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RELATIONSHIP BETWEEN DEPRESSION OF BRAIN OR PLASMA CHOLINESTERASE AND PARALYSIS IN CHICKENS CAUSED BY CERTAIN ORGANIC PHOSPHORUS COMPOUNDS*

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Abstract—A study has been made of the relationship between the inhibition of plasma or brain cholinesterase of the chicken and the paralytic syndromes caused by DFP, TOCP, malathion, ronnel, EPN, and Trithion. DDVP and Dipterex served as non-paralytic controls. TOCP was the only compound tested that was a specific inhibitor of brain cholinesterase. All compounds except EPN inhibited plasma cholinesterase. All compounds tested inhibited brain true and/or pseudocholinesterase. All paralytic compounds tested except DFP produced a prolonged inhibition of brain true and/or pseudocholinesterase which lasted for 5 to 17 days. It was concluded that no causative relationship exists between the prolonged inhibition of brain cholinesterase and the paralytic syndromes. The regeneration of brain cholinesterase after inhibition with DFP, Dipterex, or DDVP is probably due to protein synthesis *de novo*; the prolonged inhibition of these enzymes after the administration of the other compounds may be due in part to the persistence of the inhibitor in the body.

THE PARALYTIC effect of certain organic phosphorus compounds on chickens has been used as an index of possible paralytic action in man, since TOCP[1, 2] and mipafox,[3, 4] which produce paralysis in man, were found to produce a similar syndrome in chickens. As far as we are aware, no clear-cut cases of paralysis in man have been reported in connection with other paralytic compounds, even though human cases of anticholinesterase poisoning by DFP, malathion, and Trithion are known. It is true that there have been reports of temporary paralysis in five people exposed to malathion,[5–8] but the cases varied widely among themselves and occurred in people with little exposure rather than in people with massive exposure, including those poisoned by ingesting the compound.

The paralysis produced in chickens with malathion and some other organic phosphorus derivatives differs from that noted with TOCP, mipafox, or DFP in time of onset after dosing and/or in duration.[9] The most outstanding difference between the two groups of paralytic compounds is that the syndrome does not appear until about 10 to 14 days after dosing with DFP, TOCP, or mipafox,[9–14] whereas with EPN and malathion, the syndrome usually develops within 1 or 2 days.[9]

A number of investigations have been made on the effects of the neurotoxic organic phosphorus compounds on the brain and plasma cholinesterase of the chicken, but these have been restricted to TOCP,[11, 13–17] its metabolites[17] and similar aromatic

* See Table 1 for chemical names of compounds and abbreviations.

phosphates,[18] to mipafox,[15, 16] and to DFP.[15, 16] In these studies no definite correlation was found between inhibition of cholinesterase and paralytic effects, although each compound inhibited plasma or brain pseudocholinesterase *in vivo*.

However, in view of the two kinds of paralytic syndrome, it seemed worthwhile to study the effects on brain and plasma cholinesterase activity of compounds with immediate, temporary neurotoxicity in an attempt to determine whether the effects of these substances differ from the effects of TOCP, mipafox, DFP, or from nonparalytic compounds. In connection with this study, preliminary tests were conducted on the neurotoxicity of some organic phosphorus compounds not previously reported, including Dipterex, ronnel, and Trithion.

In these experiments, EPN, malathion, ronnel, and Trithion were studied as representative of the compounds that may produce paralysis within 1 or 2 days after dosing. TOCP and DFP were investigated as representative of those compounds that produce paralysis after a delay of 10 to 14 days. The studies with the latter two compounds permitted direct comparison of the results of the present investigation with those of other workers. Dipterex and DDVP were the two nonparalytic compounds investigated.

