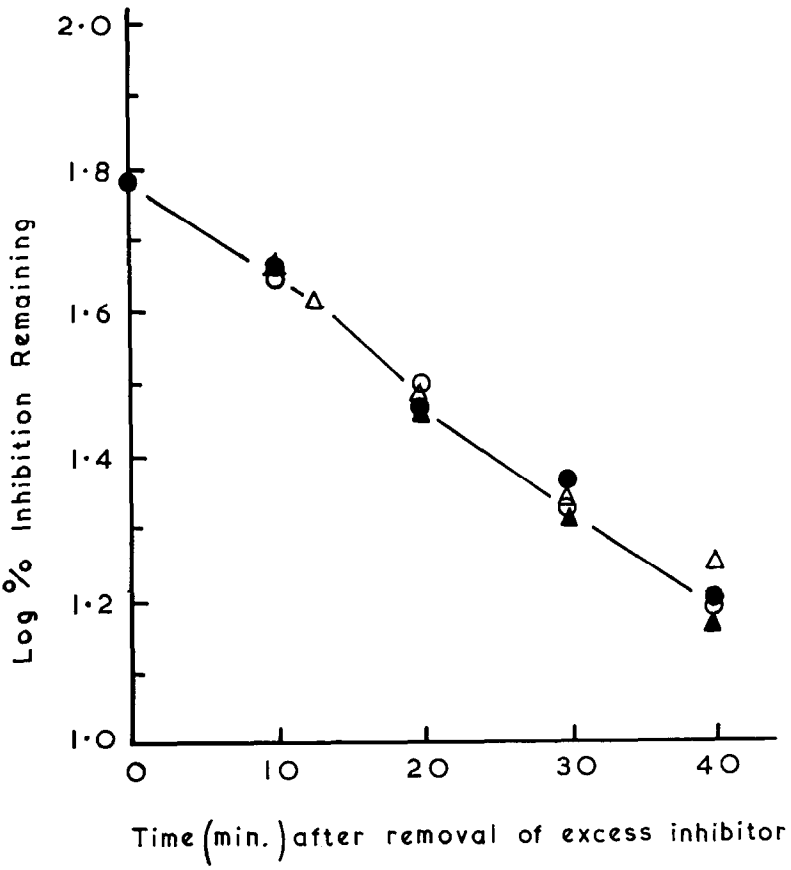


FIG. 3. The rate of reactivation of sheep erythrocyte cholinesterase after inhibition by various dichloroethyl aryl phosphates, followed by removal of excess inhibitor.

○ = dichloroethyl-4-nitrophenyl phosphate.
● = Haloxon,
△ = dichloroethyl 2,4,5-trichlorophenyl phosphate.
▲ = dichloroethyl 4-carbethoxycoumarin-7-yl phosphate.
Substrate ACh $2 \times 10^{-3}$ M, hydrolysis measured colorimetrically.

TABLE 1. UNIMOLECULAR RATE CONSTANTS FOR THE RECOVERY, AT VARIOUS TEMPERATURES, OF SHEEP ERYTHROCYTE CHOLINESTERASE, AFTER INHIBITION BY HALOXON AND DI-(2-CHLOROETHYL) 4-NITROPHENYL PHOSPHATE (DCE 4 NPP)

| Temperature °C | Compound | Unimolecular Rate Constant ($K_1$) $\times 10^3$ $min^{-1}$ |
|---|---|---|
| 18 | Haloxon | 9·3 |
| 18 | DCE 4 NPP | 9·0 |
| 27 | Haloxon | 17·0 |
| 27 | DCE 4 NPP | 17·4 |
| 36 | Haloxon | 29·0 |
| 36 | DCE 4 NPP | 29·0 |

The recovery rate of sheep brain cholinesterase, and cholinesterases from other species, was measured in the presence of $10^{-2}$M ACh, after inhibition by Haloxon. (Fig. 5). The unimolecular rate constant for sheep brain cholinesterase reactivation at 36°C was $3 \times 10^{-2}$ $min^{-1}$, which is the same as that for the erythrocyte enzyme. Similar reactivation rates were found with the cholinesterases of other species, and in no case did the halons produce a long-lasting inhibition.

