Biochemical Pharmacology, 1960, Vol. 3, pp. 184–189. Pergamon Press Ltd., Printed in Great Britain.

INHIBITION OF CHOLINESTERASE WITH METHYLFLUOROPHOSPHORYLCHOLINE AND CARBOCHOLINE: SPONTANEOUS RETURN OF ACTIVITY

T. FREDRIKSSON and G. TIBBLING

Research Institute of National Defence, Department 1,
Sundbyberg 4, Sweden

(*Received* 9 *November* 1959)

Abstract—The spontaneous reversal of cholinesterase activity has been studied following inhibition with methylfluorophosphorylcholine, methylfluorophosphorylcarbocholine (3 : 3-dimethylbutoxyphosphoryl fluoride) and *iso*propoxymethylphosphoryl fluoride (Sarin). Aceto- and butyrocholinesterase have been used as enzyme sources, and the reversal of enzyme activity has been studied at 4 °, 25 ° and 35 °. Inhibition produced by the two phosphorylcholines is readily reversible in contrast to that produced by Sarin. This is explained in terms of formation of an enzyme–inhibitor complex of another type than the one produced by Sarin. The results also supports the theory that butyrocholinesterase lacks an anionic site, and they confirm the previous observation that in intact animals methylfluorophosphorylcholines produce symptoms of shorter duration than ordinary organophosphorus cholinesterase inhibitors.

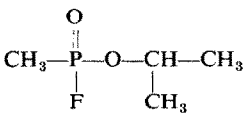
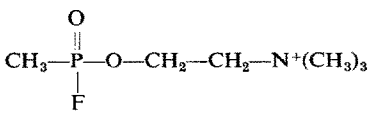
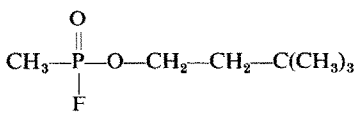
PREVIOUS studies[3, 4] of methylfluorophosphorylcholines (Table 1) gave the impression that animals intoxicated with these compounds showed symptoms which were of shorter duration than those produced by ordinary organophosphorus cholinesterase inhibitors, such as Sarin. Furthermore, cumulative effects could not be demonstrated in repeated toxicity tests on mice. It was therefore assumed that the enzyme inhibition was more reversible in the case of methylfluorophosphorylcholines, probably due to a more rapid spontaneous hydrolysis of the enzyme–inhibitor complex.

However, Enander[2] could not, in experiments *in vitro*, reactivate human erythrocyte cholinesterase inhibited by methyl fluorophosphorylcholines with the aid of a series of reactivators. On the other hand, it was possible to reverse neuromuscular block in the rat nerve–diaphragm preparation with different reactivators.[5] However, enzyme studies on homogenized rat diaphragm muscle could not reveal a reactivation of acetocholinesterase inhibited by methylfluorophosphorylcholine, while Sarin inhibited enzyme could be reactivated.[11] Thus, the reversal of the neuromuscular block seems to be produced by mechanism other than reactivation of the enzyme activity, possibly due to interaction with receptors.

Methylfluorophosphorylcholines are considered to produce a phosphorylation of the esteratic site of acetocholinesterase with liberation of fluoride ions.[10] But owing to their positively-charged nitrogen group they probably also react with the anionic site of the enzyme with the formation of an ionic bond.[10] This may explain why they are very potent acetocholinesterase inhibitors, and this interaction with the anionic site may give the enzyme–inhibitor complex particular properties in regard to stability.

In view of this it seemed to be of interest to investigate the spontaneous hydrolysis of the enzyme–inhibitor complex with methylfluorophosphorylcholine as inhibitor and make comparisons with Sarin, using both aceto- and butyrocholinesterase as enzyme sources. It was also decided to use methylfluorophosphorylcarbocholine as

TABLE 1.

Substance	Formula	pI_{50}*	
		Erythrocytes	Plasma
Sarin		8·8	8·4
Methylfluorophosphorylcholine		10·0	8·4
Methylfluorophosphorylcarbocholine		9·0	8·3

* From Tammelin[9].

inhibitor, an analogue to the former compound but containing a carbon atom instead of the quaternary nitrogen. This compound, though having a closely similar spacial arrangement, can be assumed to be considerably less attracted to the anionic site (Table 1).

