neither, and only the positions of N→O group in IX and XII become meager. The theoretical moments of 4-methylpyridazine 1-oxide and 2-oxide can be calculated by simple vector summation to be 5.58 D and 5.52 D respectively as compared to IX 5.70 D and XII 5.53 D. This calculation suggests that IX is 1-oxide and XII is 2-oxide. The observed value for IX, 5.70 D, is also in good agreement with 5.61 D, the resultant moment of 4-methylpyridine N-oxide 4.50 D[10] and pyridine 2.22 D.

Based upon the above discussion, it can be concluded that IV, VIII, and IX are 1-oxides and III, V, XI, and XII are 2-oxides.

Dipole moments of some nitroderivatives of 3-methylpyridazine N-oxides were also measured to confirm their molecular structures, the values of which were normal for the structures predicted in the previous paper.[1] These values are listed in Table I.

The authors express their appreciation to Prof. Emeritus E. Ochiai and Dr. K. Takeda, Director of the laboratory, for their helpful advices and encouragements.

Summary

Dipole moments of 3- and 4-methylpyridazine N-oxides and their related compounds were measured in order to determine the positions of N→O group in these compounds, and the following conclusions were derived.

1. It was confirmed that IV was 1-oxide and III and V were 2-oxides.
2. In 4-methyl derivatives, VIII and IX are 1-oxides and XI and XII are 2-oxides.

(Received January 17, 1962)

10) A. N. Sharpe and S. Walker: J. Chem. Soc., **1961**, 4522.

UDC 615.711.76[615.785]-015.25:547'118.5

10. Masayasu Kimura*1: Molecular Pharmacological Studies on Drug-Receptor Complexes System in Drug Action. I. Antagonism to Acetylcholine of Organophosphorous Compounds.*2

(*Faculty of Pharmaceutical Sciences, University of Toyama*)

Using organophosphorous compounds, known well as insecticides, the present studies were initiated to extend the research field further and the author had been studying on an acetylcholine (ACh)-receptor complex in drug action.

In recent years, considerable attention has been paid to the action of the organophosphorous poison against mammals, which is mainly due to the direct inhibition against cholinesterase (ChE) activity, and there exists in reality a considerable literature on the pharmacological treatment of anti-ChE poisoning by organophosphorous compounds and its detoxication. From a clinical point of view, it is now well established that atropine as well as 2-pyridinealdoxime methiodide (PAM) have to be given as the antidotes against organophosphorous compounds. Likewise, from many of the

*1 5-Okuda, Toyama (木村正康).

*2 This was presented at the 13th Annual Meeting of the Pharmaceutical Society of Japan (April 1960, in Tokyo).

pharmacological experiments, it is evident that atropine plays an important role in the treatment of the poisoning.[1~5]

In view of the above fact, whence it may be suggested that the organophosphorous compounds have some possibilites of an attack on the ACh receptor, where is inhibited the accumulated ACh by atropine. However, comparatively little is known of inhibiting ACh at the neuromuscular junction by organophosphorous compounds, although some attention has been paid to organophosphates on the action of the intestinal muscle in a report[6] and a review[7] which were published in the course of this study.

In the present paper, therefore, observations on the antagonistic action to ACh of organophosphates were described, together with the results of a comparison between the inhibitory effects against ACh and ChE. It is the object of this paper to suggest that the organophosphorous poisoning is not only due to the inhibition of ChE activity, but also somewhat to the inhibition of ACh activity. Thus this preliminary report will be invaluable also as the starting point in this series of study.

Method and Materials

(1) Procedure for Determination of Anti-ACh Activity

Using both the intestinal segments 20~25 mm. isolated from white mice, weighing 15~20 g., and the rectus abdominis of frogs, weighing about 30 g., Magnus method was adapted as in the previous report.[8]

Effect of anti-ACh activity was expressed as an inhibitory ratio to a given ACh by a single dose of 10^{-5} g./cc. organophosphates in the case of the isolated intestine, and shown as doses at 50% inhibitory effect in the other case of the rectus abdominis.

(2) Procedure for Determination of Anti-ChE Activity *in vitro*

According to Ammon's method,[9] Warburg manometric method was used for measuring the inhibition of ChE by organophosphates in the Ringer solution (0.9 g./dl-NaCl 100 cc., 1.15 g./dl-KCl 2 cc., 1.22 g./dl-$MgCl_2$ 2 cc., 1.3 g./dl-$NaHCO_3$ 20 cc.).

