aziridinyl)-1-azepinylphosphine oxide (hexadepa) (LD_{50} = 22 mg/kg). Twenty-four rats of Wistar–Furth strain were used in each of these groups, six each of male and female rats were retained as controls, and six male or female rats were injected with 1 mg/kg of III daily for 5 days and 5 mg/kg of hexadepa for 5 days. They were then mated with noninjected male or female rats (six in each group) in separate cages, fed with the same food. The total number of litters was counted after 12 weeks. The male injected group yielded a total of six (for III) and zero (for hexadepa). Both of these numbers are lower than the normal control group of 19. While the difference was obvious, no effort was made to determine either the optimum sterilizing dose or the reversibility of such treatment.

Histochemical Study. The histochemical study of the effect of III and hexadepa was carried out at the same dose level in Wistar–Furth rats and was tested for 1-, 3-, and 24-h periods. The testes or ovary was obtained when the animal was sacrificed and frozen sections were put on a cryostat. Tissues were fixed with 95% alcohol for 10 min. Acridine orange was used as a fluorescent stain for DNA (green) and RNA (red). No difference was noted between the treated and control groups. Only in the 24-h group could one find a decrease in the visible number of secondary spermatocytes but not in the mature spermatocytes. No effect on ovaries was noticed. *Corpus luteus* effect was attributed to the usual estrus cycle of female rats. This result suggested to us that the damage to testes also might be reversible and further extensive study should be necessary.

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Synthesis and Properties of 2-*S*-[2'-(*N*,*N*-Dialkylamino)ethyl]thio-1,3,2-dioxaphosphorinane 2-Oxide and of the Corresponding Quaternary Derivatives as Potential Nontoxic Antiglaucoma Agents

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A new series of cyclic organophosphorus esters, 2-*S*-[2'-(*N*,*N*-dialkylamino)ethyl]thio-1,3,2-dioxaphosphorinane 2-oxide and their quaternary derivatives, was synthesized and studied as potential antiglaucoma agents. These compounds inhibit acetylcholinesterase (E.C. 3.1.1.7) at a bimolecular rate constant (k_i) in the range of 10^3–10^4 M^{-1} min^{-1}. Values of the affinity (K) and phosphorylation (k') rate constants for this enzyme indicate that k' is responsible for the relatively low values of k_i as compared with similar data for the open-chain analogues, *O*,*O*-diethyl phosphorothiolates (10^6 M^{-1} min^{-1}). The mammalian toxicity of the new compounds in terms of acute LD_{50} values in mice is 1–3 × 10^3 less than that of phospholine, an open-chain analogue. In an initial clinical trial, one member of the new series (alkyl = C_2H_5) caused a significant decrease of intraocular pressure in aphakic glaucoma, while phospholine proved to be ineffective.

Anticholinesterase agents are used as topical agents for the treatment of ophthalmological conditions such as glaucoma.[1] For example, carbamates and organophosphates have found considerable use in the therapy of primary glaucoma (narrow and wide angle) or secondary glaucoma (aphakic glaucoma). The severe ocular and general side effects which follow the treatment with powerful AcChE inhibitors such as DFP or phospholine[2,3] have limited their application in the relief of intraocular pressure.

Recently it has been shown that AcChE (electric eel) inhibited with 1,3,2-dioxaphosphorinane 2-oxide derivatives (I, X = F, Cl, *p*-nitrophenyl) undergoes spontaneous reactivation with $t_{1/2} \simeq 12$ min at pH 7.0.[4] This conceivably contributes to the very low mammalian toxicity of these compounds (>100 mg/kg sc in mice).

Since it has been established that phosphorothiolates with leaving groups of the type $(alkyl)_2NCH_2CH_2S$– are excellent inhibitors of AcChE,[5] it seemed rational to investigate compounds of structure I, but where X is a thiocholine analogue. This approach dwells on the assumption that the proposed structure (I) may prove more reactive toward the enzyme than the cyclic analogue, where X = halogen, but at the same time, the rate of spontaneous reactivation should not be affected. Compounds abiding by these criteria would constitute a new class of relatively safe AcChE inhibitors for drug therapy of glaucoma or conditions where cholinergic function is impaired (e.g., myasthenia gravis).

