Potentiation Between Cholinesterase Inhibitors.* (23894)

PHILIP ROSENBERG AND J. M. COON
Department of Pharmacology, Jefferson Medical College

Simultaneous administration of insecticides EPN (ethyl-p-nitrophenyl thionobenzenephosphonate) and malathion (S-(1,2-dicarbethoxyethyl) 0,0-dimethyl dithionophosphate) to rats and dogs has been reported to result in potentiation of their toxicities(1). This is thought(2,3) to be due to inhibition by EPN of detoxification of malathion by the liver. Since many other organic phosphate anticholinesterases are widely used as insecticides it is important to determine whether other combinations also show potentiation to enable one to evaluate hazards involved in handling these agents and in consuming food from crops sprayed with them. Data are presented here which show that several combinations of anticholinesterase insecticides exhibit potentiated toxicities, and the results of cholinesterase studies are correlated with this phenomenon.

Methods and materials. Young adult male Swiss-Webster mice fasted 16 to 20 hours were used in toxicity experiments. The anticholinesterase agents tested were technical samples of EPN (89%)† malathion (95%),‡ chlorothion (p-nitro-m-chlorophenyl dimethyl thionophosphate)§ BFP (bis-dimethyl-amido fluorophosphate)§ and phostex (mixture of bis-(dialkoxyphosphinothioyl) disulfides).‖ All anticholinesterases were given orally in corn oil and combinations were administered simultaneously except where otherwise noted. Animals were observed at least 48 hrs following injections and times of death recorded. Brain and liver tissue used for cholinesterase estimations were obtained from Wistar rats (200-300 g). In liver incubation studies the general procedure of DuBois, Doull and Coon (4) was used. Liver slices (15 to 30 mg dry wt) were incubated for 1 hr in a Krebs phosphate medium with and without added aqueous solutions of anticholinesterase. One-tenth portions (0.3 ml) of these incubates were then added to flasks containing 2.4 ml of brain homogenate and allowed to incubate 1 hr. Cholinesterase determinations were then performed manometrically by the method of DuBois and Mangun(5) with 0.3 ml 0.1 M acetylcholine chloride as the substrate.

Results. Potentiated toxicity between 2 compounds was assumed present if non-toxic doses of each, when combined, produced a greater mortality than did twice these doses of either compound alone. Those combinations which showed potentiation by this definition are recorded in Table I. EPN, malathion, chlorothion and phostex in all possible paired combinations showed potentiation. EPN plus malathion showed greatest potentiation and EPN plus phostex the least. Potentiation was also seen with these agents in numerous other combinations of dosages not recorded in Table I. It was also observed that when EPN and malathion were given 4 hrs apart the potentiation was almost as great

TABLE I. Combinations of Anticholinesterases Showing Potentiation in Toxicity.

EPN	Malathion	Chlorothion	Phostex	No. of mice	% mortality
		mg/kg			
30				10	10
20				10	0
	500			19	21
	250			12	0
		1000		10	70
		500		10	0
			2000	9	11
			1000	11	0
10	125			11	91
10	60			10	30
10		250		10	40
20			1000	7	43
10			1000	10	20
	250	180		10	80
	125		500	8	75
		250	1000	10	60

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† EPN was supplied by E. I. du Pont de Nemours & Co.

‡ Malathion was furnished by Am. Cyanamid Co.

§ Chlorothion and BFP were given to us by Dr. K. P. DuBois, Univ. of Chicago.

‖ Phostex was provided by Niagara Chem. Division, Food Machinery and Chem. Corp.

TABLE II. Inactivation of Malathion, Chlorothion and Phostex by Rat Liver Slices.

Anticholinesterase	Molar conc.	% brain cholinesterase inhibition: Incubated without liver slices	% brain cholinesterase inhibition: Incubated with liver slices
Malathion	1×10^{-4}	64	23
	5×10^{-5}	38	0
Chlorothion	1×10^{-4}	57	36
	5×10^{-5}	40	0
Phostex	1×10^{-5}	95	14
	5×10^{-6}	40	0

as when given simultaneously, and even when EPN was administered 24 hrs before malathion this pair still showed some potentiation. This is in accord with the finding that a single dose of EPN inhibits ability of rat liver to detoxify malaoxon (oxygen analog of malathion) for as long as 72 hrs(3). Pairs of anticholinesterases tested which did not show definite potentiation were: malathion combined with OMPA (octamethyl pyrophosphoramide), with parathion (p-nitrophenyl diethyl thionophosphate) or with physostigmine; EPN plus parathion, EPN plus OMPA, and OMPA plus parathion. Malathion plus BFP appeared to be a borderline case.

