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THE TOXICITY OF HALOXON TO GEESE, DUCKS AND HENS, AND ITS RELATIONSHIP TO THE STABILITY OF THE DI-(2-CHLOROETHYL) PHOSPHORYL CHOLINESTERASE DERIVATIVES

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Abstract—Geese are very susceptible to the acute toxic effects of haloxon [di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate], 50 mg/kg being sufficient to elicit a lethal response in most birds. Haloxon is much less toxic to ducks than to geese, whilst hens can tolerate very large doses of the anthelmintic, 5000 mg/kg being the dose necessary to produce the typical symptoms of acute toxicity.

Pyridine 2-aldoxime methiodide (2-PAM) is a very effective antidote to haloxon toxicity in geese, but has much less effect in ducks.

The cholinesterase of goose brain differs from that of ducks and hens in forming a stable di-(2-chloroethyl) phosphoryl derivative after reaction with haloxon, and this stability virtually precludes spontaneous reactivation of the cholinesterase. However, the *in vitro* recovery of goose brain cholinesterase activity in the presence of 2-PAM is rapid. as is the response of the poisoned bird dosed with the oxime.

The di-(2-chloroethyl) phosphoryl cholinesterase from duck brain is much less stable than that from goose brain, but more stable than that from hen brain cholinesterase This difference is reflected in the greater toxicity of haloxon to ducks than to hens, and also in the fact that 2-PAM speeds dephosphorylation of the di-(2-chloroethyl) phosphoryl cholinesterase formed by duck brain enzyme, but has no effect on the corresponding reaction involving hen brain cholinesterase.

The plasma cholinesterases of all three species are similar in their susceptibility to inhibition by haloxon and in their rate of spontaneous reactivation (dephosphorylation) following inhibition.

HALOXON [di-(2-chloroethyl) 3-chloro-4-methylcoumarin-7-yl phosphate], and other di-(2-chloroethyl) aryl phosphates, have previously been shown to produce di-(2-chloroethyl) phosphoryl derivatives of cholinesterases, which vary in stability according to their source. Those of certain nematode parasites are stable[1–3] whereas those of other nematodes and of mammals are unstable, with a half-life of about 20 min at 37°.[3, 4]

The parasites whose cholinesterase formed stable di-(2-chloroethyl) phosphoryl derivatives were more affected by haloxon, used as an anthelmintic treatment, than were those in which the cholinesterase recovered spontaneously after haloxon inhibition.[3]

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Malone[5] reported the toxicity of haloxon to hens and sheep; in further tests, it was found that geese were more susceptible than these animals, and it therefore became of interest to discover whether geese possessed a vital cholinesterase which showed similar reactions with haloxon to those of the susceptible nematode parasites.

The poisoned geese were found to respond extremely rapidly to intramuscular injections of 2-PAM, one of the recognized antidotes for organophosphate poisoning.

The present work compares the kinetics of the inhibition of brain cholinesterase by haloxon in geese, ducks and hens. An explanation of the variation of haloxon toxicity between these three species is sought from the differences in the reactions involved in inhibition.

The efficiency of pyridine-2-aldoxime methiodide (2-PAM) as an antidote to toxicity by cholinesterase inhibition, has also been investigated *in vivo* and *in vitro*, using geese, ducks and hens, in order to find whether geese respond differently to reactivators.

In addition, the plasma cholinesterase activity of geese, ducks and hens has been examined with respect to inhibition by haloxon, and compared with the corresponding brain enzyme.

EXPERIMENTAL

Geese, ducks and hens were maintained under free range conditions until used for dosing or to provide tissues for cholinesterase activity determinations.

Haloxon in a wettable powder formulation was given orally as a suspension in water, and 2-PAM was dosed in aqueous solution by intra-muscular injection.

Blood was obtained from the birds by decapitation immediately after death, followed by bleeding into beakers containing heparin as anticoagulant. Separation of plasma and erythrocytes was achieved by centrifugation at 4000 *g* for 15 min.

Brains were dissected out immediately after decapitation and used at once or deep frozen in solid carbon dioxide until required. Homogenates were made in an all-glass tissue grinder.

Cholinesterase activity was measured colorimetrically using the Fleischer *et al.*[6] modification of the method described by Hestrin.[7] The substrate in every experiment was 4×10^{-3} M acetylcholine perchlorate; incubation was at 37° and pH 7·5.