TABLE 1. COMMON AND CHEMICAL NAMES, GRADE, AND SOURCE OF SUPPLY OF COMPOUNDS TESTED*

Common name	Chemical name	Purity and grade	Source of supply
DDVP	O,O-dimethyl 2,2-dichlorovinyl phosphate	98% Refined	Montrose Chemical Co.
DFP	Diisopropyl fluorophosphonate	99%	Aldrich Chemical Co.
Dipterex (Dylox)	Dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate	50%	Chemagro Corp.
EPN	O-ethyl O-*p*-nitrophenyl phenylphosphonothioate	Technical	E. I. DuPont Chemical Co.
malathion	O,O-dimethyl S-(1,2-dicarbethoxyethyl) dithiophosphate	Refined Practical 98%	American Cyanamid Co.
ronnel	O,O-dimethyl O-2,4,5-trichlorophenyl phosphorothioate	100%	Dow Chemical Co.
TOCP	Triorthocresol phosphate	Practical	Eastman Kodak Co.
Trithion	O,O-diethyl *p*-chlorophenylmercaptomethyl dithiophosphate	95% Technical	Stauffer Chemical Co.
	Atropine sulfate		Merck and Co.
	Acetyl β-methyl choline bromide		K and K Laboratories
	Butyrylcholine chloride		K and K Laboratories
2-PAM iodide	Pyridine-2-aldoxime methiodide		Chemical Procurement Co.

* The use of trade names is for identification only and does not constitute endorsement of these products by the Public Health Service.

METHODS

The chemical names, grade or purity, and source of supply of the compounds used in these studies are shown in Table 1.

Butyrylcholine perchlorate was prepared by a modification of the method of Bell and Carr[19] from butyrylcholine chloride, and was recrystallized twice from cold ethanol. All the salts of choline esters, with the exception of butyrylcholine chloride, evolved the theoretical amount of CO_2 when hydrolyzed in the Warburg apparatus in

bicarbonate buffer with brain homogenate as described in the next section. All inorganic chemicals were of a reagent or analytical grade.

The procedure for carrying out the preliminary tests of the neurotoxicity of Dipterex, ronnel, and Trithion was essentially the same as that described by Durham *et al.*[9]

The chickens used were females of a sex-linked strain and weighed 2·5 to 3·0 kg. They were dosed subcutaneously on the right side between the thigh and the wing with the organic phosphorus compound in peanut oil, or with peanut oil alone if in control groups, after oral administration of atropine 15 to 20 min previously at a level of 15 mg/kg. The single exception was that Dipterex, because of its poor suspensibility in peanut oil, was given as an aqueous solution. Atropine was given in order to minimize the acute effects of the toxicant. The animals were observed for symptoms and each day tested for their ability to stand or walk on bare ground as previosuly described.[9] At various intervals, individual animals used in the study of cholinesterase inhibition were bled with heparinized syringes from the wing vein and then sacrificed by decapitation.

Twenty per cent (w/v) brain homogenates were prepared from whole chicken brains in ice-cold 0·15 M sodium chloride with an ice-cold motor-driven glass homogenizer of the type described by Dounce and Beyer.[20]

Cholinesterases were assayed in plasma or homogenates of brain by the Warburg manometric procedure in bicarbonate carbonic acid buffer under conditions similar to those described by Earl and Thompson.[21] The reaction mixture for the assay of plasma cholinesterase contained 0·035 M butyrylcholine. The plasma cholinesterase was corrected for the nonenzymatic hydrolysis of butyrylcholine (0·6 μmoles/30 min at 37°). Brain pseudocholinesterase was measured at 25° with 0·035 M butyrylcholine, and brain true cholinesterase was estimated with 0·040 M acetyl-β-methyl choline at 25°.

TABLE 2. THE NEUROTOXIC EFFECT OF ORGANIC PHOSPHORUS COMPOUNDS GIVEN SUBCUTANEOUSLY IN PEANUT OIL TO ATROPINIZED FEMALE CHICKENS

Compound	Dosage range tested (mg/kg)	Lowest dosage producing		Duration of paralysis (days)	Approximate subcutaneous LD_{50} (mg/kg)
		Cholinergic signs (mg/kg)	Paralysis (mg/kg)		
Dipterex	50–800	50			125*
ronnel	100–1,600	1,600	1,600†	4–24	> 1,600
Trithion	10–640	640	640	15–49‡	640

* Dosage levels of 200 mg/kg or greater killed all the chickens tested.
† Paralysis in 5 of 8 chickens tested at this dose, but none died.
‡ All 12 chickens dosed at this level developed paralysis. It was delayed 2 days in 4, 3 days in 1, 4 days in 3, and 7 days in 1 chicken(s).