METHODS

Enzyme sources. Human erythrocytes were used as source of acetocholinesterase and separated from fresh, heparinized blood by centrifugation. They were washed three times in 0·9% saline and then haemolysed in distilled water, the original volume being diluted ten times. Finally the sample was diluted with an equal volume of Michel's buffer (0·02 M sodium barbital, 0·004 M KH_2PO_4, 0·6 M KCl and 0·1 N HCl to pH 8·14).

As source of butyrocholinesterase a preparation of Cohn's fraction 4 : VI was chosen (manufactured by Kabi AB, Stockholm, Sweden). Twenty mg were diluted in equal parts of distilled water and Michel's buffer to a final volume of 100 ml.

Inhibitors. The inhibitors used, synthetized in this institution, appear in Table 1 together with their pI_{50}-values (the negative logarithm of the molar concentration causing 50 per cent inhibition of enzyme activity). They were diluted in the enzyme solutions to a final concentration of $5 \cdot 10^{-7}$ M, a concentration found to cause complete inhibition of the enzyme activity. The disappearance of the excess of inhibitor, i.e. its hydrolysis, was controlled in the following way. Samples were withdrawn at 24 hr intervals and mixed with equal volumes of the enzyme stock solutions. The enzymic

activity of this mixture was then measured. The excess of inhibitor was regarded as lost when there was no further decrease in enzyme activity.

Enzyme activity determinations. The enzyme activities were estimated with the electrometric method described by Tammelin.[9] Acetylcholine iodide (final concentration $0{\cdot}73 \times 10^{-2}$ M) was used as substrate, and all measurements were performed at 25 ° following incubation during 30 min. All enzyme activity values were calculated as percentages of the activity of the enzyme stock solutions. Corrections for the spontaneous hydrolysis of the substrate were not found to be necessary.

General procedure. The enzyme stock solutions and the test solutions were stored in darkness at the following temperatures: 4 °, 25 ° and 35 °. At 24 hr intervals samples were withdrawn and the activities determined (two independent measurements were always performed). The pH of the solutions was also determined daily with the aid of a type pHM 23 c pH-meter (manufactured by Radiometer, Copenhagen). Two independent series of experiments were performed.

RESULTS

Spontaneous loss of enzyme activity following storage

There was a slight loss in enzyme activity of the stock solutions following storage, particularly at the two higher temperatures, and the acetocholinesterase preparation was the least stable (Table 2). For this reason daily determinations of the enzyme

TABLE 2. SPONTANEOUS LOSS OF ENZYME ACTIVITY FOLLOWING STORAGE AT DIFFERENT TEMPERATURES, EXPRESSED IN PER CENT PER 24 HR

Temperature	(°C)	4	25	35
Acetocholinestrase	(%)	1–2	6–7	8–9
Butyrocholinesterase	(%)	<1	3–4	4–5

activity of the stock solutions, stored at the same temperatures as the test solutions, were performed. These values were regarded as 100 per cent values for the relevant temperature and time and used as the basis for the other calculations.

Variations in the pH of stock solutions following storage

The stock solution of the acetocholinesterase preparation had initially a pH of 8·03–8·05, and the pH of the butyrocholinesterase preparation was 8·10–8·12. Following storage at 4 ° there was a daily decrease in pH of about 0·03–0·04 units in case of the acetocholinesterase preparation, while the decrease in pH did not amount to more than 0·04–0·06 units during 10 days in the case of the butyrocholinesterase preparation. At 25 ° the daily decrease was, respectively, 0·1 and 0·03 units, and about the same decreases were also found following storage at 35 °.

Disappearance of the excess of inhibitor (Table 3)

The excess of methylfluorophosphorylcholine was found to have disappeared within 24 hr following storage at all the different temperatures. In case of methylfluorophosphorylcarbocholine the excess was found to be lost within 48 hr at 4 ° and within 24 hr at 25 ° and 35 °. The excess of Sarin disappeared within 5 days

following storage at 4 ° and within 24 hr following storage at the other two temperatures.