The present report deals with the syntheses and some biochemical and toxicological properties of new cyclic phosphorothiolates I as compared to the open-chain analogues II.

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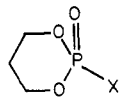
Table I. New Cyclic Phosphorothiolates

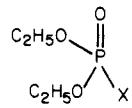
Compd	Mp or bp (mm-Hg), °C	Yield, %	Formula	Analyses[a]
1	63 (4 × 10^{-5})	10	$C_9H_{20}NO_3PS$	*b*
2	97-98	75	$C_{11}H_{26}NO_6PS_2$	C, H, N, P
3	*c*	56	$C_{11}H_{24}NO_3PS$	C, H, N, P
4	143-146	50	$C_9H_{22}NO_2PS_2$	C, H, N

[a] Unless stated, the results are within ±0.4% of the calculated value. [b] Calcd: C, 42.69; H, 7.90; N, 5.53; P, 12.25. Found: C, 42.10, H, 7.85; N, 6.34; P, 11.41. [c] Viscous oil, purified by column chromatography.



I

1, X = $SCH_2CH_2N(C_2H_5)_2$
2, X = $SCH_2CH_2{}^+N(CH_3)(C_2H_5)_2\ CH_3SO_3^-$
3, X = $SCH_2CH_2N(i\text{-}C_3H_7)_2$
4, X = $SCH_2CH_2{}^+N(CH_3)_3\ CH_3SO_3^-$



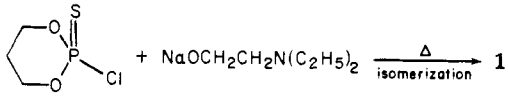
II

5, X = $SCH_2CH_2N(CH_3)_2$
6, X = $SCH_2CH_2{}^+N(CH_3)_3\ I^-$

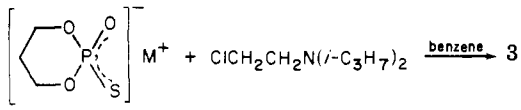
Results and Discussion

The synthesis of the new series was carried out according to the known procedures for the preparation of the open-chain analogues. The following schemes demonstrate the three major routes by which the cyclic or the open-chain compounds were prepared for this study.

route 1

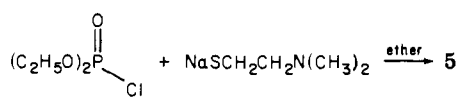


route 2



M = K, Na, dicyclohexylamine

route 3



The corresponding quaternary derivatives **2**, **4**, and **6** were obtained by reacting the tertiary bases with methyl iodide or methyl methanesulfonate. Table I summarizes some of the physical properties of compounds **1-4**. Ir and NMR spectral data are in good agreement with the proposed structures.

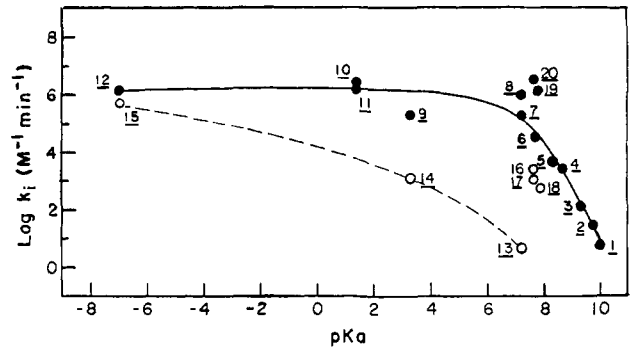


Figure 1. Bronsted plot for the inhibition of eel ChE by 1,3,2-dioxaphosphorinane 2-oxide derivatives (○-○) and diethyl phosphates (●-●). The data for compounds **1-15** were taken from Ashani et al. (ref 10). For details of compounds **16-20** refer to text.

