Biochemical Pharmacology, Vol. 34, No. 11, pp. 1937–1943, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

EFFECTS OF DIAZINON ON NUCLEOTIDE AND AMINO ACID CONTENTS OF CHICK EMBRYOS

TERATOGENIC CONSIDERATIONS*

SEM KUSHABA-RUGAAJU† and PAUL A. KITOS
Department of Biochemistry, The University of Kansas, Lawrence, KS 66045, U.S.A.

(*Received* 7 *July* 1984; *accepted* 2 *October* 1984)

Abstract—The effects of diazinon (DZN), an organophosphorus (OP) insecticide, and nicotinamide (Nam) on the pyridine nucleotide, purine and pyrimidine ribonucleotides, and free alpha amino acid contents of chick embryos were determined. The teratogen (DZN) and/or the anti-teratogen (Nam) were administered by the intravitelline route to chicken eggs at day 3 of incubation, and nucleotide and amino acid analyses were made on acid-soluble extracts of homogenates of the embryos at day 10. The results show that the amounts of both the oxidized and reduced forms of NAD and NADP were decreased by the insecticide and restored by Nam. The amounts of the purine and pyrimidine ribonucleotides in the embryos were also decreased by DZN but their changes were proportionately not as great as those of the pyridine nucleotides. The levels of the purine and pyrimidine ribonucleotides were also wholly or partially restored by Nam. Neither DZN nor Nam had any effect on the "energy charge" of the embryos. The levels of free tryptophan (TRP) and histidine (HIS) were decreased by DZN while the levels of threonine (THR) and aspartic acid (ASP) were increased. All other amino acid levels remained virtually unchanged in response to DZN or Nam. Based upon these findings, a possible involvement of TRP in OP insecticide-induced micromelia, parrot beak, and abnormal feathering in chick embryos is considered.

Some organophosphorus (OP‡) and alkylcarbamate (AC) insecticides are potent teratogens [1–8]. In the chick embryo they cause developmental failures of two types: type 1 includes micromelia, parrot beak and abnormal plumage and is alleviated by the co-administration of nicotinamide (Nam), nicotinic acid (Nac), or any of several other NAD precursors [9–16]; type 2 includes short and wry neck, and is alleviated by the co-administration of 2-pyridine-aldoxime methonium ion (2-PAM) [7, 8, 14].

Although the type 1 deformities have been attributed to decreases in the NAD contents of the embryonic tissues, the cause of the abnormal morphogenesis is not known [14–18]. The deformities can occur even if the NAD contents of the tissues are normal. If 2-PAM is administered along with the insecticide, the NAD content does not decrease, but micromelia and abnormal feathering do develop [15]. Rather than being a cause of the syndrome, the NAD deficit could simply be a correlate of it.

Casida and his colleagues [14, 19, 20] have attributed the decreases in NAD content of OP-treated avian embryos to an impairment of the conversion of tryptophan to NAD at the kynurenine formamidase (KFase) catalyzed reaction. But tryptophan prevents both the insecticide-induced lowering of the tissue NAD levels [16] and the type 1 teratisms [13, 16, 17, 21], suggesting that the insecticides do not severely inhibit KFase *in ovo*. Insufficient free tryptophan in the embryo, rather than the inhibition of KFase, could be responsible for the NAD deficit. Nam could act to elevate the tryptophan content of the tissues by maintaining the pool of pyridine nucleotides replete which, in turn, would act to diminish the tryptophan dioxygenase activity [22, 23]. Tryptophan dioxygenase is the regulatory "branch point" enzyme which provides access of tryptophan to the pathways for NAD synthesis and tryptophan catabolism [24]. The question remains, is an insufficiency of NAD or a variance in the availability of tryptophan or some other metabolite responsible for the type 1 deformities?

In searching for an answer to this question, we have examined the levels of the four principal pyridine nucleotides, the twelve principal free ribonucleotides, and the free α-amino acids in diazinon-treated and untreated chick embryos. The results indicate that there are several OP insecticide-induced changes in the nucleotide complement of the embryos and in the amounts of a few of the free α-amino acids, including tryptophan.

