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RELATIONSHIP OF THE ACYLATION OF MEMBRANE ESTERASES AND PROTEINS TO THE TERATOGENIC ACTION OF ORGANOPHOSPHORUS INSECTICIDES AND ESERINE IN DEVELOPING HEN EGGS

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Abstract—The yolk sac membrane from hen eggs contains esterases that hydrolyze phenyl phenylacetate (PPA) and are sensitive to inhibition, both *in vitro* and *in vivo*, by many organophosphorus compounds and by eserine. These esterases are the principal proteins in the membrane which are phosphorylated by ^{3}H-diisopropyl phosphorofluoridate. Inhibition of this mixture of PPA-hydrolyzing esterases *in vivo* does not correlate with the induction of teratogenesis because they react with both teratogenic and non-teratogenic acylating agents. However, electrophoresis studies indicate that inhibition *in vivo* of one or more separable components making up a very small proportion of the total membrane esterase mixture occurs with teratogenic but not with non-teratogenic treatment schedules. Thus, dialkylphosphorylation or methylcarbamoylation of this reactive membrane component(s) *in vivo* may initiate a sequence of events leading to embryonic abnormalities. This postulated mode of action is analogous to that proposed earlier by British workers in the production of delayed neurotoxicity by some organophosphorus compounds.

SOME organophosphorus insecticides and methylcarbamates are teratogenic when injected into hen eggs before or within the first few days of incubation.[1–12]† or when administered to mammals.[12–16] Chemical structure-teratogenic activity correlations and biochemical studies made with hen eggs establish that: (a) a wide variety of compounds are teratogenic;[1–12]† (b) the acetylcholine–cholinesterase components of the cholinergic system of the embryo are probably not involved;[2,8] (c) nicotinamide and nicotinamide–adenine dinucleotide cofactors diminish the abnormalities;[1–3,8,10,11] and (d) some teratogens impair membrane permeability.[2]

Many organophosphorus compounds also produce delayed neurotoxicity in hens. The mechanism of this neurotoxicity may depend on the reaction of the organophosphate with a specific nerve membrane protein which is readily phosphorylated by diisopropyl phosphorofluoridate (DFP) and which hydrolyzes phenyl phenylacetate (PPA). The esteratic activity of this protein is sensitive to inhibition by neurotoxic but not by non-neurotoxic organophosphorus compounds.[17–21] Possibly, the acylation of a similar protein in the yolk sac membrane would lead to nutritional deficiencies in the embryo and differential inhibition of embryonic development, thus causing the teratogenic effects noted with organophosphorus compounds and methylcarbamates.

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This report considers the effects of some teratogenic and non-teratogenic organophosphorus compounds and eserine on esterases and proteins acylated by these compounds acting in the yolk sac membrane of developing hen eggs.

MATERIALS AND METHODS

Chemicals, treatment of eggs, and preparation of yolk sac membrane homogenates

The sources for the chemicals were previously described[2,3] with the following exceptions: paraoxon from American Cyanamid Co., Princeton, N.J.; *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonate (EPNO) from E. M. Bellet of this laboratory; diisopropyl-(1,3-^{3}H) phosphorofluoridate (^{3}H-DFP) (sp. act. 3·6 c/m-mole) from Amersham/Searle, Arlington Heights, Ill.; α-naphthyl acetate (NA), Fast Blue RR salt and potato starch hydrolyzed for electrophoresis from Sigma Chemical Co., St. Louis, Mo. α-Naphthyl phenylacetate and PPA were synthesized according to published procedures.[22,23]

Fertile white Leghorn eggs from Kimber Farms Inc., Niles, Calif., were incubated in a Jamesway model 252B automatic egg incubator (Jamesway Manufacturing Co., Fort Atkinson, Wis.) at 37° and 73 per cent relative humidity. The toxicants were injected after 120 hr of incubation because this approximates the stage of embryo development most sensitive to the teratogenic action of organophosphorus compounds and methylcarbamates.[3] The test chemicals were injected directly into the yolk through a sterilized Swinny filter adapter containing a 0·22 μ filter pore (Millipore Filter Corp., Bedford, Mass.) headed with a sterile disposable No. 22 gauge 2·5-cm needle. Control eggs received 100 μl saline solution. Water-insoluble compounds were injected in 10–50 μl ethanol or dimethylsulfoxide (DMSO) using a microsyringe. The injection hole was resealed with paraffin wax immediately following the injection.

