Biochemical Pharmacology, Vol. 25, pp. 757–762. Pergamon Press, 1976. Printed in Great Britain.

CHICKEN EMBRYO NAD LEVELS LOWERED BY TERATOGENIC ORGANOPHOSPHORUS AND METHYLCARBAMATE INSECTICIDES

NICK H. PROCTOR,* A. DAVID MOSCIONI and JOHN E. CASIDA

Division of Entomology and Parasitology, University of California, Berkeley, Calif. 94720, U.S.A.

(*Received* 16 *June* 1975; *accepted* 30 *July* 1975)

Abstract Many organophosphorus (OP) and methylcarbamate (MC) insecticides and related compounds, when injected into the yolk sac on day 4 of incubation, markedly lower chicken embryo NAD levels at day 12 of incubation. The same compounds also produce drastic developmental defects, suggesting that the lowered level of NAD may be the cause of teratogenesis. The most potent OP compounds of those tested are diazinon and dicrotophos acting at 0·1 and 1 mg/kg, respectively, while the MC compounds, eserine sulfate and carbaryl, are effective at 1 and 4 mg/kg respectively. The injection of nicotinamide and other NAD precursors prevents the decrease in NAD levels and alleviates most of the teratogenic signs. Previous hypotheses, including that relating the inhibition of yolk sac membrane esterases to embryonic abnormalities, are less satisfactory or are inadequate to explain this type of chemically induced teratogenesis. Areas remaining to be defined are the biochemical lesion leading to decreased chicken embryo NAD levels and the relevance of these findings to mammals.

Organophosphorus (OP) and methylcarbamate (MC) insecticides are designed to inhibit acetylcholinesterase in the insect central nervous system. Their toxic effects in higher animals and man are also generally attributable to action on the cholinergic system [1, 2]. However, cholinesterase and the cholinergic system do not appear to be involved in the induction of teratogenesis in chicken embryos by a variety of OP and MC compounds [3–5]. It is therefore important to define the nature of the biochemical lesion leading to developmental defects (particularly since these insecticides are now the major weapons for insect control and therefore environmental contamination and human exposure are inevitable).

Several features of teratogenesis in the chicken embryo warrant special consideration. Despite large potency differences, teratogenic doses of a tremendous variety of OP and MC compounds, injected at day 0–5 of incubation, always produce the same or similar developmental defects, which include some or all of the following: reduced body size, micromelia, gross edema, abnormal beak, retarded down development and wry neck [3–19]. With the exception of wry neck, the teratogenic signs are almost completely alleviated by coinjection of nicotinamide (NAm), nicotinic acid (NAc) or any one of many precursors and derivatives of these two substances, including the nicotinamide-adenine dinucleotide cofactors (NAD, NADH, NADP and NADPH) [3–10]. However, the conversion of yolk-administered NAm and NAc to embryo NAD (analyzed along with NADP) is not altered by an OP teratogen [9]. The teratogenesis induced by dicrotophos injected on day 4 of incubation is partially alleviated by NAm administered at any time from day 0 to day 10 of incubation [9]. The site of the primary biochemical lesion [i.e. yolk, yolk sac membrane (YSM), embryo organ, etc.] is not as yet defined, although the following observations may be relevant: inhibition of selected esterases in the YSM appears to correlate with OP and MC teratogenesis [19], reduced transport from the yolk to the embryo of endogenous tryptophan [4] and exogenous acetylcholine [3] results from some OP and MC teratogens, and altered levels of RNA, glycogen, sulfated mucopolysaccharide and calcium are evident in the developing tibiotarsus [18].

Further experimental work is needed on the proposed teratogen-specific inhibition of YSM esterases or peptidases; the focus should be on the selection of appropriate substrates and adequate differentiation of the component enzymes. The apparent lack of any relationship between embryo NAD level and teratogenesis should also be re-evaluated, since the previous determinations were not made on the endogenous NAD but rather on the NAD formed via the more limited pathways from exogenous NAc or NAm. These two areas are re-examined in the present detailed report and in our preliminary communication [20] on these studies. A cause and effect relationship is proposed between diminished levels of embryo NAD and OP- and MC-induced teratogenesis.

