Biochemical Pharmacology, Vol. 30, No. 16, pp. 2225–2235, 1981.
Printed in Great Britain.

0006-2952/81/162225-11 $02.00/0

TERATOGENIC EFFECTS OF CHOLINERGIC INSECTICIDES IN CHICK EMBRYOS—II

EFFECTS ON THE NAD CONTENT OF EARLY EMBRYOS*

PAUL A. KITOS,† DOUGLAS S. ANDERSON, EDWIN M. UYEKI,‡ MIWA MISAWA‡ and CHARLES R. WYTTENBACH§

Departments of Biochemistry and of Physiology and Cell Biology, The University of Kansas, Lawrence, KS 66045, and the Department of Pharmacology, The University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.

(*Received* 22 *September* 1980; *accepted* 16 *January* 1981)

Abstract—Chick embryos at 72 hr of incubation were exposed *in ovo* (0.2 or 1.0 mg per egg) to the organophosphate insecticide diazinon (DZN) or dicrotophos (DCP) and examined at days 5, 7 and 10 for visible defects and changes in the protein and NAD content of the whole organism, their wings and legs. Skeletal changes of the spinal column and legs were scarcely detectable on day 5 but were readily apparent by day 10. The NAD contents of the embryos and their limbs were normal up to day 5, slightly reduced by day 7, and greatly reduced by day 10, while the wet weights and protein contents were virtually normal over this time span. Nicotinamide (8 μmoles/egg on day 3) increased the NAD content of the embryos by 20–30 per cent. If administered along with DZN or DCP, it produced an even greater increase by day 10 and minimized the insecticide-induced leg deformities. A reactivator of organophosphate-inhibited acetylcholine esterase, 2-pyridinealdoxime methochloride (2-PAM), did not affect the NAD content of control embryos during these early times. If given with the insecticide, however, it prevented the expected decrease in NAD level. Some of the effects of the cholinergic insecticides and their reversing agents on the NAD content of young chick embryos are considered in terms of their possible mechanisms of action and teratogenic implications.

Many organophosphate (OP)‖ and alkylcarbamate (AC) insecticides are potent teratogens [1–5]. They cause readily identifiable developmental failures which in avian systems fall into two general classes—those that are antagonized by exogenous nicotinamide and several of its congeners (type I), and those that are antagonized by certain cholinesterase reactivators (type II) [6, 7].

The type I teratisms include abnormal beak and feather development and micromelia and have been attributed to lower than normal tissue NAD concentrations brought on by an impairment in the conversion of L-tryptophan to nicotinic acid in the yolk sac membrane [6, 8, 9]. This metabolic failure may be due to the inactivation of one of the two major forms of kynurenine formamidase (KFase) by the insecticide during a critical period of embryonic development [8, 9]. Type II teratisms such as tibiotarsal arthrogryposis, muscular hypoplasia of the legs, and certain spinal problems—short and wry neck and rumplessness—are considered to be consequences of disruptions of the cholinergic system [6, 7, 10, 11]. Thus, the two classes of damage appear to be mediated by distinctly different biochemical processes.

Consistent with the view of differential causation, Misawa *et al.* [10] showed that the amount of one of the more potent OP insecticides, diazinon, needed to generate type I responses is much less than for type II responses. They also showed that the type II responses could be reversed by gallamine but not by atropine, *prima facie* evidence that cholinergic nicotinic but not muscarinic receptors are involved in the type II teratogenic processes.

We have examined the effects of two OP insecticides, diazinon (DZN) and dicrotophos (DCP), and of two of their antagonists, nicotinamide (NAM) and 2-pyridinealdoxime methochloride (2-PAM), on the NAD content of chick embryos up to day 10 of development. In agreement with the reports of Casida and his colleagues [4, 12, 13], we found that, if 0.2 mg or more of the insecticides was administered via the yolk at an early stage (days 3–4), the NAD content of the embryo decreased to less than 30 per cent of the control value by day 10. The decline did not occur immediately and was most noticeable 4–7 days after injection. In contrast, the acetylcholinesterase (AChE) activity of the embryonic limbs and

* Supported, in part, by Grant ES-02046 from NIEHS and KU GRF No. 3516.

† Author to whom all correspondence should be addressed: Paul A. Kitos, Department of Biochemistry, The University of Kansas, Lawrence, KS 66045, U.S.A.

‡ Present address: Department of Pharmacology, The University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.

§ Present address: Department of Physiology and Cell Biology, The University of Kansas, Lawrence, KS 66045, U.S.A.

‖ Abbreviations: OP, organophosphate; NAD, nicotinamide adenine dinucleotide; DZN, diazinon; DCP, dicrotophos; 2-PAM, 2-pyridinealdoxime methochloride; AChE, acetylcholine esterase; KFase, kynurenine formamidase; and AC, alkylcarbamate.

brain was almost totally suppressed as early as 2 days post-injection [10]. Based on this schedule, the cholinergic teratogenic responses (type II) might be expected to appear before the NAD-linked responses (type I).

A rather large dose of nicotinamide (8 μmoles/egg) elevated the tissue NAD levels and, paradoxically, the increase was augmented by the simultaneous administration of a teratogenic insecticide. Unexpectedly, 2-PAM, an antagonist of type II responses, prevented the insecticide-induced decrease in tissue NAD.

MATERIALS AND METHODS

Handling of eggs and insecticides

SPF Cofal negative eggs (Larson Lab-Vac Eggs, Inc., Gowrie, IA) from white leghorn hens were used in these studies. The eggs were candled and injected on day 3 of incubation according to the protocol of Wyttenbach *et al.* [14] and Kitos *et al.* [15]. A 50-μl volume of corn oil containing the desired amount of diazinon or dicrotophos was injected directly under the embryo. In the experiments involving the administration of NAM and/or 2-PAM, 50 μl of a sterile chick Ringer's solution of these agents was injected into the yolk a few minutes before the corn oil insecticide solution.

The insecticides diazinon (DZN; Spectracide; *O*, *O*-diethyl-*O*-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothionate) and dicrotophos (DCP; Bidrin; 3-(dimethoxyphosphinyloxy)-*N*,*N*-dimethylisocrotonamide) were weighed on an analytical balance and dissolved in corn oil at the desired concentrations. For each study these solutions were made freshly immediately before injection.

Tissue extraction and NAD analysis

General principle. NAD analyses of the embryos were performed by a modification of the enzymatic cycling procedure of Serif and Butcher [16] and of O'Dorisio and Barker [17]. It involves incubating an extract of the tissues, prepared so as to preserve the NAD and NADH, with ethyl alcohol, alcohol dehydrogenase, [1-^{14}C]α-ketoglutarate, NH_4^+ and glutamate dehydrogenase. In this system, the rate of oxidation of the ethyl alcohol is a function of the concentration of the NAD provided by the embryo extract. The NADH thus produced is used to convert the [1-^{14}C]α-ketoglutarate and NH_4^+ to [1-^{14}C]L-glutamate plus NAD through the action of the glutamate dehydrogenase. The regenerated NAD can be used again in the alcohol dehydrogenase reaction, and the significant accumulating product is [1-^{14}C]L-glutamate. The cycling is terminated by chilling, ninhydrin is introduced into the reaction medium, and the vessel is tightly sealed with a CO_2 trap inside. The vessel is then incubated at 40° for 1 hr to decarboxylate the [1-^{14}C]L-glutamate and the released $^{14}CO_2$ is collected in the CO_2 trap. The amount of radioactivity collected is proportional to the concentration of NAD plus NADH in the system.