Eggs were opened after an additional 24-hr period of incubation, unless stated otherwise, and the yolk sac membrane was carefully dissected away from the embryo and yolk. (For the purpose of this study, the yolk sac membrane is defined as the invaginated, vesicular membrane portion delineated by the *sinus terminalis*.) The membrane was washed in cold water to free it from excess yolk, blotted and immediately frozen. Samples were stored at −15° for up to 3 weeks before assays. Membranes were rapidly thawed and representative samples thereof were homogenized in 0·05 M tris (hydroxymethyl)aminomethane (Tris) buffer (pH 7·4) using a precooled glass tissue grinder with a glass pestle. This homogenate was used directly for reaction with ^{3}H-DFP or assay of esterase activity with PPA as the substrate or, for electrophoresis studies, was centrifuged at 20,000 *g* for 10 min to separate the supernatant and sediment fractions, only the supernatant fraction being used for electrophoretic analysis. The yolk sac membrane homogenates contained 333 mg fresh tissue weight equivalent/ml or 26·4 ± 0·3 mg protein/ml. Protein was determined by the method of Lowry *et al.*[24]

Assay of available phosphorylation sites using ^{3}H-DFP. This procedure is based on the method described and used extensively by Johnson, Aldridge *et al.*[17–21] The membrane homogenate (0·1 ml) was added to 0·05 M Tris buffer (pH 7·4; 0·9 ml) with or without added inhibitors and the mixture was held at 25° for 30 min. The phosphorylation assay was initiated by addition of excess ^{3}H-DFP (7·0 μμmoles) in isopropanol (50 μl) followed by a 5-min reaction period and immediate centrifugation at 20,000 *g* for 10 min. After decantation of the supernatant, the pellet was

resuspended in Tris buffer (pH 7·4; 1·0 ml) and this suspension was recentrifuged at 20,000 *g* to obtain a second supernatant which was combined with the first. The combined supernatants, containing greater than 90 per cent of the radioactivity, were treated with acetonitrile (3 ml) and the denatured protein was sedimented by centrifugation at 10,000 *g* for 10 min. The supernatant, containing the unreacted ^{3}H-DFP and, possibly, some labeled hydrolysis products, was discarded, the pellet was resuspended in water (1·0 ml) and the precipitation procedure was repeated again by addition of acetonitrile (3 ml) and centrifugation as before. The clear supernatant was discarded and the pellet, after holding in air until most of the residual acetonitrile had evaporated, was partially dissolved in 0·2 ml of Soluene® 100 (Packard Instrument Co., Downers Grove, Ill.). Toluene (1–2 ml) was added and after a further holding period of 12 hr, during which time the solution was complete, additional portions of toluene (2 × 2 ml) were used to wash the clear solution from the original centrifugation tube into pre-counted scintillation vials containing 15 ml of a liquid scintillator [0·55% 2,5-diphenyloxazole in toluene-2-methoxyethanol mixture (2 : 1, v/v)]. The radioassay results were corrected for quenching using ^3H_2O as an internal standard. Under the standard assay conditions, about one-third of the radioactivity is protein bound in control membrane preparations, representing 787 ± 80 μμmole ^{3}H-DFP equivalent/g protein. Addition of higher levels of ^{3}H-DFP, up to three times the standard level, does not alter the level of bound radioactivity.

The radioactivity determined by the ^{3}H-DFP procedure is probably covalently bound to the protein rather than occluded in the course of protein precipitation because no radioactivity was lost from the final pellets when they were dissolved in 98% formic acid (1 ml) and diluted with water (10 ml), and the protein in the solution was reprecipitated at 0° by addition of 10 M $HClO_4$ (2 ml) followed by resuspension in water and final precipitation with acetonitrile and dissolution in Soluene 100 for radioassay. As discussed later, it is likely that the bound radioactivity represents diisopropylphosphoryl esterases or proteins and that the available phosphorylation sites are titrated by this procedure.

Assay of esterase activity. The procedures for enzyme and phenol assays are based on Poulsen and Aldridge[23] and Gottlieb and Marsh[25] respectively. The membrane homogenate (0·1 ml) was added to 0·05 M Tris buffer (pH 7·4; 0·9 ml) with or without added inhibitors and the mixture was held at 25° for 30 min. (This first part of the procedure is identical to that used for the phosphorylation-site assays with ^{3}H-DFP.) The esterase assay was initiated by addition of PPA (4·7 μmoles) in acetone (50 μl) and incubation was continued for 15 min. The reaction was stopped by the addition of trichloroacetic acid (10%; 0·5 ml) and, after centrifugation at 5000 *g* for 10 min, a 0·5 ml aliquot of the clear supernatant was mixed with 1 ml of 0·025% 4-aminoantipyrine in 0·5 M Tris buffer (pH 9·0). Finally, 0·5 ml of aqueous 0·4% $K_3Fe(CN)_6$ was added and the absorbance was determined at 510 nm against a reagent blank. When necessary, the solutions were diluted 2- to 3-fold with water prior to absorbance measurements. The normal enzyme activity of the yolk sac membrane homogenate is 2·41 ± 0·20 μmoles PPA hydrolyzed/g fresh weight equivalent/min or 30·4 ± 2·5 μmoles/g protein/min. The amount of phenol released is directly proportional to both enzyme concentration (15 min, up to at least 10 mg protein) and to time (2·6 mg protein, up to at least 30 min). The enzyme activity was unchanged on holding the membrane preparations in a frozen state for up to 3 weeks.