RESULTS AND DISCUSSION

The results of basic studies establishing the neurotoxicity of Dipterex, ronnel, and Trithion in atropinized hens are shown in Table 2. The results for Trithion confirm the work of Edson, as reported by Hearn.[22] The results of similar studies carried out with DDVP, EPN, malathion, and TOCP were reported previously.[9]

Summaries of the symptoms produced in the chickens by the compounds, and of the changes in cholinesterase activities after dosing, are given in Tables 3 and 4. The effect

TABLE 3. PARALYTIC SYNDROMES FOLLOWING THE ADMINISTRATION OF CERTAIN ORGANIC PHOSPHORUS COMPOUNDS TO ATROPINIZED CHICKENS

The syndrome of immediate paralysis is defined as a muscle weakness that lasts 3 or more days so that the leg weakness noted 1 or 2 days after dosing with Dipterex, DDVP, and DFP is not considered to be the syndrome.[9]

The data in this table are summarized from the results of 5 separate experiments, each of which had its own control group and in which chickens were sacrificed for cholinesterase assay. The mortality data in this table include only those chickens that died spontaneously. The determination of the length of time the paralytic syndromes lasted is based on those chickens sacrificed last.

Compounds	Weight loss	Paralytic syndrome
Paralytic compound (delayed paralysis)		
TOCP 400 mg/kg	Large	No apparent symptoms until 12th day; no chickens died; then paralysis, starvation, weight loss
DFP 0·9 mg/kg	None	Six of 10 chickens died in 1 or 2 days; the others showed cholinergic symptoms and muscle weakness 1 or 2 days, recovery, and then developed paralysis on the 11th day after dosing
Paralytic compound (immediate paralysis)		
malathion 1,000 mg/kg	Moderate	Paralysis from 1st to 9th day; 5 of 16 chickens died 1 or 2 days after dosing; diarrhea for 3 to 4 days after dosing
EPN 60 mg/kg	Very large	Paralysis from 1st day to end of experiment; 4 of 13 chickens died between 11th and 29th day; ate very little
ronnel 1,600 mg/kg	Moderate	Paralysis from 1st or 2nd day to 7th day; no chickens died
Trithion 640 mg/kg	Large	Paralysis from 1st or 2nd day that lasted 17 days; no chickens died; ate very little; some diarrhea
Nonparalytic compound		
DDVP 18 mg/kg	Small	Paralysis for 1 or 2 days after dosing; 4 of 13 chickens died
Dipterex 90 mg/kg	Small	Paralysis for 1 or 2 days after dosing; 1 of 8 chickens died

TABLE 4. A COMPARISON OF THE CHOLINESTERASE LEVELS, 4 TO 16 DAYS AFTER DOSING, IN CHICKENS GIVEN THE NONPARALYTIC COMPOUNDS WITH LEVELS IN THOSE GIVEN THE PARALYTIC COMPOUNDS

The data are expressed as per cent of the average cholinesterase levels of the control chickens and are derived from the data in Figs. 1 through 3

Compound	No. of chickens	Days after dose	Per cent of control cholinesterase* Plasma Range	Plasma Mean	Brain true Range	Brain true Mean	Brain pseudo Range	Brain pseudo Mean
Nonparalytic compounds								
DDVP	4	4–14	64–80	74†	49–71	58	37–73	53
Dipterex	3	7–15	60–78	66	56–76	68	53–63	57
Paralytic compounds								
DFP	3	4–15			51–81	61	51–70	59
TOCP	6	5–16	24–46	32	55–78	69	25–39	33
malathion	5	4–14	80–107	95†	18–39	29	56–70	66
EPN	4	4–14	74–158	126†	9–22	16	41–77	65
ronnel	3	7–15	40–61	52	18–41	29	34–54	46
Trithion	3	7–15	15–26	20	9–18	13	19–33	27

* The number of control animals was equal to the number of experimental animals listed for each compound.