In our modification, the ninhydrin step replaced the glutamate decarboxylase step used by Serif and Butcher [16] and O'Dorisio and Barker [17]. For this purpose it was just as effective as the enzyme-catalyzed decarboxylation (data not included) and was very much less expensive, a significant factor when large numbers of determinations must be made.

Tissue extraction. All the glassware used in preparing the embryo extracts and analyzing for NAD was cleaned with chromic acid and exhaustively rinsed with distilled water. Each embryo was removed from its egg and immediately chilled in ice-cold chick Ringer's solution (6.5 g NaCl, 0.14 g KCl, 0.16 g $CaCl_2 \cdot 2H_2O$, and 0.2 g $NaHCO_3$ per liter). All adhering extraembryonic tissues were surgically removed and the embryo was damp-dried on glassine paper and weighed. It was then transferred to a chilled Potter homogenizer tube containing 1 ml of freshly prepared alkaline cysteine solution (0.024 N NaOH; 0.29 mM L-cysteine). The embryo was homogenized with ten strokes of the Teflon pestle and the volume was made to 5 ml with cold alkaline cysteine. If further dilutions were necessary, they were made with ice-cold alkaline cysteine. As an indication of the proportions needed, the homogenate of a 150-mg embryo was made to 5 ml with alkaline cysteine and for it no further dilution was necessary; 1 ml of this homogenate was used for the NAD analysis, 1 ml for a blank, and 0.1 ml for protein determination by the method of Lowry *et al.* [18]. At all times the temperature of the homogenate was maintained between 0 and 2° to minimize the destruction of NAD which is unstable at high pH.

To determine the efficiency of recovery of NAD by this extraction procedure, three studies were carried out. In the first, known amounts of NAD were mixed with the chilled alkaline cysteine and the resulting solutions were processed as though they were embryo extracts. In the second study, 5-day-old embryos were homogenized in chilled alkaline cysteine and the homogenate was divided into two portions. To one portion was added a known amount of NAD. Both portions were processed as usual. The NAD content of each was then determined and the recovery of the exogenous NAD was calculated. It was found to range from 82 to 90 per cent. In the third study, 0.2 μCi of [^{3}H]NAD (adenine[2,8-^{3}H], 3.39 Ci/mmole, new England Nuclear Corp., Boston, MA) was added to chilled chick embryo homogenates in alkaline cysteine. The extraction was carried out in the usual way and portions of the extracts were subjected to ascending chromatography on Whatman No. 1 paper, using butyric acid–58% NH_4OH–0.1 M EDTA–H_2O (66:1:1:32). The chromatograms were dried and cut laterally into strips, 1 cm in width, and the radioactivity in each was determined by scintillation spectrometry. The radioactivity profiles of the alkaline cysteine-extracted and unextracted [^{3}H]NAD samples were virtually identical, exhibiting a single peak with an R_f between 0.48 and 0.52. This R_f value corresponds to that of authentic non-radioactive NAD, detected on paper by u.v. absorption. Thus it appears that, in the extraction procedure used here, there was very little loss of NAD.

Details of cycling assay. The 1-ml blank sample was heated in a water bath at 60° for 10 min to destroy the NAD. Then 0.3 ml of 0.44 N HCl containing 0.24 M ascorbic acid was added to the sample

and it was heated again to 60° for 30 min to destroy the NADH. The sample was then chilled on ice and 2.25 ml of 0.022 M imidazole·HCl buffer, pH 6.5, was added. The solution was titrated with 0.1 N NaOH to pH 6.5, made to 5 ml with H_2O, and held at −80° prior to NAD analysis.

To the other 1-ml sample of homogenate was added 2.25 ml of 0.022 M imidazole·HCl buffer, pH 6.5. The resulting pH was approximately 7.5. In some instances, because of viscosity problems, the amount of DNA in the sample made the titration difficult. Therefore, 0.05 ml of a solution of deoxyribonuclease I (Type III, Sigma Chemical Co., St. Louis, MO, 1 mg/ml in 10 mM Tris·HCl, 1 mM $MgCl_2$, pH 7.4) was added to the ice-cold mixture a few minutes before titrating it to pH 6.5 with 0.11 N HCl. After the titration the volume of the sample was adjusted to 5 ml with H_2O and the sample was held at −80° prior to analysis.

In the NAD cycling assay, 0.25 ml of each embryo extract, prepared as described, was incubated with shaking in a 25-ml Erlenmeyer flask for exactly 1 hr at 30° with 0.75 ml of reaction mixture of the following composition: 0.2 M Na_2HPO_4, 0.4 M NH_4Cl (pH 7.8), 0.25 ml; 0.8 mg/ml crystalline bovine serum albumin; 4 mM ADP, 0.25 ml; 95% ethyl alcohol, 0.018 ml; [1-^{14}C]α-ketoglutarate (New England Nuclear Corp., 11 mCi/mmole), 2×10^6 dpm/ml in 0.1 M NH_4OH, 0.01 ml; crystalline yeast alcohol dehydrogenase (Sigma Chemical Co., 283 units/mg protein), 12 units, 0.02 ml; crystalline glutamic dehydrogenase (Boehringer-Mannheim, 2400 units/ml), 1.26 μl; and H_2O, 0.2 ml. The reaction vessels were chilled on ice and 3 ml of a freshly prepared ninhydrin solution, made as follows, was added to each: per test, 60 mg of ninhydrin was dissolved with stirring in 0.75 ml of secondary propanol; 2.4 mg of $SnCl_2$ and 3.31 mg of α-ketoglutaric acid (Sigma Chemical Co.) were dissolved in 2.25 ml of 0.2 M sodium citrate, pH 5. The $SnCl_2$–α-ketoglutarate solution was then added to the ninhydrin solution with rapid stirring, and 3 ml of the mixture was added to the chilled reaction vessel.

The vessel was closed immediately with a tight fitting rubber vial seal (Kontes, Vineland, NJ, item No. K-882310), into which was glued a rubber center plug with a small hole bored part way into it to accommodate the stem of the plastic center well. A center well (Kontes, item No. K-882320), from which a piece of the stem had been cut so that the well was held about 5 mm above the level of the reaction mixture, was inserted into the center plug. The center well, lined with a 1 cm^2 piece of glass fiber paper (Whatman, Clifton, NJ, item No. 1820.914), was loaded with 0.4 ml of Protosol (New England Nuclear Corp.) a few minutes before insertion into the flask. The stopper was inserted carefully into the chilled reaction vessel so as to avoid spilling the Protosol and any contact between the plastic well and the reaction mixture. The flange of the stopper was folded over the neck of the flask to provide additional assurance of an air-tight seal. These precautions, taken to guarantee the seal, are imperative to the success of the assay because when the reaction vessels are warmed to 40° the internal pressure increases greatly, resulting in a significant risk of gas leakage.