Electrophoretic separations of yolk sac membrane esterases. Starch gel electrophoresis was accomplished by the method of Tashian and Shaw[26] and Tashian.[27] Potato starch hydrolyzed for electrophoresis (28 g) was added directly to 0·03 M sodium borate buffer (pH 8·6; 200 ml) and the mixture was heated to boiling, deaerated under reduced pressure, and poured into a plastic tray (dimensions: 5 × 220 × 20 mm). The gel was covered with parafilm and allowed to cool to 4°. Aliquots (25 μl) of the clear supernatant of the yolk sac membrane homogenates, containing greater than 95 per cent of the esterase activity when assayed as above with PPA, were applied to pieces (10 × 4 mm) of Whatman No. 3mm filter paper, and individual pieces were inserted in each of up to six slits cut transversely in the starch block. Optimum results were obtained using two anodal trays and one cathode tray, with the bridge buffer (0·3 M borate, pH 8·0, containing 0·03 M NaCl) in the cathode tray and the first anode tray. The anode itself was placed in 10% NaCl solution, and the electrode solutions were linked to the gel by filter paper wicks saturated with bridge buffer. A voltage drop of 7–8 volts/cm was employed for 18–20 hr at 4°, resulting in 11–12 cm migration of the bromothymol blue marker. After electrophoresis, the gel slab was removed from the tray and sliced appropriately using a thin wire. The individual slices were then stained to detect esterases hydrolyzing NA according to the method of Markert and Hunter.[28] To accomplish this, NA (2% acetone solution; 1 ml) was added to a solution of 10 mg Fast Blue RR salt in 50 ml of 0·05 M Tris buffer (pH 7·0), with or without inhibitors, and the gel slice was then introduced and allowed to remain in this medium for 3 hr at 25°. The medium was changed when it became too cloudy. The reaction was stopped by decanting off the liquid and adding 7% aqueous acetic acid (50–60 ml). The gel patterns were sketched and photographed. Relative mobilities (Rm) were calculated with reference to bromothymol blue. The proteins were stained with 1% amido schwarz 10B in methanol–water–acetic acid mixture (5 : 5 : 1) for 10 min. Destaining was accomplished with the same solvent for 24–48 hr with several changes of solution. Attempts to modify this procedure for using PPA as the substrate were not satisfactory; these attempts included varying the solvent for dissolving the PPA, varying the coupling agent, and homogenization in Tris buffer of gel areas known to be active in hydrolyzing NA for direct assay of activity in PPA hydrolysis as previously described. α-Naphthyl phenylacetate was hydrolyzed only by the upper band (designated later as Z6-Z10) and at a very low rate relative to that achieved with NA; so, the former substrate was not used.

Polyacrylamide gel electrophoresis was accomplished by the method of Clarke[29] employing vertical tubes of 0·7 mm, i.d. Samples (25–50 μl) of centrifuged homogenate were mixed with 2–4 volumes of 10% sucrose in 0·005 M Tris—0·038 M glycine buffer (pH 8·3) and applied to the gel. The electropherograms were developed with the Tris–glycine solution, lacking sucrose, at 1–2 mA/tube at 4°, until the bromothymol blue marker had moved 7–8 cm. The gels were eased out of the tubes with a stiff wire and stained in a similar manner to the starch gels for esterases hydrolyzing NA and for protein. As before, PPA was not a satisfactory substrate.

RESULTS

Nature of esterases in the yolk sac membrane. Homogenates of the yolk sac membrane from eggs incubated for 144 hr rapidly hydrolyze PPA. The majority of this esterase activity is sensitive *in vitro* to inhibition by paraoxon and EPNO but Bidrin,

TABLE 1. RELATIVE POTENCY OF SEVERAL ORGANOPHOSPHORUS COMPOUNDS FOR INHIBITION *in vitro* AND *in vivo* OF PPA-HYDROLYZING ESTERASES IN YOLK SAC MEMBRANE HOMOGENATES

Compound	pI_{50}*	
	In vitro	*In vivo*†
Bidrin, technical	<3 (39%)‡	5·7
Azodrin	<3 (25%)‡	5·5
Bidrin amide	<3 (15%)‡	5·5
Paraoxon	7·7	>5·4 (69%)§
EPNO	7·8	
EPN		>>6·2 (90%)§

* Negative logarithm of molar inhibitor concentration producing 50 per cent inhibition.

† The concentration in the whole egg calculated on the assumption that each egg contains 50 ml fluid volume.

‡ Per cent inhibition at 1×10^{-3} M.

