The Reactivation of Cholinesterase after Inhibition *in vivo* by some Dimethyl Phosphate Esters

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It is generally believed that inhibition of cholinesterase by organophosphorus compounds is the result of phosphorylation of the enzyme's active centre. Aldridge (1953*a*) showed that reactivation of the phosphorylated enzyme by hydrolysis is more rapid when cholinesterase is inhibited by certain dimethyl phosphate esters *in vitro* than when it is inhibited by the corresponding diethyl esters. Davison (1955), studying the return of cholinesterase activity *in vivo* after inhibition with different organophosphorus compounds, found that reactivation of inhibited rat-brain cholinesterase took place at the same rate as *in vitro*. A change of slope at about 55% recovery was observed, suggesting that the remainder of the inactivated enzyme is more permanently inhibited.

Some preliminary observations showed that *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and some of its derivatives, injected into animals, produced prolonged symptoms with delayed recovery. This was inexplicable if the inhibited enzyme recovered its activity at the high rate indicated by Aldridge's (1953*a*) experiments *in vitro*, and we were therefore led to investigate the action of these compounds in more detail. We have shown that they persist in the body and that, in consequence, cholinesterase is inhibited in a form from which it cannot be reactivated at an appreciable rate. A brief communication has already been made outlining the results (Vandekar, 1957). The work carried out *in vitro* by Wilson (1955), Davies & Green (1956) and Hobbiger (1956) suggests how this comes about. They showed that the degree of reactivation of inhibited enzyme by nucleophilic reagents is inversely related to the time of contact between enzyme and inhibitor. It has been suggested that this inhibition is a two-stage process, an initial form of inhibited enzyme being converted at a first-order reaction rate into a second form which can no longer be reactivated. This conversion occurs more rapidly with dimethyl- and di*iso*propyl- than with diethyl-phosphorylated enzyme.

In the present work we have shown that *in vivo*, also, the two-stage process of inhibition occurs after administration of some dimethyl phosphate esters.

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The rapidly-reversible first stage of inhibition changes into the non-reversible stage whenever prolonged contact between inhibitor and enzyme takes place. Thus non-reversible inhibition may be produced either by a rapid injection of an inhibitor which persists in the body or by artificially prolonged administration of a non-persistent inhibitor such as dimethyl *p*-nitrophenyl phosphate.

We have used for this study *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate and some related compounds, and compared their toxic action with that of dimethyl *p*-nitrophenyl phosphate (Me-E 600) and a diethyl phosphate inhibitor. The reproducibility of the toxic properties of these compounds and of the differences between them result from the care taken in their purification, as described by Heath & Vandekar (1957).

MATERIALS AND METHODS

The compounds used for this study are named in Table 1.

Dimethyl *p*-nitrophenyl phosphate (Me-E 600) was prepared as follows: dimethyl phosphorochloridate (42 g.), *p*-nitrophenol (41·8 g.), Na_2CO_3 (31·8 g., dried for 0·5 hr. at 160°), acetone (200 ml.) and ethyl methyl ketone (100 ml.) (both dried over anhyd. Na_2SO_4) were refluxed gently with brisk stirring for 3 hr. The product was cooled, filtered, and concentrated *in vacuo*. The residue was shaken with Na_2CO_3 solution (15 g./500 ml. of water) and extracted with benzene. The benzene extract was shaken in turn with Na_2CO_3 solution to remove any remaining *p*-nitrophenol and twice with water, and immediately concentrated *in vacuo*. The product was purified by fractionation, b.p. 150° at 0·20 mm. Hg. The compound contained 0·07% of free *p*-nitrophenol and liberated *p*-nitrophenol equivalent to 96·5% of the theoretical on hydrolysis. The only likely impurity other than solvent is tetramethyl pyrophosphate. To remove any present, aqueous solutions used were stored for 2 hr. at room temp. before use.

The other compounds were purified as described by Heath & Vandekar (1957).