Inhibition by haloxon was achieved by incubating the enzyme preparation and inhibitor with phosphate buffer (pH 7·5) at 37° for various times prior to the addition of substrate.

Rates of reactivation after inhibition of cholinesterases by haloxon were determined by the following method. Enzyme, buffer and inhibitor in a total volume of 1 ml were incubated for 15 min at 37° until the level of inhibition reached a plateau in the cases of ducks and hens; 15 min was also the time chosen to produce a convenient inhibition level using goose brain cholinesterase.

All tubes then received 1 ml of 8×10^{-3} M acetylcholine and were further incubated at 37° for varying times. Duplicate sets of tubes for each species were removed from the water bath at 7½-min intervals after the addition of substrate, and were tested for residual acetylcholine using the method of Hestrin.[7]

Uninhibited enzyme preparations were treated in the same manner to determine whether acetylcholine hydrolysis was related directly to time of incubation. A linear relationship was found over a period of 37½ min.

From the increasing rates of hydrolysis in successive 7½ min periods, which occurred in the tubes with inhibited enzyme, the rate of spontaneous reactivation was calculated by use of the equation given by Reiner and Simeon-Rudolf[8] for the manometric method of estimating reactivation rates.

The effect of 2PAM on the rates of reactivation was assessed by adding oxime at 10^{-2} M final concentration to the inhibited cholinesterase together with the substrate, and then following the procedure outlined above.

RESULTS AND DISCUSSION

Geese, ducks and hens were dosed with haloxon to establish approximately a dose which would produce severe toxic symptoms but not death within the first 2 hr after dosing, so that antidotal therapy with 2-PAM could be investigated. The doses in Table 1 include 25, 500 and 5000 mg/kg, which regularly produced typical symptoms of organophosphate poisoning in geese, ducks and hens, respectively.

TABLE 1. THE EFFECT OF VARYING DOSES OF HALOXON ON HENS, DUCKS AND GEESE

Species	Haloxon dose (mg/kg)	Effect
Hen	500	None
Hen	5000	Toxic symptoms
Duck	50	None
Duck	500	Severe toxic symptoms
Duck	1500	Lethal
Goose	25	Severe toxic symptoms
Goose	50	Lethal

The effectiveness of 2-PAM as an antidote to haloxon toxicity was tested for geese and ducks by giving a 100 mg/kg dose by intramuscular injection when severe toxic symptoms were evident. The time for recovery of normal gait and posture was noted, and compared with the time taken by birds similarly dosed with haloxon, but not given 2-PAM.

TABLE 2. THE EFFECT OF 2-PAM ON TIME OF RECOVERY OF GEESE AND DUCKS FROM A DOSE OF HALOXON PRODUCING SEVERE TOXIC SYMPTOMS

Species	Haloxon dose (mg/kg)	2-PAM dose (mg/kg)	Recovery time
Goose	25	0	6–12 hr
Goose	25	100	10–30 min
Duck	500	0	4–6 hr
Duck	500	100	2–4 hr

The results in Table 2 indicate that 2-PAM has a rapid action in geese, all signs of toxicity having disappeared 30 min after the dose of 2-PAM, whereas control geese continued to suffer the toxic effects of haloxon for several hours.

For ducks, the difference between 2-PAM treated and control birds was much less marked, so much so that it was doubtful whether the antidote altered the time of recovery to any significant extent.

The high susceptibility of geese to poisoning by haloxon, together with the antidotal efficiency of 2-PAM, suggested that the cholinesterase of geese might differ significantly, in its reaction with haloxon and 2-PAM from that of ducks and hens.

Accordingly, experiments were carried out to determine the type of reaction between haloxon and the cholinesterases of goose, duck and hen brains.

The linear relation between the logarithm of percentage residual activity and time of inhibition indicated that the inhibition of goose brain cholinesterase was progressive over the course of the experiment (Fig. 1). No equilibrium was established between