§ Per cent inhibition when the compounds are injected in ethanol to yield the indicated negative logarithm of molar inhibitor concentration in the whole egg.

Azodrin, or Bidrin amide are only weakly inhibitory (Table 1, Fig. 1). A portion of the esterase activity (35–40 per cent) is more sensitive than the remaining portion to inhibition *in vitro* by Bidrin but this does not appear to be the case with the more potent inhibitors, paraoxon and EPNO (Fig. 1). The membrane homogenate reacts readily with ^{3}H-DFP to produce a relatively constant level of protein labeling. If the

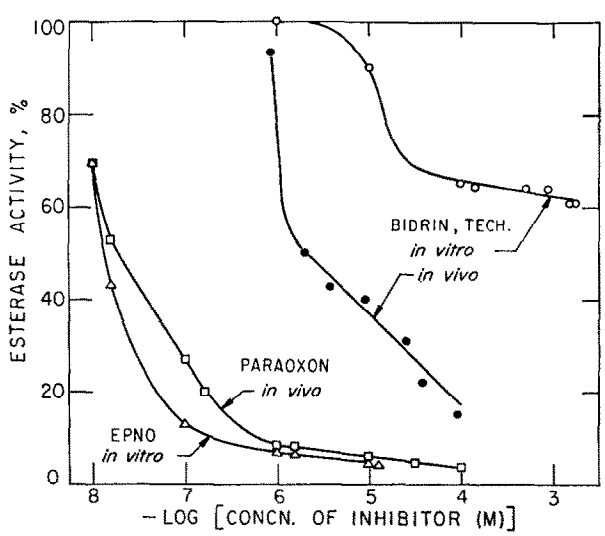

FIG. 1. Relative potency of technical Bidrin, paraoxon, and EPNO for inhibition *in vitro* and of technical Bidrin for inhibition *in vivo* of PPA-hydrolyzing esterases in yolk sac membrane homogenates. For details, see the text and Table 1.

incubation is carried out in the presence of 5×10^{-3} M aryl ester substrate, the level of phosphorylation under the standard conditions is greatly reduced (65 per cent in the case of NA and 90 per cent with PPA). This competition experiment indicates that some of the sites involved in ester hydrolysis are also those phosphorylated by DFP. Pretreatment of the homogenates with technical Bidrin (10^{-6} to 10^{-3} M) for 30 min reduces the sites available for subsequent phosphorylation by DFP. This Bidrin effect exceeds the inhibition of PPA-hydrolyzing esterase activity by Bidrin; the magnitude of difference in the two parameters increases with Bidrin concentration so that at 10^{-3} M there is 80–85 per cent blockage at the phosphorylation sites but there is only 35–40 per cent inhibition of PPA hydrolysis. Thus, there are several types of evidence, from studies *in vitro*, that PPA hydrolysis by whole membrane homogenates involves several esterases and that all the esterase components of highest activity are readily inhibited by paraoxon and EPNO or phosphorylated by DFP.

Electrophoretic separation of components in the yolk sac membrane preparations provides additional evidence that several esterases of varying sensitivity to organophosphorus compounds and methylcarbamates are involved. Starch-gel electrophoresis resolves six distinct regions of esterase activity using NA as the substrate, but no protein bands are detected in the regions of these esterases (Fig. 2). This finding establishes that the esterases are only a small fraction of the total protein amount. Better

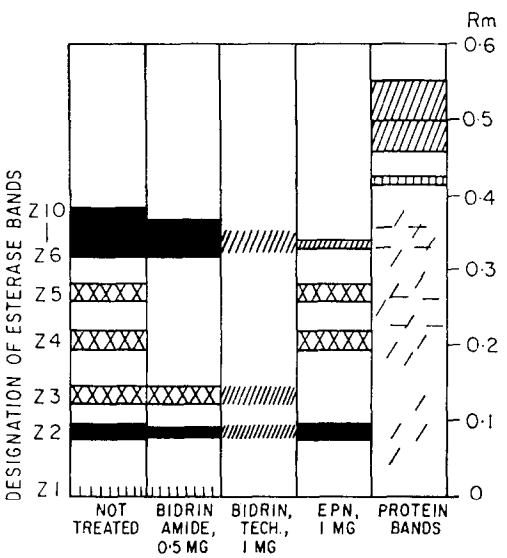

FIG. 2. Starch-gel zymogram patterns of yolk sac membrane esterases obtained from control eggs and from eggs injected 24 hr previously with various organophosphorus compounds. Relative intensities of esterase bands detected with NA and of protein bands are indicated by shading.