Enzyme preparation used was crude powders of the dried pseudo-ChE which were prepared by vacuum freeze-drying method from the blood serum of an adult rabbit of about 2 kg. weight as follows. After the blood gathered through the auricularis artery of rabbit, was kept in an ice box until the blood coagulated in a glass vessel, the cold blood was centrifuged at 3000 r.p.m. for 5~10 min. The upper solution was converted into powder by a vacuum freeze-drying apparatus for about 5 hr. The new upper solution, which was again obtained from the dry powder soluble in the Ringer solution by centrifuge at 2000 r.p.m. for 5~10 min., was used as ChE preparation.

A final concentration of 5×10^{-2} g./cc. of dried blood serum was used for hydrolysis of 2×10^{-4} g./cc. ACh, under inhibition of 10^{-5} g./cc. organophosphates at 37°C as the temperature of a manometric bath.

(3) Procedure for Determination of Anti-ChE Activity *in vivo*

In vivo experiments, effect of anti-ChE was determined indirectly by the fall of blood pressure. The arterial blood pressure was recorded by a mercury manometer at the left femoral artery of adult dogs, weighing 8~12 kg., which were intravenously anesthetized with 35 mg./kg. pentobarbital. A prepared solution 2~4 mg./kg. test compounds was infused into the right femoral vein.

(4) Organophosphorous Compounds used as Materials

Prepared organophosphates were as follows :

DDVP = Dimethyl 2,2-dichlorovinyl phosphate
DFP = Diisopropyl fluorophosphate

1) G. Wilhelmi, R. Domenjoz : Arch. intern. pharmacodynamie, **86**, 321 (1951).
2) M. W. Parkes, P. Szcra : Brit. J. Pharmacol., **9**, 299 (1954).
3) B. M. Askew : *Ibid.*, **12**, 340 (1957).
4) B. Pobertv : J. Pharmacol. Exptl. Therap., **120**, 276 (1957).
5) A. G. Karczmar, J. P. Long : *Ibid.*, **123**, 230 (1958).
6) W. D. Erdmann, D. Heye : Arch. exptl. Pathol. Pharmakol., **232**, 507 (1958).
7) B. Holmstedt : Pharmacol. Rev., **11**, 567 (1959).
8) K. Takagi, M. Kimura : This Bulletin, **4**, 444 (1956).
9) R. Ammon : Pflüger's Arch. Ges. Physiol., **233**, 486 (1933).

Dipterex = Dimethyl 1-hydroxy-2,2,2-trichloroethylphosphonate
Metasystox = O-[2-(Ethylthio)ethyl] O,O-dimethylphosphorothioate
Methylparathion = O,O-Dimethyl O-*p*-nitrophenylphosphorothioate
Parathion = O,O-Dimethyl O-*p*-nitrophenylphosphorothioate
TEPP = Tetraethyl pyrophosphate
TEP = Tetraethyl phosphate

Some compounds insoluble in water were suspended by the solubilizer of Gum Arab. (1:1).

Experimental Results

I. Effects of DFP, Dipterex, and Parathion against the Action of ACh on the Isolated Intestines of Mice

Observations shown in Fig. 1 are the results of influence upon the effect of 5×10^{-8} g./cc. ACh (estimated as doses of probable 50% response for maximum response produced by 10^{-4} g./cc. ACh) in the intestinal segments of mice by DFP, dipterex, and parathion, of which doses brought out little or no direct effect on the intestinal muscle without ACh.

