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THE PART PLAYED BY INHIBITION OF CHOLINESTERASES OF THE CNS IN PRODUCING PARALYSIS IN CHICKENS

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Bidstrup and Hunter (1952) reported two cases of poisoning with *NN'*-di*iso*propylphosphorodiamidic fluoride (mipafox) which exhibited an unexpected symptomatic picture. Three people developed acute poisoning while engaged on a pilot plant manufacturing this substance. Recovery from the acute phase of the illness followed the administration of atropine in large doses, but in the three weeks after the onset of symptoms two of the patients developed paralysis of the limbs similar to that which follows triorthocresol phosphate (TOCP) poisoning. Smith and Lillie (1931) showed that this was accompanied by a demyelination of the nerve sheath. Petry (1951) has described similar effects after poisoning with *OO*-diethyl *O-p*-nitrophenyl phosphorothioate (parathion). Paralysis in man following intoxication with TOCP has been described frequently. Thus Lorot (1899) reported neuritis in tuberculous patients after treatment with "phosphocreosote." During prohibition in the United States, TOCP was used as an adulterant in "Ginger Jake," a drink then popular. As a result, several thousand cases of paralysis occurred (Smith, Elvove, and Frazier, 1930). Ter Braak (1931) and Sampson (1938) reported several others from the continent of Europe. In 1944, Hunter, Perry, and Evans described further cases in industries using TOCP as a "plasticizer."

The appearance of these effects after poisoning with the new organophosphorus insecticides has resulted in researches designed to determine the mechanism by which the paralysis is caused.

Three compounds—TOCP, DFP, and mipafox—will produce paralysis in chickens (Barnes and Denz, 1953; Earl and Thompson, 1952; and David, 1951). Koelle, Gilman, and Binzer (1946) administered DFP over a long period to cats and dogs with similar results. Bloch (1941), and Hottinger and Bloch (1943), had attempted to explain such effects in terms of the anticholinesterase properties of TOCP. They suggested that the inhibition of cholinesterase resulted in excessive concentrations of acetylcholine at the nerve endings and that this caused the paralysis.

More recently the effects of TOCP upon a number of widely different enzyme systems have been studied by Earl, Thompson, and Webster (1953). They examined glucose and pyruvate oxidation by brain, brain amine oxidase, brain cephalinase, pancreatic lecithinase, and trypsin. All these systems were insensitive to this compound. The tributyrinase activity of the spinal cord of hens poisoned with TOCP was moderately reduced. In every experiment, however, the pseudo-cholinesterase of the spinal cord was inhibited to a greater extent than was either the true cholinesterase of the tissue or the tributyrinase.

Both DFP and mipafox, in company with TOCP, are selective inhibitors of pseudo-cholinesterase. Thus all the available evidence suggested that paralysis might well be a consequence of inhibition of pseudo-cholinesterase. Earl and Thompson (1952), in discussing these results, suggested that paralysis may result from the inhibition and continued depression of the pseudo-cholinesterase of the spinal cord. Since these authors had at that time worked with only TOCP, it was desirable to examine this hypothesis further. We have, therefore, examined the possible paralysing effects of five powerful anticholinesterases: di*iso*propyl phosphorofluoridate (DFP), *iso*propyl methylphosphonofluoridate (sarin), ethyl *NN'*-dimethylphosphoroamidocyanidate (tabun), 3:3-dimethyl-*n*-butyl 2-methylphosphonofluoridate (soman), and *iso*propyl-ethylphosphonofluoridate (ethyl sarin).

We set out to answer two questions:

