Table VI. Effect of DDT on Dieldrin Storage in Swine

Daily Treatment[b]	Tissue Dieldrin,[a] P.P.M.
5.45 mg. of dieldrin	
Males	25.5
Females	13.0
5.45 mg. of dieldrin + 22.55 mg. of DDT	
Males	5.4
Females	7.8
5.45 mg. of dieldrin + 45.10 mg. of DDT	
Males	6.7
Females	8.3

ANALYSIS OF VARIANCE

Source	Degrees of Freedom	Mean Sq.
Treatment	5	116.33 ($P < 0.01$)
DDT	2	192.89 ($P < 0.01$)
Sex	1	28.63
DDT × sex	2	83.61 ($P < 0.01$)
Error	9	8.85

[a] Values are the mean dieldrin levels in adipose tissue after 15 days of treatment. There were three males and two females in each group. [b] The insecticides were administered daily by injection of corn oil solutions.

daily with 0.15 mg. per kg. of body weight of dieldrin and either 0.60 or 2.40 mg. per kg. of body weight of injected DDT. Neither eggs, collected during the 15-day experiment, nor abdominal adipose tissue showed lowered dieldrin values resulting from DDT. A similar experiment was conducted for 21 days with young hens just beginning to lay. Dieldrin dosages were fixed at 0.075 and 0.375 mg. per kg. daily and DDT dosages were increased to 2.0 and 10.0 mg. per kg. Again, no influence of DDT on dieldrin storage was observed.

Animal variation in sensitivity to such an interaction effect may be important in determining the ecological consequences of some agricultural chemical usage. In particular, it leaves the question of possible interaction in humans quite problematical.

The findings in this report point out an important new aspect of the toxicology of a major class of environmental contaminants, the chlorinated hydrocarbon insecticides. Much more study is needed to determine the ultimate significance of the findings with those insecticides, and the principles learned may well apply to the toxicology of many other chemical pollutants being placed in our environment. An intriguing possibility is that effective agents might be developed which would safely reduce insecticide storage in animals and man. Such agents also might be used for the treatment of individuals who may become overexposed to insecticides and other foreign chemicals.

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INTERACTIONS

Toxicologic Interactions of Chlorinated Hydrocarbon and Organophosphate Insecticides

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THE first study of the influence of the chlorinated hydrocarbon insecticides on the toxicity of a subsequently administered organophosphate insecticide was that of Ball *et al.* in 1954 (*3*). They reported that rats given a single oral dose of aldrin, chlordan, or lindane were protected 4 days later against the oral toxicity of parathion, an effect which they attributed to the increase in serum aliesterase activity induced by the insecticides. Main (*18*) noted in 1956 that similar aldrin pretreatment reduced the mortality of rats challenged with parathion, paraoxon, and TEPP. He attributed this protective effect of aldrin to an increase in liver A-esterase activity. In 1958 Neubert and Schaefer (*20*) reported that pretreatment with α-hexachlorocyclohexane protected mice against the toxicity of paraoxon and OMPA, but not DFP.

As knowledge developed of the role of the liver microsomal enzymes in the metabolism of drugs and other chemicals, several groups of investigators reported findings indicating that the chlorinated hydrocarbon insecticides stimulate microsomal enzyme activity. The present authors found that a single dose of aldrin in mice markedly decreased the sleeping time due to hexobarbital, a drug which is metabolized mainly by the microsomal fraction of the liver (*27*). Hart *et al.* (*16*) and Hart and Fouts (*15*) pretreated rats with chlordan or DDT and noted an increased rate of hexobarbital metabolism by the liver microsomes. Isomers of hexachlorocyclohexane, DDT, and dieldrin have been reported to accelerate several detoxication processes of rat liver microsomes—including hydrolysis of paraoxon, oxidation of hexobarbital, *O*-dealkylation of phenacetin, and *N*-dealkylation of nikethamide—and to lower

One hour after a single oral dose of aldrin, dieldrin, or chlordan the toxicity of parathion in mice was increased; after 4 days it was decreased. Aldrin had a similar diphasic effect on hexobarbital sleeping time. Aldrin also protected mice 4 days later against paraoxon, EPN, Guthion, TEPP, TOTP, DFP, and physostigmine, but not against OMPA and neostigmine. Protection against parathion appeared after 16 hours, reached a peak after 4 days, and lasted at least 12 days. One milligram of aldrin per kg. provided significant protection. Aldrin increased liver A-esterase and plasma B-esterase, decreased plasma A-esterase, and reduced the inhibition of brain but not plasma cholinesterase (ChE) by paraoxon. The esterase increases, and the protective effect against toxicity and brain ChE inhibition by paraoxon, were all abolished by ethionine. Although the esterase increases probably account for at least part of aldrin's protective action, the difference in its influence on the inhibitory action of paraoxon on plasma and brain ChE cannot yet be explained.

the toxicity of the convulsant drugs pentylenetetrazol and scilliroside (*14*). Welch and Coon (*28*) demonstrated that pretreatment of mice for several days with drugs known to be microsomal stimulants—such as chlorcyclizine or phenobarbital (*6*)—markedly protected against the toxic action of the organophosphates parathion, malathion, and EPN.