All compounds were stored in the dark at −30° between experiments. To avoid conversion in water into other toxic-compounds *OO*-dimethyl *S*-ethylthioethyl phosphorothiolate was injected without dilution; other compounds (except Me-E 600) were diluted with water immediately before use. Except when stated otherwise, the doses injected were those given in the second column of Table 1.

Estimation of the recovery of brain and erythrocyte cholinesterase in vivo. Male and female albino rats (180–250 g.)

were given organophosphorus compounds via the tail vein. The doses were close to the LD_{50} and invariably produced severe symptoms. At selected times animals were killed by coal gas. On death, blood (5–8 ml.) was collected from the vena cava into 0·2 ml. of 1% heparin, and erythrocytes were washed twice with cold buffer (0·0357 M-$NaHCO_3$, 0·164 M-NaCl and 0·040 M-$MgCl_2$, freshly prepared from stock solutions, and cooled to 5° before use).

Estimations of cholinesterase activity were carried out on 1 ml. of brain suspension or on washed erythrocytes corresponding to 1·5 ml. of blood in a Warburg apparatus at 37° with an atmosphere of $N_2 + CO_2$ (95:5). Acetylcholine chloride was used as substrate at a final concentration of 0·0138 M. Gassing and equilibration time in the bath did not exceed 15 min.

Estimation of persistence of anticholinesterase activity of serum in rats. This was done at selected intervals after a single intravenous injection, when animals were killed by coal gas, and blood was collected as described above. Serum was separated and 2·0 ml. added to sedimented washed normal rat erythrocytes and incubated for 1 hr. at 37°. The erythrocytes were then washed twice with buffer, and the cholinesterase activity was estimated as above. The results are expressed as percentages of the activity of erythrocytes treated in the same way with serum obtained from normal rats.

Slow intravenous infusion of Me-E 600. One day before infusion, fine polythene canulae were inserted into the vena jugularis of male rats (225–275 g.) anaesthesized with ether. Different concentrations of Me-E 600 in saline were infused at 1·0 ml./hr. by means of a constant-injection apparatus as described by Church & Ridge (1955). During infusion, animals were kept in small cages, which allowed undisturbed feeding and drinking but prevented the animals from turning round. The symptoms were followed during each infusion and, at the onset of death, the positions of canulae were checked by injection of dye or air through them. Results were considered valid when no leakage of the dye (or air) was observed in the area surrounding the place of insertion of the canulae. In a satisfactory experiment the dye (or air) went only through the heart's auriole and out through the lower vena cava, which was cut. Such precautions were necessary because the convulsions which might take place during infusion sometimes displaced the canula.

The brain was dissected out and suspended in cold buffer in order to determine the return of cholinesterase activity *in vitro*.

Return of cholinesterase activity in vitro *in the brains of rats treated with Me-E 600.* The rate of return of cholinesterase activity was determined on 1 ml. of brain suspension by the method of Aldridge (1953*a*), in which excess of inhibitor is removed by a preparation of A-esterase. The activity obtained after 4 hr. incubation at 37° was taken as 89·4% of complete reactivation, a value calculated from the half-life for the reaction of 1·23 hr., obtained by experiments where inhibition was produced *in vitro* by incubation for 15 min. only at 37°. In this time very little of the enzyme is inhibited irreversibly.

RESULTS

Dimethyl phosphate esters

Response to a single acute dose. After intravenous administration of doses close to the LD_{50} all compounds produced typical symptoms of acetylcholine poisoning. Marked differences, however, were observed in the onset and duration of symptoms. The dimethylsulphonium compound and Me-E 600 produced rapid onset of symptoms and, if rats survived the critical first few minutes, they began to recover very soon. Within 1 hr. of sublethal intravenous injection they showed no symptoms except perhaps an occasional fasciculation.

