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KYNURENINE FORMAMIDASE INHIBITION AS A POSSIBLE MECHANISM FOR CERTAIN TERATOGENIC EFFECTS OF ORGANOPHOSPHORUS AND METHYLCARBAMATE INSECTICIDES IN CHICKEN EMBRYOS

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Abstract—At least two types of developmental anomalies are induced in chicken embryos by certain organophosphorus (OP) and methylcarbamate (MC) insecticides. One of them (Type I) leads to micromelia and abnormal feathering and another (Type II) involves arthrogryposis, wry neck and rumplessness. Type I but not type II teratogenesis is associated with a lowered embryo NAD level and is alleviated on restoring the NAD level by administration of intermediates in the tryptophan to NAD biosynthetic pathway. These and other observations with chicken embryos suggest but do not in themselves establish that impairment in the conversion of tryptophan to NAD, possibly by inhibition of kynurenine formamidase, leads to type I teratogenesis. This hypothesis is supported by finding that mouse liver kynurenine formamidase is extremely sensitive to *in vivo* inhibition by those OP and MC compounds which are the most potent NAD lowering agents and teratogens in the chicken embryo, *i.e.* crotonamide phosphates and pyrimidyl phosphorothionates such as dicrotophos and diazinon and MC compounds such as carbaryl. Teratological or other toxicological manifestations of kynurenine formamidase inhibition are probably restricted to species and developmental stages where reduced enzyme activity significantly impairs maintenance of normal levels of NAD or other essential biochemicals derived from kynurenine.

A variety of organophosphorus (OP) and methylcarbamate (MC) insecticides and related compounds induce pronounced teratogenic effects in chicken embryos, many of which are alleviated by coinjection of tryptophan, nicotinamide (NAm) and a variety of other NAD precursors [1–6]. One biochemical lesion correlated with teratogenesis involves lowered chicken embryo NAD levels possibly due to impaired synthesis from endogenous precursors on selective inhibition of a critical hydrolase [5, 6]. Several hydrolases are important in liberation of tryptophan from yolk stores and in its ultimate conversion to NAD [7]. Kynurenine formamidase (EC 3.5.1.9; arylformylamine amidohydrolase), a critical enzyme in this pathway, is known from studies with chicken and guinea pig liver to be sensitive to *in vitro* inhibition by *O,O*-diethyl *O*-(*p*-nitrophenyl) phosphate (paraoxon) [8, 9].

This report considers the hypothesis that one type of OP- and MC-induced teratogenesis involves inhibition of kynurenine formamidase thereby reducing the efficiency of NAD biosynthesis in chicken embryos (Fig. 1). The inability to adequately assay the inhibition of kynurenine formamidase in chicken embryos during the teratogen-sensitive period of organogenesis (up to day 8 of incubation) by the method used led to the indirect approach of examining *in vivo* inhibition of this enzyme in mouse liver by a variety of OP and MC insecticides for structure-activity correlations with their potency in lowering chicken embryo NAD levels and producing teratogenesis. This approach is based on the possibility that there is a similar inhibitor specificity for chicken embryo and mouse liver enzymes. The relevance of kynurenine formamidase inhibition to other toxicological effects of OP and MC insecticides is also evaluated.

MATERIALS AND METHODS

Chemicals. Structures for the 4 teratogens studied in greatest detail are given in Fig. 2. The sources and purities of most of the chemicals were as previously described [6]. Additional chemicals used and their sources are as follows [10]: *N*-demethyl monocrotophos (Shell Development Co., Modesto, CA); *O,O*-dimethyl *O*-(2-ethyl-6-ethoxy-4-pyrimidyl) phosphorothioate (SAN I 197) (Sandoz-Warner, Inc., Hanover, NJ); *O,O*-diethyl and *O,O*-dimethyl *O*-[2-(dimethylamino)-6-methyl-4-pyrimidyl] phosphorothioates (pirimiphos-ethyl and pirimiphos-methyl, respectively) (ICI America Inc., Goldsboro, NC); 1-methylethyl 2-[[ethoxy(1-methylethyl)amino]phosphinothioyl]oxy benzoate (BAY 92114), *O*-methyl *O*-(4-methyl-2-nitrophenyl) (1-methylethyl)phosphoramidothioate (BAY NTN 6867) and *O*-ethyl *S,S*-diphenyl phosphorodithioate (BAY Hinosan) (Chemagro Corp., Kansas City, MO); 2-phenoxy-4H-1,3,2-benzodioxaphosphoran-2-one (phenyl saligenin cyclic phosphate) (M. Eto, Kyushu University, Fukuoka, Japan); tri-*o*-cresyl phosphate (TOCP) (Eastman Organic Chemicals, Rochester, NY); 2-(dimethylamino)-5,6-dimethyl-4-pyrimidyl dimethylcarbamate (pirimicarb) (ICI America Inc.); *S,S'*-[2-(dimethyl-

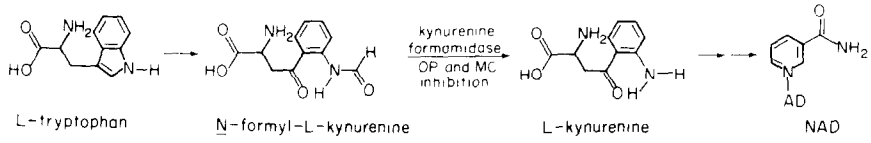

Fig. 1. Kynurenine formamidase inhibition as a possible mechanism for lowered NAD levels in chicken embryos treated with teratogenic organophosphorus and methylcarbamate insecticides.