With the ninhydrin added and the stopper in place, the reaction vessel was placed in a 40° water bath for exactly 1 hr. It was then returned to the ice bath for 10 min. The stoppers were carefully removed and the plastic center wells placed in 4-ml plastic scintillation vials with 4 ml of scintillation mixture [0.4% Omnifluor (New England Nuclear Corp.) per liter of toluene]. The radioactivity was determined using channels ratio quench correction.

Analyses of standard samples of 0, 30, 60 and 120 pmoles of NAD accompanied each set of unknowns. A linear standard curve with a regression coefficient very close to 1.000 was thus generated.

Excision of the limbs from 5-, 7- and 10-day-old embryos was done on ice with fine scissors or a scalpel. Each pair of legs and wings was placed in cold alkaline cysteine and handled in the same way as the whole embryo.

Measuring the uptake of [^{14}C]*diazinon by chick embryos*

The amount of radioactivity that accumulated in the embryos due to injection of the eggs with [^{14}C]diazinon was determined as follows. Embryos were obtained at specified times after intravitelline injection with radioactive diazinon on day 3 (*O*,*O*-diethyl-*O*-[6-methyl-2-^{14}C-(1-methylethyl)-4-pyrimidinyl]phosphorothioate, sp. act. of the undiluted sample: 36.3 μCi/mg)*. They were homogenized in the usual way, 0.2-ml aliquots of the homogenates were mixed with 1.5 ml of Protosol (New England Nuclear Corp.) in 20-ml glass scintillation vials, and the mixtures were incubated at 37° for 16 hr. The solutions were then decolorized by adding 0.1 ml of 30% H_2O_2 and incubating further for 10 hr. Next 10 ml of scintillation mixture (0.4% Omnifluor in toluene) was added, and the amount of radioactivity was measured by scintillation spectrometry.

RESULTS

Effect of diazinon and dicrotophos on the wet weight, total protein and NAD content of early chick embryos

Eggs were injected at 72 hr of incubation with 50 μl of corn oil (controls) or corn oil containing 1 mg of either diazinon or dicrotophos and then returned to the incubator. Periodically thereafter embryos were removed from eggs of each set, chilled, weighed, homogenized, and analyzed for protein and NAD (i.e. NAD + NADH) content. The results of these analyses are rendered in Fig. 1.

Even with the large dose of insecticide used here, the treated embryos increased in weight at virtually the same rate as the controls (Fig. 1A). A semi-log plot of the time-dependent increase in weight produced a biphasic curve that reflects a mass doubling time of approximately 16 hr during the first 8 days (approximately the first third of the *in ovo* period) and 55 hr from day 8 to 12. The protein content of the embryos increased likewise in a biphasic manner, although in the insecticide-treated eggs the values were generally slightly lower than those of the con-

* The [^{14}C]diazinon was provided by Dr. H. M. LeBaron, Ciba-Geigy Corp. Greensboro, NC.

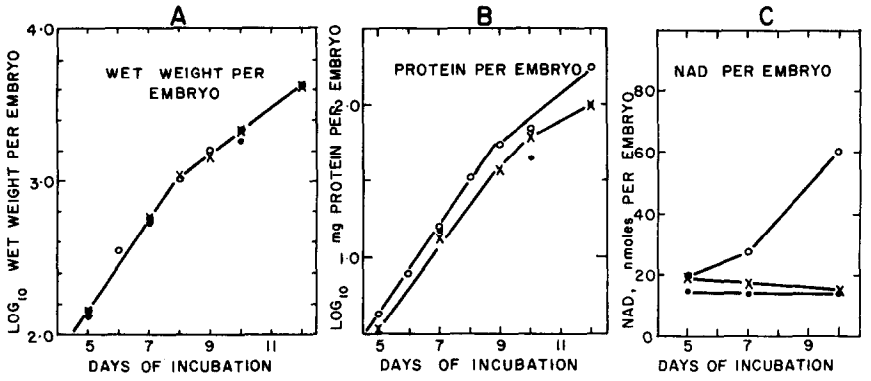

Fig. 1. Effects of organophosphate insecticides on the wet weight, protein, and NAD contents of early chick embryos. Fifty microliters of corn oil or a corn oil solution of diazinon or dicrotophos (20 mg/ml) was injected into the yolks of 72-hr chicken eggs. At each of several times thereafter the embryos were removed, weighed, homogenized, and analyzed for total protein and NAD. The values for the corn oil controls (○), diazinon-treated (×), and dicrotophos-treated (●) embryos are plotted on the ordinate as a function of the time of incubation.

trols (Fig. 1B). Similarly, the NAD content of the control embryos increased almost exponentially from 20 nmoles on day 5 to 61 nmoles on day 10, and there was little if any difference in the NAD content of the treated and control embryos on day 5. After day 5, however, the NAD content of the insecticide-treated embryos did not increase at all (Fig. 1C). Clearly, the effects of the insecticides on two of the principal aspects of early embryonic growth are very different from the effects on the NAD content of the embryo.

How much diazinon enters the embryo?

When the concentration of insecticide was increased several-fold, there was still no observable effect on the NAD content by day 5 (Table 1). The apparent lack of effect of even higher doses of organophosphate insecticides on the NAD content of the embryo after 2 days of exposure could be due to any of several possibilities. One is that the early stages of the embryos are less sensitive to the insecticide than later. Another is that there was a rate-limiting transfer of the agent from the oil droplet in the yolk to the site of its inhibitory action, the embryo or the yolk sac membrane. To measure the rate of insecticide transfer from the corn oil to the embryo we injected 1 mg of diazinon, as usual, but in this instance the injectant contained 0.2 μCi of

Table 1. Effects of diazinon concentration on wet weight and protein and NAD contents of 5-day chick embryos*

N	Diazinon conc. (mg/egg)	Embryo wet wt (mg)	Protein content of embryo		NAD content of embryo	
			(mg)	(% wet wt)	(nmoles/embryo)	(nmoles/mg protein)
10	0	179 ± 7	4.3 ± 0.2	2.43 ± 0.05	10.08 ± 0.88	2.34 ± 0.20
9	1.0	168 ± 11	3.9 ± 0.2	2.37 ± 0.08	9.12 ± 1.17	2.35 ± 0.31
9	2.5	156 ± 7	3.8 ± 0.1	2.47 ± 0.06	8.86 ± 0.58	2.33 ± 0.17
10	5.0	164 ± 4	4.0 ± 0.2	2.42 ± 0.10	10.09 ± 0.53	2.58 ± 0.17

* The specified amount of diazinon was injected into the yolk in 50 μl of corn oil at 72 hr of incubation. Values are means ± S.D.

