Cholinesterase-Inhibiting Properties of Two Vinyl-Substituted Phosphates. (20485)

M. S. MORSE, J. K. KODAMA, AND C. H. HINE.

From the Department of Pharmacology and Experimental Therapeutics, University of California School of Medicine, San Francisco.

Aromatic and halide derivatives of organo substituted phosphoric and phosphonic acids have found considerable use in agriculture and medicine. In general, their activity stems from their ability to combine with and inhibit the cholinesterases. We have studied a new series of organophosphorus compounds and have found certain unsaturated alkyl derivatives to be highly toxic to rodents(1). Corey and his associates(2) have reported the insecticidal activity of 2 of these, diethyl 2-chlorovinyl phosphate (I) and dimethyl 1-carbomethoxy-1-propen-2-yl phosphate (II). The report of Kodama(3) summarizes their mammalian toxicity.

In this paper we report on the cumulative inhibitory properties of I and II following repeated administration, the rate of regeneration or reactivation after single administration, and the inhibitory action *in vitro* of these compounds on cholinesterase. For comparative purposes, p-nitrophenyl diethyl thionophosphate (parathion) was included in the experimental design.

Method. Cumulative toxicity. Graded intraperitoneal injection. The cumulative inhibitory effect of different sublethal doses was determined by daily (except weekends) administration of each compound to female rats (100-156 g) of the Long-Evans strain. Groups of 10 rats received I, II, and parathion in amounts equal to 10, 25, and 50% of the calculated intraperitoneal LD_{50}. These LD_{50} values for female rats of this strain and weight range are, in mg/kg, for I, 9.0; for II, 1.51; and for parathion, 4.0. A group of 10 *control* rats received propylene glycol (the solvent used to facilitate administration to the experimental rats) in an amount equal to 0.2% of the body weight. Five rats from each group, or all survivors if there were fewer than 5, were sacrificed after 17 injections. The remaining rats were given a total of 22 injections before sacrifice. Twenty-four hours after their final injection, the rats were lightly anesthetized, and decapitated. The per cent of active cholinesterase present in the erythrocytes, plasma, and brain, was determined by a modification of the method of Frawley and his coworkers(4). Wherever advisable, cholinesterase values were examined by comparison of pH readings by means of the "Student" t test, or by analysis of variance. P values of greater than 0.05 were considered insignificant. *Varied number of injections.* The rate of cumulative inhibition was determined by repeated intraperitoneal administration of 25% of the LD_{50} of the compounds. Groups of 3 rats (110-140 g) were sacrificed 24 hours after 1, 2, 4, 8, and 16 daily injections. Sacrifice of the animals and evaluation of enzyme activity conformed with the procedure outlined above. *Sixty-day feeding.* Female rats (104-165 g) of the Long-Evans strain were divided randomly into groups of 6 and fed I and II at dietary levels of 6.3, 12.5, 25, 50, and 100 ppm. The phosphates were intimately mixed with a standard feed of known composition. A group of 10 rats served as control. At the end of 60 days, the surviving rats were anesthetized and decapitated, and the per cent activity of the cholinesterase in the erythrocytes, plasma, and brain, was determined.

Rate of regeneration. The rate of return of cholinesterase activity was determined in groups of rats (110-140 g) given 25% of the LD_{50} of I, II, and parathion, and sacrificed subsequently at appropriate time intervals until activity returned to normal. Five rats were used in each experimental group, and 5 in the control group. Groups were killed at ½, 1, 2, 4, 24, 48, and 96 hours after treatment, and in the case of I, also at 8 and 16 days. The cholinesterase content of erythrocytes, plasma, and brain, was determined for each rat according to the method of Frawley(4).

Inhibitory action in vitro. Inhibitory ac-

tivity of the phosphates was determined *in vitro*, and the concentration at which the compound caused 50% inhibition of normal activity (IC_{50}) was determined for human erythrocytes, and for rat erythrocytes, brain, and plasma. The cholinesterase activity of human cells was determined by the method of Michel (5) while the activity of rat tissues was measured by Frawley's method.