The authors' results show that certain chlorinated hydrocarbon insecticides alter the toxicity of parathion in a manner parallel to the changes they produce in hexobarbital sleeping time. Furthermore, aldrin pretreatment of mice resulted in changes of various tissue esterases similar to those observed by others in rats. In this study aldrin, parathion, and paraoxon were used, as representatives of these two classes of insecticides, to learn more about the characteristics of their toxicologic interactions.

Materials and Methods

Mature male Swiss-Webster mice weighing 18 to 25 grams were used. All insecticides and drugs were administered to animals in solutions, the volume of which was 1% of their body weight. Mortalities were recorded for 24 hours after the administration of the anticholinesterase agents. Only an occasional death occurred later. Mortality results were analyzed statistically by the exact chi square test (*19*). Probability values of 5% or less were considered significant. The LD_{50} values were obtained by the method of Litchfield and Wilcoxon (*17*).

The time from the intravenous injection of hexobarbital (80 mg. per kg.) to the regaining of the righting reflex was the measure of sleeping time. The mice were asleep immediately after injection, and were considered asleep until they could right themselves three times in 1 minute when placed on their backs. Significance of the difference between the mean sleeping times of the control group and the groups receiving aldrin alone, aldrin plus ethionine, and ethionine alone was determined by the *t* test.

Chemicals. The compounds administered orally, dissolved in corn oil, are parathion, EPN, Guthion, TOTP (tri-*o*-tolyl phosphate), aldrin, dieldrin, and chlordan (reference grade, a mixture consisting of α- and β-chlordan, heptachlor, hexachlor, and trichlor). The substances administered orally, dissolved in distilled water, are paraoxon, TEPP (tetraethyl pyrophosphate), DFP (diisopropyl fluorophosphate), OMPA (octamethylpyrophosphoramide), physostigmine sulfate, and neostigmine methyl sulfate. The chemicals administered parenterally, dissolved in 0.9% saline solution, are ethionine (*d,l*-ethionine) and hexobarbital sodium.

Hexobarbital Plasma Levels. The plasma levels of hexobarbital were measured by the method of Brodie *et al.* (*5*). Animals were sacrificed 20 minutes after administration of the drug. The pooled blood from six mice was used for each determination, so as to obtain 2 ml. of plasma containing the hexobarbital, which was extracted with *n*-heptane and then transferred to a phosphate buffer at pH 11. The amount of hexobarbital was determined by measuring the ultraviolet absorption of the drug at 247 mμ in the Perkin-Elmer 202 spectrophotometer.

Plasma Pseudocholinesterase. Plasma cholinesterase activity was measured colorimetrically by the method of Ellman *et al.* (*11*) modified as follows: Plasma was diluted 1 to 41 with 0.1*M* phosphate buffer at pH 8 and 0.5 ml. of this dilution was added to 1 ml. of the buffer. This mixture was brought to 25° C. in an Eberbach water bath shaker. A stock solution of the substrate butyrylthiocholine iodide (95.1 mg. per ml.) in HCl at pH 4 was kept refrigerated. Prior to use it was diluted 1 to 4 with pH 8 phosphate buffer. When 0.5 ml. of this solution was added to the test tubes containing the enzyme, the final concentration of the butyrylthiocholine iodide was $1.5 \times 10^{-2}M$ in a total of 2 ml. of reaction mixture. After incubation for 20 minutes, the reaction was stopped by the addition of 0.5 ml. of TEPP solution (3.6 mg. per ml.), bringing the final concentration of TEPP to $2.5 \times 10^{-3}M$. Then 0.1 ml. of the dye dithiobisnitrobenzoic acid (4 mg. per ml.) was added to produce a yellow color which was read at 412 mμ in a Bausch & Lomb Spectronic 20 colorimeter.

Brain Acetylcholinesterase. The assay was performed by the same general procedure as that used for plasma pseudocholinesterase. A volume of 0.05 ml. of a 10% homogenate of mouse brain in cold phosphate buffer at pH 8 was added to 1.5 ml. of the buffer. Then 0.5 ml. of acetylthiocholine iodide solution was added, so that the final concentration of substrate was 5×10^{-4} *M* in the reaction mixture. After incubation for 15 minutes, the reaction was stopped by the addition of TEPP.

Plasma B-Esterase. Total plasma esterase activity was determined by the colorimetric method of Seligman and coworkers (*22*). To adapt this method to the present purposes plasma was diluted 1 to 151 with distilled water containing $10^{-5}M$ physostigmine SO_4 to inhibit plasma cholinesterase, and incubated at room temperature for 30 minutes. One milliliter of the plasma dilution was added to 5 ml. of the buffered substrate solution of 2-naphthyl acetate, which also contained $10^{-5}M$ physostigmine SO_4. After a 30-minute incubation period at room temperature, 1 ml. of a freshly prepared cool solution of tetrazotized di-*O*-anisidine (4 mg. per ml.) was added. The liberated 2-naphthol forms a purple azo dye which may be extracted into ethyl acetate and measured in the colorimeter at 540 mμ. To distinguish B-esterase activity from that of other esterases, the diluted plasma was incubated with various concentrations of paraoxon at room temperature for 30 minutes before addition to the substrate solution. Concentrations of paraoxon as low as $10^{-6}M$ almost completely inhibited the esterase activity, indicating that the enzyme was the B-type esterase as classified by Aldridge (*1*).