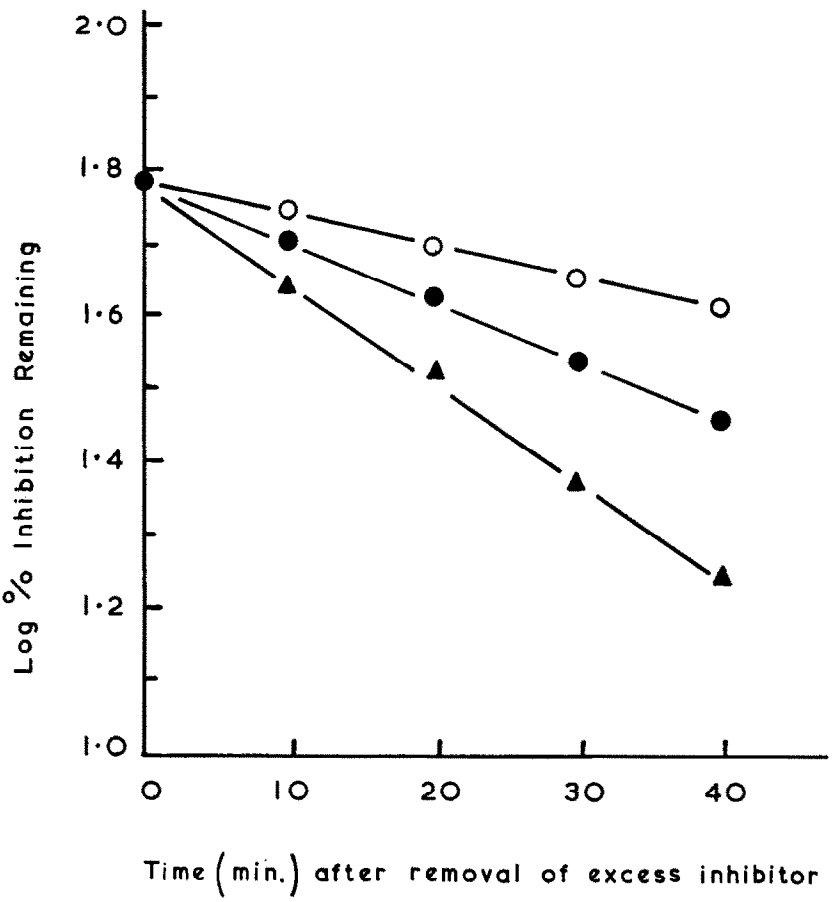


FIG. 4. The rate of reactivation at various temperatures of sheep erythrocyte cholinesterase after inhibition by Haloxon.
○ = 18°; ● = 27°; ▲ = 36°.
Substrate $2 \times 10^{-3}$ M ACh, hydrolysis measured colorimetrically.

These results support the hypothesis adopted by Lee and Hodsden,[5] that the reaction between halons and mammalian cholinesterase is spontaneously reversible, and that rapid reactivation of the enzymes takes place when substrate concentration greatly exceeds inhibitor concentration, or when excess inhibitor is removed by physical or biochemical means.

Since the reactions causing inhibition and recovery of enzyme activity are both temperature dependent, with activation energies similar to those found for other

organophosphate–cholinesterase reactions,[12] it must be concluded that chemical combination and degradation are involved in the overall reaction, which can be represented thus:

$$E + I_{AB} \rightleftharpoons EI_{AB} \longrightarrow EI_A + B \longrightarrow E + A + B$$

All the halon compounds produced phosphorylated cholinesterase ($EI_A$)[5] with identical reactivation rates (Fig. 3), so that $EI_A$ is, in this case, di-(2-chloroethyl) phosphoryl cholinesterase. It is this compound which is spontaneously hydrolysed by

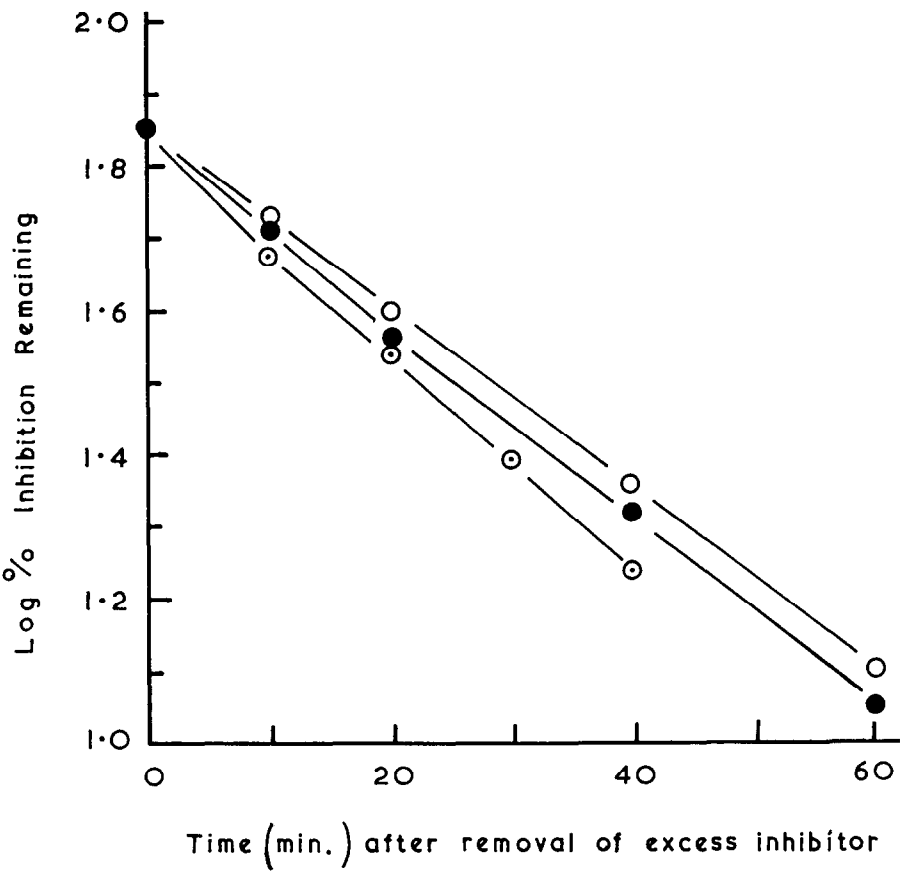


FIG. 5. The rate of reactivation at 36° of cholinesterases from three mammals.
○ = Rat brain cholinesterase.
◌ = Guineapig erythrocyte cholinesterase.
● = Sheep brain cholinesterase.
Substrate $10^{-2}$ M ACh, hydrolysis measured manometrically.

water, at the pH used in these experiments, to give regenerated cholinesterase, which may react with inhibitor again, or, if excess inhibitor has been removed, would be free to hydrolyse ACh (cf. Aldridge and Davison[13]).