amino)trimethylene]*bis*(thiocarbamate) hydrochloride (cartap) (Takada Chemical Industries, Ltd., Kyoto, Japan); *N'*-(4-chloro-*o*-tolyl)-*N*,*N*-dimethylformamidine (chlordimeform) (CIBA-Geigy Corp., Greensboro, NC); 2-pyridinealdoxime methyl methanesulfonate (P2S) (Aldrich Chemical Co., Milwaukee, WI); *N*-formyl-L-kynurenine (Calbiochem, LaJolla, CA); other tryptophan derivatives and metabolites (Sigma Chemical Co., St. Louis, MO and Aldrich Chemical Co.); 6-aminonicotinamide (6-AN), 3-acetylpyridine (3-AP), insulin and sulfanilamide (Sigma Chemical Co.); DL-[7a-^{14}C]tryptophan (ICN Pharmaceuticals, Irvine, CA).

Treatment of eggs and rating of teratogenic signs. Fertile white leghorn eggs (Western Scientific, West Sacramento, CA) treated by injecting the candidate teratogens, dissolved in methoxytriglycol (40 μl), directly into the yolk sac were incubated as previously described [4, 6]. Teratogenic signs were rated at day 19 of incubation from no effect (−) to very severe manifestations (++++) based primarily on micromelia and abnormal feathering. Embryos designated (*) displayed joint fusion anomalies such as wry neck, arthrogryposis (inflexibility of the tarso-metatarsal joint) and sometimes rumplessness as the only teratogenic signs or in addition to micromelia and abnormal feathering [6]. Although the chemicals are rated in this report only as (−) or (*) to (++++*), the detailed teratogenic signs based on observations with 10 embryos in each case are given by Moscioni [10].

Treatment of mice and chicks. Male Swiss–Webster mice (22–24 g, Simonsen Laboratories, Inc., Gilroy, CA) were treated intraperitoneally (i.p.) with various OP and MC compounds (usually 1 mg/kg) dissolved in methoxytriglycol (20 μl) and sacrificed 1 hr to 8 days later by cervical dislocation prior to removal of the liver and brain. Two-week-old leghorn chicks were treated similarly with selected OP compounds and sacrificed 24 hr later by decapitation prior to removal of the liver. These tissues were rinsed in cold 0.14 M KCl then blotted dry and weighed.

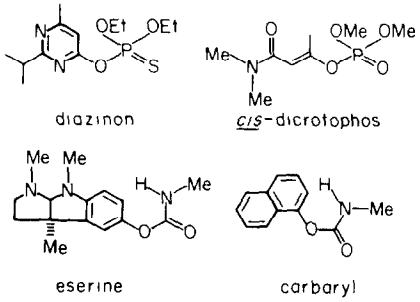

Fig. 2. Structures for organophosphorus and methylcarbamate teratogens in chicken embryos. *N*-Demethyl monocrotophos is the crotonamide analog of dicrotophos.

Analyses for NAD, NADH and products of in vivo [^{14}C]*tryptophan metabolism.* NAD was analyzed using appropriate numbers of chicken embryos or weights of tissues from the embryos or other organisms by the yeast alcohol dehydrogenase (EC 1.1.1.1; alcohol:NAD oxidoreductase) method [6, 10–12]. Four to six chicken embryos were pooled for assays at days 6–9 of incubation whereas individual embryos were analyzed at later times. Prior to homogenization, all feathers were plucked from 14-day embryos and the yolk sac and its contents were removed from 19-day embryos. NADH levels were analyzed for 12-day chicken embryos homogenized in hot 0.3 N KOH containing 0.42 mM tryptophan [10] by absorbance difference after oxidation with yeast alcohol dehydrogenase and acetaldehyde [11]. The data are given as mean values ± standard error (S.E.) based on 6 replicates. The normal pyridine nucleotide levels for 12-day chicken embryos (nmoles/g fresh weight) were 185 ± 4 for NAD (unless otherwise specified) and 55 ± 2 for NADH.

The ^{14}C content of the NAD fraction was analyzed for chicken embryos at day 13 of incubation following treatments with diazinon (1 mg/egg) at day 4 and DL-[7a-^{14}C]tryptophan (117 μg or 2 μCi) at day 9 of incubation, using methoxytriglycol for the administrations. Protein was precipitated with $HClO_4$ [6], the supernatant neutralized with 6 N KOH and the insoluble $KClO_4$ removed. A 0.3-ml aliquot (~1000 dpm) of the final supernatant, fortified with unlabeled NAD (1.5 mg), was subjected to thin-layer chromatography on silica gel chromatoplates (2 mm layer thickness), detecting the NAD region by viewing the chromatoplates under ultraviolet light. Developments were with *n*-butanol–acetic acid–water (3:1:1) (for quantitation) or *n*-propanol–water (16:9) (for tentative characterization), solvent systems which give R_f values for NAD of 0.00 and 0.46, respectively, and which separate the pyridine nucleotides from possible [^{14}C]intermediates in their biosynthesis, *i.e.* kynurenine, 3-hydroxyanthranilic acid, nicotinic acid (NAc) and NAm which give higher R_f values. The ^{14}C content of the NAD fraction was averaged for 3 control embryos and 3 diazinon-treated embryos.