The inhibition of AcChE by compounds **1-4** is represented by eq 1. E is the free enzyme, I is the inhibitor,

$$E + I \overset{K}{\rightleftharpoons} EI \overset{k'}{\rightarrow} E' \overset{k_s}{\rightarrow} E \qquad (1)$$

EI is the enzyme–inhibitor complex, and E' is the inhibited enzyme. K, k', and k_s represent affinity, phosphorylation, and reactivation constants, respectively. The second-order rate constant for inhibition, k_i, is given by k'/K. The mathematical solution of eq 1 is expressed by two sets of equations. Equation 2 is the steady-state derivation while eq 3 expresses the approach to the steady state[4] where ϵ_t

$$[\epsilon/E]_{ss} = (k_s/k')(1 + K/I) \qquad (2)$$

$$\ln\left[\frac{\epsilon_t}{E_0} - \frac{E_t'}{E_0}\left(\frac{\epsilon}{E'}\right)_{ss}\right] = \left[\frac{k'}{1 + K/I} + k_s\right]t \qquad (3)$$

is the measured activity of the enzyme at time t, E_0 is the initial activity, and E_t' is the inhibited enzyme at time t. $(\epsilon/E')_{ss}$ denotes the steady-state enzyme activity.

We have found that the inhibition of the enzyme is a second-order reaction. Table II summarizes the reaction parameters for the phosphorylation of eel ChE by compounds **1-4**. Comparison of our results with those obtained by Maglothin and Wilson[6] for compounds **5** and **6** clearly indicates that reversible binding to the enzyme in the ground state, as measured in terms of dissociation constant K (affinity), is not diminished by the presence of the ring. However, the phosphorylation step (given by k') is 10^3 times slower for the cyclic phosphorothiolates than for the open-chain derivatives. In this respect, noteworthy is the "thiolo effect"[7,8] which has recently been shown to be related to the pK_a values of the corresponding leaving groups (7.7-7.9).[6] Since all compounds in this study (**1-6**)

Table II. Summary of Kinetic Parameters and Toxicity Data for the Inhibition of Eel ChE[a] by Compounds of Series I and II

Compd	Inhibitor concn, M[b]	K, M	k', min^{-1}	k'/K, M^{-1} min^{-1}	LD_{50} in mice sc, mg/kg
1	$(0.12\text{-}5.4) \times 10^{-4}$	5.0×10^{-4}	2.08	4.1×10^3	700[c]
2	$(0.15\text{-}5.8) \times 10^{-3}$	1.4×10^{-3}	1.61	1.1×10^3	370
3	$(0.87\text{-}7.5) \times 10^{-3}$	1.2×10^{-2}	0.31	25.8	550
4	$(1.58\text{-}7.9) \times 10^{-4}$	3.3×10^{-3}	2.5	0.8×10^3	190
5[d]	$(0.5\text{-}2.0) \times 10^{-7}$	1.0×10^{-3}	1.5×10^3	1.5×10^6	0.23
6[d]	$(0.5\text{-}2.0) \times 10^{-7}$	1.0×10^{-3}	2.6×10^3	2.6×10^6	0.13

[a] pH 7.0, 25 °C, μ = 0.1 (phosphate buffer). [b] Under the same conditions but in the absence of enzyme, the half-life time of the hydrolysis of compounds **1-4** is ~60 h (see Experimental Section). [c] This value is for a preparation purified by column chromatography (see Experimental Section). [d] See ref 6.

contain the dialkyl- or trialkylaminoethanethiol moiety as a leaving group, we may ascribe the poor phosphorylation capability of the cyclic analogues to an unfavorable orientation of the phosphoryl group in the reversible complex EI. The low activity of compound **3** could be further attributed to the steric factors arising from the bulky diisopropyl groups, in addition to the unfavorable cyclic structure.

A sharp break occurs in the Bronsted plot for the inhibition of eel ChE by series II.[9] The same phenomenon has been observed in series I but the break is less marked.[4] A possible explanation for these results is a slow conformational change in the reversible complex EI into an active conformer EI* prior to the phosphorylation step. This step is assumed to be independent of the pK_a's of the leaving groups.[10] The bimolecular rate constants for **5** and **6** (points 19 and 20, respectively, in Figure 1) are higher than that which would be anticipated from the pK_a of their leaving group (by a factor of 50). Our data (Figure 1) show that compounds **1**, **2**, and **4** (represented by points 16, 17, and 18, respectively) have a bimolecular rate constant which is about 10^3 times higher than what would have been predicted from the corresponding pK_a values. It is assumed that the attachment of the alkylaminoethanethiolo group to a priori unreactive structures such as I will have a more dramatic effect than in the case of the *O,O*-diethylphosphates II, which are much better inhibitors, even where X = halogen. Presumably such thiolo side chains can accelerate conformational changes such as EI → EI* or stabilize the transition state, thus compensating the relatively high pK_a values of these leaving groups (~8).