Results. Cumulative toxicity. Graded intraperitoneal injection. There were no deaths in any of the groups receiving II, or in groups given I or parathion at the 10% level. Only one animal at the 25% level died, after 2 injections of I. Eight rats receiving 50% of the LD_{50} of I, and 8 at this level of parathion, died during the course of the experiment. There was a direct correlation between the degree of inactivation of erythrocyte and brain cholinesterase and the amount of inhibitor administered (Fig. 1). However, there were occasionally significant increases in plasma cholinesterase activity, *e.g.*, in animals receiving 10% of the LD_{50} of II and parathion. *Varying number of injections.* When 25% of the LD_{50} of the 3 phosphates was given for from one to 16 injections, the inhibition of erythrocyte cholinesterase by II was much less than that by the other phosphates, and values computed after the first, second, and fourth injections did not differ significantly from the control values. The cholinesterase activity of the erythrocytes fell progressively with increasing numbers of injections, of II and parathion; this effect was not evident with I, probably because of the original striking decrease to 20% of the control value after the first injection.

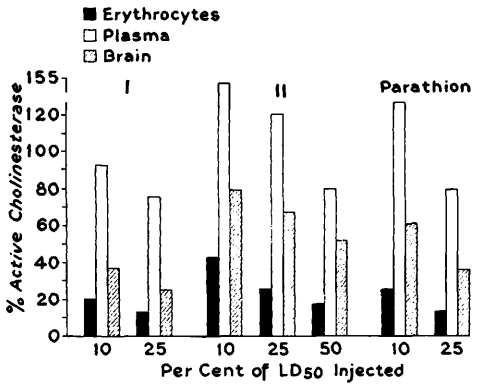

FIG. 1. % active cholinesterase of erythrocytes, plasma, and brain of female rats surviving 22 intraperitoneal injections of 10, 25, or 50% of the LD_{50} of I, II, or parathion.

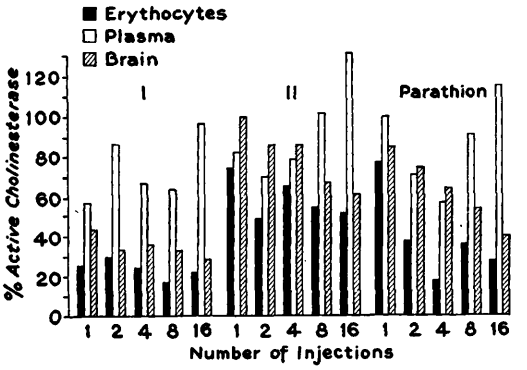

FIG. 2. % active cholinesterase in erythrocytes, plasma, and brain of female rats receiving 1 to 16 daily intraperitoneal injections of 25% of the LD_{50} of I, II, and parathion.

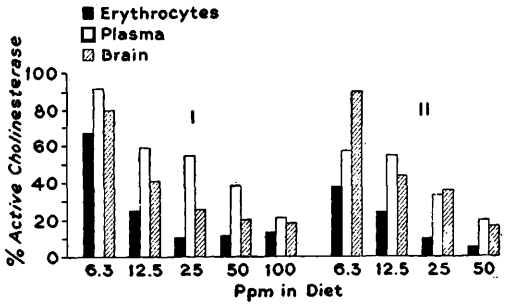

FIG. 3. % active cholinesterase in erythrocytes, plasma, and brain of female rats surviving diets containing 6.3, 12.5, 25, 50, or 100 ppm of I and II for 60 days.

There was no significant decrease in *brain cholinesterase* after the first injection of II and parathion, but all other values observed were significantly lower than those of control animals. The greatest depression followed I injections, and II had the least effect. A progressive decline in activity was seen with all 3 phosphates as the number of injections increased.