Liver and Plasma A-Esterase. The liver and plasma A-esterase was determined colorimetrically according to the method of Aldridge (*2*). The method was slightly modified by centrifuging at 600 × G a 10% liver homogenate at 0° C. for 10 minutes in phosphate buffer at pH 7.6 to obtain the supernatant as a source of enzyme. The *p*-nitrophenol liberated by 0.5 ml. of liver supernatant or 0.5 ml. of plasma from 10 ml. of the substrate solution containing 20 mg. of paraoxon was measured at 420 mμ in the colorimeter.

Results and Discussion

The LD_{50} of a single oral dose of aldrin in the strain of mice and under the conditions used in this study was 33 mg. per kg., with 95% confidence limits of 27.7 to 39.2. Mice were given 16 mg. per kg. of aldrin 4 days before they received toxic doses of various anticholinesterase agents. The data in Table I show that the aldrin pretreatment reduced the toxicity of all the agents tested except neostigmine and OMPA. Neostigmine and OMPA, in single toxic doses, have little inhibitory effect on brain cholinesterase (*9, 10*) because of their poor ability to penetrate the brain. All other anticholinesterase agents against which aldrin treatment provided protection are good inhibitors of brain cholinesterase. The fact that protection was found for only those anticholinesterase agents capable of inhibiting brain enzyme indicates that factors involving the central nervous system may play a partial role in the protective effect of aldrin. Also, several of the agents against which aldrin offered protection are weak inhibitors of cholinesterase in vitro and must be activated in vivo through the action of microsomal enzymes: parathion, EPN, Guthion, and TOTP. Aldrin was also protective, however, against paraoxon, TEPP, DFP, and physostigmine, agents not requiring in vivo activation to potent inhibitors of cholinesterase. This indicates that the protective effect of aldrin is not necessarily due to the in vivo inhibition of the conversion of organophosphates to their active metabolites.

To determine the time of onset of the observed protective action, groups of mice were given parathion at various intervals ranging from 1 hour to 4 days after single doses of the chlorinated hydrocarbon insecticides aldrin, dieldrin, and chlordan. The results presented in Table II show that 1 hour after aldrin or dieldrin, the parathion (22 mg. per kg.) produced mortalities significantly greater than those of the control groups, while at 16 hours and at 4 days the mortality was reduced to zero. Thus aldrin and dieldrin had a diphasic effect on parathion toxicity as the interval between the administration of the agents increased. With chlordan similar results were obtained, although a significant increase in mortality could not be elicited during the early phase after treatment, as was seen with aldrin and dieldrin, because of the large challenging dose of the parathion (35 mg. per kg.), which killed 88% of the control group. Although the mechanism for the increased mortality of parathion observed 1 hour after the administration of aldrin or dieldrin was not studied, it seems reasonable to attribute it to the additive gross toxic effects of the chlorinated hydrocarbons and parathion.

In order to find the time of development of the maximum degree of protection, larger doses of parathion were used. The data in Table III indicate that in a series of intervals of 1, 2, 4, and 8 days, significant protection appeared only at 4 days against a large dose of 100 mg. per kg. of parathion. With a dose of 35 mg. per kg. the resulting mortalities indicated significant protection at 12 days, but not

Table I. Oral Toxicity of Anticholinesterases 4 Days after a Single Oral Dose of 16 Mg. per Kg. of Aldrin in Mice

Pretreatment	Anticholinesterase, Mg./Kg.	No. of Mice	Mortality, %	P
Control[a]	Parathion, 22	20	35.0	<0.01
Aldrin	Parathion, 22	19	0.0	
Control	Paraoxon, 40	18	100.0	<0.001
Aldrin	Paraoxon, 40	18	44.4	
Control	TEPP, 10	20	95.0	<0.001
Aldrin	TEPP, 10	20	0.0	
Control	DFP, 50	15	66.6	<0.02
Aldrin	DFP, 50	10	10.0	
Control	EPN, 75	14	50.0	<0.02
Aldrin	EPN, 75	13	0.0	
Control	Guthion, 15	13	84.6	<0.005
Aldrin	Guthion, 15	13	15.4	
Control	TOTP, 2000	20	60.0	<0.025
Aldrin	TOTP, 2000	20	20.0	
Control	Physostigmine, 6.5	18	100.0	<0.001
Aldrin	Physostigmine, 6.5	18	33.3	
Control	Neostigmine, 10	20	50.0	N.S.
Aldrin	Neostigmine, 10	20	40.0	
Control	OMPA, 25	20	60.0	N.S.
Aldrin	OMPA, 25	20	70.0	

[a] All controls received corn oil.

