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INHIBITION OF CHOLINESTERASES BY IRREVERSIBLE INHIBITORS *IN VITRO* AND *IN VIVO*

BY

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Two main types of cholinesterase inhibitors are known: (i) reversible inhibitors, such as the carbamic esters, eserine and neostigmine, and (ii) irreversible inhibitors, such as the alkylphosphates, tetraethylpyrophosphate (TEPP) and di*iso*propyl-fluorophosphonate (DFP). The kinetics of cholinesterase inhibition by reversible inhibitors are so complex that it is impossible to correlate cholinesterase inhibition *in vitro* with pharmacological action *in vivo*. The introduction of DFP by McCombie and Saunders (1946) and of TEPP by Schrader (1947) opened therefore a new line for physiological and pharmacological research. Both these compounds have been classified as irreversible inhibitors because neither dilution nor dialysis can restore cholinesterase activity once the enzyme has been inactivated (Brauer, 1948; Aldridge, 1950). Furthermore, the addition of acetylcholine or other substrates to cholinesterase in the presence of these irreversible inhibitors merely stops the progress of enzyme inactivation, but does not, as happens in the presence of eserine or neostigmine, partly reverse it (Burgen, 1949a). Now if these compounds really block cholinesterase irreversibly, and do not alter the speed of resynthesis of the enzyme, the time-course of recovery should be the same for all members of the group; but this is not so (see review by Koelle and Gilman, 1949).

To study this problem experiments have been carried out with TEPP. The effect of this compound *in vivo* has been compared with its inhibitory effect *in vitro*, and the time-course of inhibition has been followed under both these conditions. DFP has been used in these experiments only to help in the interpretation of the experimental results obtained with TEPP.

Materials and Methods

In all experiments male guinea-pigs weighing between 200 and 300 grammes and male albino Wistar rats weighing between 150 and 200 grammes were used. Human serum and red cell esterases were also taken for *in vitro* studies.

The activity of cholinesterase was estimated by the usual Warburg technique at 37° C. (Ammon, 1933). Washed red cells, serum, and homogenized tissues were diluted in $NaHCO_3$ (final concentration 0.025 M) and equilibrated with a 95 per cent N_2 + 5 per cent CO_2 gas mixture; 0.03 M-*dl*-acetyl-β-methylcholine and 0.01 M-benzoylcholine were used as substrates for true and pseudo-cholinesterase respectively.

In experiments where the inhibition of cholinesterase was studied *in vitro* washed red cells and unpurified serum were used as described above, but the tissue homogenates were centrifuged at 2,000 r.p.m. for 20 minutes and only the supernatant taken. This procedure

was adopted because the experiments of this type were designed to determine the sensitivity towards TEPP of cholinesterase itself under conditions where interference by other tissue constituents, especially cell rests, was reduced as far as possible without use of chemical methods for purification. The cholinesterase activity of the supernatant varied between 60 and 80 per cent of the non-centrifuged enzyme preparation.

The enzyme solutions were placed in the main compartment of the vessel, and substrate and inhibitor, each dissolved in 0.2 ml. of 0.025 M-$NaHCO_3$, were placed in separate side-arms. The total volume of fluid was always 3 ml. In all experiments corrections for non-enzymic hydrolysis of the substrate were applied, taking also into account changes of gas volume not due to substrate hydrolysis.

To study the duration of cholinesterase inhibition *in vitro* washed red cells, serum, and the supernatant of centrifuged tissue homogenates were diluted with $NaHCO_3$ (final concentration 0.01 M) or NaCl (final concentration 0.9 g. per 100 ml.). TEPP or DFP was then added in a concentration which produced a marked inhibition of cholinesterase activity and the samples incubated at 30° C. The enzyme activity was estimated at intervals corresponding to the *in vivo* experiments.

In the *in vivo* experiments TEPP or DFP was given by subcutaneous or intraperitoneal injection. The animals were killed after various intervals and the cholinesterase activity estimated by the method described above. Tissue homogenates were used without centrifugation.

The duration of cholinesterase inhibition was also followed in rats by the method described by Burgen (1949b), the threshold concentration of acetylcholine necessary to produce the secretion of red tears (chromodacryorrhoea response) being used as an indicator of the extent of enzyme inhibition.

Most of the experimental figures are expressed as percentages of untreated controls. Enzyme concentrations which produced at least 30 μl. CO_2 in 10 minutes were used in order to obtain significant results.

