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# OBSERVATIONS ON THE SPECIFICITY OF THE INHIBITION OF CHOLINESTERASES BY TRI-*ORTHO*-CRESYL PHOSPHATE

BY

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In 1941 Bloch demonstrated that horse serum cholinesterase was inhibited by tri-*ortho*-cresyl phosphate (TOCP). He suggested that the characteristic flaccid paralysis observed in TOCP poisoning might be due to inhibition of the cholinesterase at the motor end-plates, leading to a local accumulation of acetylcholine. Hottinger and Bloch (1943) showed that acetylcholine hydrolysis by human and rabbit serum, liver, and brain was also inhibited by TOCP ; tributyrinase activity in serum and liver was also diminished, but alkaline phosphatase, trypsin, arginase, histidase, and pancreatic lipase were not affected. The demyelination observed peripherally and centrally in animals and men poisoned with TOCP, and the delay of about 14 days before the onset of symptoms, are difficult to explain on the basis of Bloch's suggestion that the causative lesion is cholinesterase inhibition at the motor end-plates. When this early work was done, selective substrates for the true and pseudo-cholinesterases were not employed. As Bloch's view implies an inhibition of the true cholinesterase, Earl and Thompson (1952a) studied the effect of TOCP *in vitro* on the true and pseudo-cholinesterases of blood, nervous tissue, and striated muscle from man, rabbit, hen, and rat. In man, the true cholinesterase in erythrocytes, muscle, and nervous tissue was not markedly inhibited by high concentrations of TOCP, but the pseudo-cholinesterases in nervous tissue and in serum were profoundly inhibited by relatively low concentrations. Further, in hens poisoned with TOCP the pseudo-cholinesterases of plasma, brain, and spinal cord were markedly diminished, even one day after poisoning, whereas the true cholinesterase in these tissues was relatively unaffected (Earl and Thompson, 1952b). Mendel and Rudney (1944) had also briefly reported that in the rat oral administration of TOCP lowers the serum cholinesterase but not the true cholinesterase.

From these findings, and since pseudo-cholinesterase appears to be associated particularly with the white fibre tracts in the brain (Ord and Thompson, 1952) and to be the predominant cholinesterase in peripheral myelinated nerves and in the white tracts of the spinal cord (Thompson and Webster, unpublished results), we have suggested (Earl and Thompson, 1952a) that this enzyme may be connected with the maintenance of the myelin sheaths of nerve fibres, and that its inhibition by TOCP might be causally related to the demyelination and consequent paralysis.

We have therefore examined the action of this compound on some enzymes which might be regarded as relevant to this possible action. Since defects in pyruvate metabolism may accompany peripheral neuritis due to thiamine deficiency, or certain other causes, the abilities of brain preparations from normal and TOCP-intoxicated hens to oxidize both glucose and pyruvate have been compared. As adrenaline and noradrenaline are also concerned with transmission processes, and as brain contains an amine oxidase (Pugh and Quastel, 1937; Blaschko, Richter and Schlossmann, 1937), the effect of TOCP on the ability of brain homogenates to oxidize tyramine both *in vitro* and *in vivo* has also been studied. Following the demonstration by Jansen, Nutting, Jang, and Balls (1949) that the activity of trypsin and chymotrypsin is inhibited by small concentrations of di*iso*propyl fluorophosphonate (DFP), trypsin has also been examined for TOCP-sensitivity. Finally, experiments have been carried out with lecithinases and with Sloane-Stanley's (1951) cephalin-splitting enzyme from brain tissue.

## METHODS AND MATERIALS

*Pseudo-cholinesterase* activity in homogenates of hen brain and spinal cord has been assayed as

described previously (Earl and Thompson, 1952a), using butyrylcholine perchlorate (0.03 M) as substrate.

*Glucose and Pyruvate Oxidation.*—Extra oxygen uptake of hen brain "brei" was measured by the Warburg technique, using glucose (0.014 M) or sodium pyruvate (0.018 M) as substrate.

*Amine Oxidase* activity was determined by measuring the extra oxygen uptake of hen spinal cord homogenates in the presence of 0.01 M-tyramine hydrochloride (L. Light & Co., Ltd.) as substrate.