MATERIALS AND METHODS

Embryo and injection protocols. SPF Cofal-negative fertile eggs from White Leghorn hens (Larson Eggs, Inc., Gowrie, IA) were used in this study. They were incubated at 38° for 72 hr, candled to locate the embryo, and then injected according to the procedure of Kitos *et al.* [25]. Nam was dissolved in chick Ringer's solution [15] at 20 mg/ml and sterilized by filtration through a 0.45 μm pore-size membrane filter. Diazinon [*O*,*O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate, Spectracide] (DZN), 99.7% pure, was obtained from the

* Supported in part by NIH Grant ES-02046 and KU GRF Grant 3558.
† Deceased, June 1984.

U.S. Environmental Protection Agency, Research Triangle Park, NC and was dissolved in corn oil (Sigma Chemical Co., St. Louis, MO) at 4 mg/ml. The injection of 50 μl of sterile Ringer's solution (control) or Nam solution in Ringer's was followed immediately by the injection of corn oil (control) or DZN in corn oil.

Tissue preparation for pyridine nucleotide and protein analyses. The embryos were harvested at day 10 of incubation, homogenized, [15] and made to 5 ml volume with alkaline cysteine. So as to minimize the alkali-catalyzed destruction of NAD, care was exercised to maintain the solutions at 0° and to work quickly. Under our conditions, less than 5% of the NAD of the samples was lost by alkaline destruction [15, 26]. The homogenate was then diluted 1:5 with ice-cold alkaline cysteine solution, and three 1-ml aliquots of it were taken, one to be used as a blank, another for the determination of the oxidized forms of the pyridine nucleotides, and a third for the determination of the reduced forms. The blank samples were incubated at 60° for 10 min to destroy the oxidized forms. They were then acidified with 0.3 ml of freshly-prepared HCl-ascorbate solution [15] and incubated at 60° for an additional 30 min to destroy the reduced forms. The samples were cooled to room temperature, and 2.25 ml of 0.02 M imidazole-HCl buffer, pH 6.5, was added. They were titrated with dilute HCl to pH 6.5 and the volumes made to 5 ml with water. The samples to be used for the determination of the reduced pyridine nucleotides were also incubated at 60° for 10 min. Then 2.25 ml of the imidazole buffer solution was added, and the samples were titrated to pH 6.5. A 0.5-ml portion of oxidized-glutathione: glutathione reductase solution [15] was added to each sample, and the mixtures were incubated at 37° for 30 min. The samples were then boiled for 90 sec to destroy the glutathione reductase and the volumes of the mixtures made to 5 ml with water. To the 1-ml samples to be used for determining the oxidized form of the nucleotides was added 0.3 ml of the HCl–ascorbate solution. They were heated to 60° for 30 min, cooled, and 2.25 ml of the imidazole buffer was added. The solutions were titrated to pH 6.5 with dilute NaOH, and the volume of each sample was made to 5 ml with water. All the samples thus prepared were stored at −70° prior to nucleotide analysis. An aliquot of the remaining homogenate was used for protein analysis by the method of Lowry *et al.* [27].

An independent procedure for NAD extraction and analysis was performed on some of the embryos. The extraction method was that of Goldberg *et al.* [28], and the HPLC analysis was a modification of the method of Hartwick and Brown [29] (see also the subsequent section, "Ribonucleotide analyses"). The NAD results obtained by this alternate procedure were in good agreement with those obtained by the enzymatic cycling assay.

Pyridine nucleotide analyses. The pyridine nucleotide analyses were carried out by a modification of the radiometric enzymatic cycling method of O'Dorisio and Barker [30, 31], based on the original procedure of Serif *et al.* [32, 33]. In these assays, the decarboxylation of [1-^{14}C]glutamate was accomplished with ninhydrin rather than glutamate decarboxylase [15]. The procedures for NAD and NADH analyses were essentially the same as those described by Kitos *et al.* [15]. For measuring NADP and NADPH, an amount of each of the following reagents per sample to be analyzed was pooled in a chilled flask: 0.25 ml of a solution containing 0.8 mg of bovine serum albumin and 4 μmoles of ADP per ml; 0.25 ml of 0.2 M Na_2HPO_4 + 0.4 M NH_4Cl, pH 7.8; 0.1 ml of 0.01 M glucose-6-phosphate (G6P); 0.01 ml of [1-^{14}C]α-ketoglutarate (New England Nuclear Corp., Boston, MA; 11 mCi/mmole), 2×10^6 dpm/ml in 0.1 M NH_4OH; 1 unit of yeast glucose-6-phosphate dehydrogenase (Boehringer-Mannheim Corp., Indianapolis, IN); and 3 units of beef liver glutamate dehydrogenase (Boehringer-Mannheim). The volume of this mixture was 0.63 ml. A 0.37-ml volume of the tissue extract, prepared as described, was added to the reaction vessel. The incubation and subsequent decarboxylation procedures were as described previously for NAD analyses [15].