The results of acute toxicity tests in mice are given as LD_{50} values in Table II. There is a marked difference in toxicity between the two series of compounds: all the new cyclic phosphorothiolates are less toxic than their open-chain analogues. This difference may be ascribed to a difference in the rate of phosphorylation (k_i), in the rate of reactivation (k_s), or in the combined effect of these two rates, as occurs in vivo. This is certainly true for the in vitro experiments. Of more practical relevance is the course of AcChE inhibition in vivo. To test this we determined the level of ChE activity in vivo in whole blood of beagle dogs following administration of compound **1**. Intravenous injection of 1 mg/kg, a dose which in itself causes no discernible signs of intoxication, resulted in a peak reduction of 37–39% in enzyme activity after 5 h and remained at this level throughout the next 24 h. The disparity between the rapid in vitro spontaneous reactivation and the relatively long duration of whole blood ChE inhibition in vivo could be ascribed to the slow elimination rate of compound **1** from body compartments. Aging of the inhibited enzyme is not likely since whole blood ChE regained 95% of its original activity after inhibition with I, X = Cl, in vitro. Compound **1** was used in a pilot clinical trial to relieve intraocular pressure in a case of aphakic glaucoma. The subjective feeling of the patient and the measured pressure indicated significant improvement to a level that could not be reached with phospholine, the open-chain analogue.

Experimental Section

a. **Synthesis.** Melting points were determined in open glass capillaries using a Thomas-Hoover Unimelt apparatus and are uncorrected. Ir spectra were obtained using a Perkin-Elmer Model 457 grating infrared spectrophotometer. NMR spectra were determined on a Jeol 60-MHz spectrometer. Thin-layer chromatograms were carried out on glass plates (25 × 75 mm) coated with Woelm neutral alumina or silica (without binder). Gas–liquid chromatography was done using a Packard Model 7300 gas chromatograph with a flame photometric detector.

Visible spectra (412 nm) for the kinetic and enzymatic studies were taken on a Pye Unicam SP 1800 ultraviolet spectrophotometer attached to a Unicam AR-25 linear recorder. Data processing was carried out with a Hewlett-Packard Calculator Model 9100 A using the program for linear regression and correlation coefficient (HP No. 70803).

2-*S*-[2'-(*N*,*N*-Diethylamino)ethyl]thio-1,3,2-dioxaphosphorinane 2-Oxide (1). Route 1. Small pieces of Na (4.6 g, 0.2 mol) were added to a solution of diethylaminoethanol (26.7 g, 0.3 mol) in dry benzene (300 ml) and heated until the metal completely dissolved. The mixture was stirred and cooled to room temperature with continuous stirring. 2-Chloro-1,3,2-dioxaphosphorinane 2-sulfide[11] (34.5 g, 0.2 mol) was added in portions. The mixture was refluxed for 5 h and allowed to stand overnight with continuous stirring at room temperature. The white precipitate was filtered off and the solvent was removed in vacuo (20 mm). The crude oil was dissolved in benzene (100 ml) and washed with water (2 × 50 ml). The benzene solution was dried over $MgSO_4$ and the solvent removed under reduced pressure (last traces at 0.05 mm). The residual yellow oil (40 g, 79%) was distilled at 4×10^{-5} mmHg and 63 °C to yield a colorless liquid (5 g, 10%). The distilled product was checked by GC (glass column, 0.3 cm × 1.8 m), 10% silicone oil DC-550 on Chromosorb W AW DMCS, 80–100 mesh (temperature of injector, 200 °C; column, 190 °C; detector, 200 °C) with nitrogen as a carrier gas (35 ml/min). Only one peak was observed under these conditions. TLC determinations (see later on) likewise revealed one single spot. According to free thiol analysis this preparation contains 0.7% free thiol, apparently related to *N,N*-diethylaminoethanethiol: NMR ($CDCl_3$, ppm) δ 1.00 (6 H, t, CH_3C, J = 7.5 Hz), 1.95 (2 H, m, CCH_2C), 2.55 [4 H, q, $-N(CH_2CH_3)_2$, J = 8.0 Hz], 2.80 (4 H, m, $-SCH_2CH_2N$), 4.35 (4 H, m, CCH_2O); ir (benzene, cm^{-1}) 1285 ($\nu_{P=O}$, s), 550 (ν_{PSC}, m).

