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RELATION OF YOLK SAC MEMBRANE KYNURENINE FORMAMIDASE INHIBITION TO CERTAIN TERATOGENIC EFFECTS OF ORGANOPHOSPHORUS INSECTICIDES AND OF CARBARYL AND ESERINE IN CHICKEN EMBRYOS

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Abstract—One type of teratogenic effect induced in chicken embryos by a variety of organophosphorus (OP) and methylcarbamate (MC) insecticides is associated with lowered embryo NAD levels. This appears to result from inhibition of kynurenine formamidase, which impairs conversion of tryptophan to essential pyridine nucleotide cofactors. The yolk sac membrane and the embryonic liver contain two types of kynurenine formamidase, one sensitive and the other relatively insensitive to OP and MC inhibitors. The eserine-sensitive enzyme accounts for ~26 per cent of the overall activity for hydrolysis of N^1,N^2-diformyl-L-kynurenine. In studies with twenty-one OP insecticides and two MC compounds injected at day 4 of incubation, the inhibition of the eserine-sensitive kynurenine formamidase at day 9 of incubation correlates well with the lowering of NAD levels at day 12 of incubation and the intensity of teratogenic signs. Teratogens of this type combine high potency for inhibiting the relevant kynurenine formamidase in the yolk sac membrane early in development, and possibly in the liver at later stages, with adequate *in vivo* stability to maintain the inhibition through critical stages of embryogenesis. Thus, kynurenine formamidase joins acetylcholinesterase in the list of OP- and MC-sensitive enzymes of critical physiological significance.

Several organophosphorus (OP) and *N*-methylcarbamate (MC) insecticides induce developmental anomalies in chicken embryos [1]. Two types of teratogenesis are recognized. Type I leads to micromelia and abnormal feathering and is associated with a lowered embryo NAD level [1]. Type II involves arthrogryposis, wry neck and rumplessness and may result from disruption of the cholinergic system [1–3].

The relative potency of different OP and MC compounds for producing type I teratogenic signs and lowered embryo NAD levels correlates with their activity for *in vivo* inhibition of mouse liver kynurenine formamidase (arylformylamine amidohydrolase, EC 3.5.1.9) (KFase) [1]. This indirect evidence suggests that type I teratogenesis may result from interruption of the NAD biosynthetic pathway by inhibition of KFase [1]. The present report provides more direct evidence for this hypothesis by examining the nature and inhibition of KFase in the yolk sac membrane (YSM) of eggs treated with a variety of OP and MC compounds.

MATERIALS AND METHODS

Chemicals. Sources and purities for the OP and MC compounds were as previously reported [1]. N^1,N^2-Diformyl-L-kynurenine was obtained from Cal-Biochem (La Jolla, CA). Eserine was used as the sulfate salt.

Preparation of KFase. The yolk sac membranes were blotted with filter paper and homogenized in 0.1 M sodium phosphate, pH 7.4, buffer (1:2, v/v) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 18,000 *g* for 30 min and the supernatant fraction carefully separated from the sediment and floating yolk layer. The recovered supernatant fraction was centrifuged at 100,000 *g* for 90 min followed by careful removal from the sediment and yolk. This process was repeated a third time with centrifugation at 100,000 *g* for 120 min (or longer as necessary to obtain a clear solution). This laborious procedure removes yolk components which inhibit KFase. The enzyme preparation can be stored at 0–4° for 2 days without substantial change in activity.

The procedure used for preparing YSM–KFase was modified for other portions of the egg only in the concentration on homogenization (1:2, w/v, for embryo; 1:4, w/v, for liver; 1:2, v/v, for allantois; and 1:1, v/v, for white) and in the time of centrifugation (30 min at 18,000 *g* and 60 min at 100,000 *g*). Yolk KFase was extracted by the n-butanol method [4].

