Biochemical Pharmacology, Vol. 32, No. 19, pp. 2881–2890, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

TERATOGENIC EFFECTS OF CHOLINERGIC INSECTICIDES IN CHICK EMBRYOS—IV

THE ROLE OF TRYPTOPHAN IN PROTECTING AGAINST LIMB DEFORMITIES*

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(*Received* 23 *August* 1982; *accepted* 4 *March* 1983)

Abstract—The mechanism by which organophosphate (OP) insecticides cause micromelia in embryonic chick limbs was examined using a tissue culture approach. Limb bud cells in micromass culture were assayed for their proliferative and chondrogenic activities, [^{3}H]thymidine and $^{35}SO_4^=$ incorporation, respectively, into the trichloroacetic acid-insoluble constituents of the cell masses and/or the accumulation of ^{35}S-labeled soluble macromolecular products in the culture medium. There was no obvious correlation between either the teratogenicity or toxicity of the insecticide *in ovo* and the inhibition of proliferation and chondrogenesis *in vitro*. In addition, nicotinamide, which prevents insecticide-induced micromelia *in ovo*, did not improve the proliferative and chondrogenic performance of insecticide-treated cells in culture. On the other hand, 2-pyridinealdoxime methochloride, which offers little or no protection against micromelia *in ovo*, did protect both the proliferative and chondrogenic activities of the limb bud cells in micromass culture. These observations suggest that the actions of the insecticides on the cells in culture are not the same as those that produce micromelia *in ovo*. L-Tryptophan antagonized OP insecticide-induced micromelia in the embryo. In micromass culture, a much greater concentration of tryptophan was needed to support the chondrogenic than the proliferative activities of the limb bud cells. Moreover, a greater concentration of tryptophan was needed to support the chondrogenic activities of the leg bud than the wing bud cells. These *in vitro* responses of the limb bud cells to tryptophan deprivation are analogous to the *in ovo* response of the limbs to the teratogenic OP insecticides. A possible explanation of the roles of tryptophan and nicotinamide in preventing the limb deformities is offered.

Many organophosphate (OP)‡ insecticides are teratogenic in animals [1–5]. Chick embryos exposed to such agents early in development exhibit two distinct classes of deformities, those that are antagonized by the co-administration of nicotinic acid or any number of other metabolic precursors of the pyridine ring moiety of NAD (type I) and those antagonized by the co-administration of certain non-toxic oximes such as 2-pyridinealdoxime methochloride (2PAM) (type II) [6, 7]. The first of these classes of teratisms includes, but is not limited to, a form of micromelia in which the development of the legs is impaired much more severely than that of the wings [6–8]; the second class includes the wry neck syndrome [6–9].

Moscioni *et al.* [6] and Seifert and Casida [10, 11] proposed that the OP insecticides produce a lesion in the pathway from L-tryptophan to NAD which is responsible for the type I defects. They showed that kynurenine formamidase (KFase), the enzyme which catalyzes the conversion of *N*-formylkynurenine to kynurenine, is inhibited *in vitro* by teratogenic OP insecticides. A consequence of this action *in vivo* would be to decrease the rate of NAD synthesis and the steady-state concentration of NAD. Since exogenous niacin maintains the NAD content of the tissues and prevents type I deformities [3, 4, 12], the NAD deficit would appear to be causally linked to this class of teratisms.

Inconsistent with the concept that the type I teratisms are caused by too little NAD in the tissues due to an impairment of KFase are the following observations: (1) if 2PAM, a compound that does not reactivate KFase, is administered along with the insecticide, it acts to maintain the NAD content of the tissues close to normal but does not prevent micromelia [12]; (2) exogenous tryptophan is essentially as effective as nicotinamide in preventing the type I teratogenic actions of the insecticides [13, 14]; and (3) the NAD contents of the legs and wings of the embryos are decreased equally by the insecticides but only the legs exhibit severe micromelia [12]. The first of these observations suggests that the NAD content of the tissues does not determine the course of development of the limbs; the second suggests that at least *in vivo* the OP insecticides do not

* Supported, in part, by Grant ES-02046 from the NIEHS and KU GRF No. 3558.

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‡ Abbreviations: OP, organophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; DZN, diazinon; DXN, diazoxon; MLN, malathion; MXN, malaoxon; 2PAM, 2-pyridinealdoxime methochloride; TOase, tryptophan 2,3-dioxygenase; KFase, kynurenine formamidase; FBS, fetal bovine serum; L-Trp, L-tryptophan; NAM, nicotinamide; and EC_{50}, concentration at which 50% of the maximal response is produced.

produce the type I deformities by inhibiting KFase; the third observation suggests that whatever the mechanism of limb deformation, it is appendage specific.

To examine the metabolic basis of type I teratogenesis, particularly as NAD and/or tryptophan may be involved, we used the micromass culture technique of Ahrens *et al.* [15]. Chick limb bud tissues from day 4 embryos (stages 22–24, Hamburger and Hamilton [16]) were disaggregated with trypsin to single cell suspensions and seeded in culture at high cell density. The resulting cultures exhibit many of the chemical and physical traits of limb development, including some which are characteristic of the initial stages of chondrogenesis [17–23]. The differentiation and development of the limb rudiments, isolated in this way from the rest of the embryo, its supporting membranes and the yolk, can be studied in micromass cultures in ways that are not possible *in ovo*.

Using the tissue culture approach, we observed that nicotinamide was unable to prevent the inhibitory action of a highly teratogenic OP insecticide, diazinon (DZN), on either the proliferative or chondrogenic activities of the limb bud cells. Under similar conditions, a deficiency of tryptophan in the culture medium produced an effect on the limb bud cells that resembled the micromelic action of DZN on the limbs *in ovo*. These findings are discussed in terms of the possible roles played by NAD and tryptophan in type I teratogenesis.

