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SOME OBSERVATIONS ON THE CHOLINESTERASES OF THE CENTRAL NERVOUS SYSTEM AFTER THE ADMINISTRATION OF ORGANO-PHOSPHORUS COMPOUNDS

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Three cases of paralysis in workers exposed to a new organo-phosphorus compound (mipafox, bis-mono-isopropylamino fluorophosphine oxide) have been reported (Bidstrup and Hunter, 1952). The condition was said to resemble closely the toxic effects of tri-ortho-cresyl phosphate (TOCP). Paralysis in man due to TOCP poisoning has been known since 1899 (Lorot). The lesion can be produced in certain laboratory animals ; in the chicken and rabbit it has been examined histologically (Smith and Lillie, 1931) and shown to consist of demyelination of peripheral nerves and some tracts in the spinal cord.

The effect of a number of newer organophosphorus compounds on the chicken has recently been studied. Di-isopropyl fluorophosphonate (DFP) and mipafox were found to produce demyelination identical with that produced by TOCP (Barnes and Denz, 1953). Earl and Thompson (1952b) paralysed chickens by TOCP and found that there was a marked inhibition of the pseudo cholinesterase of the central nervous system persisting until the onset of paralysis 10 days after giving the TOCP. Ord and Thompson (1952) had previously investigated the distribution of brain and spinal cord cholinesterases in man, and concluded that the pseudo cholinesterase appears to be associated with white matter. Further, TOCP, DFP, and mipafox selectively inhibit pseudo cholinesterase rather than true cholinesterase (Earl and Thompson, 1952a ; Aldridge, 1953 ; Ord and Thompson, 1950). Observations on the plasma cholinesterase levels in the human cases of poisoning by mipafox showed that the pseudo cholinesterase remained at a low level for many weeks (Callaway, Davies, and Risley, 1952). These observations led Earl and Thompson to make the interesting suggestion that pseudo cholinesterase might play a part in myelin metabolism and that inhibition of this enzyme in nervous tissue might therefore bring about demyelination.

Of the many organo-phosphorus compounds tested on chickens, only a few have been shown to be capable of producing demyelination and paralysis. There is no obvious correlation in their chemical structure or general properties as anticholinesterase poisons which distinguishes those that do from those that do not produce demyelination. An examination was therefore made of the changes in activity of the true and pseudo cholinesterase of the chicken's central nervous system and peripheral nerve after administration of both compounds which produce demyelination and those which do not, to see whether they differed in their effect upon these enzymes as suggested by Earl and Thompson's hypothesis. Their behaviour as selective inhibitors *in vitro* against chicken cholinesterase has also been studied.

METHODS

Warburg buffer and manometric methods used were as described by Aldridge (1950) and Davison (1953). Final substrate concentrations were acetylcholine perchlorate (AcCh) 0.0138 M, butyrylcholine perchlorate (BuCh) 0.03 M, and acetyl-β-methylcholine bromide (MeCh) 0.03 M. AcCh and BuCh were prepared in this laboratory, MeCh was obtained from Messrs. Light & Co. Ltd., Colnbrook.

The inhibitors employed were di-isopropyl fluorophosphonate (DFP) obtained from Dr. Saunders, Cambridge ; tri-ortho-cresyl phosphate (TOCP, Messrs. Geigy Ltd., Manchester) ; bis-mono-isopropylamino fluorophosphine oxide (mipafox, isopestox) and pyrophosphoric acid tetra (mono-isopropyl amide) (isoompa) from Pest Control Ltd., Cambridge ; O.O di-isopropyl O.p-nitrophenyl phosphate (iso-E600) and tetra-isopropyl pyrophosphate (TIPP) from Albright & Wilson Ltd., Birmingham.