On the other hand, the sulphide and its two oxidative products, the sulphoxide and sulphone, produced symptoms which developed relatively slowly. Pronounced fasciculations, tremor, gasping, salivation and chromodacryorrhea lasted for 3–4 hr., and moderate fasciculations and prostration were present for more than 5 hr. (Table 1). Even 24 hr. later, the animals were still weak and dirty, with staring coats, and had lost 10–15% of their weight.

Table 1. *Duration of symptoms after sublethal intravenous injection of different dimethyl phosphate esters and one diethyl phosphate ester into rats*

Except for Me-E600, the values for intravenous LD_{50} are taken from Heath & Vandekar (1957).

	Intravenous LD_{50} (mg./kg.)	Amount administered intravenously (mg./kg.)	Duration of symptoms (hr.)
$(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SEt$ (Sulphide) (*OO*-dimethyl *S*-ethylthioethyl phosphorothiolate)	64·6 (♀)	50·0 (♀)	> 5
$(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SO \cdot Et$ (Sulphoxide) (*OO*-dimethyl *S*-ethylsulphinylethyl phosphorothiolate)	47·2 (♂)	40·0 (♂)	> 5
$(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot SO_2 \cdot Et$ (Sulphone) (*OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate)	23·7 (♂) 21·7 (♀)	— 17·3 (♀)	— > 5
$(MeO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot \overset{+}{S}MeEt$ (Dimethylsulphonium) (*OO*-dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate)	0·0615 (♀)	0·040 (♀)	Approx. 1
$(MeO)_2PO \cdot O\text{-}p\text{-}C_6H_4 \cdot NO_2$ (Me-E 600) (dimethyl *p*-nitrophenyl phosphate)	0·410 (♀)	0·30 (♀)	1 or less
$(EtO)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot \overset{+}{S}MeEt$ (Diethylsulphonium) (*OO*-diethyl *S*-ethylsulphonioethylmethyl phosphorothiolate)	0·01 (♀)	0·01 (♀)	4–5

Reactivation of cholinesterase in vivo. The difference in the duration of symptoms between the two groups of compounds suggested that the inhibited enzyme was reactivated at different rates. We therefore investigated the rate at which cholinesterase activity returned *in vivo* in the brain and erythrocytes of rats given single injection of these two groups of compounds.

The activity of brain and erythrocyte cholinesterase was estimated at selected intervals after injection of sublethal doses. As shown in Fig. 1*a* intravenous injection of the dimethylsulphonium derivative produced a considerable inhibition of erythrocyte cholinesterase 10 min. after injection, after which enzyme activity returned rapidly. Brain-cholinesterase activity was only slightly reduced. Similarly, Fig. 1*b* shows that erythrocyte cholinesterase inhibited by Me-E 600 recovers its activity at the same rapid rate. This compound also inhibited brain cholinesterase, the activity of which returned just as rapidly.

Sulphide, sulphoxide and sulphone reduced both brain- and erythrocyte-cholinesterase activity to 30 % of normal, but in this group the reduction was maintained for the first 24 hr. After this period the activity returned slowly and reached about 75 % in 28 days (Fig. 1*d–f*).

Reactivation of cholinesterase in vitro. These results showed that the different durations of symptoms are paralleled by different rates of reactivation of the enzyme *in vivo*. The high rates of reactivation found after injection of Me-E 600 and the dimethylsulphonium compound are consistent with the rates of reactivation *in vitro* found by Aldridge (1953*a*) for dimethylphosphorylated enzymes. The sulphide, sulphoxide and sulphone inhibit far more permanently, which might imply that not all dimethyl phosphate esters are able to produce reversible inhibition. We therefore determined the rate of reactivation of cholinesterase inhibited *in vitro* with all five compounds. Aldridge's technique was used, in which the inhibitor and rabbit erythrocytes are left in contact for 15 min. only at 37°, after which the return of cholinesterase activity is followed. There was no distinction between the five compounds when tested in this way. The return of activity took place at the same high rate (half-life, 1·3 hr.) characteristic of cholinesterase inhibited with dimethyl phosphate esters.