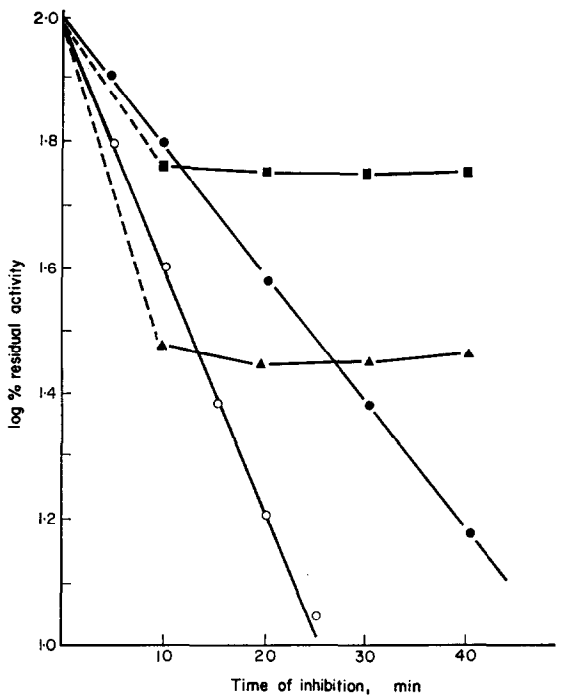

FIG. 1. The inhibition of goose, duck and hen brain cholinesterases by haloxon.
○ = Goose brain : 2×10^{-8} M haloxon.
● = Goose brain : 10^{-8} M haloxon.
■ = Duck brain : 10^{-8} M haloxon.
▲ = Hen brain : 10^{-6} M haloxon.
Inhibition carried out at 37°, pH 7·5

rate of inhibition and rate of spontaneous reactivation, as occurs with sheep erythrocyte and other acetylcholinesterases.[4] The bimolecular rate constant of the reaction was 6×10^6 l./mole/min at 37° and pH 7·5, which is similar to that for the cholinesterase of the parasitic nematode *Haemonchus contortus*.[1]

Using duck brain cholinesterase, inhibition by 10^{-8} M haloxon was rapid initially but after 10 min no further decrease in activity was observed (Fig. 1). Similar results

were obtained with hen brain cholinesterase, except that 10^{-6} M haloxon was necessary to give a comparable level of inhibition at equilibrium.

A similar equilibrium for sheep red cell cholinesterase was satisfactorily explained[4] by postulating the same route of inhibition as in the case of progressive inhibition but followed by spontaneous reactivation at a much greater rate:

$$E + I_{AB} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI_{AB} \xrightarrow{k_3} EI_A + B \xrightarrow{k_4} E + A + B,$$

where A and B are the di-(2-chloroethyl) phosphoryl and the 3-chloro-4-methyl-coumarinyl constituents of the haloxon molecule, respectively.

The rate constants (k_1–k_4) determine the speed of inhibition and reactivation; where k_4 is large, spontaneous recovery is rapid as with hen brain cholinesterase, and haloxon can be regarded as a substrate although the hydrolysis rate is slow compared with that for acetylcholine. For duck brain cholinesterase, k_4 is small but still capable of returning significant quantities of free enzyme to the system, so that an equilibrium is established, although at much lower concentrations of haloxon than occurs with either hen or sheep brain cholinesterase.

For goose brain cholinesterase, k_4 is so small that EI_A can be regarded as stable; thus, no reactivation occurs during the experimental period, and first order kinetics can be applied to describe the inhibition.

The "inhibiting" rate constants (k_1–k_3) may also vary between species, and the rate of formation of EI_A combined with the value of k_4, will determine the level of enzyme activity at equilibrium according to the concentration of inhibitor.

This postulate of differing reactivation rates for the di-(2-chloroethyl) phosphoryl derivatives of goose, duck and hen brain cholinesterase was tested by measuring the rate of spontaneous recovery of these enzymes after inhibition by haloxon (Fig. 2). The unimolecular rate constants calculated from the plots in Fig. 2 are given in Table 3, together with those for reactivation in the presence of 10^{-2} M 2-PAM (Fig. 3). These results show that spontaneous recovery of phosphorylated hen brain cholinesterase is five times more rapid than the recovery of duck brain cholinesterase. The spontaneous reactivation of goose brain cholinesterase was too slow to allow any estimate to be made.

In the presence of 2-PAM, however, the goose brain cholinesterase has the fastest rate of recovery, a marked improvement on the spontaneous rate, whereas the rate of reactivation of duck brain cholinesterase was raised fourfold, and that of hen brain cholinesterase was unaffected.

These results strongly suggest that the difference in toxicity of haloxon to hens, ducks and geese can be accounted for by the differences in the rates of spontaneous reactivation of the di-(2-chloroethyl) phosphorylated cholinesterases from the species. Also, the remarkable therapeutic effect of 2-PAM in geese can be attributed to the great increase in reactivation rate of the phosphorylated cholinesterase which 2-PAM produces in this species.

