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STRUCTURE–ACTIVITY AND METABOLISM STUDIES ON ORGANOPHOSPHATE TERATOGENS AND THEIR ALLEVIATING AGENTS IN DEVELOPING HEN EGGS WITH SPECIAL EMPHASIS ON BIDRIN*

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Abstract—Bidrin (3-hydroxy-*N*,*N*-dimethyl-*cis*-crotonamide dimethyl phosphate) is teratogenic in hen eggs when injected into the yolk sac at a dosage of 0·03 mg per egg or higher; the *cis*-crotonamide isomer is the active teratogenic component. The response is dose dependent and the effect is at a maximum with injection on or about day 4 of incubation. Signs include straight legs, micromelia, short spine, wry neck, parrot beak, abnormal feathering, edema and, more rarely. syndactyly and visceral hernia. Many other organophosphates are teratogenic, including the Bidrin metabolites that are not hydrolysis products, but no simple structure–activity relationship is evident.

Nicotinic acid, nicotinamide and certain of their precursors, analogs and derivatives are active alleviating agents for Bidrin-induced teratogenesis. The active nicotinic acid analogs are those that may be converted to nicotinic acid or nicotinamide, while the inactive analogs probably are not converted to these products. Treatment with nicotinamide prior to incubation and up to day 10 of incubation greatly alleviates teratogenesis. At equimolar levels, nicotinamide and pyridine nucleotide coenzymes show the same alleviating activity, the only exception to this relationship being the 3-acetylpyridine analog of the coenzyme. It is not known whether the active alleviating agent is a pyridine nucleotide or any one of several 3-pyridyl compounds.

Bidrin is rapidly biodegraded in the egg; successive *N*-demethylation occurs through the *N*-hydroxymethyl analogs to yield the unsubstituted amide. Extensive hydrolysis is also involved. Bidrin metabolism between days 4 and 10 of incubation is not affected by nicotinamide; thus, the alleviating agents do not appear to act by altering the metabolism of the teratogenic agents. The metabolism of nicotinic acid is unaffected by the teratogen, Bidrin. Therefore, the alleviating action of nicotinic acid analogs for Bidrin teratogenesis results from mechanisms different from those involving altered rates of metabolism of the teratogen or the alleviating agents.

MANY CHEMICALS and drugs produce a morphologic disturbance of such a nature that the embryo does not develop normally but survives to the later stages of development. Certain organophosphate insecticide chemicals show teratogenic activity when injected into hen eggs before or within the early period of incubation,[1–7] and the teratogenic signs are often relieved by administration of either nicotinamide or nicotinamide-adenine dinucleotide (NAD).[1–3] The cholinergic system does not appear

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to be involved either in the teratogenesis induced by anticholinesterases and other neuroactive agents or in the relief of the teratogenesis by nicotinamide or NAD. However, anticholinesterase agents create a permeability barrier which prevents the transport of unhydrolyzed acetylcholine from the yolk to the embryo. The growth abnormalities resulting from certain esterase inhibitors are possibly the result of nutritional deficiencies of the embryo, due to failure of the embryo to obtain ester hydrolysis products from the yolk.[2]

With emphasis on vinyl phosphate insecticide chemicals, and particularly on 3-hydroxy-*N*-*N*-dimethyl-*cis*-crotonamide dimethyl phosphate (Bidrin), studies were undertaken on the time sequence and structural requirements for teratogenesis using hen eggs. Similar studies were made on those compounds that alleviate Bidrin-induced teratogenesis. The effect of a teratogenic agent (Bidrin) on the metabolism of two alleviating agents (nicotinic acid and nicotinamide) and the effect of nicotinamide on the metabolism of Bidrin were examined in an attempt to elucidate the mode of teratogenic action of organophosphate insecticide chemicals.

MATERIALS AND METHODS

Chemicals and treatment of eggs. Bidrin samples, labeled with ^{32}P or *N*-methyl-^{14}C, were provided as the *cis*-crotonamide isomer by the Shell Development Co., Modesto, Calif., and by R. E. Menzer.[8] Unless stated otherwise, the technical grade of Bidrin (75% *cis*-crotonamide isomer) was used in the teratogenesis tests; the other major constituent of the technical mixture, the *trans*-crotonamide isomer, has little teratogenic activity. Nicotinic acid -7-^{14}C, nicotinamide-7-^{14}C, NAD and NADP (nicotinamide-adenine dinucleotide phosphate) were from Calbiochem Corp., Los Angeles, Calif. Dowex 1 × 8 resin was converted from the chloride form to the formate form by batch washing with 4 N sodium formate. Sources for most of the compounds involved in the teratogenesis testing are given in the footnotes to Tables 1 and 3, while those for other chemicals used are given in earlier reports from this laboratory.[2, 3]

The methods used for treatment of the fertile white leghorn eggs, by injection of the compounds into the yolk, and for assay of teratogenic activity were, in general, those previously reported.[2, 3] Ten eggs were involved in each treatment and at least two replicates were made in each case. Since rating of teratogenic signs as to type and intensity is difficult, photographs were taken and used for comparison and reevaluation of the gross appearance of the 18- to 21-day-old embryos and of young chicks within and between experiments. Partial quantification was achieved with the embryos by measurements, as follows: (1) embryo weight, (2) leg length, from the femoraltibial joint to the tip of the toes, (3) body length, from the tip of the spine to the top of the skull. Records were maintained on the incidence of parrot beak, "straight legs" in which the joints are rigid and the legs extended, poor feathering, gross edema and wry neck. Results are also presented in the following manner, based on the measurements and photographs: —, signs same as control; + to +++, intermediate degrees of teratogenesis; ++++, most severe manifestation.

Metabolism of Bidrin. Each group of eggs was injected on day 4 of incubation with 0·1 ml of a saline solution containing either 1 mg radioactive Bidrin or 1 mg radioactive Bidrin plus 1 mg nicotinamide. The radioactive dose per egg was about 0·35 μc Bidrin-^{32}P and 0·35 μc Bidrin-*N*-methyl-^{14}C; these two labeled components were

administered as a mixture in the same solution. A comparable series of eggs within the same experiment was injected with unlabeled materials, so that embryos at 21 days of incubation were available for observation of signs as they relate to alleviation by nicotinamide and possible effects of the labeled materials on the interaction.

Analyses were made on eggs immediately after injection (on day 4 of incubation) and at days 6, 8 and 10 of incubation. Radiophosphorus levels were determined for the shell and for fractions derived from both the yolk and the embryo, except for the group immediately after treatment where the yolk and the embryo were combined for analysis. Replication of the analyses was as follows, for each of the appropriate groups with or without nicotinamide: two replicates of one egg each for the combined yolk and embryo; three replicates of one egg each for the yolk alone; two replicates of three eggs each for the embryo alone; four or six replicates of one egg each for the shell.

For extraction, water was added to the yolk or yolk plus embryo of individual eggs, or to a group of three embryos to make a total volume of 100 ml. The mixture was homogenized in a Lourdes Multi-Mix blender, after which 150 ml acetonitrile and 10 g Celite (Johns Manville's Hyflo Super Cel) were added and the mixture was again blended; acetonitrile and Celite aid in protein precipitation and flocculation of the precipitate respectively. After filtration through Whatman No. 2 filter paper, the precipitate was blended with 100 ml chloroform and again filtered to yield the "insoluble fraction" and a filtrate which was added to the original aqueous acetonitrile filtrate. Mixing and equilibration of the combined filtrates in a separatory funnel resulted in an acetonitrile–chloroform phase and an aqueous phase which were separated. The aqueous phase was re-extracted with 70 ml acetonitrile–chloroform (1:1) mixture and the organic phase was added to the previous acetonitrile–chloroform mixture. The "organosoluble" products appear in the acetonitrile–chloroform mixture and the "water-soluble" products are in the aqueous phase. The acetonitrile–chloroform mixture was dried over sodium sulfate, filtered and concentrated to a 10-ml volume. Total radiophosphorus determinations were made on each of these fractions and on the pulverized shells (which were not extracted) with an end-window Geiger–Müller counter.