This sequence of events explains why the di-(2-chloroethyl) aryl phosphates are much less toxic than the corresponding diethyl compounds to mammals, and why the

depression of blood cholinesterase after dosing with halon compounds is a transitory one.[18]

From these results, and those of Aldridge[12] and of Davison[3], the sequence of stability of mammalian phosphorylated cholinesterases can now be extended to include the di-(2-chloroethyl) phosphoryl enzyme which is the least stable of the group, having a half-life of 22 min at 36°C (Fig. 4) for sheep cholinesterase. This sequence of stability does not necessarily apply to cholinesterases other than mammalian,[5] but on electronic theory it is the expected one.

2. *The reaction between sheep liver B-esterases and di-(2-chloroethyl) aryl phosphates*

The hydrolytic activity of sheep liver esterases was tested against various substrates (Table 2). No hydrolysis of ACh was observed, but ethyl acetate, tributyrin, methyl butyrate, 4-nitrophenyl butyrate and 4-nitrophenyl acetate were all hydrolysed.

TABLE 2. THE HYDROLYSIS OF ESTERS BY SHEEP LIVER AND ITS INHIBITION BY PARAOXON

| Substrate | Hydrolysis rate $\mu$l $CO_2$/mg/hr | Inhibition by paraoxon (%) $10^{-7}$M for 30 min | $10^{-4}$M for 30 min |
|---|---|---|---|
| ACh. | 0 | — | — |
| Et. Ac. | 94 | 98 | — |
| Me. Bu. | 434 | 100 | — |
| TB | 240 | 98 | — |
| 4-NPB | 1365 | 100 | — |
| 4-NPA | 1045 | 51 | 52 |

Liver 0·4 mg/flask; Substrates 30 mg/flask.

Paraoxon ($10^{-7}$M for 30 min) almost completely inhibited the hydrolysis of all substrates except 4-nitrophenyl acetate, the hydrolysis of which was 50 per cent inhibited under these conditions (Table 2). The remaining 50 per cent activity could not be further reduced by paraoxon concentrations of up to $10^{-4}$M.

This finding eliminated 4-nitrophenyl acetate as a suitable substrate for sheep liver B-esterases; methyl butyrate was also rejected for manometric work because of its high volatility. Of the remaining substrates, 4-nitrophenyl butyrate was most rapidly hydrolysed and this compound was used for all B-esterase activity determinations.

Various halons were tested for their ability to inhibit sheep liver B-esterase activity; the pattern of inhibition obtained was compared with that found with paraoxon and TOCP. (Fig. 6).

The results indicate that maximum inhibition, by any one concentration of a halon compound, was achieved within a short period (Fig. 6) after which recovery of the B-esterase occurred when the substrate was introduced into the system, preventing further reaction between enzyme and inhibitor. This recovery was confirmed by the manometric technique (Fig. 7), the evolution of $CO_2$ increasing with time after addition of substrate.

Inhibition of sheep liver B-esterase by paraoxon was a first order, bimolecular reaction, with no recovery of activity during the time course of the experiment. (Fig. 6).

When TOCP was the inhibitor, the results were less straightforward. The maximum inhibition obtained with both $10^{-7}$ and $2 \times 10^{-6}$M TOCP was 51 per cent, although

the time required to reach this maximum was greater with the lower concentration. Using $10^{-7}$M and $10^{-8}$M TOCP, there was an initial rapid inhibition of the esterase activity followed by a slower reaction. This pattern of inhibition could be due to impurities in the TOCP which were exhausted rapidly, or to the presence of two