1. Would all five substances produce paralysis in chickens?

2. Could the cholinesterase changes be related to the possible ability of these substances to cause paralysis?

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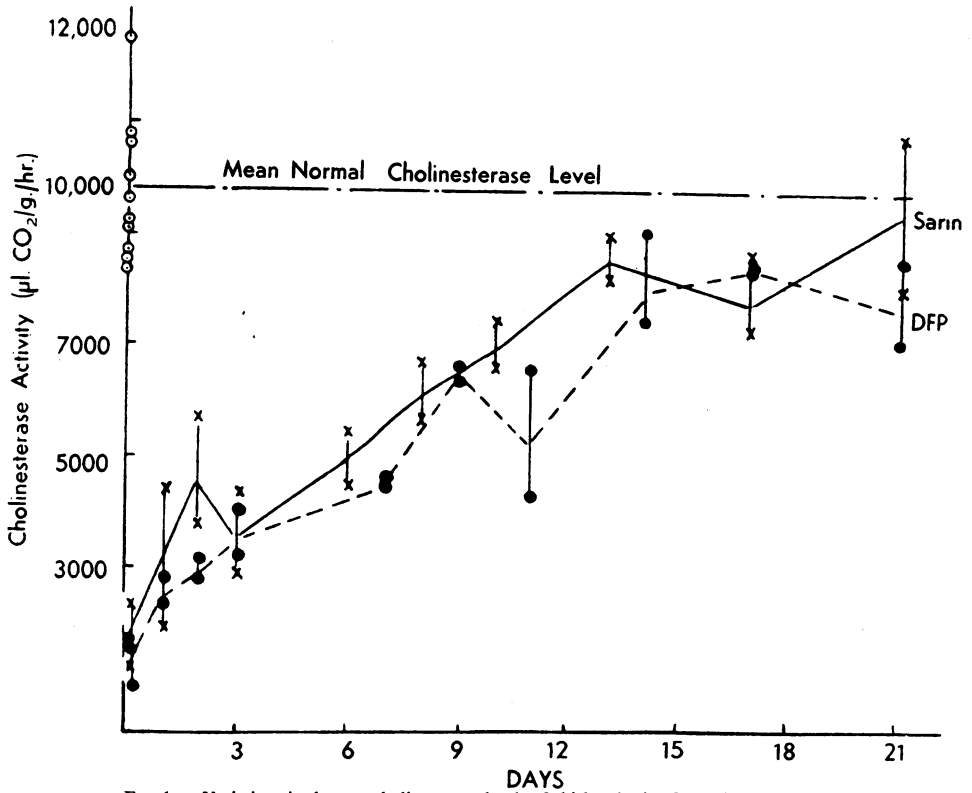

FIG. 1a.—Variations in the true cholinesterase levels of chicken brain after poisoning with DFP and sarin.

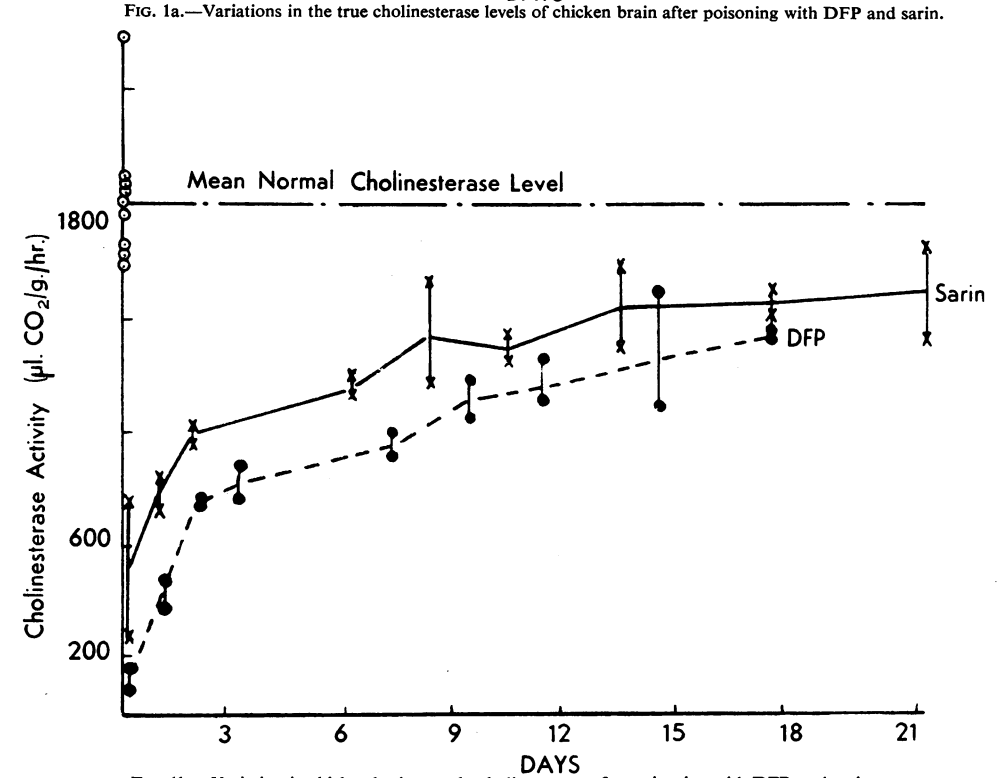

FIG. 1b.—Variation in chicken brain pseudo-cholinesterase after poisoning with DFP and sarin.