Table II. Oral Toxicity of Parathion after Pretreatment with Single Oral Doses of Aldrin (16 Mg./Kg.), Dieldrin (16 Mg./Kg.), and Chlordan (150 Mg./Kg.) in Mice

Pretreatment	Time before Parathion, Hours	Anticholinesterase, Mg./Kg.	No. of Mice	Mortality, %	P
Control	...	Parathion, 22	20	25.0	...
Aldrin	1		20	85.0	<0.001
	6		19	52.6	N.S.
	16		20	0.0	<0.05
	4 days		22	0.0	<0.05
Control	...	Parathion, 22	20	30.0	...
Dieldrin	1		20	70.0	<0.05
	6		20	75.0	<0.025
	16		20	0.0	<0.025
	4 days		20	0.0	<0.025
Control	...	Parathion, 35	17	88.2	...
Chlordan	1		20	100.0	N.S.
	6		20	95.0	N.S.
	16		19	15.8	<0.01
	4 days		16	0.0	<0.001

Table III. Oral Toxicity of Parathion after Pretreatment with Single Oral Doses of Aldrin

(16 mg./kg. in mice)

Pretreatment	Interval, Days	Anticholinesterase, Mg./Kg.	No. of Mice	Mortality, %	P
Control	...	Parathion, 100	20	100.0	...
Aldrin	1	Parathion, 100	20	95.0	N.S.
	2	Parathion, 100	20	90.0	N.S.
	4	Parathion, 100	20	65.0	<0.01
	8	Parathion, 100	20	90.0	N.S.
Control	12	Parathion, 35	20	90.0	<0.005
Aldrin	12	Parathion, 35	17	29.4	
Control	24	Parathion, 35	15	93.3	N.S.
Aldrin	24	Parathion, 35	15	66.7	
Control	30	Parathion, 35	18	88.9	N.S.
Aldrin	30	Parathion, 35	17	64.7	

as long as 24 days, although the groups of aldrin-treated mice given parathion showed a mortality suggestive of some persisting degree of protection at 24 and 30 days after treatment.

On the basis of the results thus far described, which indicate that protection against parathion appeared to be at its maximum approximately 4 days after aldrin, this time interval was selected to measure more quantitatively the magnitude of the protection by determining the LD_{50}'s of several anticholinesterase agents in control and aldrin-treated mice. The results presented in Table IV show that aldrin treatment had the greatest protective effect against the toxicity of parathion, with a 5.7 ratio of LD_{50} change, followed by paraoxon and physostigmine with ratios of 4.8 and 2.4, respectively. In the case of neostigmine and OMPA, however, there was no significant change in the LD_{50}'s, confirming the absence of protection for these agents as shown in Table I. At variance with these results (Tables I and IV) is the fact that Ball *et al.* (*3*) did not observe a protective effect in rats with aldrin against physostigmine. Also, Neubert and Schaefer (*20*) did not obtain a protection of mice against DFP, whereas they did find a protection against OMPA with α-hexachlorocyclohexane. Thus, different species or different chlorinated hydrocarbons may give different results in regard to their toxicologic interactions with a given anticholinesterase agent.

Since 16 mg. per kg. of aldrin exerted some toxic action in mice, and killed an occasional animal, the effect of larger doses was not tested. Smaller doses, however, were used to find the minimal dose that would provide significant protection against parathion. The results in Table V show that a progressive decrease in the dose of aldrin resulted in an increasing mortality when parathion was administered 4 days later. One milligram per kg. of aldrin was the smallest dose tested that provided a significant protection against 35 mg. per kg. of parathion.

The foregoing results showed an increase in the toxicity of parathion 1 hour after aldrin treatment, but at 16 hours and for as long as 12 days mice were significantly protected against this organophosphate. With dieldrin or chlordan pretreatment a similar diphasic effect on the toxicity of parathion was observed. In regard to the delayed phase in which protection develops against the toxicity of parathion and several other anticholinesterase agents, there is evidence that adaptive enzymes are involved. Numerous chlorinated hydrocarbon insecticides have been reported to increase the rate of metabolism of several drugs by increasing liver microsomal enzyme activity (*13–16, 26*). If the protective action of the chlorinated hydrocarbon insecticides is based on an increased

Table IV. Oral LD_{50}'s of Anticholinesterases in Mice after Treatment 4 Days Previously by 16 Mg. per Kg. of Aldrin

Pretreatment	Anticholinesterase	LD_{50}, Mg./Kg.	95% Confidence Limits	Ratio of LD_{50} Change
Control	Parathion	17.5	14.8–20.7	
Aldrin	Parathion	100.0	75.8–132.0	5.7
Control	Paraoxon	15.5	12.6–19.1	
Aldrin	Paraoxon	74.0	52.9–103.6	4.8
Control	Physostigmine	4.8	3.5–6.6	
Aldrin	Physostigmine	11.5	8.5–15.5	2.4
Control	Neostigmine	14.0	11.0–17.8	
Aldrin	Neostigmine	13.5	11.1–16.5	0.96[a]
Control	OMPA	21.8	19.0–25.0	
Aldrin	OMPA	21.0	17.9–24.6	0.96[a]

[a] Not significant.