MATERIALS AND METHODS

Chemicals. The OP and MC compounds were analytical reference standards obtained from the Pesticides and Toxic Substances Effects Laboratory (Environmental Protection Agency, Research Triangle Park, N.C.) or from the manufacturers. Although referred to here by their common names, they are chemically defined in relevant listings of pesticide chemicals [21, 22]. Other chemicals were used in the purest forms available from commercial sources.

* Present address: Kaiser Foundation International, Oakland, Calif. 94666, U.S.A.

Treatment of eggs and rating of teratogenic signs. Fertile white leghorn eggs (45–55 g) were incubated at 37° and 73 per cent relative humidity. Each test compound (candidate teratogen or alleviating agent) was administered, using sterile technique, by injecting a methoxytriglycol solution (30 μl) into the yolk after a 4- or 5-day incubation period and then sealing the injection hole with paraffin wax [3, 9, 19]. A portion of the eggs from each treatment group was used for biochemical tests at day 6 to 12 of incubation, and the remainder (at least 10 eggs) were incubated for 19 days and then opened to observe the teratogenic signs. These signs are rated from − (no effect) to + + + + (most severe manifestation) or as * to designate embryos having wry neck and occasionally tibiotarsal arthrogryposis but not the other signs normally associated with OP and MC teratogenesis [9, 20].

Appropriate controls were run in each experiment to confirm that the carrier solvent and treatment conditions did not alter the normal biochemical parameters and developmental cycle. Embryos from typical control eggs at day 19 of incubation showed a zero incidence of straight legs, abnormal feathers and wry neck. Their size was as follows (mean ± S. E.): body weight, 25·4 ± 1·3 g; body length, 8·3 ± 0·2 cm; and leg length, 6·4 ± 0·2 cm.

Inhibition in vivo of YSM hydrolases. The supernatant fraction (100,000 *g*, 60 min) of YSM homogenates prepared according to Flockhart and Casida [19] was used for polyacrylamide gel electrophoretic separation at 4° and detection of esterases hydrolyzing α-naphthyl acetate (NA) at 37°. Disc gel electrophoresis by the method of Hedrick and Smith [23] involved 5% gel in vertical tubes of 0·7 mm i.d., 50-μl enzyme samples mixed with 50 μl of 0·06 M imidazole-HCl and 50% glycerol, pH 5·7, for application to the gel, and development with 0·034 M asparagine Tris buffer, pH 7·3, at 1–2 mA/tube until the bromophenol blue marker had moved 7–8 cm. For slab gel electrophoresis, the Gradipore 4–26 per cent linear gradient preparations of Isolab Inc. (Akron, Ohio) were used with a Tris borate buffer, pH 8·3 (similar to that of Margolis and Kenrick [24] but lacking ethylenediaminetetraacetic acid), applying the supernatant samples (15 μl) mixed with 15 μl of 40% sucrose and developing for 3 hr at 400 V and 30 mA. The method of Markert and Hunter [25] was employed for esterase detection as follows: equilibration of the gel in 0·04 M Tris–Cl buffer, pH 6·6, for 5 min; replacement of the buffer with fresh solution containing 0·1% (w/v) Fast Blue RR salt and 0·04% (w/v) NA, the latter added in acetone to 4 per cent (v/v); agitation for 15 min; replacement of the staining solution with a 4:1 mixture of 10% acetic acid and 95% ethanol; and sketching and photography of the zymograms, calculating the relative mobilities (R_m) with reference to bromophenol blue (tube gels) or the length of the slab.

Dipeptidase activity was examined by measuring tryptophan release from glycyl-tryptophan. A mixture consisting of 80 μl enzyme (supernatant of YSM homogenate in 2 parts 30% glycerol and centrifugation at 6000 *g* for 10 min; 1·7 mg protein [26]) and 2×10^{-4} M glycyl-tryptophan in 2·0 ml McIlvaine's buffer, pH 7·0, was incubated for 0, 5, 10 and 20 min at 37° with determination of released tryptophan by the method of Saifer and Gerstenfeld [27]. Normal preparations liberated 80 nmoles tryptophan/mg of protein/5 min.