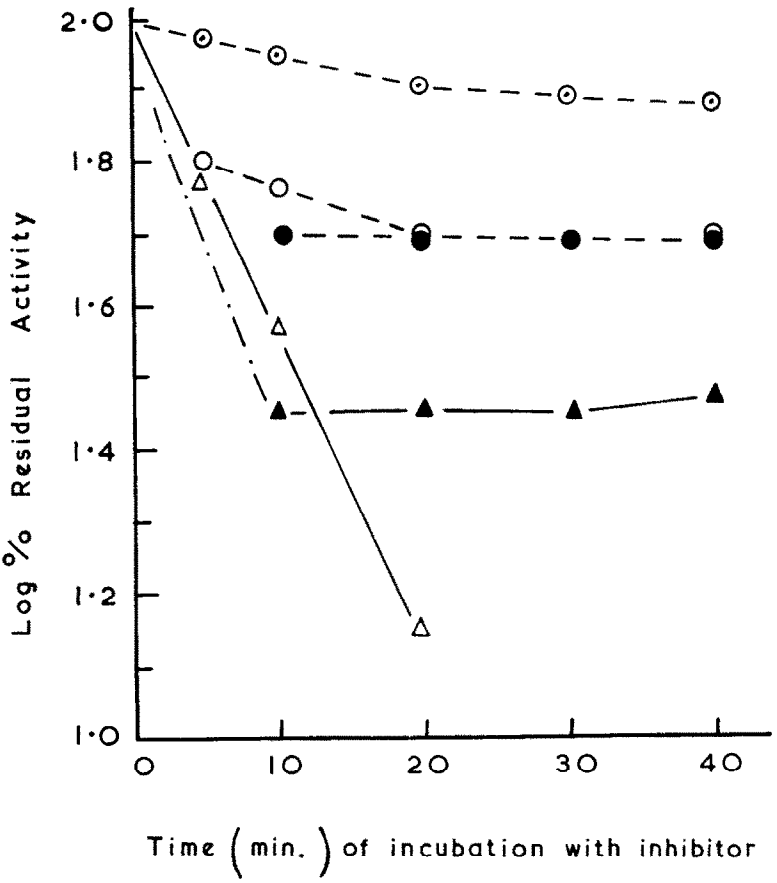


FIG. 6. The inhibition of B-esterase activity from sheep liver by paraoxon, TOCP and Haloxon at 37°.
⊙ = $10^{-8}$ M TOCP
○ = $10^{-7}$ M TOCP
● = $2 \times 10^{-6}$ M TOCP
△ = $10^{-7}$ M paraoxon
▲ = $10^{-7}$ M Haloxon; other halons gave similar results.
Substrate $10^{-3}$ M NPB, hydrolysis measured manometrically.

TOCP-sensitive esterases. Nevertheless, it is apparent that at least two esterases were present in sheep liver, one of which was not susceptible to TOCP. Both TOCP-susceptible and other esterases were susceptible to inhibition by paraoxon ($10^{-7}$M) and Haloxon, since both these compounds produced more than 50 per cent inhibition of 4-nitrophenyl butyrate hydrolysis.

These results were interpreted as indicating the presence of at least three B-esterases in sheep liver; one was not inhibited by $2 \times 10^{-6}$M TOCP, but was susceptible to low concentrations of paraoxon ($10^{-7}$M), the rest of the hydrolytic activity was susceptible to low concentrations of both TOCP and paraoxon, although the inhibition by $10^{-7}$M and $10^{-8}$M TOCP suggested that two enzymes were involved.

The halon compounds were capable of reacting with all three enzymes, since medium concentrations of these inhibitors produced more than 50 per cent inhibition of 4-nitrophenyl butyrate hydrolysis, whereas TOCP did not.

However, inhibition by halons was again readily reversible in the presence of substrate, and the mechanism of reaction between halons and B-esterases is concluded to be the same as that for halons and cholinesterases. (Fig. 7).

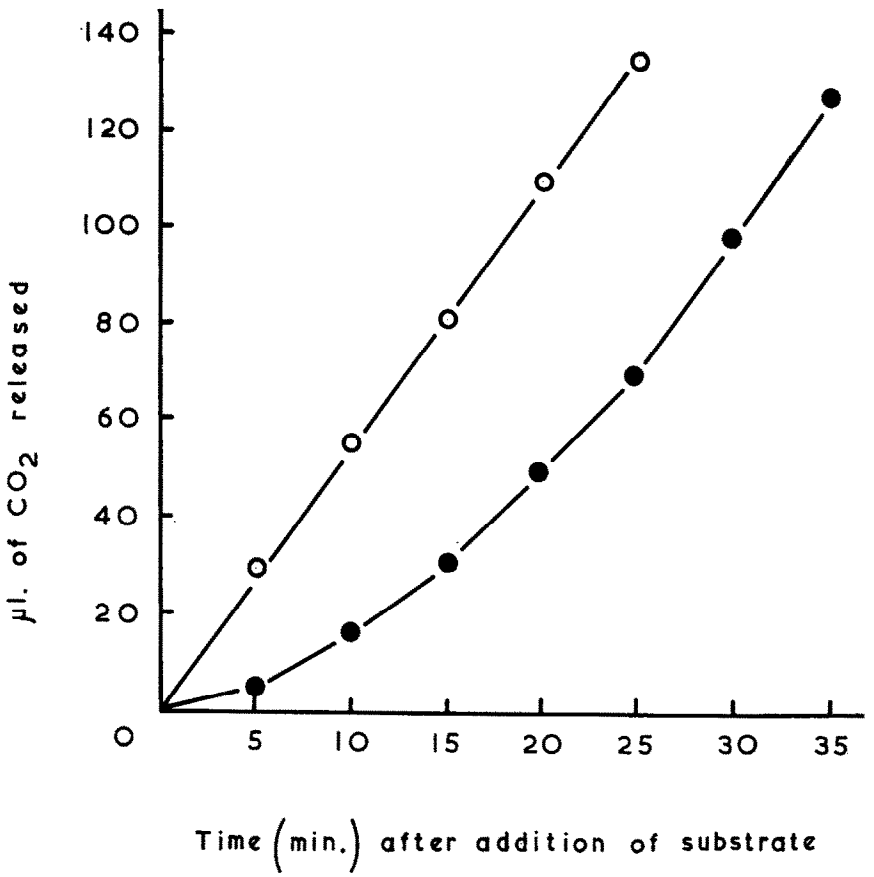


FIG. 7. The pattern of $CO_2$ evolution by sheep liver B-esterase in the presence of $10^{-2}$ M NPB. ○ = Control ● = 15 min incubation with $10^{-6}$ M Haloxon prior to the addition of substrate. (Other halons gave similar results).