Table 2. Time course of [^{14}C]diazinon incorporation into young chick embryos*

N	Day of analysis	Embryo wet wt (mg)	Incorporated radioactivity			Specific activity of tissue (dpm/mg wet wt)
			(dpm/embryo)	(% of administered radioactivity)	Diazinon equivalent (μg)	
5	5	197 ± 9	268	0.20	2.0	1.36 ± 0.06
4	7	657 ± 20	953	0.71	7.1	1.45 ± 0.11
4	8	1132 ± 40	1098	0.82	8.2	0.97 ± 0.09
5	10	2237 ± 37	3028	2.26	22.6	1.33 ± 0.07

* Eggs were injected at 72 hr of incubation with 50 μl of corn oil containing 1 mg of diazinon (sp. act. 0.2 μCi/mg). At each of the indicated days of analysis embryos were removed, washed and homogenized, and representative portions of the homogenates were digested with Protosol. The amount of radioactivity in each embryo was determined by scintillation spectrometry. Values are means ± S.D.

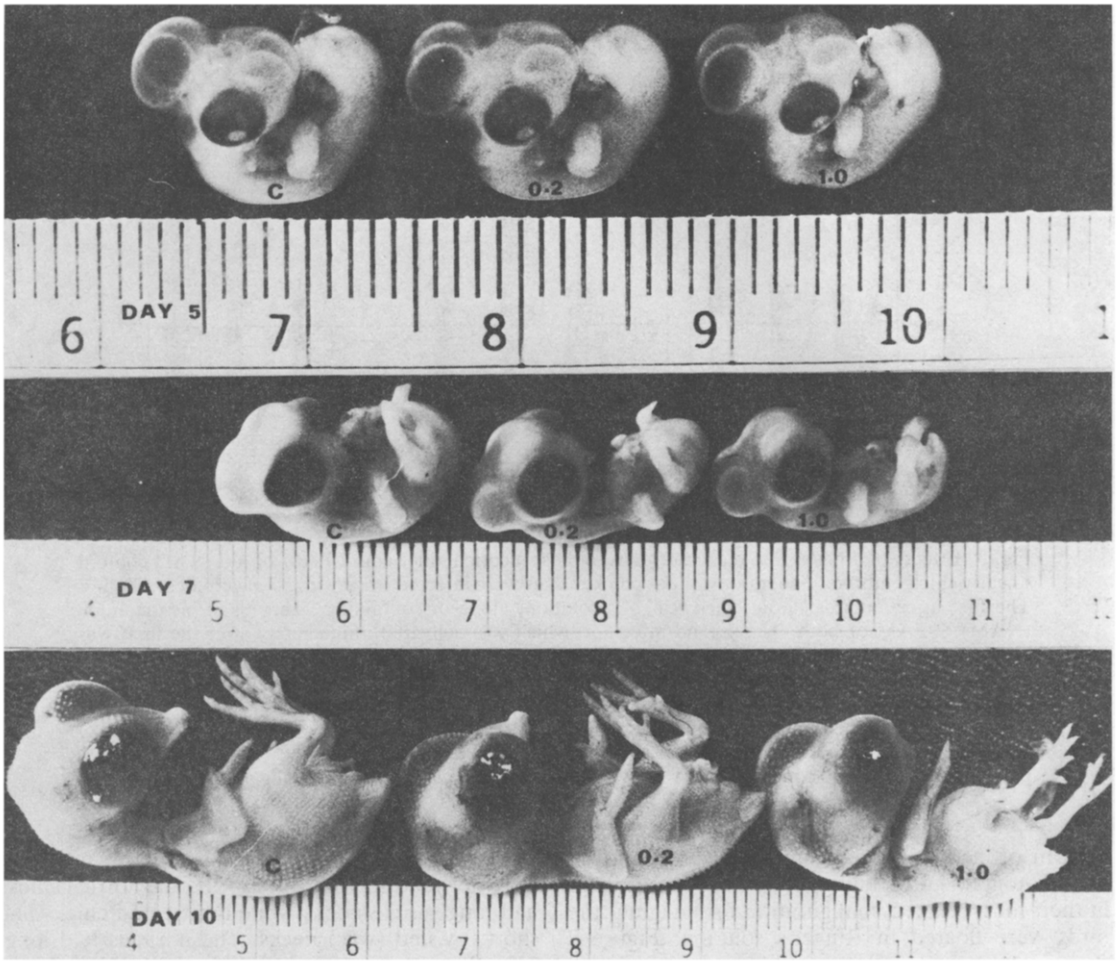

Fig. 2. Diazinon-induced teratogenic signs in early chick embryos. Chicken eggs at day 3 of incubation were injected intravitelline with 50 μl of corn oil or corn oil containing either 0.2 or 1.0 mg of diazinon or dicrotophos. The eggs were incubated further to day 5, 7 or 10 and the embryos were carefully removed, floated right side up in chick Ringer's solution, and photographed. Since the results from the diazinon and dicrotophos sets were virtually identical, only the diazinon set is shown here. A millimeter ruler underlies the pictures. Upper row, day 5; middle row, day 7; and bottom row, day 10. From left to right: control; 0.2 mg diazinon/egg; and 1.0 mg diazinon/egg.

[^{14}C]diazinon. At times thereafter, embryos were isolated and homogenized. Representative portions of the homogenates were digested with Protosol, and the radioactivity in them was measured.

The fraction of the administered radioactivity found in the embryos was small, reaching only 2.26 per cent by day 10 (Table 2). This represented 22.6 μg of diazinon, assuming that during the period of the study there was no degradation of the insecticide. Since degradation almost certainly did occur, these values must be considered maximal. On day 5, when the NAD response was negligible, only 0.2 per cent of the radioactivity, equivalent to 2 μg of diazinon, was detected in the embryo. Even though the amount of radioactivity in the embryo increased with time, the specific activity (dpm/mg wet wt) remained approximately constant over the 5-day course of the study. Thus, the amount of insecticide and its breakdown products in the embryo increased with the length of the incubation period but the concentration of radioactive products (radioactive material/unit wet wt) did not change between days 5 and 10. The diazinon must move from the corn oil into the embryo at a rate that reflects the tissue mass. A more detailed pharmacokinetic study of this system was not undertaken, so a reliable estimate of the actual insecticide content of the embryos at any time cannot be made. However, even though the amount of diazinon that entered the embryo was extremely small, it was sufficient under these test conditions to inhibit totally the increase in NAD per embryo (Fig. 1C) and the acetylcholine esterase of the limbs and brain even during the very early times (days 6–8) [10]. It also inhibited the increase in protein per embryo, but to a lesser extent (Fig. 1B).

How early do the teratogenic signs appear?

