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THE INHIBITORY ACTION OF TRI-*ORTHO*-CRESYL PHOSPHATE ON CHOLINESTERASES

BY

C. J. EARL AND R. H. S. THOMPSON

From the Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.1

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The toxic effects of tri-*o*-cresyl phosphate (TOCP) in man have been observed since the end of the last century. Lorot (1899) described six cases of multiple neuritis in patients with pulmonary tuberculosis treated with preparations of "phosphocreosote" which were subsequently found to contain TOCP. Further outbreaks of paralysis associated with poisoning by this compound have since been reported. The TOCP ingested in these cases was found to have been present as an adulterant in drinks prepared from extracts of Jamaica ginger (Smith and Elvove, 1930a, b), in apiol (Ter Braak, 1931 ; Germon, 1932), and in edible oils (Sampson, 1938 ; Hotston, 1946). TOCP is now used in the plastics industry and cases of poisoning among workers in this country have been described by Hunter, Perry, and Evans (1944).

The way in which this compound produces these paralytic symptoms is at present unknown. Smith and Lillie (1931) have described the histopathology of the condition in man and also the lesions produced by TOCP in the peripheral nerves and the central nervous system of experimental animals. They found evidence of demyelination of the peripheral nerves, degenerative changes in the anterior horn cells, and "fatty degeneration" in the white substance of the spinal cord.

On the assumption that the motor paralysis induced by TOCP might be associated with a lesion of the motor endplates in the affected muscles, Bloch in 1941 began a study of the action of this compound on cholinesterase activity. He first showed that the cholinesterase of horse serum was inhibited by low concentrations of this compound. Two years later Hottinger and Bloch (1943) demonstrated that the hydrolysis of acetylcholine by human and rabbit serum, liver, and brain was also inhibited by TOCP ; in addition, the splitting of tributyrin by serum and liver was found to be diminished by this compound, although serum phosphatase and pancreatic lipase were unaffected. No attempt was made at that time to study the different types of cholinesterase by means of selective substrates.

In a brief report it was next stated (Mendel and Rudney, 1944) that the oral administration of TOCP to rats caused a lowering of the serum pseudo-cholinesterase, whereas the true cholinesterase appeared to be unaffected ; Myers and Mendel (1949) later claimed that the hydrolysis of tributyrin by rat brain was insensitive to TOCP.

The experiments described below were therefore carried out to investigate this inhibition of cholinesterases in greater detail.

It seemed desirable in the first instance to compare the sensitivities to TOCP of " true " and " pseudo " cholinesterases from different sources. As substrate for the true cholinesterase present, for example, in human erythrocytes and in the grey matter of the central nervous system, we have used acetyl-β-methylcholine (Mendel, Mundell, and Rudney, 1943); for the pseudo-cholinesterase we have in most cases used butyrylcholine (Stedman, Stedman, and Easson, 1932), since this compound is hydrolysed much more rapidly than benzoylcholine by human serum cholinesterase, and since Adams (1949) has reported that it is hydrolysed by the true cholinesterase of human erythrocytes at only 5 per cent of the rate at which acetyl-β-methylcholine is hydrolysed. From the symptomatology of TOCP poisoning the esterases of the central nervous system and of the motor endplates seemed specially worthy of study, and, in view of the finding by Ord and Thompson (1951) of a relatively high level of pseudo-cholinesterase in the white matter of the nervous system, samples of grey and white matter from the human cerebrum were among the tissues employed. In addition to human material, tissues were taken from the chicken, rabbit, and rat. The chicken and rabbit were chosen, since previous workers had succeeded in producing paralysis with TOCP in these animals, whereas the rat had been found insensitive (Smith, Engel, and Stohlman, 1932; Sampson, 1938).

METHODS

Estimation of esterase activity was carried out manometrically at 38° and at *p*H 7.4, the Warburg apparatus being used. All measurements were done in duplicate, and corrections were made for non-enzymic hydrolysis of the substrate. Esterase activity of serum is expressed as μl. CO_2/ml./min.

Tissue preparations

Human serum or oxalated plasma was diluted with 3 vol. of 0.025 M-$NaHCO_3$; 0.2 ml. of this dilution was added to each flask.