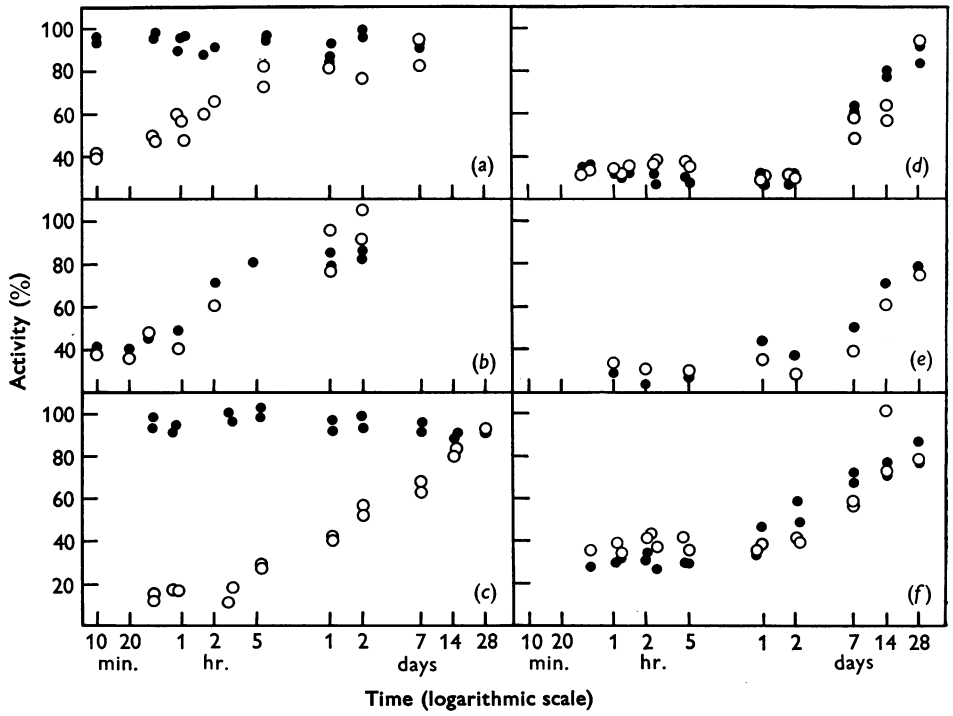

Fig. 1. Return of brain (●) and erythrocyte (○) cholinesterase activity in rats after single sublethal intravenous injection of (*a*) dimethylsulphonium derivative, (*b*) Me-E 600, (*c*) diethylsulphonium derivative, (*d*) sulphide, (*e*) sulphoxide and (*f*) sulphone. Each point represents one female rat except (*e*), where each point represents the mean of four male rats.

Persistence of inhibitor in the body. It seemed possible that the prolonged symptoms as well as the exceedingly slow rate of return of cholinesterase activity in animals following administration of the sulphide, sulphoxide and sulphone might be explained if these inhibitors persisted in the body. As no microchemical methods exist for determining these compounds and their products in animals, an attempt was made to detect the presence of free inhibitor by measuring the inhibitory activity of the circulating blood.

The anticholinesterase activity of serum was estimated at selected times after intravenous injection of sublethal doses of all five compounds (Fig. 2). After injection with the dimethylsulphonium derivative the rats' serum had lost most of its anticholinesterase activity within 2 hr. No inhibitor was detectable in serum 10 min. after injection with Me-E 600 (Fig. 2*a*). On the other hand, injection with sulphide, sulphoxide and sulphone produced an inhibitor which persisted for several hours in the blood. Some inhibitor was detectable even after 16 or 24 hr. (Fig. 2*b*).

These experiments showed that those dimethyl compounds which produced long-lasting inhibition of enzyme are those that persisted in the body.