Plasma activity was not significantly decreased by II, and a significant increase appeared after the 16th injection. Plasma activity following parathion injection followed the same pattern, but with a greater initial depression, significant after the 4th injection. The subsequent rise was not significant by the

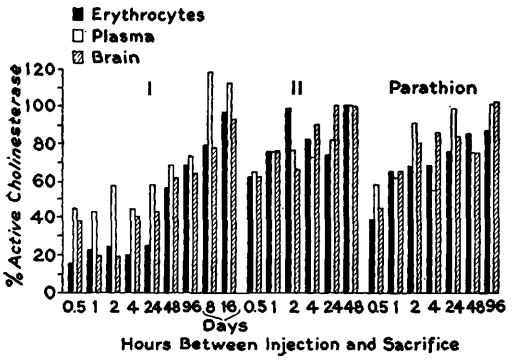

FIG. 4. % active cholinesterase in erythrocytes, plasma, and brain of female rats receiving one intraperitoneal injection of 25% of the LD_{50} of I, II, and parathion.

16th injection. Plasma activity was lowest after I injection, with a significant decrease after the first injection. There were no spontaneous deaths among the animals. Comparative cholinesterase values are shown in Fig. 2.

Subacute feeding. No animals survived the feeding of II at a level of 100 ppm for 60 days, but 2 of the 6 fed 100 ppm of I survived. There were no deaths at lower levels. This experiment showed the most decided progressive decrease in cholinesterase activity correlated with increase in dosage, and all 3 tissues were affected (Fig. 3). The erythrocyte cholinesterase showed the greatest depression by both compounds. Brain cholinesterase was inhibited to the same extent by the 2 compounds, the only significant difference appearing at the 25% level, where II was less inhibitory ($p = 0.01$). Plasma cholinesterase showed the least inhibition.

Rate of regeneration. Cholinesterase activity of all tissues had returned to values not significantly different from control values by 24 hours after the injection of 25% of the LD_{50} of II and parathion. Depression was more severe and lasting with I. In general, the lowest values were observed within an hour after injection, and the rise thereafter was irregular rather than steadily progressive. The erythrocyte enzyme seemed to be inhibited to the greatest extent, and to regenerate a little more rapidly.

Inhibitory action in vitro. The results of the tests for activity *in vitro* did not follow the findings *in vivo*. Compound II was 10 times as active as I against all tissues studied. It was slightly more active than parathion against the enzyme of rat brain, however, and more active against rat plasma cholinesterase. The data are presented in Table I.

Discussion. The present studies indicated that I and II exhibit properties similar to the cholinesterase-inhibiting compound parathion. Inhibition of the activity of the cholinesterase of erythrocytes, brain, and plasma occurred to varying degrees, and was usually least and of shortest duration with II. Both vinyl phosphates and parathion showed greatest activity against the enzyme of erythrocytes, and least against the "pseudo" enzyme of plasma; the erythrocyte enzyme appeared to regenerate most rapidly. The plasma enzyme seemed to show a rise after the phosphates had been given intraperitoneally over a period of time, but not when fed. *Cholinesterase* inhibition by parathion resembled that of II after a single injection, but was more similar to that of I after multiple injections.

Close comparisons and absolute statements are difficult, because of the rather striking individual differences in the response of the rats to all 3 compounds, and the variations encountered in normal animals. Altered cholinesterase values were sometimes found after repeated injections of the supposedly innocuous propylene glycol, administered to control animals when used as a solvent for injection of experimental rats. The statistical analysis (analysis of variance and "Student" t test) presumably clarifies the data and enhances the reliability of our results.