A partition column was used to resolve Bidrin and its organosoluble metabolites.[8] To individual columns were added each of the organosoluble fractions and, for co-chromatography, unlabeled samples of Bidrin, des-*N*-methyl Bidrin and Bidrin amide (compounds 2, 6 and 5 respectively, Table 1). Elution from the Celite-water column was accomplished with, in order, 200 ml hexane, 400 ml hexane–chloroform (2:1) mixture, 300 ml hexane–chloroform (1:1) mixture, 400 ml chloroform, and 200 ml methanol; 20-ml fractions were collected. Radiocarbon and radiophosphorus were separately counted on an aliquot from each tube, by using the appropriate channel settings on the Tricarb liquid scintillation spectrometer (model 314EX, Packard Instrument Co., La Grange, Ill.), and the total organophosphate amount in each fraction was determined colorimetrically.[8] Certain fractions, representing a resolved metabolite, were analyzed for formaldehyde-^{14}C released on addition of acid by a procedure involving dicoumarol-methylene-^{14}C formation as previously described.[8] Also, co-chromatography patterns and the $^{14}C/^{32}P$ ratio in individual fractions were determined for use in metabolite characterization.

Metabolism of nicotinic acid-7-^{14}C and nicotinamide-7-^{14}C. Living eggs, previously treated on day 2 of incubation with 0·1 ml water or water containing 1 mg Bidrin were injected on day 4 or 5 of incubation with 10 or 1010 μg nicotinic acid-7-^{14}C or 6·5 μg nicotinamide-7-^{14}C in 0·1 ml water per egg. Each egg within each experiment received an identical level of total radioactivity, which generally was about 1 μc. On termination of the incubation period with the radiolabeled substrate, the embryo was quickly excised free of attached membranes, washed to remove yolk material, blotted to remove surface liquid and finally the isolated embryo was weighed. Two to five embryos were immersed in 3 ml of ice-cold 70% perchloric acid (w/v) per embryo, quickly sliced with scissors and homogenized in a precooled glass tissue grinder with a Teflon pestle. This technique was used in order to inactivate rapidly all enzymes that might degrade NAD or intermediary metabolic products. After sedimentation of the insoluble material by centrifugation at 10,000 *g* for 20 min at 0° and re-extraction of the residue with 5 ml of 70% perchloric acid, the combined supernatant fractions were assayed for total radioactivity. The extracts were then either rapidly frozen and stored at − 10° or were immediately purified[9] for further analysis by paper or column chromatography.

Paper chromatography was used to separate metabolites from eggs treated with either nicotinic acid-7-^{14}C or nicotinamide-7-^{14}C. Portions of the purified extracts were concentrated and applied as a band to Whatman No. 1 chromatography paper strips of 2·5 × 30 cm. Development of the chromatograms in a descending direction for 25 cm gave the following range of R_f values with the indicated solvent systems:

Compounds	Solvent systems	
	95% Ethanol–1 M ammonium acetate adjusted to pH 5 with hydrochloric acid (7:3) mixture[9]	Water–butanol-acetone (10:9:1) mixture, upper phase[10]
NAD and/or NADP	0·15–0·24	0·00
Nicotinamide mononucleotide (NMN)	0·37–0·47	0·00–0·01
Nicotinic acid	0·74–0·83	0·33
Nicotinamide	0·74–0·83	0·60–0·80

Resolved radiolabeled products on the chromatograms were detected and quantitatively analyzed by using a Vanguard model 880 Autoscanner with the Automatic Data System (Vanguard Instrument Co., La Grange, Ill.). Known compounds were detected for co-chromatography purposes as follows: pyridinium compounds (NAD, NADP and NMN) but not nicotinic acid or nicotinamide fluoresce under ultraviolet light when treated with aqueous ammonia–2–butanone (1:1) mixture, while only nicotinic acid and nicotinamide, but not the pyridinium compounds, give a yellow color with cynanogen bromide and *p*-aminobenzoic acid reagents.[11]

Ion-exchange column chromatography with Dowex 1 × 8 resin (formate form), involving stepwise elution with increasing concentrations of formate, was used for more complete identification of the metabolites of nicotinamide-7-^{14}C. This system separates nicotinamide, NMN, and NAD plus NADP into three distinct fractions, eluting in the indicated order. The labeled nucleotide fractions were subsequently

hydrolyzed with acid or alkali; the presence of nicotinic acid-^{14}C or nicotinamide-^{14}C or of both in the hydrolysates was analyzed by radioactive scanning and co-chromatography with known compounds, by using the spot tests described above.[9, 11]

In the study with nicotinamide-7-^{14}C, the results are the average from two experiments with five embryos per treatment in each experiment. In the nicotinic acid-7-^{14}C study, two embryos were combined for extraction and analysis at each time interval and with each treatment.

RESULTS

Structure–activity and time-sequence relations for teratogenesis. The organophosphates assayed and the results obtained are given in Tables 1 and 2. Table 1 gives the structures and the numbering system used. Percentage values are rounded off to the closest ten because of the small number of embryos involved in each experiment. None of the injection solvents, including dimethyl sulfoxide and ethanol, is teratogenic under the conditions and doses used in these tests.

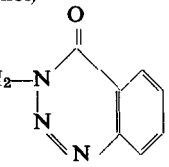
Injection of technical Bidrin on day 4 of incubation results in the following signs: reduced hatch and shortening of the legs at 0·03 mg per egg; shortening of the body and appearance of parrot beak, straight legs, abnormal feathering and edema at 0·10 mg; increased incidence and intensity of these abnormalities at higher doses. At these higher doses, visceral hernia and syndactyly also appear but these signs are not tabulated because, at the doses generally used throughout this study, their occurrence is not common. The length of the legs gives the best graded response to increasing dosages of Bidrin, the active ingredient of which is the *cis*-crotonamide isomer. The *trans*-crotonamide isomer does not produce gross abnormalities even at 10 mg per egg. The chloro analog (compound 4), a potential impurity in technical Bidrin, is of lower teratogenic activity than Bidrin. The *cis*-crotonamide and technical preparations of Bidrin amide and des-*N*-methyl Bidrin (compounds 5 and 6, respectively) are of a potency similar to that of Bidrin; therefore, only the results for the *cis*-crotonamide isomers are tabulated. Replacement of the dimethylamide group by the diethylamide group reduces the activity both in the Bidrin series (compound 7 vs. compound 1) and the chloro Bidrin series (compound 8 vs. compound 4), while the diethylphosphate analog of Bidrin (compound 9) appears to be even more active than Bidrin in shortening the length of the body. Teratogenesis does not result with a 1-mg dose of the carboxylic acid analog of Bidrin (compound 10) or with its α-methylbenzyl ester (compound 12), but does occur at high doses of its methyl ester (compound 11). The carboethoxy ester, as the diethyl phosphate analog (compound 13), is also teratogenic at high doses. Chlorovinyl phosphates (compounds 14 and 15) are teratogenic, but only at the higher dosage levels.

Each of the tributyl phosphate and phosphorotrithioate compounds (16 and 17 respectively) is slightly teratogenic at high levels. TOCP (compound 18) does not induce embryonic abnormalities and no muscular weakness or other abnormalities are observed among the chicks hatching from eggs treated with 50 mg TOCP at days 4, 7, 11 or 13 of incubation, although a rather high mortality occurs. At the doses used, the thionophosphorus compounds, malathion, Guthion and EPN (compounds 19, 20 and 21 respectively), are not teratogenic. Parathion (compound 22), a phosphorothionate, is highly teratogenic and its effect is particularly evident in the marked shortening of the spine. The various acetoacetamides (compounds 23–26, which are

TABLE 1. TERATOGENIC SIGNS OF EMBRYOS AT DAY 21 FROM EGGS INJECTED ON DAY 4 OF INCUBATION WITH BIDRIN AND RELATED CHEMICALS