Spontaneous return of enzyme activity following inhibition

These results are summarized in Figs. 1–3. Following storage at 4 ° for 8 days there is full spontaneous return of enzyme activity in only two cases; i.e. with butyrocholinesterase and methylfluorophosphorylcholine and -carbocholine as inhibitors

TABLE 3. CALCULATED HALF-LIFE PERIODS ($t_{\frac{1}{2}}$) AND TIME UNTIL DISAPPEARANCE OF EXCESS OF INHIBITOR AT DIFFERENT TEMPERATURES

	4 °		25 °		35 °	
	$t_{\frac{1}{2}}$	Disappearance of excess	$t_{\frac{1}{2}}$	Disappearance of excess	$t_{\frac{1}{2}}$	Disappearance of excess
Methylfluorophosphorylcholine	2–2·5 hr	<24 hr	6–8 min	<24 hr	2–2·5 min	<24 hr
Methylfluorophosphorylcarbocholine	55–70 hr	<48 hr	2–2·5 hr	<24 hr	30–40 min	<24 hr
Sarin	70–90 hr	<5 days	4–5 hr	<24 hr	1–1·5 hr	<24 hr

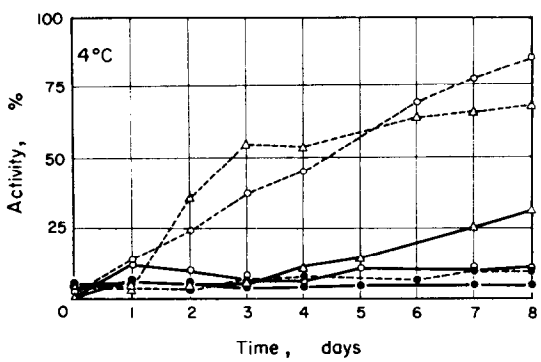


FIG. 1. Spontaneous return of enzyme activity following storage at 4 °: ——— acetocholinesterase, - - - - - butyrocholinesterase. ● Sarin (*iso*propoxymethylphosphoryl fluoride). ○ methylfluorophosphorylcholine. ▲ methylfluorophosphorylcarbocholine.

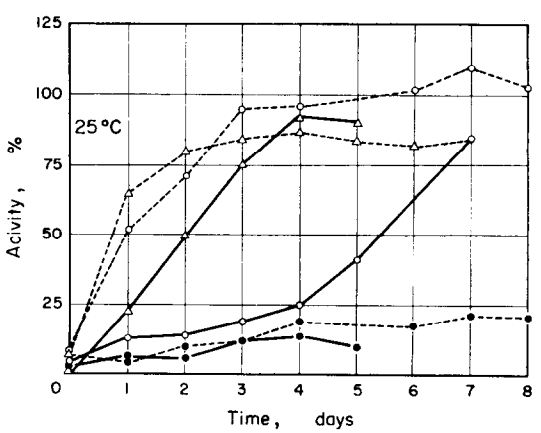


FIG. 2. Spontaneous return of enzyme activity following storage at 25 °. Symbols as in Fig. 1.

(Fig. 1). Acetocholinesterase inhibited with the carbocholine analogue shows a partial return of activity. Following storage at 25 ° there is return of activity except when Sarin is used as inhibitor (Fig. 2), and the reversal of inhibition is rapid in the other four cases with one exception, i.e. acetocholinesterase inhibited with methylfluorophosphorylcholine. The same tendency is found following storage at 35 °, but here is also some return of the enzyme activity in the case of butyrocholinesterase inhibited with Sarin (Fig. 3).

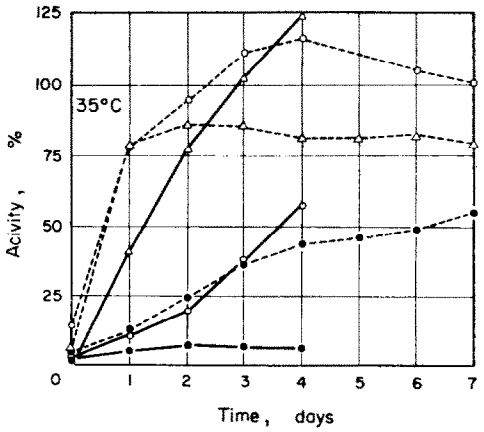


FIG. 3. Spontaneous return of enzyme activity following storage at 35 °. Symbols as in Fig. 1.