Since chlorothion and phostex have relatively low toxicities, the possibility was considered that they, like malathion, might be inactivated by the liver. As shown in Table II we confirmed the inactivation of malathion by liver and also found that chlorothion and phostex are partially inactivated. The results recorded are the averages of 2 to 4 determinations. It appears that phostex, the least toxic of all of the anticholinesterases employed, is also the most extensively inactivated by the liver. It was anticipated that EPN would inhibit inactivation of chlorothion and phostex, as it does in the case of malathion(2,3), thereby accounting for potentiation observed with these agents. Since malathion, chlorothion and phostex are all inactivated to some extent by liver a competition for sites of inactivation seems possible when combinations of these 3 inhibitors are administered to mice. This competition could explain the observed potentiation in toxicity. It was first seen, in confirmation of an earlier report(1), that addition of EPN and malathion to brain homogenates without prior incubation with liver slices did not cause a potentiation of cholinesterase inhibition. *In vitro* liver incubation studies were then performed to see whether potentiation of toxicity by various combinations of cholinesterase inhibitors could be correlated with *in vitro* inhibition of inactivation of certain of these agents by liver slices. When anticholinesterases were first incubated with liver slices, either alone or combined, potentiation of brain cholinesterase inhibition was observed with several combinations (Table III). The results are averages of 2 determinations. Each combination that exhibited potentiated inhibition of cholinesterase also showed potentiated toxicity (Table I). None of the combinations tested showed as great a potentiation of enzyme inhibition as did EPN plus malathion, and this pair also produced the most marked potentiation of toxicity (Table I). Preliminary results indicate that OMPA does not inhibit inactivation of malathion by liver slices since this combination showed no potentiated inhibition of brain cholinesterase after incubation with liver slices. This agrees with the observed lack of potentiation in toxicity when OMPA and malathion were combined.

TABLE III. Potentiation of Brain Cholinesterase Inhibition by Combinations of Anticholinesterases Previously Incubated with Liver Slices.

Anticholinesterases	Molar conc.	% cholinesterase inhibition
EPN	1×10^{-5}	0
Malathion	1×10^{-4}	0
EPN + malathion	1×10^{-5} 1×10^{-4}	78
EPN + malathion	1×10^{-5} "	48
EPN	1×10^{-6}	0
Chlorothion	5×10^{-5}	17
EPN + chlorothion	1×10^{-6} 5×10^{-5}	32
EPN	1×10^{-6}	0
Phostex	1×10^{-5}	14
EPN + phostex	1×10^{-6} 1×10^{-5}	45
Malathion	1×10^{-4}	9
Chlorothion	1×10^{-5}	0
Malathion + chlorothion	1×10^{-4} 1×10^{-5}	65

Discussion. The data presented demonstrate that combinations of anticholinesterases other than EPN and malathion exert potentiated toxic action in mice. In each combination showing potentiation at least 1 of 2 compounds is inactivated by liver. Inactivation of malathion, chlorothion and phostex by liver is undoubtedly the reason for their low toxicity since they are fairly strong cholinesterase inhibitors when added directly to brain homogenates. This inactivation of chlorothion by liver slices is not in accord with the report(2) that this compound is unaltered by liver homogenates, a discrepancy which may be due to differences in methods used. We observed that EPN inhibits inactivation of chlorothion and phostex by liver as has been reported for malathion(2,3). Combinations of malathion and chlorothion showed potentiation in toxicity and cholinesterase inhibition possibly because they mutually inhibit their inactivations through competition for the same enzyme site. Thus in order for potentiation to occur it appears that one or both compounds must inhibit inactivation of the other.

It is not yet possible to explain why EPN inhibits inactivation of malathion whereas OMPA does not. Purified EPN and OMPA are oxidized by liver from weak cholinesterase inhibitors to potent inhibitors(4,6), and one might expect them to affect inactivation of malathion similarly. Our technical sample of EPN differs from purified EPN in that it possesses some *in vitro* anticholinesterase activity, but after incubation with liver slices it is a more potent inhibitor. OMPA and other anticholinesterases in high concentrations may inhibit inactivation of malathion, chlorothion or phostex as does EPN, but the concentrations required may produce enough cholinesterase inhibition as to be themselves toxic, thereby masking any interference with the inactivation of malathion. This could account for the apparent lack of potentiation between OMPA and malathion.