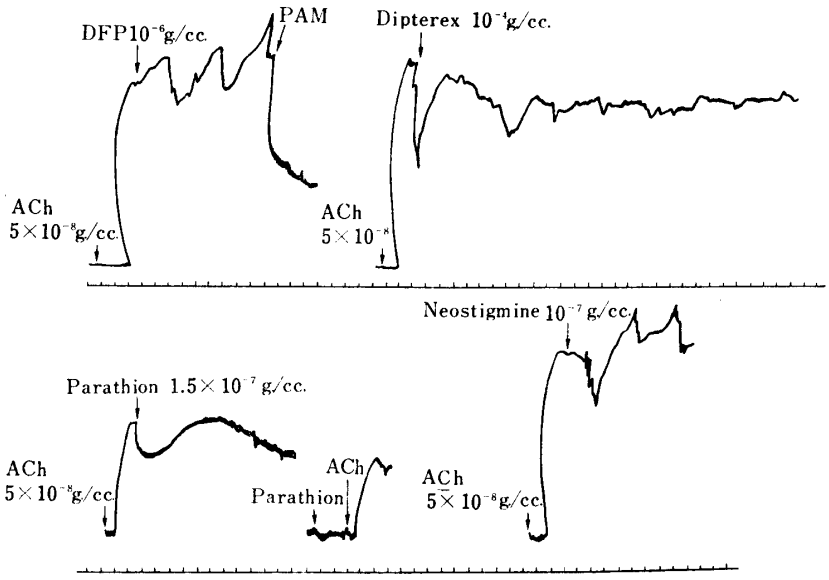


Fig. 1. Effects of ACh (5×10^{-8} g./cc.) and Organophosphates upon the Intestinal Segments of Mice

Upper : an Appearance of Peristalsis by DFP (10^{-6} g./cc.) and Dipterex (10^{-4} cc./cc.), and its Disappearance by PAM.
Lower : an Effect of Parathion (1.5×10^{-7} g./cc.) before and after the Administration of ACh, and an Appearance of a Peristalsis by Neostigmine (10^{-7} g./cc.).

In Fig. 1 is recorded that both 10^{-6} g./cc. DFP and 10^{-4} g./cc. dipterex made an appearance of peristalses on the contracted intestine by ACh and this appearance resembled to an effect of 10^{-7} g./cc. neostigmine in a pendular motility, but the peristalsis of DFP were disappeared by PAM.

On the contrary, parathion made an appearance of the slack pendular motility at doses of 1.5×10^{-7} g./cc., but an appearence of the inhibitory effect against ACh at doses greater than 8×10^{-7} g./cc. (see Fig. 2). Parathion was administered before or after the effect of ACh and it made little difference in the inhibitory effect (see Fig. 1).

Effects of DFP and parathion upon intestinal muscle were rapidly failed under PAM, or reversed by washing out with fresh Tyrode solution. However, even after the effect was thoroughly washed out, their influence was left behind on isolated intestine for sometime.

Fig. 2 shows that the intestinal muscle, after washing out 2×10^{-7} g./cc. DFP with Tyrode solution increased in some sensitivity more than response by a same dose of ACh (5×10^{-8} g./cc.) and recovered slowly to yield the usual response in 55 min., while in the case of parathion at total doses of 2.4×10^{-6} g./cc., the muscle was recovered in 30 min. after washing out, and then also that the muscle with a full recover was gradually contracted in proportion to higher doses (e.g. 10^{-6} g./cc.) of ACh. These observations clearly demonstrate that the effect of parathion did not bring any change in quality to the intestinal muscle.

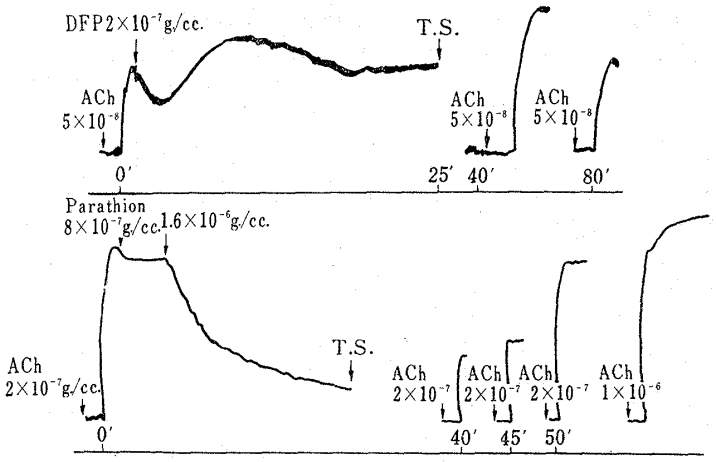
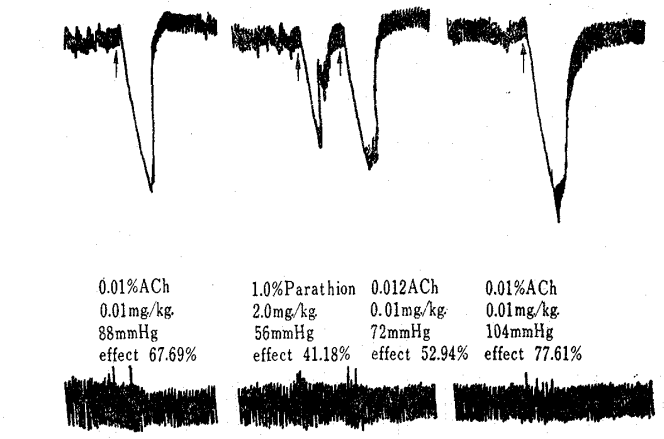