MATERIALS AND METHODS

Micromass cultures

Fertile SPF Cofal negative eggs (Larson Lab-Vac Eggs Inc., Gowrie, IA) from White Leghorn hens were incubated for 4 days (to stage 22–24, Hamburger and Hamilton [16]), the embryos were removed and the limb buds were dissected into sterile chick Ringer's solution [12]. Each limb type was pooled separately into a sterile, flat bottom shell vial containing a Teflon-coated magnetic flea. The pooled material from twenty to twenty-four embryos was rinsed twice with 5- to 10-ml portions of Ringer's solution and digested at 37° to a single-cell suspension with trypsin: EDTA [0.1% crystalline porcine trypsin, type IX (Sigma Chemical Co., St. Louis, MO) plus 0.1% EDTA in calcium- and magnesium-free phosphate buffered saline (PBSA); NaCl, 0.137 M; KCl, 2.68 mM; Na_2HPO_4, 10.6 mM; and KH_2PO_4, 1.47 mM; pH 7]. Two digestion periods, 15 min and then 10 min, with vigorous stirring disrupted all the limb bud tissues. Two milliliters of the trypsin solution was used for the first digestion and 1 ml for the second. The digests were passed through a No. 20 (20 μm) Nitex screen and collected in an ice-chilled tube containing at least 0.1 vol. of fetal bovine serum (FBS; KC Biologicals, Inc., Lenexa, KS) to inactivate the trypsin. The single cell suspensions were diluted and counted with a hemocytometer and then centrifuged for 1 min at 800 *g*. The supernatant fractions were discarded and the cells were suspended at 25×10^6 cells/ml in Ham's F12 medium (KC Biologicals) supplemented with 0.12 g $NaHCO_3$, 1 mg ascorbic acid, 5 mg Gentamycin sulfate (Sigma Chemical Co.) per 100 ml (designated Ham's F12A medium), and 10% FBS.

Micromass cultures [15] were then prepared by spotting 20 μl of the cell suspension in each well of a 24-place cluster dish (Costar, Cambridge, MA). The cultures were incubated for 1–2 hr at 37° in a 5% CO_2:95% air atmosphere to allow the cells to attach to the substratum, and then an additional 0.5 ml of medium containing 10% FBS was added to each well. On the following day, the medium was exchanged for 0.9 ml of fresh medium containing 1% FBS and either 1 μCi/ml of $^{35}SO_4^=$ (43 Ci/mg S, ICN, Irvine, CA) or 0.1 μCi/ml of [methyl-^{3}H]thymidine (53 Ci/mmole, Research Products International, Mount Prospect, IL). At the same time any additional test substances were also included in the medium. The media, prepared freshly at this time, were dispensed in aliquots sufficient for one day's refeeding and stored at −60°, to be thawed just prior to use. In the experiments involving the use of other than the normal concentration of tryptophan or nicotinamide, the basal culture medium was KU-1 [24], lacking the identified nutrient.

During the usual 5 days of culture, the media were renewed daily to avoid nutritional insufficiency and to ensure the continuous presence of the test substances. In most instances, the media were collected each day and assayed for their content of ^{35}S-containing proteoglycans.

Quantitating the sulfated proteoglycans in the medium

From day 2 through 5 days of culture the used media containing $^{35}SO_4^=$ were collected and stored individually at −20° for subsequent radioactive acid polysaccharide content by a modification of the method of Castor *et al.* [25]. In the assay, 50 μl samples of used medium were applied to DEAE cellulose discs, 2.4 cm in diameter (Whatman, Clifton, NJ, item no. 3658-024). The discs, up to 50 at a time, were allowed to dry for 10 min at 37° and then were immersed as a group for 5 min in 0.1% (w/v) cetylpridinium bromide (Sigma Chemical Co.) at room temperature. They were then washed batch-wise six times in 400 ml of 0.1 N NaOH, once in 400 ml of water, once in 200 ml of 95% ethanol, and once in 100 ml of anhydrous ethyl ether. The discs were dried in a fume hood and placed in 7 ml plastic scintillation vials with 4 ml of 0.4% (w/v) Omnifluor (New England Nuclear Corp., Boston, MA) in toluene for radioactivity measurement. A negligible amount of free $^{35}SO_4^=$ bound to these filters, as indicated by comparable application of 50 μl of the $^{35}SO_4^=$-containing medium which had not been exposed to the cells.

Quantitating the $^{35}SO_4^=$ and [^{3}H]thymidine incorporation into the micromasses

On day 5 of culture, the media were removed, and each well was rinsed twice with 1 ml of 0.85% (w/v) sodium chloride. The culture dishes were floated on ice-slush, and 1 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA) was added to each well. After 30 min the TCA was removed, and the micromass cultures were rinsed four additional times with 0.5 ml of cold 5% TCA. They were then rinsed three times with 0.5 ml of cold 80% (v/v) ethanol and allowed

to dry at room temperature. The cell mass in the well was then dissolved in 0.3 ml of 0.5 N NaOH at room temperature for 30 min. The total digest was transferred to a 7 ml plastic scintillation vial, and the well was rinsed with 0.21 ml of 0.5 N NaOH, which was also added to the vial. One-tenth volume of 5 N HCl was added to neutralize the base and 4.5 ml of Bray's scintillation mixture [26] was added for radioactivity measurement.