*Trypsin* (Hopkin & Williams, Ltd.) activity was measured both by the method of Charney and Tomarelli (1947), and by micro-Kjeldahl estimation of trichloroacetic acid-soluble nitrogen liberated from casein (British Drug Houses, Ltd., light white soluble), using the Markham apparatus (1942).

*Lecithinases.*—(1) A dry preparation of the α-toxin of *Cl. Welchii* was used as enzyme source. Activity was measured by a modification of the technique of Zamecnik, Brewster, and Lipmann (1947), using a bicarbonate buffer (*p*H 7.3) containing 0.016 M-$NaHCO_3$ gassed with 95% $N_2$+5% $CO_2$. Approximately 10 LD50 (mouse) of toxin were used in each Warburg flask with a final concentration of 3% ovolecithin as substrate. $CaCl_2$ was not used to activate the system, as it was found that the $CO_2$ output became non-linear in less than 5 min. in the presence of $Ca^{++}$.

(2) A 1-in-6 (w/v) homogenate of rat pancreas in $NaHCO_3$ buffer at *p*H 7.5 with 1% ovolecithin as substrate was used as an alternative enzyme source.

*Brain Cephalinase.*—A 1-in-50 homogenate of guinea-pig brain in Krebs bicarbonate buffer (with $KH_2PO_4$ omitted) was used as enzyme source. Activity was measured under the conditions described by Sloane Stanley (1951), using 4% (w/v) crude cephalin as substrate. All enzyme estimations were carried out in duplicate.

*Tributyrinase* activity was estimated in homogenates of hen spinal cord as described previously (Earl and Thompson, 1952a).

*Butyrylcholine Perchlorate* (BuCh).—Choline perchlorate, prepared from choline chloride (Hopkin & Williams, Ltd.), was refluxed with excess of freshly distilled butyryl chloride (British Drug Houses, Ltd.). Recrystallized from ethanol: m.p. 72–74° (Found: C, 39.5; H, 7.2; N, 5.4; Cl, 12.8. Calc. for $C_9H_{22}O_6NCl$: C, 39.5; H, 7.4; N, 5.1; Cl, 13.0). Micro-analysis by Drs. Weiler and Strauss, Oxford.

*Sodium pyruvate* was prepared from crude pyruvic acid (British Drug Houses, Ltd.) by the method of Robertson (1942).

*Sulphanilamide-azocasein* was prepared by the method of King (1951).

*Lecithin* was prepared from egg yolk according to Macfarlane and Knight (1941).

*Cephalin* was prepared from human brain by the method of Folch (1942).

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

*Diisopropyl fluorophosphonate* (DFP) was kindly provided by the Experimental Station, Porton.

## Results

*Glucose and Pyruvate Metabolism.*—Experiments were first carried out to determine the effect of TOCP *in vitro* on the oxidation of glucose and pyruvate by brain. The human brain tissue (cerebral cortex) was obtained fresh from operation; whole hen and pigeon brains were used, about 100 mg. of the mixed minced tissue from each species being added to each Warburg flask. No significant effect on oxygen uptake was produced by the addition to the system of TOCP in concentrations up to 167 μg./ml., whereas a concentration of 67 μg./ml. produced about 90% inhibition of pseudo-cholinesterase activity in human brain (Earl and Thompson, 1952a).

The results of the *in vivo* experiments are given in Table I. Estimations of the levels of glucose

TABLE I

OXIDATION OF GLUCOSE AND PYRUVATE BY BRAIN *BREI* FROM HENS POISONED WITH TOCP

(Dose: 1 ml./kg. *per os.*) ($O_2$ uptake measured over 0–30 min.)

| | Days after Poisoning | Extra $O_2$ Uptake (μl. $O_2$/g./hr.) in presence of | |
|---|---|---|---|
| | | Glucose | Pyruvate |
| Normal hens .. | — | 1,365* | 1,515* |
| | — | 1,140 | 1,115 |
| | — | 790 | 1,175 |
| | — | 940 | 1,095 |
| | — | 1,028 | 1,465 |
| | — | 1,125 | 1,245 |
| | — | 1,315 | 1,655 |
| | Mean: | 1,100 | 1,325 |
| TOCP-poisoned.. | 1 | 1,205 | 1,310 |
| | 2 | 1,415 | 1,390 |
| | 5 | 965 | 1,160 |
| | 8 | 1,295 | 1,325 |
| | 12† | 1,217 | 1,385 |
| | 15‡§ | 985 | 957 |
| | 19§ | 1,495 | 1,705 |
| | Mean: | 1,225 | 1,320 |