TEPP and DFP were made up as 5 per cent (v/v) stock solution in dry propylene glycol and from these an aqueous solution was made immediately before use.

RESULTS

Inhibition of cholinesterase in blood and tissues in vivo

Guinea-pig.—TEPP is a very potent inhibitor of cholinesterase, and its action *in vivo* has been investigated in detail by Burgen, Keele, and Slome (1949). In my experiments a dose of 0.05–0.1 mg./kg. produced marked symptoms of the muscarinic type of acetylcholine poisoning such as bradycardia, salivation, and diarrhoea, but only slight nicotinic symptoms, such as muscular twitching. The minimal lethal dose was about 0.1 mg./kg. if the inhibitor was given by subcutaneous injection, and 0.25 mg./kg. after intraperitoneal administration. A dose of 2 mg./kg. of atropine sulphate given half an hour before TEPP reduced the toxicity so that doses of TEPP up to 0.4 mg./kg. did not then cause death, in spite of very marked impairment of neuromuscular transmission. Atropine in such a dose blocked the muscarinic actions of acetylcholine, but had no inhibitory effect on cholinesterase as was shown in control experiments *in vitro*.

In Fig. 1 the reduction in the cholinesterase activity in blood and various organs of the guinea-pig after subcutaneous injection of TEPP is shown. Small doses of the inhibitor, e.g., 0.13 mg./kg., caused a variable degree of cholinesterase inhibition in the various tissues. These differences generally became less with higher concen-

trations of the inhibitor, when a state of saturation was reached. Brain cholinesterase was, however, much less inhibited than that in other organs; this might be due partly to the intervention of the " blood-brain barrier," and partly to the poor lipoid solubility of TEPP.

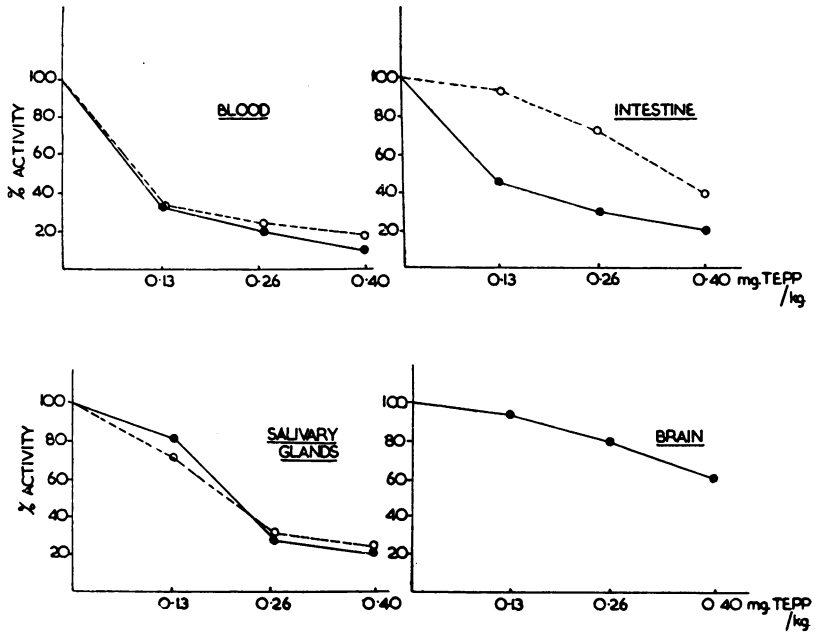

FIG. 1.—*In vivo*. Inhibition of cholinesterase in various tissues in guinea-pigs by TEPP. Ordinates: cholinesterase activity expressed as per cent of the cholinesterase activity of untreated animals. Abscissae: doses of TEPP in mg./kg. The inhibitor was given by subcutaneous injection 30 minutes after injection of 2 mg. atropine sulphate/kg. The animals were killed 30 minutes after the injection of TEPP. Each point represents the average cholinesterase activity of tissues from two animals, two untreated animals serving as controls. ●——● true cholinesterase. O- - - -O pseudo-cholinesterase.

Rat.—In rats the minimal lethal dose of TEPP was 0.2 mg./kg. for subcutaneous and 0.5 mg./kg. for intraperitoneal injection. The protecting effect of atropine was less marked. As far as the inhibition of cholinesterase by various concentrations of TEPP was concerned the result was similar to that obtained in guinea-pigs, except that the brain was inhibited to a somewhat greater extent.