Determination of KFase activity. N^1,N^2-diformylkynurenine is an appropriate substrate for assay of KFase activity [5]. The reaction was initiated by adding 25 μl of 1.5×10^{-2} M N^1,N^2-diformylkynurenine to 0.6 ml of KFase preparation at 25°. N^2-formylkynurenine release was followed spectrophotometrically at 365 nm [6] and expressed as kynurenine equivalents. The reaction proceeded according to zero-order kinetics for at least 7 min.

Determination of in vitro *and* in ovo *FKase inhibition.* For *in vitro* inhibition studies, an aqueous solution of the OP or MC compound was added to YSM–KFase from day 9 embryos for preincubation

as stated prior to substrate addition. An additional internal-control method was used for eserine with essentially the same results as for the procedure just described. For the internal-control assay, 25 μl substrate was added to 0.6 ml KFase, the change of absorbancy at 365 nm was followed for 2.5 min, then 10 μl of eserine solution was added and, after a period of 1–2 min for equilibration, the change of absorbancy was followed for a further 3 min.

In ovo inhibition of the YSM eserine-sensitive KFase (referred to as B-KFase for reasons given later) was measured by two methods based on those just described. In the preincubation method, the total KFase activity was compared to that for the same preparation preincubated for 2 min with 10^{-3} M eserine. In the internal-control procedure, the total KFase activity was followed as above for 2.5 min, then eserine solution (10 μl) was added to give a final concentration of 10^{-3} M and the enzyme reaction after equilibration was followed for a further 3 min. The zero-order kinetic constants before eserine addition (k_0 for control eggs and k_I for OP- and MC-treated eggs) were compared to those for the same enzyme preparations after eserine (e) addition (k_{0+e} and k_{I+e} respectively). The k_{I+e} value of treated eggs was identical to k_{0+e} of control eggs. With control eggs, k_0 was found to equal (1.35 ± 0.06 standard deviation) × k_{0+e}. The percentage inhibition of B-KFase in OP- or MC-treated eggs or enzyme preparations is therefore derived by the equation:

$$\%I = 100\left[1 - \left(\frac{k_I - k_{I+e}}{1.35k_{I+e} - k_{I+e}}\right)\right] = 100\left[1 - \left(\frac{k_I - k_{I+e}}{0.35k_{I+e}}\right)\right]$$

Effect of various OP and MC compounds as in ovo *inhibitors of YSM B-KFase.* Fertile white leghorn eggs were injected at day 4 of incubation with the OP or MC compound dissolved in 10 μl methoxytriglycol. Control eggs were injected with solvent only. The YSM was removed at the designated time and the KFase prepared and assayed as described above. Each assay utilized YSMs from four to five eggs except those in studies on the time course of inhibition, where eight to ten eggs were used. The KFase and B-KFase activities of OP- or MC-treated eggs were always compared to control eggs in the same experiment.

RESULTS

Distribution of total KFase in the egg. YSM preparations exhibit much higher total KFase activity than other portions of the egg (Table 1). This activity is present only in the soluble fraction. Attempts to recover enzyme from the particulate material at 800, 18,000 and 100,000 *g* by homogenization in a high-speed Polytron and extraction with sodium deoxycholate (0.1%, w/v) and Triton X-100 (1%, v/v) proved unsuccessful.

The total YSM KFase activity increases with incubation time (Fig. 1), in a manner generally paralleling that for the increased amount of total YSM during development [7].

Table 1. Distribution of total kynurenine formamidase (KFase) activity in different portions of the egg at day 4 of incubation

Portion	KFase activity (% of total)
Yolk sac membrane	96*†
Embryo	3*
Yolk	1
White	0

* Average values from four experiments, each with ten eggs.

† The KFase activity of the allantois is 19 per cent of that for the YSM, including the allantois at day 9 of incubation.