Assay of kynurenine formamidase and tryptophan pyrrolase activities. Kynurenine formamidase was assayed at 25° by the increase in absorbance at 365 mμ due to liberation of kynurenine from *N*-formyl-L-kynurenine [13]. Tissues were homogenized in 0.14 M KCl–2.5 mM NaOH using a cold glass homogenizer with a teflon pestle. The supernatant from centrifugation (11,000 *g* for 20 min) of the homogenate was filtered through glass wool (to remove floating lipid material) and diluted with 0.14 M KCl as appropriate for assay (*e.g.*, 1.25% fresh weight equivalent with mouse liver). The reaction medium in a 1-cm silica cuvette consisted of the following components

added in sequence: 0.2 ml 0.01 M *N*-formyl-L-kynurenine; 0.8 ml distilled H_2O; 1.0 ml 0.2 M sodium phosphate buffer, pH 7.5; 1 ml enzyme preparation. The absorbance change was compared with that for a blank containing all components except substrate, and was linear over a period of 3–4 min (unless otherwise specified). The data reported are based on duplicate determinations of enzymes from 2 different animals. The kynurenine formamidase activity of normal mouse liver was 46 ± 2 μmoles kynurenine liberated/mg protein/min. The activity resides entirely in the soluble (105,000 *g* supernatant) fraction.

In *in vitro* kynurenine formamidase inhibition studies, the OP or MC compound in ethanol (7 μl) was preincubated with the enzyme preparation (1.0 ml, 11,000 *g* supernatant fraction) at 25° for various times prior to assay as above. The sensitivity to *in vitro* OP and MC inhibition was identical for the 11,000 *g* and 105,000 *g* supernatant fractions.

Tryptophan pyrrolase (EC 1.13.1.12; L-tryptophan: oxygen oxidoreductase) was assayed at 25° by the accumulation of *N*-formyl-L-kynurenine and L-kynurenine (measured at 321 and 365 mμ, respectively) on incubation of tryptophan with mouse liver homogenate [10, 13].

RESULTS

Teratogens that block NAD synthesis in chicken embryos. The remarkably good correlation between embryo NAD levels at day 12 of incubation and the severity of micromelia and abnormal feathering at day 19 of incubation following administration of 26 OP and MC compounds at 1 mg/egg on day 4 of incubation (Table 1) establishes that micromelia and abnormal feathering are associated with severe lowering of embryo NAD levels. Other teratogenic signs (*) (*e.g.* arthrogryposis, wry neck and rumplessness) lack association with lowered NAD levels and may appear together with or independently of the micromelia and abnormal feathering. Five OP compounds (**1–4** and **6**) and eserine administered at 1 mg/egg on day 4 of incubation give the complete set of NAD-associated and non-associated teratogenic effects. With dicrotophos at 1 mg/egg, the severest manifestations of micromelia and abnormal feathering occur

Table 1. Effects of various organophosphorus compounds and methylcarbamates administered to eggs at 1 mg/egg on day 4 of incubation on the chicken embryo NAD levels at day 12 and the teratogenic signs at day 19 of incubation compared with their effect following intraperitoneal administration to mice at 1 mg/kg on the mouse liver kynurenine formamidase activity at 1 and 24 hr after treatment

Compound		Chicken embryo*		Mouse liver kynurenine formamidase activity rel. to control (%)		
No.	Name	NAD level rel. to control (% ± S.E.)†	Teratogenic signs, rating‡	1 hr	24 hr	Ave
1	*N*-Demethyl monocrotophos§	n.d.¶	++++*	11	51	31
2	Monocrotophos§	20 ± 3	++++*	18	56	37
3	Dicrotophos	20 ± 3	++++*	19	61	40
4	Diazinon	21 ± 3	++++*	23	34	29
5	SAN I 197	22 ± 3	++++	n.d.	49	49
6	Pirimiphos-ethyl	26 ± 4	+++*	n.d.	17	17
7	Pirimiphos-methyl	30 ± 4	+++	43	60	52
8	Carbaryl	28 ± 4	++	20	n.d.	20
9	Phosphamidon	49 ± 2	++	61	52	57
10	Methamidophos	53 ± 4	++	15	91	53
11	BAY 92114	77 ± 8	+*	n.d.	91	91
12	Parathion	78 ± 11	+*	80	94	87
13	Phorate	80 ± 6	+	n.d.	88	88
14	Methyl parathion	77 ± 7	−	n.d.	82	82
15	Coumaphos	80 ± 10	−	n.d.	88	88
16	Mevinphos	88 ± 5	−	72	68	70
17	Malathion	87 ± 6	−	82	88	85
18	Dimethoate	87 ± 6	−	n.d.	93	93
19	Dichlorvos	97 ± 1	−	n.d.	93	93
20	Phenyl saligenin cyclic phosphate	98 ± 2	−	n.d.	99	99
21	TOCP	>80	−	97	99	98
22	Chlorpyriphos	103 ± 2	−	n.d.	100	100
23	EPN	109 ± 4	−	90	105	98
24	Leptophos	107 ± 2	−		105	105

* Additional studies establish that the OP compounds BAY Hinosan and BAY NTN 6867 and the MC compound pirimicarb do not lower embryo NAD levels and are not teratogenic (−).