Human erythrocytes were obtained from oxalated blood; the cells were centrifuged, washed twice with 0.9 per cent (w/v) NaCl, and lysed with a volume of glass-distilled H_2O equal to twice the volume of the original sample of blood; 0.2 ml. of this haemolysate was added to each flask.

Human brain, spinal cord, sciatic nerve, and muscle were obtained fresh from autopsies. The grey matter from the cerebral cortex or white matter from the subcortical tracts was dissected out, washed in 0.9 per cent NaCl, and homogenized in 0.025 M-$NaHCO_3$. A segment of spinal cord was dissected free from meninges and treated similarly. Since it was found difficult to prepare a satisfactory homogenate of sciatic nerve on account of the fibrous elements present in this tissue, a weighed portion of the nerve was ground with washed sand and 0.025 M-$NaHCO_3$, centrifuged, and re-ground twice; the supernatant after each centrifugation was pipetted off and the volume made up to give a known dilution of the original weight of the nerve. The intrinsic muscles of the tongue and the adductor pollicis were chosen as sources of motor endplate cholinesterase, since these muscles, subserving fine movements, are richly innervated and might therefore be expected to show a high overall content of true cholinesterase resulting from a relatively high concentration of motor endplates within the muscle.

Nerve tissues from other animals were prepared in the same way, the animals being killed by air-embolism or decapitation.

Chicken and rat serum were obtained from blood collected by decapitation; 0.2 ml. of undiluted serum was added to each flask.

Substrates

(1) Acetylcholine chloride (ACh) (British Drug Houses, Ltd.);

(2) Butyrylcholine chloride (BuCh) (British Drug Houses, Ltd.);

(3) Acetyl-β-methylcholine chloride (MCh) (Savory & Moore, Ltd.);

(4) Benzoylcholine chloride (BCh) prepared by Dr. A. H. Ford-Moore, Experimental Station, Porton;

(5) Tributyrin (TB) (British Drug Houses, Ltd.).

The choline esters were dissolved in 0.025 M-$NaHCO_3$ immediately before use to give a final concentration of 0.015 M for ACh and BCh and 0.03 M for BuCh and MCh. The tributyrin was pipetted directly into the side-arm of the Warburg flask (0.03 ml. per flask). The zero reading was always taken 3 min. after tipping in the substrate from the side-arm.

Inhibitors

(1) Tri-*o*-cresyl phosphate (TOCP) prepared from pure *o*-cresol (Geigy Pharmaceutical Laboratories, Ltd.);

(2) Di-*iso*propyl fluorophosphonate (DFP), kindly provided by the Experimental Station, Porton.

Owing to the low solubility of TOCP in water, only the lowest concentrations that we have used have given optically clear solutions. With the higher concentrations, in most experiments, a fine, stable suspension of TOCP in 0.025 M-$NaHCO_3$ was used although in some of our earlier experiments it was dissolved in *iso*propanol before adding to the flasks. Since *iso*propanol has a potentiating effect on cholinesterase activity (Todrick, Fellowes, and Rutland, 1951), an equal volume of *iso*propanol (0.05 ml.) was added to the control flask in these experiments. A given concentration of TOCP, however, was found to produce roughly the same degree of inhibition whether dissolved in *iso*propanol or suspended in $NaHCO_3$.

Since, with the higher concentrations of TOCP, we have not been using a true solution of our inhibitor we have expressed our results in terms of the total amount of TOCP added to each Warburg flask; assuming complete solution, 100 μg. per flask (3 ml.) = 0.91×10^{-4}M.

The DFP was dissolved in 0.025 M-$NaHCO_3$ immediately before use. In all experiments 25–35 min. elapsed between the addition of the inhibitor to the enzyme preparation and the time at which the substrate was tipped in.