Effect of slow infusion of Me-E 600. To provide further evidence that the production of permanent inhibition was due solely to the persistence of some dimethyl phosphate esters in the body, we investigated the type of inhibition produced when prolonged contact was arranged between a transient inhibitor and cholinesterase *in vivo*.

Me-E 600 was infused at a constant slow rate into rat's vena jugularis, and the return of cholinesterase activity was determined *in vitro* as described under Methods. Table 2 shows the amount of irreversible inhibition produced by such treatments, which is compared with that produced by a single intravenous injection into rat's tail vein. The rate of reactivation after a single rapid injection (which killed the animal within 5 min.) approached closely that of a first-order reaction such that, during the 4 hr. incubation, most of the enzyme was recovered.

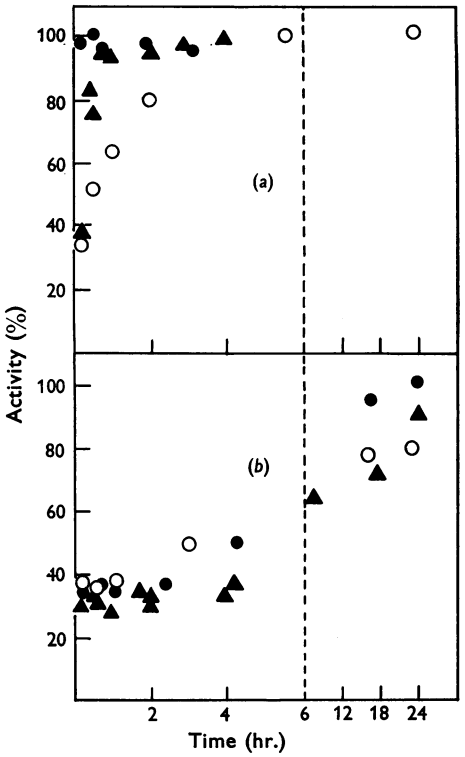

Fig. 2. Anticholinesterase activity of serum after intravenous injection of a single sublethal dose into male rats with (*a*) Me-E 600 (●), dimethylsulphonium derivative (○), diethylsulphonium derivative (▲), and (*b*) sulphide (○), sulphoxide (▲) and sulphone (●). Each point represents one rat.

Table 2. *Effect of slow intravenous infusion of Me-E 600 into rats*

Me-E 600 was infused at a constant slow rate into rat's vena jugularis or injected rapidly by a single intravenous injection into rat's tail vein. The return of cholinesterase activity was determined *in vitro* as described under Methods.

Rat no.	Rate and amount administered			Percentage of brain cholinesterase inhibited		
				At zero time	After 4 hr. of reactivation	Irreversibly
1	Rapid injection of 1·1 LD_{50}			63·6	10·2	3·9
	Infusion					
	Rate (LD_{50}/hr.)	Time of survival (hr.)	Dose tolerated (LD_{50})			
2	6·06	0·2	1·21	62·0	20·4	15·5
3	1·31	6·7	8·74	66·6	30·1	25·8
4	0·27	>34·0*	>7·43	82·3	66·8	65·0

* Killed by coal gas.

After slow infusion the amount of enzyme reactivated was much less, and the amount of irreversibly inhibited enzyme increased with the duration of infusion.

The total amounts of Me-E 600 tolerated at different rates of injection are shown in Fig. 3, and are expressed in terms of intravenous LD_{50} (= 0·410 mg./kg.). A decrease in the rate of infusion below 1·5 LD_{50}/hr. was accompanied by a remarkable increase in tolerance and survival time. All infused rats showed the typical symptoms of poisoning by acetylcholinesterase inhibitors, and passed through a stage in which they developed successively more severe symptoms, culminating in severe convulsions, dyspnoea with râles and tremor. Those that survived reached an 'equilibrium stage' which was characterized by constancy of symptoms (very pronounced tremor and fasciculations but only occasional slight convulsions) and lasted for several hours—only after which the animals became more and more prostrate, dyspnoea developing with increasingly laboured costal respiration. Death usually followed within half an hour after the onset of terminal dyspnoea, during which period fasciculations diminished and ceased. Convulsions (probably due to anoxia) and cessation of respiration always preceded death. The effects of different rates of infusion of other cholinesterase inhibitors have been studied also with results to be published in more detail elsewhere.

