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RESPONSE OF RABBIT WHOLE-BLOOD CHOLINESTERASE *IN VIVO* AFTER CONTINUOUS INTRAVENOUS INFUSION AND PERCUTANEOUS APPLICATION OF DIMETHYL ORGANOPHOSPHATE INHIBITORS

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Abstract—The *in vivo* reactions of three dimethyl organophosphates with rabbit whole-blood cholinesterase were studied during continuous intravenous infusion or percutaneous application of these compounds. 3-(Dimethoxyphosphinyloxy)-N,N-dimethylcrotonamide (Bidrin) was poorly absorbed when applied as an undiluted liquid; 1-carbomethoxy-1-propen-2-yl dimethyl phosphate (Phosdrin) and DDVP, 2,2-dichlorovinyl dimethyl phosphate (Vapona) were absorbed more readily. The absorption of Bidrin, Phosdrin, and Vapona was enhanced when the compounds were applied with dimethylsulfoxide. Whole-blood cholinesterase was progressively inhibited during intravenous infusion of the compounds, followed by rapid but incomplete reversal of enzyme inhibition during an immediate postinfusion period. Differences in the enzyme inhibition and recovery curves are discussed in relation to possible mechanisms of *in vivo* inhibition. Theoretical equations, derived from the experimental data and programmed into an analog computer, successfully described the inhibition process.

INCREASED use of organophosphate insecticides constitutes a widespread problem in industrial toxicology. Although these materials generally are highly toxic, sublethal doses can cause quantitative inhibition of the cholinesterase enzymes. Since toxic reactions in mammals can be elicited by ingestion, percutaneous application, or inhalation, it is important to understand the factors affecting *in vivo* reactions of these agents when attempting to assess their hazards to humans, domestic animals, and wildlife.

McPhail and Adie[1, 2] measured the penetration and distribution of ^{32}P-labeled Sarin vapors through the skin of rabbits. Absorption was shown to be influenced by vapor concentration, surface area, and exposure time. Also, there appeared to be a direct relationship between ^{32}P concentration in the blood and the degree of cholinesterase inhibition. Fredriksson[3] has extensively studied the onset and severity of symptomatology after percutaneous application of this compound. Sarin absorption was measured by the disappearance of the labeled material from the site of application.

The use of radioisotopes to follow a compound's absorption is a sensitive and accurate technique, but many compounds in the early stages of development are not available in labeled form. Acute percutaneous toxicity studies, therefore, are frequently conducted with new, unlabeled compounds as an approximate measure of absorption. However, such factors as surface area, the solvent or vehicle, and exposure

time can markedly affect the toxicity. Hadgraft and Somers[4] stated that organic solvents such as ether, chloroform, benzene, and acetone may alter percutaneous absorption to such an extent that the toxicity data of a particular compound may be significantly modified.

In this study, cholinesterase depression curves following intravenous infusion or percutaneous application of a compound were compared as a measure of the amount of compound present in the blood.

MATERIALS AND METHODS

Three registered organophosphate compounds were used: Bidrin, 3-(dimethoxyphosphinyloxy)-N,N-dimethylcrotonamide, a technical material containing 75 per cent of the active *cis*-isomer; Phosdrin, 1-carbomethoxy-1-propen-2-yl dimethyl phosphate, a technical material containing 60 per cent of the active α-isomer; and Vapona, DDVP, 2,2-dichlorovinyl dimethyl phosphate, a product of 94 per cent purity.

For i.v. administration, each compound was diluted to the desired concentration with physiological saline solution. In percutaneous studies, each liquid material was applied to the skin as an undiluted material or in dimethylsulfoxide (DMSO).

In the infusion studies, polyethylene cannulas, i.d. 0·86 mm, were surgically inserted into the external jugular vein and/or carotid artery of New Zealand white rabbits weighing 2·3 to 2·5 kg, according to the procedure described by Popovic and Popovic.[5] Cannulas were implanted, while the animal was anesthetized with 5% sodium pentothal, 3 to 4 days before an experiment.

The compound solutions were infused into the external jugular vein with a Harvard model 600–900 single infusion-withdrawal pump throughout a predetermined interval. Whole-blood samples were taken from the carotid artery at intervals to determine whole-blood cholinesterase activity; to maintain fluid balance, a volume of saline solution equivalent to the volume of blood removed was reinfused into the animal. Additional blood samples were taken during the posttreatment recovery periods. Cholinesterase inhibition and recovery curves were plotted as a function of time; average pretreatment enzyme activity levels were used as a point of reference. Comparison of the enzyme inhibition rates obtained by percutaneous and i.v. routes were also plotted as log per cent enzyme activity versus time.