Tissue extract preparation for purine and pyrimidine ribonucleotide and amino acid analyses. The embryos were harvested at 10 days of incubation and then homogenized according to a modification of the method of Goldberg *et al.* [28]. Each embryo was transferred to a chilled Potter homogenizing tube containing 1 ml of 5% trichloroacetic acid (TCA) and homogenized with ten strokes of a Teflon pestle, and the volume was made to 7 ml with 5% TCA. In making the dilution, the tissue was considered to have a specific gravity of 1.0. The homogenate was vortexed, centrifuged at 1900 *g* at 4°, and the supernatant solution transferred to a polypropylene tube. The pellet was resuspended in 3 ml of cold TCA and centrifuged as before, the supernatant fraction being pooled with the initial extract. The TCA was then removed from the pooled aqueous sample by three extractions with 10 ml of ethyl ether. The residual ether was removed from the aqueous layer by a stream of air. The 5 ml of each extract was lyophilized and the dried sample was dissolved in 0.5 ml of 0.01 M KH_2PO_4, pH 2.5, and centrifuged in a microfuge of 8000 *g* for 2 min to remove any insoluble material. Appropriate dilutions were made to produce samples with A_{260} values of 0.10 to 0.30. These extracts were kept at −20° prior to analysis.

Ribonucleotide analyses. The ribonucleotide concentrations of the prepared extracts were determined by a modification of the method of Hartwick and Brown [29]. Aqueous buffers were prepared from KH_2PO_4–0.01 M, pH 2.5, and 0.1 M, pH 4.0 and filtered through a 0.45 μm membrane filter. The nucleotides in the embryo extracts were resolved on a 4.6 × 250 mm Altex AX-78 Ultrasil HPLC column (ion exchange) with a short silica Pregel column. A 25-μl sample was loaded, and the column was developed over a period of 20 min using a linear gradient, from 0.01 M buffer to a 30:70 mixture of the 0.01 M:1.0 M respectively. The gradient was constructed using a Beckman model 110 gradient maker with electronic programmer. The A_{260} of the column effluent was followed using a Gilson Holochrome photometer and chart recorder. The material in each absorption peak was identified by its elution time, and the amount of the nucleotide was deter-

mined by measurement of the areas of the eluted peaks, taking the extinction coefficients into consideration.

Amino acid analyses. Total amino acid analysis was made on the tissue extracts that had been prepared for ribonucleotide quantitation (see previous section) using the colorimetric ninhydrin procedure of Moore and Stein [34]. The amounts of most of the amino acids were obtained by the automatic method of Spackman *et al.* [35] using a Glenco custom modular amino acid analyzer (Glenco Scientific, Texas). The amounts of tryptophan in the extracts were too small to measure by this procedure. However, the procedure for ribonucleotide analysis described in the previous section was also used to resolve tyrosine and tryptophan and to measure their abundance by light absorption at 280 nm. The tyrosine values thus obtained agreed well with those from the automatic amino acid analyzer. By this HPLC method the values for both tyrosine and tryptophan were quite reproducible.

RESULTS

Effects of diazinon and Nam on the pyridine nucleotide contents of chick embryos. The effects of the OP insecticides on the tissue contents of the major pyridine nucleotides other than NAD (NADH, NADP, and NADPH) have not been reported in detail. Therefore, we examined the tissue levels of all four of the pyridine nucleotides in response to a teratogenic dose (200 μg/egg; 0.66 μmole/egg) of DZN and a protective dose (1 mg/egg, 8.2 μmoles/egg) of Nam. These agents were administered at day 3, and the amounts of the pyridine nucleotides in the embryos were determined at day 10. At day 10 the diazinon-treated embryos were slightly smaller than the controls, as indicated by their wet weights and protein contents (Table 1), and they exhibited the expected teratogenic changes, types 1 and 2 [7, 14]. In addition, their NAD and NADH levels were greatly reduced, as was the fractional contribution of NADH to this pool of pyridine nucleotides.