Fig. 2. Difference of ACh Effect upon the Intestinal Muscle of Mice after washing out DFP (upper) and Parathion (lower) with Tyrode Solution (T. S.)



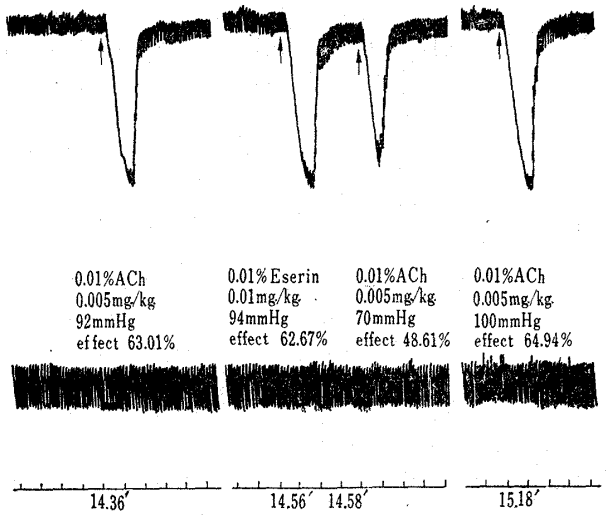


Fig. 3. Similarity of ACh Effect upon the Blood Pressure of Dog treated with Parathion (upper) and Eserine (lower)

In addition to experiments *in vitro*, observations were made on the effect of parathion upon the fall by ACh in the blood pressure of dog. The results are shown in Fig. 3.

In vivo experiments anticholinergic effect of parathion was compared very poorly with above results *in vitro*, but the observations showed that parathion as well as eserine brought some inhibitory influence to the effect of ACh administered immediately, and then after 30 min. the fall effect was increased. This phenomenon may be easily considered as the anti-ChE effect by parathion *in vivo*.

II. Relationship between the Activity of Anti-ACh and Anti-ChE by Organophosphates

The anti-ACh activity of 10^{-5} g./cc. organophosphates was measured as the inhibitory ratio for parathion 100 calculated from the means of the experimental value using 8 segments of intestine in mice. On the other hand, anti-ChE activity was measured as follows; *in vitro* from the inhibitory ratio by 10^{-5} g./cc. organophosphate in manometric experiment, which was treated with the means of 6 measurements after the mean time between 30 and 40 min. of reaction, *in vivo* from the fall ratio in the blood pressure of dog by 3 mg./kg. organophosphates. The results of experiments described above are brought together for ready comparison in Fig. 4.