The physical abnormalities of insecticide-treated embryos were readily seen and evaluated just prior to hatching. However, if the insecticide was administered on day 3, at least one gross structural change was detectable as early as day 5. In this study, 50 μl of corn oil containing 0, 0.2 or 1.0 mg of diazinon or dicrotophos was injected into the yolks of eggs

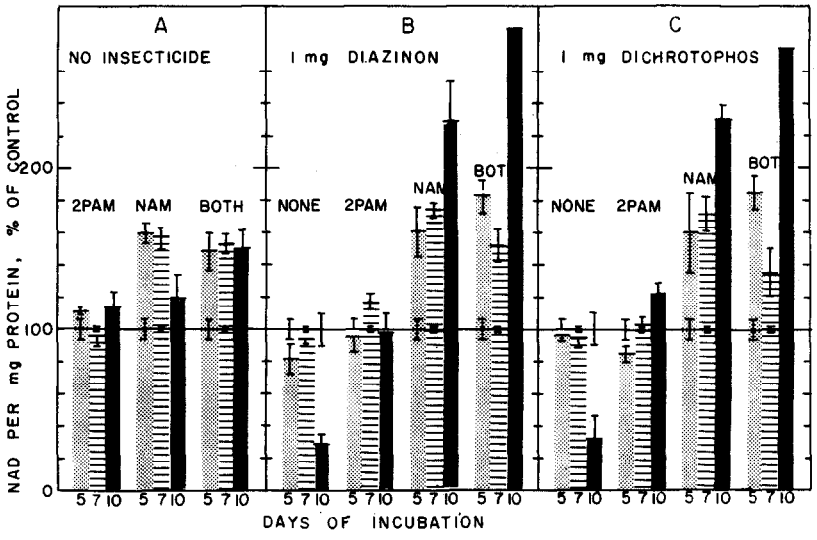

Fig. 3. Effects of 1 mg/egg of diazinon, dicrotophos, nicotinamide, and 2-PAM on the NAD content of early chick embryos. Seventy-two-hour chicken eggs were injected twice with 50-μl volumes of liquid. The first injectant was sterile chick Ringer's solution, alone or containing 20 mg/ml of nicotinamide (NAM), 2-PAM or both. The second injectant, which was delivered immediately after the first, was corn oil, alone or containing 20 mg/ml of diazinon or dicrotophos. The eggs were incubated further for another 2, 4 or 7 days, at which times representatives (five to ten per group) were taken, the embryos were removed, weighed and homogenized, and the protein and NAD contents were determined. The mean value for the NAD per mg embryonic protein is reported here as the percentage of the control (which received only Ringer's solution and corn oil), the error bars are ± the S.D. of the mean in percentage terms. The control values were: at 5 days, 1.98 ± 0.11 nmoles NAD/mg embryonic protein; at 7 days, 1.55 ± 0.02 nmoles; and at 10 days, 1.02 ± 0.10 nmoles.

at 72 hr of incubation. The embryos were removed and photographed on days 5, 7 and 10. To put them in their most relaxed configuration for imaging, the birds were floated in Ringer's solution. Figure 2 shows the control and insecticide-treated embryos at each of the three indicated times. The hind limbs of the diazinon-treated 10-day-old embryos were less well developed than those of the controls, and the retardation was greater in those treated with 1 mg rather than 0.2 mg of the agent. Feathering problems were not yet discernible at day 10. The normal chicks had long supple necks while the treated chicks had short, twisted (wry) necks. The normals had long, smoothly contoured backs and well-developed rumps; the treated chicks had less well extended backs and poorly developed rumps. The neck, back and rump differences were also visible at day 7. At day 5 the only hint of morphological difference was

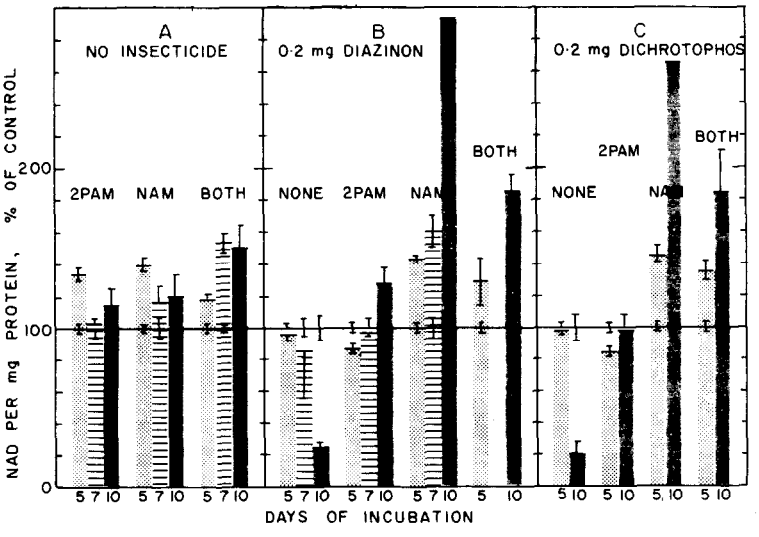

Fig. 4. Effects of 0.2 mg/egg diazinon or dicrotophos on the NAD content of early chick embryos. The experimental protocol is the same as that for Fig. 3 except that 0.2 mg of insecticide per egg was administered rather than 1 mg per egg. The control (100 per cent) values were: at 5 days, 2.42 ± 0.07 nmoles/mg protein; at 7 days, 1.76 ± 0.10 nmoles; and at 10 days, 1.02 ± 0.01 nmoles.

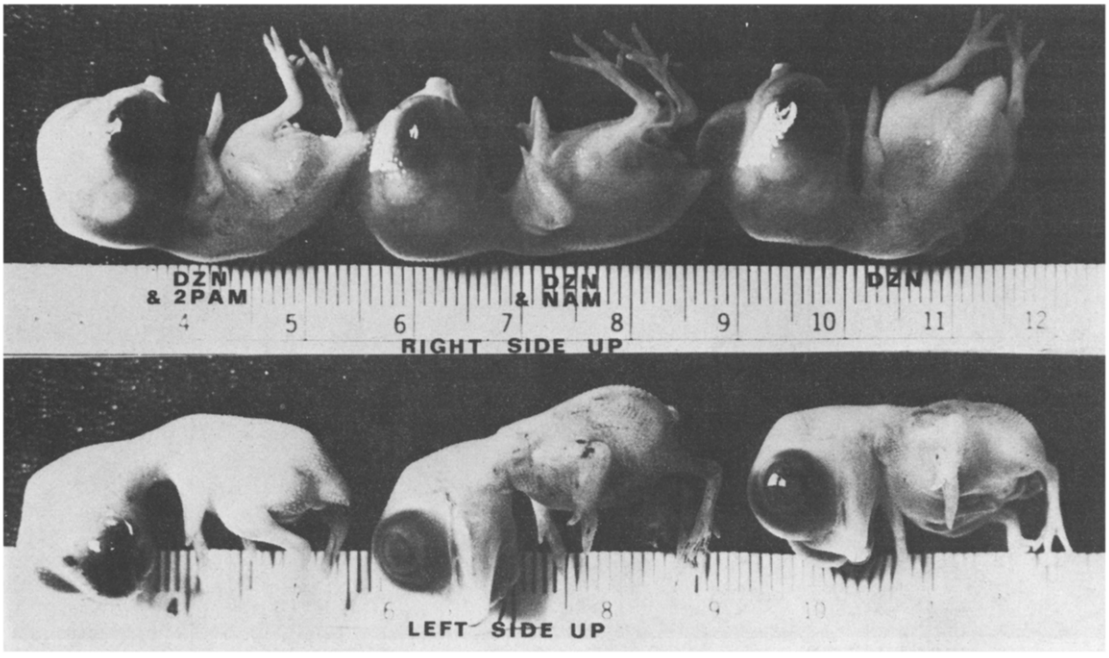

Fig. 5. Effects of nicotinamide and 2-PAM on insecticide-induced morphological changes in early chick embryos. Chicken eggs at day 3 of incubation were injected intravitelline with 1 mg per egg of nicotinamide or 2-PAM in 50 μl of chick Ringer's solution and then with 50 μl of corn oil or corn oil containing 0.2 mg of diazinon or dicrotophos. The eggs were incubated further until day 10 and then the embryos were removed, floated right side up and left side up in Ringer's solution, and photographed. Upper row, right side up; lower row, left side up. From right to left: diazinon-treated alone; diazinon plus nicotinamide-treated; and diazinon plus 2-PAM-treated. Since the results from the diazinon and dicrotophos-treated eggs were virtually identical, only the diazinon set is shown here.

a slightly less well-developed tail region in the insecticide-treated embryos. Frequently at days 7 and 10 the viscera of the treated birds were not within the abdominal wall, and at day 10 the abdominal region often appeared bloated or edematous. The diagnostic features shown in Plate 1 for diazinon-treated eggs were virtually the same as those for their dicrotophos-treated counterparts, so pictures of the latter group are not shown.