Embryo NAD levels. On day 12 of incubation, after injection on day 4, each of six embryos from each treatment group was removed from the egg, weighed, suspended in 0·5 vol. of cold distilled water, and homogenized for 5 sec with a model PT 10 Polytron (Kinematica, GmbH, Luzern, Switzerland) at maximum speed. A volume of 20% $HClO_4$ equal to the volume of distilled water was mixed with each homogenate, and the mixture was centrifuged (30,000 *g*, 10 min). An aliquot of the supernatant was adjusted to pH 7·0 by the addition of a neutralizing agent (3 M KOH–0·5 M phosphate buffer, pH 7·0–water; 1·1:1·9:2·0, by vol.) [28]. After 10 min at 5° for precipitation of insoluble $KClO_4$, the samples were again centrifuged for 10 min. The NAD content in this supernatant was assayed against a standard curve for NAD by the alcohol dehydrogenase method, determining the NADH formed by its absorbance at 340 nm [29]. The level of NAD in embryos from typical control eggs was 185 ± 4 nmoles/g of embryo fresh weight.

RESULTS

Inhibition in vivo of YSM hydrolases. Preliminary studies by Flockhart and Casida [19] indicated a possible association of teratogenesis with inhibition *in vivo* of YSM esterases. These relationships were re-examined using improved conditions for electrophoretic esterase separation on polyacrylamide disc and slab gels. The comparisons involved injections at day 5 of incubation, YSM removal at day 6 of incubation, four teratogenic treatments (selected from 1 mg dicrotophos, 1 mg parathion, 10 mg mevinphos and 1 mg eserine sulfate), and four treatments that did not yield teratogenic signs [selected from 1 mg dicrotophos plus 1 mg NAm, 10 mg ethyl *p*-nitrophenyl thionobenzenephasphonate (EPN), 1 mg mevinphos and 50 mg tri-*o*-cresyl phosphate (TOCP)].

The tube gel studies with normal embryos revealed eight NA-hydrolyzing bands, which were designated in order of increasing R_m values as follows: 1–0·21, 2–0·39, 3–0·49, 4–0·59, 5–0·74, 6–0·76, 7–0·78 and 8–0·83. Investigations with treated embryos establish that dicrotophos strongly inhibits esterase bands 1, 2, 4 and 8 and partially blocks bands 5, 6 and 7. Mevinphos at both 1 and 10 mg strongly blocks bands 2, 3, 4 and 8 and partially inhibits bands 5, 6 and 7. The nonteratogenic compound EPN inhibits bands 5, 6, 7 and 8. These results would implicate either band 2 or 4 in the teratogenesis, but this is not supported by tests with dicrotophos plus NAm, which resulted in the same inhibition pattern obtained with dicrotophos alone and particularly with eserine sulfate in which none of the bands were blocked.

In studies with slab gels, normal embryo YSM preparations gave seven NA-hydrolyzing bands designated by their R_m values as follows: 1–0·40, 2–0·58, 3–0·62, 4–0·70, 5–0·77, 6–0·85 and 7–0·94. Dicrotophos treatment completely inhibited bands 1, 5 and 7 and partially blocked bands 2, 3, 4 and 6. Mevinphos at either a teratogenic dose (10 mg) or a nonteratogenic

one (1 mg) blocked band 1 and partially inhibited bands 6 and 7. Parathion blocked band 1 without other inhibition. EPN partially inhibited band 6, and TOCP inhibited none of the bands. Thus, inhibition of band 1 appears to correlate best with teratogenesis. A teratogenic dose of eserine sulfate, however, inhibited none of the bands. On considering the full set of results, it appears there is no esterase band on either tube or slab gels that is consistently blocked by teratogens but not by nonteratogens. Therefore, these further investigations do not lend support to the hypothesis of Flockhart and Casida [19] that inhibition of YSM esterases is involved in the teratogenic mechanism.

The dipeptidase activity of YSM homogenates is similar or identical with eggs at day 10 of incubation, which were pretreated by injection at day 5 of incubation with either teratogenic compounds (dicrotophos or eserine sulfate at 1 mg/egg) or nonteratogenic compounds (controls and EPN at 10 mg/egg). Thus, if protease inhibition occurs, the enzyme involved is not the one assayed with glycyl-tryptophan as the substrate.