RESULTS

Human tissues

The effects of different concentrations of TOCP on the esterase activities of a number of different human tissues are shown in Table I. All the figures given for percentage inhibitions in this Table are the means of at least two experiments. It will be seen that a very striking difference exists between the sensitivities to this compound of the true and pseudo-cholinesterases, when MCh and BuCh respectively are used as substrates. The pseudo-cholinesterase activities of serum, white and grey matter from the cerebrum, and spinal cord were each inhibited approximately 50 per cent by 50 μg. TOCP per 3 ml. reaction mixture; sciatic nerve appears to be somewhat more sensitive, a mean inhibition of 59 per cent being caused by 25 μg. TOCP per 3 ml., i.e., approximately 2×10^{-5}M. Using a purified

TABLE I

INHIBITION BY TOCP OF HUMAN CHOLINESTERASES AND TRIBUTYRINASE

Enzyme and source	Percentage inhibition of human esterases by TOCP at								
	25	50	100	150	200	300	500	1,000	2,000
	μg./3 ml.								
Pseudo-cholinesterase (substrate 0.03 M-BuCh)									
Serum	27	55	73	82	93	—	—	—	—
White matter (cerebrum)	38	65	81	87	91	—	—	—	—
Grey matter (cerebrum)	33	51	66	72	86	—	—	—	—
Spinal cord	39	55	64	64	74	—	—	—	—
Sciatic nerve	59	81	92	97	99	—	—	—	—
True cholinesterase (substrate 0.03 M-MCh)									
Erythrocytes	—	7	4	1	7	10	4	—	—
White matter	—	—	—	9	9	—	—	—	—
Grey matter	5	4	9	6	8	—	—	—	—
Spinal cord	—	—	2	8	7	6	2	13	22
Striated muscle	—	3	8	—	10	15	11	31	—
Tributyrinase (substrate 0.03 ml. TB/3 ml.)									
White matter	—	21	21	—	21	—	31	—	—
Grey matter	—	—	—	—	—	—	28	30	40
Spinal cord	—	21	30	—	33	—	—	—	—

human plasma cholinesterase (Fraction IV-6) Brauer (1948) reported 50 per cent inhibition of activity by approximately 10^{-7}M-TOCP. Experiments with BCh as substrate for the pseudo-cholinesterase of serum, cerebrum (white matter), and sciatic nerve gave inhibitions of the same order as those obtained in the presence of BuCh.

The true cholinesterase in erythrocytes, white and grey cerebral matter, spinal cord, and striated muscle, on the other hand, was not significantly inhibited by concentrations of this order, and only 2–25% inhibition was produced by concentrations as high as 500–2,000 μg. per 3 ml. Tributyrinase activity, as reported earlier by Hottinger and Bloch (1943), is also inhibited by TOCP, although to a very much smaller extent than the pseudo-cholinesterase, concentrations of TOCP as high as 2,000 μg. per 3 ml. producing only up to 40 per cent inhibition.

Animal tissues

Similar *in vitro* experiments have been carried out with tissues from rabbits, chickens, and rats as a preliminary to extending these observations to a study of *in vivo* intoxication in laboratory animals. The results are summarized in Table II.

With rabbit brain and spinal cord there is also preferential inhibition of the pseudo-cholinesterase as indicated by BuCh hydrolysis. Thus, 200 μg. TOCP per 3 ml. inhibits BuCh hydrolysis 50 per cent and MCh hydrolysis only 10–18 per cent. The true cholinesterase in skeletal muscle is also insensitive to inhibition by these concentrations of TOCP. Comparison of these results with those given in Table I will show that the pseudo-cholinesterase in the rabbit central nervous system is somewhat less sensitive to inhibition by TOCP than the corresponding enzyme in

