Biochemical Pharmacology, Vol. 17, pp. 1529–1542. Pergamon Press. 1968. Printed in Great Britain

BIOCHEMICAL STUDIES ON THE TERATOGENIC ACTION OF BIDRIN AND OTHER NEUROACTIVE AGENTS IN DEVELOPING HEN EGGS*

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(*Received* 18 *December* 1967; *accepted* 15 *March* 1968)

Abstract—Bidrin (3-hydroxy-*N*,*N*-dimethyl-*cis*-crotonamide dimethyl phosphate), eserine, and certain other neuroactive agents induce embryonic abnormalities when injected into hen eggs early in development. The effect of Bidrin and other teratogenic and nonteratogenic neuroactive agents on esterase activity, acetylcholine (ACh) levels, and labeled ACh distribution and metabolism within the egg was examined. The inhibition of the activity of acetylcholinesterase and other esterases resulting from injection of Bidrin and other organophosphate esters does not correlate directly with the degree of teratogenesis, and agents that alleviate teratogenesis, such as nicotinamide and nicotinamide adenine dinucleotide, do not significantly alter the magnitude of esterase inhibition. Embryonic levels of ACh do not appear to be related to the degree of teratogenesis, particularly in the presence of an alleviating agent, further indicating that ACh and the cholinergic system do not play a major role in the differentiation processes involved in organophosphate-induced teratogenesis. A permeability barrier appears to be operative between the yolk and the embryo, preventing the transfer of unhydrolyzed ACh but not of its hydrolysis products. Growth abnormalities possibly occur in the presence of certain esterase inhibitors due to nutritional deficiency of the embryo resulting from failure of the embryo to obtain ester hydrolysis products from the yolk.

SEVERAL neuroactive materials, including certain acetylcholinesterase (AChE) inhibitors, produce morphogenic defects when injected into the developing hen egg.[1–4] Embryonic abnormalities are also induced by certain organophosphorus insecticide chemicals,[5, 6] including Bidrin (3-hydroxy-*N*,*N*-dimethyl-*cis*-crotonamide dimethyl phosphate) and several of its analogs, when injected into the egg during the first 9 days of development.[7] The signs induced by Bidrin can be largely alleviated by pre-, post-, or simultaneous injection of nicotinamide and certain of its analogs.[7, 8]

The primary lesion involved in acute poisoning of mammals by many organophosphates is considered to be inhibition of AChE at the neuromuscular junction and at the cholinergic synapses. This results in prolonged depolarization of the postsynaptic cholinergic receptor site due to the elevated acetylcholine (ACh) levels because of the failure to hydrolyze ACh.[9] However, the delayed neurotoxic effect in hens induced by tri-*o*-cresyl phosphate and many related compounds,[10] and the

* Supported in part by grants from the United States Public Health Service, National Institutes of Health (Grant No. GM-12248), the United States Atomic Energy Commission (Contract AT(11-1)-34, Project Agreement No. 113) and the Shell Chemical Company, New York, N.Y.

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narcotic effects of tri-*n*-butyl phosphate[11] cannot be explained solely on the basis of AChE or pseudocholinesterase inhibition.[12] Similarily, the teratogenic effects of cholinomimetic agents and AChE inhibitors are difficult to explain purely on the basis of AChE inhibition.

Studies on amphibia suggest that some facet of the cholinergic system may be involved directly or indirectly with the trophic or inductive mechanism of tissue regeneration.[13–15] Acetylcholine induces AChE synthesis in explants of chick embryo intestine,[16, 17] but not in chick embryo skeletal muscle[18] or in the embryonic brain after injection into the yolk of developing eggs.[19] The cholinesterase (ChE) of intestine seems to be neither the classical specific nor the non-specific type of enzyme.[16] Burdick and Strittmatter speculate that permeability barriers limit the penetration of injected ACh into the central nervous system.[19]

In the present study, the interaction between the cholinergic system and embryonic development is examined in the hen egg by the use of Bidrin and other neuroactive agents as teratogens and by analyses for esterase activity, ACh levels and labeled ACh distribution and metabolism. In those cases where biochemical lesions are evident, the significance of the lesions is evaluated, in part, by comparison of untreated and Bidrin-treated eggs with those treated with Bidrin and an alleviating dose of nicotinamide or nicotinamide adenine dinucleotide (NAD).

MATERIALS AND METHODS

Chemicals and treatment of eggs. Insecticide chemicals were obtained from the following sources: Bidrin (technical grade, contining 75% *cis*-isomer), Azodrin (3-hydroxy-*N*-methyl-*cis*-crotonamide dimethyl phosphate), Bidrin amide (3-hydroxy-*cis*-crotonamide dimethyl phosphate), mevinphos (methyl 3-hydroxycrotonate dimethyl phosphate), OS 1808 (ethyl 3-hydroxycrotonate diethyl phosphate) and dichlorvos (2,2-dichlorovinyl dimethyl phosphate) from Shell Development Co., Modesto, Calif.; phosphamidon (2-chloro-2-diethylcarbamoyl-l-methylvinyl dimethyl phosphate) from Chevron Chemical Co., Richmond, Calif.; dioxathion (2,3-*p*-dioxanedithiol *S,S*-bis[*O,O*-diethyl phosphorodithioate]) from Hercules Powder Co., Wilmington, Del.; parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate) and malathion (*S*-[1,2-bis(ethoxycarbonyl)ethyl] *O,O*-dimethyl phosphorodithioate) from American Cyanamid Co., Princeton, N.J.; EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate) from E. I. Du Pont de Nemours & Co., Wilmington, Del.; azinophosmethyl (*O,O*-dimethyl phosphorodithioate *S*-ester with 3-[mercaptomethyl]-1,2,3-benzotriazin-4[3H]-one) from Chemagro Corp., Kansas City, Mo.; *l*-nicotine from I. Yamamoto, Tokyo University of Agriculture, Setagaya, Tokyo.