Compound*	Dose (mg/egg)	Embryo† No. of surviv.	Embryo† Wt. (g)	Embryo† Length (cm) Body	Embryo† Length (cm) Legs	Incidence (%) of† Parrot beak	Incidence (%) of† Str. legs	Incidence (%) of† Abnormal feathers	Hatch (%)†	Teratogenic signs‡
Control (saline)		40	26 ±1·5§	9·1 ±0·8§	7·1 ±0·4§	0	0	0	95	–
1. $(CH_3O)_2P(O)OC(CH_3)=CHC(O)N(CH_3)_2$ (Bidrin, technical mixture of isomers)	0·01	23	22	8·5	7·0	0	0	0	80	–
	0·03	25	18	8·0	6·0	0	0	0	60	+
	0·10	25	21	7·2	5·2	20	10	10	30	+++
	0·30	42	21	6·3	3·9	50	30	60	0	++++
	1·00	10	14	5·0	3·2	70	40	100	0	++++
2. $(CH_3O)_2P(O)OC(CH_3)\overset{c}{=}CHC(O)N(CH_3)_2$ (Bidrin, alpha or *cis* isomer)	0·01	16	27	8·4	6·8	0	0	0	90	–
	0·10	10	23	7·4	4·3	90	20	40	0	+++
	1·00	15	20	5·0	3·3	60	40	100	0	++++
3. $(CH_3O)_2P(O)OC(CH_3)\overset{t}{=}CHC(O)N(CH_3)_2$ (Bidrin, beta or *trans* isomer)	0·01	16	22	8·3	7·3	0	0	0	90	–
	0·10	17	18	8·0	7·4	0	0	0	80	–
	1·00	14	29	8·5	7·5	0	0	0	90	–
	10·00	12	22	8·0	6·5	0	0	0	80	–
4. $(CH_3O)_2P(O)OC(CH_3)=C(Cl)C(O)N(CH_3)_2$ (SD 5562)	1·00	16	25	6·9	4·3	70	60	50	0	+++
5. $(CH_3O)_2P(O)OC(CH_3)\overset{c}{=}CHC(O)NH_2$ (Bidrin amide, alpha or *cis* isomer)	0·01	15	20	7·5	6·7	0	0	0	90	–
	0·10	10	20	6·6	4·2	70	50	50	0	+++
6. $(CH_3O)_2P(O)OC(CH_3)\overset{c}{=}CHC(O)NHCH_3$ (Des-*N*-methyl Bidrin, alpha or *cis* isomer)	0·01	16	23	8·0	6·8	0	0	0	90	–
	0·10	19	20	7·0	4·2	60	50	30	10	+++
7. $(CH_3O)_2P(O)OC(CH_3)=CHC(O)N(C_2H_5)_5$ (SD 5911)	1·00	18	21	5·6	4·0	80	30	90	0	++++
8. $(CH_3O)_2P(O)OC(CH_3)=C(Cl)C(O)N(C_2H_5)_2$ (Phosphamidon)	3·00	21	23	5·8	4·7	40	30	0	0	+++
	7·00	10	20	6·0	3·6	100	100	10	0	++++
9. $(C_2H_5O)_2P(O)OC(CH_3)=CHC(O)N(CH_3)_2$ (SD 2244)	1·00	10	13	3·8	3·4	80	40	80	0	++++
10. $(CH_3O)_2P(O)OC(CH_3)=CHC(O)OH$ (SD 4455)	1·00	14	29	8·4	7·5	0	0	0	90	–
11. $(CH_3O)_2P(O)OC(CH_3)=CHC(O)OCH_3)_2$ (Mevinphos or Phosdrin)	1·00	10	20	7·5	6·2	0	0	0	50	–
	10·00	13	19	6·0	4·6	50	40	10	0	+++
12. $(CH_3O)_2P(O)OC(CH_3)=CHC(O)OCH(CH_3)C_6H_5$ (Ciodrin) (ethanol)	1·00	14	25	9·0	7·0	0	0	0	90	–
13. $(C_2H_5O)_2P(O)OC(CH_3)=CHC(O)OC_2H_5$ (OS 1808) (ethanol)	1·00	11	23	8·5	6·6	0	0	0	60	–
	3·00	10	19	6·2	4·6	0	0	0		++
14. $(CH_3O)_2P(O)OCH=CCl_2$ (Dichlorvos or DDVP)	10·00	10	20	6·9	4·0	80	70	20	0	+++

TABLE 1.—*continued*

Compound*	Dose (mg/egg)	Embryo† No. of surviv.	Wt. (g)	Length (cm) Body	Legs	Incidence (%) of† Parrot beak	Str. legs	Abnormal feathers	Hatch (%)†	Teratogenic signs‡
15. $(C_2H_5O)_2P(O)OCH{=}CHCl$ (OS 1836) (ethanol)	1·00	11	24	6·8	4·5	50	40	0	50	+++
16. $(CH_3CH_2CH_2CH_2O)_3P(O)$ (Tributylphosphate)	5·00	10	15	7·1	5·0	0	0	0	20	+
17. $(CH_3CH_2CH_2CH_2S)_3P(O)$ (DEF)	5·00	10	12	6·8	4·8	0	0	0	20	++
18. $(2\text{-}CH_3\text{-}C_6H_4O)_3P(O)$ (TOCP)	10·00	16	22	9·3	6·7	0	0	0	60	—
19. $(CH_3O)_2P(S)SCH[C(O)OC_2H_5][CH_2C(O)OC_2H_5]$ (Malathion) (ethanol)	1·00	17	20	8·2	6·2	0	0	0	70	—
20.  (Guthion) (DMSO)	1·00	13	19	8·0	6·2	0	0	0		—
21. $(C_6H_5)(C_2H_5O)P(S)OC_6H_4\text{—}4\text{—}NO_2$ (EPN) (DMSO)	1·00	23	18	8·0	6·2	0	0	0	60	—
22. $(C_2H_5O)_2P(S)OC_6H_4\text{—}4\text{—}NO_2$ (Parathion) (alcohol)	1·00	10	15	5·0	4·5	10	0	30	0	++++
23. $CH_3C(O)CH_2C(O)N(CH_3)_2$ (SD 6167)	1·00	10	28	8·0	7·2	0	0	0	90	—
24. $CH_3C(O)CH(Cl)C(O)N(CH_3)_2$ (SD 5722) (DMSO)	1·00	14	29	8·5	7·8	0	0	0	100	—
25. $CH_3C(O)C(Cl)_2C(O)N(CH_3)_2$ (SD 6166) (DMSO)	1·00	15	29	8·5	7·5	0	0	0	90	—
26. $CH_3C(O)CH_2C(O)NHCH_3$ (SD 9112)	1·00	15	29	7·9	7·0	0	0	0	90	—

* Sources: compounds 1–15 and 23–26 from Shell Development Co., Modesto, Calif.; 16 and 18 from Eastman Organic Chemicals, Rochester, N.Y., 17 and 20 from Chemagro Corp., Kansas City, Mo.; 19 and 22 from American Cyanamid Co., Princeton, N.J.; and 21 from E. I. Du Pont de Nemours Co. Inc., Wilmington, Del. Solvents other than saline are given in parentheses after test chemical.

† Each treatment was replicated at least twice, with 10 eggs in each replicate. Measurements and percentages are based on the number of surviving embryos that developed to hatching time, as indicated (No. surviv). Because of the low number of embryos, percentages should be interpreted as nil, low or high activity of the compound. A percentage hatch of zero indicates that the embryos developed to hatching time but did not hatch. Str. legs = straight legs.

‡ Signs are rated from not detectable (−) to severe manifestation (++++).

§ Average values and S.D.'s.

TABLE 2. EFFECT OF TIME OF ADMINISTRATION ON THE TERATOGENIC SIGNS AT DAY 21 FROM EGGS INJECTED WITH BIDRIN OR BIDRIN PLUS NICOTINAMIDE