For the percutaneous work, small glass rings of known surface area were glued to the closely clipped skin in the mid-shoulder region of the rabbit with Plybon cement. Each ring was carefully checked to assure a tight seal, and the glue was prevented from seeping into the enclosed surface area. After the test materials were applied to the skin within the ring, a glass cover slip was placed on the ring and sealed with stopcock grease. Each test fluid was dispersed throughout the enclosed surface area. Blood samples were removed at intervals from the carotoid artery and analyzed for cholinesterase activity. The data obtained for each animal—three usually were used in each trial—were plotted as described for the infusion studies.

Whole-blood cholinesterase was determined electrometrically by automatic constant pH titration of acetic acid liberated from the acetylcholine substrate. The instrumentation and the basic procedure for analysis of cholinesterase in brain homogenates were described previously by Krech *et al.*[6] The method was modified for whole blood as follows: 0·1 ml of heparinized whole blood is added to a small reaction vial containing 0·2 ml of 0·1% heparin in physiological saline solution; 1·8 ml of a 0·1%

Triton X solution in distilled water is then added to hemolyze the erythrocytes. After incubation for 10 min at 37°, the reaction medium is adjusted to pH 7·8 with dilute NaOH. Acetylcholine bromide (0·1 ml of a 1% solution in distilled water) is added and the reaction medium again quickly adjusted to pH 7·8. Enzymatic hydrolysis of the substrate is followed for 3 to 5 min by measuring the amount of 0·0003 N NaOH required to maintain a constant pH.

RESULTS

The effect of continuous infusion of Bidrin on rabbit whole-blood cholinesterase is shown in Fig. 1. Infusion at rates of 0·051 and 0·106 mg/min progressively inhibited

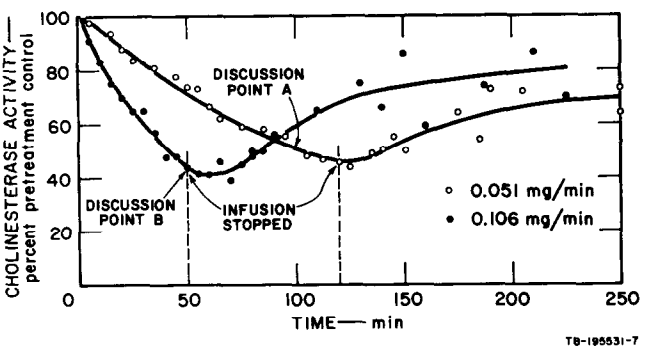


FIG. 1. Rabbit whole-blood cholinesterase inhibition after i.v. infusion of Bidrin (discussion points A and B indicate times when nearly equal quantities of compound had been infused).

the whole-blood enzyme to levels of 44 and 46 per cent of the pretreatment control. After infusion there was consistent but incomplete recovery of enzyme activity to a level of 70 to 80 per cent of the pretreatment controls; this effect was more pronounced at the 0·106 mg/min level. At point A on the 0·051 mg/min curve and at point B on the 0·106 mg/min curve, nearly equal quantities of compound had been infused; the enzyme activities were 50 and 44 per cent of the control periods, respectively, a difference of only 6 per cent.

Phosdrin at 0·005 and 0·011 mg/min progressively inhibited whole-blood cholinesterase during the infusion, followed by a definite but incomplete recovery during the posttreatment period (Fig. 2). Nearly equal quantities of compound had been infused at point A where the infusion was stopped on the 0·005 mg/min curve and at point B before the infusion was stopped on the 0·011 mg/min curve with the enzyme activities at 65 and 54 per cent of pretreatment controls respectively. This 11 per cent difference suggests there may be less enzyme inhibition when the same amount of Phosdrin is infused into the animal over a longer period of time. This effect was not observed with Bidrin.

The effects of Vapona on rabbit whole-blood cholinesterase after i.v. infusion are shown in Fig. 3. The shapes of the curves are similar to those of Bidrin and Phosdrin, showing progressive inhibition with marked but incomplete recovery during the posttreatment period. However, at point A on the 0·026 mg/min curve and at point B on the 0·052 mg/min curve (where the same total quantities of compound have been infused) the enzyme activities were 54 and 19 per cent of pretreatment controls

respectively. The markedly greater differential in cholinesterase inhibition with Vapona at two infusion rates than was observed with Phosdrin or Bidrin suggests a basic difference in the *in vivo* behavior of this compound.