Sources for other chemicals and radiochemicals used were as follows: unlabeled choline esters, hexamethonium and nicotinamide from Nutritional Biochemicals Corp., Cleveland, Ohio; esters of glycerol and phenol from Eastman Organic Chemicals, Rochester, N.Y.; carbamyl choline and hemicholinium from Aldrich Chemical Co., Milwaukee, Wis.; tetramethylammonium chloride from Matheson, Coleman & Bell, Cincinnati, Ohio; decamethonium bromide from K & K Laboratories, Long Island City, N.Y.; Triton X-100 from Rohm and Haas Co., Philadelphia, Pa.; NAD, eserine sulfate, *d*-tubocurarine, atropine sulfate, sulfanilamide, homocysteine, glucose-U-^{14}C (28·5 mc/m-mole), glucose-1-^{14}C (100 mc/m-mole), and glucose-6-^{14}C (13·6 mc/m-mole) from Calbiochem Corp., Los Angeles, Calif.; acetyl-l-^{14}C-choline

iodide (ACh-acetyl-1-^{14}C, 5·0 mc/m-mole), acetylcholine-*N*-methyl-^{14}C iodide (ACh-*N*-methyl-^{14}C, 3·9 mc/m-mole), choline-*N*-methyl-^{14}C (4·8 mc/m-mole), sodium carbonate-^{14}C (5·0 mc/m-mole), sodium formate-^{14}C (4·0 mc/m-mole), methionine-*S*-methyl-^{14}C (8·8 mc/m-mole) and betaine-*N*-methyl-^{14}C (1·7 mc/m-mole) from New England Nuclear Corp., Boston, Mass.; sodium acetate-1-^{14}C (7·8 mc/m-mole) from Volk Radiochemical Co., Burbank, Calif.; glycine-2-^{14}C (1·1 mc/m-mole) from Tracerlab, Waltham, Mass.

Fertile, white Leghorn eggs obtained from Kimber Farms Inc., Niles, Calif., were incubated in a Jamesway model 252B automatic egg incubator (James Manufacturing Co., Fort Atkinson, Wis.) at 37·5° and 73 per cent relative humidity. After varying periods of incubation, test chemicals were injected into the yolk of the eggs,[8, 20] by using standard sterile procedures via sterilized Swinny filter adapters having a filter pore size of 0·45 μ (Millipore Filter Corp., Bedford, Mass.) and headed with No. 22 gauge 3·8-cm needles. Each egg received 0·1 ml water, either alone or as part of the solution of the test chemical, and the hole through the shell into the air sac was resealed with collodion before placing the treated egg into the incubator. Water-insoluble compounds were injected in 10 μl ethanol with a microsyringe.

Distribution and fate of ^{14}C-labeled substrates in vivo. Eggs, previously treated with water or with water containing NAD or various unlabeled toxicants or with combinations of these materials, were candled on day 5 of incubation, and the living eggs were injected with the ^{14}C-labeled compound (0·5 or 1·0 μc) in 0·1 ml water solution in such a manner that each egg in each experiment received an equivalent level of total radioactivity. In order to facilitate gas exchange between the air sac and the atmosphere, the hole in the shell was not resealed after injection of the radioisotope. In some experiments, the neuroactive agent under test was injected into a group of 10 eggs, of which 2 were used for radiolabeled investigation and the remaining eggs were permitted to complete development so that the possible inducement of embryonic abnormalities and toxic signs, if any, could be observed.

Two eggs injected with the same compounds were immediately transferred to a polyethylene sack (1-lb Freezer-Refrigerator Bags, Kordite Corp., Macedon, N.Y.), fitted in a quart glass jar and sealed with a rubber stopper in such a manner as to yield an air volume of approximately 400 ml prior to introducing the eggs. Tubes through the rubber stopper allowed an inlet for air and an outlet to a CO_2-absorption trap.[21, 22] The CO_2 absorbant was replaced after 2, 4, 6, 12 and 24 hr and the $^{14}CO_2$ liberated in each interval was determined.[21]

At the termination of the $^{14}CO_2$-trapping period on day 6 of incubation, each embryo was quickly removed, washed free of yolk, blotted free of surface liquid and weighed. The shell was discarded, the embryo and remaining egg contents (referred to as yolk) were fractionated at 4° and the radiocarbon content of the fractions was determined. Liquid samples were counted directly as 0·2-ml aliquots and 50-mg representative portions of the solid samples were combusted for total radiocarbon analysis.[21, 23] Cold trichloroacetic acid (TCA, 10% w/v) was used at 4 ml/g of embryo fresh weight or 75 ml/yolk for extraction. The embryo was homogenized in TCA solution for 1 min in a glass tissue grinder with a Teflon pestle and the yolk for 1 min at high speed in a Waring–Blendor. The embryo and yolk homogenates were centrifuged at 2000 *g* for 15 and 30 min, respectively, to yield residue and supernatant fractions. Each residue fraction was washed with 10% TCA solution by rehomogenization and recentrifugation

to yield the final residue fraction. The combined supernatant fractions (TCA-soluble) were further fractionated into acid-volatile and nonvolatile materials; the supernatant was counted directly (acid-volatile and nonvolatile) or after adding 0·1 ml of 0·1 N hydrochloric acid to 0·2 ml of extract, followed by heating at 105° for 20 min with subsequent cooling before radioassay (acid-nonvolatile); the acid-volatile radiocarbon was determined by difference.