The tissue contents of NADP and NADPH were much smaller than those of NAD and NADH and predominantly in the reduced form (Table 1). DZN acted to decrease the amounts of both forms of NADP although these decreases were relatively smaller than those of the corresponding forms of NAD.

Nam increased the dimensions of the pyridine nucleotide pools by approximately 50%. Paradoxically, when DZN was administered along with Nam, the NAD and NADH levels increased by even greater margins (Table 1).

Effects of DZN and Nam on the purine and pyrimidine ribonucleotide pools of chick embryos. Because of the relationships that exist between the pyridine nucleotides and energy transduction, we sought to determine whether DZN and/or Nam have any effect on the purine and pyrimidine ribonucleotide contents of the embryos. DZN and/or Nam were administered on day 3, and the purine and pyrimidine ribonucleotide contents of the trichloroacetic acid-soluble fractions of the embryos were determined on

Table 1. Effects of DZN and Nam on the pyridine nucleotide contents of chick embryos*

Additives		Embryo		Pyridine nucleotide content						
						Ratio NADH				Ratio NADPH
DZN	Nam	Wet weight (g)	Protein (% of wet weight)	NAD (nmoles/mg protein)	NADH (nmoles/mg protein)	NAD + NADH	NADP (nmoles/mg protein)	NADPH (nmoles/mg protein)	NADP + NADPH	
−	−	2.36 ± 0.02	2.61 ± 0.03	0.80 ± 0.02	0.31 ± 0.00	0.28	0.05 ± 0.00	0.12 ± 0.01		0.70
+	−	2.05 ± 0.01	2.71 ± 0.03	0.26 ± 0.01	0.06 ± 0.00	0.18	0.04 ± 0.01	0.05 ± 0.00		0.54
−	+	2.31 ± 0.02	2.57 ± 0.05	1.12 ± 0.01	0.59 ± 0.02	0.34	0.08 ± 0.00	0.17 ± 0.00		0.68
+	+	2.04 ± 0.02	2.69 ± 0.04	1.88 ± 0.03	0.80 ± 0.03	0.29	0.09 ± 0.00	0.15 ± 0.00		0.62

* Chicken eggs were injected by the intravitelline route at day 3 of incubation with 200 μg DZN, 1 mg Nam, or both, and the embryos were harvested at day 10. All values are means obtained from eight to ten individual experiments ± the standard deviation.

Table 2. Effects of DZN and Nam on the total free purine and pyrimidine ribonucleotide contents of chick embryos*

Additives		Total ribonucleotide contents (nmoles/g embryo)		
DZN	Nam	Purine ribonucleotides	Pyrimidine ribonucleotides	N
−	−	272 (100)†	125 (100)	4
+	−	206 (76)	72 (58)	10
−	+	299 (110)	102 (82)	9
+	+	268 (98)	87 (70)	10

* The injection protocol was the same as that for Table 1.
† Values in parentheses are the calculated percentages of the control.

Table 3. Effects of DZN and Nam on the individual free purine ribonucleotide contents of chick embryos*

Additives		Ribonucleotide contents (nmoles/g embryo)			Energy charge	Ribonucleotide contents (nmoles/g embryo)			
DZN	Nam	ATP	ADP	AMP		GTP	GDP	GMP	N
−	−	161 ± 17	43 ± 4	13.4 ± 4.0	0.84 ± 0.04	38.4 ± 7.0	17.0 ± 6.0	ND†	4
+	−	123 ± 12	38 ± 6	9.9 ± 3.3	0.85 ± 0.02	27.4 ± 4.0	8.0 ± 2.0	ND	10
−	+	185 ± 11	39 ± 7	13.4 ± 4.0	0.86 ± 0.02	46.7 ± 8.0	15.2 ± 2.0	ND	9
+	+	166 ± 21	33 ± 5	12.0 ± 3.0	0.87 ± 0.01	43.2 ± 7.0	14.0 ± 5.0	ND	10

* The injection protocol was the same as that for Table 1. The embryos were extracted on day 10. HPLC analyses of the ribonucleotides of the deproteinized extracts were performed as described. The energy charge was calculated from the adenine ribonucleotide values as follows:

$$\text{Energy charge} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

All values are means ± the standard deviations.
† Not detectable.

day 10. When DZN was administered alone, there was a decrease in the amounts of both classes of ribonucleotides (Table 2). Exogenous Nam lessened the severity of these responses. A breakdown of the distributions of the purine ribonucleotides among the mono-, di-, and triphosphate subclasses is presented in Table 3. The triphosphates were the most abundant species (70–79%) followed by the diphosphates (16–22% of the adenine ribonucleotides and 23–30% of the guanine ribonucleotides). The amounts of AMP were small, but measurable, while the amounts of GMP were too small to be detected by this method.