Table V. Oral Toxicity of 35 Mg. per Kg. of Parathion 4 Days after Single Oral Doses of Aldrin in Mice

Pretreatment	Aldrin, Dose, Mg./Kg.	No. of Mice	Mortality, %	P
Control	...	20	95.0	...
Aldrin	16.0	20	0.0	<0.001
	4.0	20	40.0	<0.001
	1.0	20	65.0	<0.05
Control	...	20	100.0	...
Aldrin	0.5	19	89.5	N.S.

synthesis of enzymes, such as the esterases suggested by others (*3, 18*), this effect would hasten the metabolism of certain organophosphate insecticides, thus reducing their toxicity.

The observed diphasic effect on the toxicity of parathion after treatment with the chlorinated hydrocarbon insecticides is a property common to many liver microsomal enzyme stimulants. For example, Serrone and Fujimoto (*23*) have shown that administration of proadifen (SKF-525A), iproniazid, or nikethamide less than 12 hours before hexobarbital prolonged sleeping time, but if pretreatment were as long as 24 to 48 hours before, hexobarbital sleeping time was shortened. The latent period for the shortening of sleeping time represents the time required for the synthesis of the microsomal enzymes. The change in sleeping time and metabolism of hexobarbital is a test used by many (*6, 15, 16, 23*) to determine whether a drug increases liver microsomal enzyme activity, since hexobarbital is metabolized mainly by the microsomal fraction of the liver (*8*). The ability of a drug to increase the activity of drug-metabolizing enzymes in liver microsomes may also be determined by the use of ethionine (*7*), which is known to inhibit protein synthesis (*25*). Evidence for induction of enzyme synthesis is obtained when ethionine is administered simultaneously with the inducing agent, and the stimulatory effect on the synthesis of drug metabolizing enzymes is abolished. The reversal of the protective effect of the chlorinated hydrocarbon insecticides on the toxicity of the organophosphates by ethionine would indicate that protection was due to enzyme induction.

On the basis of these considerations it was of interest to determine whether ethionine abolished the protective effect of aldrin on organophosphate toxicity, and also to study the effect of aldrin pretreatment on the sleeping time and metabolism of hexobarbital. As shown in Table VI, the protective effect of aldrin against parathion and paraoxon was abolished by the administration of two daily doses of 200 mg per kg. of ethionine. The animals pretreated only by aldrin were well protected against the toxicities of parathion and paraoxon, whereas mice pretreated by both aldrin and ethionine were no longer significantly protected. The interpretation of this effect of ethionine, however, is not clear, because the dose of ethionine that was necessary to abolish the protective effect of aldrin itself increased the toxicity of parathion and paraoxon. Smaller doses of ethionine that did not increase the toxicity of either parathion or paraoxon did not abolish the protective effect of aldrin against these organophosphates.

The results in Table VII show that hexobarbital sleeping time in mice was prolonged at 1 hour after aldrin treatment, but decreased at 16 hours and at 4 days. This diphasic effect of aldrin on hexobarbital sleeping time is similar to that seen in the mortality data previously obtained (Table II) after the administration of parathion in mice pretreated by aldrin, dieldrin, and chlordan. It was observed further that the decreased sleeping time which occurred 2 days after aldrin treatment was abolished by the administration of ethionine (Table VIII). As in the case of the toxicity of parathion and paraoxon, the decreased sleeping time seen after aldrin could be abolished

Table VI. Effect of Ethionine on Protective Action of Aldrin on Toxicity of Parathion and Paraoxon

Pre-treatment	Anticholinesterase, Mg./Kg.	No. of Mice	Mortality, %	P
Control	Parathion, 25	20	50.0	...
Aldrin[a]	Parathion, 25	20	0.0	<0.001
Ethionine[b]	Parathion, 25	19	97.4	<0.005
Aldrin + ethionine[c]	Parathion, 25	18	22.2	N.S.
Control	Paraoxon, 10	20	45.0	...
Aldrin[a]	Paraoxon, 10	19	0.0	<0.005
Ethionine[b]	Paraoxon, 10	20	95.0	<0.005
Aldrin + ethionine[c]	Paraoxon, 10	19	73.7	N.S.

[a] Single dose of 16 mg./kg. of aldrin given orally 2 days before anticholinesterase agent.
[b] Two daily doses of 200 mg./kg. of ethionine i.p.; anticholinesterase given 24 hours after second dose.
[c] Aldrin given 15 min. after first of two daily doses of ethionine.