Bidrin, when applied as undiluted material at a dose of 200 mg/kg on a skin surface area of 4·54 cm^2, was poorly absorbed. Enzyme activity at 300 min after compound

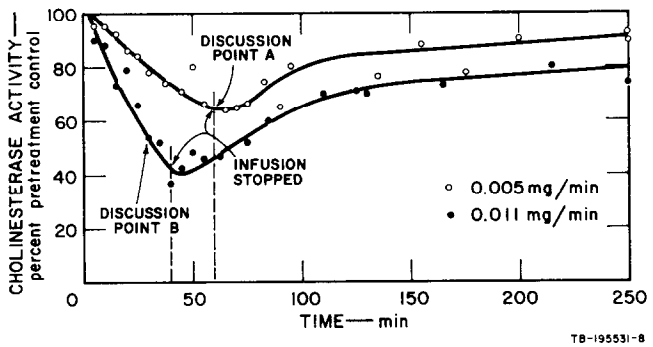


FIG. 2. Rabbit whole-blood cholinesterase inhibition after i.v. infusion of Phosdrin (discussion points A and B indicate times when nearly equal quantities of compound had been infused).

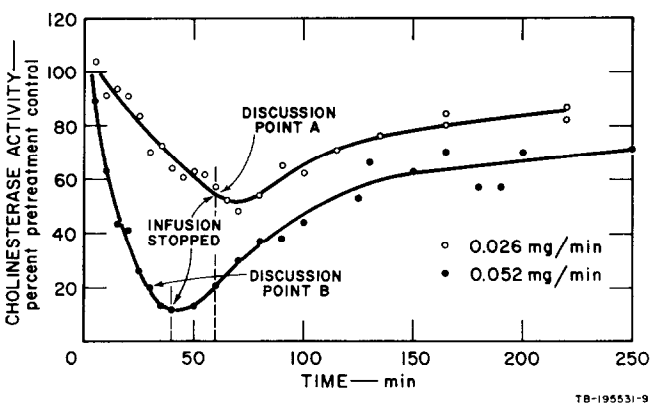


FIG. 3. Rabbit whole-blood cholinesterase inhibition after i.v. infusion of Vapona (discussion points A and B indicate times when nearly equal quantities of compound had been infused).

application was 80 per cent of the pretreatment controls, and then 0·1 ml of DMSO was added to the skin treatment area. Within 50 min the whole-blood enzyme was completely inhibited, indicating a marked absorption of compound. Subsequently, Bidrin was applied to other rabbits at 200 mg/kg in 0·1 ml DMSO on a surface area of 4·54 cm^2; 15 min after application there was progressive whole-blood enzyme inhibition to 20 per cent of normal.

The depression portion of the whole-blood enzyme inhibition curves obtained after i.v. infusions and percutaneous application of Bidrin was redrawn as log per cent activity against time. This method of expressing the initial enzyme inhibition gave essentially straight lines. Based on a comparison of the slopes of these lines, the

calculated absorption of Bidrin applied in DMSO was 37 μg/min/cm^2. As undiluted material, the estimated absorption of Bidrin was no greater than, and probably less than, 1 μg/min/cm^2. These results demonstrate an enhancement of absorption in the presence of DMSO.

Similar results were obtained with Phosdrin and Vapona. Percutaneous application of undiluted Phosdrin at 30 mg/kg on a surface of 2·54 cm^2 produced measurable cholinesterase inhibition within 50 min. The calculated absorption was 1·4 μg/min/cm^2, as estimated from the enzyme inhibition slopes. In the presence of DMSO, the calculated absorption of Phosdrin was 7·8 μg/min/cm^2. The calculated percutaneous absorption of undiluted Vapona applied at a dose level of 78 mg/kg on a surface of 3·81 cm^2 was 3·9 μg/min/cm^2, and in DMSO the calculated absorption was 35 μg/min/cm^2.

DISCUSSION

These results demonstrate that whole-blood cholinesterase inhibition curves attained after continuous i.v. infusion or percutaneous application of a cholinesterase inhibitor can provide an estimate of the quantity of an active compound in the blood. Factors such as compound structure, surface area, and solvent can be studied readily by this procedure, and it offers some advantages over isotope studies. The loss of labeled material from the skin or the increase of an isotope in the body may be a measure of gross absorption of a particular compound, but such a procedure does not ensure that the material reaching the blood stream is the unmetabolized or active compound. Also, metabolism or detoxification of the initial compound to a material of lesser toxicity during the absorption process cannot be ascertained without considerable effort. In addition, the procedure used in these studies is applicable to compounds that may not be readily available with radio-labels. A combined study to compare isotope absorption and cholinesterase inhibition would give the most information.