† Data from this study (compounds **5–7**, **11** and **20**), from Proctor *et al.* [6] (compounds **2**, **8**, **10**, **13–19** and **21–24**) or from both of these studies (compounds **3**, **4**, **9** and **12**).

‡ Data from this study (compounds **5–7**, **11** and **20**), from Proctor *et al.* [6] (compounds **2**, **13–19** and **21–24**), from both of these studies (compounds **3**, **4**, **8–10** and **12**) or from Upshall *et al.* [2] (compound **1**, administered at 0.3 mg/egg).

§ The *cis*-crotonamide was used. Other crotonamides (**3** and **9**) were *cis*/*trans* isomer mixtures.

¶ Not determined.

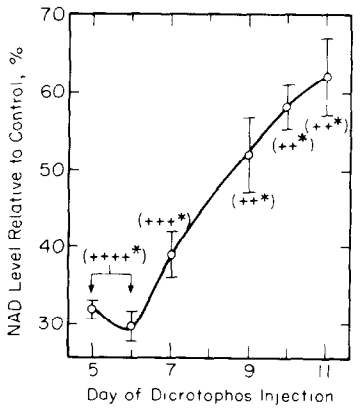

Fig. 3. Effect of dicrotophos (1 mg/egg) administered at day 5–11 of incubation on the chicken embryo NAD levels at day 12 and the teratogenic signs at day 19 of incubation.

when administration is at day 5 or 6 of incubation while administration at day 7–11 produces primarily arthrogryposis and wry neck and gives less extreme lowering in embryo NAD levels (Fig. 3). Two *O,O*-dimethyl OP compounds (**5** and **7**) administered at 1 mg/egg and carbaryl even at 3 mg/egg yield only the NAD-associated developmental anomalies. In contrast, BAY 92114 and parathion are selective in producing primarily joint fusion anomalies.

The NAD level of control embryos increases from day 6–13 of incubation and decreases thereafter when expressed as nmoles/g (Fig. 4). If expressed as nmoles/embryo, the NAD level would continue to increase throughout development. In eggs treated with 30 or 60 μg diazinon at the 4th day, embryo NAD levels (nmoles/g) are progressively lowered until day 10–13 of incubation then they rise to near control values by day 18 (Fig. 4). Measurements of tissue

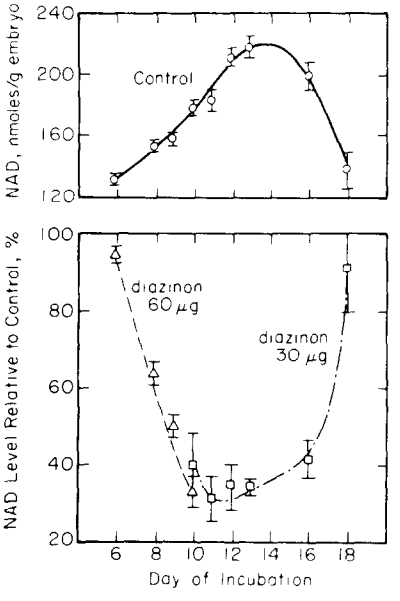

Fig. 4. Effect of diazinon (0, 30 or 60 μg/egg) administered at day 4 of incubation on the chicken embryo NAD levels at day 6–18 of incubation.

NAD levels at day 19 for embryos from eggs administered either diazinon (30 μg) or carbaryl (3 mg) at day 4 of incubation demonstrate the persistence of the lowered NAD levels especially in brain and muscle (Table 2). The embryo NADH level at day 12 is lowered as drastically as the NAD level following treatment with 30 μg diazinon at day 4 of incubation [10] and the ratio between oxidized and reduced pyridine nucleotides is not greatly altered.

A teratogenic dose (0.3 μmole) of 6-AN administered at day 6, 8, 10 or 11 of incubation causes micromelia and abnormal feathering, the former anomaly decreasing in severity when treatment is at later days of development, but in no case is there a lowering in NAD level per unit of embryo fresh weight at day 12 [10]. Teratogenic doses of sulfanilamide (2 mg/egg) and insulin (82 μg or 2 units/egg) administered at day 4 do not lower the NAD level at day 12 and non-teratogenic doses of chlordimeform (1 mg/egg) and cartap (0.1 mg/egg) under the same conditions also yield normal NAD levels. Cartap, however, at 1 mg/egg is lethal to all embryos before day 12 of incubation [10].