Insecticides

The insecticides used in this study were analytical reference standards, provided by the U.S. Environmental Protection Agency, Research Triangle Park, NC, U.S.A. They are: diazinon (DZN, Spectracide), *O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphorothioate, 99.7% pure; diazoxon (DXN, diazinon oxygen analog), *O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl)phosphate, technical grade, undetermined purity; malathion (MLN), diethylmercaptosuccinate, *S*-ester with *O,O*-dimethyl phosphorodithioate, 98% pure; malaoxon (MXN, malathion oxygen analog), *O,O*-dimethyl *S*-[1,2-di(ethoxycarbonyl)ethyl]phosphorothioate, 95% pure; dicrotophos (DCP, Bidrin), 3-(dimethoxyphosphinyloxy)-*N,N*-dimethylisocrotonamide, 98% pure; and monocrotophos (MCP, Azodrin), dimethyl phosphate of 3-hydroxy-*N*-methyl-*cis*-crotonamide, 98% pure.

Method used to count the cells in micromass culture

The culture medium was removed from the chambers of a 24-well culture dish and the micromass in each well was washed with 1 ml of PBSA. The wash was discarded and 0.5 ml of 0.2% (w/v) collagenase (Type I, Sigma Chemical Co.) in PBSA was added. The plate was incubated for 10 min at 37° at which time the collagenase solution was discarded. Then 0.5 ml of 0.1% trypsin (Type IX, porcine pancreas, Sigma), 0.1% EDTA was added to each well, and the plate was incubated for another 10 min at 37°. The micromasses were disaggregated by the trypsin and the cells dispersed uniformly by repeated aspiration through the tip of a Pasteur pipette. The cells in the suspension were counted using a hemocytometer.

At the same time, replicate micromasses were washed with PBSA and digested at room temperature in 0.5 ml of a solution containing 2% Na_2CO_3 (w/v) in 0.1 N NaOH. Representative portions of the resulting solutions were used to assay the protein content by the method of Lowry *et al.* [27].

RESULTS

Some characteristics of embryonic chick cells in micromass culture

During the first 2 days of incubation, the micromass cultures from the wing and leg buds of stage 22–24 chicken embryos were similar in appearance. They were circular and their cells multilayered at the center and monolayered at the periphery. At the center of the culture the cells were tightly packed in a three-dimensional order and were polygonal; at the edges they were in two-dimensional contact and fusiform.

By at least day 3, the limb bud cultures began to exhibit distinctive features. The cells organized into a multitude of discrete chondrogenic nodules or foci, the appearance of which was been described in detail by others [15, 20, 21, 23, 28]. This pattern of development is partly suppressed by certain organophosphate insecticides in the culture medium. Also, visible cell damage and death, especially at the edges of the circular aggregates, become apparent.

By day 4, the formation of chondrogenic nodules in the limb bud cultures was pronounced, though less so when an inhibitory insecticide was present in the medium (Table 1). The monolayer edges of diazinon-treated cultures appeared necrotic. Nicotinamide, which is known to counteract the adverse effect of organophosphate insecticides on limb development *in ovo* [4, 6, 12, 29], did not diminish the extent of the apparent cell damage *in vitro*, nor did it favor the formation of chondrogenic nodules within the micromasses. 2PAM did not counteract the effect of diazinon on limb development *in ovo* but did decrease the extent of necrosis and augment

Table 1. Effects of nicotinamide, 2PAM and diazinon on the number of leg bud cells and chondrogenic nodules per micromass*

Group	NAM (1 mM)	2PAM (1 mM)	DZN (0.16 mM) (50 μg/ml)	Mean cell number 10^{-5}	Mean number of nodules per micromass ± S.D.
I	−	−	−	4.28 (1.00)†	210 ± 27 (1.00)†
	−	−	+	2.57 (0.60)	187 ± 8 (0.89)
II	+	−	−	4.43 (1.00)	218 ± 30 (1.00)
	+	−	+	2.85 (0.64)	196 ± 18 (0.90)
III	−	+	−	3.82 (1.00)	205 ± 13 (1.00)
	−	+	+	3.25 (0.85)	215 ± 33 (1.05)

* The cultures were exposed for the last 4 days of the 5-day culture period to Ham's F12A medium containing 1% fetal bovine serum plus diazinon (DZN), nicotinamide (NAM) and/or 2-pyridinealdoxime methochloride (2PAM).

† The number of cells or nodules per micromass of each diazinon-treated group relative to that of its appropriate control is expressed in parentheses. The cell population determinations were performed on triplicates and the number of nodules on quadruplicates.