Relationship of teratogenic signs to embryo NAD levels. Studies with 36 OP and 12 MC compounds injected at doses of 0·003 to 5 mg/egg (0·06 to 100 mg/kg) establish an association between the embryo NAD levels and the severity of teratogenic

Table 1. Effect of various organophosphorus compounds and methylcarbamates injected at day 4 of incubation on the NAD levels at day 12 and the teratogenic signs at day 19 of incubation†

Candidate teratogen, name and type	Dose (mg/egg)	NAD level rel. to control (% ± S. E.)	Teratogenic signs: Rel. to control (%) Body weight	Body length	Leg length	Incidence (%) Str. legs	Abnormal feathers	Wry neck	Rating
Dicrotophos (OP)	1	10 ± 1	56	53	40	90	100	83	+ + + +
Eserine sulfate (MC)	1	19 ± 1	59	61	44	67	100	100	+ + + +
Monocrotophos (OP)	1	20 ± 3	61	52	46	84	100	100	+ + + +
Dicrotophos (OP)	0·3	20 ± 1	78	87	55	84	100	100	+ + +
Diazinon (OP)	1	21 ± 3	60	70	48	33	100	56	+ + +
Eserine sulfate (MC)	0·3	22 ± 2	80	86	51	88	100	25	+ + +
Phosphamidon (OP)	5	23 ± 2	65	65	56	75	100	100	+ + + +
Carbaryl (MC)	3	23 ± 3	75	90	61	50	100	0	+ +
Carbofuran (MC)	5	26 ± 1	55	75	40	75	100	25	+ + +
Diazinon (OP)	0·3	26 ± 5	69	82	45	45	100	39	+ + +
Carbaryl (MC)	1	28 ± 4	86	96	72	28	72	0	+
Diazinon (OP)	0·03	29 ± 5	98	92	54	49	89	0	+ +
Diazinon (OP)	0·1	33 ± 4	74	89	49	63	91	3	+ +
Eserine sulfate (MC)	0·1	38 ± 2	79	93	57	84	100	0	+ +
Dicrotophos (OP)	0·1	41 ± 4	81	94	62	50	60	0	+ +
Diazinon (OP)	0·01	41 ± 10	89	96	78	17	50	0	+
Carbaryl (MC)	0·3	45 ± 4	91	95	87	11	22	0	+
Phosphamidon (OP)	1	45 ± 3	89	88	71	25	15	0	+
Carbofuran (MC)	1	52 ± 3	94	94	90	0	0	0	−
Methamidophos (OP)	1	53 ± 4	90	97	67	0	69	0	+
Meobal (MC)	1	58 ± 5	65	80	64	0	100	0	+ +
Diazinon (OP)	0·003	59 ± 9	100	99	90	0	7	0	−
Metalkamate (MC)	1	62 ± 8	97	97	87	18	18	0	+
Methidathion (OP)	5	62 ± 7	98	97	85	0	0	0	−
Parathion (OP)	1	63 ± 15	66	71	75	0	0	75	+ +
Azinphosmethyl (OP)	5	69 ± 11	94	103	94	0	0	0	−
Dicrotophos (OP)	0·03	69 ± 6	95	97	88	0	0	0	−
Pyramat (MC)	1	73 ± 4	48	72	71	0	0	0	+
Carbaryl (MC)	0·1	75 ± 3	93	94	92	0	11	0	−
Phosmet (OP)	1	76 ± 5	87	94	96	0	0	0	−
Crufomate (OP)	1	77 ± 17	85	91	94	0	0	0	−
Methyl parathion (OP)	1	77 ± 7	111	107	102	0	0	0	−
Eserine sulfate (MC)	0·03	77 ± 4	104	98	87	0	0	0	−
Isolan (MC)	1	78 ± 4	66	68	80	0	0	0	+
Phorate (OP)	1	80 ± 6	88	78	84	0	0	61	+
Coumaphos (OP)	1	80 ± 10	95	98	96	0	0	0	−
Dimetilan (MC)	1	92 ± 3	56	75	70	0	0	0	+
Aldicarb (MC)	1	100 ± 2	111	84	108	0	0	0	−