METHODS

Chickens were used, since several workers have described techniques for the production of paralysis in these with TOCP and DFP (Smith, Elvove, and Frazier, 1930; Smith and Lillie, 1931; Smith, Engel, and Stohlman, 1932; Earl and Thompson, 1952; and Barnes and Denz, 1953). Birds of mixed breeds weighing 1,800–3,500 g. were used.

The organophosphorus compounds were given subcutaneously in a 10^{-3} dilution in physiological saline. DFP, however, was used in a 1% w/v solution, in physiological saline containing 10% ethyl alcohol to aid solution.

The doses used were within the lethal range, so atropine sulphate (1 mg./kg.) was given subcutaneously immediately after each dose of the toxic substance.

Enzyme determinations were made upon the brain, spinal cord, and blood. After removal the brain and spinal cord were quickly homogenized in 0.25 M-sodium bicarbonate. For assay of the *true* enzyme of the brain 1 g. of tissue was homogenized with 150 ml. of the bicarbonate solution; for the *pseudo*-cholinesterase of the brain, and for both *true* and *pseudo*-cholinesterases of the spinal cord, 1 g. was homogenized with 25 ml. Blood was obtained from a wing vein and collected into dry heparin, the plasma separated off and diluted with 11.5 volumes of bicarbonate solution.

The activity of the enzymes was determined in the Warburg apparatus using acetyl-β-methylcholine chloride (0.03M) for the true enzyme and butyrylcholine iodide (0.03M) for the pseudo enzyme. The results are expressed as $\mu l.CO_2$ produced in 1 hr. by 1 g. tissue (or 1 ml. plasma).

RESULTS

Doses within the lethal range were used and, despite the administration of atropine, some deaths occurred.

With DFP, paralysis was produced regularly; immediately after dosing each bird exhibited signs of ACh poisoning which persisted for 24–48 hr. They recovered from this; but 10 to 14 days after dosing they showed signs of progressive weakness of the legs. The subsequent picture was identical with that described by Earl and Thompson (1952) after TOCP poisoning, and by Barnes and Denz (1953) after treatment with some insecticides. After the onset of paralysis the general health of the birds deteriorated. They lost weight rapidly and, about a week later, many showed cyanosis of the combs and gaping of the beak. At this stage many died. Histological examination showed evidence of demyelination of the ascending and descending tracts of the spinal cord.

In no chicken was there any sign of paralysis with sarin, tabun, soman, or ethyl sarin, although both large and small doses were repeated for several weeks. Sarin, for example, was administered twice a week for five weeks. Despite this long period there was no evidence of paralysis, although a number of the birds were observed for as long as three months. This was in marked contrast to DFP. The doses used were such that many of the birds died after two or three weekly injections.

Changes in the Cholinesterase Levels of Tissues and Plasma in Relation to the Onset of Paralysis

As indicated in the introduction, Earl and Thompson (1952) have suggested that paralysis might result from prolonged inhibition of pseudocholinesterase. Because of our failure to produce paralysis with the agents described, it was important to compare the changes in the true and pseudocholinesterases of the brain, spinal cord, and plasma in groups of chickens injected with DFP and sarin.

Normal levels for both the true and the pseudoenzymes of the brain and spinal cord were obtained by examining the tissues of a group of 10 normal birds. Mean values, together with the standard deviations of the individual values, are shown in Table I.

TABLE I

NORMAL LEVELS OF TRUE AND OF PSEUDO-CHOLINESTERASE IN CHICKEN BRAIN AND SPINAL CORD

(Mean values from 10 birds. The substrates were acetyl-β-methylcholine (0·03M) and butyrylcholine (0·03M). Cholinesterase activity is expressed as $\mu l.CO_2/g./hr.$)

	Brain		Spinal Cord	
	True	Pseudo	True	Pseudo
Mean ..	9,828	1,810	1,689	1,247
Range ..	8,400–12,600	1,600–2,400	1,360–2,280	1,020–1,520
Standard deviation	1,260	229	393	153

Ten chickens were injected with DFP and 10 with sarin. Each bird received atropine sulphate. They were then sacrificed in pairs, one pair from each group, the first pair being killed 2 hr. after dosing. The levels of enzyme activity at this time are shown in Table II. Further pairs were killed at varying times over a period of three weeks, as illustrated in Fig. 1a and Fig. 1b, which show the variations in enzyme levels.