* $O_2$ uptake measured over 0–54 min. † Slight weakness of feet and legs. ‡ $O_2$ uptake measured over 0–45 min. § Severe leg weakness; wings slightly affected.

and pyruvate oxidation were carried out in brain preparations from normal hens and from hens killed at intervals up to 19 days after poisoning; in the birds killed 1–8 days after poisoning there were no abnormal signs; in one killed after 12 days there was slight leg weakness, while those killed after 15 and 19 days showed severe weakness of the legs, the wings also being slightly weak. It will be seen that the rate of glucose

oxidation in the poisoned brains was not significantly affected, 2 of the 7 levels found in the normal birds being lower than any in the poisoned series. Apart from one of the birds killed at 15 days the rate of pyruvate oxidation also agrees well with the rates found for the normal tissue.

In order to ensure that the TOCP had in fact been absorbed and had reached the nervous system, especially in those birds which were killed before the stage of paralysis became manifest, estimation of the pseudo-cholinesterase activity of the brain was also carried out in the poisoned birds. It was found to be significantly diminished in all poisoned birds as compared with previously determined normal values (Earl and Thompson, 1952b).

In support of these findings it should be mentioned that in earlier experiments in collaboration with Dr. C. L. Joiner it was found that the blood pyruvate level was normal in pigeons poisoned with TOCP and showing signs of paralysis. A preliminary report on these observations on pyruvate metabolism in TOCP poisoning has already been made (Thompson, 1952).

*Amine Oxidase.*—Three experiments were carried out on the amine oxidase activity of hen spinal cord, when tyramine hydrochloride was used as substrate. A concentration of TOCP sufficient to produce 95% inhibition of pseudo-cholinesterase activity in this tissue (Earl and Thompson, 1952a) produced no inhibition of amine oxidase activity which could be regarded as significant. In support of these *in vitro* observations we have measured the amine oxidase activity of the spinal cords of three hens poisoned with TOCP; in two of these, poisoned 24 hours before killing by the oral administration of 1 ml. TOCP/kg., the extra $O_2$ consumption found was 184 and 283 µl./g./hr., and in the third, poisoned 48 hours before killing, 226 µl./g./hr., i.e., the levels of oxidation were in each hen above the mean found for the three normal birds used for the *in vitro* experiments.

*Trypsin.*—Six experiments were carried out with an impure trypsin preparation (Hopkin & Williams, Ltd.) in order to compare its sensitivities to DFP and TOCP. Varying concentrations of DFP ranging from $10^{-6}$ to $10^{-3}$M were added to tubes containing a final concentration of 0.25% (w/v) trypsin and 1.25% (w/v) sulphanilamide-azocasein in 0.5% $NaHCO_3$ (final *p*H 8.1), and incubated for five minutes at 38°. The rate of proteolysis was determined colorimetrically by addition of 0.5N-NaOH to trichloroacetic acid filtrates of the reaction mixture (Charney and Tomarelli, 1947). The $I_{50}$ value of DFP for trypsin under these conditions was found to be $2.5 \times 10^{-5}$M. Amounts of TOCP up to 2,000 µg./ml. under the same conditions were found to cause no inhibition.

Two further experiments were carried out using the micro-Kjeldahl technique for the estimation of trichloroacetic acid-soluble N, in which DFP and TOCP were added to a reaction mixture containing 0.025% trypsin and 1.25% casein in 0.5% $NaHCO_3$ (final *p*H 8.4), which was then incubated for 10 minutes at 38°. $10^{-4}$M-DFP produced 86% and 91% inhibition of activity in the two experiments, TOCP again giving no inhibition.

In agreement with Kies and Schwimmer (1942) we have been unable to demonstrate detectable tryptic activity in our brain preparations; our experiments were carried out at *p*H 8.1, and we have not studied the proteolytic system with an optimal activity at *p*H 7.4 described by Ansell, Williams, and Richter (1952).