DISCUSSION

Following storage there is a spontaneous loss of enzyme activity and a decrease in pH of the stock solutions; the exact nature of these two processes will not be discussed in this paper. These changes are, however, of importance for the present measurements. The decrease in pH means that the sensitivity of the method used to record enzyme activity also decreases, but only in the case of acetocholinesterase.[6] Errors resulting from this have been reduced by performing daily measurements of the activity of the stock solutions and using these activity values as a basis for the calculations. These corrections account also for spontaneous loss of enzyme activity.

Larsson[7] has determined the second-order rate constants for the alkaline hydrolysis (pH 8·5) of the compounds at 25 ° and 35 °. From these values the half-life periods have been calculated, corrected for actual pH, temperature and ionic strength (Table 3). In those cases, where our method of estimating the rate of disappearance of the excess of inhibitor is sensitive enough, the loss is somewhat faster than the corresponding calculated values indicate. It can be assumed that this is due to the presence of enzymes catalysing the hydrolysis, i.e. the cholinesterase itself and/or phosphorylphosphatases.

Osterbaan *et al.*[8] have shown that when DFP reacts with butyrocholinesterase the fluorine is first split off and then one of the *iso*propoxy groups, presumably resulting in two linkages between the phosphorus atom and the enzyme. It is reasonable to assume that an analogous reaction also may occur in the case of Sarin, i.e. the fluorine and the alkoxy group are split off. The methyl group is less reactive than the alkoxy group, and it is therefore unreasonable to suppose that it will be displaced.

If the two phosphorylcholines react in the same way, i.e. by splitting off the flourine and the choline or carbocholine group, this would result in a phosphorylated enzyme, being the same for both compounds and identical with the one produced by Sarin. This assumption means that the spontaneous return of enzyme activity would occur at the same rate in all the three cases. As this is not the fact, the phosphorylcholines must produce a phosphorylated enzyme of different structure. This interpretation is also supported by the fact that acetocholinesterase inhibited with phosphorylcholines could not be reactivated with PAM, while Sarin inhibited enzyme could be reactivated.[2] It thus seems as if the phosphorylcholines only react with the enzyme by splitting off the flourine, leaving the alkoxy group intact, which was originally suggested by Tammelin.[10] This simple linkage to the enzyme may explain the comparatively rapid spontaneous return of enzyme activity.

The rate of return of enzyme activity is the same for both phosphorylcholines, when butyrocholinesterase is used as enzyme source. This indicates that the compounds react with this enzyme in an analogous way. When acetocholinesterase, however, is used as enzyme source, the return of enzyme activity occurs at different rates, suggesting a different type of reaction with the enzyme. This may be explained by the choline analogue forming an ionic bond with the anionic site of the enzyme, owing to its quaternized structure. The results thus also support the theory that butyrocholinesterase lacks an anionic site.[1] This conclusion can also be drawn from the pI_{50}-values[9] (Table 1). The slight difference in rate of return of activity between carbocholine inhibited butyro- and acetocholinesterase may depend on its polarized structure, resulting in a weak attraction to the anionic site of the latter enzyme.

Finally, the present results are consistent with the initial observation[3, 4] that phosphorylcholines produce symptoms of considerably shorter duration than other organophosphorus cholinesterase inhibitors.

Acknowledgement—We wish to express our thanks to Prof. G. Ljunggren for his kind interest in this investigation and to Miss Britt Nilsson for skilful technical assistance.

REFERENCES

1. D. H. Adams and V. P. Whittaker, *Biochem. Biophys. Acta* **4**, 543 (1950).
2. J. Enander, *Acta Chem. Scand.* **12**, 780 (1958).
3. T. Fredriksson, *Arch. Int. Pharmacodyn.* **113**, 101 (1957).
4. T. Fredriksson, *Arch. Int. Pharmacodyn.* **115**, 474 (1958)'
5. T. Fredriksson and G. Tibbling, *Biochem. Pharmacol.* **2**, 63 (1959).
6. E. Heilbronn, *Acta Chem. Scand.* **8**, 1368 (1954).
7. L. Larsson, *Acta Chem. Scand.* **11**, 1131 (1957).
8. R. A. Osterbaan *et al. Fourth International Congress on Biochemistry* 1958.
9. L.-E. Tammelin, *Scand. J. Clin. Lab. Invest.* **5**, 269 (1953).
10. L.-E. Tammelin, *Svensk Kem. Tidskr.* **70**, 157 (1958).
11. G. Tibbling. Unpublished.