† The following compounds (name, type, mg/egg, NAD level as percent of control) gave >80% NAD level and >90% body weight, body length and leg length relative to the control and no signs of straight legs, abnormal feathers and wry neck: Abate (OP, 1, 103); carbophenothion (OP, 1, 93); chlorpyrifos (OP, 1, 103); DEF (OP, 1, 99); dichlorvos (OP, 1, 97); dimethoate (OP, 1, 87); dioxathion (OP, 1, 95); disulfoton (OP, 1, 112); EPN (OP, 1, 109); ethion (OP, 1, 96); fenitrothion (OP, 1, 89); fenthion (OP, 1, 94); fonofos (OP, 1, 86); leptophos (OP, 1, 107); malathion (OP, 5, 83); methomyl (MC, 1, 89); mevinphos (OP, 1, 88); mexacarbate (MC, 1, 102); naled (OP, 1, 93); oxydemetonmethyl (OP, 1, 94); phosalone (OP, 1, 105); propoxur (MC, 1, 103); ronnel (OP, 1, 97); Salithion (OP, 1, 108); tepp (OP, 1, 99); trichlorfon (OP, 1, 94).

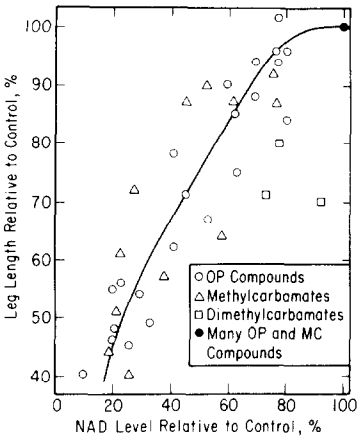

Fig. 1. Relation of the leg length at day 19 of incubation to the NAD level at day 12 after injection of various organophosphorus compounds, methylcarbamates and dimethylcarbamates at day 4 of incubation. This plot is based on the data given in Table 1.

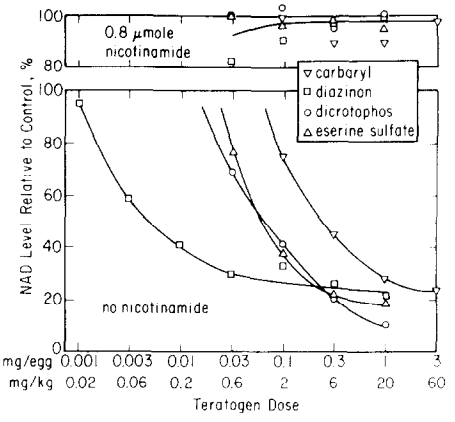

Fig. 2. Effect of varying doses of carbaryl, diazinon, dicrotophos and eserine sulfate injected alone or in combination with nicotinamide at day 4 of incubation on the NAD levels at day 12 of incubation. For comparable data on the teratogenic signs, see Tables 1 and 2.

signs (Table 1). Each of the 18 treatment schedules that gave NAD levels less than 45 per cent of normal yielded mild to severe teratogenic signs. In the treatment schedules with the range of 52–63 per cent of normal NAD levels, the teratogenic signs varied from normal (three examples) to slight or moderate (four examples). In those with NAD levels between 69 and 100 per cent of normal, only four treatment schedules of 39 tested gave mild teratogenic signs. Three of these were dimethylcarbamates of heterocyclic enols (dimetilan, isolan and pyramat), which gave large reductions in body weight without other marked abnormalities, while the fourth was the OP compound, phorate, which gave wry neck as the only prominent teratogenic sign. Thus, based on an overall rating of the teratogenic signs, there appears to be a reasonably good correlation between the embryo NAD levels and the severity of teratogenesis.

Of the teratogenic signs that appear to bear a direct relationship to the NAD level, the leg length is the one most easily quantitated (Fig. 1). All of the OP and MC compounds except the dimethylcarbamates (see above) gave results which follow nearly the same curve relating NAD level to leg length. Regardless of the compound or dose involved, a treatment reducing the NAD level below 40 per cent of normal is associated with a marked reduction in leg length.