Route 2. *N,N*-Diethylaminoethyl chloride (34.5 g, 0.25 mol) was added dropwise into a heterogeneous mixture of the sodium salt of 2-thiolo-1,3,2-dioxaphosphorinane 2-oxide[12] (44 g, 0.25 mol) in dry benzene (400 ml). The mixture was refluxed 2.5 h and cooled to room temperature. The white precipitate was filtered off and the benzene was removed in vacuo (20 mm) to afford a pale yellow oil (27 g, 30%). The oil was purified by column chromatography. A glass column was packed with Merck Kieselgel 60 (80 cm height, 5 cm i.d.) in a mixture of acetone–benzene–methanol (5:4:1, respectively) which was also used as the eluent. The separated compound was checked for purity on silica gel chromatoplates [chloroform–methanol (9:1), R_f 0.40; acetone–benzene–methanol (5:4:1), R_f 0.70].

2-*S*-[2'-(*N*,*N*,*N*-Diethylmethylammonio)ethyl]thio-1,3,2-dioxaphosphorinane 2-Oxide Methanesulfonate (2). Compound **1** (4.2 g, 0.011 mol) was dissolved in dry benzene (30 ml) and methyl methanesulfonate was added in excess. The mixture was refluxed for 2 h to yield a brown oily precipitate. After decanting most of the mother liquid and allowing to stand overnight a yellowish solid was obtained. The product was triturated with dry acetone (3 × 10 ml) and dried over P_2O_5 in vacuo (20 mmHg) at 45 °C for 3 h: mp 97–98 °C.

2-*S*-[2'-(*N*,*N*-Diisopropylamino)ethyl]thio-1,3,2-dioxaphosphorinane 2-Oxide (3). *N,N*-Diisopropylaminoethyl chloride (4.9 g, 0.032 mol) was added dropwise at room temperature into a stirred solution of the sodium salt of 2-thiolo-1,3,2-dioxaphosphorinane 2-oxide (5.3 g, 0.03 mol) in dry benzene (100 ml). The mixture was refluxed for 2 h and cooled to room temperature. The white precipitate was filtered off and the benzene was removed in vacuo. The residue was washed with 1 M NaCl solution (20 ml). The extracted benzene solution was dried over $MgSO_4$, filtered, and concentrated to give a yellowish oil. The crude oil was purified on Kieselgel 60 (Merck), packed in a glass column (35 cm × 2 cm), using chloroform–methanol (10:1) as the eluent. The purity was checked on silica gel chromatoplates [chloroform–methanol (10:1), R_f 0.30]: NMR ($CDCl_3$, ppm) δ 0.98 (12 H, d, CH_3C, J = 7.5 Hz), 2.00 (2 H, m, CCH_2C), 2.50–3.30 (6 H, m, SCH_2CH_2N + $NCHC_2$), 4.30 (4 H, m, CCH_2O); ir (benzene, cm^{-1}) 1280 ($\nu_{P=O}$, s), 550 (ν_{PSC}, m).

2-Thiolo-1,3,2-dioxaphosphorinane 2-Oxide Dicyclohexylammonium Salt. Treatment of the sodium salt of 2-thiolo-1,3,2-dioxaphosphorinane 2-oxide (10 g, 0.06 mol) with Dowex 50W (100–200 mesh, H^+ form) and the eluate with di-

cyclohexylamine in acetone yielded the dicyclohexylammonium salt (10.5 g, 90%): mp 227–229 °C. Anal. ($C_{15}H_{30}NO_3PS$) C, H, N, P.

2-S-[2'-(*N*,*N*,*N*-Trimethylammonio)ethyl]thio-1,3,2-dioxaphosphorinane 2-Oxide Methanesulfonate (4). *N*,*N*-Dimethylaminoethyl chloride (2.15 g, 0.02 mol) was added to a solution of the corresponding dicyclohexylammonium salt (6.7 g, 0.02 mol) in dry methylene chloride (60 ml). The mixture was allowed to stand 3 days at room temperature. After filtration of the white precipitate and concentration under reduced pressure, the residue was dissolved in dry acetone (20 ml). Methyl methanesulfonate (2.5 g, 0.022 mol) was added dropwise and the mixture was kept at room temperature for 48 h. The white solid was collected and dried over P_2O_5 in vacuo (20 mm) at 40 °C for 48 h: mp 143–146 °C.