OO-*Diethyl* S-*ethylsulphonioethylmethyl phosphorothiolate*

Analogous experiments were carried out with the diethylsulphonium compound, to compare the previous results with those obtained on a diethyl phosphate ester.

After sublethal intravenous injection the onset of symptoms was rapid but their duration prolonged. The reactivation of cholinesterase inhibited *in vitro* took place at the slow rate typical of diethyl phosphorylated enzymes (Aldridge, 1953*a*). The rate of reactivation of erythrocyte cholinesterase *in vivo* was similar (Fig. 1*c*), showing that little or none of the enzyme had been inhibited irreversibly. Brain cholinesterase was not inhibited appreciably.

The compound did not persist in the serum (see Fig. 2*a*).

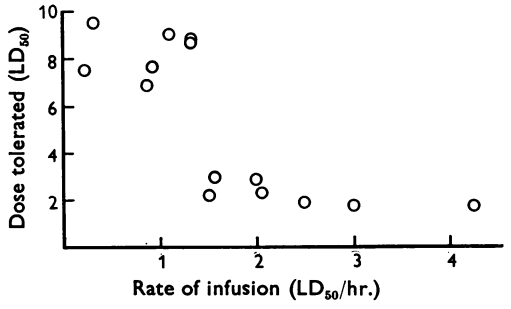

Fig. 3. Tolerance of Me-E 600 at different rates of infusion into vena jugularis of male rats (225–275 g.).

DISCUSSION

The dimethyl phosphate esters we have studied fall into two groups with very different biochemical actions *in vivo*: Me-E 600 and the dimethylsulphonium compound; and the sulphide, sulphoxide and sulphone.

Me-E 600 and the dimethylsulphonium derivative, when given intravenously in single sublethal injections, produced symptoms of short duration, which were shown to be associated with rapid recovery of cholinesterase activity *in vivo*. The inhibitor persisted in the blood only a very short time. These findings agree with those of Aldridge (1953*a*), who showed that cholinesterase inhibited *in vitro* by short contact with dimethyl phosphate esters recovered its activity rapidly when the inhibitor was removed.

The sulphide, sulphoxide and sulphone, on the other hand, administered in the same way, produced symptoms of long duration, which were associated with a very slow rate of return of cholinesterase activity *in vivo*. Inhibitors could be found in the blood in appreciable concentrations for several hours after injection. Delayed return of cholinesterase activity might be expected from the results obtained *in vitro* by other workers. Thus Wilson (1955) showed that prolonged contact between cholinesterase and dimethyl phosphate esters led to irreversible inhibition. Inhibition by organophosphorus esters is a two-stage process, in which the first, reversible, form of the inhibited enzyme is converted at a first-order reaction rate into a second form, which cannot be reactivated by nucleophilic reagents (Davies & Green, 1956; Hobbiger, 1956). Davies & Green (1956) have suggested that this reaction may be due to an initial phosphorylation of histidine followed by migration of the phosphoryl group to the hydroxyl group of serine. In our experiments prolonged contact between enzyme and inhibitor was achieved *in vivo*, apparently with the same results. The striking differences in the duration of symptoms observed in animals poisoned by single doses of dimethyl phosphate esters can therefore be reconciled with *in vitro* observations on the reactions of these compounds with cholinesterase. The difference between the two groups of compounds is determined by their different degrees of persistence in blood. If a non-persistent inhibitor is made to persist artifically by prolonged slow infusion, an irreversible inhibition of cholinesterase can be produced (see Table 2).