Esterase inhibition. Embryos excised from untreated or treated eggs were homogenized in bicarbonate buffer [0·036 M sodium bicarbonate and 0·164 M sodium chloride saturated with nitrogen–carbon dioxide (95:5) gas mixture just before use] at 5° to yield concentrations appropriate for manometric esterase assays in a Warburg apparatus (Precision Scientific Co., Chicago, Ill.). Each substrate was adjusted to a 0·01 M final concentration. For substrates that were not water-soluble, Triton X-100 was used to emulsify the ester in the buffer solution; the amount of Triton X-100 was such that 40 μg were present in each flask during the assays. Warburg flasks, with one sidearm and of about 10-ml total volume, were used with 1·6 or 1·8 ml of the homogenate in the main compartment and 0·4 or 0·2 ml, respectively, of substrate preparation in the sidearm. The flasks with their unmixed contents at 37·5° were gassed with the nitrogen–carbon dioxide for 3 min with shaking and allowed to equilibrate while shaking for an additional 7-min period. Manometric readings were taken at 5-min intervals for a 30-min period, starting immediately after tipping of the flask to mix the homogenate and the substrate; the results were plotted to determine the specific esterase activity, expressed as μl CO_2/embryo/hr. Corrections were made for nonenzymatic hydrolysis of the substrate and for the endogenous activity of the homogenate in the absence of substrate.

Acetylcholine levels. Embryos were extracted by procedures appropriate to recover free and total ACh,[24] and ACh levels were assayed[25] with the rectus abdominis muscle of medium-sized frogs (*Rana pipiens*) obtained from Dahl Co., Berkeley, Calif.

Bioassay was accomplished by determining the degree of contraction of the rectus abdominis muscle with the test solution as compared with frog Ringer solution of known ACh concentration. For free ACh determination, embryos (five at day 6 and three at day 9 of incubation) were homogenized in 10^{-4} M eserinized Ringer solution at 5°, the homogenates were centrifuged (1000 *g* for 30 min at 2°) and the supernatant liquid was diluted to 10 ml with 10^{-5} M eserinized Ringer solution for bioassay. For total ACh determination, embryos (three from day 6 and two from day 9 of incubation) were homogenized in 10% TCA at 5°, the homogenates were centrifuged (1000 *g* for 10 min at 2°) and the supernatant liquid was extracted 3 times with 15-ml portions of ether. The residual ether was evaporated from the aqueous phase and it was then neutralized with sodium bicarbonate and diluted with 10^{-4} eserinized Ringer solution (to 10 ml at day 6 and to 20 ml at day 9 of incubation). Standard ACh concentrations were assayed in eserinized Ringer solution and the response of the muscle to ACh was found to be the same for this solution as for that of each of the extracts indicated above. The degree of contraction was determined after 1 min in the test solution; then the preparation was washed with and allowed to relax for 8 min in fresh eserinized Ringer solution before subsequent assays. During the bioassay, oxygen was constantly bubbled through the 10-ml test medium at 20°; the same muscle was used for all extracts within each experimental series, which included free and total ACh and two or more standard levels of ACh inserted in the assay sequence.

Free ACh was determined by direct analysis and bound ACh by the difference between the total and the free ACh. All results were expressed as μg ACh/embryo. The sensitivity of the assay varies considerably from one muscle preparation to another and usually decreases as the muscle tires. The decrease in sensitivity is linear with time and corrections are made when necessary. Standard curves, established with known levels of ACh in frog Ringer solution, or dilution curves, established for free ACh in extracts, are linear for concentrations below about 3 μg/10 ml, which produces near maximal contraction, but linear dilution curves with extracts for total ACh are consistently obtained only at 1:1 or greater dilution of the extract with eserinized Ringer solution. Alkaline hydrolysis destroys the activity of the embryo extracts, indicating that inorganic ions are not the contraction-inducing agents.

RESULTS

Metabolism and distribution of ^{14}C-labeled acetylcholine and its hydrolysis products in Bidrin-treated incubating eggs. Table 1 shows the $^{14}CO_2$ expiration and radiocarbon

TABLE 1. DISTRIBUTION OF RADIOCARBON ON DAY 6 AFTER INJECTION OF ACETYL-1-^{14}C-CHOLINE, ACETYLCHOLINE-*N*-METHYL-^{14}C, ACETATE-1-^{14}C AND CHOLINE-*N*-METHYL-^{14}C ON DAY 5 WITH DEVELOPING EGGS PRETREATED ON DAY 2 WITH BIDRIN OR NAD OR BOTH

Pretreatment compound (1 mg each/egg)	Total recovered radiocarbon* after 24 hr (%)					
		Embryo		Yolk		
				TCA-soluble		
	CO_2	TCA-soluble	Residue	Volatile	Nonvolatile	Residue
			Acetyl-1-^{14}C-choline			
None	20	0·8	2·5	62	4	11
NAD	16	0·6	2·4	63	7	11
Bidrin	3	0·3	0·6	64	25	7
Bidrin + NAD	3	0·2	0·6	60	30	6
			Acetylcholine-*N*-methyl-^{14}C			
None	0	1·6	2·0	5	67	24
NAD	0	1·5	2·0	10	64	22
Bidrin	0	0·4	0·4	8	79	12
Bidrin + NAD	0	0·5	0·5	2	76	21
			Acetate-1-^{14}C			
None	13	0·6	1·7	67	6	11
NAD	13	0·6	1·8	74	3	7
Bidrin	13	0·5	1·8	70	5	10
Bidrin + NAD	13	0·7	2·0	72	4	8
			Choline-*N*-methyl-^{14}C			
None	0	1·7	2·3	12	61	23
NAD	0	1·6	2·0	10	64	23
Bidrin	0	2·2	2·8	4	64	27
Bidrin + NAD	0	1·9	2·6	8	64	23