separations, of ten or more esterase bands, are sometimes obtained on starch-gel by the method of Mendoza and Hatina[30] and on polyacrylamide gel, but these methods are not adequately reproducible or the gel is too fragile for routine use in comparative inhibition studies. The mobility sequence of the esterase bands in these latter two

systems, as compared with that shown in Fig. 2, indicates that the Rm 0·32–0·38 region represents a mixture of at least five esterases. Therefore, in designating the esterase bands in Fig. 2, this upper region is indicated as Z6-Z10. When potential esterase inhibitors are added with NA to the gel after electrophoretic development, there is preferential inhibition of activity in the various bands. Zymogram band Z3 (Fig. 2) is most sensitive to inhibition by eserine (10^{-4} M) whereas, with DFP (10^{-7} to 10^{-5} M) and Bidrin (10^{-4} to 10^{-3} M), the order for sensitivity of the esterase bands to inhibition is as follows: Z8-Z10 region > Z3 and Z4 > the other bands. DFP, at 10^{-3} M, completely inhibits the esterase activity in all bands. It is evident that homogenates of the yolk sac membrane contain many esterases which are capable of hydrolyzing NA but which vary in sensitivity to inhibition *in vitro* by eserine and different organophosphorus compounds; a similar situation is expected for esterases hydrolyzing PPA.

Effects in vivo *of organophosphorus compounds and other chemicals on esterases and available phosphorylation sites in the yolk sac membrane*

A variety of organophosphorus compounds and eserine, injected into eggs after 120 hr of incubation, inhibit the PPA-hydrolyzing esterases of the yolk sac membrane when it is removed after 144 hr of incubation. These chemicals also reduce, to almost the same extent, the remaining sites available for phosphorylation by ^{3}H-DFP (Table 2). Thus, the degree of inhibition of PPA hydrolysis appears to be directly proportional to the reduction in available phosphorylation sites (Fig. 3). This suggests that all of the sites in the membrane that react with DFP, and no other sites, are involved in PPA hydrolysis. Although it is possible that a portion of the esterases hydrolyzing PPA are selectively inhibited by some of the compounds acting *in vivo*, this is not evident from the cumulative data (Table 2, Fig. 3).

Bidrin and two of its metabolites formed in the hen eggs, Azodrin and Bidrin amide,[3] are almost equipotent in reacting with membrane esterases and phosphorylation sites. It appears therefore that either the terminal crotonamide phosphate (Bidrin amide) in the metabolic sequence is the actual inhibitor or, more likely, that each compound is active *per se*. Bidrin and its crotonamide phosphate metabolites probably react more slowly than EPNO or paraoxon with the PPA-hydrolyzing esterase(s) and the phosphorylation site(s) because Bidrin, Azodrin, and Bidrin amide prove to be more potent inhibitors *in vivo*, where the exposure time is 24 hr, as opposed to 30-min *in vitro*. This is probably not the case with EPNO as compared to *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN) and with paraoxon (Table 1, Fig. 1).

Technical Bidrin (1 mg) injected at 120 hr of incubation reduces the available phosphorylation sites at various times after administration as follows: 1 hr — 50 per cent; 2 hr — 60 per cent; 5, 7·5, 24 and 48 hr — 60–80 per cent. On this basis, and for convenience, the standard assays were made at a 24-hr post-treatment interval. *trans*-Bidrin not only fails to inhibit PPA hydrolysis and reduce the available phosphorylation sites at doses of 0·05–0·25 mg/egg but may actually increase the level of available phosphorylation sites (Table 2; points below the origin in Fig. 3). Technical Bidrin does not appear to be as active as anticipated, on the basis of its *cis*-isomer content; so, it is possible that the *trans*-isomer in the technical mixture either offers some protection of the esterases and phosphorylation sites against blockage by the *cis*-isomer or it results in higher levels of these sites.

TABLE 2. EFFECTS OF VARIOUS COMPOUNDS, INJECTED INTO EGGS INCUBATED FOR 120 HR, ON THE SITES AVAILABLE FOR PHOSPHORYLATION BY ^{3}H-DFP AND ON THE ACTIVITY OF ENZYMES HYDROLYZING PPA IN HOMOGENATES OF THE YOLK SAC MEMBRANE REMOVED FOR ASSAYS AFTER 24 HR OF ADDITIONAL INCUBATION, COMPARED TO THE TERATOGENIC SIGNS FOUND AT DAY 21 AFTER INJECTION OF THE COMPOUNDS AT 96 HR OF INCUBATION