Brain.—The true cholinesterase of the brain was depressed equally by the two agents to approximately 20% of the mean normal value.

The rate of recovery was similar in each group (Figs. 1a and 1b). The level reached 50% of

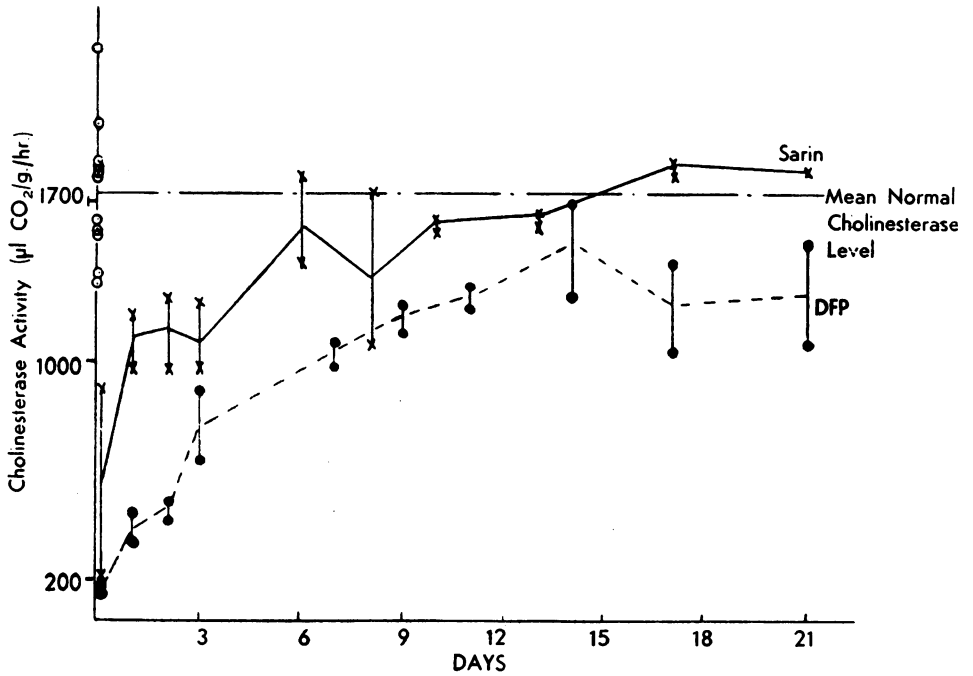

FIG. 2a.—The variation in true cholinesterase of chicken spinal cord after poisoning with DFP and sarin.