† Not statistically significant. Other values significant at 95% level or higher.

on brain and plasma cholinesterase (expressed as per cent of normal) for the non-paralytic compounds, for those that produced the delayed syndrome, and for those that caused paralysis without delay are summarized in Figs. 1, 2, and 3, respectively.

In confirmation of previous work it was found that DFP [15, 16] and TOCP[11, 14–16] produced paralysis 11 to 12 days after dosing, whereas a neurotoxic effect was noted within a short time after dosing with malathion and EPN.[9] No paralysis occurred after DDVP[9] was administered. The preliminary studies summarized in Table 2 were also confirmed in the separate tests to study cholinesterase activity. No correlation between structure of compound and paralytic effect was detected.

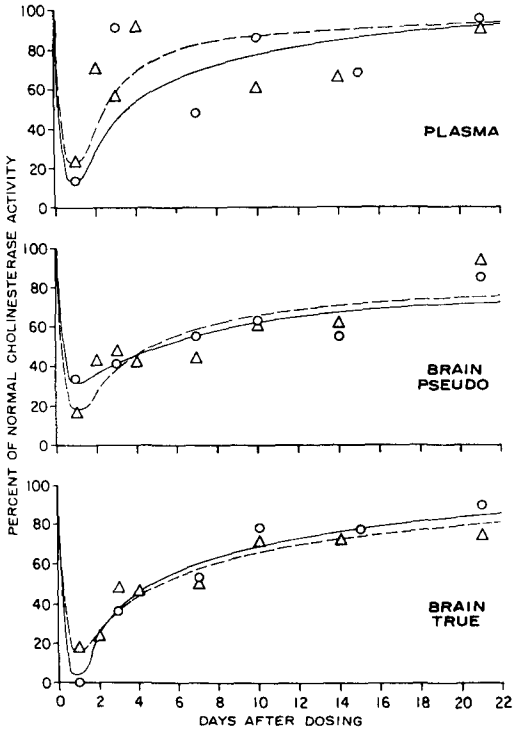

FIG. 1. Effect of DDVP (△) and Dipterex (○), nonparalytic compounds, on the plasma and brain cholinesterases of the chicken. The results are expressed as percentage of the cholinesterase activity found in a control chicken killed on the same day. Each point is calculated on the basis of one chicken in the experimental and control groups respectively.

As shown in Tables 3 and 4 and Figs. 1 through 3, all the organic phosphorus compounds tested in this report inhibited chicken plasma cholinesterase *in vivo* with the exception of EPN, a paralytic compound.

The effects of DFP on this enzyme were not investigated in the present studies, but Davison[15, 16] has shown that this organic phosphorus derivative does indeed inhibit this enzyme. The present results do not confirm the general rule that paralytic compounds inhibit plasma cholinesterase.[11, 13, 14, 16] Furthermore, no correlation was found between the degree or duration of the inhibition of this enzyme and the neurotoxicity of the compounds. The amount of inhibition of the plasma cholinesterase

and its rate of recovery did not necessarily parallel effects noted with the brain enzymes.

All the organic phosphorus compounds tested inhibited brain true and/or pseudocholinesterase. In confirmation of previous reports,[13, 17, 23] TOCP was found to be a more or less specific inhibitor of brain pseudocholinesterase, as shown in Table 4 and Fig. 2. However, this was not true with the other compounds because the level of brain true cholinesterase also was decreased to 10–20 per cent of the control level. The results obtained with DFP agree with those published previously.[15, 16] Thus it appears that specific inhibition of brain pseudocholinesterase is not characteristic of the paralytic compounds whether they are of the type that produces an immediate paralysis or a paralysis after about 10 to 14 days.