The exact function of the liver B-esterase group of enzymes is not known, and the effect of their inhibition *in vivo* cannot be assessed; it seems clear, however, that the inhibition of B-esterases caused by halon compounds will be as transitory as that of cholinesterases so that no lasting damage to the animals is likely.

3. *The reaction between sheep plasma A-esterases and halon organophosphates*

(*a*) *Two rates of halon hydrolysis in sheep tissues.* An investigation of the rate of hydrolysis of various organophosphates showed that all the sheep plasma samples tested had similar rates for paraoxon and Coroxon, two diethyl aryl phosphates,

but that a certain proportion of the samples hydrolysed di-(2-chloroethyl) aryl phosphates at a much faster rate than the remainder.

Those individuals of the sheep population whose plasma was capable of high rates of hydrolysis of halons were termed 'Halon-high', the rest being designated as 'Halon-low'. (Table 3).

Samples of liver were also taken from sheep of high and low plasma activity; the rate of hydrolysis of halons by sheep liver was found to coincide with the activity of the plasma in being either high or low. (Table 3).

All hydrolyses of organophosphates by sheep tissues were linear with respect to time and concentration of enzymes.

TABLE 3. THE HYDROLYSIS OF SOME OXONS AND HALONS BY SHEEP PLASMA AND LIVER SAMPLES

| Enzyme Source | Substrate | No. of samples tested | Rate of Hydrolysis (μM/g./hr) Mean | Range |
|---|---|---|---|---|
| Halon-low Plasma | Paraoxon | 12 | 10·5 | 10·0 – 11·0 |
| | Coroxon | 12 | 2·2 | 1·8 – 2·15 |
| | Haloxon | 12 | 0·2 | 0·1 – 0·5 |
| | DCE 4 NPP | 12 | 1·4 | 1·2 – 1·7 |
| | DCE 4 ECP | 12 | 0·2 | 0·1 – 0·3 |
| Halon-high Plasma | Paraoxon | 12 | 9·4 | 9·0 – 10·0 |
| | Coroxon | 12 | 2·3 | 1·8 – 2·5 |
| | Haloxon | 12 | 2·7 | 2·1 – 3·4 |
| | DCE 4 NPP | 12 | 9·9 | 9·8 – 10·0 |
| | DCE 4 ECP | 12 | 4·0 | 3·1 – 4·6 |
| Halon-low Liver | Paraoxon | 6 | 15·3 | 14·2 – 15·7 |
| | Coroxon | 6 | 2·0 | 1·7 – 2·6 |
| | Haloxon | 6 | 0·3 | 0·2 – 0·4 |
| | DCE 4 NPP | 6 | 2·0 | 1·4 – 3·0 |
| | DCE 4 ECP | 2 | 0·4 | — |
| Halon-high Liver | Paraoxon | 6 | 14·0 | 12·8 – 16·1 |
| | Coroxon | 6 | 2·1 | 1·6 – 2·6 |
| | Haloxon | 6 | 2·3 | 1·9 – 2·9 |
| | DCE 4 NPP | 6 | 14·7 | 14·2 – 15·2 |
| | DCE 4 ECP | 2 | 3·8 | — |

DCE 4 NPP is dichloroethyl 4-nitrophenyl phosphate. DCE 4 ECP is dichloroethyl 4-ethoxycarbonylcoumarin-7-yl phosphate.

TABLE 4. THE RATE OF HYDROLYSIS OF HALOXON BY PLASMA AND LIVER SAMPLES OF VARIOUS ANIMAL SPECIES

| Species | No. tested | Hydrolysis of Haloxon (Range, μM/g/hr) Plasma | Liver |
|---|---|---|---|
| Guinea pig | 8 | 0·03 – 0·12 | 1·5 – 2·0 |
| Rabbit | 6 | 0·05 – 0·20 | 0·4 – 0·6 |
| Rat | 10 | 0·15 – 0·53 | 0·2 – 0·3 |
| Mouse | 10 | 0·05 – 0·14 | 0·1 – 0·3 |
| Cat | 4 | 0·03 – 0·13 | — |
| Dog | 10 | 0·31 – 0·49 | — |
| Horse | 15 | 0·05 – 0·24 | — |
| Cow | 10 | 0·05 – 0·12 | 0·1 – 0·2 |
| Goat | 32 | 0·10 – 0·30 | — |
| Human | 40 | 0·18 – 0·76 | — |
| Chicken | 18 | 0·06 – 0·16 | 0·2 – 0·3 |

Plasma and liver samples from other species were also tested for their ability to hydrolyse Haloxon (Table 4) and, within the limits of the numbers tested, all activities have corresponded to that of the 'Halon-low' group of sheep, even where paraoxon hydrolysis is extremely rapid in, for example, rabbit plasma.[14] Plasma samples from different breeds of sheep were tested to obtain an estimate of the incidence of 'Halon-high' individuals in the various breeds. (Table 5).