* Results are the average of those from 2 experiments with 2 eggs in each experiment.

distribution in the day 6 developing egg, after injection on day 5 of ACh-acetyl-1-^{14}C, ACh-*N*-methyl-^{14}C, acetate-1-^{14}C and choline-*N*-methyl-^{14}C into eggs pretreated with 1 mg each of Bidrin or NAD or both along with 0·1 ml water on day 2 of incubation. The radiocarbon distribution is that resulting from a 24-hr period. It is expected that,

on fractionation of the yolk TCA extract into acid-volatile and acid-nonvolatile fractions, acetate will appear in the acid-volatile fraction and choline and ACh will appear in the acid-nonvolatile fraction; however, these fractions from the developing egg cannot be categorized as representing only these labeled compounds because of the presence of other materials that prevent such an interpretation.

Injection of Bidrin or NAD has little effect on the production of $^{14}CO_2$ and the radiocarbon distribution from acetate-1-^{14}C and choline-*N*-methyl-^{14}C, the hydrolysis products of ACh-^{14}C. Approximately 13 per cent of the radiocarbon from acetate-1-^{14}C injected on day 5 is expired as $^{14}CO_2$ within the 24-hr period, some 70 per cent remaining in the yolk volatile fraction. About 63 per cent of the radiocarbon from choline-*N*-methyl-^{14}C remains in the yolk nonvolatile fraction after 24 hr and no $^{14}CO_2$ is expired. (Yolk and embryo residues are probably a measure of the reincorporation of the substrate, being unmodified by Bidrin or NAD treatment.)

Comparisons of the acetate-1-^{14}C and choline-*N*-methyl-^{14}C data with those of ACh-acetyl-1-^{14}C and ACh-*N*-methyl-^{14}C, respectively, clearly indicate that Bidrin injection severely inhibits the production of expired $^{14}CO_2$ from ACh-acetyl-1-^{14}C and also decreases the transfer of radiocarbon from the yolk to the embryo with ACh labeled either in the 1 or *N*-methyl position. In the case of ACh-acetyl-1-^{14}C, $^{14}CO_2$ expiration decreases on Bidrin injection by 80–82 per cent and embryo radiocarbon (TCA-soluble and residue) by 73 per cent; with the ACh-*N*-methyl-^{14}C-treated eggs, embryo radiocarbon decreases by 71–78 per cent. An unexpected finding is the similar high levels of acid-volatile radiocarbon (60–64 per cent) in the yolk of all eggs injected with ACh-acetyl-1-^{14}C, whether pretreated with Bidrin or not. The nonvolatile yolk fraction with ACh-acetyl-1-^{14}C shows an increase in the presence of Bidrin of 4–6 times, but with ACh-*N*-methyl-^{14}C the increase is small (1·2 times). In no instance does the injection of 1 mg NAD greatly modify the distribution of radiocarbon. The fact that the decreases induced by Bidrin in both $^{14}CO_2$ expiration and embryo radiocarbon content are of the same order of magnitude suggests, but does not necessarily establish, that the $^{14}CO_2$ expired originates for the most part from the embryo.

Metabolism of acetyl-1-^{14}C-choline in incubating eggs pretreated with various neuroactive agents. The distribution of radiocarbon, including that amount expired as $^{14}CO_2$, on day 6 in eggs pretreated with various neuroactive agents on day 2 and day 4, followed by ACh-acetyl-1-^{14}C injection on day 5, is shown in Table 2.

Only the known AChE inhibitors, Bidrin and eserine, produce great modification of the pattern of radiocarbon expiration and distribution; in all other cases, the results do not differ importantly from those found with the control eggs. Bidrin depresses $^{14}CO_2$ expiration by 80 per cent and eserine depresses it by 74 per cent; the respective embryo radiocarbon contents are also depressed 74 and 69 per cent. In line with the results given in Table 1, the chemicals tested have little, if any, effect on the yolk acid-volatile fraction, whereas Bidrin or eserine injection results in a marked increase of the yolk nonvolatile fraction (8·8 times with Bidrin and 7·2 times with eserine).

All incubated eggs treated with atropine, *d*-tubocurarine and saline solution only on day 2 or 4 develop and hatch normally. Embryonic abnormalities induced by Bidrin are markedly similar to those induced by eserine (described in earlier reports of Landauer[1, 2]). These abnormalities include shortening of the spine, parrot beak, micromelia, syndactyly, growth inhibition, almost complete feather inhibition and

gross edema. Carbamyl choline induces growth inhibition and wryneck, but at the dosage levels selected this effect is slight; eggs so treated do not hatch and the embryos show slight edema. Decamethonium induces severe shortening of the spine and wryneck, but gives no decrease in embryo weight. Hemicholinium injected on day 4 is nonteratogenic and all eggs so treated hatch normally; however, when hemicholinium is injected on day 2, hatch is reduced and wrynecks appear. Hexamethonium

TABLE 2. DISTRIBUTION OF RADIOCARBON ON DAY 6 AFTER INJECTION OF ACETYL-1-^{14}C-CHOLINE ON DAY 5 AND TERATOGENIC EFFECTS WITH DEVELOPING EGGS PRETREATED ON DAY 2 AND DAY 4 WITH VARIOUS TOXICANTS