We cannot say whether those compounds producing long-lasting symptoms exert their action themselves, or whether they are converted *in vivo* into other active compounds. The sulphone is fairly stable in water and has no groups that we would expect to be readily attacked in the body. It is probable, however, that the sulphide and sulphoxide are oxidized *in vivo* to the sulphone, which is more toxic. March, Metcalf, Fukuto & Maxon (1955) have shown that such reactions take place *in vivo* with the diethyl homologues. Possibly the sulphide is converted into its much more toxic dimethylsulphonium derivative, a reaction known to take place rapidly *in vitro* (Heath & Vandekar, 1957). This reaction is bimolecular *in vitro*, and would be very slow at the concentrations present in the blood, unless other methylating agents act on the compound *in vivo*. We found that both the dimethyl- and diethyl-sulphonium derivatives did not inhibit brain cholinesterase when injected into animals (possibly because their ionic nature hindered passage through the blood–brain barrier). The sulphide inhibited brain cholinesterase considerably. Thus if the sulphide is converted into the sulphonium derivative this conversion must take place in the brain. The chemical conversions of these compounds *in vivo* are being studied at present.

When we consider why some compounds persist and not others, we can find a clear answer only in one instance. Me-E 600 is rapidly hydrolysed by A-esterase, which is present in serum (Aldridge, 1953*a*, *b*), and hence cannot be found in the blood 10 min. after injection. The sulphonium compounds do not persist *in vivo*, although they are fairly stable *in vitro*. They may be removed either by losing methyl groups to potential acceptors normally present in the body, with the formation of the much less toxic sulphides, or, in view of the minute quantities injected, they may be excreted rapidly in urine, removed by reaction with other esterases or irreversibly adsorbed on surfaces in the body. The sulphide and sulphoxide are probably converted into the sulphone. Apparently there is no biochemical system *in vivo* that is capable of degrading the sulphone rapidly. The relatively large amounts injected and conversions into still more toxic compounds (sulphone and possibly sulphonium compound) may also contribute to the persistence. Until much more is known of the factors involved, it will be impossible to predict the persistence of any given compound which is fairly stable in water; also there is no reason to believe that persistence *in vivo* is rare. Consequently, how long a compound persists must be found in every instance. Where it persists, the possibility of accumulation during repeated exposures, as well as the effect on the type of inhibition produced, must be borne in mind.

The comparison between persistent and non-persistent dimethyl phosphate esters shows that they produce different biochemical lesions after sublethal intravenous injection. Thus LD_{50} doses, although equilethal by definition, cannot be regarded as equitoxic. There is no doubt that the ability to tolerate a further dose would be different in surviving animals if in one case inhibition were rapidly reversed and in the other prolonged for days. When the toxicity of such compounds is compared, it is not enough to determine the LD_{50} dosages, but the persistence of the inhibitor in the body must be investigated. The duration of symptoms after a single sublethal intravenous injection of dimethyl phosphate esters gives some indication of the persistence of the inhibitor (see Table 1).

The non-persistent inhibitor, Me-E 600, can be made to act as a persistent inhibitor by slow infusion. When injected for several hours into the rat's jugular vein, conversion into the second irreversible stage of inhibition took place. A decrease in the rate of infusion was followed by a remarkable increase in tolerance of inhibitor, with a parallel increase in production of irreversibly inhibited enzyme. Oral and skin absorption in certain circumstances may be analogous to slow infusion. In such cases the enzyme inactivated will be mainly inhibited irreversibly, and the animal will be more susceptible to further exposure than after intravenous injection or administration via the lungs, where absorption is also very rapid. Thus although the LD_{50} is much higher when the compound is absorbed slowly, the biochemical lesion is more serious. This conclusion applies to all dimethyl phosphate inhibitors; prolonged exposure of any kind will lead to irreversible inhibition.