***S*-[2-(*N*,*N*-Dimethylamino)ethyl] *O*,*O*-Diethyl Phosphorothiolate (5).** Caution: the open-chain phosphorothiolates are extremely toxic (see Table II) and adequate safety precautions were taken during their preparation. A mixture of the sodium salt of *N*,*N*-dimethylaminoethanethiol (12.7 g, 0.1 mol) in dry ether was added in portions to a solution of *O*,*O*-diethyl phosphorochloridate (17.2 g, 0.1 mol) in dry ether (80 ml). The mixture was refluxed for 3 h, cooled to room temperature, filtered, and concentrated in vacuo. Distillation of the crude product yielded a colorless liquid (10 g, 60%): bp 96–100 °C (0.4 mm) [lit.[13] 90 °C (0.1 mmHg)].

***S*-[2-(*N*,*N*,*N*-Trimethylammonio)ethyl] *O*,*O*-Diethyl Phosphorothiolate Iodide (Phospholine) (6).** Methyl iodide (5 g, 0.035 mol) was added to a solution of **5** (2.1 g, 0.01 mol) in dry ether (50 ml). A white solid precipitated instantaneously and was recrystallized from ethanol–ether: mp 132–134 °C (lit.[5] 138 °C).

b. Hydrolytic Stability of the Cyclic Phosphorothiolates (pH 7.0, μ = 0.1, 25 °C). An aliquot of 50 μl of the cyclic ester solution (40 mg in 5.0 ml) was transferred to a quartz cuvette (Pye Unicam, 3 ml, 1 cm) containing phosphate buffer, pH 7.0 (3.0 ml), and 100 μl of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). All solutions were prepared under nitrogen from nitrogen-purged buffers. The thiol obtained during the hydrolysis was determined following the increase in optical density (OD) at 412 nm. The calculation of the amount of thiol liberated into solution was based on the molar absorption coefficient of the corresponding thiophenol (ϵ 13600).

c. Inhibition of Acetylcholinesterase. Acetylcholinesterase from the electric organ of *Electrophorus electricus* (Worthington, specific activity 150 units/mg of protein) was used. The stock solution was made in phosphate buffer (pH 7.0, μ = 0.1) containing 0.05% gelatin. The residual activity of the enzyme during inhibition was measured by the Ellman procedure[14] with acetylthiocholine (Sigma, 1.0 mM) and DTNB (0.3 mM) at pH 7.0 and 25 °C. The rate of formation of the corresponding thiophenol was monitored at 412 nm using a Pye Unicam SP 1800 spectrophotometer.

The enzyme was assayed (after inhibition) for a period that did not exceed 1 min in order to reduce as much as possible any error introduced by the spontaneous reactivation of the phosphoryl enzyme. To a 1-ml aliquot of enzyme solution ($\sim 5 \times 10^{-10}$ M in phosphate buffer (pH 7.0, μ = 0.1), 20–25 μl of the cyclic ester solution in water (10^{-3}–10^{-5} M) was added. The residual activity of the enzyme was followed until reaching the steady state. The slopes of the linear curves obtained at steady state represent the various concentrations of free enzyme and were taken for calculating the kinetic parameters.

d. Toxicology. Acute toxicity of all the compounds was determined in white female mice (20–22 g) of the "Ness-Ziona" strain by subcutaneous injection. Final solutions were made up in physiological saline (0.9% NaCl) and at concentrations adjusted so that in all cases the volume of injection was 0.1–0.2 ml/22 g. For each dose level a group of at least six mice was used and final mortality was recorded after 24 h. LD_{50} values were calculated according to the method of Reed and Muench.[15] For studies of in vivo cholinesterase inhibition, compound 1 was injected intravenously to beagle dogs at various doses (0.1, 0.5, and 1.0 mg/kg) with three animals per dose level. Venous blood samples were withdrawn prior to and at different time intervals following administration of the compound and changes in the level of whole blood ChE activity were determined by the colorimetric method of Fleisher and Pope.[16]

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