In ducks, however, a fourfold increase of reactivation rate by 2-PAM *in vitro* did not correspond to any observed increase of recovery rate in the live birds.

It should be noted that the source of cholinesterase for the *in vitro* experiments was brain tissue, so that, unless 2-PAM has a central nervous effect in geese, the correlation

between *in vitro* and *in vivo* results depends upon the assumption that the cholinesterase of the peripheral nervous system has the same inhibition characteristics for haloxon as that of the brain.

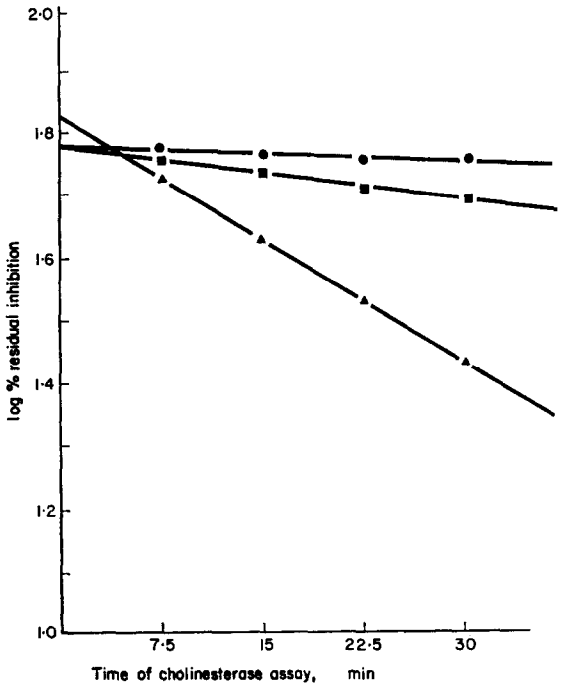

FIG. 2. Rate of spontaneous reactivation of Di-(2-chloroethyl) phosphoryl cholinesterases from goose, duck and hen brain
● = Goose brain.
■ = Duck brain.
▲ = Hen brain.

TABLE 3. UNIMOLECULAR RATE CONSTANTS FOR THE REACTIVATION OF GOOSE, DUCK AND HEN BRAIN CHOLINESTERASES FOLLOWING INHIBITION BY HALOXON

Species	Spontaneous reactivation k (first order) (H_2O) min^{-1}	Induced reactivation k (first order) (2-PAM) min^{-1}
Hen	$3{\cdot}1 \times 10^{-2}$	$3{\cdot}1 \times 10^{-2}$
Duck	$0{\cdot}67 \times 10^{-2}$	$2{\cdot}7 \times 10^{-2}$
Goose	Undetectable	$5{\cdot}9 \times 10^{-2}$

In order to determine whether goose brain cholinesterase activity is greatly affected after a 25 mg/kg dose of haloxon, and also whether treatment with 2-PAM can reverse any such effects, four geese were dosed and two of these given antidotal treatment at 30 and 90 min after dosing. All four birds were sacrificed at 2 hr after dosing, and the

heads deep frozen immediately in solid carbon dioxide. The cholinesterase activities of each brain were determined and compared with those of undosed birds (Table 4).

The results in Table 4 suggest that brain cholinesterase of geese is affected when a toxic dose of haloxon is administered, and that antidotal treatment with 2-PAM has no effect on the loss of activity of brain cholinesterase during the period when this

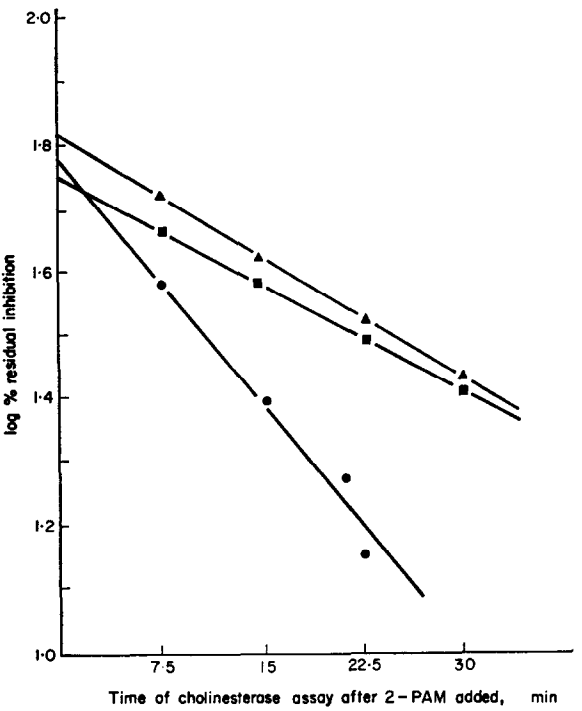

FIG. 3. Rate of 2-PAM induced reactivation of di-(2-chloroethyl) phosphoryl cholinesterases from goose, duck and hen brain