Compounds that alleviate teratogenic signs in chicken embryos. NAm (0.8 μmole/egg) coadministered with the teratogen (1 mg/egg) at day 4 of incubation completely alleviates the lowered NAD levels and the micromelia and abnormal feathering due to diazinon, dicrotophos, carbaryl, eserine and Meobal (3,4-dimethylphenyl methylcarbamate) but it is not effective under the same conditions in relieving arthrogryposis, wry neck and rumplessness caused by diazinon, dicrotophos, eserine or parathion [10]. NAm sometimes appears to accentuate joint fusion anamolies, possibly because on relieving the other teratogenic signs these become more apparent. The lowered NAD level is quickly restored by NAm administration since, following a 1 mg/egg dose of dicrotophos at day 4 of incubation, NAm at 0.8 μmole/egg even at day 8–11 elevates the NAD to near normal levels by day 12 [10]. NAm alleviates the lowered NADH level as well as the NAD level at day 12 following administration of diazinon at 30 μg/egg at day 4 of incubation [10].

Table 2. Effects of diazinon and carbaryl administered to eggs on day 4 of incubation with or without simultaneous administration of nicotinamide at 0.8 μmoles/egg on the chicken embryo brain, liver and leg muscle NAD levels at day 19 of incubation

	NAD level rel. to control (% ± S.E.)*		
Tissue	OP or MC	OP or MC + NAm	NAm
	Diazinon (30 μg/egg)		
Brain	60 ± 7	95 ± 14	98 ± 1
Liver	87 ± 6	95 ± 5	99 ± 6
Muscle	65 ± 9	98 ± 6	96 ± 4
	Carbaryl (3 mg/egg)		
Brain	55 ± 2	96 ± 3	97 ± 4
Liver	73 ± 6	85 ± 7	88 ± 4
Muscle	44 ± 6	98 ± 8	96 ± 6

* Control NAD levels for brain, liver and muscle, respectively, were 365 ± 11, 292 ± 22 and 206 ± 13 nmoles/g in the diazinon experiment and 331 ± 6, 398 ± 26 and 236 ± 10 nmoles/g in the carbaryl experiment.

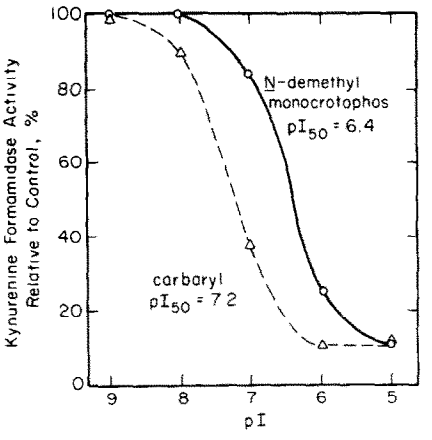

Fig. 5. *In vitro* inhibition of mouse liver kynurenine formamidase activity by *N*-demethyl monocrotophos and carbaryl with 60 and 15 min preincubation, respectively, of inhibitor and enzyme prior to substrate addition. pI_{50} is the negative logarithm of the molar concentration for 50 per cent inhibition. *N*-Demethyl monocrotophos is a major teratogenic metabolite of dicrotophos and probably of monocrotophos in chicken embryos [3].

It also alleviates the lowered NAD levels in brain, liver and leg muscle at day 19 of incubation following treatment with diazinon or carbaryl at day 4 of incubation (Table 2).

The relative effectiveness of various compounds in the NAD biosynthetic pathway as alleviating agents for lowered NAD levels and the associated micromelia and abnormal feathering was examined with diazinon administered at day 4 of incubation using a dose which yields about 30 per cent of the normal 12-day embryo NAD level. L-Tryptophan derivatives with *C*-terminal substituents (L-tryptophanamide and L-tryptophylglycine) or a *N*-terminal substitutent (glycyl-L-tryptophan) are as effective as L-tryptophan at 30 μmoles/egg in alleviating the lowered NAD level and teratogenic signs. L-Tryptophan at 3 μmoles/egg is more effective than D-tryptophan at 30 μmoles/egg in alleviating the lowered NAD level and abnormal feathering so the epimer that is presumably not nutritionally effective is also without alleviating action, except perhaps in ameliorating the micromelic condition. The dose (given in parenthesis as μmole/egg) of compounds in the NAD biosynthetic pathway required to elevate the NAD level to 50 per cent of normal decreases in the following order: tryptophan (8), quinolinic acid (4, a high level attributed to its insolubility), *N*-formyl-L-kynurenine (2.5), L-kynurenine (1.2), 3-hydroxyanthranilic acid (1.1) and NAm (0.3) [10].

The reactivator for phosphorylated acetylcholinesterase, P2S, administered at 1 mg/egg on day 4 does not alleviate the lowered NAD level, micromelia or abnormal feathering resulting from parathion, dicrotophos or eserine administered at 1 mg/egg on day 4 of incubation. However, P2S may decrease the incidence of arthrogryposis, wry neck and rumplessness induced by parathion and eserine but this is not apparent with dicrotophos [10].

Micromelia and abnormal feathering induced by 6-AN (0.3 μmole/egg), which does not lower the embryo NAD level, are not alleviated by NAm (0.8 μmole/egg) or tryptophan (5 μmoles/egg).

Effect of teratogens on chicken embryo [^{14}C]tryptophan metabolism and kynurenine formamidase activity. In comparing metabolism of a non-alleviating dose of [7a-^{14}C]tryptophan, administered at day 9 of incubation to control eggs and to diazinon-treated eggs (1 mg/egg, day 4), the embryo NAD level was lowered by diazinon to a similar extent based either on ^{14}C content (34 per cent) reflecting NAD formed from exogenous [^{14}C]tryptophan or on enzymatic assay (22 per cent) which determines NAD formed from endogenous precursors [10]. This apparent teratogen-induced inhibition of tryptophan conversion to NAD must be considered as preliminary because of the low numbers of embryos and non-optimal chromatographic conditions involved.