TABLE II

EFFECT OF TOCP ON ANIMAL CHOLINESTERASES

Enzyme and source	Percentage inhibition of animal esterases by TOCP at									
	50	100	200	400	500	800	1,000	1,200	1,500	2,000
	μg./3 ml.									
Pseudo-cholinesterase (substrate 0.03 M-BuCh)										
Rabbit Brain	—	36	49	62	—	75	81	—	82	82
,, Spinal cord	—	37	52	72	—	80	86	—	89	—
Chicken Serum	39	60	62	94	—	—	—	—	—	—
,, Brain	—	—	33	48	57	68	72	—	—	—
,, Spinal cord	8	19	39	50	60	88	94	—	—	—
,, Nerve	—	26	28	38	46	—	—	—	—	—
Rat Brain	—	—	9	27	—	39	41	51	54	62
,, Serum	—	5	8	—	8	14	15	—	—	—
True cholinesterase (substrate 0.03 M-MCh)										
Rabbit Brain	—	6	18	12	—	17	9	—	15	—
,, Spinal cord	—	4	10	16	—	16	14	—	15	—
,, Muscle	—	—	0	3	—	—	3	—	9	22
Chicken Brain	—	—	1	—	10	—	12	—	24	—
,, Spinal cord	—	—	18	—	25	—	31	—	41	—
Rat Brain	—	7	—	0	—	0	8	—	—	—
,, Spinal cord	—	—	—	—	0	—	10	—	—	4
Tributyrinase (substrate 0.03 ml. TB/3 ml.)										
Rabbit Brain	—	—	24	32	—	35	47	—	51	43

human tissues ; this agrees with the earlier finding by Hottinger and Bloch (1943) concerning overall hydrolysis of acetylcholine in the tissues of these two species. Tributyrinase activity of rabbit brain is only partially inhibited by high concentrations of TOCP.

Hydrolysis of BuCh by chicken serum, brain, spinal cord, and peripheral nerve was also markedly inhibited by concentrations of 100–500 μg. TOCP per 3 ml., although with this species also these tissues were rather less sensitive to TOCP than the corresponding human ones. It was found, however, that chicken serum hydrolyses both BuCh and MCh. Summation experiments with these two substrates gave no evidence of any increased rate of hydrolysis over that observed with BuCh alone ; thus in two experiments the rates in μl. CO_2 per ml. per min. were : with MCh alone 9.4 and 8.3 ; with BuCh alone 21.6 and 21.0 ; and with MCh plus BuCh 20.6 and 17.8. This failure to observe summation in the presence of both substrates might be due to inhibition of the MCh hydrolysis by the BuCh, as indeed has been found to be true with MCh and BCh hydrolysis by rat serum (unpublished observations by Ord and Thompson) or to the hydrolysis of both substrates by a single enzyme. If, however, two distinct enzymes were concerned with the hydrolysis of these two substrates it might be expected that, as with the hydrolysis of BuCh and MCh by the tissues of other species, they would show different degrees of sensitivity to inhibition by DFP. Experiments were therefore carried out to compare the inhibition of hydrolysis of these two substrates by both DFP and TOCP. It was found that with both these inhibitors, which in all other species so far studied have

proved themselves to be selective in their actions on the true and pseudo-cholinesterases, almost identical degrees of inhibition of the hydrolysis of BuCh and MCh were obtained ; thus with 3×10^{-9}M-DFP the percentage inhibitions were, for BuCh, 79 and, for MCh, 78 ; similarly with 200 μg. TOCP per 3 ml. the inhibitions were, for BuCh, 55 and, for MCh, 58 per cent. This suggests that these two substrates are hydrolysed by the same enzyme. Augustinsson (1948) has also reported on the relatively high rate of hydrolysis of MCh by fowl plasma, although he did not carry out summation or inhibition experiments to characterize the enzyme concerned. It would seem therefore that chicken serum contains a cholinesterase which does not conform to either the " true " or " pseudo " type, as judged by its action on selective substrates, inasmuch as it appears to hydrolyse both BuCh and MCh. However, since it hydrolyses BuCh more rapidly than MCh, and since it is DFP-sensitive, it is proposed for present purposes to regard it as a pseudo-cholinesterase.

By analogy with human tissues, in which the pseudo-cholinesterase in the brain and in the serum are similar in respect of substrate specificity, it might be expected that the pseudo-cholinesterase in chicken brain would also hydrolyse both BuCh and MCh, and that MCh hydrolysis by this tissue would therefore also be sensitive to TOCP. It will be seen from Table II, however, that MCh hydrolysis by chicken brain is hardly affected by concentrations of TOCP that cause over 50 per cent inhibition of BuCh hydrolysis.

