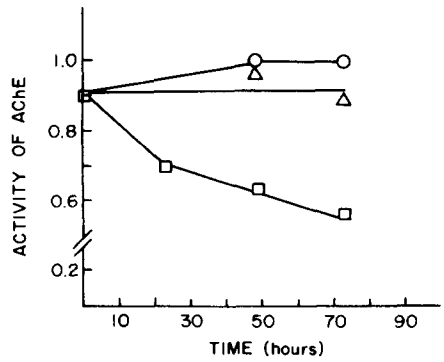

Fig. 2. Activity of lung acetylcholine esterase from paraquat-poisoned mice. Four groups of mice, five animals in each group, were used in each experiment. Experimental conditions were similar to those described in Fig. 1. Injections were given intraperitoneally with 1 hr intervals. The first group (control) was injected five times with sterile water (not shown). The second group (○) was treated with desferrioxamine (three injections of 5 mg followed by two injections of 2 mg of Desferal). The third group (□) was treated with a single dose of paraquat (20 mg/kg), and the fourth group (△) was treated with a combination of paraquat and desferrioxamine (a single dose of paraquat (20 mg/kg) followed by multiple treatment of desferrioxamine, 3 injections of Desferal 5 mg each, two injections of Desferal 2 mg each). Lungs from each group were excised daily and individually analysed for the enzymatic activity of acetylcholine esterase, determined according to Brown and Mailing [22] and Ellman *et al.* [26].

It has been suggested that the toxicity of paraquat is due to oxygen-derived free radicals such as the superoxide anion [10]. We have shown in a bacterial model that transition metal ions are an essential requirement for paraquat toxicity, in a mechanism analogous to that of superoxide or ascorbate [23, 21]. In this mechanism we hypothesize that hydroxyl radicals, produced via the "site-specific metal-mediated Haber-Weiss" reaction, are highly efficient in destroying biological macromolecules. The results in this communication are in full accord with the mechanism suggested for the bacterial system. Thus, it is anticipated that chelation therapy should have a marked beneficial effect on paraquat intoxicated human subjects.

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Enzymatic synthesis of sarin and soman

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There are two groups of organophosphate inhibitors that inactivate cholinesterases, namely G-group inhibitors $CH_3(RO)P(O)F$ and V-group inhibitors $CH_3(RO)P(O)$-$SCH_2CH_2NR_2'$. Hydrolysis of G-group organophosphate inhibitors is specifically catalyzed by G-group organophosphate hydrolase (phosphorylphosphatase) [1–4]. According to the theory of catalysis, enzymatic reactions might proceed in both directions. In this paper, enzymatic syn-

theses *in vitro* of sarin and soman from their corresponding hydrolytic products [1] were studied.

Materials and methods

Cholinesterase preparation. Rabbit brain was homogenized in 0.5% Triton X-100–1/15 M sodium phosphate buffer, pH 7.2, and thoroughly dialyzed against saline at 4°. The dialyzed homogenate was centrifuged at 13,000 *g* for 20 min, and the resulting supernatant fraction was stored at −20°.

G-group organophosphate hydrolase preparation. Rabbit plasma was frozen and thawed three times to inactivate, if any, the V-group organophosphate oxidase.* The G-group organophosphate hydrolase was not affected significantly by freezing and thawing. Plasma cholinesterase activity was inhibited completely and aged in 30 min by submitting the plasma to 1,2,2′,-trimethylpropyl-*S*-(β-di-ethylamino-ethyl)-methylphosphonothiolate at a final concentration of 1 mM. The free organophosphate inhibitor was removed by dialyzing against saline for 150 hr at 2–4°. This "treated plasma" did not inhibit the added cholinesterase, indicating that it was free from organophosphate inhibitor. The "treated plasma" lost all its cholinesterase activity, whereas almost 90% of the somanase activity (131–133 μl CO_2/hr/mg protein) remained (data not shown). "Treated plasma" was kept at −20°.

G-group organophosphate hydrolase activity was assayed manometrically at 37° in a bicarbonate buffer medium, pH 7.2, using 38 mM soman as substrate. Cholinesterase activity was measured by a microassay modified from Hestrin [5]. Protein content was estimated by ultraviolet spectrometry [$Conc_{protein}$ (mg/ml) = $1.45\,A_{280} - 0.74\,A_{260}$].