Attempts were made to determine the *in vivo* sensitivity to selected OP compounds of the kynurenine formamidase from chicken embryo liver and chick liver. The assay method used was not adequate to detect formamidase activity in the combined embryo and extra-embryonic tissues from eggs on the 5th day of incubation even with a reaction time of 1 hr. The chicken embryo liver at days 12, 16 and 20 of incubation displays kynurenine formamidase-like activity which is 1.3 per cent of that in mouse liver. The liver enzyme from 16-day embryos is inhibited 65 per cent *in vitro* by 10^{-3} M paraoxon with a 10 min preincubation of the enzyme and OP compound prior to substrate addition. With diazinon administered at day 4 of incubation, a 60 μg/egg dose (with or without NAm at 0.8 μmoles/egg) reduces the chicken embryo liver formamidase activity by 40 per cent at day 12 of incubation, while a 1 mg/egg dose gives 20 per cent reduction in enzyme activity at day 20 of incubation [10]. Diazinon administered at 60 μg/egg on day 9 gives little if any reduction in formamidase activity at day 12 of incubation. The liver kynurenine formamidase from 2-week-old chicks is more active (58 per cent of that in mouse liver) and it undergoes definite inhibition by diazinon, monocrotophos, pirimiphos-methyl and dicrotophos, *i.e.* 61, 36, 32 and 26 per cent, respectively, 24 hr after i.p. administration at doses of 1 mg/kg [10].

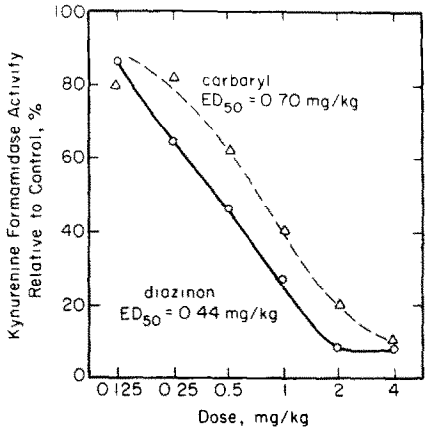

Fig. 6. *In vivo* inhibition of liver kynurenine formamidase activity 1 hr after intraperitoneal administration to mice of diazinon and carbaryl. ED_{50} is the effective dose for 50 per cent inhibition.

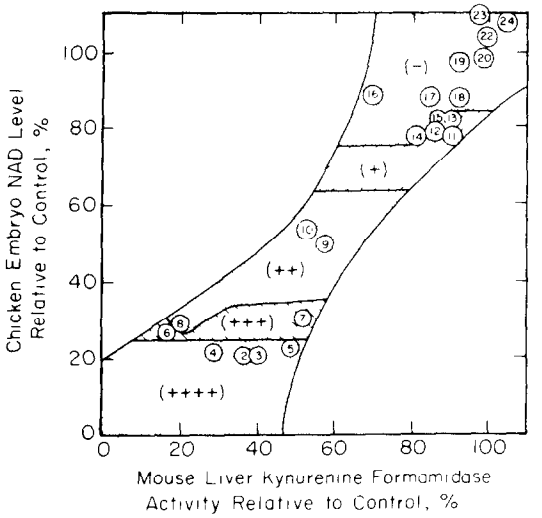

Fig. 7. Correlation of potency of various organophosphorus compounds and carbaryl for producing lowered NAD levels and teratogenesis in chicken embryos and for inhibiting mouse liver kynurenine formamidase activity. For original data and conditions, see Table 1.

Effect of certain OP and MC compounds on mouse liver kynurenine formamidase activity. Mouse liver kynurenine formamidase is very sensitive to *in vitro* inhibition by *N*-demethyl monocrotophos and carbaryl (Fig. 5). Other inhibition values with a 10 min preincubation of 10^{-3} M compound and enzyme prior to substrate addition are: paraoxon—96%; eserine—48%; 6-AN and 3-AP—0%. A separate study [10] established that eserine at 10^{-3} M inhibits the conversion of tryptophan to kynurenine resulting in accumulation of *N*-formyl-L-kynurenine; accordingly, the second step in tryptophan breakdown mediated by kynurenine formamidase is selectively inhibited relative to the first tryptophan pyrrolase-catalyzed reaction.

The most extensive studies were made *in vivo* with i.p.-treated mice to evaluate inhibition from the administered compounds and/or their bioactivation products. Mouse liver kynurenine formamidase is inhibited by very low doses of diazinon and carbaryl (Fig. 6). Diazinon inhibition is relatively persistent with a 1 mg/kg dose, *i.e.* 77, 66, 22 and 9 per cent inhibition at 1 hr and 1, 4 and 8 days, respectively, following treatment [10].