Adult hens of about 2 kg. weight and male albino rats of about 200 g. were used. Hens were killed by

injection of sodium pentobarbitone (Abbott Laboratories Ltd.) into the wing vein and rats by coal gas. The tissues were dissected out, chicken cerebrum and cerebellum separated, washed free from blood in saline, blotted dry, weighed, and homogenized with buffer in an all-glass homogenizer (Potter–Elvehjem type). Sciatic nerve was minced finely with scissors before homogenizing, and the cholinesterase activity was then determined manometrically in micro flasks (capacity about 1 ml.). Corrections were made for non-enzymic hydrolysis of substrates.

RESULTS

Determination of True and Pseudo Cholinesterase in the Chicken.—MeCh, which is usually regarded as a specific substrate for true cholinesterase (Mendel, Mundell, and Rudney, 1943), has been shown by Earl and Thompson (1952a) to be hydrolysed both by the true and pseudo cholinesterases of the chicken, therefore MeCh hydrolysis does not give an accurate measure of true cholinesterase activity except in tissue such as the cerebrum, which contains very little pseudo cholinesterase. True and pseudo cholinesterases have therefore been estimated by a method depending upon the selective inhibition of pseudo cholinesterase with mipafox (Davison, 1953). The activity remaining after incubation of homogenates of brain and spinal cord with 1×10^{-7} M mipafox for 30 min. at 37° C. has been taken as true cholinesterase. The difference between this and the original uninhibited activity is the activity of pseudo cholinesterase. At a concentration of 1×10^{-7} M mipafox all the pseudo cholinesterase (brain, spinal cord, and plasma) is inhibited, while the activity of the true cholinesterase remains virtually unaffected. Thus the activity of true and pseudo cholinesterase of different tissues against AcCh and BuCh was determined. It is interesting to note that under these conditions the residual activity of chicken brain will only hydrolyse one part of BuCh for every 100 of AcCh, but in the spinal cord and sciatic nerve it hydrolyses 7 parts of BuCh for every 100 of AcCh. In the brain, spinal cord, and sciatic nerve, pseudo cholinesterase was found to hydrolyse 110 parts of BuCh for every 100 of AcCh. For the cholinesterases of spinal cord and sciatic nerve, the ratio of AcCh to BuCh hydrolysis ranged from 14.3 for 100% true to 0.91 for 100% pseudo cholinesterase. When both true and pseudo cholinesterase were active, intermediate values were found for this ratio, and these could be calculated arithmetically and were plotted on a calibration curve. From the AcCh/BuCh ratio determined for any time, the relative contribution of true and pseudo cholinesterase can be determined and its composition expressed in terms of hydrolysis of either AcCh or BuCh.

Inhibition of Chicken Cholinesterases in vitro.—As a preliminary to experiments in the living animal the inhibitor concentrations producing 50% inhibition of true and pseudo cholinesterase have been determined. The inhibitor concentration producing 50% inhibition of true cholinesterase, after incubation for 30 minutes at 37° C., divided by that for pseudo cholinesterase gives an indication of the selectivity of the organophosphorus compound. The results are presented in Table I, and show that all the compounds tested inhibit pseudo cholinesterase preferentially, but the degree to which they do so varies over a wide range.

TABLE I

CONCENTRATIONS OF DRUGS WHICH CAUSE 50% INHIBITION OF CHOLINESTERASES *IN VITRO*

Chicken brain and plasma preparations were incubated with different concentrations of inhibitors for 30 min. at 37° C. Cholinesterase activity was determined with MeCh for the true cholinesterase of brain and BuCh for the pseudo cholinesterase of plasma

Inhibitor	Molar Concentration Required to Cause 50% Inhibition		Ratio of These Concentrations
	True	Pseudo	
Mipafox	3×10^{-5}	1×10^{-9}	30,000
DFP	$1{\cdot}6 \times 10^{-7}$	2×10^{-9}	80
TOCP	—	—	67*
Iso-ompa ..	$4{\cdot}1 \times 10^{-5}$	1×10^{-6}	41
Iso-E600 ..	$3{\cdot}2 \times 10^{-7}$	$6{\cdot}3 \times 10^{-8}$	5
TIPP	$2{\cdot}4 \times 10^{-7}$	$1{\cdot}3 \times 10^{-8}$	18

* Calculated from Earl and Thompson's (1952a) data.