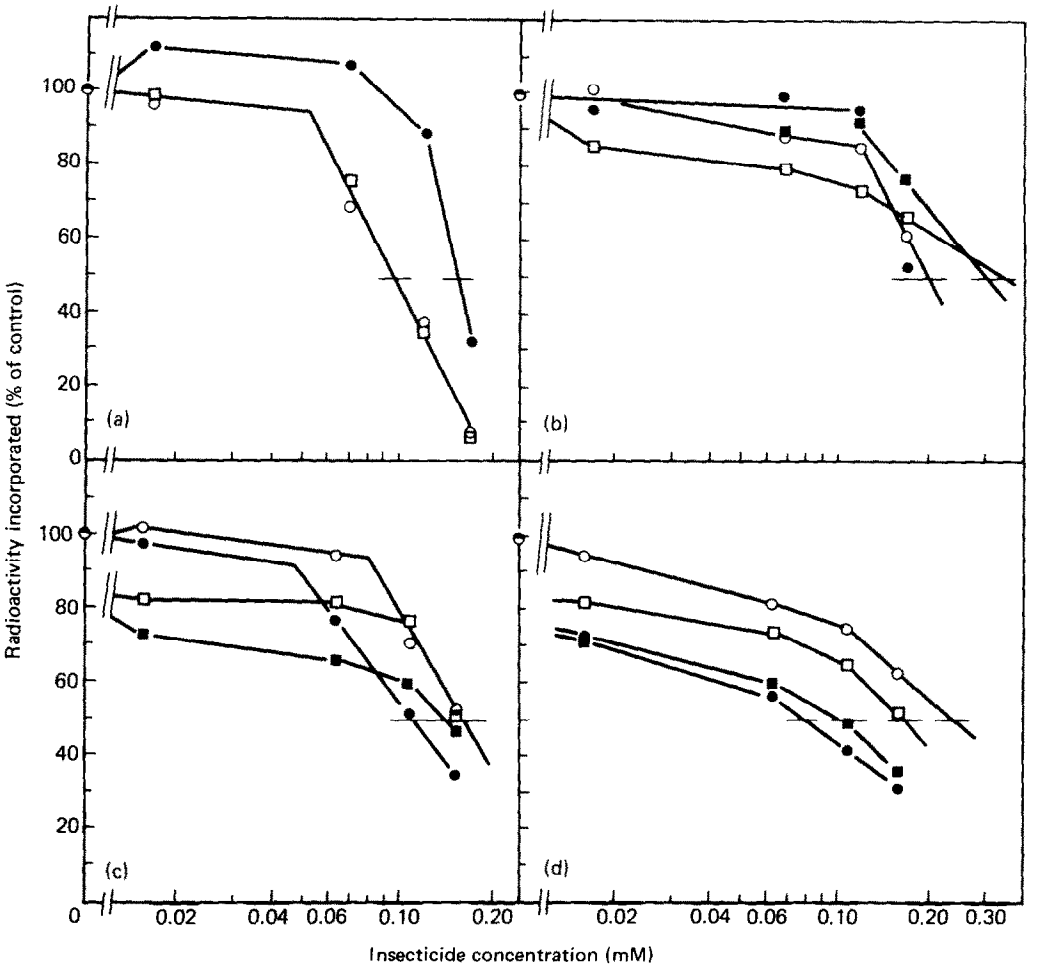

Fig. 1. Organophosphate (OP) toxicity studies on chick limb bud cells in culture. (A) diazinon (DZN); (B) diazinon oxygen analog (diazoxon, DXN); (C) malathion (MLN); and (D) malathion oxygen analog (malaoxon, MXN). Open symbols: [^{3}H]thymidine incorportion; closed symbols: $^{35}SO_4^=$ incorporation; circles: leg bud cells; squares: wing bud cells. Except for the OP-free controls, all values are averages of independent duplicated. The OP-free controls (100% values) are averages of quadruplicates. Their radioactivity values and the S.D. of the means for them are: [^{3}H]thymidine incorporation—leg 3,130 ± 290 cpm, wing 3,993 ± 291; $^{35}SO_4^=$ incorporation—leg 14,424 ± 612 cpm, wing 15,442 ± 767 cpm.

the formation of nodules in the insecticide-treated micromass cultures.

Effects of various organophosphate insecticides on the proliferation and proteoglycan synthesis of cells in micromass culture

[^{3}H]Thymidine incorporation into the trichloroacetic acid-insoluble fraction of the cell masses was used as an index of the proliferative activities of variously treated cultures since we have observed that the level of [^{3}H]thymidine incorporation reflects an increase or decrease in cell number (Table 1 and unpublished data). $^{35}SO_4^=$ incorporation into the same fraction was used as a measure of the chondrogenic activities. Several insecticides were tested for their actions in these respects, and the results are presented in Fig. 1. Diazinon, a phosphorothioate which is very teratogenic *in ovo* [4, 8, 12], inhibited growth ($EC_{50} \simeq 0.09$ mM) and chrondrogenesis ($EC_{50} \simeq 0.15$ mM) in micromass culture (Fig. 1A). Its oxygen analog diazoxon was much more toxic *in ovo* but somewhat less antiproliferative ($EC_{50} \simeq 0.25$ mM) and antichondrogenic ($EC_{50} \simeq 0.20$ mM) in culture (Fig. 1B). Malathion, a phosphorodithioate, was relatively non-teratogenic and non-toxic *in ovo* but, like diazinon, was strongly antiproliferative ($EC_{50} \simeq 0.15$ mM) and antichondrogenic ($EC_{50} \simeq 0.12$ mM) in culture (Fig. 1C). Its oxygen analog malaoxon, a comparatively toxic but only weakly teratogenic compound *in ovo*, was slightly less antiproliferative ($EC_{50} \simeq 0.20$ mM) but at least as antichondrogenic ($EC_{50} \simeq 0.09$ mM) in culture (Fig. 1D). Dicrotophos and monocrotophos, both of which are highly teratogenic phosphotriesters, had no effect on cell proliferation *in vitro* and only a small effect on chondrogenesis (unpublished data). Thus, there do not appear to be any consistent correlations between the observed responses of the limb bud cell cultures to the various insecticides *in vitro* and the toxic and teratogenic responses of the