Bidrin		Nicotinamide		Embryo*				Incidence (%) of*				
Dose (mg)	Day of inject.	Dose (mg)	Day of inject	No. surviv.	Wt. (g)	Length (cm)		Parrot beak	Str. legs	Abnormal feathers	Hatch (%)*	Teratogenic Signs†
						Body	Legs					
0·10	0			10	20	7·0	6·5	0	0	0	50	−
0·10	2			14	23	7·0	6·3	10	10	0	50	+
0·10	4			23	21	6·9	5·1	20	20	10	40	+++
0·10	6			19	23	7·0	5·6	0	0	0	50	+
0·10	8			14	21	7·5	7·0	0	0	0	70	−
0·10	10			15	27	8·3	7·6	0	0	0	70	−
1·00	0			10	21	5·8	3·3	90	70	100	0	++++
1·00	4			18	15	5·0	3·2	90	60	100	0	++++
1·00	10			11	25	7·1	5·7	0	0	0	0	++
1·00	12			8	24	7·8	6·3	0	0	0	0	+
0·30	4			42	21	6·3	3·9	50	30	60	0	++++
0·30	4	0·10	0	10	27	7·1	6·5	0	0	0	0	+
0·30	4	0·10	2	11	26	6·7	6·5	0	0	0	0	+
0·30	4	0·10	4	13	25	6·9	6·3	0	0	0	0	+
0·30	4	0·10	6	14	25	6·9	6·4	0	0	0	0	+
0·30	4	0·10	8	16	27	6·9	6·1	0	0	0	0	+
0·30	4	0·10	10	16	26	6·2	6·1	0	0	0	0	+
0·30	4	0·10	12	15	23	6·2	4·8	40	30	20	0	+++
0·30	4			13	23	6·4	4·2	60	40	70	0	++++
0·30	4	0·01	4	16	24	6·8	5·3	0	0	50	0	++
0·30	4	0·03	4	15	24	6·9	5·9	0	0	10	0	++
0·30	4	0·10	4	25	25	7·2	6·5	0	0	0	0	+
0·30	4	0·30	4	13	26	7·2	6·5	0	0	0	0	+
0·30	4	1·00	4	23	24	7·0	6·5	0	0	0	0	+

* Each treatment was replicated at least twice, with 10 eggs in each replicate. Measurements and percentages are based on the number of surviving embryos that developed to hatching time, as indicated (No. surviv.). Because of the low number of embryos, percentages should be interpreted as nil, low or high activity of the compound. A percentage hatch of zero indicates that the embryos developed to hatching time but did not hatch. Str. legs = straight legs.

† Signs are graded from not detectable (−) to severe manifestation (++++).

Bidrin metabolites and related derivatives) are not teratogenic under the conditions of this test.

Appropriate timing of Bidrin injection is critical for maximal teratogenic effects (Table 2). When the lower dose (0·1 mg per egg) is injected, day 4 of incubation is the critical time at which teratogenic signs are most severe. On day 0 to day 2 of incubation, the effect of Bidrin on the leg length is negligible, but parrot beaks and straight legs are observed on injection after day 2 of incubation. On day 4, the full complement of tabulated signs is induced, but on day 6 only micromelia is produced. A 0·1-mg dose of Bidrin is no longer teratogenic after day 8 of incubation. With a 1-mg Bidrin dose, teratogenic signs are produced with injections up until day 12 of incubation (induction of wry neck) and, with this dose, the effects are almost the same for injection at day 0 or 4 of incubation.

In preliminary studies with other compounds, a similar critical period for maximal effects at day 4 occurs with Bidrin amide and des-*N*-methyl Bidrin. Chemicals which are unstable in the egg (based on esterase inhibition studies with such compounds as mevinphos and dichlorvos[2]) produce little if any abnormality when injected prior to incubation even at a level of 10 mg per egg, whereas, when injected on the fourth day of incubation (Table 1), they produce marked abnormalities. Teratogenic dose levels for other compounds injected prior to incubation are as follows: 0·3 mg for Bidrin and compounds 6, 7 and 9; 3 mg for compounds 4 and 15; 10 mg for compounds 8, 10 and 13; inactive at 10 mg for compound 12 and for 2,2-dichloro-1-methoxyvinyl dimethyl phosphate and 2-chloro-1-(2,4-dichlorophenyl)vinyl diethyl phosphate. Other inactive compounds, when injected at a dose of 10 mg per egg prior to incubation, are: des-*O*-methyl Bidrin (a product from Bidrin hydrolysis); diethyl phosphate and ethyl phosphate, a hydrolysis product and its analog, respectively, of compound 9, a potent teratogenic Bidrin analog.

Daily intramuscular injection of adult hens with sublethal Bidrin doses, ranging from 0·8 to 6·4 mg per hen, in the presence of 2 mg atropine for the highest dose, results in cessation of egg-laying within 4 days. Only two eggs were collected which developed to hatching time and these embryos were normal; the Bidrin doses for the hens which laid these eggs were 0·8 and 1·6 mg per hen per day.

Other neuroactive materials and known teratogens were also assayed for comparison. No teratogenic effect results from injection of 10 mg atropine on day 4 of incubation. Ten mg pilocarpine on day 4 of incubation produces parrot beak, straight legs and micromelia. Tetramethylammonium chloride (10 mg, day 4) and decamethonium bromide (5 mg, day 4) affect mainly the length of the body, but carbamylcholine chloride (5 mg, day 4) affects both the spine length and the leg length. Sulfanilamide at a dose of 2 mg per egg at day 4 of incubation results in parrot beak, straight and short legs (3·8 cm), feather inhibition and the embryos have a very long neck. A dose of 0·2 mg at day 4 of incubation results only in a shortening of the legs. Eserine at a dose level of 0·5 mg per egg at day 0, 2 or 4 of incubation causes abnormalities similar to those produced by 0·3 mg Bidrin at day 4 of incubation. The same eserine dose does not produce abnormalities when injected on day 6 or later.

Structure–activity and time-sequence relations for alleviating agents. The alleviating agents assayed and the results obtained are given in Tables 2 and 3. Table 3 gives the structures and numbering system used. Nicotinic acid, nicotinamide and certain of their analogs, precursors and derivatives are active alleviating agents (Table 3).

TABLE 3. EFFECT OF NICOTINAMIDE ANALOGS AND OTHER POSSIBLE ALLEVIATING AGENTS ON TERATOGENIC SIGNS AT DAY 21 FROM EGGS INJECTED SIMULTANEOUSLY ON DAY 4 WITH 0·3 mg BIDRIN AND THE TEST CHEMICAL

Test chemical*	Teratogenic signs†	Test chemical*	Teratogenic signs†
Control (saline)	−		
Bidrin alone	++++		
3-Pyridyl compounds, with 3-substituent as indicated (0·8 μmole/egg)			
1. C(O)OH (aqu. $NaHCO_3$)	+	16. $C(O)N(C_2H_5)_2$	+
2. $C(O)OCH_3$ (DMSO or ethanol)	+	17. $C(O)NHCH_2C(O)OH$ (aqu. $NaHCO_3$)	++++
3. $C(O)OC_2H_5$ (DMSO or ethanol)	+	18. C(O)NHOH	+
4. $C(O)OC_3H_7$ (DMSO or ethanol)	+	19. $C(O)CH_3$	++++
5. $C(O)OCH(CH_3)_2$ (DMSO or ethanol)	+	20. C(O)H	+
6. $C(O)OC_4H_9$ (DMSO or ethanol)	+	21. CH_2OH	+
7. $C(O)OC_6H_{13}$ (DMSO or ethanol)	+	22. CH_3	++++
8. $C(O)OCH_2C_6H_5$ (DMSO or ethanol)	+	23. CH_2NH_2	+
9. $C(O)OC_6H_5$ (DMSO or ethanol)	+	24. CH_2NHCH_3‡	+
10. $C(O)OC_6H_4$-2-OCH_3 (DMSO or ethanol)	+	25. $CH_2NR_1R_2$§	++
11. $C(O)NH_2$	+	26. $CH_2C(O)OH$(aqu.$NaHCO_3$)	++++
12. $C(O)NHCH_3$	+	27. CH_2Cl(hydrochloride)	++++
13. $C(O)NHC_2H_5$	+	28. CH=NOH	++
14. $C(O)NHCH_2C_6H_5$ (ethanol)	+	29. CH=NOR‖	++++
15. $C(O)N(CH_3)_2$	+	30. C≡N¶	++++
		31. $S(O)_2OH$ (sodium salt)	++++
2- and 4-Pyridyl compounds (0·8 μmole/egg)			
32. 4—C(O)OH (aqu. $NaHCO_3$)	++++	35. 2-C(O)OH (aqu. $NaHCO_3$)	++++
33. 4—$C(O)NH_2$	++++	36. 2—CH=NOH	++++
34. 4—CH=NOH	++++		
N^1-substituted 3-pyridyl compounds (0·8 μmole/egg)			
37. N^1-CH_3-3-C(O)OH (hydrochloride)	++++	39. N^1-Oxide-3-$C(O)NH_2$	+
38. N^1-CH_3-3-$C(O)NH_2$ (iodide)	++++	40. N^1-Oxide-3-CH_3	++++
		41. N^1-Oxide-3-$C(O)NHNH_2$	++++
Ring-substituted nicotinamide analogs (0·8 μmole/egg)			
42. 6-Cl-3-$C(O)NH_2$ (DMSO)	++++	43. 4-Hydroxy-3-C(O)OH (aqu. $NaHCO_3$)¶	++++
		44. 6-Hydroxy-3-C(O)OH (aqu. $NaHCO_3$)	++++