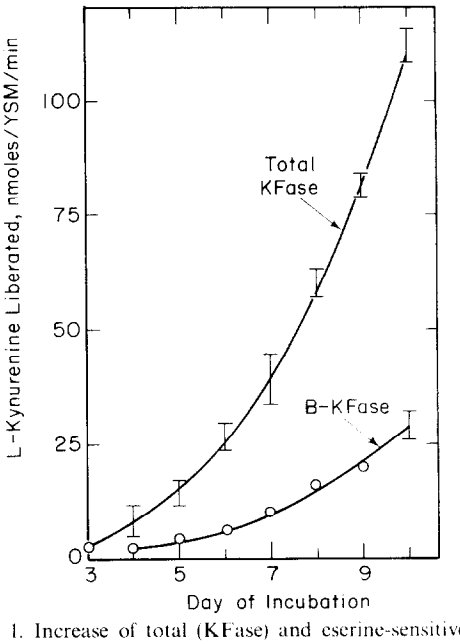

Fig. 1. Increase of total (KFase) and eserine-sensitive (B-FKase) yolk sac membrane kynurenine formamidase activity during initial development of incubating hen eggs. Each point represents a group of ten eggs or the range for two to six experiments on groups of ten eggs.

In vitro *inhibition of YSM KFase.* Only 26 per cent of the total KFase is readily inhibited by diazoxon, *N*-demethyl-monocrotophos and eserine (Fig. 2). This sensitive portion is referred to as B-KFase, in analogy with Aldridge's OP-sensitive B-esterases [8]. Similarly, the nonsensitive portion of the total KFase is designated as A-KFase [8]. The negative logarithm of the molar concentration for 50 per cent inhibition (pI_{50}) of B-KFase is 5.4 for diazoxon, 4.8 for *N*-demethyl-monocrotophos and 4.1 for eserine. Essentially complete inhibition of B-KFase is obtained with 2×10^{-5} M diazoxon, 1×10^{-4} M *N*-demethyl-monocrotophos and 1×10^{-3} M eserine, under the experimental conditions used. The inhibition of B-KFase is dependent on the preincubation time before substrate addition, e.g. eserine at 10^{-6} M inhibits 4 per cent after 2 min and 77 per cent after 225 min and *N*-demethyl-monocrotophos at 10^{-7} M inhibits 8 per cent after 5 min and 39 per cent after 225 min.

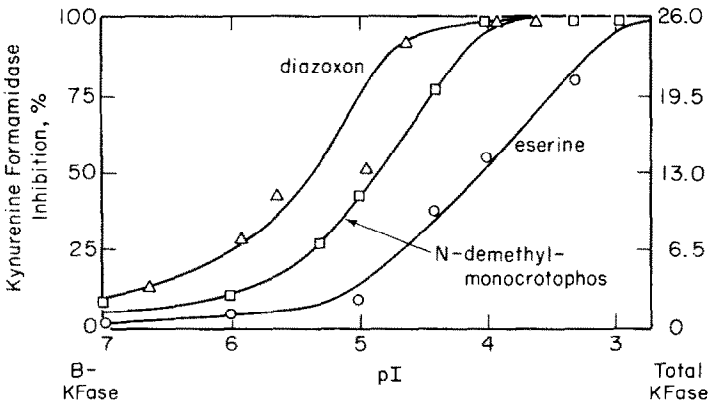

Fig. 2. *In vitro* inhibition of total (KFase) and eserine-sensitive (B-KFase) yolk sac membrane kynurenine formidase by diazoxon, *N*-demethyl-monocrotophos and eserine with 10, 5 and 2 min of preincubation, respectively, of enzyme and inhibitor prior to substrate addition. pI is the negative logarithm of the molar concentration of inhibitor. Inhibition is shown relative to both B-KFase (left ordinate) and total KFase (right ordinate).

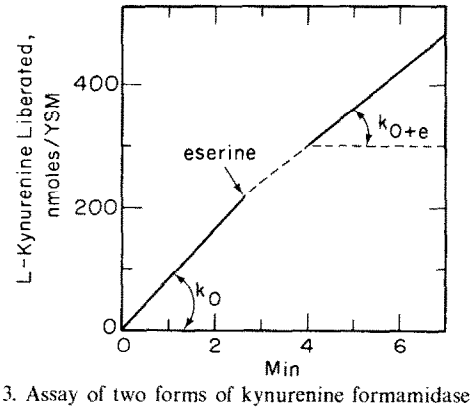

Fig. 3. Assay of two forms of kynurenine formamidase by the internal control method. Constants: k_0 = zero order kinetic constant for control enzyme corresponding to combined activity of A-KFase and B-KFase; k_{0+e} = zero order kinetic constant for control enzyme corresponding to A-KFase only.