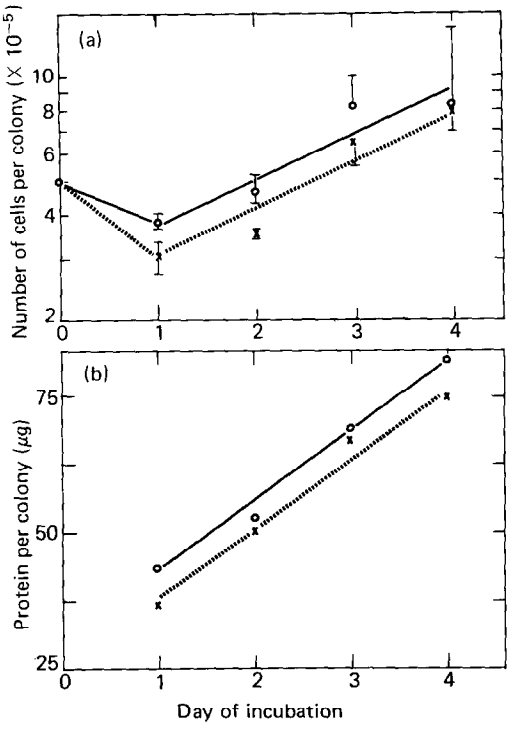

Fig. 2. Growth kinetics of chick limb bud cells in micromass culture. Micromass cultures containing 5×10^5 leg or wing bud cells of stage 22–24 (day 4) chick embryos were prepared on day zero in 24-well Costar cluster dishes and incubated in Ham's F12A plus 10% FBS for 24 hr. The medium was then changed to F12A plus 1% FBS and thereafter was exchanged daily with fresh homologous nutrient medium until day 4 of incubation. The colonies, three per time point for each tissue, were disaggregated to single cell suspensions by sequential treatment with collagenase and trypsin, and the cells were counted using a hemocytometer. Replicate colonies at the same time points were solubilized with 2% Na_2CO_3 in 0.1 N NaOH, and the protein content of each was determined by the method of Lowry *et al.* [27]. (A) Cell population per colony; as a function of time of incubation. The circles represent the mean wing bud cell population per micromass and the vertical spread is the S.D. of the mean. The Xs represent the leg bud cell populations per micromass. (B) Protein per micromass colony. The circles and Xs represent the protein content per micromass colony of the wing and leg bud cells respectively.

embryo to the same substances *in ovo*. It would seem unlikely, therefore, that in the avian embryo the observed teratogenic actions of the insecticides on the developing limb buds are due solely, or even principally, to the antiproliferative and antichondrogenic expressions seen in these *in vitro* studies.

Antagonists of diazinon in culture. From days 2 to 6 of micromass culture in Ham's F12A medium supplemented with 1% fetal bovine serum, the cell populations increased exponentially with a doubling time of 50–70 hr (Fig. 2). Diazinon inhibited both the proliferative and chondrogenic activities of these cells, as indicated by the incorporation of [^{3}H]thymidine and $^{35}SO_4^=$, respectively, into the acid-insoluble fraction (Fig. 1A and Table 2). These effects of diazinon were partly antagonized by 2PAM but not at all by nicotinamide. The relief by the oxime and lack thereof by the vitamin was contrary to that seen *in ovo*; in the developing chick embryo, nicotinamide prevents the leg deformities due to diazinon but 2PAM does not [6, 8, 12]. The effects of nicotinamide and 2PAM on the proliferative and chondrogenic responses of the cells to diazinon were consistent with the previously mentioned results of the morphological studies in culture; the damage done to the limb bud cultures by the insecticide does not appear to be counteracted by agents which protect against micromelia in embryonic chickens.

Low levels of ^{35}S-labeled macromolecules were released into the medium during the second day of culture and the output increased through day 5. In the presence of diazinon, the output at the later times was decreased significantly (Fig. 3). Nicotinamide did not counteract the effect of diazinon on the release of sulfated proteoglycans into the medium but 2PAM did, restoring the level of proteoglycan secretion close to normal (Fig. 3).

Roles of nicotinamide and tryptophan in the nutrition and differentiation of chick limb bud cells. The chick embryo *in ovo* does not need preformed nicotinic acid or nicotinamide to synthesize NAD and NADP [30–33]. It normally synthesizes the pyridine ring portion of these coenzymes from tryptophan. We sought to determine whether the isolated limb bud cells require exogenous nicotinamide for growth and chondrogenic function. Micromass cultures of leg and wing bud cells were established and, after 1 day, refed with medium KU-1 from which nicotinamide and tryptophan had been omitted and to which 1% dialyzed fetal bovine serum had been added. Supplements of nicotinamide and/or tryptophan were added to representative cultures, and after 4 days the cumulative incorporation of [^{3}H]thymidine and $^{35}SO_4^=$ into the acid-insoluble fraction of the cell masses was measured. The results in Table 3 show that neither the proliferative nor chondrogenic functions of the cells were affected by deleting nicotinamide from the medium. However, both of these functions were strongly impaired by deleting L-tryptophan. In fact, the cells died. If undialyzed serum was used in place of dialyzed, the cultures responded similarly although the damage to the cells due to the omission of tryptophan was less severe. Fetal bovine serum contributed a small but obviously not insignificant amount of tryptophan to the system.

Since the availability of tryptophan to these inchoate tissues seemed critical while the availability of nicotinamide did not, we examined the effect of the availability of L-tryptophan on both proliferation and chondrogenesis of the limb bud cells in micromass culture. In this study, nicotinamide was omitted from, and 1% undialyzed fetal bovine serum was added to, all the test media. Tryptophan was added to the culture media at concentrations ranging from 196 μM to less than 0.08 μM. The highest tryptophan concentration shown in Fig. 4, 19.6 μM (4 μg/ml), supported growth and chrondrogenesis at a level slightly greater than did the more generous supplements (data not shown).