TABLE 3.—*continued*

Test chemical*	Teratogenic signs†	Test chemical	Teratogenic signs†
Nicotinamide Precursors			
45. Tryptophan (aqu. $NaHCO_3$)		46. Anthranilic acid (aqu. $NaHCO_3$)	
5 μmole/egg	+	7 μmole/egg	+++
50 μmole/egg	+	70 μmole/egg	++
		47. Indole (DMSO)	
		8 μmole/egg	++++
		40 μmole/egg	++++
N^1-methyl pyridones (0·8 μmole/egg)			
48. N^1-CH_3-2-pyridone-3-carboxamide	++++	51. N^1-CH_3-2-pyridone-3-phenylcarboxylate (DMSO)	++++
49. N^1-CH_3-4-pyridone-3-carboxamide	++++	52. N^1-CH_3-6-pyridone-3-phenylcarboxylate (DMSO)	++++
50. N^1-CH_3-6-pyridone-3-carboxamide	++++		
Nicotinamide coenzymes and analogs (0·8 μmole/egg)			
53. NAD	+	59. 3-Acetylpyridine NAD	++++
54. NADH	+	60. Deamino NAD	+
55. NADP	+	61. 3-Pyridinealdehyde deamino NAD	+
56. NADPH	+	62. 3-Acetylpyridine deamino NAD	++++
57. Thionicotinamide NAD	+	63. Adenosine-5-mononicotinate	+
58. 3-Pyridinealdehyde NAD	+	64. Nicotinamide mononucleotide	+

* Sources: compounds 1–4, 6, 9, 11–16, 20–24, 26–30 and 32–41 from Aldrich Chemical Co., Inc., Milwaukee, Wisc.; 5, 7, 8 and 10 from International Chemical and Nuclear Corp., City of Industry, Calif.; 17–19, 31, 45–47 and 63 from Nutritional Biochemicals Corp., Cleveland Ohio; 25 from Dr. I. Yamamoto) Dept. of Entomology, University of California, Berkeley; 26, 42, 44, 50, 53–57, 59 and 62 from Calbiochem, Los Angeles, Calif.; 58, 60 and 61 from Mann Research Laboratories, Inc., New York, N.Y.; 43, 48, 51 and 52 from Dr. H. W. Chambers, Dept. of Entomology, University of California, Berkeley; 49 from Professor T. Wieland, Dept. of Chemistry, University of Frankfurt, Germany; and 64 from Pabst Laboratories, Milwaukee, Wis. Solvents other than saline, as well as salt forms, are given in parentheses after test chemical.

† Signs are rated from not detectable (−) to severe manifestation (++++). Each treatment was replicated twice, with 10 eggs in each replicate.

‡ In a single experiment, the *N*-ethyl analog was active but apparently less active than the *N*-methyl analog.

§ Average of results with one experiment each on 3-picolyldimethylamine, 3-picolyldiethylamine, *N*-(3-pyridylmethyl)piperidine and *N*-(3-pyridylmethyl)-morpholine, each of which was of similar activity. Nicotine was inactive.

‖ Average of results, with one experiment each on propyl, isoamyl, benzyl and phenethyl ethers, each of which was not active as an alleviating agent.

¶ Based on the general appearance of the embryos and on their leg length, the signs for the test compound and Bidrin appear to be more severe than with Bidrin alone; the test chemicals were not assayed for teratogenesis in the absence of Bidrin.

The following vitamins are not active as alleviating agents when injected at 1 mg per egg simultaneously with 0·3 mg Bidrin on day 4 of incubation: thiamine hydrochloride, riboflavin-5-monophosphate diethanolamine, pyridoxine hydrochloride, sodium D-pantothenate, biotin, folic acid, inositol and α-tocopheryl acetate.[3] Under the same test conditions, the following purines, pyrimidines and nucleosides at 10 mg per egg are not active as alleviating agents: uracil, orotic acid, cytosine, thymine, adenine, guanine hydrochloride, xanthine, uridine, thymidine, adenosine, guanosine, cytidine and deoxycytidine hydrochloride; deoxyguanosine is lethal at this assay level.[3]

There are also other untabulated results for assays on potential alleviating agents. Adenosine triphosphate at a level of 10 mg per egg does not alleviate the effect of 0·3 mg Bidrin by simultaneous injection on day 4 of incubation. A dose of 10 mg per egg of choline or acetylcholine, injected simultaneously with 0·1 mg Bidrin, 0·15 mg eserine or 10 mg mevinphos on day 4 of incubation, results in no change in the teratogenic signs produced. When injected in the absence of Bidrin, acetylcholine or choline at 10 mg per egg on day 4 of incubation does not induce abnormalities of the embryos. A 10-mg dose of betaine or betaine aldehyde or betaine aldehyde diethylacetal, injected simultaneously on day 4 with 0·3 mg Bidrin, induces no alleviation of the abnormalities. Also inactive under these conditions are L-methionine and certain of its analogs (D-methionine, DL-methionine sulfone, L-methionine DL-sulfoxide, *N*-acetyl-DL-methionine, S-methyl-L-cysteine). Injection on day 0 of incubation with glutamine or glutamate, at the level of 4 mg per egg, does not relieve the teratogenesis induced by 1 mg Bidrin injected on the same day. A dose of 2 mg per egg of pyridine-2-aldoxime methiodide or 1,3-*bis*(pyridinium-4-aldoxime)propane dibromide, injected on day 2 or day 4 of incubation, does not relieve the effects due to 0·3 mg Bidrin injected on day 4.

Nicotinamide activity, with injection on day 4 of incubation, is dose dependent, but a level of 0·1 mg per egg is adequate to alleviate most of the tabulated signs (Table 2). With Bidrin administration at day 4 of incubation along with 0·1 mg nicotinamide at varying times, maximum alleviation occurs during the period from day 0 to day 10 of incubation. Limited testing indicates that 0·8 μmole NAD gives its full alleviating effect when injected as late as day 7, but that its alleviating action is markedly diminished when the injection is on day 9 of incubation. The alleviating effect of nicotinamide is never complete. Parrot beak, micromelia and abnormal feathering can be completely relieved, but the wry neck is unaffected and the body length is not greatly improved. Severe edema, a common sign in Bidrin-treated embryos, is relieved by nicotinamide, but this effect is not tabulated because of difficulties in evaluating mild edema from only gross inspection of the embryos. Syndactyly and visceral hernia are also eliminated by nicotinamide. Nicotinamide does not increase the hatch of eggs treated with 0·3 mg Bidrin.

Nicotinic acid and each of the esters of nicotinic acid (compounds 1–10) which were tested show the same alleviating potential toward Bidrin-induced teratogenesis. The same is true for nicotinamide and its various *N*-alkyl or *N*,*N*-dialkyl derivatives (compounds 11–16). Other 3-substituents of 3-pyridyl compounds, with activity comparable to the 3-carboxamide, include the following: C(O)NHOH, CHO, CH_2OH, CH_2NH_2 and CH_2NHR (compounds 18, 20, 21, 23 and 24 respectively). Pyridine analogs substituted in the 3-position with $CH_2NR_1R_2$ or an aldoxime group (compounds 25 and 28 respectively) are active, but not as active as the compounds previously

mentioned. Substituents in the 3-position which are inactive under the conditions of the test include: $C(O)NHCH_2C(O)OH$, $C(O)CH_3$, CH_3, $CH_2C(O)OH$, CH_2Cl, $CH{=}NOR$ $C{\equiv}N$ and $S(O)_2OH$ (compounds 17, 19, 22, 26, 27, 29, 30 and 31 respectively).

Inactive analogs result on shifting the ring substituent to the 2- or 4-position (compounds 32–36) and on methylation of the N^1-position to give the pyridinium analog (compounds 37 and 38). The N^1-oxides (compounds 39–41) are of comparable activity to their analogs lacking the N^1-substituent (compounds 11 and 22 respectively). Ring substituents on nicotinamide destroy its alleviating action (compounds 42–44). Tryptophan, a nicotinic acid precursor, at a level of 5 μmole per egg alleviates the Bidrin-induced teratogenesis. Anthranilic acid, a more indirect precursor for nicotinic acid, requires a high level (70 μmole per egg) and shows only partial alleviation. Indole does not alleviate the signs and is toxic at a level of 80 μmole per egg. N^1-methyl pyridones, as carboxamides or phenyl carboxylates, are not active alleviating agents (compounds 48–52).