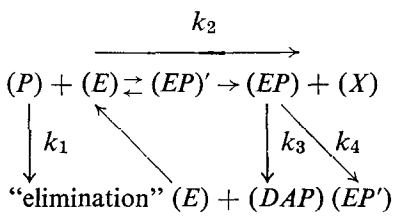
The responses of rabbit whole-blood cholinesterase during and after i.v. infusion of the three dimethylphosphates were generally similar, i.e. progressive enzyme inhibition during infusion followed by rapid but incomplete recovery during the immediate posttreatment period. Previously, Vandekar and Heath[7] reported that inhibited rat erythrocyte cholinesterase recovered rapidly after a sublethal i.v. injection of certain dimethylphosphates. They also showed that the amount of enzyme reactivation was much less with slow i.v. infusion of compound and that the amount of irreversibly inhibited enzyme increased with the duration of infusion.

Although the shapes of the enzyme inhibition–recovery curves are basically similar, the anomalous behavior between compounds suggests different *in vivo* activities. In Fig. 1, the total enzyme inhibition attained when equal quantities of Bidrin were were infused into rabbits was approximately the same even though the solutions of the compound differed twofold in concentration, and the infusion period for the more dilute solution was twice as long. With Phosdrin there was 11 per cent greater inhibition of cholinesterase when the total amount of compound was infused in half the time (Fig. 2). For Vapona with the same conditions, the differential inhibition was 35 per cent, a difference too large to be due to biological or analytical variations (Fig. 3.)

Inhibition of cholinesterase by organophosphates is thought to occur by direct phosphorylation at the active enzyme site. According to Davies and Green,[8] this enzyme inhibition is a two-stage process, initially producing an enzyme complex that is reversible by reactivators. This complex is then converted by a first-order reaction to a second form that can no longer be reactivated. The latter step is referred to as the "aging" process.

The inhibition of whole-blood cholinesterase by continuous i.v. infusion of a compound and the subsequent recovery of enzyme activity during the posttreatment period suggest a technique to study *in vivo* mechanisms of enzyme inhibition. Progressive inhibition during infusion may be related to rate of enzyme inhibition; recovery during the posttreatment period should reflect spontaneous reversal of the phosphorylated enzyme. Incomplete recovery may be related to the aging process, while the degree of inhibition at different infusion rates could be related to various non-specific losses of compound, which might include metabolism in blood, liver, and other tissues, renal excretions, partition or storage in a body tissue such as brain or fat; inhibition of enzymes other than cholinesterase; noninhibitory reactions with proteins, including enzymes; and compound instability and, hence, spontaneous hydrolysis in aqueous biological systems.

The overall *in vivo* reactions of an organophosphate inhibitor with whole-blood cholinesterase may be visualized by the following simplified sequence of chemical reactions.



(P) = added organophosphate inhibitor
(E) = whole-blood cholinesterase
$(EP)'$ = intermediate enzyme–inhibitor complex
(EP) = dialkylphosphorylated enzyme
(X) = an anion substituent from the inhibitor
(DAP) = dialkyl phosphate
(EP') = "aged" enzyme
k_1 = reaction rate constant for "elimination", assumed to be first order
k_2 = rate constant for enzyme inhibition, formation of (EP), assuming that the formation of $(EP)'$ is rate limiting
k_3 = rate constant for spontaneous reversal
k_4 = rate constant for formation of "aged" enzyme
"elimination" = nonspecific compound loss.

A series of three differential equations can be written which, if the assumptions are reasonable, describe these reactions between (E), (P), and (EP) as follows:

$$-\frac{d(P)}{dt} = R - k_1(P) - k_2(E)(P) \tag{1}$$

$$\frac{d(E)}{dt} = -k_2(E)(P) + k_3\,(EP) \tag{2}$$

$$\frac{d(EP)}{dt} = k_2(E)(P) - (k_3 + k_4)\,(EP) \tag{3}$$

$(P) = (EP) = 0$ and
$(E) = (E)_0$ at $t = O$,
$R =$ rate of addition of inhibitor (P)

Solution of equations (1), (2), and (3), a function of R and t, should provide numerical values for the various enzyme reaction rate constants (k_1–k_4) at each infusion rate.