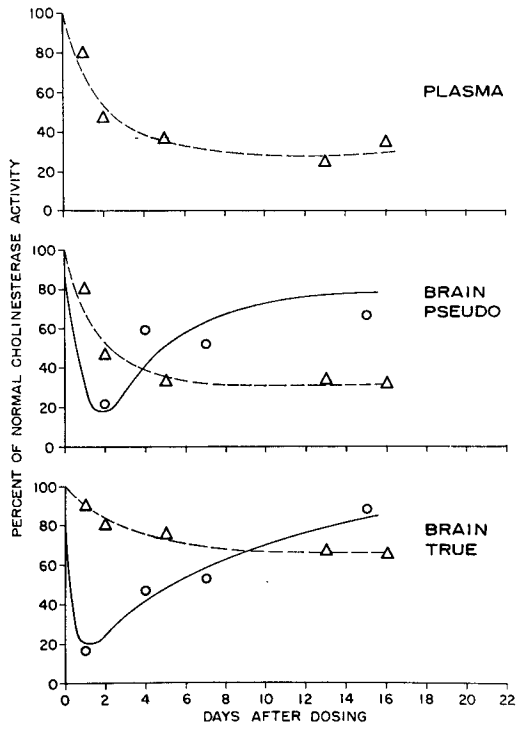

FIG. 2. Effects of DFP (○) and TOCP (△), which produced delayed paralytic syndrome, on the plasma and brain cholinesterases of the chicken. The results are expressed as percentage of the cholinesterase activity in control chickens killed on the same day as the poisoned chicken or chickens. One chicken in each group was sacrificed to obtain the data for the points in the curve for DFP. The average obtained from the enzyme activities of two chickens in each group was used to calculate the points in the curve for TOCP.

Although all the compounds tested inhibited brain true and/or pseudocholinesterase, the duration of the inhibition was different with each class of compounds tested, as is shown in Table 4 and Figs. 1 through 3. With the nonparalytic compounds, DDVP and Dipterex, a rapid recovery of inhibited brain cholinesterases took place (Fig. 1). However, in the case of the second group of organic phosphorus derivatives—those that produce paralysis after a delay of 10 to 14 days (Fig. 2)—it was found that with DFP a rapid recovery of the inhibited brain true or pseudocholinesterase occurred,

whereas with TOCP a prolonged depression of brain pseudocholinesterase was observed. These results with DFP and TOCP confirm previous work.[10, 11, 13–18] On the other hand, a prolonged depression of brain true and/or pseudocholinesterase was noted with each of the compounds (ronnel, malathion, EPN, and Trithion were

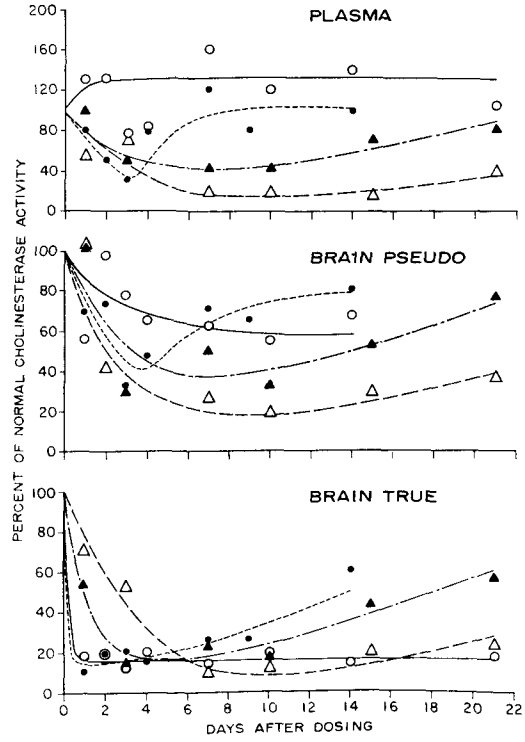

FIG. 3. Effect of malathion (●), EPN (○), Trithion (△), and ronnel (▲), which produce immediate paralytic condition, on plasma and brain cholinesterases of the chicken. The results are expressed as the percentage of the cholinesterase activity in a control chicken killed on the same day. Each point is calculated on the basis of one chicken in the control and experimental groups respectively.

tested) of the third group—those that produced paralysis without a prolonged delay.