Several of the compounds are very potent in altering the embryo NAD levels, as indicated by the following approximate mg/kg doses yielding a 50 per cent decrease: diazinon (0·1), dicrotophos and eserine sulfate (1) and carbaryl (4) (Fig. 2).

Effect of candidate alleviating agents on the teratogenic signs and the NAD levels. The abnormalities induced by teratogenic doses of carbaryl, diazinon, dicrotophos and eserine sulfate (or even by doses 10 to 30 times higher than the threshold teratogenic levels) are almost completely alleviated by 0·8 μmole NAm (Table 2). Exceptions are that wry neck and tibiotarsal arthrogryposis are not reversed at very high teratogen doses, and several of the other signs are only partially alleviated at the 1 mg/egg level of diazinon. The reversal of teratogenic signs is associated with an almost complete return of the NAD levels to the normal region, even with teratogen doses 30- to 300-fold greater than would be necessary to cause reduction of NAD levels in the absence of NAm treatment (Fig. 2). NAm also markedly increased the NAD level and alleviated most or all of the teratogenic signs, except for the wry neck, when administered with 1 mg methamidophos, monocrotophos and parathion or 5 mg carbofuran and phosphamidon.

Candidate alleviating agents other than NAm were also tested for their effect on the teratogenic signs and recovery from the lowered NAD levels, which resulted from dicrotophos treatment (Table 3). Four compounds which do not serve as NAD precursors were ineffective in alleviating the teratogenic signs and elevating the NAD levels. Tryptophan, a precursor of NAc and NAD, was partially effective at 5 μmoles/egg in alleviating both the teratogenic signs

Table 2. Effect of varying doses of carbaryl, diazinon, dicrotophos and eserine sulfate injected alone or in combination with nicotinamide at day 4 of incubation on the teratogenic signs at day 19 of incubation

Teratogen (name and type)	Dose (mg/egg)	Teratogenic signs with indicated NAm level (μmoles) 0	0·8
Carbaryl (MC)	3	+ +	
	1	+	
	0·3	+	
Diazinon (OP)	1	+ + +	+ *
	0·3	+ +	
	0·1	+ +	
	0·03	+	
	0·01	-	
Dicrotophos (OP)	1	+ + + +	*
	0·3	+ + +	*
	0·1	+ +	
Eserine sulfate (MC)	1	+ + + +	*
	0·3	+ + +	*
	0·1	+ +	

Table 3. Effect of dicrotophos injected alone or in combination with candidate alleviating agents at day 4 of incubation on the NAD levels at day 12 and the teratogenic signs at day 19 of incubation

Alleviating agent	NAD level rel. to control (% ± S. E.)	Teratogenic signs: Rel. to control (% ± S. E.) Body weight	Body length	Leg length	Incidence (%) Straight legs	Abnormal feathers	Wry neck	Rating
		Dicrotophos (1 mg/egg)						
None	10 ± 1	56 ± 3	53 ± 1	40 ± 2	90	100	83	+ + + +
Four ineffective compounds†	19 ± 2	64 ± 3	60 ± 2	38 ± 3	89	100	100	+ + + +
One partially effective compound‡	53 ± 7	71 ± 4	64 ± 1	69 ± 3	0	10	80	+*
Three effective compounds§	94 ± 2	87 ± 4	73 ± 4	83 ± 2	0	0	100	*
		Controls (no dicrotophos)						
None	100	100	100	100	0	0	0	–
Eight compounds¶	101 ± 3	103 ± 4	98 ± 2	100 ± 2	0	0	0	–

† Isonicotinic acid, β-picoline, picolinic acid or 3-pyridylacetic acid (0·8 μmole), administered singly.
‡ Tryptophan (5 μmoles).
§ NAm, NAc or NAD (0·8 μmole).
¶ Each of the alleviating agents referred to above.

and the diminished NAD levels. Other more direct NAD precursors (NAm and NAc) and NAD itself were very effective at 0·8 μmole/egg in elevating the NAD level to the normal region and in alleviating the teratogenic signs.