Time-course study of the effects of the organophosphate insecticides and their antagonists on the NAD content of early chick embryos

The effects of diazinon, dicrotophos, nicotinamide, and 2-pyridinealdoxime methochloride on the NAD content of chick embryos at days 5, 7 and 10 were determined. The agents, individually or in specific combinations, were injected into the yolks of eggs at 72 hr of development. The antagonists, NAM and 2-PAM (1 mg/egg), were injected first in 50 μl of Ringer's solution, and the insecticides (1 mg/egg) were injected immediately thereafter in 50 μl of corn oil. The results, reported as the NAD content per mg of embryonic protein, are presented in Fig. 3. The values are expressed as a percentage of the control for that particular experimental period. The controls were injected with 50 μl of both Ringer's solution and corn oil.

In the absence of insecticide, 2-PAM had virtually no effect on the NAD content of the embryos at days 5, 7 and 10 (Fig. 3A). Nicotinamide, on the other hand, caused a 10–30 per cent increase in the NAD content of the embryos. A combination of these two agents produced an increase differing little from that produced by nicotinamide alone. Up to day 10 the whole body wet weights and the protein contents, the latter calculated as percentage of wet weight, were nearly the same as those of the controls (data not shown).

At the early times, days 5 and 7, the effects of diazinon (Fig. 3B) and dicrotophos (Fig. 3C) on the NAD content of the embryos were not great, but by day 10 the NAD content was reduced to 20–30 per cent of the control values. The simultaneous presence of 2-PAM antagonized this response, restoring the NAD content to the control level. Exogenous nicotinamide produced an increase in the NAD content of the insecticide-treated embryos, not unlike that of the controls on days 5 and 7, but substantially greater on day 10 (Fig. 3). Together, nicotinamide and 2-PAM exerted an effect that was similar to that of nicotinamide alone, elevated somewhat on days 5 and 7, and greatly on day 10. If the NAD contents were expressed per embryo, rather than per mg protein as given in Fig. 3, the pattern of the results would be the same (data not shown).

At 0.2 mg per egg, diazinon and dicrotophos elicited NAD responses that were quantitatively the same as those caused by 1 mg of the agents (Fig. 4). Without either of the reversing agents they caused large decreases in the NAD content of the embryos by day 10. As before, 2-PAM restored the NAD level to normal, whereas, nicotinamide or nicotinamide plus 2-PAM caused unusually large accumulations of NAD in these tissues by day 10.

As shown on Fig. 5, nicotinamide or 2-PAM,

Table 3. Effects of diazinon, dicrotophos, nicotinamide and 2-PAM on the NAD content of the limbs of chick embryos

Variables				(nmoles NAD*/mg limb protein)					
				Wings			Legs		
NAM (1 mg)	2-PAM (1 mg)	DZN (0.2 mg)	DCP (0.2 mg)	Day 5	Day 7	Day 10	Day 5	Day 7	Day 10
–	–	–	–	1.85 ± 0.13 (100)	2.21 ± 0.11 (100)	1.90 ± 0.04 (100)	1.80 ± 0.14 (100)	2.00 ± 0.20 (100)	1.44 ± 0.08 (100)
+	–	–	–	NT†	3.60 ± 0.04 (162)	2.54 ± 0.10 (134)	NT	3.42 ± 0.06 (171)	1.92 ± 0.10 (133)
–	+	–	–	NT	2.20 ± 0.11 (104)	1.60 ± 0.27 (84)	NT	2.27 ± 0.10 (114)	1.23 ± 0.04 (86)
+	+	–	–	NT	2.97 ± 0.21 (134)	2.07 ± 0.09 (109)	NT	3.25 ± 0.12 (163)	1.71 ± 0.09 (119)
–	–	+	–	1.74 ± 0.14 (94)	1.57 ± 0.32 (71)	0.64 ± 0.11 (34)	1.75 ± 0.12 (97)	1.58 ± 0.35 (79)	0.55 ± 0.08 (38)
+	–	+	–	NT	3.75 ± 0.15 (169)	3.10 ± 0.27 (163)	NT	3.81 ± 0.14 (190)	2.36 ± 0.09 (164)
–	+	+	–	NT	1.95 ± 0.09 (88)	1.72 ± 0.07 (91)	NT	1.97 ± 0.09 (98)	1.35 ± 0.06 (94)
+	+	+	–	NT	3.13 ± 0.18 (142)	2.55 ± 0.06 (134)	NT	3.33 ± 0.28 (167)	1.90 ± 0.05 (132)
–	–	–	+	1.77 ± 0.18 (96)	1.54 ± 0.27 (70)	0.40 ± 0.05 (21)	1.77 ± 0.14 (98)	1.56 ± 0.29 (78)	0.33 ± 0.07 (23)
+	–	–	+	NT	3.39 ± 0.30 (153)	2.80 ± 0.18 (147)	NT	3.19 ± 0.14 (159)	1.82 ± 0.04 (127)
–	+	–	+	NT	2.06 ± 0.10 (93)	1.33 ± 0.16 (70)	NT	2.03 ± 0.14 (102)	1.02 ± 0.09 (71)
+	+	–	+	NT	3.28 ± 0.20 (148)	2.59 ± 0.10 (136)	NT	3.07 ± 0.20 (154)	1.91 ± 0.09 (133)

* Values are means ± S.D.; N ⩾5. Numbers in parentheses are mean NAD values expressed as a percentage of the appropriate control value (first line of the column).

† Not tested.

injected on day 3 along with diazinon, prevented at least some of the insecticide-induced morphological damage that was otherwise seen on day 10. In this study, eggs were injected at 72 hr of incubation with either diazinon or dicrotophos (0.2 mg/egg) and with 1 mg of either of the reversing agents; the embryos were removed on day 10 and photographed in each of two positions, left and right sides uppermost. In all cases the responses to the two OP insecticides were sufficiently alike so that only one set, the diazinon-treated, is shown here. The short and wry neck syndrome was not reversed by NAM and was somewhat, but not completely, reversed by 2-PAM. The rumplessness was much less exagerated in the 2-PAM-treated embryos but was still quite evident in the NAM-treated birds. Underdevelopment of the hind limbs due to the insecticides seemed less pronounced in both the NAM and 2-PAM treatments. Clearly, some of the gross morphological manifestations of both types I and II teratogenesis were reversed by these antagonists to the extent that they were evident by day 10 of development.