In vitro *inhibition of cholinesterases prepared from blood and tissues*

Guinea-pig.—The next step was to correlate these *in vivo* findings with the inhibition achieved by TEPP *in vitro*. The results of experiments designed for this purpose are summarized in Table I.

The concentrations of TEPP which produce a 50 per cent inhibition in 20 minutes at 37° C. in equiactive enzyme preparations (for a given substrate) were chosen for comparison. As can be seen from the results in Table I the true cholinesterases in red cells, brain, and salivary glands were inhibited to the same extent by roughly

TABLE I

INHIBITION OF CHOLINESTERASES *in vitro*

Guinea-pig enzyme preparations		Concentrations of TEPP required to produce 50% inhibition in 20 min. at 37° C.	
		True cholinesterase Substrate: 0.03 M-acetyl-β-methylcholine	Pseudo-cholinesterase Substrate: 0.01 M-benzoylcholine
Red cells	0.2 ml.	7.8×10^{-9}	—
	0.4 ml.	8.7×10^{-9}	—
Serum	0.3 ml.	—	4.5×10^{-9}
	0.6 ml.	—	1.25×10^{-8}
Brain	50 mg.	5.3×10^{-9}	—
	100 mg.	6.0×10^{-9}	—
Salivary glands	7 mg.	—	6.4×10^{-10}
	14 mg.	—	1.0×10^{-9}
	80 mg.	5.2×10^{-9}	—
	160 mg.	6.2×10^{-9}	—
Intestine	65 mg.	—	2.7×10^{-8}
	130 mg.	6.4×10^{-8}	6.1×10^{-8}
	260 mg.	7.6×10^{-8}	—

equal concentrations of TEPP. Serum esterase was less sensitive to TEPP than the pseudo-cholinesterase of salivary glands. If the amount of enzyme was doubled higher concentrations of TEPP were necessary to produce the same degree of inhibition. This was particularly marked with the pseudo-cholinesterases.

The cholinesterases obtained from the intestine behaved quite differently, much higher concentrations of the inhibitor being necessary to achieve a 50 per cent inhibition. Such a difference might arise in the following ways:

(*a*) The enzymes in the different organs, though all classified as true or pseudo-cholinesterases, might differ in some respects, e.g., in their sensitivity to inhibition by TEPP.

(*b*) The inhibitor might be destroyed before combination with cholinesterase; this process of destruction might occur at different speeds in various organs.

(*c*) The inhibitor might combine not only with cholinesterase but also with other tissue constituents; such an " unspecific " combination could reduce the concentration of TEPP available for cholinesterase inhibition to different degrees in different organs.

A closer analysis of these three points gave the following results:

(*a*) The terms " true cholinesterase " and " pseudo-cholinesterase," as defined by Mendel and his co-workers (1943, 1944), are strictly applicable to the enzymes mentioned in Table I. For example, the pseudo-cholinesterase preparations from serum, salivary glands, and intestine showed identical properties, in that they all split acetylcholine about twice as fast as benzoylcholine, and that higher concentrations of acetylcholine had no depressant effect on the enzyme activity (taking

into account the effect of true cholinesterase which was present in these unpurified enzyme preparations). The true cholinesterase preparations from brain, salivary glands, and intestine behaved just like human red cells when *dl*-acetyl-β-methylcholine was used as substrate in various concentrations (Augustinsson, 1948).

Thus there is nothing to suggest that any significant differences exist among the true and pseudo-cholinesterases derived from the various sources, and there is no direct evidence that the enzymes in the preparations from different tissues are differentially inhibited by TEPP.

(*b*) In order to study the capacity of tissue homogenates to destroy TEPP it was first necessary to measure the rate of spontaneous hydrolysis of this substance in solution. TEPP, therefore, was made up in a concentration of 3×10^{-7} in 0.01 M-$NaHCO_3$ and incubated at 37° C. for varying periods. The activity of the inhibitor was reduced by 50 per cent after 3½ hours, and after 24 hours no significant anti-cholinesterase effect could be detected. Higher dilutions of TEPP showed a similar breakdown.