Compound*	Dose (mg/egg)	Phosphorylation sites available, % reduction†	Esterase activity, % inhibition†	Teratogenic signs‡
Vinyl phosphates $(CH_3O)_2P(O)OCR_1 = CR_2R_3$				
Bidrin, technical mixture of isomers $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)N(CH_3)_2$	0·010	7 ± 8	7 ± 8	−
	0·025	50 ± 3	50 ± 3	+
	0·050	51 ± 7	57 ± 7	
	0·125	55 ± 4	60 ± 4	+++
	0·25	60 ± 5	69 ± 5	++++
	0·50	76 ± 5	78 ± 5	++++
	1·0	77 ± 4§	85 ± 4§	++++
Bidrin, *cis*-isomer $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)N(CH_3)_2$	0·010	41 ± 2	58 ± 2	−
	0·025	64 ± 3	71 ± 2	
	0·050	73 ± 7	75 ± 3	
	0·10	75 ± 2	76 ± 5	+++
	0·50	85 ± 2	84 ± 2	
	1·0	87 ± 3	88 ± 3	++++
Bidrin, *trans*-isomer $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)N(CH_3)_2$	0·05	−7 ± 7	1 ± 4	
	0·10	−8 ± 9	1 ± 3	−
	0·25	−15 ± 23	2 ± 9	
	1·0	10 ± 18	23 ± 6	−
Azodrin, *cis*-isomer $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)NHCH_3$	0·0125	19 ± 9		−
	0·025	31 ± 4	44 ± 2	
	0·050	37 ± 12	66 ± 2	
	0·10	63 ± 6	64 ± 2	+++
	0·25	64 ± 4	70 ± 2	
	0·50	72 ± 2	73 ± 2	
	1·0	85 ± 2	87 ± 1	
Bidrin amide, *cis*-isomer $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)NH_2$	0·0125	18 ± 9	21 ± 8	
	0·025	31 ± 6	38 ± 5	−
	0·050	63 ± 12		
	0·10	74 ± 6		+++
	0·25	73 ± 1	70 ± 2	
	0·50	76 ± 3	83 ± 3	
	1·0	91 ± 1	87 ± 4	
Chloro-Bidrin $R_1 = CH_3$, $R_2 = Cl$, $R_3 = C(O)N(CH_3)_2$	0·25	23 ± 2	38 ± 4	
	0·50	68 ± 1	68 ± 2	
	1·0	72 ± 1	79 ± 2	+++
Mevinphos $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)OCH_3$	1·0	50 ± 2	63 ± 5	−
	2·0	67 ± 3	71 ± 3	
	5·0	84 ± 1	87 ± 2	
	10	92 ± 4	93 ± 6	+++
Ciodrin $R_1 = CH_3$, $R_2 = H$, $R_3 = C(O)OCH(CH_3)C_6H_5$	0·5	17	0	
	1·0	29	33	−
Dichlorvos $R_1 = H$, $R_2 = R_3 = Cl$	1·0	43 ± 7	68 ± 2	
	2·0	51 ± 3	76 ± 3	
	5·0	68 ± 1	82 ± 1	
	10	78 ± 2	78 ± 1	+++

TABLE 2. *cont.*

Compound*	Dose (mg/egg)	Phosphorylation sites available, % reduction†	Esterase activity, % inhibition†	Teratogenic signs‡
p-Nitrophenyl phosphorus compounds $C_2H_5O(R)P(X)OC_6H_4$–4–NO_2				
Paraoxon (C_2H_5OH)	0·05	60 ± 3	69 ± 2	
R = C_2H_5O, X = O	0·10	65 ± 3	77 ± 3	
	0·25	90 ± 2	88 ± 2	(+++)
	0·50	92 ± 1	87 ± 1	(++++)
	1·0	94 ± 1	89 ± 1	(++++)
EPN (C_2H_5OH)	0·010	75 ± 4	90 ± 12	
R = C_6H_5, X = S	0·025	83 ± 3	92 ± 2	
	0·050	93 ± 1	95 ± 1	(−)
	0·10	91 ± 1	93 ± 1	
	0·25	92 ± 2	95 ± 1	(±)
	0·50	90 ± 2	95 ± 1	
	1·0	93 ± 2	95 ± 1	(±)
EPN (DMSO)	0·05	71 ± 13	56 ± 8	(—)
R = C_6H_5, X = S	0·10	82 ± 6	67 ± 4	
	0·25	91 ± 2	92 ± 3	(±)
	0·50	90 ± 2	91 ± 5	
	1·0	90 ± 2	93 ± 2	(±), —
EPNO (C_2H_5OH)	0·25	91	95	
R = C_6H_5, X = O	0·50	95	94	
	1·0	95	94	
Other organophosphorus compounds‖				
Malaoxon (C_2H_5OH)	1·0	25 ± 12	28 ± 10	
TOCP (C_2H_5OH)	10	52 ± 6	44 ± 6	—
Guthion (DMSO)	1·0	27 ± 8	18 ± 3	—
Methylcarbamate				
Eserine (sulfate)	0·025	51 ± 2	42 ± 3	(—)
	0·05	52 ± 3	43 ± 6	
	0·10	58 ± 3	48 ± 7	
	0·25	59 ± 2	54 ± 2	
	1·0	65 ± 9	60 ± 3	++++

* Solvents other than saline, as well as salt forms, are given in parentheses after test chemical. The following compounds at the indicated dose are not teratogenic and do not produce significant (i.e. < 20 per cent) or dose-dependent reduction in the phosphorylation sites available or inhibition of esterase activity: $(CH_3O)_2P(O)OC(CH_3) = CHC(O)OH$ (0·1–1·0 mg/egg) in saline, $CH_3C(O)CH_2C(O)NHCH_3$ or $CH_3C(O)CH_2C(O)N(CH_3)_2$ (1·0 mg/egg) in saline, and thalidomide (0·01–0·50 mg/egg) in DMSO.