The differential equations were programmed into a model TR-48 analog computer (Electronic Assiciates, Inc., Palo, Alto Calif.). Details of the computer program and results obtained with another phosphate inhibitor have been reported by Shellenberger and Sarros.[9] The enzyme reaction constants were represented by variable resistors, which were varied manually to change the shape of the computer-derived curves. The experimental conditions, rate, and time of compound infusion were then inserted into the program.

Computer-drawn inhibition curves are shown in Figs. 4–6; selected points from experimentally derived curves have been superimposed to indicate the degree of fit that was attained with this procedure. Although these curves describe the inhibition

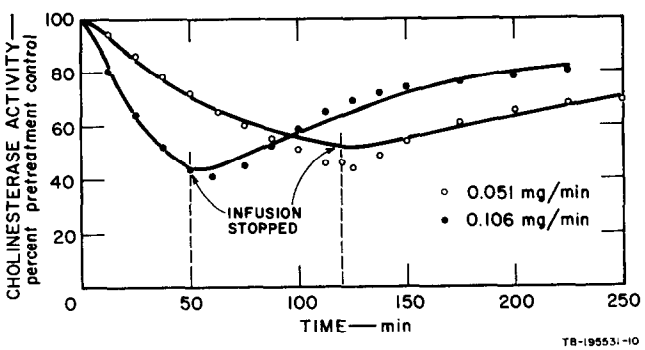


FIG. 4. Computer-derived rabbit whole-blood cholinesterase inhibition and recovery curves simulating experimental results after i.v. infusion of Bidrin (experimental points have been superimposed on computer curves to indicate degree of simulation).

and recovery of enzyme activity, the computer also provides simultaneous information on the status of the phosphorylated enzyme and the concentration of phosphate inhibitor during the infusion process.

In general, there is good agreement between the computer-derived and the experimental enzyme activity curves. There was least agreement in the Bidrin curves (Fig. 4); with Phosdrin and Vapona (Figs. 5, 6) the close agreement between the computer-derived and experimental curves is within biological variability.

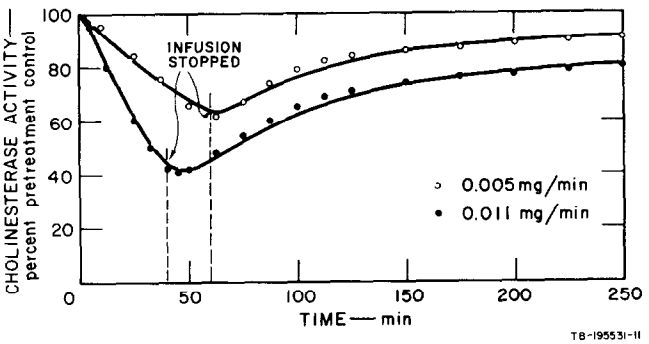


FIG. 5. Computer-derived rabbit whole-blood cholinesterase inhibition and recovery curves simulating experimental results after i.v. infusion of Phosdrin (experimental points have been superimposed on computer curves to indicate degree of simulation).

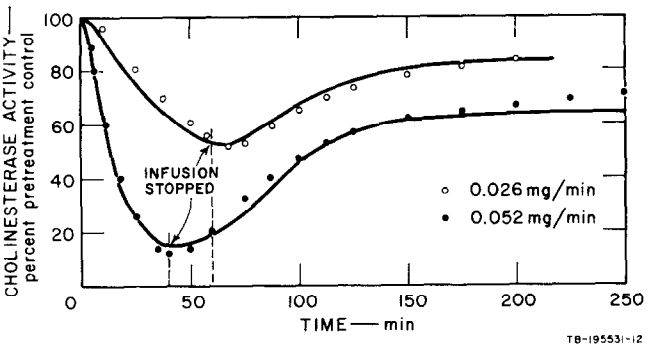


FIG. 6. Computer-derived rabbit whole-blood cholinesterase inhibition and recovery curves simulating experimental results after i.v. infusion of Vapona (experimental points have been superimposed on computer curves to indicate degree of simulation).