Table VII. Alteration of Hexobarbital (80 Mg./Kg.) Sleeping Time at Various Intervals after Single Oral Dose of 16 Mg./Kg. of Aldrin in Mice

Pretreatment	Time before Hexobarbital, Hours	No. of Mice	Sleeping Time, Min. (Mean ± S.D.)	P
Control	1	14	40.6 ± 11.3	<0.02
Aldrin	1	12	53.5 ± 14.8	
Control	6	18	34.9 ± 11.0	N.S.
Aldrin	6	18	30.1 ± 10.8	
Control	16	18	40.9 ± 13.4	<0.005
Aldrin	16	18	26.6 ± 10.6	
Control	4 days	12	38.9 ± 15.3	<0.001
Aldrin	4 days	15	11.4 ± 5.6	

Table VIII. Influence of Ethionine on Effects of Aldrin on Hexobarbital Sleeping Time and Hexobarbital Plasma Level in Mice

Pre-treatment	Sleeping Time,[a] Min. (Mean ± S.D.)	P	Plasma Levels,[b] μG./Ml. (Mean ± S.D.)	P
Control	20.8 ± 10.7	...	36.7 ± 8.0	...
Aldrin[c]	8.1 ± 4.6	<0.001	19.6 ± 3.7	<0.001
Ethionine[d]	42.7 ± 15.6	<0.001	50.3 ± 5.1	<0.005
Aldrin + ethionine[e]	29.0 ± 20.5	N.S.	42.4 ± 3.7	N.S.

[a] Each value is mean obtained from 18 mice, each receiving 80 mg./kg. of hexobarbital i.v.
[b] Each value is mean of eight determinations in each of which pooled blood of 6 mice was used. Animals sacrificed 20 min. after administration of 80 mg./kg. of hexobarbital i.v.
[c] Single dose of 16 mg./kg. of aldrin given orally 2 days before hexobarbital.
[d] Two daily doses of 200 mg./kg. of ethionine i.p. Hexobarbital given 24 hours after second dose.
[e] Aldrin given 15 min. after first of two daily doses of ethionine.

only with doses of ethionine that when given alone significantly prolonged hexobarbital sleeping time. The plasma levels of hexobarbital administered to aldrin- and ethionine-treated mice, shown also in Table VIII, paralleled the changes in sleeping time. The fact that plasma levels of hexobarbital were lower in aldrin-treated animals than in controls indicates that aldrin decreased the sleeping time by increasing the in vivo metabolism of hexobarbital, and not by the well-known tendency of many chlorinated hydrocarbon insecticides to stimulate the central nervous system. This is in accord with the work of Ghazal and associates (*14*), who found that dieldrin, formed in vivo from aldrin (*4*), increased the metabolism of hexobarbital by liver microsomes in rats.

It is pertinent to inquire whether the reported increases in rat serum aliesterase (*3*) and liver A-esterase (*18*) as a result of aldrin treatment are responsible for the protective effect against paraoxon toxicity, or whether factors involving the central nervous system may also play a role in explaining the mechanism of protection, as suggested by the finding that aldrin pretreatment protected against only anticholinesterase agents that are relatively good inhibitors of brain cholinesterase. Parathion is first activated by the liver microsomal enzymes (*21*) to its potent anticholinesterase form, paraoxon (*12*), which is then at least partially detoxified by A-esterase (*2*). If aldrin pretreatment increases the enzymes responsible for the activation of parathion without also increasing the detoxifying enzymes, the toxicity of parathion would be expected to increase. Since, on the contrary, aldrin pretreatment protected against the toxicity of both parathion and paraoxon, the mechanism of the protective action against the toxicity of parathion may be explained in relation to its activated form, paraoxon.

The toxicity of paraoxon is limited by the cholinesterases and B-esterase, which are inhibited by paraoxon, and by A-esterase, which hydrolyzes paraoxon (*2*). It was of interest to determine whether mice showed the same rise of plasma aliesterase (B-esterase) and increase in liver A-esterase as other investigators, previously cited, observed in rats after aldrin treatment. The B-esterases are microsomal enzymes, but A-esterase has been shown in rats to be a lysosomal enzyme (*24*). Since increased levels of these esterases would reduce the toxicity of paraoxon, we tested the effect of aldrin on these enzymes in mice, and how such effect might be modified by ethionine. The results in Table IX show that the aldrin treatment increased liver A-esterase by about 38%, while simultaneously decreasing the plasma enzyme by about 50%, findings similar to those of Main in rats (*18*). However, the enzyme activity of the livers from animals receiving both aldrin and ethionine, and ethionine alone, did not differ significantly from controls. The plasma A-esterase activity in all experimental groups was significantly decreased to approximately 50% of that in controls. In the case of B-esterase in the plasma of mice a moderate increase after aldrin treatment is again in accord with the finding of Ball *et al.* (*3*) in rats. Table X shows that after a single dose of aldrin, plasma B-esterase activity was increased by approximately 24%. Also, as in the case of the increased liver A-esterase (Table IX), the 24% increase seen here was offset by the addition of ethionine to the pretreatment.