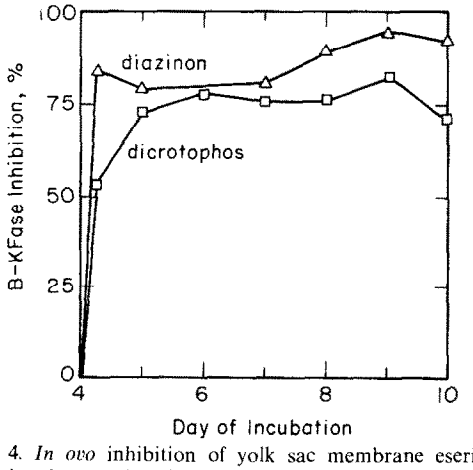

Fig. 4. *In ovo* inhibition of yolk sac membrane eserine-sensitive kynurenine formamidase (B-KFase) at various times after administering diazinon and dicrotophos at 50 μg/egg on day 4 of incubation.

The internal-control method used to differentiate A- and B-KFase is illustrated in Fig. 3. The initial rate of the enzyme reaction (k_0) corresponds to the sum of A- and B-KFase activity and the residual enzyme activity after addition of 10^{-3} M eserine (k_{0+e}) to A-KFase. The change in the slope when eserine is added represents B-KFase activity. Both the A- and B-KFase activities are proportional to protein concentration under the experimental conditions used.

The ratio of B-KFase to total KFase activity remains essentially constant during development (Fig. 1).

In ovo *course of YSM B-KFase inhibition by OP and MC compounds.* The most potent teratogens at 50 μg/egg give strong B-KFase inhibition until at least day 10 of incubation (Fig. 4). In contrast, the less- or non-teratogenically active compounds, even at 1 mg/egg, give lower or less persistent inhibition (Table 2). Only with parathion and methyl parathion is there a significant increase in B-KFase inhibition between days 8 and 10 of incubation.

Structure–activity and dose–dependency correlations of potency for inhibiting YSM B-KFase and for producing lowered embryo NAD levels and type I teratogenesis. Figure 5 gives the results with twenty-three OP and MC compounds injected (1 mg/egg) on day 4 of incubation, plotting the arithmetic average of B-KFase activities at days 8 and 10 (Table 2) against embryo NAD levels at day 12 and type I teratogenic signs at day 19 [1]. A good correlation is evident in this structure–activity study between B-KFase activity, embryo NAD level and type I teratogenic signs.

The dose–dependence for inhibition of YSM B-KFase is shown in Fig. 6 and is related to the lowering of embryo NAD levels in Table 3. The concentrations for 50 per cent inhibition in the two assays generally agree within a magnitude of about 2-fold, and the potency in both cases decreases in the order: diazinon > dicrotophos = eserine > Carbaryl. This dose–dependency study supports the structure–activity study in relating B-KFase inhibition to lowered embryo NAD levels and type I teratogenesis (Fig. 7).

Table 2. Potency of various organophosphorus compounds and of carbaryl and eserine administered at 1 mg/egg on day 4 of incubation for inhibiting yolk sac membrane eserine-sensitive kynurenine formamidase (B-KFase) at days 8 and 10 of incubation