Any assumption that dimethyl phosphate esters always produce a transient effect, because the cholinesterase inhibited by them is rapidly reactivated, is not justified, for two reasons. Some persist. Others, although transient after single injection, produce the same durable lesion by prolonged aministration, for example by feeding. In fact the lesion produced is more permanent than that produced by a transient diethyl phosphate ester, as can be seen by comparing Figs. 1*d–f* with Fig. 1*c*. The inactivated enzyme in the first case is irreversibly inhibited, and gain in activity is probably achieved by synthesis of fresh enzyme; in the second case the enzyme is inactivated reversibly, although the reversal is slow.

We conclude that both the degree of the persistence of the inhibitor in the body and the rate at which the inhibitor is administered will decide to what extent inhibition by dimethyl phosphate esters *in vivo* will pass from the reversible to the irreversible stage.

SUMMARY

1. Marked differences in the duration of symptoms were observed when sublethal single doses of different dimethyl phosphate esters were injected intravenously into rats. The compounds inducing prolonged symptoms (*OO*-dimethyl *S*-ethylthioethyl phosphorothiolate, *OO*-dimethyl *S*-ethylsulphinylethyl phosphorothiolate and *OO*-dimethyl *S*-ethylsulphonylethyl phosphorothiolate) either persisted themselves, or produced an inhibitor which persisted for several hours in the blood. Those compounds inducing symptoms of short duration (*OO*-dimethyl *S*-ethylsulphonioethylmethyl phosphorothiolate and dimethyl *p*-nitrophenylphosphate) showed short persistence in the blood.

2. *In vivo* the rate of return of brain and erythrocyte cholinesterase activity differed markedly between the two groups of inhibitors. The persistent inhibitors produced irreversible inhibition of practically all of the inactivated enzyme, whereas most of the enzyme inhibited by the non-persistent inhibitors recovered its activity rapidly.

3. There was no distinction between the persistent and non-persistent inhibitors when tested *in vitro* for the return of cholinesterase activity by Aldridge's method. Return of activity took place at the same high rate in all instances, with a half-life of about 1·3 hr.

4. When the non-persistent inhibitor, Me-E 600, was made persistent by slow infusion for several hours into the rat's jugular vein, conversion into the second irreversible stage of inhibition took place. Decrease in the rate of infusion was followed by a remarkable increase in tolerance of inhibitor, with a parallel increase in production of irreversibly inhibited cholinesterase.

5. Because the conversion from the reversible into the irreversible stage occurs rapidly *in vivo*, it is concluded that both the persistence of the inhibitor and the route, or speed, or both, by which the dimethyl phosphate esters are administered will decide the extent to which this conversion will take place in the animal.

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Aspects of Vitamin A Deficiency in the Rat

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McCollum & Davis (1913) and Osborne & Mendel (1913*a*) independently proved that fat-soluble material was indispensable in the diet of the growing rat. Since that time great progress has been made in many aspects of the study of vitamin A, but only in respect of scotopic vision is much known about its mode of action. Research into the systemic mode of action is halted for want of a useful clue.

Lowe, Morton & Harrison (1953), using chromatography and ultraviolet-absorption spectroscopy, detected a new constituent in the unsaponifiable fraction from the livers of vitamin A-deficient rats. The substance, designated SC pending elucidation of its nature, is characterized by absorption maxima at 275 and 332 mμ, with sharp inflexions at 233 and 283 mμ (solvent, *cyclo*hexane).

If SC appears as a direct result of vitamin A deficiency its chemical nature should throw light on processes regulated by the vitamin. If, however, SC occurs as an indirect consequence of vitamin A deprivation its immediate relevance will be less obvious.

The present paper is concerned with comparing (qualitatively and quantitatively) the unsaponifiable fractions of certain tissues from vitamin A-deficient rats and from similar rats given an adequate supplement of vitamin A. (For a preliminary report, see Heaton, Lowe & Morton, 1955.) These experiments lead to conclusions which are, in a limited sense, quite clear-cut, but experiments on chicks and on adult hens and cockerels (Lowe, Morton, Cunningham & Vernon, 1957) showed that