● = Goose brain.
■ = Duck brain.
▲ = Hen brain.

TABLE 4. ACETYLCHOLINE HYDROLYSIS BY GOOSE BRAIN TAKEN FROM CONTROL BIRDS AND FROM BIRDS DOSED WITH HALOXON PLUS 2-PAM

Treatment	Number of birds	Brain ChE activity μ moles/g/hr $\pm$ S.E.M.
None	3	811 $\pm$ 49
Haloxon (25 mg/kg)	2	363 $\pm$ 136
Haloxon (25 mg/kg) plus 2-PAM (100 mg/kg) at 30 min and 50 mg/kg at 90 min after the haloxon	2	264 $\pm$ 69

treatment eliminates all superficial toxic symptoms. These symptoms must be assumed to be associated with cholinesterase inhibition in the peripheral nervous system. There is no reason to suppose that this peripheral cholinesterase differs in any way from that of the brain in its reactions with inhibitors and reactivators.

For comparative purposes, the cholinesterases of the plasma from hens, ducks and geese were examined for type of reaction with haloxon. With each, an equilibrium between inhibition and reactivation was rapidly established, only slight differences between species being observed (Table 5). The low activity in goose plasma was the main reason for the choice of brain tissue as a cholinesterase source for experimental work.

TABLE 5. INHIBITION OF HEN, DUCK AND GOOSE PLASMA CHOLINESTERASE BY HALOXON EXPRESSED AS A pI_{50} VALUE

Species	Haloxon pI_{50}
Hen	6·58
Duck	6·30
Goose	6·45

N.B. The pI_{50} is calculated from the concentration of haloxon which produced 50 per cent inhibition under the conditions of assay of cholinesterase activity (0·2 ml plasma incubated with 4×10^{-3} M acetylcholine for 15 min at 37° and pH 7·5).

It is concluded that goose brain cholinesterase differs from all other vertebrate cholinesterases so far investigated, in forming a stable di-(2-chloroethyl) phosphoryl derivative. The only other cholinesterases known to form similar stable derivatives occur in certain nematode parasites of the sheep.[13]

Goose brain cholinesterase inhibited by haloxon is reactivated to a greater extent by 2-PAM than the enzymes from duck and hen brain. It is suggested that this difference exists in the peripheral nervous system, and accounts for the high efficiency of 2-PAM as an antidote for haloxon poisoning in geese.

Duck brain cholinesterase forms a di-(2-chloroethyl) phosphoryl derivative which is less stable than that formed by goose cholinesterase, but more stable than the phosphorylated cholinesterase produced in hens. Consequently, it might be expected that ducks would be more susceptible to haloxon poisoning than hens, and less susceptible than geese. In practice, ducks are unlikely to encounter a toxic dose of haloxon (> 300 mg/kg), whereas geese might accidently acquire a lethal dose (50 mg/kg) by ingesting haloxon intended for hens.

REFERENCES

1. R. M. LEE and M. R. HODSDEN, *Biochem. Pharmac.* **12**, 1241 (1963).
2. C. O. KNOWLES and J. CASIDA, *Expl Parasit.* in press.
3. R. J. HART and R. M. LEE, *Expl Parasit.* **18**, 332 (1966).
4. R. M. LEE, *Biochem. Pharmac.* **13**, 1551 (1964).
5. J. C. MALONE, *Res. vet. Sci.* **5**, 17 (1964).
6. J. H. FLEISHER, E. J. POPE and S. F. SPEAR, *A.M.A. Archs ind. Hlth.* **11**, 332 (1955).
7. S. HESTRIN, *J. biol. Chem.* **180**, 249 (1949).
8. E. REINER and V. SIMEON-RUDOLF, *Biochem. J.* **98**, 501 (1966).