Inhibition of Cholinesterases after Injection of the Inhibitors in vivo.—The activity of true and pseudo cholinesterase, determined as described above by AcCh and BuCh hydrolysis, in the various parts of the nervous system (cerebrum, cerebellum, cervical and lumbar spinal cord, and sciatic nerve) of the normal chicken was measured in 6 birds. The results are given in Table II. Similar measurements were also made on chickens killed at different times after they had been injected with the various organo-phosphorus compounds. The dose of each compound was either that shown to produce paralysis within 10 days, or, where no paralysis was produced, it was the maximum dose tolerated by the chickens. All the birds except those given TOCP received one or more injections of atropine to suppress the muscarinic symptoms in the acute stages of poisoning. There were no symptoms of acute poisoning after TOCP, but after all the other compounds severe symptoms with collapse of the birds persisted for 12–48 hours. After iso-ompa these symptoms were less than after the other compounds. After administration

TABLE II

NORMAL VALUES FOR AcCh 0·0138 M and BuCh 0·03 M HYDROLYSIS BY CHICKEN BRAIN, SPINAL CORD, SCIATIC NERVE, AND PLASMA

Corrected values for true and pseudo cholinesterases determined as in text. Nervous tissue is expressed as μl. CO_2/g. wet weight/hr. and plasma as μl./CO_2/0·5 ml./min. with the standard deviation

Tissue	AcCh (Total Activity)	True ChE (AcCh) (Corrected)	% True	BuCh (Total Activity)	Pseudo ChE (BuCh) (Corrected)	No. of Animals
Cerebrum	30,066±5,000	29,066±4,840	96	1,393±254	1,093±202	6
Cerebellum	51,313±22,920	50,013±22,700	96·4	1,940±410	1,427±302	6
Cervical spinal cord	3,642±504	3,148±432	89	781±185	530±174	5
Lumbar cord	4,886±724	4,217±251	84	1,154±156	895±63	7
Sciatic nerve	1,655±431	1,287±389	77	501±54	406±38	8
Plasma	8·02±1·52	—	0	—	8·8±1·68	8

of both TOCP and iso-ompa it was found that the true cholinesterase was only partially inhibited; this, therefore, probably accounts for the fewer symptoms.

The doses of the compounds given were as follows:

TOCP: 1 ml./kg. per os, undiluted—paralysis.
DFP: 1 mg./kg. subcut., in 10% ethanol—paralysis.
Mipafox: 20 mg./kg. subcut., in ethanol—paralysis.
TIPP: 8 mg./kg. subcut., undiluted—no paralysis.
Iso-E600: 15 mg./kg. subcut., in ethanol—no paralysis.
Iso-ompa: 300 mg./kg. subcut., in propylene glycol—no paralysis.

Pairs of birds were killed at intervals after administering the compounds, and the activity of the true and pseudo cholinesterase in different parts of the brain, spinal cord, sciatic nerve, and plasma was determined. Only 2 birds were studied after TOCP, and the findings agree with the much larger series published by Earl and Thompson (1952b). Thus true cholinesterase was only slightly inhibited, while the pseudo cholinesterase was reduced to about 20% of its initial activity for at least 10 days. The true cholinesterase of birds treated with iso-ompa was only slightly inhibited, but the pseudo cholinesterase was inhibited by about 70% after 2 days and remained low for at least 6 days. A similar finding with iso-ompa was obtained by Thompson (personal communication). Despite large variations in individual values, the pattern of recovery of cholinesterases after injection of all compounds other than TOCP and iso-ompa was progressive and faster. True cholinesterase was found to be inhibited about 95% 2 hours after injection; it was inhibited 80% 2 days later, and 60% 6 days after injection. Likewise, after 2 hours pseudo cholinesterase was inhibited from 90–100%, about 70% after 2 days, and about 40% after 6 days. Both DFP and mipafox initially inhibited pseudo cholinesterase more completely than the other compounds, although recovery was similar to that after administration of the other compounds. Lumbar spinal cord and sciatic nerve pseudo cholinesterase recovered more slowly than the other pseudo cholinesterases examined.