TABLE 5. THE INCIDENCE OF HALON-HIGH INDIVIDUALS IN VARIOUS BREEDS OF SHEEP

| Breed | No. tested | Incidence of Halon-high individuals (%) |
|---|---|---|
| Dorset Down | 70 | 3 |
| South Down | 58 | 9 |
| Herdwick | 88 | 10 |
| Blackface | 61 | 20 |
| Dorset Horn | 25 | 20 |
| South Down/Kent 1st Cross | 102 | 20 |
| Kerry | 95 | 25 |
| Suffolk/Clun 1st Cross | 86 | 25 |
| Cheviot | 126 | 27 |
| Welsh Mountain | 108 | 30 |
| Kent | 38 | 37 |

TABLE 6. THE HYDROLYSIS OF HALOXON BY A MIXTURE OF HALON-HIGH AND HALON-LOW PLASMA FROM SHEEP

| Plasma type | No. of samples | Hydrolysis of Haloxon $\mu$M/ml/hr. (Mean) |
|---|---|---|
| Halon-low | 4 | 0·16 |
| Halon-high | 4 | 1·80 |
| Mixture of plasmata (50% of each) | 4 | 1·00 |

The possibility that an inhibiting factor preventing hydrolysis of halons was present in the plasma of the 'Halon-low' group of sheep was tested by mixing the two types of plasma and measuring the hydrolytic activity with Haloxon as substrate.

The results in Table 6 indicate that no factor is present in 'Halon-low' sheep plasma which directly inhibits the hydrolysis of Haloxon.

Since paraoxon hydrolysis proceeded at a similar rate in both types of sheep plasma, this activity was tested in the presence of an equal amount of Haloxon (Table 7). The rate of hydrolysis of paraoxon by 'Halon-high' sheep plasma was reduced under these circumstances, whereas the hydrolysis of paraoxon by 'Halon-low' plasma was unaffected. This result suggested competition by paraoxon and Haloxon for the same enzyme site in the Halon-high plasma, and this hypothesis was supported by the unsuccessful attempt to separate the two activities by various treatments (Table 8). One such treatment was the investigation of the effect of $10^{-3}$M $Mn^{2+}$ions, which inhibited the hydrolysis of both paraoxon and Haloxon by 50 per cent. This result agrees very well with that of Main,[7] and strongly suggests that the enzyme designated

paraoxonase by Main is the one responsible for hydrolysis of the oxons and halons used in the present study.

(*b*) *Genetic determination of rate of halon hydrolysis.* The modification of paraoxonase, to include a high rate of hydrolysis of halons in certain sheep, was further investigated to discover whether a simple genetical basis could be found for this phenomenon.

TABLE 7. THE EFFECT OF HALOXON ON THE HYDROLYSIS OF PARAOXON BY HALON-LOW AND HALON-HIGH SHEEP PLASMA

| Type of plasma | Organophosphates present ($5 \times 10^{-4}$M) | Hydrolysis of paraoxon ($\mu$M/g/hr) | | S.D. |
|---|---|---|---|---|
| Halon-low | Paraoxon | 10·3 | ± | 0·5 |
| | Haloxon + Paraoxon | 10·2 | ± | 0·5 |
| Halon-high | Paraoxon | 9·8 | ± | 0·8 |
| | Haloxon + Paraoxon | 6·5 | ± | 1·0 |

Each figure is the mean of six experiments.

TABLE 8. THE EFFECT OF VARIOUS TREATMENTS ON THE HYDROLYSIS OF HALOXON, AND PARAOXON BY HALON-HIGH PLASMA SAMPLES FROM SHEEP

| Treatment | Hydrolysis rate (% of Control) Haloxon | Paraoxon |
|---|---|---|
| 55°C for 15 min | 36 | 36 |
| 45°C for 60 min | 79 | 81 |
| pH 6·0 phosphate buffer | 52 | 55 |
| pH 9·0 (tris-HCl buffer) | 87 | 86 |
| Addition of equal volume of 50% $(NH_4)_2SO_4$ followed by overnight dialysis | 74 | 71 |
| Ditto, 75% $(NH_4)_2SO_4$ | 29 | 22 |
| $10^{-4}$M $Mg^{2+}$ ions | 100 | 100 |
| $10^{-3}$ M $Mn^{2+}$ ions | 50 | 50 |

Blood samples were tested from flocks where matings and parentage were recorded (Table 9). In the experimental flocks from Compton and Edinburgh, each ram was mated with his own batch of ewes; in the Southdown flock much inbreeding had taken place.

The results from each flock showed that when a 'Halon-low' ram was mated with a 'Halon-low' ewe, all the lambs were 'Halon-low', whereas when one of the parents was 'Halon-high', a proportion of the offspring was also 'Halon-high'. The simplest explanation of these results was that the 'Halon-high' condition was due to a dominant allele of a single pair.

On this hypothesis genotypes can be assigned to most of the animals tested (*hh* for homozygous low, *Hh*, for heterozygotes, and *HH* for homozygous high). In Table 9 all sheep which are low have genotype *hh* and all those which are high, except for the figures in brackets, are *Hh* by virtue of the Halon-low offspring born to them;

those in brackets may be *Hh* or *HH*, but if they are homozygotes their hydrolytic activity is no greater than that of the heterozygotes. (Table 10).

In the summary of Table 9, the High × High matings were all from the Herdwick flock, and only one ewe could be genotype *HH*. The expected percentage Halon-high offspring, if all parents were heterozygous, is 75 per cent, the figure obtained was 71 per cent, which agrees with this postulate. ($0{\cdot}3 < P < 0{\cdot}5$).