DISCUSSION

Success has been achieved, for the first time, in correlating an important biochemical parameter with OP- and MC-induced teratogenesis in chicken embryos. There is a remarkable association between embryo NAD levels and the teratogenic signs. On the other hand, the results of the present study do not support the hypothesis [19] that acylation of YSM esterase(s) or dipeptidase(s) leads to abnormal chicken embryo development. This is so because no esterase band(s) was consistently inhibited by teratogens but not by nonteratogens, low doses of teratogens or teratogens administered with NAm.

The oxidative mechanisms for activation of phosphorothionate insecticides (e.g. parathion, EPN and others) are clearly present in the developing hen egg, since these indirect inhibitors are very effective in producing inhibition *in vivo* of YSM esterases and embryo cholinesterase [3, 19, this study].

The association of diminished NAD levels with teratogenesis extends over a sufficient number of test conditions to propose a cause and effect relationship. It holds for a large variety of OP and MC compounds and correlates with their dose–response curves. A few compounds may be exceptions, however, since they are rated here as teratogenic despite relatively high NAD levels. They give only a small proportion, though, of the teratogenic signs rated, i.e. reduced body size with Meobal, parathion and the dimethylcarbamates and additional signs of abnormal feathers with Meobal and wry neck with parathion and phorate. If these are more than random variations, the indicated compounds may differ somewhat from the others in the locus or persistence of the biochemical lesion.

Any mechanism proposed for the teratogenesis must include a role for the alleviating action of NAm, NAc and their precursors and derivatives. When NAm is administered with teratogenic doses of several OP and MC compounds, the NAD levels are elevated to near normal values and the developmental abnormalities (except for the wry neck and sometimes the tibiotarsal arthrogryposis) are reversed. Candidate alleviating agents that serve as NAD precursors are effective in alleviating the teratogenic signs, whereas those that do not serve as NAD precursors are ineffective in this respect. Thus, the OP and MC compounds appear to produce a metabolic block at an undetermined step in the pathway for conversion of tryptophan within yolk protein to embryo NAD, and this block can be bypassed by direct administration of some NAD precursors.

It is proposed that the OP and MC teratogens diminish the embryo NAD levels and that the imbalance of NAD in the limb during its development results in abnormal growth and micromelia. The present study establishes that the leg length bears a direct relationship to the embryo NAD level. Two teratogenic nicotinamide antagonists, 6-aminonicotinamide (6-AN) and 3-acetylpyridine (3-AP), are stated to act, at least in part, by diminishing the NAD levels in chicken embryos[30, 31], chicken limb mesodermal cell cultures [32, 33] and mammals [34]. We were unable to reproduce the reported [31] lowering of chicken embryo NAD levels by administering 3-AP (0·9 mg) or a highly teratogenic dose of 6-AN (40 μg) at day 4 of incubation with NAD analyses at day 12. It is of interest that 6-AN produces several teratogenic signs in common with OP and MC teratogens (i.e. micromelia, abnormal beak, reduced body size, and retardation of down development), whereas 3-AP gives different teratogenic signs (muscle hypoplasia, most noticeable in the legs) [30]. The importance of NAD levels in the control of muscle and cartilage

development is evident from studies with embryonic chicken limbs and cultures of their mesodermal cells, since chondrogenic cells are seen when NAD levels are low and myogenic cells are observed when NAD levels are high [35].

The structure activity relationships of OP and MC compounds in blocking the synthesis or maintenance of adequate levels of embryo NAD appear to be greatly different from those involved in inhibition of acetylcholinesterase. Inhibition of acetylcholinesterase and other sensitive hydrolases by OP and MC compounds generally proceeds by phosphorylation and carbamoylation, respectively, of a serine residue in the active site of the enzyme [1, 2]. It appears likely that the biochemical lesion leading to teratogenesis is initiated by similar phosphorylation and carbamoylation reactions but at an undefined enzyme in the pathway of NAD biosynthesis.

Acknowledgements—Supported in part by the National Institute of Environmental Health Sciences (Fellowship 1 F22 ES00314 to N. H. P. and Grant 5 P01 ES00049 to J. E. C.) and The Rockefeller Foundation. We thank Judith L. Engel of this laboratory for advice and assistance.