Evidence indicates that detoxification of malathion involves hydrolysis of ester linkages in the 1,2-dicarbethoxyethyl side chains (2,7). It has been suggested, therefore, that EPN may inhibit detoxification of other drugs, besides malathion, which contain ester groups (3). Chlorothion and phostex do not contain ester groupings but do contain alkoxy groups which suggests that the inactivation of these agents may involve dealkylation. The possibility thus arises that EPN also inhibits dealkylations in the body.

The observations described here suggest the necessity of focusing broader attention upon hazards which may be involved in simultaneous exposure to, or ingestion of, through any source, 2 or more anticholinesterase agents which are used as insecticides. Extensive manufacture and wide dissemination of a great variety of these agents has created the complex and important problem of protecting workers and the food-consuming public against the potential dangers of these materials in their environment.

Summary. Organic phosphate insecticides EPN, malathion, chlorothion and phostex, in all possible paired combinations administered simultaneously to mice showed potentiation in their oral toxicities. Some potentiation was observed even when EPN was given 24 hours before malathion. Malathion plus BFP showed borderline potentiation, whereas malathion combined with OMPA, parathion or physostigmine showed no potentiation, nor did EPN plus parathion, EPN plus OMPA or OMPA plus parathion. Malathion, chlorothion and phostex were partially inactivated by incubation with rat liver slices. EPN inhibited inactivation of malathion, chlorothion and phostex, producing an *in vitro* potentiation of cholinesterase inhibition. Potentiation in all cases found so far appears to be caused by interference by one compound with inactivation of the other by liver, or by a mutual interference with inactivation through a competition for a specific enzyme site.

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Tissue Culture and Circulating Myeloblasts of Avian Leukemia Studied in Electron Micrographs of Ultrathin Sections.* (23895)

D. F. PARSONS, J. C. PAINTER, G. S. BEAUDREAU, C. BECKER AND J. W. BEARD
Department of Surgery, Duke University School of Medicine, Durham, N. C.

In blood of chickens with virus-induced myeloblastosis, there occur primitive cells of myeloid origin(1,2). Recent studies(3) have shown that such cells can be maintained for long periods in tissue culture. Under these conditions virus particles characteristic of the disease are liberated in large numbers into the culture fluid, as estimated by direct particle count in electron microscope and by measurement of enzymatic activity of the agent to dephosphorylate adenosine triphosphate. The source of the virus was presumably the primitive cells. In this report there are described the results of studies made by means of technics of ultrathin sectioning† with the object of obtaining information about the cell-virus relationships involved in the process. The principal materials examined were myeloblasts taken directly from the circulation and the cells after various intervals of culture. For purposes of comparison, there were investigated, also, sections of spleen, bone marrow and liver.

Material and methods. The materials were obtained from birds with avian myeloblastosis produced with virus of the B.A.I., a strain studied(4) for several years in this laboratory. Experimental induction of the disease was effected by intravenous injection of 0.1-ml volumes of filtered whole or diluted plasma from previous passage chickens. Myeloblasts were obtained as already described(3) by centrifugation of heparinized blood. Donor birds, in advanced stages of the disease, were selected for high blood content of myeloblasts (about 1 to 2 million/mm^3) as judged from routine blood smears and high plasma virus concentration (about 10^{12} particles/ml) determined by enzyme activity(5). Tissue cultures of the myeloblasts were established and maintained in the usual way(3) in medium consisting of Gey's saline solution containing 5 to 20% chicken serum and added glucose in 0.5% concentration. The cultures were prepared in 5-ml volumes with about 10^7 cells/ml in 50-ml flasks and incubated at 37°C on the gyratory shaker. Fluids were changed at 2 to 4-day intervals. Two kinds of samples of cells were taken for thin sectioning; namely, free floating cells collected by centrifugation of culture fluid; and attached cells scraped from walls of flasks. Samples were taken from 4 different series of cultures at intervals of 0 to 77 days. Each sampling was from 2 or 3 flasks carried in parallel in each series. All were fixed for 3 hours in the cold in 2 ml of 1% osmic acid buffered to pH 7.4. Myeloblasts from centrifuged blood were delivered in drops of about 1 mm diameter from vaselined fine-tipped pipettes directly into the osmic acid solution. Pieces of spleen, bone marrow and liver of about 1 mm^3 were taken from 5 diseased birds in late stage of disease. Studies were made, also, on spleen, liver, bone marrow and white blood cells from 2 untreated birds of comparable age. Procedures for preparation of all specimens for thin sectioning were essentially those of Palade(6). The fixed samples were dehydrated with alcohol, imbedded in methacrylate and sectioned with

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