The figures for the activities of true and pseudo cholinesterase of the cervical spinal cord after each of the inhibitors are given in Table III. The pattern of recovery of chicken cerebrum and lumbar spinal cord after inhibition with demyelinating and non-demyelinating compounds (other than TOCP) is shown in Figs. 1 and 2. There is no clear difference between the rates of recovery after any of the compounds.

TABLE III

CHOLINESTERASE VALUES IN THE CERVICAL SPINAL CORD OF HENS POISONED WITH ORGANO-PHOSPHORUS COMPOUNDS

Activity is expressed as μl. CO_2/g./hr. true cholinesterase in terms of AcCh and pseudo cholinesterase in terms of BuCh (both corrected)

Time After Injection	DFP	TOCP	Mipafox	TIPP	Iso-ompa	Iso-E600
Pseudo Cholinesterase (Normal: 530±174)						
2 hrs.	15		0	150		40
	0		118	213		
1 day	0					
	0					35
2 days	305	0	278	166	122	391
	156		71	46		353
6 ,,	298	0	588	435	409	381
	119		352	312		335
8 ,,			425			
			450			
11 ,,	492					
	529					
True Cholinesterase (Normal: 3,148±432)						
2 hrs.	11		1,170	30		67
	240		950	41		
1 day	610					
	525					330
2 days	450	1,830	1,500	950	3,140	334
	790		1,580	595		356
6 ,,	1,700	3,680	1,890	1,870	2,840	937
	1,430		2,290	1,020		1,395
8 ,,			2,680			
			2,510			
11 ,,	1,810					
	2,330					

Experiments in the Rat in vivo.—Earl and Thompson (1952a) suggested that TOCP failed to produce demyelination in rats because the pseudo cholinesterase of the central nervous system

was insensitive to this inhibitor. Rats were given DFP (0.5 mg./kg.) intravenously. The activity of pseudo cholinesterase in both brain and spinal cord was reduced to zero and returned at a similar rate (40% in 2 days and 60% in 10 days). There were no signs of paralysis, and histologically there was no demyelination.

Discussion

The observations reported here were undertaken as part of an attempt to distinguish between those organophosphorus compounds that do produce demyelination and those that do not. The work was stimulated by the interesting hypothesis put forward by Earl and Thompson (1952a and b) that demyelination might be related to inhibition of the pseudo cholinesterase activity of nervous tissue. A number of compounds, having certain features in common, were chosen for study. Structurally, the compounds were related by the possession of at least two isopropyl groups (except TOCP). Secondly, they all showed preferential inhibition of pseudo cholinesterase rather than true cholinesterase of the chicken when examined *in vitro*. Finally, all the compounds were recognized as being practically "irreversible" inhibitors of cholinesterase in the sense that rates of recovery after inhibition in man and in the rat (Callaway, Davies, and Risley, 1952; Freedman, Willis, and Himwich, 1949; Koelle and Gilman, 1946) approached that of enzyme resynthesis and were indeed very slow when compared to recovery rates after inhibition by E600 or Me-E600 (Aldridge, 1953; Davison, 1953). Such compounds might therefore be expected to produce a profound and a prolonged reduction of pseudo cholinesterase in the hen. It will be seen, however, that in contrast to the prolonged inhibition of pseudo cholinesterase induced by TOCP poisoning all the five remaining compounds produce a similar pattern of changes in the brain, spinal cord, and sciatic