TABLE 1. *IN VIVO* REACTION RATE CONSTANTS CALCULATED FROM COMPUTER VALUES OF RABBIT WHOLE-BLOOD CHOLINESTERASE INHIBITION AFTER INTRAVENOUS INFUSION OF DIMETHYL ORGANOPHOSPHATE INHIBITORS

Compound	Infusion rate (mg/min)	Infusion time (min)	Reaction constant (min^{-1})			
			k_1	k_2	k_3	k_4
Bidrin	0·051	120	$2{\cdot}9 \times 10^{-5}$	$5{\cdot}6 \times 10^{-6}$	$1{\cdot}7 \times 10^{-3}$	$2{\cdot}0 \times 10^{-5}$
	0·106	50	$1{\cdot}9 \times 10^{-4}$	$1{\cdot}2 \times 10^{-5}$	$1{\cdot}7 \times 10^{-3}$	$6{\cdot}1\frac{1}{2} \times 10^{-5}$
Phosdrin	0·005	60	$6{\cdot}2 \times 10^{-5}$	$1{\cdot}6 \times 10^{-4}$	$2{\cdot}7 \times 10^{-4}$	$4{\cdot}0 \times 10^{-5}$
	0·011	40	$6{\cdot}2 \times 10^{-5}$	$1{\cdot}6 \times 10^{-4}$	$2{\cdot}3 \times 10^{-4}$	$5{\cdot}8 \times 10^{-5}$
Vapona	0·026	60	$7{\cdot}5 \times 10^{-4}$	$2{\cdot}3 \times 10^{-5}$	$4{\cdot}9 \times 10^{-4}$	$8{\cdot}2 \times 10^{-5}$
	0·052	40	$2{\cdot}8 \times 10^{-4}$	$8{\cdot}5 \times 10^{-5}$	$7{\cdot}9 \times 10^{-4}$	$11{\cdot}0 \times 10^{-5}$

Reaction rate constants, as defined in the *in vivo* inhibition model, were calculated from the appropriate computer resistor values (Table 1). The values of these constants appear to be characteristic of the compound and its infusion rate. The elimination constant value, k_1, varied between compounds. For Bidrin, k_2, the inhibition constant, was approximately 20 times greater at the higher infusion rate but was generally smaller than values calculated for Phosdrin and Vapona. This indicates that Bidrin may be a less effective *in vivo* enzyme inhibitor for the species studies.

If the assumptions used to establish the enzyme inhibition model are correct, the spontaneous reversal reaction constant, k_3, and aging constant, k_4, should be approximately the same order of magnitude for each compound—the dimethylphosphorylated enzyme should be identical in each case. For Phosdrin and Vapona this was essentially true. With Bidrin, k_4 was somewhat lower than with the other compounds but still of the same general order of magnitude. The reversal reaction constant, however, was 2 to 7 times greater for Bidrin than was calculated for Phosdrin and Vapona; this may be a meaningful difference in view of the generally lower inhibition constant and the higher rates of infusion required to produce enzyme inhibition with this compound. If the formation of a dimethylphosphorylated enzyme from the initial enzyme–compound complex is a rate-limiting reaction for Bidrin, cholinesterase reactivation may be due in part to a breakdown of the initial enzyme–inhibitor complex. With further experimental data, modification of the inhibition model may be necessary.

The computer-derived curves were matched to the experimental curves by varying appropriate reaction constant resistors to give a reasonable fit. With greater experience, a more sophisticated computer program could be written that would allow programming the experimental data directly into the computer for selecting the best reaction constants and producing faster and more reliable comparisons.

Initial emphasis in this program was to investigate the percutaneous absorption of the inhibitors. Although only whole blood was studied, it would be desirable to expand this work to study both the true and pseudo-cholinesterase of whole blood to obtain individual inhibition constants. This might best be accomplished with substrates specific for each enzyme.

The effect of other dialkyl phosphate structures on blood cholinesterase can be studied with this infusion technique. Preliminary data obtained with a diethylphosphate indicate that little, if any, spontaneous reversal of inhibited whole-blood enzyme occurs during the immediate postinfusion period, in contrast to the rapid reversal with the dimethyl compounds. Injection of 2-pyridine aldoxime methiodide (2-PAM) after such an infusion, however, causes an immediate partial recovery of enzyme activity; the extent of recovery appears to be directly related to the amount of injected reactivator. This suggests that the aging reaction is not enhanced with diethylphosphates and that the spontaneous reversal reaction is negligible. Based on the effect of 2-PAM, this technique could also provide a convenient *in vivo* method to study potential reactivators.

The technique used in this work is a sensitive method for *in vivo* studies of organophosphate biochemistry, i.e. evaluation of factors affecting percutaneous absorption, cholinesterase inhibition, and the structure–activity relationships of organophosphate inhibitors and enzyme reactivators. Additional studies are needed to demonstrate the full potential of this dynamic *in vivo* system.

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