In his studies of reversibility, DuBois and his coworkers(6) administered parathion intraperitoneally at a dose level of 5 mg/kg (71% of the stated LD_{50}), and determined that while the brain cholinesterase was depressed to 6% of the normal activity at one-half hour, it returned to 100% in 4 hours. Our experiment, using 25% of the calculated LD_{50}, showed less severe depression, to 57% of the control value. However, activity returned in the same length of time, 4 hours, to a value of 92% (not significantly different from the control value). These results compare satisfactorily, considering the comparatively small

TABLE I. Molar Concentration of Compounds I, II, and Parathion Required to Produce 50% Inhibition *In Vitro* of Cholinesterase from Various Sources.

Compound	Human erythrocytes	Rat erythrocytes	Rat brain	Rat plasma
I	2.1×10^{-5}	1.7×10^{-5}	1.1×10^{-5}	5.4×10^{-5}
II	2.4×10^{-6}	1.5×10^{-6}	2.0×10^{-6}	3.0×10^{-6}
Parathion	2.6×10^{-6}	3.2×10^{-6}	2.0×10^{-6}	3.0×10^{-6}

numbers of animals used and the unavoidable variations within groups.

Frawley(4), using the same dose as DuBois (16.6% of his stated LD_{50}) but peroral administration, found no inhibition at the end of 4 hours, and return to 90% of normal at the end of 14 hours. The values were no higher at the end of one week, and 2 weeks were required for the return of 100% activity of the brain enzyme. Lehman(7), on the other hand, using a peroral "sublethal" dose, found a maximum inhibition to 51%, and recovery in 72 hours. Erythrocyte cholinesterase in Lehman's experiment was reduced to 15% and did not return to normal for more than 4 weeks. According to Lehman, this prolonged recovery time shows that the enzyme-inhibitor complex is irreversible in erythrocytes, since the average life of a rat erythrocyte is 4 weeks. In our experiment, using intraperitoneal injection, recovery time was approximately 2 weeks, which, according to Lehman's theory, would seem to indicate reversibility.

Our results indicate that I and II resemble parathion more closely than DFP. The slower action and prolonged recovery time with I would suggest a slower absorption, transport, or metabolism.

Summary. 1. Comparative cholinesterase-inhibiting activity of 2 new vinyl-substituted phosphates and parathion was studied in respect to their cumulative inhibitory properties, the rate of regeneration of the enzyme following single administration, and their inhibitory action *in vitro*. 2. While qualitatively resembling parathion in inhibitory action against cholinesterase, quantitatively the diethyl 2-chlorovinyl phosphate was found to be more active both *in vitro* and *in vivo* than parathion; and the dimethyl 1-carbomethoxy-1-propen-2-yl phosphate was less active *in vivo*. 3. By examination of the cumulative inhibitory properties of these 3 phosphates, the observation of others has been confirmed, that on increasing the dosage or duration of exposure to the cholinesterase inhibitor, remarkably low levels of enzyme activity can occur without fatal termination. 4. Erythrocyte cholinesterase was usually depressed to a greater extent than that of brain or plasma. There was some indication of a tolerance developing on the part of the "pseudo" cholinesterase with one of the compounds.

1. Hine, C. H., Shack, E. R., Kodama, J. K., and Morse, M. S., *Fed. Proc.*, 1952, v11.

2. Corey, R. A., Dorman, S. C., Hall, W. E., Glover, L. C., and Whetstone, R. R., *Science,* in press.

3. Kodama, J. K., Morse, M. S., Hine, C. H., Anderson, H. H., and Dunlap, M. K., in press.

4. Frawley, J. P., Hagan, E. C, and Fitzhugh, O. G., *J. Pharmacol. and Exp. Therap.*, 1952, v105, 156.

5. Michel, H. O., *J. Lab. and Clin. Med.*, 1949, v34, 1564.

6. DuBois, K. P., Doull, J., Salerno, P. R., and Coon, J. M., *J. Pharmacol. and Exp. Therap.*, 1949, v95, 79.

7. Lehman, A. J., *Assoc. Food and Drug Officials U. S. Quart. Bull.*, 1952, v14, 85.

Received June 22, 1953. P.S.E.B.M., 1953, v83.