The chickens given either EPN or Trithion refused food after dosing. Starvation has been shown to reduce the content of enzymes in the tissues under certain conditions.[24, 25] However, control experiments reported in Table 5, in which chickens were starved for as long as 12 days, show that there was no drop in the levels of the brain or plasma cholinesterases and that the animals did not develop characteristic muscle weakness. Therefore, the results obtained in the present experiment were not a result of starvation.

It also seems unlikely that the prolonged inhibition of cholinesterase found in the present experiments was an artifact caused by the reaction of previously separated enzyme and inhibitor during the process of homogenization of the brain. The most pronounced difference in levels of cholinesterase found between various compounds was obtained in those assays run 5 to 20 days after dosing, at which time any tissue level of inhibitor, if present, should be approaching a minimum. In addition, separate experiments in which malathion and DDVP were used as model compounds indicated

that homogenization artifacts did not occur. No inhibition of the brain cholinesterase in unpoisoned chickens was noted when homogenates of these brains were assayed in the presence of homogenates of the brains of chickens poinsoned 18 hr previously with malathion. Since maximal inhibition of brain cholinesterase was observed at this time with these two compounds, the conditions were most favorable for the presence of an excess of inhibitor. Hobbiger[26] and Seume *et al.*[27] also failed to obtain evidence of homogenization artifacts.

TABLE 5. EFFECTS OF STARVATION ON BRAIN AND PLASMA CHOLINESTERASES OF THE CHICKEN

Condition	No. of chickens	Brain true cholinesterase Range μmoles/g brain	Brain true cholinesterase Average ± S.D.	Brain pseudo-cholinesterase Range μmoles/g brain	Brain pseudo-cholinesterase Average ± S.D.	Plasma cholinesterase Range μmoles 0·3 ml	Plasma cholinesterase Average ± S.D.
Starvation 8 to 12 days	3	163–176	174 ± 13	20–21	21 ± 0·71	6·4–9·9	8·5 ± 1·8
Normal	4	153–173	161 ± 9	19–23	21 ± 2·1	5·5–10·0	7·3 ± 2·0

No causative relationship exists between the delayed paralytic syndrome and the depression of brain cholinesterase since one of the compounds, DFP, that produces this syndrome did not cause a prolonged depression of this enzyme. On the other hand, a prolonged depression of brain true or pseudocholinesterase appears to be associated in some way with the syndrome of muscle weakness brought about by the compounds that produce paralysis without prolonged delay after dosing. In this case also, a cause-and-effect relationship apparently does not exist between the paralytic syndrome and the inhibition of brain cholinesterases. For example, the chickens sacrificed on the 15th or 21st day after the administration of ronnel, or the 14th day after administration of malathion, had recovered from the muscle weakness after 8 and 6 days, respectively, and yet the activity of brain cholinesterase was only 40 per cent of normal.

The results obtained in these studies with a variety of organic phosphorus compounds are in agreement with, and extend to other compounds, the conclusion reached in earlier studies with DFP, TOCP, and mipafox,[15, 16] that there is no causative relationship between the depression of the activities of brain cholinesterases and paralysis.

Vandekar and Heath[28] have shown that differences in rates of reactivation of cholinesterase can arise *in vitro* between compounds that form the same dialkyl phosphorylated enzyme if one compound persists longer than the other in the body. Their studies, together with those of Blaber and Creasey,[29] have shown that the rate of regeneration of cholinesterase *in vivo* depends on several competing reactions. The two that promote reactivation are: (1) spontaneous reactivation of the enzyme by hydrolysis and (2) synthesis *de novo* of new cholinesterase molecules. The two that oppose reactivation are: (1) persistence of the inhibitor in the body, causing re-inhibition of any reactivated enzyme, and (2) conversion of the inhibited form of the enzyme to a type that cannot be reactivated spontaneously or with oximes.