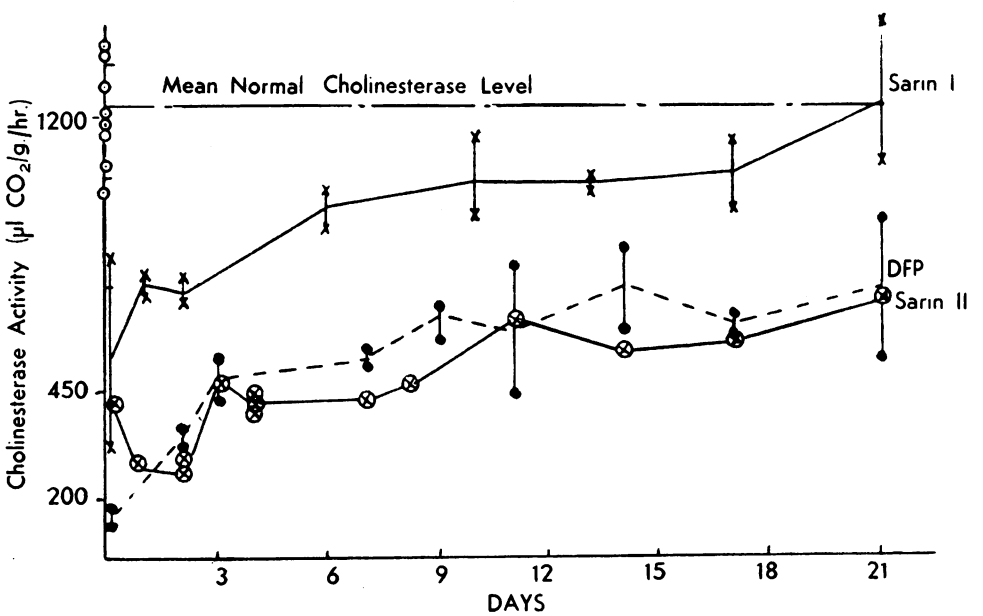

FIG. 2b.—The variation in pseudo-cholinesterase of chicken spinal cord after poisoning with DFP and sarin. Curves Sarin I and DFP show the rate of reappearance of enzyme after a single dose of each agent. Curve Sarin II shows corresponding changes after repeated dosing with smaller doses of sarin, to reduce cholinesterase to level comparable with that obtained with DFP.

TABLE II

CHOLINESTERASE ACTIVITY (% OF NORMAL) IN CHICKENS TWO HOURS AFTER POISONING WITH DFP AND SARIN

(Each estimate is the mean of observations upon four birds)

Tissue	True		Pseudo	
	DFP	Sarin	DFP	Sarin
Plasma ..	—	—	0	19
Brain ..	12·5	17·5	6	28
Spinal cord	6	33·5	10	47

normal in approximately a week and was back to within the lower normal limits in two to three weeks. The pseudo-cholinesterase of the brain was inhibited rather more strongly by DFP than by sarin.

The pseudo-cholinesterase of the DFP-treated birds was reduced to 5% or normal, whereas with sarin 25% activity remained. Rates of recovery were again similar. There was a rapid initial rise to about the 50% level, then the recovery rate slowed down, and even at the end of three weeks the enzyme level was not back within the normal range.

Spinal Cord.—Neither the true nor the pseudo-cholinesterases of the spinal cord was inhibited by sarin to the same extent as with DFP (Fig. 2). The true esterase was inhibited by sarin to only 30%, whereas with DFP it was reduced to 8%. The pseudo enzyme was reduced to 40% by sarin and to 10% by DFP. Again, the rates of recovery were essentially the same as for the true enzyme, the levels returning to within normal limits in approximately a fortnight after both agents. The level of the pseudo-cholinesterase in the DFP-treated birds was, however, substantially below normal for the whole three weeks of the experiment; this was in marked contrast to the sarin-treated birds, in which the enzyme reached normal limits in approximately a week after dosing. In view of the suggestions of Earl and Thompson (1952), this observation was felt to be significant. It was therefore decided to carry out another experiment in which the pseudo esterase of the spinal cord would be reduced to less than 20% and kept at a level comparable with that found in the DFP-treated birds of the previous experiment.

The essential problem was the administration of sufficient sarin to depress the enzyme to less than 20% of normal, to maintain it at or near this level for a prolonged period, and still keep the bird alive. If sarin is given in a series of appropriately spaced small doses, the animal can tolerate considerably more than if it were given in a single large dose (Berry and Davies, 1951). By giving sarin in this way the necessary degree of inhibition of the pseudo-cholinesterase of the spinal cord was obtained.

Birds were killed at intervals for the assay of the tissue enzyme levels. The remainder were then kept under observation for a further three weeks. The changes in the spinal cord pseudo-cholinesterase are shown in Fig. 2, from which it can be seen that the enzyme level in this group of birds was: (i) reduced to a level comparable with that which obtained in the DFP group of the previous experiment, and (ii) was maintained at a low level