Pretreatment		Total recovered radiocarbon* after 24 hr (%)						
			Embryo		Yolk			
					TCA-soluble			
Toxicant	Dose (mg/egg)	CO_2	TCA-soluble	Residue	Volatile	Non-volatile	Residue	Teratogenic signs†
None		22	1·0	2·5	61	4	9	—
AChE inhibitors								
Bidrin	1	5	0·3	0·6	48	35	11	++++
Eserine sulfate	1	6	0·4	0·7	55	29	9	++++
Other neuroactive agents								
Atropine sulfate	5	24	0·8	2·6	58	3	11	—
Carbamyl choline	5	21	0·7	1·7	64	3	9	+
Decamethonium bromide	1	16	0·6	1·7	66	3	13	+++
Hexamethonium	5	31	0·8	2·2	54	3	9	—
Hemicholinium	1	19	0·7	2·0	62	4	12	—
l-Nicotine	5	18	0·6	1·9	71	2	7	toxic
Tetramethylammonium chloride	10	25	1·2	2·1	58	4	9	+++
d-Tubocurarine	2·5	22	0·8	2·2	59	5	11	—

* Results are the average of those from 2 experiments with 2 eggs in each experiment. In one set of these experiments, the toxicant was administered on day 2 and in the other set on day 4 of incubation. Results of the experiments are averaged because the results in the 2 sets of experiments were not markedly different, except in the case of Bidrin where the effect was greater when administered on day 2 than on day 4 of incubation.

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

is more toxic when injected on day 4 than on day 2; embryo mortality is increased, hatch is reduced and there is a possible growth retardation (mean weight of 22·0 g for treated and 24·0 g for control). *l*-Nicotine is highly toxic, all embryos being dead on day 10 of incubation. Tetramethylammonium chloride is definitely teratogenic, greatly reducing the body length.

No correlation between $^{14}CO_2$ expiration, radiocarbon distribution and the teratogenic signs is apparent.

Effect of Bidrin on distribution and metabolism of various labeled substrates. Table 3 summarizes the effect on metabolism of 1 mg Bidrin or 1 mg NAD or 1 mg Bidrin plus 1 mg NAD in comparison with saline-treated control eggs as measured by $^{14}CO_2$ expiration and radiocarbon distribution after injection of various labeled substrates on day 5. (Table 3 contains the average of all radiocarbon recoveries found for these different pretreatment conditions, because the individual results obtained for a given substrate, after injection of the pretreatment compounds, do not differ appreciably.)

In no instance did the injection of Bidrin or NAD or of Bidrin plus NAD on day 2 significantly alter the $^{14}CO_2$ expiration or radiocarbon distribution. In all cases, except with sodium carbonate-^{14}C, the embryo and yolk extracts were further fractionated into acid-volatile and nonvolatile parts, but in no case did the pretreatment schedule modify the distribution of radiocarbon. Thus, while Table 3 gives much information in regard to the fate of the radiocarbon from various substrates, it does not aid materially in elucidating the teratogenic action of Bidrin.

TABLE 3. DISTRIBUTION OF RADIOCARBON ON DAY 6 AND DAY 7 AFTER INJECTION OF VARIOUS ^{14}C-LABELED SUBSTRATES ON DAY 5 FOR DEVELOPING EGGS PRETREATED ON DAY 2 WITH BIDRIN OR NAD OR BOTH

Treatment			Total recovered radiocarbon* (%)				
				Embryo†		Yolk†	
^{14}C-labeled substrate	Dose (μmoles/egg)	Incubation period after treatment (hr)	CO_2	Extract	Residue	Extract	Residue
Pretreatment with Bidrin, NAD, Bidrin + NAD or no toxicant							
Choline-*N*-$^{14}CH_3$	0·60	24	0·0	1·9	2·4	72·0	23·8
Glycine-2-^{14}C	0·41	24	0·3	1·1	3·1	77·6	17·7
Sodium acetate-1-^{14}C	0·14	24	13·1	0·6	1·9	75·8	8·8
Sodium carbonate-^{14}C	1·0	24	84·3	0·3	0·6	1·5	13·4
Sodium formate-^{14}C	1·0	24	1·9	3·2	5·4	80·4	9·9
Pretreatment with Bidrin, Bidrin + NAD or no toxicant							
Glucose-U-^{14}C	0·02	24	12·5	2·5	4·0	66·8	12·2
Pretreatment with Bidrin or no toxicant							
Betaine-*N*-$^{14}CH_3$	0·53	48	1·2	8·2	3·7	84·8	2·6
Betaine-*N*-$^{14}CH_3$ + 1 mg homocysteine	0·53	48	1·3	6·8	3·5	85·4	3·2
Choline-*N*-$^{14}CH_3$ + 1 mg homocysteine	0·53	48	0·3	5·4	4·9	86·8	2·9
Glucose-1-^{14}C	1·0	24	5·6	1·5	2·4	89·1	1·5
Glucose-6-^{14}C	1·0	24	6·6	2·0	3·2	86·9	1·3
Methionine-*S*-$^{14}CH_3$	0·59	48	0·2	3·0	5·3	83·8	5·3

* Results are the average of those from 4, 3 or 2 sets of individual comparison pretreatment experiments respectively; pretreatment dose: 1 mg Bidrin or NAD or both.

† Extracted with 10% TCA in all experiments except that with sodium carbonate-^{14}C in which ethanol was used.

Esterase inhibition and acetylcholine levels. Cholinesterase activity of the homogenates of whole embryos increases markedly with time of incubation of the eggs, and this increase is only slightly affected, if at all, by nicotinamide (Table 4). Although it is a teratogen, sulfanilamide has little, if any, significant inhibiting effect on the enzyme activity. Mevinphos gives complete ChE inhibition initially, but recovery of activity is complete by day 15 of incubation. Eserine and particularly Bidrin give marked and persistent ChE inhibition, but nicotinamide, simultaneously injected at a level which alleviates the teratogenesis, does not alter the inhibition with either compound or the rate of recovery of ChE activity.