The pyridine nucleotide coenzymes (compounds 53–56) all show alleviating activity and are indistinguishable in activity from one another or from nicotinamide under the conditions of the test. With the exception of the 3-acetylpyridyl analogs (compounds 59 and 62), the analogs of the various pyridine nucleotide cofactors (compounds 57–64) also alleviate the Bidrin effect.

More limited tests, which are not tabulated, indicate that the pyridine nucleotides that are active in alleviating Bidrin teratogenesis are also active with Bidrin amide. A 1-mg dose of NAD or a 0·5-mg dose of nicotinamide injected at day 4, 6 or 8 of incubation relieves most of the teratogenic signs (but not the wry neck) induced by 0·5 mg eserine given at day 4 of incubation. The teratogenic effects of tetramethylammonium chloride, decamethonium chloride and carbamylcholine are not alleviated by nicotinamide.

Effect of nicotinamide on Bidrin metabolism. Bidrin metabolism in the egg yields organosoluble products which resolve on the Celite chromatographic column into six major peaks as detected by ^{32}P-analysis and five major peaks as detected by ^{14}C-analysis (Fig. 1, Table 4). Co-chromatography serves to characterize three of the major components as Bidrin, des-*N*-methyl Bidrin and Bidrin amide. The following average $^{14}C/^{32}P$ ratios support these identifications; 2·0 for Bidrin with two *N*-methyl-^{14}C groups per ^{32}P; 1·09 for des-*N*-methyl Bidrin which has lost one of the *N*-methyl-^{14}C groups; 0·0 – 0·2 for Bidrin amide in which both of the *N*-methyl-^{14}C groups are removed on metabolic *N*-demethylation. Occasionally, a minor ^{14}C-containing component is found in the region of Bidrin amide, but this component does not appear consistently and is not characterized. A fourth major component probably is *N*-hydroxymethyl Bidrin, because the radiocarbon of one methyl group is lost ($^{14}C/^{32}P = 0{\cdot}98$) and on acid degradation it yields formaldehyde-^{14}C, although the amount released is below the theoretical value (dicoumarol-methylene-^{14}C, cpm: theory, 3700; found, 1980). The chromatographic position of each of these metabolites is in accord with a detailed study on the behavior of Bidrin analogs.[8] An additional minor component sometimes appears between the des-*N*-methyl Bidrin and the Bidrin amide peaks; on analogy with an earlier study,[8] this appears to be a trace amount of 3-hydroxy-*N*-methyl-*N*-hydroxymethyl-*cis*-crotonamide dimethyl phosphate. The nature of metabolites A and B, which contain both ^{32}P and ^{14}C, is not known. No

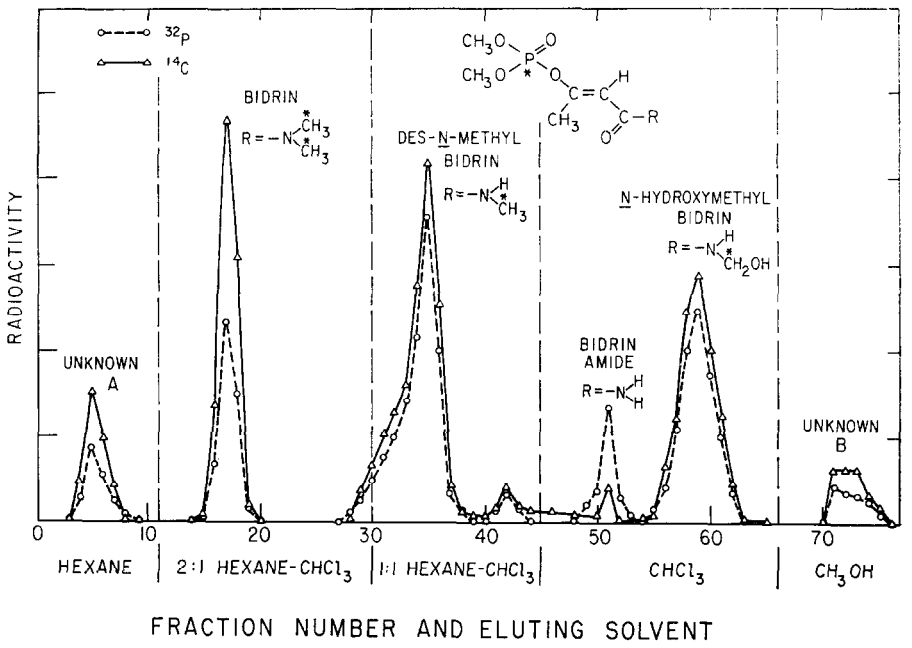


FIG. 1. Chromatographic characteristics of Bidrin organosoluble metabolites on a Celite column based on partition between water and hexane–chloroform mixtures. Metabolites are from the combined yolk and embryo of an egg on day 6 of incubation after injection of a mixture of Bidrin-^{32}P and Bidrin-*N*-methyl-^{14}C on day 4 of incubation.

TABLE 4. RADIOLABELED METABOLITES ON DAYS 4, 6, 8 AND 10 OF INCUBATION AFTER INJECTION OF 1 mg BIDRIN-^{32}P ON DAY 4 OF INCUBATION

	Bidrin-^{32}P equivalents in each fraction (μg)*						
Day of incubation:	4	6		8		10	
Days after Bidrin injection:	0	2		4		6	
Portion of egg analyzed	Yolk + embryo	Yolk	Em-bryo	Yolk	Em-bryo	Yolk	Em-bryo
Fraction							
Organosoluble							
Bidrin	811 ± 33	74 ± 34	0·6	8 ± 4	0·2	1 ±1	0·5
Des-*N*-methyl Bidrin	27 ± 13	165 ± 26	3·2	34 ± 12	1·1	5 ± 4	0·0
N-hydroxymethyl Bidrin	0 ± 0	76 ± 26	4·0	72 ± 20	1·1	33 ± 11	3·0
Bidrin amide	0 ± 0	23 ± 6	1·2	41 ± 10	1·7	36 ± 13	2·0
Unknown A	7 ± 4	7 ± 10	0·1	1 ± 1	1·1	2 ± 1	1·5
Unknown B	36 ± 15	55 ± 26	1·7	48 ± 20	6·5	18 ± 9	5·0
Total	881 ± 30	400 ± 25	10·8	205 ± 36	11·7	95 ± 30	12·0
Water soluble	87 ± 28	447 ± 31	23·0	581 ± 43	21·0	578 ± 34	33·0
Insoluble	16 + 6	90 ± 13	5·0	146 ± 33	8·0	228 ± 35	30·0
Shell	16 ± 10	25 ± 7		28 ± 15		25 ± 13	

* The average values and standard deviations are given when results are based on 4 or 6 replicates; averages alone are given when only 2 replicates are involved. The radiophosphorus content for each fraction is adjusted proportionally so that the total Bidrin-^{32}P equivalents per egg summate to 1000 for each time of injection.

information is available on the identity of the water-soluble or insoluble metabolites and of the radiophosphorus material in the shell, but these probably comprise both hydrolysis products and natural-labeled materials resulting from the incorporation of phosphate-^{32}P arising from the complete hydrolysis of the Bidrin metabolites.

Table 4 gives the levels of Bidrin metabolites present in the yolk and embryo at days 4–10 of incubation. No differences in the amount or ratio of any of the organosoluble radioactive metabolites are evident between the Bidrin-treated and Bidrin-plus-nicotinamide-treated eggs.[3] In addition, water-soluble or insoluble breakdown products and radiophosphorus in the shell are not altered by nicotinamide treatment.[3] Thus, Bidrin oxidation and hydrolytic detoxication are not affected by an antidotal level of nicotinamide (1 mg). Accordingly, the results for Bidrin metabolism in the presence and in the absence of nicotinamide are averaged for presentation in Table 4.