TABLE 9. THE HYDROLYSIS OF HALON ORGANOPHOSPHATES BY PLASMA FROM SHEEP OF KNOWN HEREDITARY RELATIONSHIP

(a) HERDWICK BREED

| Parent Phenotypes ♂ Halon high | ♂ Halon low | ♀ Halon high | ♀ Halon low | Offspring Phenotypes ♂ Halon high | ♂ Halon low | ♀ Halon high | ♀ Halon low |
|---|---|---|---|---|---|---|---|
| A | — | (1) | — | 2 | 0 | 0 | 0 |
| | | — | 13 | 7 | 2 | 4 | 3 |
| B | — | 2 | — | (2) | 1 | (1) | 1 |
| | | — | 10 | 2 | 1 | 3 | 4 |
| C | — | — | 6 | 2 | 2 | 1 | 1 |
| — | D | 1 | — | 0 | 0 | 0 | 1 |
| | | — | 11 | 0 | 7 | 0 | 4 |
| — | E | (4) | — | 3 | 0 | 2 | 0 |
| | | — | 11 | 0 | 6 | 0 | 6 |
| | | a | — | 0 | 1 | 0 | 0 |
| | | (b) | — | 1 | 0 | 0 | 0 |
| — | F | c | — | 0 | 1 | 0 | 0 |
| | | d | — | 0 | 1 | 0 | 0 |
| | | | 14 | 0 | 8 | 0 | 6 |

Figures in brackets indicate those sheep whose genotype could be either heterozygous or homozygous dominant. Capital letters indicate individual males, and lower case letters individual females.

TABLE 9. (b) MIXED MATINGS

| Parents ♂ Breed | ♂ Phenotype | ♀ Breed | ♀ Phenotype | No. | Offspring (all twins) Phenotypes Halon-high | Halon-low |
|---|---|---|---|---|---|---|
| Merino | Halon-low | Blackface | Halon-low | 6 | 0 | 12 |
| | | Blackface | Halon-high | 1 | 0 | 2 |
| Merino | Halon-low | Blackface | Halon-low | 7 | 0 | 14 |
| Dorset Horn | Halon-low | Blackface | Halon-low | 5 | 0 | 10 |
| | | Blackface/ Swaledale | Halon-low | 1 | 0 | 2 |
| Dorset Horn | Halon-low | Blackface | Halon-low | 5 | 0 | 10 |
| | | Blackface/ Swaledale | Halon-high | 1 | 2 | 0 |

In the High × Low crosses, five ewes could be genotype *HH* (4 Herdwick, 1 Blackface/Swaledale cross). If all the high animals were heterozygotes, 50 per cent of the offspring should be genotype *Hh*, the figure obtained was 55 per cent, which is not statistically significantly different from the expected ($0{\cdot}3 < P < 0{\cdot}5$), but leaves the possibility that a few of the parents could have been genotype *HH*.

TABLE 9. (c) SOUTHDOWN BREED—RELATIONSHIP OF THE FIVE HALON-HIGH PHENOTYPES

| Parent mating | | | | Offspring | | | |
|---|---|---|---|---|---|---|---|
| ♂ No. | ♂ Phenotype | ♀ No. | ♀ Phenotype | ♂ No. | ♂ Phenotype | ♀ No. | ♀ Phenotype |
| F101 | Unknown | C9 | Halon-high | — | — | 50/9 | Halon-high |
| G10 | Halon-low | C9 | Halon-high | 52/2 | Halon-high | — | — |
| G10 | Halon-low | G39 | Unknown | W65 | Halon-low | W66 | Halon-high |
| F101 | Unknown | G39 | Unknown | — | — | 65/9 | Halon-low |
| W65 | Halon-low | C9 | Halon-high | (165 | Halon-low | — | — |
| | | | | (166 | Halon-low | — | — |
| H14 | Halon-low | W66 | Halon-high | (83 | Halon-high | — | — |
| | | | | (84 | Halon-low | — | — |
| H378 | Halon-low | 50/9 | Halon-high | 192 | Halon-low | — | — |

Thirty-eight other matings were 'Halon-low × Halon-low' and produced forty Halon-low offspring. The unknown phenotypes were of sheep which had died or been sold. G39 should be heterozygous, together with all the other Halon-high phenotypes.

TABLE 9. SUMMARY OF RESULTS

| Type of mating | No. of matings | Offspring | | |
|---|---|---|---|---|
| | | Halon-high | Halon-low | High (%) |
| Halon-high × Halon-high | 3 | 5 | 2 | 71 |
| Halon-high × Halon-low | 44 | 29 | 23 | 55 |
| Halon-low × Halon-low | 98 | 0 | 125 | 0 |

Low × Low phenotype crosses produced only low offspring, which is the strongest evidence that the low phenotype is governed by the presence of two recessive alleles (*hh*).

The results in Table 9 indicate that no linkage of sex with ability to hydrolyse halons is involved; no other characteristics were observed which distinguished Halon-low from Halon-high sheep.