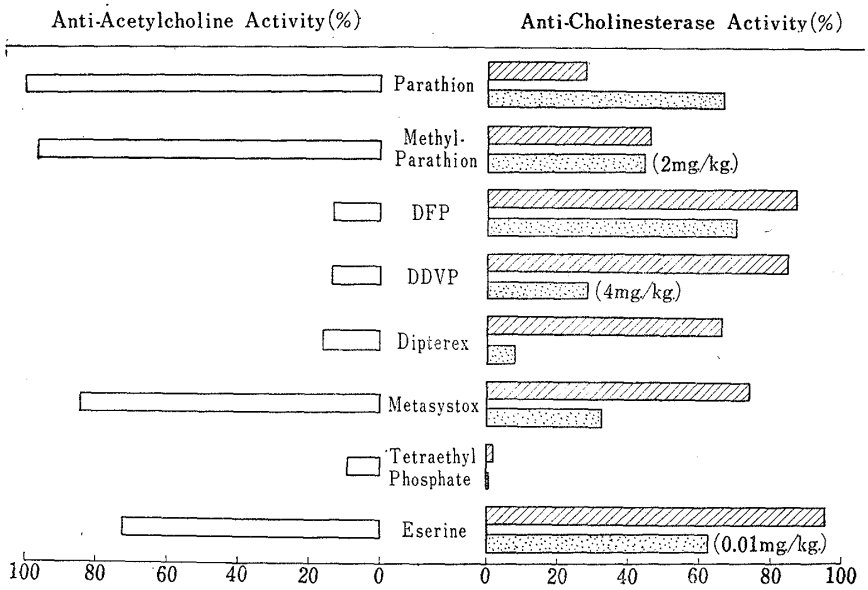


Fig. 4. Comparison between Anti-ACh Activity and Anti-ChE Activity of Organophosphates : ▭ anti-ACh activity, ▨ anti-ChE activity *in vitro*, ▩ anti-ChE activity *in vivo*

In the next place, an experiment similar to those described for anti-ACh effects by some of organophosphate was made in further detail with the rectus abdominis of frog. Table I shows the data, which have been measured in the condition without the intracellular ChE activity in the muscle by the presence of 10^{-6} g./cc. neostigmine in Ringer solution.

In another experiment, was recorded that TEPP at doses greater than 4×10^{-5} g./cc, made the muscle of the rectus abdominis irreversibly contract with one large slack pendular motility during response, while the doses less than 4×10^{-6} g./cc. did not only make such a phenomenon, but also even the inhibitory effect against ACh.

TABLE I. The Inhibitory Effects (50% inhibited doses) of 2×10^{-6} g./cc. ACh by some Organophosphates upon Rectus Abdominis of Frog treated with 10^{-6} g./cc. Neostigmine

Organophosphates	ID_{50}(g./cc.)	Confidence limits
Parathion	1.95×10^{-5}	$8.71\times10^{-6}\sim4.37\times10^{-5}$
Methylparathion	2.25×10^{-5}	$4.67\times10^{-6}\sim1.08\times10^{-4}$
Dipterex	3.64×10^{-4}	$1.85\times10^{-4}\sim7.17\times10^{-4}$
Metasystox	2.29×10^{-5}	$9.62\times10^{-6}\sim5.45\times10^{-5}$

Discussion and Conclusions

The intestinal segments contracted by ACh (5×10^{-8} g./cc.) were responded with rhythmical motility and intense peristalsis by DFP (10^{-6} g./cc.), dipterex (10^{-4} g./cc.) and parathion (10^{-7} g./cc.) of doses producing no discernible effects on the intestinal muscle in itself. This appearance resembles to one by neostigmine (10^{-7} g./cc.). This phenomenon seems possible, therefore, to illustrate the fact that the remarkable appearance is produced by DFP, dipterex and DDVP, which are more effective inhibitor to ChE

than parathion and methylparathion. Also the other experiment[10] shows that DFP (10^{-7}～10^{-6} g./cc.) increased the effect of ACh upon the isolated intestine of rabbit. Thus, the experimental results obtained on TEPP in this study can be considered as an extreme case of these phenomena. These findings, therefore, support the view that the potentiation of motility and peristalsis was interpreted by Erdmann and Heye[6] as a result of inhibition of a critical quantity of intestinal ChE in relation to the autonomic ganglia.

On the other hand, the action of organophosphate has been generally described as an irreversible response to ChE *in vitro*, but regarding the response to intestinal muscle, probably to ACh receptor, both DFP and parathion were readily reversed by washing out with fresh Tyrode solution and of course by PAM. This is similar to observation in the phrenic nerve diaphragm preparation.[11,12] This reversible response gives a residual after-effect, which was caused by the slow recovery of tonus and sensibility, more than atropine to intestinal muscle, that is to say, DFP enhances the sensibility of intestine for ACh but parathion reduces to the contrary. From these results, it may be suggested that DFP has stronger affinity for ChE, than parathion for ACh receptor. In the case of experiments *in vivo*, however, the effect of parathion was produced mainly as the fall of blood pressure by anti-ChE effect, together with a slight anti-ACh effect. This confusion appears in parallel with Toman's suggestion[13] that DFP acts by decreasing the sensitivity of the nerve by some effect other than inhibition of ChE. Finally, in other words, this fact means that the action of organophosphates is caused at such two different points of action as ChE and ACh receptor.

As a natural consequence, this discussion has been centering on relationship between anti-ACh activity and anti-ChE activity of organophosphates. Fig. 4 indicates the tendency that the more effective anti-ACh activity is, the less effective anti-ChE activity is, and then the anti-ChE activity *in vitro* is parallel to one *in vivo*.