In two experiments TEPP in a concentration of 2×10^{-9} was incubated with 100 mg. intestine at 37° C. for 20 minutes. The mixture was then brought to a *p*H of 3.0–4.0, heated for 3 minutes at 95° C. (to destroy all cholinesterase activity), then cooled and the *p*H adjusted to 7.2. It was found that 12 and 20 per cent respectively of the original amounts of TEPP had disappeared as a result of these procedures. Control experiments showed a disappearance rate of about 5 per cent. The concentration of TEPP required to produce 50 per cent inhibition of the pseudo-cholinesterase in 65 mg. intestinal homogenate in 20 minutes was a hundred times that required to produce 50 per cent inhibition of the enzyme in 0.2 ml. of human serum in the same time. The relatively small increase in rate of breakdown of TEPP in the presence of intestinal tissue can only explain a very small fraction of this observed hundredfold difference.

(*c*) The experiments under (*a*) and (*b*) suggest that the small inhibitory effect of TEPP on the enzyme preparations obtained from intestine might be due to com-

TABLE II

EFFECT OF NON-SPECIFIC COMBINATION ON THE APPARENT SENSITIVITY OF UNPURIFIED ENZYME PREPARATIONS TO TEPP

Source of enzyme	Substrate	Concentration of TEPP which produced 50% inhibition in 20 min.
0.2 ml. human serum	Benzoylcholine	2.2×10^{-10}
0.04 ml. human red cells ..	Acetyl-β-methylcholine	3.2×10^{-9}
65 mg. guinea-pig intestine ..	Benzoylcholine	2.7×10^{-8}
65 mg. guinea-pig intestine + 0.2 ml. human serum	Benzoylcholine	3.0×10^{-8}
65 mg. guinea-pig intestine + 0.04 ml. human red cells ..	Acetyl-β-methylcholine	5.3×10^{-8}

bination of a large amount of inhibitor with other tissue constituents before it comes in contact with the cholinesterase. Experiments were therefore performed to study this possibility. From Table II it will be seen that a concentration of 2.2×10^{-10} TEPP is required to inhibit serum pseudo-cholinesterase by 50 per cent in 20 minutes. A concentration of 2.7×10^{-8} TEPP is required to inhibit a homogenate of 65 mg. intestine to the same degree. This difference in sensitivity is about a hundredfold. Since equiactive concentrations of both enzyme preparations were chosen, a concentration of roughly 2.5×10^{-9} TEPP would be expected to give a 50 per cent inhibition for a mixture of both. Actually 3×10^{-8} TEPP is necessary to produce this effect. Table II also shows that a similar result was obtained when human red cells were used instead of serum.

Similar results have been obtained with other enzyme preparations of the guinea-pig and with rats.

Duration of cholinesterase inhibition by TEPP in vivo

Most of the work on the actions of cholinesterase inhibitors *in vivo* has been concerned with the recovery of enzyme activity in blood. Only few authors have extended their work to the study of other organs. The only available information about the alkylphosphates relates to the duration of enzyme inhibition in blood, brain, and muscle (Freedman, Willis, and Himwich, 1949; Mazur and Bodansky, 1946). This work has therefore been extended, and the results are given in Fig. 2. It will be seen that after inhibition by TEPP cholinesterase reactivation *in vivo* proceeds rather rapidly, and at about the same rate in blood, brain, and intestine; in salivary glands, however, it is at least three times as fast.

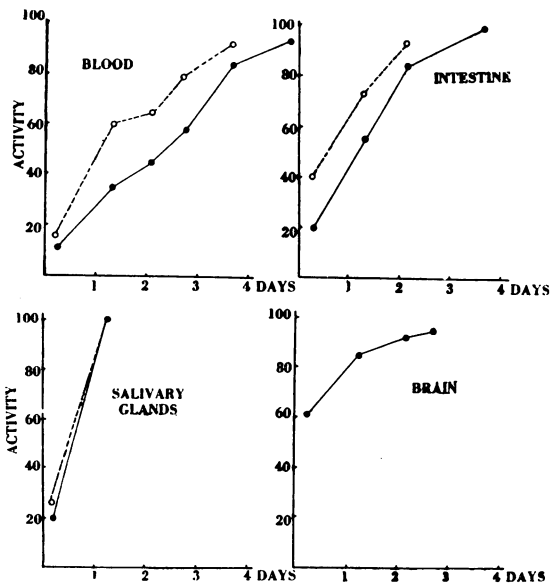

FIG. 2.—Duration of cholinesterase inhibition in guinea-pigs after subcutaneous injection of 0.4 mg. TEPP/kg.; 2.0 mg. atropine sulphate was given 30 minutes before the inhibitor. Ordinates: cholinesterase activity as per cent of control. Abscissae: days of observations. ●——● true cholinesterase. O- - - - - O pseudo-cholinesterase.