When ChE activity is separately assayed for the head, trunk of the body and the whole embryo, the contribution of the head to the total ChE activity of the embryo diminishes from 50 per cent at day 8–40 per cent at day 12 of incubation. The results of this study (which are not given in Table 4) show that the degree of ChE inhibition is almost the same for the head and trunk of the body with eserine, Bidrin, EPN, mevinphos, parathion and phosphamidon.

Cholinesterase inhibition at day 12 of embryos from eggs injected with various organophosphorus compounds on day 4 of incubation, with one exception, correlates approximately with the overall teratogenic signs and with the shortening of the body and legs (Table 5). The exception to this relationship is EPN, a nonteratogenic compound, which results in ChE inhibition as severe as that found with teratogenic compounds such as Bidrin, Azodrin, Bidrin amide and parathion. At 1 and 3 mg per egg, EPN fails to give abnormal embryos or reduced hatch, but at both levels the chicks show severe nervous signs with the head held abnormally over the back, and they die within a few days after hatching. Parathion gives a marked shortening of the spine, but is less effective than Bidrin in reducing the length of the legs.[8]

TABLE 4. CHOLINESTERASE ACTIVITY ON VARIOUS DAYS OF INCUBATION OF NORMAL EMBRYOS AND OF EMBRYOS FROM DEVELOPING EGGS INJECTED WITH VARIOUS COMPOUNDS ON DAY 4 OF INCUBATION

Treatment		Days of incubation				
Compound	Dose (mg/egg)	6	9	12	15	21
		ACh hydrolysis (μl CO_2/embryo/hr)				
None		843	3024	13,455	26,180	
		ChE activity relative to control (%)				Teratogenic signs*
Nicotinamide	1·0	95†	98†	84†	85†	—
Bidrin	0·3	7	11	19	55	++++
Bidrin nicotinamide	0·3 1·0	4	10	18	47	+
Eserine	0·5	20	24	50	86	++++
Eserine nicotinamide	0·5 1·0	16	25	48	92	+
Mevinphos	10	0†	17†	81†	97†	++
Sulfanilamide	15	78†	63†	76†	90†	++++‡

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

† Results are the average of those from 2 replicates in one experiment. In all other cases, the result is an average of those from 2 replicates in one experiment and 3 replicates in a second experiment.

‡ Very long neck.

Esterase activity of the homogenates prepared from normal embryos at day 7 of incubation is greatest with ACh as the substrate, in the case of the choline esters, and with triacetin, in the case of the glycerol esters; this activity is very great with phenyl acetate and phenyl propionate alike (Table 6). Inhibition resulting from Bidrin injection on day 4 of incubation is 87–96 per cent with choline esters, but is only 56–78 per cent with the glycerol and phenol esters. In no case does NAD appreciably alter the esterase activity or greatly relieve the Bidrin-induced inhibition found with any of the test substrates.

Injection of Bidrin at 300 μg or 1 mg/egg on day 3 of incubation does not significantly alter the level of bound ACh in the embryo on day 6 or day 9 of incubation, but it markedly increases the free ACh level in a dose-dependent manner (Table 7). Nicotinamide is without significant effect on the ACh level, either in the free or bound form, in control eggs as well as in those treated with Bidrin.

TABLE 5. COMPARISON OF CHOLINESTERASE ACTIVITY ON DAY 12 WITH TERATOGENIC SIGNS ON DAY 21 OF EMBRYOS FROM DEVELOPING EGGS INJECTED WITH VARIOUS TOXICANTS ON DAY 4 OF INCUBATION

Treatment		ChE activity relative to control* (%)	Length of embryo parts† (cm)		Teratogenic signs‡
Compound	Dose (mg/egg)		Body	Legs	
None		100	8·5	6·0	—
Malathion	1	107	8·0	6·0	—
Dichlorvos	1	106	8·0	5·5	+
Mevinphos	1	105	7·5	6·0	—
OS 1808	3	98	6·2	5·6	+
OS 1808	1	94	8·0	6·0	—
Dioxathion	1	80	8·0	6·0	—
Bidrin	0·03	76	8·0	6·0	—
Bidrin	0·10	51	6·7	5·5	+ +
Mevinphos	10	51	6·0	4·6	+
Azinophosmethyl	1	50	7·5	5·5	+
Phosphamidon	3	42	5·6	5·3	+ +
Phosphamidon	7	40	5·5	3·9	+ + +
Bidrin	0·30	28	5·6	3·3	+ + + +
EPN	1	18	8·0	6·3	—
EPN	3	15	8·0	6·3	—
Bidrin	1	10	5·0	3·5	+ + + +
Bidrin amide	0·30	4	4·5	3·0	+ + + +
Azodrin	0·30	3	5·0	4·0	+ + + +
Parathion	1	0	5·0	4·0	+ + + +

* Results are the average of those from 2–4 replicates.
† Results are the average of measurements of 8–25 embryos.
‡ Signs are graded from not detectable (—) to severe manifestation (+ + + + +).