Compound*		Inhibition of YSM B-KFase activity (%)		
No.	Name	Day 8	Day 10	Average
1	Diazinon	100	82	91
2	SAN 1 197	86	87	87
3	Dicrotophos	93	68	81
4	Monocrotophos	100	58	79
5	Pirimiphos-methyl	76	74	75
6	Carbaryl	86	59	73
7	Eserine	72	70	71
8	Pirimiphos-ethyl	57	71	64
9	Phosphamidon	68†	57	63
10	Phorate	69	39	54
11	Mevinphos	52†	50	51
12	Parathion	36	64	50
13	Methamidophos	42†	57	50
14	Methyl parathion	30	67	49
15	Coumaphos	27	50	39
16	Chlorpyriphos	50	23	37
17	EPN	68	12	35
18	Dichlorvos	41	22	32
19	Dimethoate	40	21	31
20	TOCP	24	22	23
21	Leptophos	15†	25	20
22	Phenyl saligenin cyclic phosphate	10	26	18
23	Malathion	23	0	12

* For chemical identity, see Ref. 1.
† YSM at day 7.

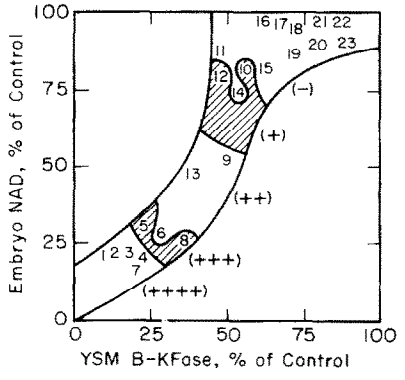

Fig. 5. Correlation of potency of various organophosphorus compounds and of Carbaryl and eserine administered at 1 mg/egg on day 4 of incubation for inhibiting yolk sac membrane eserine-sensitive kynurenine formamidase (B-KFase) and for producing lowered embryo NAD levels and type I teratogenesis. Number designations for compounds and original data for B-KFase inhibition are given in Table 2. Teratogenic ratings at day 19 of incubation from most to least severe are + + + + > + + + > + + > + > − [1]. NAD levels are at day 12 of incubation [1].

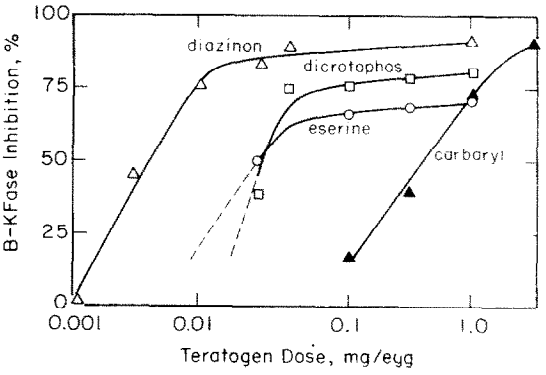

Fig. 6. *In ovo* inhibition of yolk sac membrane eserine-sensitive kynurenine formamidase (B-KFase) at day 9 of incubation by diazinon, dicrotophos, eserine and carbaryl administered to eggs at various doses on day 4 of incubation.

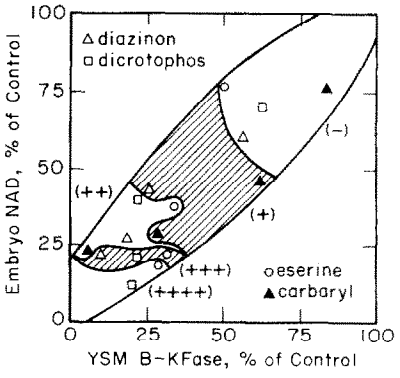

Fig. 7. Correlation of potency of diazinon, dicrotophos, eserine and carbaryl administered to eggs at various doses on day 4 of incubation for inhibiting yolk sac membrane eserine-sensitive kynurenine formamidase (B-KFase) and for producing lowered embryo NAD levels and type I teratogenesis. Original data for B-KFase inhibition are given in Fig. 6 and for NAD levels and teratogenic signs in Ref. 9.

Table 3. Potency of diazinon, dicrotophos, eserine and carbaryl administered at various doses on day 4 of incubation for inhibiting yolk sac membrane eserine-sensitive kynurenine formamidase (B-KFase) at day 9 of incubation and for lowering embryo NAD levels at day 12 of incubation

Compound	Dose for 50% effect (μg/egg)	
	Inhibition of YSM B-KFase*	Lowering of embryo NAD†
Diazinon	4	5
Dicrotophos	34	66
Eserine	31	60
Carbaryl	410	240

* Data from Fig. 6.
† Data from Ref. 9.