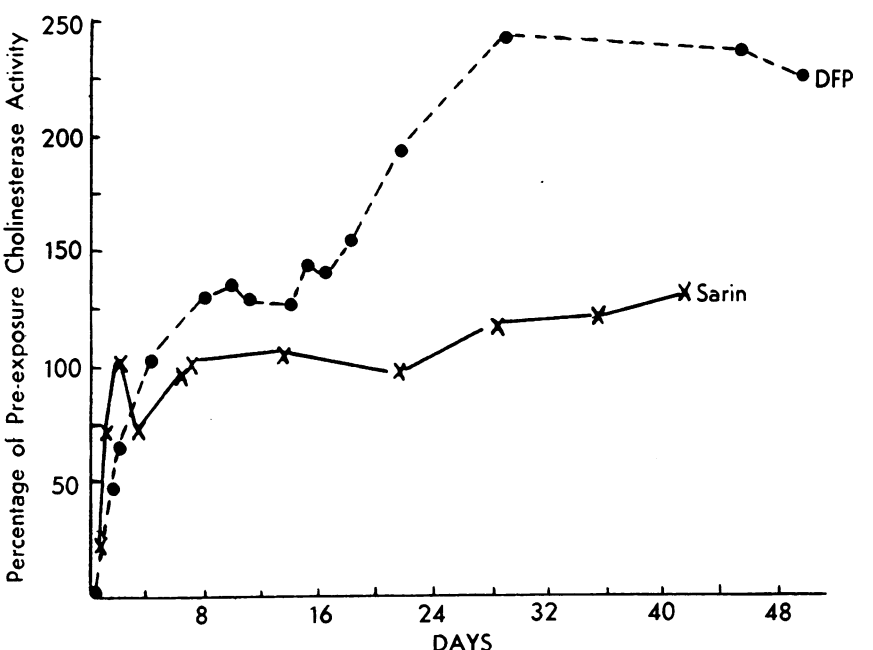

FIG. 3.—Typical recovery curves of chicken plasma cholinesterase, after treatment with DFP and sarin. Note the early recovery and the subsequent slow and prolonged rise above normal after DFP. A similar but much less marked rise occurs after sarin.

for an equally long time. There were no signs of paralysis in this group.

Variations in the Level of Plasma Cholinesterase in Fowls After Dosing

Observations were also made upon variations in the levels of plasma esterase. With the doses used, the enzyme was substantially inhibited. With DFP all the enzyme was destroyed, but with sarin there was only 85% inhibition.

The rate of return of the plasma esterase to pre-exposure levels was rapid with all the agents. Complete recovery took only 2–5 days, but the plasma activity increased substantially beyond normal in the succeeding three weeks, and remained at this level for a further 30 days (Fig. 3). Thus, with DFP, there was a mean increase of 150% above the normal (5 birds); with the other agents there was a 20–50% increase. The possibility that this increased activity, since it was measured against butyrylcholine, was due to another type of cholinesterase—a butyrylcholinesterase which did not hydrolyse acetylcholine—has been considered. This type of phenomenon has been noticed by Ellis (1947), who showed that guinea-pig kidney contains a benzoylcholinesterase which does not hydrolyse acetylcholine. He showed that this enzyme also occurs in some, but not all, rabbits, and that even in one rabbit its occurrence varies from time to time (Ellis, 1947).

The nature of the plasma cholinesterase was therefore investigated both before and one week after dosing. At the latter time it was significantly above the pre-exposure value. Substrate specificity patterns against acetylcholine, acetyl-β-methylcholine, benzoylcholine, butyrylcholine, and propionylcholine were determined. There were no differences. The behaviour of selective inhibitors was also examined. Austin and Berry (1953) described two inhibitors which were highly selective towards each enzyme: 1:5 bis (4 allyldimethylammonium phenyl) pentan-3-one dibromide (284C51, Wellcome), a reversible inhibitor highly selective for the true enzyme, and *NN'di*isopropylphosphorodiamidic anhydride (DPDA), an irreversible inhibitor which is highly selective for the pseudo enzyme. Tests with these two inhibitors were carried out on the plasma before and after exposure. No differences were seen. Thus it is highly probable that this increased activity is due to an increase in concentration of the normal plasma enzyme.

Discussion

The purpose of this investigation has been twofold. Firstly, to find whether all anticholinesterases cause paralysis under laboratory conditions; and secondly, to observe the changes in the true and the pseudo-cholinesterases of brain, spinal cord, and blood in chickens. The results have been clear-cut. Chickens were used because they had been shown to be particularly susceptible to the paralysing action of TOCP. Furthermore, Earl and Thompson (1952), Barnes and Denz (1953) and David (1951) had already shown with DFP, TOCP, and mipafox that paralysis and demyelination could be produced relatively easily in this species. We have confirmed this repeatedly with DFP. We have, however, been unable to produce paralysis with tabun, sarin, soman, or ethyl sarin, not only with large single doses but also with repeated large doses at weekly and twice weekly intervals over periods varying from two to nine weeks. No other effects which could be interpreted as resulting from demyelination developed. This aspect of the investigation may thus be summarized as follows: in a particularly susceptible species, it has not been possible to produce paralysis with any of the substances examined except DFP.