Bidrin is quite unstable in the egg, being partially broken down even within the time necessary to take the "zero time" samples and being more than 90 per cent broken down within 2 days after injection. Fortification of the combined yolk plus embryo from an untreated egg with Bidrin-^{32}P immediately before homogenizing in water, followed by subsequent extraction, results in 97 per cent recovery of ^{32}P, of which 89 per cent is in the organosoluble, 5 per cent is in the water-soluble and 3 per cent is in the insoluble fractions. The dominant organosoluble product in the egg is initially Bidrin; after 2 days it is des-*N*-methyl Bidrin; after 4 days it is *N*-hydroxymethyl Bidrin, and after 6 days it is Bidrin amide and unknowns.

Effect of Bidrin on nicotinic acid and nicotinamide metabolism. Injection of Bidrin at 1 mg per egg on day 2 of incubation does not affect the transfer to the embryo and the subsequent metabolism of nicotinamide-7-^{14}C injected on day 4 of incubation (Table 5). The total levels of NAD, NMN and nicotinamide are almost the same for control and Bidrin-treated eggs. Nicotinic acid-7-^{14}C was not detected as a nicotinamide-7-^{14}C metabolite in any case.

TABLE 5. EFFECT OF BIDRIN INJECTED ON DAY 2 OF INCUBATION ON LEVELS OF RADIOLABELED NICOTINAMIDE METABOLITES IN CHICK EMBRYOS ON DAY 6 AFTER INJECTION OF 6·5 μg NICOTINAMIDE-7-^{14}C ON DAY 4 OF INCUBATION

Nicotinamide-^{14}C and ^{14}C-metabolites	Nicotinamide-^{14}C equiv. (mμg) Control (water)	Bidrin (1 mg)
Nicotinamide	65	55
Nicotinic acid	<10*	<10*
Nicotinamide mononucleotide	45	40
NAD/NADP	430	495
Total	540	590

* Not detected. Sensitivity limit of the method is indicated.

Nicotinic acid-7-^{14}C transferral into the embryo and subsequent metabolism in the embryo are also unaffected by Bidrin administration (Fig. 2). Nicotinic acid and NAD plus NADP (designated as NAD/NADP) are the major products found in the embryo, and occasionally a material is detected in the position of nicotinamide-7-^{14}C, but the level of this material is always less than 1 per cent of that of either nicotinic acid or NAD/NADP. This experiment, which was carried out on a single group of eggs to minimize variability due to season and diet of the hens, also compared the fate of a

trace amount (10 μg) of nicotinic acid-7-^{14}C with a level (1010 μg) sufficiently high to alleviate the teratogenic signs of Bidrin treatment. The high level of nicotinic acid greatly increases the nicotinic acid and NAD/NADP content of the embryos, but reduces the proportionate rate of NAD/NADP formation from nicotinic acid. At the lower level of nicotinic acid-7-^{14}C, the major part of the nicotinic acid equivalents that is transferred to the embryo is quickly converted to NAD/NADP (80–83 per cent on day 6, 90–92 per cent on day 7 and 78–93 per cent on day 9). At the higher dose of nicotinic acid, there is a much slower proportionate conversion to NAD/NADP (21–23 per cent on day 6, 41–46 per cent on day 7 and 63–69 per cent on day 9). Thus, increasing the injected dose of nicotinic acid-7-^{14}C from 10 μg to 1010 μg gives a differential effect in increasing embryo NAD/NADP-^{14}C equivalents as compared to embryo nicotinic acid-^{14}C equivalents. Embryo NAD/NADP-^{14}C-equivalents at 6–9 days of incubation are increased by the 1010-μg dose of nicotinic acid to about 20 times the level resulting from the 10-μg dose. On the other hand, nicotinic acid-^{14}C equivalents in the embryo are increased more markedly by the 1010-μg dose to about 180–290 times on day 6 and to 30–140 times on day 9 over the levels resulting from the injection of the 10-μg dose of nicotinic acid. An upper limit to the level of nicotinic acid accumulated in the embryo is evident from the change in slope of the plot in Fig. 2.

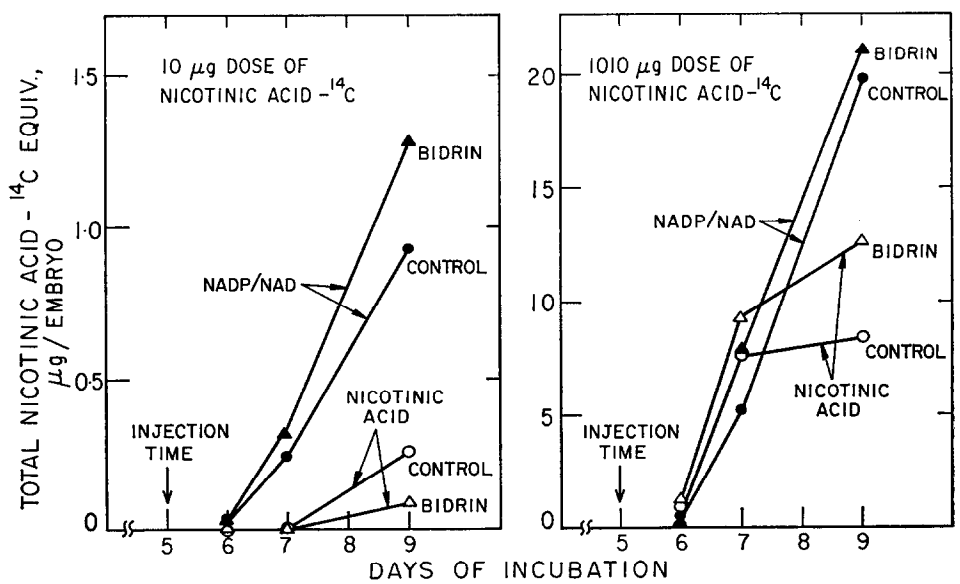


FIG. 2. Effect of Bidrin (1 mg) injected on day 2 of incubation on levels of radiolabeled nicotinic acid metabolites in chick embryos on days 6, 7 and 9 after injection of 10 or 1010 μg of nicotinic acid-7-^{14}C on day 5 of incubation.

DISCUSSION

Chick embryo assays are convenient and sensitive for studies on organosphosphate-induced teratogenesis, but care must be taken in interpreting the results of the present experiments. First, they were carried out at different times of the year and there are some seasonal fluctuations as well as variations with the diet of the hens in the susceptibility of the embryo to teratogenesis induction. Second, the number of eggs

used was relatively small, so the values given should be viewed as semiquantitative rather than as precise measurements. Third, only gross examination of the embryo was involved and it is probable that disruptions at a histological level went unnoticed. Assays with hen eggs are not necessarily indicative of effects on mammalian fetuses because considerable variation occurs with species in teratogenesis induction and because, with the hen egg, the toxicant is injected in close proximity to the embryo, which is not protected by maternal detoxication mechanisms and the placetal barrier. However, such studies with hen eggs are useful in suggesting critical mode-of-action experiments to be made subsequently with mammals.

Bidrin is a very potent teratogen, producing obvious gross malformations at a level of 0·03 mg per egg, which is approximately 0·6 ppm based on the total weight of the egg. The *cis*-crotonamide isomer is the active teratogenic ingredient, the *trans*-isomer being essentially inactive, and the products of *N*-demethylation of Bidrin are teratogenic but its hydrolysis products are not. Bidrin induces teratogenesis when injected prior to incubation or up until day 12 of incubation, at which time a 1-mg dose produces wry neck. The time of greatest teratogenic activity for low dosage levels of Bidrin occurs with injection on or about day 4 of incubation. Since the studies on the metabolism of Bidrin show that Bidrin and its organosoluble metabolites reach their highest concentration within the embryo 2 days after injection, the period at which Bidrin is the most potent in inducing teratogenesis is beteween days 4 and 6 of incubation. Many crotonamide phosphates are teratogenic in hen eggs, but diazinon is also active at a similar dosage level and many other unrelated phosphates are active at higher dosage levels.[4, 6, 7] The signs are not the same with all compounds; for example, parathion gives gross effects different from those found with Bidrin in that only the spine is severely affected. EPN does not give grossly abnormal embryos, but the chicks which hatch from the treated eggs show a lack of equilibrium and extend the head backward. On the other hand, eserine, a carbamate, is teratogenic and its signs are similar to those induced by Bidrin.[2, 3, 12] No simple structure–activity relationship is thus evident, even if limited to organophosphate-induced teratogenesis.