The results of anti-ChE activity described above will be valid to discuss the comparison with the anti-ACh activity, because the pseudo-ChE using in this study is generally inhibited stronger by organophosphate than the true ChE. In order to discuss whether there is a striking parallelism between this tendency and the lethal doses of the organophosphates, there have been brought together for detailed comparison in Table II, which were collected the results of Table I and the result obtained from the other reference data.[7,14,15]

Table II. Summary of the Organophosphate Potency as Anti-ACh Effects, Anti-ChE Effects, and Lethal Doses

	Anti-ACh (pI 50)	Anti-ChE (pI 50)	LD 50 mg./kg. (mice, s.c.)	LD 50 mg./kg. (rat, oral)
Parathion	4.10	4.6 (horse serum)	10～12	4～4.4
Methylparathion	4.04	4.0 (horse serum)	30	15.2
Dipterex	2.85	5.7 (rat brain)	124	450
Metasystox	4.02	5.4 (rat brain)	2.9～3.3	250

From the data given in Table II it will be seen that the order of toxic effects between dipterex and the others is parallel to one of anti-ACh effects, but is opposite to one of anti-ChE effects, although the relation to the order of the other one another is not so clearly. Therefore, the possibility that the organophosphorous poisoning in

10) Chihara : Folia Pharmacol. Japon., **51**, 531 (1955).
11) W.K. Berry, C.L. Evans : "Annual Review of Physiology," **14**, 375 (1952).
12) J.A.B. Barstad : Arch. intern. pharmacodynamie, **107**, 21 (1956).
13) J.E.P. Toman, *et al.* : J. Neurophysiol. **10**, 429 (1947).
14) Edited by W.S. Spector : "Handbook of Toxicology," Vol. I (1956).
15) R. Yamamoto, *et al.* : "Shin-Nōyaku-Kenkyūhō," (1958), (Nankōdō).

mammals is produced also by blocking ACh in the neuromuscular junction must be admitted, besides inhibiting ChE. And then, at the peripheral nervous system, it may be concluded that the ACh receptor must be selectively attacked by some of organophosphates, above all parathion.

This conclusion supports O'brien's view[16] that neuromuscular transmission seems to be blocked by high levels of organophosphate by some mechanism unrelated to ChE inhibition, and it must be considered as a distinct possibility that some of the consequences of organophosphate poisoning in mammals are caused by combination between organophosphate and the ACh receptor.

The author with to express his gratitude to Professor Haruo Kitagawa of this Department for his generous help. He is also indebted to Dr. Mitsuru Uchiyama of Tohoku University for his invaluable suggestion, and then to MessrsTsuneo Wakabayashi, Motonori Hirao, and Yoshinobu Igarashi, and Miss AtsukoMatsuo for their technical assistance.

Summary

The inhibitory action of organophosphates against acetylcholine (ACh), and cholinesterase (ChE) *in vitro* and *in vivo* has been observed using parathion, methylparathion, DFP, DDVP, dipterex, metasystox and TEPP. These experiments gave the following results :

1) The investigated organophosphates produced more and less a reversible inhibitory effect against ACh upon the isolated intestinal segments of mice. The stronger anti-ChE agent (e.g. DFP, TEPP) produced a terrible rhythmical motility, but was remarkably less effective inhibiting against ACh than parathion which was found as a comparatively strong anti-ACh agent.

2) The after-effect of DFP and parathion was observed in intestinal muscle and its difference produced by having an affinity for such two different points of action as ChE and ACh receptor was discussed.

3) Anti-ACh activity and anti-ChE activity *in vitro* (by Warburg method) and *in vivo* (by the fall effect of blood pressure) of the above organophosphates were measured and compared with each other. The more effective anti-ACh activity was, the less effective anti-ChE activity was.

4) The antiacetylcholine action by some of organophosphates were measured using the rectus abdominis of frog treated with neostigmine, and then were compared with anti-ChE effects and lethal doses (LD 50) to suggest the possibility that the organophosphorus poisoniong in mammals is also produced by blocking ACh, besides inhibiting ChE.

(Received January 20, 1962)

16) R.D. O'brien : "Toxic Phosphorous Esters," 154, 160 (1960)(Academic Press Inc.).