Davison (1953) has come to the same conclusion. He studied a number of organophosphorus compounds which were all selective inhibitors of pseudo-cholinesterase; recovery from the effects was rapid—and had a similar pattern—whether paralysis was produced or not.

Reference has been made in the introduction to the ideas of Bloch (1941), Hottenger and Bloch (1943), and Earl and Thompson (1952) upon the possible significance of cholinesterase inhibition in relation to the aetiology of demyelination. Our results indicate that the inhibition of neither true nor pseudo-cholinesterase of chicken brain or spinal cord can be the direct causative factor, since with only one—DFP—of five powerful anticholinesterases was it possible to produce paralysis. In a carefully designed experiment, illustrated in Fig. 2, similar enzyme changes were brought about by DFP and sarin; although paralysis developed in the DFP-treated birds, there was none in the sarin group. Thus, whatever the cause of paralysis, it is unlikely that the inhibition and continued depression of the pseudo-cholinesterase of the brain or spinal cord plays a direct part in its development.

The plasma enzyme changes are interesting. The esterase activity returns to pre-exposure levels in 2–5 days. This is in marked contrast to the rate of recovery after TOCP in chickens, and mipafox in man. With TOCP it took at least three weeks to return to within normal limits (Earl and Thompson, 1952). In poisoning with mipafox in man the return to normal levels was significantly delayed (Callaway, Davies, and Risley, 1952).

An interesting observation is the post-intoxication increase of plasma esterase to levels very significantly higher than normal. This was particularly marked following DFP. Koelle and Gilman (1946) and Locker and Siedek (1952) have reported a significant increase in blood and tissue cholinesterase over pre-exposure levels after dosing with anticholinesterases. Koelle and Gilman also noted this in rats, but did not comment on it. Locker and Siedek, however, have made a separate study of the phenomenon, following the administration to guinea-pigs and rats of *NNN'N'*-tetramethylphosphorodiamidic anhydride (Pestox III), *NNN'N'*-tetramethylphosphorodiamidic fluoride (Pestox XIV), parathion, and mipafox. They found a rapid increase of 40–60% in the cholinesterase activity of plasma, erythrocytes, liver, and brain. The increase was more marked with small doses and occurred comparatively rapidly—in 1–3 days after dosing. They suggested that these effects might be due to a different distribution of the alkylphosphates in the body and to a stimulatory effect of low concentrations upon the formation or activity of the enzymes.

The observations here reported with chickens appear to differ from those in the literature in at least three respects. Firstly, the rise after DFP is very much greater than that reported by Locker and Siedek. Secondly, in guinea-pigs intoxicated with insecticides, the increase occurred within 1–3 days. In our experiments the rise was progressive up to three weeks, and the higher level of plasma cholinesterase was maintained in some birds for at least another month. Thirdly, Locker and Siedek suggest that the effect is due to a stimulatory effect of low concentrations of the anticholinesterases. We used only one dose level of DFP, but this was well within the lethal range. The response to other phosphorus compounds was much less than that to DFP. Thus it may be that this stimulation of esterase production or activity is dependent upon the agent initially used to inhibit the enzyme.

It is interesting to recall in this connexion that Hollinger, Rossiter, and Upmalis (1952), and Hollinger and Rossiter (1952), have found considerable increases in acid-phosphomonoesterases, 5-nucleotidase, and glucuronidase in the cat's sciatic nerve after nerve section. These changes reached their peak in the same time that maximal effects were observed in our experiments with cholinesterase, and over the same interval that it took for the toxic effects to develop.

Summary

1. Five potent anticholinesterases, DFP, sarin, tabun, soman, and ethyl sarin, have been examined for their chronic effects in chickens.

2. Paralysis was produced by DFP only.

3. It is improbable that paralysis following DFP is due directly to the inhibition and prolonged depression of either the true or the pseudocholinesterase of CNS or blood.

4. The plasma cholinesterase was markedly inhibited after poisoning with each of the compounds. The rate of return to normal levels was always rapid (3–5 days). The cholinesterase level of the plasma continued to rise to levels substantially higher than normal; this was particularly striking with DFP.

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