Effects of the insecticides and their antagonists on the NAD content of the wings and legs of early chick embryos

Since micromelia of the legs, but not of the wings, is a common type I teratogenic response of chicks to the OP insecticides [10], we sought to determine the NAD contents of these tissues at times prior to the appearance of this class of deformity. Chick embryos were treated as before with 0.2 mg of either diazinon or dicrotophos and/or the reversing agents nicotinamide and 2-PAM. The pairs of wings and legs from single embryos were analyzed for their NAD contents at days 5, 7 and 10. As indicated in Table 3, no change in the NAD content of the limb buds was evident by day 5. By day 7, however, the NAD contents of the wings and legs were only 70 and 80 per cent, respectively, of the untreated controls. By day 10 the NAD concentrations were still lower, reminiscent of the whole body effects shown in Fig. 3. Here, also, 2-PAM reversed the effects of the insecticides on the NAD content of both sets of limbs, restoring them to near control levels, and nicotinamide caused a major increase in the NAD levels to values much greater than normal.

DISCUSSION

The biochemical mechanisms by which the OP and AC insecticides interfere with the normal course of embryonic development are not yet understood. Proctor and coworkers [4, 12] have shown that large doses of the insecticides administered early in the development of the chick cause as much as an 80–90 per cent decrease in the NAD content of the embryos by days 12 to 14. A similar effect was seen with as little as 30 μg of diazinon per egg [6]. In our studies, diazinon (a phosphorothioate) and dicrotophos (a phosphotriester), injected at 0.2 or 1.0 mg per egg in corn oil at 72 hr of incubation, caused similar decreases that occurred mainly after day 7 and reached 20–30 per cent of the control values by day 10 (Figs. 3 and 4). Only a single morphological expression of treatment was detected by day 5, a few by day 7, and several by day 10 (Fig. 2).

Several lines of evidence suggest that the NAD deficit is responsible for at least some of the observed teratogenic changes. For example, exogenous nicotinamide and certain other NAD precursors and exogenous NAD itself counteract both the OP- and AC-induced decrease in tissue NAD concentrations and the appearance of type I teratisms [4, 6, 12, 13]. In general, there is a good correlation between the reduced concentrations of NAD in avian embryos and the defects in feather and leg development [4]. Proctor *et al.* [4] showed that, if the NAD content on day 12 was greater than 69 per cent of the control value, little or no teratogenic change was detected, but if it was less than 45 per cent of the control the teratogenic signs were mild to severe [4]. Similarly, two toxic nicotinamide analogs, 6-aminonicotinamide (6-AN) and 3-acetylpyridine (3-AP), substances that interfere with NAD synthesis and function, cause the NAD content of avian embyros to decrease and produce teratogenic changes analogous to those brought on by the insecticides [19, 20]. The effects of these two agents are likewise manifest on the NAD complement of chondrogenic chick limb mesodermal cells in culture [21, 22]. Moscioni *et al.* [6] and Seifert and Casida [8, 9] provide evidence which indicates that the OP and AC insecticides act on chick embryos, at least in part, by inhibiting the conversion of tryptophan to NAD at the KFase step. Thus, intermediates in the pathway from tryptophan to nicotinic acid prior to the KFase reaction, including tryptophan itself, should have little or no ability to overcome the impairment of NAD synthesis, whereas those following the KFase step, and substances that can be mobilized into this metabolic sequence at points after the inhibition, should be effective in reversing the NAD deficiency. These responses have, in fact, been realized [6, 8, 9]. Proctor *et al.* [4] and Moscioni *et al.* [6] have shown that 0.8 μmole of nicotinamide per egg does reverse the suppressive effects of the OP and AC insecticides on the NAD concentration of chick embryos, restoring it to its normal level.

Our experience is consistent, in part, with the above findings which implicate NAD in the teratogenic process. Diazinon and dicrotophos severly affect the normal development of chick embryos (Fig. 2) and cause the NAD content to decrease to less than one-third of the normal level (Figs. 3 and 4). Nicotinamide alone, at 8 μmoles per egg (ten times that used in the above mentioned studies [4, 6]), produces up to a 30 per cent increase in the NAD of control embryos. Not only does it prevent the NAD content of insecticide-treated embryos from decreasing, but by day 10 it actually causes greater accumulation of this coenzyme than with nicotinamide alone. As seen here, nicotinamide also lessens the expression of micromelia (Fig. 5).

It is interesting to note that these bone development problems are seen in the legs but not the wings [10]. If insufficient NAD is indeed responsible for the problems of micromelia, perhaps the insecticides disproportionately affect the NAD level of the legs. To test this hypothesis the NAD contents of the wings and legs were examined as a function of treat-

ment with the teratogens and the potential reversing agents. The results (Table 3) show that the effects of both diazinon and dicrotophos on the wings and legs were similar and that they were very much like the effects on the whole embryo (Fig. 4). Furthermore, the NAD levels of both sets of limbs were enhanced by nicotinamide, especially at day 10 in the presence of insecticide, and were maintained at the normal level by 2-PAM. A time–course study of the effects of diazinon on the AChE activity of these same tissues shows that as early as day 6 the enzyme was strongly inhibited in both the wings and legs and that by day 13 the enzyme level had returned to normal [10]. Thus, between the wings and legs there was neither a difference in the NAD levels nor a differential cholinergic response that might serve to explain the observed inequalities of limb development occasioned by the insecticides.

Type II defects (tibiotarsal arthrogryposis, wry and short neck, rumplessness, and hypoplasia of the legs) are caused by a number of cholinomimetic compounds, including the OP and AC insecticides and a group of quaternary ammonium compounds, including succinyl choline, decamethonium, and others [7, 10, 11]. These teratogenic effects are not counteracted by nicotinamide but in many instances are reversed by cholinergic blocking agents including certain oximes, especially 2-PAM, and gallamine [10, 23]. As expected, 2-PAM (6 μmoles/egg) did not prevent the expression of micromelia by day 10 but did lessen the severity of wry neck and rumplessness that was otherwise so evident at that time (Fig. 5). Unexpectedly, and in contrast to the findings of Moscioni *et al.* [6], 2-PAM did maintain the tissue NAD levels at or near the control values (Figs. 3 and 4). If the type I teratogenic changes were actually caused by NAD deficiencies, this effect of 2-PAM should have been capable of preventing them. But 2-PAM was ineffective against type I teratisms. Thus, any explanation of the teratogenic process that is based on an NAD deficit should take this fact into consideration.

The difference between our results and those of Moscioni and his colleagues [6] with respect to the effect of 2-PAM on the NAD content of the embryos is not easily explained. One possible relevant factor is the carrier solvent used to administer the agent. Moscioni *et al.* [6] and Proctor *et al.* [4] used methoxytriglycol, whereas we used Ringer's solution for the reversing agents and corn oil for the insecticides. In our hands methoxytriglycol was slightly toxic to the embryos. Another possible factor is agent delivery to the embryos. We injected using a devise that delivers the liquid sample directly underneath the embryo [14, 15] rather than to the center of the yolk.