DISCUSSION

This study evaluates the hypothesis that OP- and MC-induced inhibition of KFase and therefore of NAD formation early in embryogenesis leads to type I teratogenic signs. This hypothesis is consistent with the teratogen-induced block in conversion of [^{14}C]tryptophan to [^{14}C]NAD [1] and the alleviating activity of different compounds in the L-tryptophan to NAD biosynthetic pathway [1, 9, 10].

The KFase of chicken eggs is localized in the soluble fraction of the YSM and liver. The YSM substitutes metabolically for the liver and many other non-developed organs at early stages of chicken embryo development [11]. OP and MC compounds inducing type I teratogenic signs are most potent when injected at days 4–6 of incubation [1], indicating that they may act initially on enzymes of the YSM [9, 12] and perhaps later on liver enzymes. The studies focused on KFase isolated from the YSM, but additional studies* show that embryo liver KFase has similar properties.

Inhibition studies suggest that two types of KFase are present in the YSM and liver. The one referred to as B-KFase is sensitive to inhibition by OP and MC compounds, and the other, A-KFase, is much less sensitive to inhibition. The present study assays these enzymes on a relative basis, i.e. B-KFase as the eserine-sensitive portion of the total KFase activity. It is likely, for reasons given below, that B-KFase is of greater importance than A-KFase for *in ovo* NAD biosynthesis.

The inhibited forms of a B-KFase are not readily reactivated, based on *in vitro* studies. B-KFase, inhibited by an excess of Carbaryl (to 96 per cent inhibition), does not spontaneously recover at 25°, even after 120 min. Similar results are observed with the enzyme inhibited *in ovo* by OP compounds. No detectable reactivation occurs on holding inhibited KFase preparations overnight at 4°.

Teratogenic OP and MC compounds appear to be those with a high affinity for YSM B-KFase and sufficient persistence in the egg to maintain *in ovo* inhibition throughout critical stages of development. Both potent teratogens and many teratogenically inactive compounds, injected at 1 mg/egg on day 4 of incubation, inhibit YSM B-KFase during the period of days 4–8 of incubation. For example, although *O*-ethyl-*O*-*p*-nitrophenyl phenylphosphonothioate (EPN) at 0.1 mg/egg does not inhibit B-KFase at any time after injection, a 1 mg/egg dose inhibits 82 per cent at day 5 and 68 per cent at day 8 of incubation. However, only compounds and doses that are teratogenic cause prolonged B-KFase inhibition. The total YSM B-KFase increases markedly during the first few days of development, so it is necessary for a single OP or MC dose to persist for several days as a reservoir for inhibition, either directly or via a metabolite (e.g. parathion and methyl parathion), of B-KFase newly synthesized between days 4 and 10 of incubation. It is conceivable that the OP and MC compounds might affect the synthesis of B-KFase rather than inhibit the enzyme once formed. This is not likely since the OP and MC compounds are potent *in vitro* B-KFase inhibitors. Further, separate studies with diazinon (1 mg/egg injected at day 4 of incubation) establish that when the metabolic block is circumvented by administering nicotinamide (0.8 μmole/egg injected at day 4 of incubation), the teratogenic signs and the lowered NAD levels are alleviated without change in the degree of B-KFase inhibition.

Type I teratogenesis in chicken embryos appears to result from inhibition of YSM B-KFase by >50 per cent at day 9 of incubation, which leads to lowering of the embryo NAD level by 50 per cent at day 12 of incubation and ultimately results in micromelia and abnormal feathering. These relationships are applicable to a variety of OP and MC compounds administered at a standard dose and to varying doses of some of the most potent teratogens. They indicate the importance of a better understanding of KFase as a target site for phosphorylating and carbamylating agents.

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* J. Seifert and J. E. Casida, unpublished results (1978).

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