The incorporation of [^{3}H]thymidine (Fig. 4, open symbols) decreased as the tryptophan concentration

Table 2. Effects of nicotinamide, 2PAM and diazinon on the proliferation and chondrogenesis of chick limb bud cells in micromass culture*

Additive			Isotope incorporation into the acid-insoluble constituents of the micromass (% of control)			
			Leg bud cells		Wing bud cells	
NAM (1 mM)	2PAM (1 mM)	DZN (0.16 mM)	[^{3}H]Thymidine	$^{35}SO_4^=$	[^{3}H]Thymidine	$^{35}SO_4^=$
			100	100	100	100
−	−	−	(8,194)†	(4,854)	(10,288)	(3,553)
+	−	−	120	116	97	83
−	+	−	92	103	90	89
+	+	−	98	103	98	91
−	−	+	23	26	26	62
+	−	+	16	35	23	63
−	+	+	62	76	56	99
+	+	+	52	83	53	97

* The micromasses were exposed for the last 4 days of the 5-day culture period to Ham's F12A medium containing 1% fetal bovine serum and the indicated radioactive tracer. The trichloroacetic acid-insoluble radioactivity in the tissue mass was then determined.

† Values in parentheses are the actual mean cpms for the control (100%) samples. All values are means of independent duplicates.

dropped to less than 2.45 μM. In this regard, the responses of the leg bud (triangles) and wing bud cells (circles) were very much alike. At 0.08 μM, both limb systems incorporated about half as much thymidine as their tryptophan replete controls (19.6 μM tryptophan). As tryptophan became less available, the incorporation of $^{35}SO_4^=$ into the micromasses also decreased (closed symbols), the effect being much more pronounced in the leg bud than in the wing bud cultures. At virtually every concentration of tryptophan below 19.6 μM, the $^{35}SO_4^=$ incorporation into the tissues was proportionally smaller than that of [^{3}H]thymidine. For example, at a tryptophan concentration where thymidine incorporation into the leg bud cells decreased 10–15%, sulfate incorporation decreased approximately 60%. The concentration of tryptophan needed for half-maximal [^{3}H]thymidine incorporation into the limb cells was approximately 0.1 μM while that needed for half-maximal $^{35}SO_4^=$ incorporation was approximately 1.4 μM for the legs and 0.8 μM for the wings. These findings suggest that sulfate incorporation, a chondrogenic expression of these tissues, is more sensitive to tryptophan deficiency than is proliferation and that sulfate incorporation is somewhat more sensitive to tryptophan deprivation in the leg bud than in the wing bud cells.

DISCUSSION

The micromass culture system has been used extensively to study differentiation and development of embryonic chick limb buds [15, 20, 23, 34–41]. The high cell densities that characterize these cultures seem to be necessary for the differentiative responses; monolayer cultures of the same cells fail to produce them [15, 23, 42]. The environment within the micromasses may be analogous to that experienced by the pluripotent cells of the relatively avascular core of the limb buds during stages 22–24 of embryogenesis [38]. During these stages, the cells can be primed to produce cartilage. The differentiative changes that are seen in the micromass cultures are at least partly a consequence of the ontogeny of the cells. Cultures prepared from cells of the whole body of 4-day-old embryos do not exhibit tissue-specific features whereas cultures from the cells of at least some selected functional regions, including the wing and leg buds, heart and neck, do.

The studies reported here were intended, in part, to determine whether some of the physiological changes that occur during inhibition of development of the legs by diazinon can be simulated in micromass cultures of limb bud cells. Diazinon-induced micromelia of the wings of the chick embryo is not as severe as that of the legs [8, 13]. In culture, an analogous diazinon-induced distinction was not evident. We have seen that diazinon partially inhibited cell multiplication, chondrogenic nodule formation, and another chondrogenic expression of the limb bud cultures, $^{35}SO_4^=$ incorporation (Figs. 1 and 3, Tables 1 and 2). In contrast to its antiteratogenic effect *in ovo* [6, 12], exogenous nicotinamide *in vitro* had little or no ability to counteract the diazinon poisoning. On the other hand, 2PAM did reverse the inhibition of growth and nodule formation in the micromass cultures and caused near normal accumulation of sulfated polysaccharides in both the medium and the cells (Tables 1 and 2, Fig. 3).

The results of the above described studies with nicotinamide and 2PAM suggest that the inhibitory action of diazinon on the leg bud cells in micromass culture is not the same as that which causes micromelia *in ovo*. Possibly the nicotinamide-sensitive defect that we recognize as micromelia is not detected in culture by these criteria. Alternatively, in causing the limb deformities diazinon may not act directly on the limb tissues. For example, the insecticide might produce a change in the yolk, the yolk sac membrane, or some other part of the embryonic system which, in turn, causes the limb deformity.

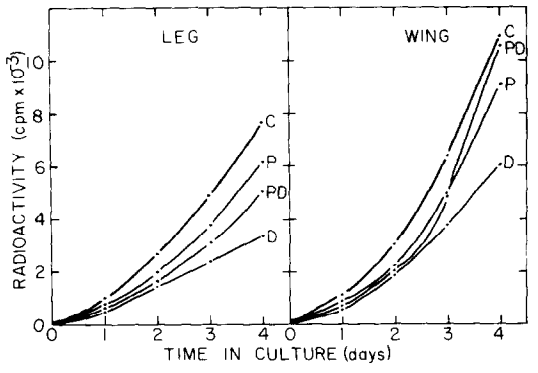

Fig. 3. Cumulative incorporation of $^{35}SO_4^=$ into the proteoglycans of the tissue culture medium. The cultures were initiated as described under Fig. 2. Each day, the refeeding medium was supplemented with 1 μCi/ml of $^{35}SO_4^=$ plus the indicated chemical(s): (C) control, i.e. no additional ingredient; (P) 1 mM 2PAM; (D) 50 μg/ml (0.16 mM) diazinon; and (PD) 2PAM plus diazinon, 1 and 0.16 mM respectively. At each time point the used media were collected, and the amount of radioactive sulfated proteoglycans in them was measured. Each datum point is the mean of independent duplicates. On the average the deviation from the mean was 3.8%.