The difference in duration of action between DFP and TEPP *in vivo* was studied by means of the chromodacryorrhoea response in rats (Burgen, 1949b). Fig. 3 shows that, starting with comparable degrees of cholinesterase inhibition, recovery of normal sensitivity to acetylcholine occurred in six days after TEPP, but that after DFP only 50 per cent recovery occurred in ten days. If it is accepted that neither

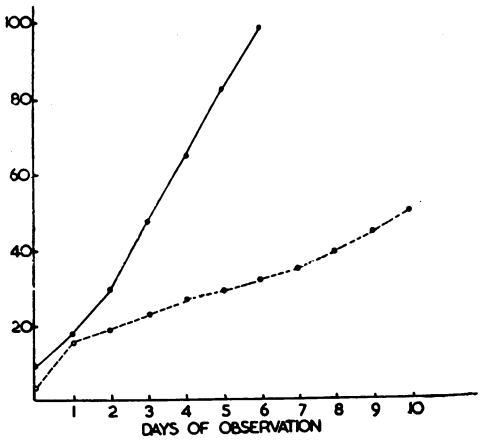

FIG. 3.—Duration of cholinesterase inhibition by DFP and TEPP in rats *in vivo* (chromodacryorrhoea response). 0.4 mg. TEPP/kg. and 4.0 mg. of DFP/kg. respectively were given by intraperitoneal injection. Ordinates: the amount of acetylcholine (as per cent of that in three tests before injection of inhibitor) which had to be given to stimulate the secretion of red tears. Abscissae: days of observations. ●———● acetylcholine threshold after TEPP. O- - - -O acetylcholine threshold after DFP.

DFP nor TEPP has any influence on cholinesterase resynthesis, this difference in enzyme recovery can only be explained by assuming that the TEPP-enzyme complex is not stable, or that the cholinesterase molecule (or another enzyme) can destroy TEPP after this complex has been formed. It is not possible to study this question further *in vivo*, so *in vitro* experiments were carried out to obtain further evidence. TEPP and DFP are both unstable in aqueous solutions, and, if red cells, serum, or tissue homogenates are incubated with an amount of TEPP or DFP sufficient to produce a marked reduction of esterase activity, any inhibitor not combined with active enzyme centres will be completely inactivated after incubation for twenty-four hours; it is thus possible to observe reactivation of the enzyme without previous dialysis. The activity of the control samples remained relatively constant during the period of observations, not more than 10 per cent loss of activity being observed. The results of such experiments are shown in Tables III and IV. It will be seen in Table III that in the presence of DFP no enzyme reactivation occurred *in vitro*. If TEPP, however, was used as an inhibitor (Table IV) the inhibition of cholinesterase

TABLE III

GUINEA-PIG CHOLINESTERASES INCUBATED WITH 10^{-7} DFP FOR VARIOUS PERIODS AT 30° C.

Source of enzyme	Activity as per cent of control after			
	1 hr.	24 hr.	48 hr.	72 hr.
Brain	22	8	7	8
Red cells	18	2	5	4

TABLE IV

REACTIVATION OF CHOLINESTERASES FROM VARIOUS SOURCES AFTER INHIBITION BY TEPP *in vitro*

Source of enzyme	Activity as per cent of control after incubation at 30° C. for				Activity expressed in μl. CO_2 produced in 10 min.	
	1 hr.	24 hr.	48 hr.	72 hr.	At beginning of experiment	After 72 hours' incubation
Guinea-pig:						
(1) 0.3 ml. red cells ..	15	33	49	65	7.1	33.2
(2) 100 mg. brain ..	5	22	45	58	4.4	46.4
(3) 0.3 ml. serum ..	8.5	31	43	55	3.1	19.8
(4) 20 mg. salivary glands	13	57	75	—	11.6	60.0 (after 48 hours' incubation)
Human blood:						
(5) 0.06 ml. red cells ..	2	26	42	55	0.96	24.75
(6) 0.2 ml. serum ..	11	14	17	16	5.1	7.3

proved to be reversible (except with human serum esterase). The reactivation was a real one and not due to a slowing down of enzyme inactivation as can be seen from Table IV. This confirms the results of Grob and Harvey (1949), who showed that the inhibition of red cell esterase by TEPP was reversible for the first twenty-four hours. Enzyme preparations obtained from rats, and human red cell esterase, behaved like those from guinea-pigs. The process of recovery was always fastest with homogenates of salivary glands, a finding which correlates well with the *in vivo* experiments. Mg^{++} ions activated this recovery process and Cu^{++} ions had an inhibitory effect. The enzyme reactivation is not related to bacterial action, because neither thymol nor other bacteriostatic agents had any significant influence.