*Lecithinase.*—In two experiments carried out with a type C lecithinase present in *Cl. Welchii* α-toxin under the conditions described above it was found that the addition of 1,000 µg. TOCP/3 ml. produced no effect. The lecithinase present in rat pancreas was also unaffected by TOCP.

Rat brain was also examined for lecithinase, but we were unable to demonstrate any activity under our conditions.

*Cephalinase.*—We have confirmed the finding of Sloane-Stanley (1951) that guinea-pig brain homogenates contain a cephalin-splitting enzyme; in two experiments we obtained activities, over the first five minutes of the reaction, corresponding to 1,100 and 1,200 µl. $CO_2$/g. brain/hr., which are of the same order as that reported by Sloane-Stanley. These high rates of reaction declined rapidly, and we found almost linear activities of 42 and 83 µl. $CO_2$/g./hr. when measured over 20–80 minutes from the start of the reaction.

TOCP in a concentration of 1,000 µg./3 ml. caused no inhibition of either the initial or the later rates of reaction.

*Tributyrinase.*—In view of Hottinger and Bloch's (1943) report that tributyrin hydrolysis by liver and serum is sensitive to TOCP, Earl and Thompson (1952a) compared the inhibition *in vitro* by TOCP of the tributyrinase of the central nervous system of man and the rabbit with the inhibition of pseudo-cholinesterase. They found that with human tissues concentrations of TOCP which produce 75–99% inhibition of pseudo-cholinesterase in the

nervous system produce only 20–30% inhibition of tributyrinase in these tissues; using rabbit brain, tributyrinase activity was also less sensitive to inhibition than was the pseudo-cholinesterase.

To support these *in vitro* observations, however, we thought it desirable to study tributyrinase levels in poisoned animals. After poisoning hens by a single dose of TOCP (1 ml./kg. by mouth), the birds were killed, the spinal cords removed, and the levels of pseudo-cholinesterase and tributyrinase determined. These levels were then compared with those found in normal birds. The results are shown in Table II. It will be seen that, although the cord tributyrinase is affected, there was never more than 57% inhibition from a "normal" level obtained on the cords from six normal birds. Further, in the same animals the cord pseudo-cholinesterase was always inhibited to a greater degree, the mean of the six experiments giving 45% inhibition of tributyrinase as compared with 70% inhibition of pseudo-cholinesterase.

TABLE II

COMPARISON OF LEVELS OF ACTIVITY OF PSEUDO-CHOLINESTERASE (ChE) AND TRIBUTYRINASE (TB) IN SPINAL CORDS OF HENS POISONED WITH TOCP

(1 ml./kg. *per os*)

| | Days after Poisoning | Activity ($\mu$l. $CO_2$/g./hr.) | | % Reduction from Mean Normal Values | |
|---|---|---|---|---|---|
| | | ChE | TB | ChE | TB |
| Normal (mean) | — | 1,700* | 1,894† | — | — |
| Poisoned | 1 | 786 | 1,232 | 53 | 35 |
| | 1 | 525 | 1,120 | 67 | 41 |
| | 2 | 295 | 812 | 79 | 57 |
| | 2 | 348 | 1,014 | 76 | 46 |
| | 3 | 407 | 1,150 | 73 | 39 |
| | 9 | 471 | 910 | 69 | 52 |
| | | | Mean: | 70 | 45 |

* Mean value obtained from 18 normal birds.
† Mean value obtained from 6 normal birds.

It should be added that we have obtained evidence indicating that the tributyrinase of hen nerve tissue is a distinct enzyme from the pseudo-cholinesterase. Thus, $0.9 \times 10^{-6}$M-eserine produces 82% inhibition of butyrylcholine hydrolysis but no inhibition of tributyrin hydrolysis; further, butyrylcholine and tributyrin together are hydrolysed by brain homogenates at a rate which is 87% of the sum of the rates of hydrolysis of the two substrates separately.