Were this the case, exposing the limb cells in micromass culture to the insecticide would not result in an *in vitro* expression of that teratism. The results of Greenberg and LaHam [14] and of Henderson and Kitos [13] are consistent with this latter possibility. They show that organophosphate-induced micromelia in the chick embryo can be prevented at least as well by tryptophan as by niacin. The implication of this finding is that, in causing micromelia, diazinon does not antagonize an enzyme in the metabolic pathway from tryptophan to NAD, as proposed by Moscioni *et al.* [6] and Seifert and Casida [10, 11]. Rather, it would appear to affect the availability of tryptophan which, for qualitative or quantitative reasons, is very important to the process of normal limb development.

Caplan [43] has shown that 6-aminonicotinamide (6AN), a congener of nicotinamide, causes bone malformations in developing chick embryos and "specific and unusual degradation of (limb bud) cells near or in the areas of most intense chondrogenic activity" in culture. He showed that both of these actions are reversible by the administration of nicotinamide. This action of 6AN may be due, at least in part, to its incorporation into 6-amino NAD and consequent interference with NAD-linked processes of ATP synthesis [44]. Our findings (data not reported here) agree that 6AN is a potent cytotoxic agent and that its actions on the cells in culture are effectively antagonized by nicotinamide. In contrast, we find that the inhibitory actions of diazinon on the limb bud cells in culture are not antagonized by nicotinamide.

To test whether tryptophan is of particular importance to chondrogenesis, we examined the nicotinamide and tryptophan requirements for growth and chondrogenesis of chick limb bud cells in micromass culture. The results (Table 3) indicate that exogenous nicotinamide was not needed for either of these processes. The limb cells are fully capable of fulfilling their needs for the pyridine ring portion of NAD from L-tryptophan. These findings are consistent with those of Levy and Young [45], Snell and Quarles [46], Briggs *et al.* [47], and Schweigert *et al.* [48] in which the niacin requirements of chick embryos and chickens are shown to be satisfied almost totally by tryptophan. As expected, the chick limb bud cells are totally dependent upon an exogenous supply of tryptophan. However, the tryptophan requirements of the limb bud cells for the process of proliferation and chondrogenesis were found to be quite different. This was determined by measuring the incorporation of [^{3}H]thymidine and $^{35}SO_4^=$ into the micromasses

Table 3. Effects of L-tryptophan and nicotinamide on the proliferative and chondrogenic activities of chick limb bud cells in micromass culture*

Supplements			Isotope incorporated into the acid-insoluble fraction of the cell mass (% of control)			
			Leg bud cells		Wing bud cells	
Serum	NAM (8 μM)	L-Trp (20 μM)	[^{3}H]Thymidine	$^{35}SO_4^=$	[^{3}H]Thymidine	$^{35}SO_4^=$
Dialyzed	+	+	100 (16,686)†	100 (2,547)	100 (19,191)	100 (879)
	−	+	111	109	108	93
	+	−	22	2	14	3
	−	−	12	2	8	2
Undialyzed	+	+	100 (26,284)	100 (1,843)	100 (25,195)	100 (870)
	−	+	104	101	101	100
	+	−	53	22	69	64
	−	−	60	21	74	70

* The basal medium used in these cultures was KU-1 lacking both L-Trp and NAM and supplemented to 1% with undialyzed fetal bovine serum. The culture media were renewed daily for 5 days with the indicated supplements plus either [^{3}H]thymidine or $^{35}SO_4^=$.

† The values in parentheses are the actual mean cpms for the control (100%) samples.

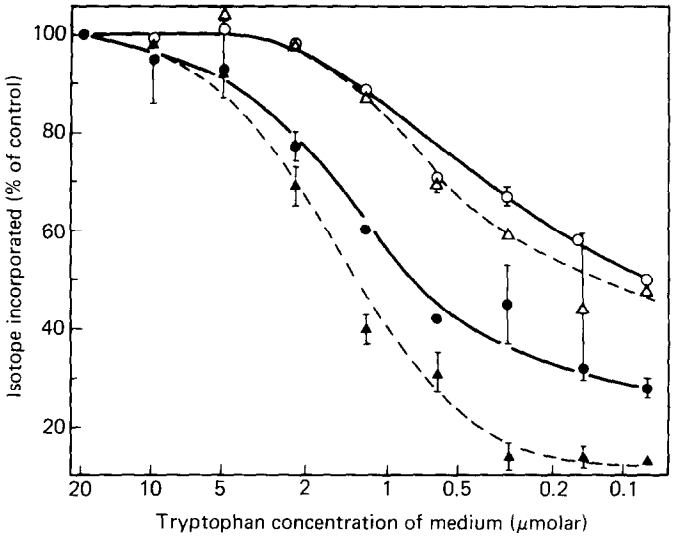

Fig. 4. Effect of L-tryptophan concentration of the nutrient medium on [^{3}H]thymidine and $^{35}SO_4^=$ incorporation into micromass cultures of chick limb bud cells. The micromass cultures were established as described in Fig. 2. After 1 day of incubation, the medium was replaced with 1% FBS-supplemented medium KU-1 lacking nicotinamide and containing the concentration of L-tryptophan indicated on the abscissa. The medium of each culture was replaced daily with the homologous nutrient fluid, and at the end of day 5 in culture the radioactivity due to the [^{3}H]thymidine and $^{35}SO_4^=$ in the trichloroacetic acid-insoluble fraction of the tissue mass was measured. The circles represent the values for the wing bud cultures and the triangles, for the leg bud cultures. The open symbols indicate the [^{3}H]thymidine incorporation values and the closed symbols, the $^{35}SO_4^=$ incorporation values. The L-tryptophan concentration values (abscissa) are presented on logarithmic scale.

over a wide range of concentrations of L-tryptophan in the otherwise sufficient nutrient medium (Fig. 4). The concentration of tryptophan needed for half-maximal chondrogenesis was approximately ten times that needed for half-maximal proliferation ([^{3}H]thymidine incorporation). Thus, one might expect the chondrogenic process to be somewhat more vulnerable than the proliferative process to a limitation in the availability of tryptophan. Consequently, proper limb formation, which depends greatly upon chondrogenesis, would be particularly sensitive to the availability of this amino acid.