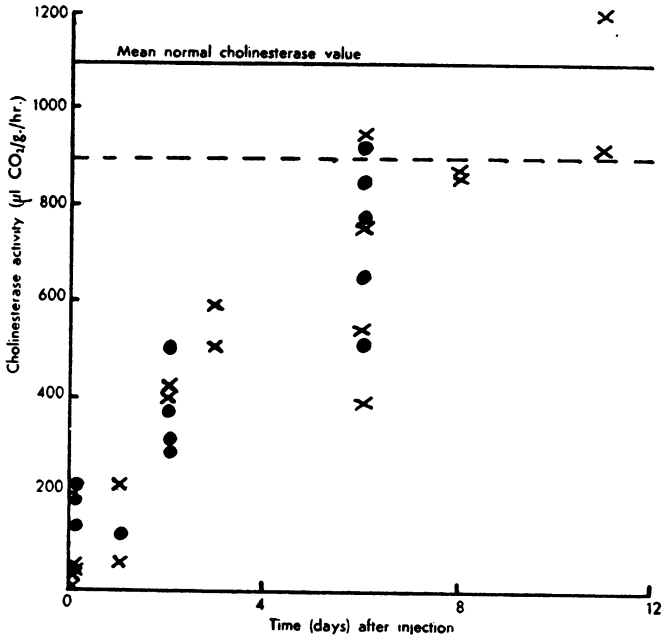

FIG. 1.—Pseudo cholinesterase levels in the chicken cerebrum after injection of organo-phosphorus compounds. Activity is expressed in terms of BuCh, corrected for hydrolysis by true cholinesterase. × demyelinating compounds (DFP, mipafox). ● non-demyelinating compounds (iso-E600, TIPP, iso-ompa). The deviation from the mean is denoted by the broken line.

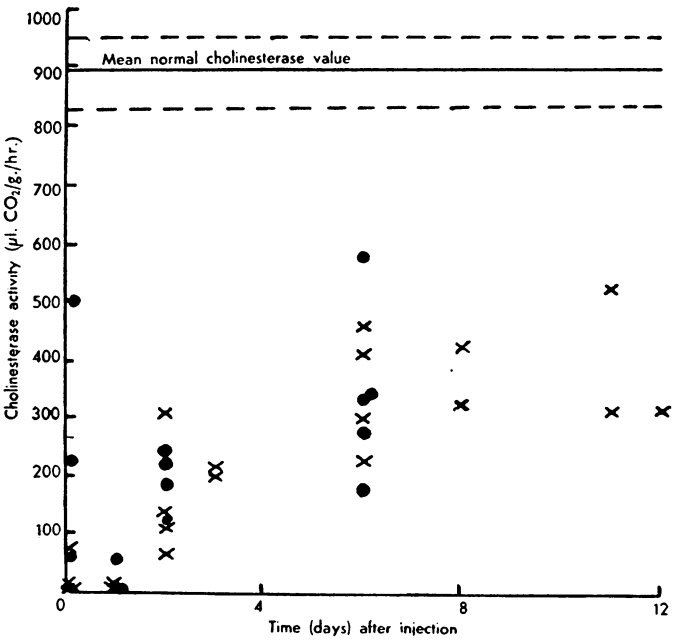

FIG. 2.—Pseudo cholinesterase levels in the chicken lumbar spinal cord after injection of organo-phosphorus compounds. Activity is expressed in terms of BuCh, corrected for hydrolysis by true cholinesterase. × demyelinating compounds (DFP, mipafox). ● non-demyelinating compounds (iso-E600, TIPP, iso-ompa). The deviation from the mean is denoted by the broken line.

nerve cholinesterase levels after the injection of a single dose. There is an immediate lowering of activity to nearly zero within 2 hours, followed by a fairly rapid, but not complete, recovery within 6–8 days. This is faster than the rate of recovery (*in vivo*) of rat's brain cholinesterase, inhibited with DFP (Koelle and Gilman, 1946; Freedman, *et al.*, 1949) and considerably faster than the recovery of rabbit brain cholinesterases (Mazur and Bodansky, 1946) after inhibition with DFP. At least two factors may be responsible for the fast recovery of cholinesterase activity in the chicken: (i) reactivation by hydrolysis of the inhibited enzyme, (ii) synthesis of fresh cholinesterase. Since the recovery rate is rapid and indeed faster than that of other species examined it would seem unlikely that fresh cholinesterase was being synthesized so quickly. Further, the rates of return of cholinesterase activity in plasma and brain are similar, although plasma proteins are known to regenerate more quickly than in other tissues (Sprinson and Rittenberg, 1949).