The increase in the activities of liver A-esterase and plasma B-esterase induced by aldrin would be expected to reduce the effect of paraoxon on cholinesterase activity. To ascertain whether this occurs, paraoxon was given orally to animals pretreated with aldrin and ethionine. The effects of aldrin and ethionine on plasma cholinesterase activity were also determined in groups of mice not given paraoxon. In mice treated with two daily doses of ethionine alone, there was a small but significant increase in plasma cholinesterase activity. After the administration of 1.0 mg. per kg. of paraoxon to animals pretreated by ethionine alone, or by aldrin with ethionine, cholinesterase activity was reduced to 19.7 and 21.9%, respectively, of the control levels, as compared to 35.1% in the untreated group (Table XI). However, when paraoxon was administered to aldrin-treated mice the

Table IX. Effects of Aldrin and Ethionine on Liver and Plasma A-Esterase in Mice

Pretreatment	p-Nitrophenol Liberated from Paraoxon, μMoles/G./30 Min. (Mean ± S.D.) Liver, g.[a]	P	Plasma, ml.[b]	P
Control	31.5 ± 6.9	...	7.9 ± 0.3	...
Aldrin[c]	43.4 ± 6.9	<0.02	4.0 ± 0.5	<0.001
Ethionine[d]	31.5 ± 10.4	N.S.	3.9 ± 0.8	<0.001
Aldrin + ethionine[e]	28.9 ± 5.8	N.S.	3.7 ± 0.4	<0.001

[a] Each value is mean of six determinations in each of which pooled livers of two animals were used.
[b] Each value is mean of six determinations in each of which pooled blood of two animals was used.
[c] Single dose of 16 mg./kg. of aldrin given orally 2 days before enzyme assay.
[d] Two daily doses of 200 mg./kg. of ethionine i.p. Enzyme assay performed 24 hours after second dose.
[e] Aldrin given 15 min. after first of two daily doses of ethionine.

Table X. Effect of Aldrin and Ethionine on Plasma B-Esterase in Mice

Pretreatment	2-Naphthol Liberated, μMoles/Ml./30 Min. (Mean ± S.D.)[a]	P
Control	188 ± 37	...
Aldrin[b]	233 ± 22	<0.05
Ethionine[c]	158 ± 17	N.S.
Aldrin + ethionine[d]	145 ± 38	N.S.

[a] Each value is mean of six determinations in each of which pooled blood of two animals was used.
[b] Single dose of 16 mg./kg. of aldrin given orally 2 days before enzyme assay.
[c] Two daily doses of 200 mg./kg. of ethionine i.p. Enzyme assay performed 24 hours after second dose.
[d] Aldrin given 15 min. after first of two daily doses of ethionine.

Table XI. Effects of Aldrin and Ethionine on Anticholinesterase Action of Oral Paraoxon[a] in Plasma and Brain of Mice

Pretreatment	% of Control[b] Cholinesterase Activity: Plasma	Brain
Untreated	35.1	50.3
Aldrin[c]	29.3	84.0
Ethionine[d]	19.7	26.7
Aldrin + ethionine[e]	21.9	54.7

[a] Animals sacrificed 40 min. after administration of 1 mg./kg. of paraoxon for plasma cholinesterase activity. Animals sacrificed 30 min. after administration of 15 mg./kg. of paraoxon for brain cholinesterase activity.
[b] Controls represent untreated and groups of animals pretreated with aldrin, ethionine, and aldrin plus ethionine not given paraoxon.
[c] Single dose of 16 mg./kg. of aldrin given orally 2 days before paraoxon.
[d] Two daily doses of 200 mg./kg. of ethionine i.p. Paraoxon given 24 hours after second dose.
[e] Aldrin given 15 min. after first of two daily doses of ethionine.

extent of inhibition of plasma cholinesterase was essentially the same as in the untreated mice. The plasma enzyme activity of aldrin-treated animals was reduced to 29.3% of the control level, an only slightly greater reduction than that seen in the untreated group (35.1%).

Since it was not possible to demonstrate a substantial difference between the plasma cholinesterase activities of control and aldrin-treated animals given paraoxon, an experiment similar to that described above was carried out to determine the effects of 15 mg. per kg. of paraoxon on brain cholinesterase activity. Neither aldrin or ethionine treatment affected the brain cholinesterase levels in mice not given paraoxon. Table XI shows that the dose of paraoxon used reduced brain cholinesterase activity to 50.3% of the control level, whereas in the aldrin-treated animals the activity remained at 84.0% of the control level. The addition of ethionine to the aldrin pretreatment offset the apparent protective effect of the latter against the inhibiting action of paraoxon. In animals receiving ethionine alone, however, paraoxon decreased enzyme activity significantly more than in the corresponding untreated group.

General Discussion

These studies show that aldrin treatment first increased and later decreased both the toxicity of parathion and the sleeping time of hexobarbital in mice. The delayed influence of aldrin on hexobarbital sleeping time was abolished by the administration of ethionine. Plasma levels of hexobarbital were lower in aldrin-treated than in control animals and tended to parallel sleeping times. Aldrin thus appears to decrease hexobarbital sleeping time by stimulating the activity of liver microsomal enzymes to accelerate its in vivo metabolism.