Although diazinon causes pronounced micromelia of the legs of the chick embryo, it has relatively little effect on the wings [8, 13]. A possibly related response was seen in the micromass cultures in which chondrogenesis appeared to be more sensitive to the availability of tryptophan in the leg bud than in the wing bud cells (Fig. 4). In both of these tissues, proliferation was less sensitive than chondrogenesis to a limitation in the concentration of free tryptophan in the medium.

One interpretation of the results presented here is that the organophosphate insecticides cause micromelia by limiting the availability of free tryptophan to the chick embryo during the early stages of embryonic development. A deprivation of this kind could be produced in any of several ways—by inhibition of proteases in the yolk or yolk sac membrane, by impairing the transport of tryptophan into the embryo, by accelerating the destruction of tryptophan, etc.—but the net result would be the same, a deficiency of this essential amino acid. The tryptophan concentration of the chicken egg is small compared to that of any of the other essential amino acids (Table 4). In addition, during the early stages of embryogenesis, at least two major metabolic processes draw heavily upon the pool of available tryptophan, protein synthesis and NAD synthesis (Fig. 5). Were free tryptophan to become limiting as a result of the action of the insecticide, both of these metabolic processes would be adversely affected. One logical result of this condition would be a lower-than-normal tissue NAD content.

The administration of nicotinamide to the insecticide-treated embryo elevates the NAD level of the tissues above normal [12]. In rat liver, the conversion of tryptophan to NAD is under tight feedback regulation (see Fig. 5) [50, 51]. If the same

Table 4. 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

* Data from Ref. 49.

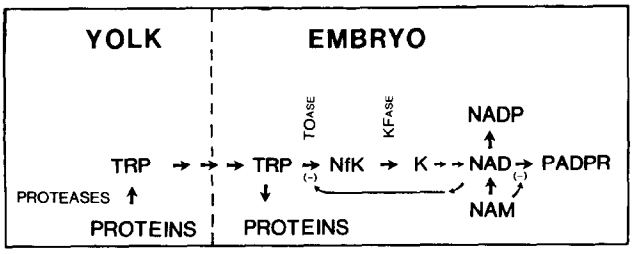

Fig. 5. A representation of selected metabolic events in the chicken egg. Abbreviations: TRP, L-tryptophan; TOase, tryptophan 2,3-dioxygenase, KFase, kynurenine formamidase; NfK, *N*-formylkynurenine; K, kynurenine; NAD and NADP, pyridine nucleotides; NAM, nicotinamide; and PADPR, poly(ADP-ribose).

regulatory expression exists in the embryonic chick tissues, these high levels of NAD would be expected to decrease the activity of tryptophan 2,3-dioxygenase to a very low value and, therefore, to extinguish the *de novo* synthesis of this coenzyme. The amount of tryptophan that would then be available for the other of the two major functions, protein synthesis, would be correspondingly increased. According to this model, the action of nicotinamide in preventing type I teratogenesis would be indirect, through its ability to maintian a close-to-normal tissue level of free tryptophan.

How, then, might 2PAM act to maintain the normal level of NAD in the tissues of diazinon-treated embryos but not prevent the type I teratisms [12]? This question may be answered if the insecticides act upon some of the yolk proteases in much the same way as they act upon acetylcholine esterase [52] and, perhaps, some yolk sac membrane esterases [30]. Ordinarily, the yolk proteins are digested by proteases to release their constituent amino acids, by far the least abundant of which is tryptophan (Table 4). Some of these proteases may be inhibited by the insecticide. The inhibition could occur within either the yolk or the yolk sac membrane, the source of at least some of the yolk proteases [31]. One effect of such an action would be to decrease the amino acid availability. 2PAM may have an effect on the organophosphate insecticide-inhibited yolk proteases similar to that which it has on insecticide-inhibited acetylcholinesterase, i.e. it may reactivate the enzyme, wholly or partially. This would tend to restore the free amino acid complement of the embryo.

Due to the low abundance of tryptophan in the tissues, even under normal circumstances, a small decrease in the amount of it due to the effect of the insecticide could be far more detrimental to the organism than a corresponding decrease in the amount of any of the other essential amino acids. Having a small K_m for tryptophan [32, 33, 53], tryptophan oxygenase would be expected to appropriate a large share of the limited pool of this amino acid. Compounding the protein synthesis problem is the fact that tryptophanyl tRNA synthetase is unstable at low concentrations of tryptophan [54]. The net result of the tryptophan deficit would be to handicap any system for which protein synthesis is quantitatively important. Unclear in this model is the basis of selective inhibition by the insecticide of leg, feather and beak development, the type I teratisms [6]. Perhaps it is due to the fact that in all three of these systems very large amounts of structural proteins must be synthesized for extracellular functions.

Acknowledgements—The authors would like to thank Ms. Hae-Young Kim for her excellent technical assistance and the U.S. Environmental Protection Agency, Research Triangle Park, NC, U.S.A., for the insecticides.

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