Sodium salts of isopropyl methylphosphonic acid (IMPA·Na), 1,2,2′-trimethylpropyl methylphosphonic acid (PMPA·Na) and methylphosphonic acid (MPA·Na_2) were prepared in our laboratory. All reagents used were chemical pure grade.

Results and discussion

Enzymatic synthesis of sarin and soman. Enzymatic reactions were carried out at 37°, pH 7.2, for 30 min. The reaction mixture contained 50 μl of 10 mM IMPA·Na or PMPA·Na, 50 μl of 10 mM NaF, 200 μl of treated plasma, 150 μl of dialyzed 1:4 rabbit brain as source of the added cholinesterase, and 1/15 M phosphate buffer, pH 7.2, to a total volume of 500 μl. Five controls were set up, as shown in Tables 1 and 2. Added cholinesterase activities in all five controls remained intact, whereas the enzyme activity in the tubes labeled "enzymatic synthesis" was inhibited by about 93–94% (Tables 1 and 2). This suggested that organophosphate inhibitors were formed in each case. Sarin and soman were synthesized catalytically by G-group organophosphate hydrolase from their corresponding hydrolytic derivatives, IMPA·Na or PMPA·Na and NaF, in the presence of the added cholinesterase. The time course for the enzymatic synthesis of soman could be followed up.

* Unpublished work.

Table 1. Enzymatic synthesis of sarin *in vitro**

	10 mM IMPA·Na (μl)	10 mM NaF (μl)	"Treated plasma" (μl)	1:4 Rabbit brain, dialyzed (μl)	Buffer (μl)	Cholinesterase activity (μmoles ACh/30 min)	(%)
Normal ChE			200†	150	150	4.31 ± 0.09	100
IMPA·Na	50		200†	150	100	4.15 ± 0.18	96
NaF		50	200†	150	100	4.0 ± 0.3	93
IMPA·Na + NaF	50	50	200†	150	50	3.94 ± 0.01	91
"Treated plasma"			200	150	150	4.50 ± 0.06	104
Enzymatic synthesis	50	50	200	150	50	0.28 ± 0.03	6‡

* Experimental conditions: 1/15 M sodium phosphate buffer, pH 7.2; incubation at 37° for 30 min. ChE activity was estimated by a microassay modified from Hestrin [5]. Values are expressed as mean ± S.D., N = 2.

† Added after the addition of alkaline hydroxylamine.

‡ $P < 0.01$, compared with the normal ChE activity.

Table 2. Enzymatic synthesis of soman *in vitro**

	10 mM PMPA·Na (μl)	10 mM NaF (μl)	"Treated plasma" (μl)	1:4 Rabbit brain, dialyzed (μl)	Buffer (μl)	Cholinesterase activity (μmoles ACh/30 min)	(%)
Normal ChE			200†	150	150	4.31 ± 0.09	100
PMPA·Na	50		200†	150	100	4.2 ± 0.3	97
NaF		50	200†	150	100	4.0 ± 0.3	94
PMPA·Na + NaF	50	50	200†	150	50	3.87 ± 0.10	90
"Treated plasma"			200	150	150	4.50 ± 0.06	104
Enzymatic synthesis	50	50	200	150	50	0.29 ± 0.22	7‡

* Conditions were the same as in Table 1. Values are expressed as mean ± S.D., N = 2.

† Added after the addition of alkaline hydroxylamine.

‡ $P < 0.01$, compared with the normal ChE activity.

Table 3. 1,2,2′-Trimethylpropyl-*S*-(β-dimethylaminoethyl)thiomethylphosphonate negative control experiment*

	10 mM PMPA·Na (μl)	10 mM R·SH (μl)	"Treated plasma" (μl)	1:4 Rabbit brain, dialyzed (μl)	Buffer (μl)	Cholinesterase activity (μmoles ACh/30 min)	(%)
Normal ChE			200†	150	150	4.37 ± 0.14	100
PMPA·Na	50		200†	150	100	4.33 ± 0.27	90
R·SH		50	200†	150	100	3.5 ± 0.6	80
PMPA·Na + R·SH	50	50	200†	150	50	3.0 ± 0.7	69‡
"Treated plasma"			200	150	150	4.54 ± 0.09	104
Synthetic control	50	50	200	150	50	3.3 ± 0.4	75§

* Conditions were the same as in Table 1. Values are expressed as mean ± S.D., N = 3.
† Added after the addition of alkaline hydroxylamine.
‡ $P < 0.05$, compared with the normal ChE activity.
§ $P < 0.01$, compared with the normal ChE activity.