† Average and S.D. are based on four or more replicates involving at least two independent experiments made at different times. The only exceptions are Ciodrin and EPNO where the averages of duplicate determinations are given. In every case where data are presented on both phosphorylation sites and esterase activity, the determinations were made on the same homogenate.

‡ Teratogenic signs are those noted by Roger *et al.*[3] except ones in parentheses that were determined in this study. The signs are rated from not detectable (—) to severe manifestation (++++).

§ The results are not significantly changed when 20 μl of DMSO or 1 mg of nicotinamide in saline are injected in the same solution as technical Bidrin.

‖ The structures are given by Roger *et al.*[3] Malaoxon is the phosphorothiolate or oxygen analog of malathion.

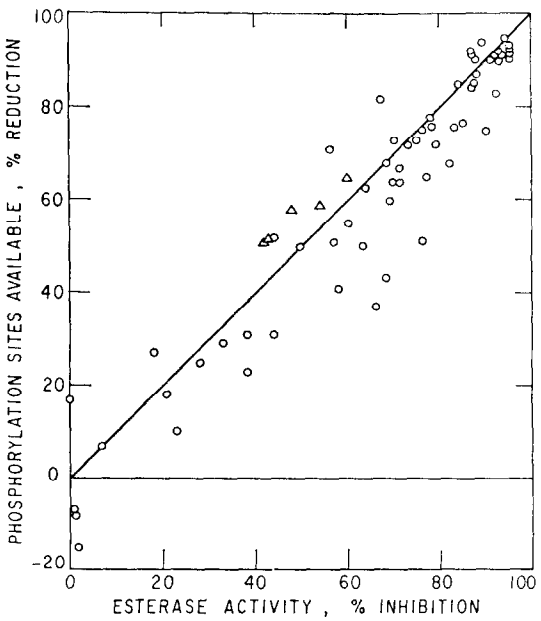

FIG. 3. Comparison of the effects of various organophosphorus compounds and eserine, injected into eggs incubated for 120 hr, on the available site(s) for phosphorylation by ^{3}H-DFP and the activity of esterase(s) hydrolyzing PPA in the yolk sac membrane removed for assays after 24 hr of additional incubation. The results incorporate all of the data in Table 2. Legend: ○, organophosphorus compounds; △, eserine. The line is the theoretical line for complete correlation.

Electrophoretic studies confirm many of the phenomena noted in direct assays made on whole homogenates of the yolk sac membrane. *cis*-Bidrin, at 0·05 mg/egg, and Azodrin, at 0·25 mg/egg, markedly or completely inhibit bands Z4 and Z5 as well as the upper portion of the Z6–Z10 region. Bidrin amide at 0·5 mg/egg and technical Bidrin at 1 mg/egg also markedly inhibit these same bands (Fig. 2). At 1 mg/egg, technical Bidrin at first inhibits the Z4 and Z5 bands and the upper region of the Z6-Z10 band (2-hr post-treatment), but, at a later stage (5- and 24-hr post-treatment), it inhibits almost all esterase activity. Mevinphos, at 1 mg/egg, acts only on the upper portion of the Z6-Z10 region but, at 10 mg/egg, it also inhibits the Z4 and Z5 bands. Eserine acts in much the same way, at 0·25 mg/egg, inhibiting the Z4 and Z5 bands as well as the upper portion of the Z6-Z10 region. EPN at 1 mg/egg differs from the other compounds in inhibiting preferentially the Z3 band and almost all of the Z6-Z10 region without marked inhibition of the Z4 and Z5 bands (Fig. 2). *trans*-Bidrin at 0·025–0·05 mg/egg does not inhibit the esterase activity of any band but results in a consistent increase in activity of the Z6-Z10 region.

Some of the compounds studied are teratogenic and others are not under the dosage schedules used (Table 2). The teratogenic effects do not correlate with the degree of inhibition of PPA hydrolysis or the reduction in available phosphorylation sites, when the comparisons are made with the mixture of enzymes present in the whole homogenate. This is particularly evident on comparing Bidrin and paraoxon, potent teratogens in the hen egg, with EPN which gives similar degrees of blockage without teratogenic signs. However, this does not rule out the possibility that one or more of

the component esterases or membrane proteins may be related to the initiation of teratogenesis. In fact, the zymogram studies show inhibition of activity of esterases in the Z4 and Z5 bands with all compounds that are teratogenic at the administered dose (i.e., *cis*-Bidrin, technical Bidrin, Azodrin, Bidrin amide, eserine, and a high dose of mevinphos) but not with the compounds that are not teratogenic (i.e., EPN, *trans*-Bidrin, and a low dose of mevinphos). This correlation is based on the results obtained for the stated series of compounds which, in each case, were tested in at least duplicate experiments.