The potency of various OP and MC compounds as *in vivo* kynurenine formamidase inhibitors 1 and 24 hr after administration at 1 mg/kg is shown in Table 1. The most potent inhibitors are crotonamide phosphates (compounds **2** and **3**), pyrimidyl phosphorothionates (compounds **4**–**7**) and carbaryl. The compounds vary greatly in their persistence of kynurenine formamidase inhibition. Diazinon and phosphamidon, two *O,O*-diethyl phosphorus compounds, give similar inhibition values at 1 and 24 hr. A number of *O,O*-dimethyl phosphorus compounds (*i.e.* **1**–**3** and **7**) and particularly the *O,S*-dimethyl phosphorus compound methamidophos give somewhat to markedly greater inhibition at 1 hr than at 24 hr. In considerations given below, the values of inhibition at 1 and 24 hr are arbitrarily averaged since it was not feasible with the large number of compounds to determine the times after treatment for maximum inhibition in each case.

Structure-activity correlation of potency for inhibiting mouse liver kynurenine formamidase activity and for producing lowered NAD levels and teratogenesis in chicken embryos. There is a reasonably good correlation between the potency of 22 OP compounds and carbaryl for inhibiting mouse liver kynurenine formamidase activity and for producing lowered NAD levels and micromelia and abnormal feathering in developing chicken embryos (Table 1 and Fig. 7). The crotonamide phosphates (compounds **2** and **3**), pyrimidyl phosphorothionates (compounds **4**–**7**) and carbaryl are the most potent teratogens in eggs and they are also the most effective inhibitors of mouse liver kynurenine formamidase. OP compounds that are highly teratogenic in chicken embryos either lower mouse liver formamidase activity by more than 75 per cent 1 hr after administration or sustain an inhibition of at least 40 per cent until 24 hr after treatment. Eleven other OP compounds (**14**–**24**) do not inhibit formamidase activity by more than 32 per cent and are not teratogenic at 1 mg/egg. As pointed out earlier, BAY 92114 and parathion yield teratogenic signs that differ from those produced by the other teratogens.

Effect of OP compounds and carbaryl on NAD levels and kynurenine formamidase activity in mouse liver, brain and blood, in adult hen liver and brain, and in rat fetuses. In studies reported in thesis form [10] but not detailed here, the possible relevance was examined of lowered NAD levels and kynurenine formamidase inhibition to other aspects of OP toxicology. In mice fasted for 24 hr, treated i.p. with diazinon (1, 4 and 10 mg/kg) and held for an additional 24 hr with only water available *ad lib.*, the liver kynurenine formamidase activity is strongly inhibited (64, 76 and 86 per cent, respectively) without alteration in the liver NAD level (283 ± 10 nmoles/g fresh weight). The blood NAD level (206 ± 11 nmoles/g blood) is unaffected in mice maintained for 1 week on diets containing 300–1000 ppm diazinon. The liver kynurenine formamidase activity is essentially identical for male white mice and adult hens. Both the mouse and hen liver enzymes are inhibited by 56–91 per cent at 1 and 3 days following oral administration of TOCP (1 ml/kg) and by 8–44 per cent at 1 and 3 days after ip administration of phenyl saligenin cyclic phosphate (2 mg/kg). With this ataxic dose of TOCP the liver enzyme is still inhibited by 68 per cent at 8 days after treatment. However, the liver enzyme in the cyclic phosphate-treated hens returns to normal values by 8 days, well before the ataxic signs of the delayed neurotoxicity are evident. Tryptophan (4 m-moles/kg) administered orally in an aqueous suspension to hens 1 day prior to and for 14 days following administration of TOCP does not alleviate the TOCP-induced delayed neurotoxicity. Mouse brain preparations slowly hydrolyze *N*-formyl-L-kynurenine (activity 0.8 per cent of that in mouse liver) but the responsible component although sensitive to heat is insensitive to *in vitro* OP inhibition. Brains from mature hens contain a similar enzyme component but the activity is 0.3 per cent of that in mouse liver. These brain enzymes are not inhibited *in vivo* by TOCP or phenyl saligenin cyclic phosphate as above at 1–8 days

after treatment. Fetuses from rats treated by intra-amniotic injection [14] with either diazinon (0.45–3.6 μg/embryo) or carbaryl (2.3–18 μg/embryo) on day 14 or 15 of gestation appear essentially normal at day 19 of gestation and they display unaltered NAD levels (265 ± 5 nmoles/g fetal fresh weight). No kynurenine formamidase activity was apparent in rat placental tissues on day 20 of gestation.

DISCUSSION

OP and MC compounds produce at least two distinct types of teratogenesis in the developing chicken embryo. One type (referred to here as Type I) expressed as micromelia and abnormal feathering involves a lowering in embryo NAD levels and is alleviated by NAm and other NAD precursors [5, 6]. Tests with 44 OP and 13 MC compounds [5, 6 and this study] establish that Type I teratogenesis is produced when the NAD level is lowered to less than 50 per cent of normal at day 12 of incubation. Highly teratogenic doses of Type I compounds lower the embryo NAD level to about 20 per cent of normal which may be the lower limit that still permits maintenance of vital functions of the embryo. Compounds producing only Type I teratogenesis, even at high doses, are carbaryl, SAN I 197 and pirimiphos-methyl. The most potent teratogens of this type are crotonamide phosphates, pyrimidyl phosphorothionates and eserine. Another teratogenic pattern (Type II) involving wry neck, arthrogryposis and rumplessness is not related to NAD levels and is not alleviated by NAm. These deformities are considered to be associated with disruption of the cholinergic system [15, 16]. Two OP insecticides, parathion [3, 16] and BAY 92114, give only Type II teratogenesis. Many OP and MC compounds give a combination of Type I and Type II teratogenesis, *i.e.* dicrotophos [16 and this study] and eserine at all teratogenic doses and diazinon and pirimiphos-ethyl at high doses. In comparisons of dicrotophos versus its *O,O*-diethyl analog [3] and pirimiphos-methyl versus pirimiphos-ethyl, it appears that both types of teratogenesis are more severe with the *O,O*-diethyl compounds.