The presence of a true cholinesterase in chicken brain, which like that in human and rabbit brain hydrolyses MCh and is insensitive to TOCP, would explain this failure of TOCP to inhibit MCh hydrolysis, particularly if, as in other species, it is present in much greater amount than the pseudo-cholinesterase. A comparison of the effects of DFP on the hydrolysis of these two substrates by chicken brain shows that a true cholinesterase is present in addition to the enzyme hydrolysing BuCh ; thus, BuCh hydrolysis by chicken brain is inhibited 72 per cent by 3×10^{-9}M-DFP, but MCh only 2 per cent.

In this connexion it is interesting to note (Table II) that although MCh hydrolysis by chicken brain, a tissue containing a large preponderance of the true cholinesterase, is only very slightly inhibited by TOCP, MCh hydrolysis by the spinal cord is significantly more sensitive ; this increased sensitivity to inhibition by TOCP may be explained by differences in the relative amounts of true and pseudo-cholinesterase in the brain and spinal cord. Thus BuCh is hydrolysed at 65–80 per cent of the rate of MCh by chicken spinal cord, whereas the corresponding figure for chicken brain is only 15–20 per cent ; in chicken spinal cord therefore the TOCP-sensitive pseudo-cholinesterase will contribute very appreciably to the hydrolysis of MCh.

We can therefore conclude that in the central nervous system of the chicken, as in other species, two cholinesterases are present, one hydrolysing BuCh and MCh and sensitive to TOCP and DFP, and the other relatively insensitive to these inhibitors, active against MCh, and therefore presumably a true cholinesterase.

Lastly a few experiments have been carried out with tissues from albino rats. In contrast to our findings with human, rabbit, and chicken tissues, the pseudo-cholinesterase of rat brain and serum, as indicated by BuCh hydrolysis, is not very sensitive to TOCP, and in no experiment did we obtain more than 62 per cent inhibition.

Discussion

Although TOCP has been described as an inhibitor of cholinesterase (Bloch, 1941), the experiments reported above indicate that in the species studied by us the true cholinesterase of nerve tissue, striated muscle, and erythrocytes is not inhibited to any major degree by amounts of TOCP even far in excess of its solubility. On the other hand, the pseudo-cholinesterases of human, rabbit, and chicken serum and nerve tissues are inhibited by relatively low concentrations of this compound *in vitro* ; of these three species the enzyme in human tissues is rather more sensitive to TOCP than that in the rabbit and chicken. The pseudo-cholinesterase of the rat nervous system is, however, very much less sensitive. These species variations of *in vitro* sensitivity of pseudo-cholinesterase to inhibition by TOCP are of interest when compared with the differing sensitivities of these species to *in vivo* intoxication by TOCP ; thus, whereas flaccid paralysis and death can be readily brought about in the rabbit and the chicken by orally or parenterally administered TOCP, the albino rat does not appear to be susceptible to this poison (Smith, Engel, and Stohlman, 1932 ; Mendel and Rudney, 1944). De Vaal (1948) has stated that 1 ml. of TOCP given orally to " rats of our own breed " causes motor disturbances (convulsions, spastic paralysis) and death after 14–16 days. We have, however, administered 0.5 ml. subcutaneously to albino rats weighing 150–200 g. without adverse effect.

The selective inhibition of pseudo-cholinesterase by TOCP *in vitro* raises the question of the possible significance of these findings in connexion with the toxicology of this compound. Koelle and Gilman (1949) have recently revived the earlier suggestion that the flaccid paralysis produced in man by this compound might be due to prolonged inhibition of the cholinesterase at the motor endplates with a resulting neuromuscular block. Our finding that the true cholinesterase in skeletal muscle is not significantly inhibited by concentrations of TOCP which cause almost complete inhibition of the pseudo-cholinesterase does not support this view. Further, if this were the explanation it is not easy to understand why a period of approximately 10–20 days normally elapses before the onset of the paralysis. It must be remembered also that, with the possible exception of some symptoms of gastro-intestinal upset shortly after the oral administration of this compound, no typical signs or symptoms of cholinergic over-activity are produced by TOCP, whereas these are usually striking after intoxication with DFP or the organo-phosphorus insecticides.