It may therefore be concluded that a prolonged inhibition of pseudo cholinesterase activity of nerve tissue of the central nervous system and sciatic nerve (e.g. TOCP) is not a prerequisite for the production of paralysis. Paralysis is easily produced by a single dose of DFP or mipafox, but the pseudo cholinesterase is completely inhibited for only a few hours, after which recovery is progressive. An indistinguishable pattern of change is also produced by other compounds which do not produce demyelination or paralysis.

Further, demyelination is not produced in rats either by TOCP (Myers and Mendel, 1952) or by DFP, despite complete inhibition of the pseudo cholinesterase of brain and spinal cord by the latter.

The relationship between inhibition of true and pseudo cholinesterase of nerve tissue and demyelination produced by certain organo-phosphorus compounds remains obscure. All that can be said is that demyelination has not yet been produced by a compound that did not also depress the activity of pseudo cholinesterase, but a depression of activity is not enough.

SUMMARY

1. The effect of the injection of five organophosphorus compounds upon the true and pseudo cholinesterase of the brain, spinal cord, and sciatic nerves of chickens is described.

2. Two compounds produced demyelination and three did not. There was no essential difference in the degree or duration of the inhibition of true and pseudo cholinesterase produced by these agents, and none resembled TOCP.

3. DFP will reduce the pseudo cholinesterase of rats, but does not produce demyelination.

4. These observations do not support the theory that prolonged depression of pseudo cholinesterase is an essential prerequisite of demyelination in fowls.

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REFERENCES

Aldridge, W. N. (1950). *Biochem. J.*, **46**, 451.
—— (1953). Ibid., **53**, 62.
Barnes, J. M., and Denz, F. (1953). *J. Path. Bact.* (in the press).
Bidstrup, P. L., and Hunter, D. (1952). *Lancet*, **262**, 262.
Callaway, S., Davies, D. R., and Risley, J. E. (1952). *Biochem. J.*, **50**, xxx.
Davison, A. N. (1953). Ibid. (in the press).
Earl, C. J., and Thompson, R. H. S. (1952a). *Brit. J. Pharmacol.*, **7**, 261.
—— —— (1952b). Ibid., **7**, 685.
Freedman, A. M., Willis, A., and Himwich, H. E. (1949). *Amer. J. Physiol.*, **157**, 80.
Koelle, G. B., and Gilman, A. (1946). *J. Pharmacol.*, **87**, 421.
Lorot, C. (1899). *Les Combinaisons de la Creosote dans le Traitment de la Tuberculose Pulmonaire.* Thèse de Paris. Quoted by Hunter, D. (1944). *Industrial Toxicology*, Oxford University Press.
Mazur, A., and Bodansky, O. (1946). *J. biol. Chem.*, **163**, 261.
Mendel, B., Mundell, D. B., and Rudney, H. (1943). *Biochem. J.*, **37**, 473.
Myers, D. K., and Mendel, B. (1952). *Nature, Lond.*, **170**, 928.
Ord, M. G., and Thompson, R. H. S. (1950). *Biochem. J.*, **46**, 346.
—— —— (1952). Ibid., **51**, 245.
Smith, M. I., and Lillie, R. D. (1931). *Arch. Neurol. Psychiat., Chicago*, **26**, 976.
Sprinson, D. B., and Rittenberg, D. (1949). *J. biol. Chem.*, **180**, 715.