Nicotinamide (1 mg/egg) injected at 120 hr along with Bidrin (1 mg/egg) does not significantly alter the effect of Bidrin on PPA-hydrolyzing esterases and available phosphorylation sites (Table 2). This contrasts with the anti-teratogenic effect of nicotinamide. At 20 μl/egg, DMSO does not significantly alter the effect of Bidrin on the membrane parameters studied but greatly decreases the potency of EPN when compared with EPN administered in ethanol (Table 2). It is not known whether the effect of DMSO results from differences in localization or distribution of the EPN when administered in different solvents or from other factors.

DISCUSSION

It appears that the biochemical lesion(s) leading to teratogenesis involves acylation (i.e. dialkylphosphorylation or methylcarbamoylation) of a specific membrane locus thereby preventing either the normal acyl transfer at this site or its esteratic function. There are two convenient ways for assay of such a site: one is indirect in which enzymatic activity is measured; the other is direct and measures the formation of a stable acyl protein, such as that formed by reaction with radiolabeled DFP. Others have used this combined analytical approach to investigate, in adult hens, the nature of the neural membrane site that is phosphorylated by neurotoxic organophosphates.[17–21] The active sites of many or most of the membrane esterases are probably phosphorylated by DFP under the conditions used. Therefore, measurement of phosphorylated sites is non-specific, whereas the esterase assay is more specific because it measures the relative activities of the component esterases. The ^{3}H-DFP procedure for assaying available phosphorylation sites probably determines the concentration of diisopropylphosphoryl protein assuming that there is no *O*-depropylation (aging)[31] of the phosphorylated sites during preparation and analysis. When the membrane preparations are exposed to organophosphorus compounds either *in vitro* or *in vivo* or to eserine *in vivo* prior to addition of ^{3}H-DFP, the reduction in subsequent ^{3}H-binding approximates the inhibition of esterase activity. This indicates that the ^{3}H-DFP reacts with esteratic sites not already occupied by other relatively stable acyl groupings.

Phenyl phenylacetate (PPA) was the substrate selected for esterase assays with whole homogenates because other workers[18–21,23] have found it useful in differentiating, along with selective inhibitors, the nerve esterases or proteins reacting with neurotoxic organophosphorus compounds. It is unfortunate that technical difficulties required the replacement of PPA by NA in the studies on electrophoretic separation of the component esterases because, even though it is likely that the same esterases hydrolyze both substrates, the relative activity of the esterase components probably differs on the two substrates.

The relevance of the findings to teratogenesis was evaluated by experiments *in*

vivo using various toxicants and treatment schedules which lead to different degrees of embryonic abnormalities. Also studied were nicotinamide and DMSO, chemicals that are known to decrease the teratogenic abnormalities.[1-3,8,10,11*] The yolk sac membrane homogenate contains a mixture of esterases which differ in their sensitivity *in vitro* to inhibition by Bidrin when PPA is used as a substrate. These esterases are separable by electrophoresis into multiple components with varying sensitivities to inhibition by organophosphorus compounds and eserine, when assayed with NA as the substrate. The most active esterase(s) in the whole homogenate for hydrolyzing PPA probably contains most of the active site(s) phosphorylated by DFP but the degree of inhibition of or reaction with this esterase(s) does not correlate with the teratogenicity of the compounds studied. If the inhibition of only one component of the esterase mixture is involved in the initiation of teratogenesis, then this esterase must represent a very small proportion of the total esterase activity on PPA and the total available phosphorylation sites. Esterases appearing in zymogram bands Z4 and Z5 are candidates for the reactive site relating to teratogenicity because they are not inhibited by administration of non-teratogenic compounds but are inhibited (sometimes selectively) by teratogenic-acylating agents. However, this correlation is qualified by the limited studies made to date. The normal substrate or function of this esterase or protein remains unknown and, in fact, the possibility that it is a cholinesterase cannot be ruled out with the available evidence. Further studies are needed on the nature of these membrane esterases and phosphorylation sites if their relationship with the teratogenicity of certain organophosphorus compounds and methylcarbamates is to be ascertained.

On analogy with the proposed involvement of a reactive protein in nerve membranes in the mode of action of neurotoxic agents,[17-21] it is possible that teratogenic agents act by acylating a similar reactive protein in the yolk sac or other membrane. However, the nature and function of these proteins remain to be defined.

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