Only the first 7 days of embryogenesis and particularly the period between days 2 and 6 are sensitive to prominent Type I OP and MC teratogenesis. NAm administered at any time up until about day 11 of incubation alleviates the lowered NAD levels and the associated teratogenic signs. The lowered NAD level starts to recover after day 12–13 of incubation even without NAm administration. Joint fusion anomalies predominate when teratogens which give a combination of Type I and Type II effects are administered at day 7 of incubation or later. The developmental pattern for the vertebral column is probably completed within the first 8 days of incubation since rumplessness is not evident with teratogens administered at later times.

The present investigation considers three possible mechanisms for the lowered NAD level involved in Type I OP and MC teratogenesis. It rules out an alteration in the ratio of oxidized and reduced pyridine nucleotides thereby favoring a block in NAD biosynthesis. Tryptophan, presumably from enzymatic hydrolysis of yolk protein, serves as the precursor for embryo NAD [17, 18] at the developmental stages sensitive to teratogenic effects. A decreased rate of tryptophan release from yolk stores is probably not involved since dipeptides with *N*- and *C*-terminal tryptophan residues are similar to tryptophan itself in alleviating the teratogenic effects [see also ref. 6]. Radiotracer experiments indicate that the teratogen interferes with *in vivo* conversion of tryptophan to NAD [this study] but not of NAc or NAm to NAD [3] nor of NAc to desamido NAD*. These findings, supplemented by the relative potency of pathway intermediates as alleviating agents, suggest that the teratogen-induced metabolic block occurs early in the tryptophan to NAD biosynthetic pathway. Attention was therefore focused on kynurenine formamidase in the yolk sac membrane or embryonic liver as a possible site of teratogen inhibition but unfortunately this enzyme in the teratogen-sensitive stage of development (*e.g.* 5 days of incubation) is of very low activity [9, 19, 20] and is not conveniently assayed by the method used. Liver kynurenine formamidase activity increases dramatically shortly after hatching, however the formamidase in both the newly hatched chick and the mature hen is immunologically unrelated to that in the liver of the embryo [9]. The formamidase activity of embryonic liver is moderately lowered at days 12 and 20 of development following OP teratogen administration at day 4 while the kynurenine formamidase activity of newly hatched chicks and mature hens is very sensitive to inhibition. The available information is not adequate to define if the lowering of embryonic liver kynurenine formamidase activity following teratogen administration is due to enzyme inhibition or to alteration in enzyme expression during development.

The most important observation supporting kynurenine formamidase inhibition as the Type I teratogenesis mechanism is the similar OP structure–activity relationship for mouse liver kynurenine formamidase inhibition and the lowering of chicken embryo NAD levels and production of teratogenesis. Liver kynurenine formamidase appears to be more sensitive in mice than nerve acetylcholinesterase to inhibition by most Type I teratogenic OP and MC compounds. The high inhibitor specificity for kynurenine formamidase inhibition suggests that, as with acetylcholinesterase, there are one or more binding sites near the critical serine hydroxyl group of the esteratic site that assist in orienting the inhibitors for phosphorylation or carbamoylation. *O,O*-Diethyl phosphorus compounds are expected to give more prolonged kynurenine formamidase inhibition than *O,O*- or *O,S*-dimethyl phosphorus compouunds due to less rapid detoxification or to formation of a more stable phosphorylated form of kynurenine formamidase with diethyl as compared with dimethyl OP compounds [21].

Other chemicals (*i.e.*, 6-AN, sulfanilamide and insulin) which produce micromelia and abnormal feathering similar to Type I OP and MC compounds and which are alleviated in these actions by NAm administered at appropriate levels do not lower the embryo

* N. H. Proctor and J. E. Casida, unpublished results (1975).

NAD level. A previous report [22] that 6-AN lowers embryo NAD levels involves expressing the NAD content per embryo, which is drastically reduced in size, rather than per unit of embryo weight. It is clear that the mechanisms of teratogenesis with 6-AN, sulfanilamide and insulin are completely different than that with OP and MC compounds.

Toxicological manifestations of kynurenine formamidase inhibition should appear only in those developmental stages or species where the reduced enzyme activity impairs maintenance of normal levels of NAD or other essential biochemicals derived from kynurenine. This block in NAD biosynthesis will be circumvented when there are adequate amounts of alternative NAD precursors, such as NAm or NAc from the mother or the diet, which may be the case with the rat fetus and the mouse and hen systems examined. The only syndrome attributable at present to kynurenine formamidase inhibition is the Type I OP and MC teratogenesis in avian embryos.

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