The comparatively rapid recovery of brain cholinesterase in chickens given DFP, DDVP, or Dipterex can be explained on the basis of the above theories. First of all, a linear plot of the logarithm of per cent inhibition of brain true cholinesterase,

calculated from data in Figs. 1 and 2 against time after dosing, showed that the half-time for the regeneration of this cholinesterase was 6 to 7 days with DDVP, Dipterex, or DFP. A similar plot for brain pseudocholinesterase revealed that the half-time of regeneration of this cholinesterase is 13 to 15 days for the same three organic phosphorus compounds. These half-times for the regeneration of brain cholinesterases are of the same order of magnitude as those calculated for the overall synthesis of brain proteins in other species.[30–32] Furthermore, the rate of synthesis varies with the cytological localization of individual protein and probably with the individual protein in the brain.[32, 33] Also, the regeneration of brain cholinesterases after inhibition with DFP *in vivo* has been considered the result of synthesis of new protein.[29, 34] Secondly, the ability of true cholinesterase in brain homogenates to be reactivated with 2-PAM, which is considered a measure of the reactivatable enzyme,[35–37] was completely gone 18 hr after dosing with DDVP.[41] Dipterex should form the same dimethyl phosphorylated enzyme as DDVP since both are dimethyl compounds. Therefore, the inhibited enzyme probably was in the irreversibly inhibited form before appreciable recovery of activity began in the brains of chickens given Dipterex. The same thing undoubtedly is true of the chickens given DFP since the half-time for the formation of the irreversible form of the diisopropyl phosphorylated cholinesterase is 4 hr.[26] These results suggest that at the dosages of DFP, Dipterex, or DDVP administered, brain cholinesterase was kept from reactivating by continued reinhibition with active inhibitor until all the inhibited enzyme had been converted to the unreactivatable form. Then regeneration of cholinesterase activity occurred by synthesis of new enzyme. It seems probable that what little spontaneous reactivation may have taken place was complete before the first test was made 1 day after dosing.

The rates of regeneration of brain true cholinesterase following dosing with those compounds that produced a prolonged inhibition of brain cholinesterase are more difficult to explain on the basis of the theories of Vandekar and Heath[28] and Blaber and Creasey[29] since it is necessary to indicate why protein synthesis did not regenerate the enzyme. In the case of the dimethyl compound, malathion, studies of the reactivation of brain homogenates with 2-PAM[41] showed that the enzyme was in the irreversible form 18 hr after dosing. Hobbiger[26] has shown that the half-time *in vivo* for the formation of the irreversible form of the diethyl phosphorylated brain cholinesterase is 36 hr. This is the alkyl phosphorylated enzyme that would be produced by the other compounds that cause prolonged inhibition of brain cholinesterase. Under these circumstances the enzyme regeneration must occur by protein synthesis.

The rate at which the plasma cholinesterase was depressed indicates that the compounds or their active derivatives that caused prolonged depression of brain cholinesterase were present in the body of the chicken longer than those that did not. Thus, as shown in Figs. 1, 2, and 3, a minimal level of plasma cholinesterase was reached one day after dosing with DDVP or Dipterex, whereas the time required to reach the minimal level ranged from 3 days with malathion to 10 days with Trithion. In all cases except that of the chickens dosed with TOCP, a slow recovery of the activities of brain cholinesterases began shortly after the time that the plasma cholinesterase activity had reached a minimum. No correlation of the rate of depression of plasma and brain cholinesterases could be made with EPN since the plasma enzyme was not depressed in chickens given this compound. These data are in accord with the concepts of Vandekar and Heath[28] and Blaber and Creasey,[29] and indicate that the brain

cholinesterase in the chickens given malathion, Trithion, TOCP, or ronnel may have been kept in the inhibited state by continued titration of any newly synthesized enzyme. On the other hand, it seems unlikely that this can be the complete explanation, particularly for the chickens given EPN or TOCP, since virtually no recovery of brain cholinesterase was observed. Repression of enzyme synthesis by substrate or related compounds has been demonstrated for several enzymes that metabolize drugs.[38–40] As a working hypothesis it is suggested that repression of the mechanisms for the synthesis of new cholinesterase molecules may also have contributed to the prolonged inhibition of brain cholinesterase noted with these compounds. However, no direct experimental evidence is available for this hypothesis.

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