Resolution of the pyrimidine ribonucleotides by this chromatographic method is not as good as that of the purine ribonucleotides. Although the diphosphates of uridine and cytidine are clearly resolved, the tri- and monophosphates are not. To estimate the abundance of UTP plus CTP, we used the mean value of their extinction coefficients since the coefficients, themselves, are similar (6.5 and 6.4 × 10^3 at 254 nm, pH 7, respectively). Like the purine nucleotides, the pyrimidine nucleotides of both the treated and untreated embryos were principally (76–81%) in the triphosphate forms (Table 4). UDP accounted for 15–20% of the pyrimidine ribonucleotide pool

Table 4. Effects of DZN and Nam on the individual free pyrimidine ribonucleotide contents of chick embryos*

Additives		Ribonucleotide contents (nmoles/g embryo, wet weight)				
DZN	Nam	UTP + CTP	UDP	CDP	CMP + UMP	N
−	−	99.0 ± 39.0	23 ± 9	3.2 ± 2.0	ND†	4
+	−	58.0 ± 12	11 ± 4	2.7 ± 2.0	ND	10
−	+	83 ± 10	16 ± 3	2.5 ± 1.2	ND	9
+	+	66 ± 11	17 ± 5	3.8 ± 1.0	ND	10

* The injection protocol was the same as that for Table 1. All values are the arithmetic means ± the standard deviation.
† Not detectable.

Table 5. Effects of DZN and Nam on the essential free L-amino acid contents of chick embryos*

Additives		Free L-amino acid contents (nmoles/g wet weight of embryo)									
DZN	Nam	TRP	HIS	ARG	PHE	THR	LEU	ILE	VAL	LYS	N
−	−	65 ± 15	225 ± 30	290 ± 75	236 ± 21	579 ± 150	215 ± 43	236 ± 43	641 ± 109	579 ± 86	4
+	−	38 ± 10	92 ± 43	367 ± 80	264 ± 46	998 ± 160	229 ± 46	241 ± 57	734 ± 115	619 ± 57	10
−	+ +	50 ± 10	221 ± 84	347 ± 105	263 ± 42	777 ± 220	252 ± 63	273 ± 63	735 ± 84	809 ± 242	9
+	+ +	44 ± 19	154 ± 88	352 ± 132	242 ± 66	661 ± 231	209 ± 66	242 ± 88	914 ± 253	639 ± 154	10

* *The injection protocol was the same as that for Table 1. All values are the arithmetic means ± the standard deviations.*

and CDP for only 3–4%. The monophosphates were insufficiently abundant to be detected by this method.

Effects of DZN and Nam on the free alpha amino acid contents of chick embryos. There are some indications that the type 1 defects may be due to deficiencies of L-tryptophan rather than of NAD [13, 16, 17, 21]. If this is the case, the afflicted embryos should contain less free tryptophan per unit tissue mass than their normal counterparts. To test this possibility we administered DZN and/or Nam at day 3 of incubation and extracted the embryos with cold trichloroacetic acid at day 10. The amounts of the free amino acids in the TCA extracts were determined by automatic amino acid analysis and the amounts of tryptophan by ion exchange HPLC with ultraviolet absorption detection at 280 nm. The tyrosine content could also be measured this way. The results of these analyses are reported in Tables 5 and 6.

Not available are the individual values for glutamine, glutamic acid and asparagine, as well as values for proline, methionine and cysteine. Even so, the data show that, in response to DZN, the contents of most of the free amino acids remained essentially unchanged. The amounts of tryptophan and histidine (Table 5) were lowered by DZN, as were the amounts of glycine and alanine (Table 6). The levels of threonine and aspartic acid were increased by the insecticide. Exogenous Nam produced no changes in the concentrations of any of the amino acids.