The essential histological change in the motor neuritis brought about by TOCP is a degeneration of the myelin sheaths of the peripheral nerves together with a fatty degeneration of the white matter of the cord, and Smith and Lillie (1931) have described this compound as a " specific myelin poison." It might therefore be expected that from the biochemical point of view also the toxicological action of TOCP might be centred round the myelin-containing regions of the nervous system, and Ord and Thompson (1952) have previously shown that the pseudo-cholinesterase of the central nervous system appears to be associated, particularly with the white fibre tracts. The possibility must therefore be considered that the pseudo-cholinesterase of nerve tissue may be connected in some way with the maintenance of the myelin sheaths of nerve fibres, and that inhibition of this enzyme by TOCP may play a

part in the production of the demyelination and consequent paralysis. Further *in vivo* experiments are now in progress in the hope of testing this hypothesis.

Since pseudo-cholinesterase is widely distributed in different tissues throughout the body (Ord and Thompson, 1950), it might be expected that widespread evidence of tissue dysfunction, though not necessarily with cholinergic symptoms, would be present in TOCP intoxication if the underlying mechanism of its toxic action was simply an inhibition of this enzyme. It must be pointed out, however, that TOCP differs very markedly from eserine, DFP, and most of the organo-phosphorus insecticides in its solubility properties ; it is only very slightly soluble in water, but readily soluble in lipoid solvents. Its distribution in the body and its access to the active centre of the tissue pseudo-cholinesterases may therefore be influenced by this, and the fatty nature of the white matter of the nervous system may facilitate its uptake by this tissue.

The insolubility of TOCP in water has indeed presented some practical problems in the course of this work. Preliminary observations have shown that TOCP is soluble in 0.025 M-$NaHCO_3$ to the extent of about 8 μg. per ml., as judged by optical clarity of the resulting solution. In the Warburg flasks containing 25 μg. per 3 ml. the TOCP may therefore be expected to be all in true solution ; with the higher concentrations of TOCP which we have used, the compound was added as a suspension. However, in view of the fact that we do not know what fraction of the amount added ultimately dissolves in the fat of the tissue preparation present as our enzyme source, it is not possible to form any quantitative opinion as to the partition of the added TOCP between the different phases of the heterogeneous systems with which we have been working. The fact that Bloch (1943) demonstrated that the relatively non-toxic tri-*m* and tri-*p*-cresyl phosphates, whose solubility properties closely resemble those of TOCP, were also very much less powerful cholinesterase inhibitors suggests strongly that the inhibition which we have observed is not due merely to some physical effect of the suspended TOCP. In order to obtain further evidence that we were not dealing with a non-specific physical process we have studied the effect of the addition of the non-ionic surface-active substance Lubrol W (a cetyl alchohol-polyoxyethylene condensate) on the inhibitory action of TOCP ; the Lubrol caused a marked clearing of the emulsion, but was without effect on the degree of inhibition produced.

Finally, we have made a few preliminary observations on the degree of reversibility of the inhibition of pseudo-cholinesterase by TOCP, and have shown that dialysis for sixteen hours causes no diminution in the degree of inhibition.

SUMMARY

1. A study has been made of the action of tri-*ortho*-cresyl phosphate (TOCP) on the true and pseudo-cholinesterases of a number of tissues of man, the rabbit, the chicken, and the rat.

2. With human tissues TOCP was found to be a selective inhibitor of the pseudo-cholinesterase, concentrations which caused 75–99 per cent inhibition of this enzyme in cerebrum, spinal cord, sciatic nerve, and serum causing only 7–10 per cent inhibition of the true cholinesterase in cerebrum, spinal cord, striated muscle, and erythrocytes.

3. With rabbit and chicken tissues a selective inhibition of the pseudo-cholinesterase was also observed, although tissues from these species were rather less sensitive to TOCP than those from man.

4. The pseudo-cholinesterase of the albino rat, an animal apparently insensitive to poisoning by TOCP, was only partially inhibited even by very high concentrations ; the true cholinesterase in this species was also insensitive.

5. The possible significance of these results is discussed in connexion with the demyelination and motor paralysis which characterize TOCP poisoning.

6. The cholinesterase of chicken plasma differs from those of other species studied in that it hydrolyses both butyrylcholine and acetyl-β-methylcholine.

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