If red cells, or one of the other enzyme preparations used for these experiments, were incubated in the presence of TEPP, and after 24 or 48 hours the same or a different type of cholinesterase was added, the process of enzyme recovery was not enhanced.

Discussion

The experiments here described have shown that TEPP inhibits both true and pseudo-cholinesterase activity *in vitro* to varying degrees in different tissues. The factors which influence the degree of inhibition *in vitro* are:

1. The concentration of inhibitor.

2. With irreversible inhibitors, the concentration of enzyme (Ackermann and Potter, 1949; Bain, 1949; Augustinsson and Nachmansohn, 1949).

3. Reduction in the effective concentration of inhibitor by the action of tissue constituents other than cholinesterase. This factor has been demonstrated by the finding that equiactive enzyme preparations from blood or tissues of the same animal differ widely in their sensitivity to TEPP. There is no evidence that any significant breakdown of TEPP occurs before it combines with the tissue constituents (including cholinesterase), and the reduction in the effective concentration of the inhibitor is most probably due to non-specific combination with tissue components (probably proteins) other than cholinesterase. Such a mechanism might

be of importance in relation to other anticholinesterases, and might account for the relative insensitivity of frog brain esterase to eserine (Hawkins and Mendel, 1946). Mazur and Bodansky (1949) concluded from their experiments with DFP that non-specific binding by proteins or other tissue constituents is of no importance in relation to cholinesterase inhibition, but their results were obtained on heat-denatured brain mixtures only and are therefore of doubtful significance.

The conditions of cholinesterase inhibition *in vivo* must take account of still other factors, such as distribution via the blood stream, passage from blood to tissue (especially important in the case of the brain), localization of cholinesterase in the tissue, and physical properties, such as the lipoid solubility of the inhibitor. With these points in mind, we are not surprised to find that there are discrepancies between the *in vitro* and *in vivo* measurements of the activity of TEPP.

Recovery of cholinesterase activity

The time-course of recovery of cholinesterase activity depends on the nature of the inhibitor used (Koelle and Gilman, 1949). With DFP the inhibition is long-lasting *in vivo* and irreversible *in vitro* (Fig. 3; Table III). With TEPP the duration of cholinesterase inhibition *in vivo* is much shorter than with DFP, and the action *in vitro* is reversible (Figs. 2 and 3; Table IV). The *in vitro* experiments have shown that the reactivation after TEPP is due either to dissociation of the enzyme-inhibitor complex or to destruction of the inhibitor by cholinesterase; there is no evidence for the intervention of a second enzyme, such as phosphatase. Mg^{++} ions have an activating effect and Cu^{++} ions slow down the process of enzyme reactivation. The true and pseudo-cholinesterases of blood, brain, intestine, and salivary glands of guinea-pigs and rats, and human red cells, all show reactivation after inhibition by TEPP. *In vivo* some differences are seen and salivary glands always recover first, in spite of the marked initial inhibition (Fig. 2).

Human serum esterase is exceptional. No significant recovery from inhibition by TEPP was observed *in vitro*, and *in vivo* little difference exists between the time-course of recovery after inhibition by TEPP and DFP respectively (Freedman, Willis, and Himwich, 1949; Mazur and Bodansky, 1946).

SUMMARY

1. The degree of *in vivo* inhibition of cholinesterase in guinea-pigs and rats produced by small concentrations of TEPP varies widely among different tissues. With higher concentrations of the inhibitor these differences disappear almost completely, brain being an exception.

2. The inhibition of cholinesterase by TEPP *in vitro* depends on the concentration of this inhibitor, the concentration of enzyme, and the amount of inhibitor which combines with tissue constituents other than cholinesterase (e.g., proteins). The correlation between *in vivo* and *in vitro* experiments is discussed.

3. The inhibition of cholinesterase by DFP *in vitro* is irreversible. The inhibition by TEPP *in vitro* is reversible, except with the enzyme of human serum. This reactivation is thought to be due either to a dissociation of the enzyme-inhibitor

complex or to destruction of the inhibitor after combination with cholinesterase. Enzyme recovery is favoured by Mg^{++} and hindered by Cu^{++}; it proceeds at different speeds in enzyme preparations from different organs.

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