REFERENCES

1. G. B. Koelle (Ed.), *Handbuch der Experimentellen Pharmakologie*, Vol. XV, 1220 pp. Springer, Berlin (1963).
2. M. Eto, *Organophosphorus Pesticides: Organic and Biological Chemistry*, 387 pp. CRC Press, Cleveland, Ohio (1974).
3. D. G. Upshall, J.-C. Roger and J. E. Casida, *Biochem. Pharmac.* **17**, 1529 (1968).
4. J. Greenberg and Q. N. LaHam, *Can. J. Zool.* **48**, 1047 (1970).
5. N. E. Walker, *Toxic. appl. Pharmac.* **19**, 590 (1971).
6. W. Landauer, *J. exp. Zool.* **136**, 509 (1957).
7. J.-C. Roger, H. Chambers and J. E. Casida, *Science, N.Y.* **144**, 539 (1964).
8. J. P. Marliac, M. J. Verrett, J. P. McLaughlin and O. G. Fitzhugh, Paper presented at Soc. of Toxicology, Fourth Meeting, Williamsburg, Va., March 8-10 (1965).
9. J.-C. Roger, D. G. Upshall and J. E. Casida, *Biochem. Pharmac.* **18**, 373 (1969).
10. W. Landauer and N. Salam, *Devl Biol.* **28**, 35 (1972).
11. I. P. Agarwal, *J. Anim. Morph. Physiol.* **3**, 63 (1956).
12. J.-P. Marliac and M. K. Mutchler, *Fedn Proc.* **22**, 188 (1963).
13. J.-P. Marliac, *Fedn Proc.* **23**, 105 (1964).
14. J. F. Dunachie and W. W. Fletcher, *Nature, Lond.* **212**, 1062 (1966).
15. K. S. Khera, *Toxic. appl. Pharmac.* **8**, 345 (1966).
16. K. S. Khera and S. Bedok, *Fd Cosmet. Toxic.* **5**, 359 (1967).
17. J. Greenberg and Q. N. LaHam, *Can. J. Zool.* **47**, 539 (1969).
18. M. Ho and M. A. Gibson, *Can. J. Zool.* **50**, 1293 (1972).
19. I. R. Flockhart and J. E. Casida, *Biochem. Pharmac.* **21**, 2591 (1972).
20. N. H. Proctor and J. E. Casida, *Science, N.Y.* **190**, 580 (1975).
21. E. E. Kenaga and C. S. End, *Entomol. Soc. Am.* (Special Publication), 74-1 (1974).
22. D. E. H. Frear, *Pesticide Index*, 4th Edn, 399 pp. College Science Publ., State College, Penn. (1969).
23. J. L. Hedrick and A. J. Smith, *Archs Biochem. Biophys.* **126**, 155 (1968).
24. J. Margolis and K. G. Kenrick, *Analyt. Biochem.* **25**, 347 (1968).
25. C. L. Markert and R. L. Hunter, *J. Histochem. Cytochem.* **7**, 42 (1959).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. A. Saifer and S. Gerstenfeld, *Clin. Chem.* **10**, 970 (1964).
28. M. Shimoyama, S. Shigehisa and I. Ueda, *Biochim. biophys. Acta* **237**, 329 (1971).
29. M. M. Ciotti and N. O. Kaplan, *Meth. Enzym.* **3**, 890 (1957).
30. W. Landauer and E. M. Clark, *J. exp. Zool.* **151**, 253 (1962).
31. I. H. Krakoff, C. R. Lacon and D. A. Karnofsky, *Nature, Lond.* **184**, 1805 (1959).
32. A. I. Caplan, *J. exp. Zool.* **180**, 351 (1972).
33. A. I. Caplan, *Expl Cell Res.* **70**, 185 (1972).
34. W. J. Johnson, in *Metabolic Inhibitors* (Eds. R. M. Hochster and J. H. Quastel), Vol. II, Chap. 16, pp. 1–21. Academic Press, New York (1963).
35. M. J. Rosenberg and A. I. Caplan, *Devl Biol.* **38**, 157 (1974).