TABLE 6. ESTERASE ACTIVITY WITH VARIOUS SUBSTRATES ON DAY 7 OF NORMAL EMBRYOS AND OF EMBRYOS FROM DEVELOPING EGGS INJECTED WITH BIDRIN OR NAD OR BOTH ON DAY 4 OF INCUBATION

Substrate (0·01 M)	ChE activity of control* (μl CO_2/ embryo/hr)	ChE activity relative to control* after injection of indicated compound(s) (%)		
		NAD	Bidrin	Bidrin + NAD
Acetylcholine chloride	1104 ± 140	104 ± 12	9 ± 1	4 ± 1
Propionylcholine *p*-toluene sulfonate	444 ± 100	103 ± 21	5 ± 1	6 ± 4
Butyrylcholine *p*-toluene sulfonate	247 ± 20	104 ± 20	4 ± 2	4 ± 2
Acetyl-β-methylcholine chloride	382 ± 16	90 ± 5	13 ± 2	8 ± 5
Benzoylcholine chloride	0 ± 0			
Triacetin	950 ± 21	98 ± 25	29 ± 2	22 ± 1
Tripropionin	847 ± 120	116 ± 6	25 ± 5	33 ± 3
Tributyrin	641 ± 120	101 ± 26	42 ± 10	44 ± 6
Phenyl acetate	3826 ± 390	111 ± 30	33 ± 15	26 ± 11
Phenyl propionate	3700 ± 330	97 ± 7	34 ± 6	36 ± 3

* Average and S.D. are based on 3 replicates; S.D. is established from the absolute values and then transformed to a percentage for tabulation, when appropriate. Bidrin dose is 300 μg/egg and that of NAD is 1 mg/egg.

TABLE 7. FREE AND BOUND ACETYLCHOLINE ON DAY 6 AND DAY 9 OF NORMAL EMBRYOS AND OF EMBRYOS FROM DEVELOPING EGGS INJECTED WITH BIDRIN AND NICOTINAMIDE ON DAY 3 OF INCUBATION

Treatment		Acetylcholine (μg/embryo)			
		Day 6		Day 9	
Compound	Dose (mg/egg)	Free*	Bound†	Free*	Bound†
None		0·03 ± 0·02	0·29 ± 0·06	0·36 ± 0·24	1·40 ± 0·20
Nicotinamide	1·0	0·02 ± 0·01	0·25 ± 0·04	0·36 ± 0·17	1·10 ± 0·16
Bidrin	0·3	0·13 ± 0·04	0·25 ± 0·03	0·75 ± 0·08	1·60 ± 0·46
Bidrin nicotinamide	0·3 1·0	0·12 ± 0·07	0·27 ± 0·08	0·76 ± 0·08	1·15 ± 0·17
Bidrin	1·0	0·22 ± 0·05	0·23 ± 0·07	0·98 ± 0·23	1·40 ± 0·16
Bidrin nicotinamide	1·0 1·0	0·23 ± 0·03	0·24 ± 0·07	0·96 ± 0·16	1·10 ± 0·33

* Average and S.D. are based on 4 replicates.

† Average of difference between free and total ACh in each of 4 paired replicates. S.D. is calculated from individual difference for each pair.

DISCUSSION

Toxic effects from organophosphate insecticide chemicals are usually attributed to AChE inhibition and subsequent disruptions resulting from ACh accumulation. The critical period for studying teratogenesis induction by Bidrin in hen eggs is between day 4 and day 12 of incubation, preferably between day 6 and day 8.[7, 8] In the present study, attention was focused on the possible involvement of the ACh–AChE system in teratogenesis. The approaches included the use of teratogenic and nonteratogenic organophosphates and other neuroactive agents and of an alleviating dose of nicotinamide or NAD, along with a level of Bidrin or eserine that would be teratogenic in the absence of the alleviating agent, and analyses for labeled ACh metabolites, ACh distribution, esterase inhibition and metabolism of certain ^{14}C-labeled biochemicals.

Acetylcholine injected into the egg early in development is rapidly hydrolyzed to acetate and choline, with subsequent metabolism of the acetate to CO_2. Bidrin and eserine, which are known to act as ChE inhibitors in other situations, substantially reduce the $^{14}CO_2$ expiration resulting from injection of ACh-acetyl-1-^{14}C. Levels of ChE inhibition *in vivo*, calculated from the $^{14}CO_2$ data, are lower than those determined by manometric ChE assays directly on embryo homogenates. This may be due in part to the release of organophosphate into solution by homogenization and the subsequent reaction *in vitro* of the liberated inhibitor with embryo ChE during enzyme preparation and assay. Alternatively, the embryo ChE may not be responsible for all of the ACh hydrolysis in the egg. It may be that the yolk ChE contributes and is less susceptible than the embryo ChE to inhibition by Bidrin and eserine or that nonenzymatic reactions also hydrolyze ACh because the residual radiocarbon in the yolk 24 hr after treatment with ACh-acetyl-1-^{14}C is largely in the acid-volatile form, whereas unhydrolyzed ACh logically should appear in the acid-nonvolatile material. The described procedure using ACh-acetyl-1-^{14}C to follow ChE inhibition *in vivo* appears to be useful in studies concerned with the degree or persistence of ChE inhibition *in vivo* in the whole organism rather than at a specific site within the organism.

Inhibition by Bidrin of $^{14}CO_2$ expiration after ACh-acetyl-1-^{14}C injection is similar in degree to the inhibition of radiocarbon transfer from the yolk to the embryo. Bidrin also inhibits transfer of radiocarbon from ACh-*N*-methyl-^{14}C into the embryo. In addition, it results in a higher proportion of residual acid-nonvolatile radiocarbon in the yolk from both ACh-acetyl-1-^{14}C and ACh-*N*-methyl-^{14}C. The distribution and metabolism of the products of ACh-^{14}C hydrolysis, acetate-1-^{14}C and choline-*N*-methyl-^{14}C, are unaffected by Bidrin treatment, indicating that a permeability barrier is operative, which prevents transfer of unhydrolyzed ACh from the yolk into the embryo but does not alter the passage of the hydrolysis products. A more effective transport of the acetate from ACh than of free acetate into the embryo is indicated by the fact that acetyl-labeled ACh yields more expired $^{14}CO_2$ than acetate-1-^{14}C in the absence of Bidrin treatment. Hydrolysis of glycerol and phenol esters is also inhibited by Bidrin, but in these cases it is not known whether a permeability barrier for the esters is operative. Growth abnormalities early in the development of the chick embryo can result from nutritional deficiencies.[26] It is possible that failure of the embryo to obtain essential alcohols and acids that are normally transferred into the embryo from the yolk might contribute to the teratogenesis. It seems likely that this could result either from the inhibition of esterases normally effecting ester hydrolysis so that the free acid and alcohol are not available or from the existence of barriers to transport of the esters in the unhydrolyzed form.