Although more than 90 compounds, including vitamins, were assayed for possible action in alleviating Bidrin-induced teratogenesis, only nicotinic acid, nicotinamide and some of their precursors, analogs and derivatives were found to be active. Nicotinamide, at 0·01 mg per egg, is beneficial and this level increases the total nicotinamide equivalents within the egg, as cofactor or otherwise, by only about 6–20 per cent.[13] Nicotinamide doses up to 0·1 mg or more afford greater, but never complete, relief of the teratogenesis caused by Bidrin doses of 0·1 mg per egg or higher. Comparable alleviation is obtained on injection of nicotinamide on any day between day 0 and day 10 of incubation and some relief is still evident with injections on day 12 of incubation. By day 10 of incubation, a high dose of Bidrin induces only minor abnormalities and it is striking that on day 10 nicotinamide still is very effective in alleviating the Bidrin signs induced probably 4–5 days earlier. At any rate, the embryos already show abnormal signs by day 8 when the eggs are injected with Bidrin on day 4. This indicates that the lesion caused by Bidrin is not permanent and is reversible until day 10–12 of incubation. The period between day 10 and day 12 of incubation or shortly thereafter may be a critical period, since it is the time after which Bidrin-induced signs become irreversible in that nicotinamide no longer effects an alleviating action. In the normal embryo, differentiation processes are almost complete by day 12, which is

also the time at which nicotinamide ceases to act as an alleviating agent. Alternatively, the alleviating action of nicotinamide until day 12 may indicate that Bidrin interferes with growth regulation processes rather than with differentiation. Not all of the Bidrin-induced lesions are reversed by nicotinamide, so it appears that these chemicals interact, only in part, at the same sites or through the same metabolic pathway, either directly or indirectly.

Bidrin probably does not affect the pathways for nicotinic acid synthesis or the interconversions involved with the nicotinic acid analogs because of the wide variety of related compounds that are active as alleviating agents. Tryptophan, a nicotinic acid precursor in the chick embryo, and to a lesser extent anthranilic acid are active alleviating agents. The active 3-pyridyl esters, amides and picolyl amines as well as the other active compounds such as 3-pyridylaldehyde,-carbinol and nicotinyl hydroxamic acid are probably readily converted to nicotinic acid or nicotinamide within the egg. Nicotinamide-N^1-oxide, an active alleviating agent, can be a precursor for nicotinamide, while nicotinuric acid, a normal nicotinic acid metabolite in some organisms, and 3-acetylpyridine possibly are not susceptible to such conversions. This is supported by the fact that 3-acetylpyridine *per se* is teratogenic and that this teratogenesis is reversed by nicotinamide.[14] Pyridone metabolites of nicotinamide are not alleviating agents, even though certain of these compounds are useful in minimizing the toxic effects of neuromuscular blocking agents.[15] Compounds that cannot be converted to nicotinic acid or incorporated into pyridine nucleotide coenzymes, such as the 2- and 4-pyridyl derivatives and the N^1-methylated derivatives, are not active in alleviating Bidrin teratogenesis. Similarly, ring-substituted analogs, such as 6-chloronicotinamide and hydroxy nicotinic acids, are inactive.

It is difficult to determine whether a pyridine nucleotide cofactor or nicotinamide or nicotinic acid is the actual alleviating agent because their alleviating effect is indistinguishable on a molecular equivalent basis. Each of the NAD analogs is active, except for 3-acetylpyridine NAD and 3-acetylpyridine deamino NAD. Most of the synthetic NAD analogs, including 3-acetylpyridine NAD, can serve with varying degrees of effectiveness as cofactors for certain dehydrogenases.[16, 17] It is thus possible that NAD and its analogs are hydrolyzed before reaching the embryo and that only the potential transformation *in vivo* of the pyridyl moiety into nicotinic acid or nicotinamide and perhaps again into pyridine nucleotides is of significance.

Nicotinamide alleviates the embryonic abnormalities induced in hen eggs by many compounds,[12, 14, 18–20] but the mechanism of this action is not understood. It is suggested that nicotinamide injection returns the levels of NAD or NADP to normal, thus permitting the embryo to carry out its normal metabolic functions. However, there is little correlation between the nature of the teratogenesis induced and the level of NAD present in the embryo, and injection of 1-mg levels of nicotinamide into the egg does not significantly increase NAD levels in the embryo.[21]

The present studies indicate that Bidrin has no effect on the metabolism or quantity of nicotinamide-7-^{14}C or nicotinic acid-7-^{14}C in the embryo. However, the amount of nicotinic acid-^{14}C equivalents transferred to the embryo is markedly increased at the 1010-μg injected dose. When the lower level of nicotinic acid, 10 μg, is injected into the egg, only a small portion of the nicotinic acid transferred to the embryo remains as such, the major part being quickly converted to NAD or NADP. When an alleviating dose of nicotinic acid is injected, much less of the transferred nicotinic

acid is converted to NAD or NADP and the percentage of conversion to coenzyme only reaches a high level at day 9. This finding may be correlated with an apparent maximum 'saturation' level of nicotinic acid in the embryo indicated by the decreased level on day 9.

Superimposed on the injected levels of nicotinic acid, however, are the normally occurring levels of nicotinic acid in the eggs; these levels range from 55 μg per 50 g on day 4 to 145 μg per 50 g on day 10.[13] If the results are reassessed on this basis, it becomes apparent that NAD and NADP levels in the embryo are not greatly modified by the 1-mg injected dose of nicotinic acid; however, the levels of nicotinic acid transferred to the embryo are increased by 6-fold. These findings are in good agreement with those of Krackoff[21] and suggest that, although Bidrin has no effect on nicotinamide or nicotinic acid metabolism, these compounds *per se*, at their greatly increased levels in the embryo, may be the actual compounds involved in the alleviating effect against various teratogenic agents, including Bidrin.

Labeled nicotinamide mononucleotide (NMN) is always detected in small amounts in the embryos of eggs treated with nicotinamide-7-^{14}C. Because the levels of NMN are low and somewhat variable, its presence may result from an artifact, due to the hydrolytic cleavage of NAD or NADP during extraction; however, an alternate possibility is that NMN serves as an intermediate in the biosynthesis of NAD or NADP in the chick embryo.

Bidrin metabolism in the egg involves *N*-methyl hydroxylation and *N*-demethylation by a pathway similar to that in other organisms.[8, 22] The sequence of metabolite appearance in the egg suggests a precursor–product relationship as follows: Bidrin → des-*N*-methyl Bidrin → *N*-hydroxymethyl Bidrin → Bidrin amide. It is probable that *N*-methyl-*N*-hydroxymethyl Bidrin is formed but this compound is not detected, possibly because of its rapid transformation to des-*N*-methyl Bidrin. Hydroxylation reactions of this type are usually catalyzed by the microsome-NADPH enzyme system, but an additional amount of cofactor, from nicotinamide injection, does not accelerate the metabolism of Bidrin through *N*-demethylation or its detoxication through hydrolysis.

Enzyme systems capable of rapidly metabolizing foreign organic compounds develop to a high level of activity early in the development of the chick embryo. This appears to be the case because of the wide variety of compounds related to nicotinic acid that are active as alleviating agents, probably involving a number of oxidative mechanisms in their conversion to the actual alleviating agent. It is also clear that the oxidase system for rapid *N*-demethylation of Bidrin is present in the 4- to 6-day-old embryo. The early appearance of detoxication mechanisms in the developing chick embryo should be considered when chicken eggs are used for routine assay of possible teratogens.

The mechanism of organophosphate-induced teratogenesis in the chick embryo remains to be defined. No simple structure–activity relationship is evident for the organophosphate teratogens. The alleviating agents may be active after metabolic conversion to nicotinic acid or nicotinamide, but it is also possible that the active alleviating agent is a pyridine nucleotide cofactor or another derivative of nicotinic acid. The metabolism of Bidrin is unaffected by an alleviating level of nicotinamide, and the metabolism of nicotinamide or nicotinic acid is also unaffected by a teratogenic level of Bidrin; consequently, it is necessary to look to biochemical systems

other than those investigated to explain the mode of action of these compounds as they relate to teratogenesis mechanisms.

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