Table 4. Enzymatic synthesis of methylphosphonofluoridate *in vitro**

	10 mM MPA·Na (μl)	10 mM NaF (μl)	"Treated plasma" (μl)	1:4 Rabbit brain, dialyzed (μl)	Buffer (μl)	Cholinesterase activity (μmoles ×10 ACh/30 min)	(%)
Normal ChE			20†	10	20	4.60 ± 0.13	100
MPA·Na_2	5		20†	10	15	4.87 ± 0.06	106
NaF		5	20†	10	15	4.0 ± 0.4	87
MPA·Na_2 + NaF	5	5	20†	10	10	3.88 ± 0.27	84
"Treated plasma"			20	10	20	4.89 ± 0.11	106
Enzymatic synthesis	5	5	20	10	10	1.57 ± 0.13	34‡

* Conditions were the same as in Table 1. Values are expressed as mean ± S.D., N = 2.
† Added after the addition of alkaline hydroxylamine.
‡ $P < 0.01$, compared with the normal ChE activity.

Effects of substrate concentration on enzymatic synthesis of soman. Various concentrations of hydrolytic products of soman were used in the reaction mixtures, and the suppression of activities of the added cholinesterase was estimated. Soman formation was closely related to substrate concentrations with an optimum at 10–30 mM PMPA·Na (data not shown). At higher substrate concentrations, substrate inhibition was observed. The Michaelis constant was about 0.75 mM.

Negative control experiment of 1,2,2′-trimethylpropyl-S-(β-diethylaminoethyl)methylphosphonothiolate. 1,2,2′-Trimethylpropyl-*S*-(β-diethylaminoethyl)methylphosphonothiolate is one of the V-group organophosphate inhibitors. It is not hydrolyzed by the G-group organophosphate hydrolase, and it is not expected to be synthesized by the same enzyme from PMPA and *S*-(β-diethylaminoethyl) thiol (R·SH). This was shown to be the case (Table 3). PMPA·Na and R·SH caused 20–30% inhibition of the added cholinesterase activity, i.e. the same extent as in the synthetic control (25%). These results give further support to the enzymatic synthesis of sarin and soman.

Formation of aged cholinesterase. Of interest is the enzymatic synthesis of methylphosphonofluoridate from NaF and disodium methylphosphonate (MPA·Na_2). The produced organophosphate inhibitor caused 66% inhibition of the added cholinesterase activity (Table 4). The immediate outcome of phosphorylation was the formation of an aged enzyme which was not activated by pyridine-2-aldoxime methochloride.

Authentic methylphosphonofluoridate has been studied for its acute toxicity. The LD_{50} in mice is 77.59 mg/kg intraperitoneally, while the pI_{50} for human brain cholinesterase is 4.4, indicating moderate anticholinesterase potency.

Enzymatic synthesis of sarin and soman and methylphosphonofluoridate prompted us to investigate the reaction *in vivo* using a more purified enzyme. We suspected that the reaction might play a role in the intoxication mechanism *in vivo* and have practical as well as theoretical significance.

In summary, incubation of sodium salts of isopropylmethylphosphonic acid, 1,2,2′-trimethylpropyl methylphosphonic acid, and methylphosphonic acid with sodium fluoride in the presence of G-group organophosphate hydrolase preparation from rabbit plasma at 37°, pH 7.2, caused pronounced inhibition of the added cholinesterase. The results indicate that the hydrolytic products of organophosphate inhibitors can serve as substrates for the *de novo* synthesis of inhibitors *in vitro*. G-group organophosphate hydrolase failed, as expected, to catalyze the synthesis of V-group organophosphate inhibitors.

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