No firm conclusion as to the spread of the dominant allele through sheep populations can be drawn from the results obtained so far. If breeding in flocks of sheep is assumed to be random, then a lack of any selection pressure for or against the ability to hydrolyse halons, or any character linked to this hydrolysis, would render the population stable in its numbers of homozygotes and heterozygotes, and each generation should repeat the pattern $1:x:\frac{1}{2}x^2$ for homozygous dominants: heterozygotes: homozygous recessives. Alternatively, the heterozygotes may have advantages, which

are selected for by natural or line-breeding pressure, whereas the homozygous Halon-high sheep are constantly eliminated or prevented from breeding in some way. Further experiments are planned to attempt a solution of this problem.

If homozygous dominants were present in the populations tested, they showed no increase in enzyme activity over the heterozygotes and the dominance would therefore be complete (Table 10). If this is so, it contrasts with the case of atropinesterase in

TABLE 10. A COMPARISON OF THE RATE OF HALOXON HYDROLYSIS BY PLASMA FROM KNOWN HETEROZYGOTES AND FROM POSSIBLE HOMOZYGOUS DOMINANTS

| Enzyme Source Genotype | Breed | No. | Hydrolysis of Haloxon (μM/ml/hr) Mean | Range |
|---|---|---|---|---|
| Heterozygotes | Herdwick Ewes | 4 | 3·7 | 3·3 – 4·0 |
| | Herdwick Lambs | 4 | 3·4 | 3·3 – 3·6 |
| | Blackface Ewe | 1 | 4·0 | — |
| Possible Homozygous dominants | Herdwick Ewes | 5 | 3·9 | 3·7 – 4·0 |
| | Herdwick Lambs | 5 | 3·4 | 3·3 – 3·5 |
| | Blackface/Swaledale Ewe | 1 | 3·8 | — |

rabbit serum,[15] the presence of which is controlled by a partially dominant allele, and with the case of atypical pseudocholinesterase in human serum, where partial dominance also occurs.[16]

No evidence of a multiple allelic system of inheritance, such as exists in pigs for plasma arylesterase,[16] was obtained. Unfortunately, no pig plasma has been available for comparison of its halon hydrolysing abilities.

TABLE 11. THE INCIDENCE OF ATAXIA IN SHEEP AFTER DOSING WITH HALOXON, AND ITS RELATIONSHIP TO THE RATE OF HALOXON HYDROLYSIS BY THE PLASMA

| Breed | Dose mg/kg | No. | Halon-high plasma Ataxic | Halon-high plasma Normal | Halon-low plasma Ataxic | Halon-low plasma Normal |
|---|---|---|---|---|---|---|
| Welsh Mountain | 132 | 1 | 0 | 1 | — | — |
| Welsh Mountain | 150 | 23 | 0 | 8 | 0 | 15 |
| Welsh Mountain | 265 | 20 | 0 | 5 | 1 | 14 |
| Welsh Mountain | 375 | 60 | — | — | 10 | 50 |
| Welsh Mountain | 530 | 35 | 0 | 11 | 12 | 12 |
| Welsh Mountains | 1500 | 1 | 0 | 1 | — | — |
| Welsh Mountain | 3000 | 1 | 0 | 1 | — | — |
| Kent | 265 | 100 | 0 | 40 | 15 | 45 |
| Kerry | 375 | 30 | — | — | 18 | 12 |
| Suffolk/Clun | 375 | 50 | 0 | 5 | 25 | 20 |
| Cheviot | 375 | 15 | — | — | 4 | 11 |
| Cheviot | 3 × 1000 daily | 1 | 0 | 1 | — | — |
| Crossbreds | 265 | 9 | 0 | 3 | 2 | 4 |
| Crossbreds | 530 | 8 | 0 | 4 | 4 | 0 |

(*c*) *Effect of rate of halon hydrolysis on toxicity studies.* An investigation[18] into the toxic symptoms produced in sheep by doses of Haloxon, greatly in excess of the anthelmintic dose, has shown that a delayed response occurs in the form of an ataxia similar to that produced in chickens by TOCP and DFP (di-isopropyl phosphorofluoridate).[1, 19]

This ataxic response could not be elicited in all sheep, and the possibility that a high rate of hydrolysis of the Haloxon might protect those animals which possessed it, was investigated. (Table 11).

The results demonstrated that Halon-high sheep could not be rendered ataxic by doses of Haloxon as high as 3,000 mg/kg, whereas a number of Halon-low sheep became ataxic after treatment with moderately large amounts of Haloxon (>150 mg/kg). The response was dependent upon the dose of Haloxon, and, probably, upon the breed of sheep.

This protection, afforded to Halon-high sheep, against the effects of an organophosphorus compound is an excellent example of a built-in resistance mechanism, possessed by a large proportion of a population which has not been exposed to any selection pressure. Such mechanisms must be taken into account in any assessment of the possible toxic effects of drugs given to animals, and the possibility of breeding animals to suit the drugs is an intriguing one.

The biochemical lesion which leads to the ataxic conditions after large doses of halons is not known, nor is there sufficient evidence to suggest whether the halon itself or a metabolite is responsible. No metabolism such as that of TOCP[19, 20] can be visualized, and it is possible that the failure of the halons to inhibit cholinesterase leaves the animal alive to suffer the later ataxic conditions, while the hydrolysis of halons cholinesterases, B-esterases and A-esterases may account for the high doses needed to bring an effective concentration of the drug into contact with the susceptible system.

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