The NAD content of 12-day embryos was reported by Proctor *et al.* [4] and Moscioni *et al.* [6] to be 185 nmoles per gram wet weight of embryo. Taking into consideration the small losses (12–18 per cent) experienced in the extraction procedure we found only 29 nmoles of NAD per g at day 10. We used a highly sensitive radiometric enzymatic NAD cycling assay which, as used here, measured the sum of the NAD plus NADH of representative portions of extracts of individual embryos, and even of the wings or legs of 5-day embryos. The results were very reproducible. Moscioni *et al.* [6] measured the formation of NADH at 340 nm using ethyl alcohol and alcohol dehydrogenase, a procedure that measures the amount of NAD but not the NADH in the sample. In some instances we measured the NADH content of the sample after selective alkaline destruction of its oxidized counterpart. Generally the NADH:NAD ratio is very small (less than 0.05). Ten-day embryos from eggs injected on day 3 with 1 mg of nicotinamide had a ratio of 0.06 and those also given 0.2 mg of diazinon had a ratio of 0.18. Thus, the insecticide did cause a significant accumulation of the reduced form of NAD, but the amount was not great enough to account for the excess of NAD that we found.

The concentrations of NAD seen in the chick embryos in this study may be rationalized in terms of what is already known about the *de novo* synthesis of this coenzyme and the effects of the cholinergic insecticides on embryonic development. The conversion of tryptophan to NAD is normally a well-regulated metabolic process, subject to feedback regulation so that over-accumulation of the end product acts to decrease the activity of tryptophan oxygenase, the first enzyme in the pathway [24, 25]. Exogenous nicotinamide may also be utilized to produce NAD, but such a process would not be under the regulatory umbrella of the *de novo* pathway. Our results show that in the presence of exogenous nicotinamide the embryos did produce a measurable excess of NAD. How might this explanation accommodate the large over-production of NAD seen on day 10 when either diazinon or dicrotophos was also present (Figs. 3 and 4)?

NAD is metabolically dynamic. It turns over in the cell at a significant rate, being synthesized from tryptophan via nicotinic acid or nicotinamide and used to form NADH, NADP and poly(ADP-ribose). The latter process is a major route of NAD utilization and in eukaryotic cells it occurs principally in the nucleus [26]. As already indicated, the OP insecticides block the synthesis of nicotinic acid from tryptophan at the KFase reaction. If the insecticides were to interfere with poly(ADP-ribose) synthesis, as has been proposed by Caplan *et al.* [27, 28], the NAD level should be maintained more readily. If nicotinamide were also present, NAD synthesis would be expected to be rapid and its utilization slow. Consequently, as seen in our studies, the intracellular NAD pool would expand.

How might our observations of the effect of 2-PAM on the NAD level of the insecticide-treated embryos be explained? This analog of hydroxylamine may act on KFase, as it does on AChE that has been inactivated by an OP insecticide, by eliminating the insecticide from its site of attachment to the enzyme, thereby restoring the *de novo* pathway of nicotinic acid synthesis and the implicit feedback relationships. Insecticide inhibition of the utilization of NAD may or may not also be reversed by 2-PAM. In either event, an over-accumulation of NAD would be unlikely because of the surveillance that the feedback regulation imposes on NAD synthesis.

These observations and interpretations do not explain how the insecticide-induced variations in

NAD concentration of the embryos are related to the type I morphological anomalies. Rather, they indicate that the cause and effect relationships between the NAD content and the structural deformities are complex. We are continuing to explore the problems that the OP and AC insecticides pose for the normal processes of NAD synthesis and utilization in embryos, particularly as they relate to the processes of teratogenesis.

Acknowledgements—The authors would like to thank Dennis Byrne, Bradley Keller, Sem Kushaba-Rugaaju, Cathy Mih, David Prentice and William Strinden for their excellent technical assistance in this project. The authors would also like to thank Dr. H. M. LeBaron, Ciba-Geigy Corp., Greensboro, NC, for contributing the [^{14}C]diazinon used in this study.

REFERENCES

1. W. Landauer, *J. cell. comp. Physiol* **43**, 261 (1954).
2. J. McLaughlin, Jr., J-P. Marliac, M. J. Verrett, M. K. Mutchler and O. G. Fitzhugh, *Toxic. appl. Pharmac.* **5**, 760 (1963).
3. J-C. Roger, D. G. Upshall and J. E. Casida, *Biochem. Pharmac.* **18**, 373 (1969).
4. N. H. Proctor, A. D. Moscioni and J. E. Casida, *Biochem. Pharmac.* **25**, 757 (1976).
5. M. Eto, J. Seifert, J. L. Engel and J. E. Casida, *Toxic. appl. Pharmac.* **54**, 20 (1980).
6. A. D. Moscioni, J. L. Engel and J. E. Casida, *Biochem. Pharmac.* **26**, 2251 (1977).
7. R. Meiniel, *Experientia* **32**, 920 (1976).
8. J. Seifert and J. E. Casida, *Biochem. Pharmac.* **27**, 2611 (1978).
9. J. Seifert and J. E. Casida, *Pestic. Biochem. Physiol.* **12**, 273 (1979).
10. M. Misawa, J. Doull, P. A. Kitos and E. M. Uyeki, *Toxic. appl. Pharmac.*, **57**, 20 (1981).
11. W. Landauer, *Teratology* **12**, 125 (1975).
12. N. H. Proctor and J. E. Casida, *Science* **190**, 580 (1975).
13. J-C. Roger, H. Chambers and J. E. Casida, *Science* **144**, 539 (1964).
14. C. R. Wyttenbach, S. C. Thompson, J. C. Garrison and P. A. Kitos, *Toxic. appl. Pharmac.*, in press.
15. P. A. Kitos, C. R. Wyttenbach, K. Olson and E. M. Uyeki, *Toxic. appl. Pharmac.*, in press.
16. G. S. Serif and F. R. Butcher, *Analyt. Biochem.* **15**, 278 (1966).
17. M. S. O'Dorisio and K. L. Barker, *Endocrinology* **86**, 1118 (1970).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. W. Landauer and E. M. Clark, *J. exp. Zool.* **151**, 253 (1962).
20. I. H. Krakoff, C. R. Lacon and D. A. Karnofsky, *Nature, Lond.* **184**, 1805 (1959).
21. A. I. Caplan, *J. exp. Zool.* **180**, 351 (1972).
22. A. I. Caplan, *Expl. Cell Res.* **70**, 185 (1972).
23. W. Landauer, *Teratology* **15**, 33 (1977).
24. C. Wagner, *Biochem. biophys. Res. Commun.* **17**, 668 (1964).
25. Y. S. Cho-Chung and H. C. Pitot, *J. biol. Chem.* **242**, 1192 (1967).
26. O. Hayaishi and K. Ueda, *A. Rev. Biochem.* **46**, 95 (1977).
27. A. I. Caplan and M. J. Rosenberg, *Proc. natn. Acad. Sci. U.S.A.* **72**, 1852 (1975).
28. A. I. Caplan, C. Niedergang, H. Okazaki and P. Mandel, *Devl. Biol.* **72**, 102 (1979).