DISCUSSION

The studies reported here were undertaken to explore some of the biochemical variables that might be involved in producing the leg, beak, and feather deformities in avian embryos that are caused by the OP insecticides. Included in the survey are quantitative assessments of the four pyridine nucleotides, the twelve purine and pyrimidine ribonucleotides, and the free α-amino acids.

The NAD content of 10-day-old chick embryos was decreased by the prior administration of DZN, a response that was antagonized by Nam (Table 1). These results agree with those of others [6, 9–16]. The NADH level was also lowered by the insecticide, perhaps relatively more so than the NAD. The reason for the apparent greater effect of the insecticide on NADH is not obvious. Possibly the malaise that accompanies OP intoxication of the embryos involves a decrease in the rate of pyridine nucleotide reduction without a corresponding change in the rate of terminal electron transport, one consequence of which could be an exaggerated lowering of the steady-state concentration of NADH. The combined presence of DZN and exogenous Nam elevated the levels of NAD and NADH well above normal and even much above those which occurred with exogenous Nam alone (Table 1). In no instance, however, was the NADH portion of this pool greater than it was in the control embryos. Thus, terminal respiration would not appear to be inhibited by the insecticide.

There is no obvious explanation for the extra large

Table 6. Effects of DZN and Nam on the non-essential free amino acid contents of chick embryos*

Additives		Free amino acid contents (nmoles/g wet weight of embryo)						
DZN	Nam	TYR	GLY	ALA	ASP	GLU + GLN + ASN	SER	N
−	−	412 ± 22	653 ± 231	869 ± 165	1117 ± 240	3346 ± 734	4140 ± 1937	4
+	−	415 ± 45	374 ± 134	580 ± 175	2171 ± 392	2954 ± 359	3083 ± 1041	10
−	+	392 ± 24	782 ± 324	1086 ± 278	1399 ± 220	4141 ± 450	3990 ± 683	9
+	+	426 ± 38	891 ± 256	961 ± 281	1433 ± 370	4401 ± 1054	4148 ± 1164	10

* The injection protocol was the same as that for Table 1. All values are arithmetic means ± the standard deviations.

accumulation of NAD when DZN and Nam were co-administered. Possibly this combination favors the mobilization of Nam to form NAD or antagonizes poly(ADP ribose) synthesis, the principal pathway of NAD turnover in eukaryotic cells [36]. Nam, itself, is known to be a product inhibitor of poly-(ADP-ribose) polymerase [37]. However, this extra large accumulation of NAD probably has nothing to do with the protective action of Nam against type 1 teratisms. This inference is made from the observation that the co-administration of tryptophan and DZN results in normal levels of NAD in the embryo and provides complete protection against the leg, beak and feather deformities [16]. In the chick embryo, the pyridine ring of NAD is formed almost exclusively from tryptophan. As in rat liver, the pathway from tryptophan to NAD may be subject to feedback inhibition of tryptophan dioxygenase [22, 23]. NAD formation from Nam would not be regulated by this feedback system and perhaps would not be regulated at all. Even so, if Nam is administered, one would not expect the accumulation of NAD to be greater in the presence of DZN than in its absence, as is seen. The action of DZN in this overproduction of NAD needs to be resolved.

In the chick embryo NADP existed principally in its reduced form (Table 1). DZN decreased the amounts of both NADP and NADPH but the changes were proportionately smaller than those for the corresponding forms of NAD. Exogenous Nam augmented the tissue levels of NADP and NADPH but the combined presence of it and DZN did not exaggerate this response as it did for NAD and NADH. In general, the patterns of change of the four pyridine nucleotides in response to DZN and Nam were similar and probably reflect the availability of NAD which is a precursor of the other three.