## DISCUSSION

Although only a few enzymes have been studied, this brief survey has demonstrated that, unlike the demyelinating lesions that can accompany vitamin $B_1$ deficiency (Swank, 1940; Street, Zimmerman, Cowgill, Hoff, and Fox, 1941; Swank and Prados, 1942), the development of the demyelination produced by tri-*ortho*-cresyl phosphate is not accompanied by any demonstrable defect in the ability of the nerve tissue to oxidize pyruvate or glucose. It would seem therefore that a fundamentally different biochemical disturbance must initiate and accompany the development of the histological changes.

TOCP was without effect on lecithinase and cephalinase activity, enzymes which might be presumed to play some role in connection with the metabolism of myelin lipids.

Apart from pseudo-cholinesterase, tributyrinase is the only other enzyme which has so far been found to be inhibited by TOCP, but, although homogenates of the spinal cords of hens poisoned with this compound show a partial inactivation of this enzyme, pseudo-cholinesterase activity of the cord appears to be regularly inhibited to a greater degree. Moreover, in human tissues (and man appears to be highly sensitive to poisoning by TOCP) brain tributyrinase is very much less sensitive than pseudo-cholinesterase to inhibition *in vitro* by this substance. Although pseudo-cholinesterase inhibition by TOCP cannot yet be regarded as playing a causative role in the demyelination and paralysis brought about by the ingestion of this compound, it may be concluded that, of the various enzyme systems so far studied in nerve tissue, pseudo-cholinesterase is the one most sensitive to inactivation by tri-*ortho*-cresyl phosphate.

## SUMMARY

1. The effect of varying concentrations of tri-*ortho*-cresyl phosphate on a number of widely different enzyme systems has been studied under both *in vitro* and *in vivo* conditions.

2. Glucose and pyruvate oxidation by brain preparations has been shown to be unaffected.

3. Trypsin, brain amine oxidase, pancreatic lecithinase, and brain cephalinase have also been found to be insensitive to this compound.

4. The tributyrinase activity of the spinal cords of hens poisoned with TOCP has been found to be moderately reduced, but in every experiment the pseudo-cholinesterase of the spinal cord has been inhibited to a greater degree.

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### References


Ansell, G. B., Williams, R. B., and Richter, D. (1952). *Biochem. J.*, **50**, xxix.

Blaschko, H., Richter, D., and Schlossmann, H. (1937). Ibid., **31**, 2306.

Bloch, H. (1941). *Helv. med. Acta*, **8**, Suppl. 7, 15.

Charney, J., and Tomarelli, R. H. (1947). *J. biol. Chem.*, **171**, 501.

Earl, C. J., and Thompson, R. H. S. (1952a). *Brit. J. Pharmacol.*, **7**, 261.

—— —— (1952b). Ibid., **7**, 685.

Folch, J. (1942). *J. biol. Chem.*, **146**, 35.

Hottinger, A., and Bloch, H. (1943). *Helv. chim. Acta*, **26**, 142.

Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K. (1949). *J. biol. Chem.*, **179**, 189.

Kies, M. W., and Schwimmer, S. (1942). *J. biol. Chem.*, **145**, 685.

King, E. J. (1951). *Micro-analysis in Medical Biochemistry*, 2nd ed., p. 158. London: Churchill.

Macfarlane, M. G., and Knight, B. C. J. G. (1941). *Biochem. J.*, **35**, 884.

Markham, R. (1942). Ibid., **36**, 790.

Mendel, B., and Rudney, H. (1944). *Science*, **100**, 499.

Ord, M. G., and Thompson, R. H. S. (1952). *Biochem. J.*, **51**, 245.

Pugh, C. E. M., and Quastel, J. H. (1937). Ibid., **31**, 2306.

Robertson, W. B. (1942). *Science*, **96**, 83.

Sloane-Stanley, G. H. (1951). *Biochem. J.*, **49**, iv.

Street, H. R., Zimmerman, H. M., Cowgill, G. R., Hoff, H. E., and Fox, J. C. (1941). *Yale J. Biol. Med.*, **13**, 293.

Swank, R. L. (1940). *J. exp. Med.*, **71**, 683.

—— and Prados, M. (1942). *Arch. Neurol. Psychiat. Chicago*, **47**, 97.

Thompson, R. H. S. (1952). *Proc. roy. Soc. Med.*, **45**, 664.

Zamecnik, P. C., Brewster, L. E., and Lipmann, F. (1947). *J. exp. Med.*, **85**, 381.