In regard to the delayed phase of aldrin's action on the toxicity of paraoxon and several other anticholinesterase agents, the findings that ethionine abolished the protective effect of aldrin against the brain cholinesterase inhibitory action of paraoxon, the aldrin-induced increase of esterase that can limit the action of paraoxon, and the protective effect of aldrin against the toxicity of paraoxon, are in accord with the conclusion that aldrin's protective effect is mediated at least partially through the production of adaptive enzymes. However, we are not able to explain why paraoxon inhibits brain cholinesterase less in aldrin-treated than in control mice, while at the same time it produces essentially the same inhibition of the enzyme in the plasma of the two groups. In aldrin-treated animals the observed 50% decrease in plasma A-esterase might be expected to offset to some extent the effects of the elevated plasma B-esterase and liver A-esterase, but if this occurs the question arises why it would not be reflected in the brain as well as in the plasma cholinesterase picture.

The protective effect of aldrin against the toxicity of paraoxon is undoubtedly related to the fact that paraoxon inhibited brain cholinesterase less in the aldrin-treated animals than in the controls. In accord with this is the observation that aldrin failed to protect against neostigmine and OMPA, which are relatively poor inhibitors of brain cholinesterase. Among the anticholinesterases that we tested aldrin provided protection against all those that are relatively active inhibitors of brain cholinesterase in vivo. Therefore, it seems reasonable to conclude that factors other than the A- and B-esterases considered here may play a role in the apparent protective effect of aldrin pretreatment against the inhibitory action of paraoxon on brain cholinesterase.

The protective effect described for the chlorinated hydrocarbon insecticides would theoretically have a beneficial significance for man in reducing the possibilities of poisoning by the organophosphate insecticides. The practical significance to public health of a toxicologic interaction of this kind, however, is not known, since the low levels of chlorinated hydrocarbons accumulated in the population on a chronic basis may not be sufficient to exert a significant stimulating effect on organophosphate-metabolizing enzymes. This protective effect may be more likely to occur among wildlife, where levels of exposure are frequently higher, or among insecticide sprayers working with both types of these agents. However, adaptive enzymes potentially induced by the chlorinated hydrocarbons in man's environment may play some role in protecting him against the adverse effects of other environmental chemicals to which he may be exposed.

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SYNERGISM

Mode of Action of Carbamate Synergists

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The insecticidal carbamates are synergized by a wide variety of methylenedioxyphenyl compounds. These act as inhibitors of phenolase enzymes which detoxify the carbamates largely by ring hydroxylation. The active inhibitors appear to require a three-point attachment to the phenolase enzyme to orient the methylene carbon so that interaction with a nucleophilic group at the enzyme active site takes place. Tyrosinase, which is abundant in the housefly, has served as a model enzyme for study of the kinetics of this interaction. Soluble housefly tyrosinase has been highly purified and accepts insecticidal carbamates as substrates for hydroxylation. The susceptibility of individual carbamates to enzymatic detoxication is greatly influenced by the nature of the aryl ring. The methylenedioxynaphthalenes and piperonyl carbamates are exceptionally active carbamate synergists.

ALTHOUGH THE ACTION of methylenedioxyphenyl compounds, such as piperonyl butoxide, sulfoxide, sesamex, and propyl isome, as synergists for the pyrethrins has been studied for many years, Moorefield's (*38*) demonstration of the remarkable activating properties of these synergists when used with the *N*-methyl- and *N,N*-dimethylcarbamates, such as carbaryl and isolan, provided the impetus for detailed studies of the mode of action of synergists in relation to the metabolism of the relatively simple structures of the carbamate insecticides. Moorefield (*39*) found that a combination of five parts of piperonyl butoxide (3,4 - methylenedioxy - 6 - propylbenzyl butyldiethyleneglycol ether), and either carbaryl or 3-*tert*-butylphenyl *N*-methylcarbamate, straightened and steepened the dosage mortality curve to the housefly and displaced it some 50-fold to the left. Sesoxane [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane] was found by Eldefrawi, Miskus, and Sutcher (*9*) to enhance the activity of carbaryl and other carbamates not only against susceptible houseflies but also to strains resistant to DDT and parathion and at ratios of synergist to insecticide as low as 1:10. Georghiou, Metcalf, and March (*18*) selected susceptible houseflies with 3-isopropylphenyl *N*-methylcarbamate and found that, after seven generations, the female flies had developed complete tolerance to this carbamate and to a variety of related substituted phenyl *N*-methylcarbamates. However, synergism with piperonyl butoxide almost completely restored the effectiveness of the carbamates to the resistant strain. Georghiou (*14, 16*) showed that the carbamate resistant R_{MIP} flies lost their resistance after 54 generations without selection pressure, and that the carbamate resistance results from a partially dominant single factor inheritance.

Georghiou and Metcalf (*17*) demonstrated that the R_{MIP} flies metabolized 3-isopropylphenyl *N*-methylcarbamate about 10 times as fast as the S_{NAIDM} flies, and that pretreatment with piperonyl butoxide substantially reduced the rate of metabolism of the carbamate. Similar studies with C^{14}-labeled Zectran (4-dimethylamino-3,5-xylenyl *N*-methylcarbamate) (*33*) also demonstrated the rapid metabolism of the carbamate in the R_{MIP} flies and the inhibition of this detoxication by pretreatment with piperonyl butoxide.

The mode of carbamate detoxication is complex as the studies of Dorough and