The cumulative levels of the purine and pyrimidine ribonucleotides were also diminished by teratogenic doses of DZN (Table 2), even though the changes were proportionately smaller than those for the pyridine nucleotides (Table 1). Nam partially counteracted these losses (Tables 2 and 3). The insecticide caused decreases in the total amounts of both the adenine and guanine ribonucleotides but did not alter the energy charge, a value that is calculated from the amounts of ATP, ADP and AMP [38]. The normal energy charge range for animal tissues is 0.80 to 0.95. The range of values obtained for untreated chick embryos and those treated with DZN and/or Nam was from 0.84 to 0.87 (Table 3). Thus, even though DZN lowered the concentrations of the adenine ribonucleotides in the embryo, it did not seriously jeopardize the processes of generation and utilization of ATP. Nor is this ledger modified by Nam supplements. In like manner, the amounts of the guanine ribonucleotides reflected a positive energy balance (Table 3). DZN also caused a reduction in the size of the pyrimidine ribonucleotide pool (Table 2) but did not greatly alter the di- to triphosphate ratios in this pool (Table 4). Nor did Nam, either with or without DZN, greatly influence this distribution. It would seem that DZN causes a decrease in the total amount of ribonucleotides in the embryos but does not directly interfere with energy generation or utilization. The basis of the decrease in the levels of the purine and pyrimidine ribonucleotides has not been addressed in this study. However, since the pyridine nucleotides are involved in the *de novo* synthesis of both of these classes of nucleotides, it would not be surprising if NAD and NADP insufficiency adversely influenced them.

Table 7. Essential amino acid composition of the proteins of the chicken egg*

Amino acid	Available nitrogen (% of total)
L-Tryptophan	0.46
L-Histidine	1.22
L-Cysteine	1.41
L-Methionine	1.92
L-Tyrosine	2.22
L-Phenylalanine	3.00
L-Threonine	3.65
L-Lysine	3.70
L-Isoleucine	4.22
L-Valine	5.32
L-Leucine	6.11

* Values from Ref. 39.

The free amino acid complements of the insecticide-treated and untreated 10-day chick embryos revealed that DZN lowered the amounts of only a few of the amino acids, including tryptophan and histidine (Table 5), both of which are available in low proportion in yolk proteins (Table 7). Tryptophan, the least abundant of all the amino acids, is unique among them in its ability to protect against the OP insecticide-induced type 1 malformations [13, 16, 21]. It plays at least two major roles in the developing embryo: it is an essential raw material for protein synthesis and pyridine nucleotide synthesis. Circumstances that reduce the already small natural abundance of this amino acid could handicap one or

both of these processes; supplementary tryptophan should and does relieve the handicap.

A tryptophan deficiency rather than an NAD deficiency has been proposed as responsible for the type 1 deformities [16, 17]. The results in Table 5 are consistent with this possibility. The tryptophan deficit in the embryo could be due to a reduced rate of tryptophan release from the yolk sac proteins and/or uptake into the embryo or to an unusually large rate of its catabolism. With respect to the former of these, the insecticide could inhibit one or more proteases of the yolk sac or yolk sac membrane. Flockhart and Casida [40] have shown such actions by OP and AC insecticides on a family of esterases, enzymes that are generically related to some proteases. Wenger and Wenger [41, 42] reported that at least two yolk sac proteases are targets of OP and AC insecticides. An effect which lowers the free amino acid content of the embryo might be most dramatic on the amino acids that are least available. Such an action should be antagonized by supplements of the limiting constituent. Supplementation with histidine does not ameliorate the type 1 response (unpublished results), but with tryptophan the amelioration is complete.

If a tryptophan deficiency is the underlying basis of the OP insecticide-induced type 1 deformities *in avia*, there are several questions that need to be addressed. Is an inhibition of yolk sac proteases actually responsible for the tryptophan deficit? Why do Nam and some of its congeners act to prevent the deformities? How does 2-PAM sustain the normal tissue levels of NAD but not prevent the teratisms? Our continuing research deals with these questions.

Acknowledgements—The amino acid analyses in this study were performed by the Enzyme Laboratory of the University of Kansas (Charles J. Decedue, Director). The analytical grade diazinon was provided by the Pesticides and Industrial Chemicals Repository of the U.S. Environmental Protection Agency, Research Triangle Park, NC, U.S.A. The authors thank Dr. D. Byrne for his constructive criticism of this manuscript.