A mechanism can be envisioned wherein a portion of the injected ACh is bound to the vitelline membrane where it is hydrolyzed and the hydrolysis products are transferred to the embryo while the remaining portion is free in the yolk and is hydrolyzed either chemically or by Bidrin-resistant esterases to liberate products that are not readily transferred to the embryo. If this is the case, then selective binding of the type indicated and selective inhibition of the membrane ChE might explain in part the differences between the fate of acetate-^{14}C and ACh-acetyl-^{14}C, the effect of Bidrin on ACh distribution and metabolism and the lower degree of ChE inhibition *in vivo* for the eggs than that found *in vitro* for the embryo.

It is unlikely that inhibition of ACh transfer from the yolk to the embryo is the cause of Bidrin-induced teratogenesis, because the transfer of choline and acetate *per se* is unhindered, even in the presence of a ChE inhibitor, and the embryo possesses its own system for ACh synthesis.[19] Further, neither ACh nor choline is teratogenic and they do not potentiate or alleviate the action of Bidrin. The inability to induce premature AChE synthesis in the chick embryo brain by injection of massive ACh doses into the yolk sac[19] is possibly the result in part of rapid hydrolysis of ACh in the yolk so that no persisting level of ACh in the embryo is achieved. As a result of ChE inhibition by Bidrin, an accumulation of free, but not of bound, ACh occurs in the embryo and, at least up to day 9 of incubation, this ACh accumulation is not affected by nicotinamide at dosage levels sufficient to alleviate teratogenesis.

During the critical period for teratogenesis induction, ChE and esterases that hydrolyze glycerol and phenol esters are inhibited by Bidrin, but neither nicotinamide nor NAD affects the inhibition even though they largely alleviate the teratogenesis. In the absence of alleviating agents, there seems to be a partial correlation between persistent ChE inhibition in the embryo and the subsequent teratogenesis; however, exceptions such as that of EPN suggest that it is not a simple cause-and-effect relationship. Although no supporting data are available, it is possible that only Bidrin- and

eserine-sensitive esterases are involved in teratogenesis, while esterases inhibited by EPN administration are not.

The total ChE activity measured consists of two components: enzyme present at the time of toxicant injection into the egg and enzyme synthesized during subsequent embryo development. Prolonged ChE inhibition in the egg indicates that the organophosphate involved is of high biological stability because it persists to inhibit the additional ChE synthesized as the embryo grows. If this is so, there is an increased opportunity for interference with other systems and this may be a reason for the correlation between persistent ChE inhibition and teratogenesis. Alternatively, it is possible that the biochemical lesions leading to teratogenesis are localized, for example in the limb buds, and that they are not discernible when assayed using a whole embryo homogenate; however, the organophosphates have the same effect on ChE levels in the head as in the body alone or in the whole embryo.

Of the many neuroactive agents examined, only the ChE inhibitors, eserine and Bidrin, altered the distribution or metabolism *in vivo* of ACh-acetyl-1-^{14}C. Embryonic abnormalities induced by carbamyl choline, decamethonium and tetramethylammonium chloride, which do not alter ACh metabolism, are of a different morphological type from those induced by Bidrin or eserine. Some of these neuroactive agents possibly block critical ACh receptor sites in the embryo to induce abnormalities, although the present studies were not designed to examine this point.

Bidrin has no effect on the metabolism or distribution within the developing egg of many radiolabeled substrates which were studied. These results rule out several possible biochemical lesions as contributory factors or secondary effects of Bidrin-induced teratogenesis. However, several interesting phenomena take place. The radiocarbon of sodium carbonate-^{14}C is rapidly eliminated from the egg, indicating that little or no permeability barrier exists against $^{14}CO_2$ expiration. Oral administration of sodium formate-^{14}C to adult mice results in rapid elimination of the radiocarbon as $^{14}CO_2$ within several hours, the major part (70 per cent) being expired within 24 hr;[27] however, in the chick embryo only a very small portion (1·9 per cent) is eliminated in this manner. The relatively high embryo radiocarbon content indicates that no permeability barrier exists against formate transfer from the yolk to the embryo, and it appears that the low $^{14}CO_2$ expiration is a reflection of the high requirement of formate for protein and nucleic acid synthesis in the developing embryo. The embryo radiocarbon content observed with methionine, betaine and choline, where the *S*-methyl or *N*-methyl carbons presumably enter the same "formate" pool, supports this hypothesis.

Tissue regeneration, as well as embryonic differentiation, is affected by certain neuroactive agents. Atropine, procaine and tetramethylammonium chloride delay regeneration in fully innervated amphibian limb stumps, while eserine salicylate, pilocarpine nitrate, methylcholine iodide and ACh iodide are without effect.[14] In chick embryos, eserine, pilocarpine and tetramethylammonium chloride are teratogenic, while the other compounds do not produce abnormalities.[1–3] Such studies on neuroactive agents that are also teratogenic suggest involvement of the cholinergic system as a whole, or in part, in both tissue regeneration and embryonic development. The present investigation fails to support this hypothesis and suggests that ACh and the cholinergic system do not play a major role in the differentiation processes involved in organophosphate-induced teratogenesis.

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