REFERENCES

1. W. Landauer, *J. cell. comp. Physiol.* **43**, 261 (1954).
2. J-C. Roger, D. G. Upshall and J. E. Casida, *Biochem. Pharmac.* **18**, 373 (1969).
3. R. Meiniel, Y. Lutz-Ostertag and H. Lutz, *Archs Anat. microsc. Morph. exp.* **59**, 167 (1970).
4. W. Landauer and N. Salam, *Devl Biol.* **28**, 35 (1972).
5. N. H. Proctor and J. E. Casida, *Science* **190**, 580 (1975).
6. N. H. Proctor, A. D. Moscioni and J. E. Casida, *Biochem. Pharmac.* **25**, 757 (1976).
7. R. Meiniel, *Experientia* **32**, 920 (1976).
8. M. Misawa, J. Doull, P. A. Kitos and E. M. Uyeki, *Toxic. appl. Pharmac.* **57**, 20 (1981).
9. J-C. Roger, H. Chambers and J. E. Casida, *Science* **144**, 539 (1964).
10. J-P. Marliac, M. J. Verrett, J. McLaughlin, Jr. and O. G. Fitzhugh, *Toxic. appl. Pharmac.* **7**, 490 (1965).
11. D. G. Upshall, J-C. Roger and J. E. Casida, *Biochem. Pharmac.* **17**, 1529 (1968).
12. J. Greenberg and Q. N. LaHam, *Can. J. Zool.* **47**, 539 (1969).
13. J. Greenberg and Q. N. LaHam, *Can. J. Zool.* **48**, 1047 (1970).
14. A. D. Moscioni, J. L. Engel and J. E. Casida, *Biochem. Pharmac.* **26**, 2251 (1977).
15. P. A. Kitos, D. S. Anderson, E. M. Uyeki, M. Misawa and C. R. Wyttenbach, *Biochem. Pharmac.* **30**, 2225 (1981).
16. M. Henderson and P. A. Kitos, *Teratology* **26**, 173 (1982).
17. D. Byrne and P. Kitos, *Biochem. Pharmac.* **32**, 2881 (1983).
18. A. Nishio, S. Nakanishi, J. Doull and E. M. Uyeki, *Biochem. biophys. Res. Commun.* **111**, 750 (1983).
19. J. Seifert and J. E. Casida, *Biochem. Pharmac.* **27**, 2611 (1978).
20. J. Seifert and J. E. Casida, *Pestic. Biochem. Physiol.* **12**, 273 (1979).
21. B. S. Wenger and E. Wenger, *Proc. Can. Fedn biol. Soc.* **16**, 61 (1973).
22. C. Wagner, *Biochem. biophys. Res. Commun.* **17**, 668 (1964).
23. Y. S. Cho-Chung and H. C. Pitot, *J. biol. Chem.* **242**, 1192 (1967).
24. B. Schepartz, *Regulation of Amino Acid Metabolism in Animals*, pp. 174–9. W. B. Saunders, Philadelphia (1973).
25. P. A. Kitos, C. R. Wyttenbach, K. Olson and E. M. Uyeki, *Toxic. appl. Pharmac.* **59**, 49 (1981).
26. H. B. Burch, M. E. Bradley and O. H. Lowry, *J. biol. Chem.* **242**, 4546 (1967).
27. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
28. N. D. Goldberg, S. B. Dietz and A. G. Toole, *J. biol. Chem.* **244**, 4458 (1969).
29. R. A. Hartwick and P. R. Brown, *J. Chromat.* **112**, 651 (1975).
30. M. S. O'Dorisio and K. L. Barker, *Endocrinology* **86**, 1118 (1970).
31. M. S. O'Dorisio and K. L. Barker, *Biology Reprod.* **15**, 504 (1976).
32. G. S. Serif and F. R. Butcher, *Analyt. Biochem.* **15**, 278 (1966).
33. G. S. Serif, L. Schmotzer and F. R. Butcher, *Analyt. Biochem.* **17**, 125 (1966).
34. S. Moore and W. H. Stein, *J. biol. Chem.* **176**, 367 (1948).
35. D. H. Spackman, W. H. Stein and S. Moore, *Analyt. Chem.* **30**, 1190 (1958).
36. O. Hayaishi and K. Ueda, *A. Rev. Biochem.* **46**, 95 (1977).
37. J. L. Sims, G. W. Sikorski, D. M. Catino, S. J. Berger and N. A. Berger, *Biochemistry* **21**, 1813 (1982).
38. D. E. Atkinson, *Cellular Energy Metabolism and its Regulation*. Academic Press, New York (1977).
39. R. W. McGilvery, *Biochemistry: A Fundamental Approach*, p. 667. W. B. Saunders, Philadelphia (1970).
40. I. R. Flockhart and J. E. Casida, *Biochem. Pharmac.* **21**, 2591 (1972).
41. B. S. Wenger, *Am. Zool.* **14**, 1305 (1974).
42. B. S. Wenger and E. Wenger, *Teratology* **21**, 74a (1980).