The Mode of Recovery of Cholinesterase Activity *in vivo* after Organophosphorus Poisoning

2. BRAIN CHOLINESTERASE

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Previous investigators (Frawley, Hagan & Fitzhugh, 1952; Davison, 1955; Kewitz & Nachmansohn, 1957) have observed that the rate of recovery *in vivo* of inhibited brain true cholinesterase depended on which inhibitor was used. With diisopropyl phosphorofluoridate (Frawley *et al.* 1952; Davison, 1955; Kewitz & Nachmansohn, 1957) recovery was slow, whereas with tetraethyl pyrophosphate (Davison, 1955) and diethyl *p*-nitrophenyl phosphate (Davison, 1955; Kewitz & Nachmansohn, 1957) recovery was considerably more rapid and occurred in two stages, the first being faster than the second. Davison (1955) suggested that the two-stage recovery could be explained by the existence of two kinds of inhibited cholinesterase with differing stabilities.

In the previous paper (Blaber & Creasey, 1960) it was shown that the changes *in vivo* in sheep-erythrocyte cholinesterase activity after inhibition by a variety of organophosphorus compounds could be explained in terms of (i) spontaneous reactivation of the inhibited enzyme by hydrolysis, (ii) aging, i.e. the gradual conversion of the inhibited enzyme from a form which can be reactivated by oximes into one which cannot, and (iii) new enzyme synthesis.

In the present paper a similar analysis has been made of the behaviour of inhibited rat-brain cholinesterase; this is a more complicated system since both true cholinesterase and pseudocholinesterase are present and cannot easily be separated. The combined effects of spontaneous reactivation, aging, and new enzyme synthesis on the recovery of inhibited brain cholinesterase have been investigated by comparing the behaviour *in vivo* and *in vitro* of the inhibited enzyme. The inhibitors were those used previously in the study of inhibited erythrocyte cholinesterase activity (Blaber & Creasey, 1960), namely dimethyl *p*-nitrophenyl phosphate, diisopropyl phosphorofluoridate, tetraethyl pyrophosphate and isopropyl methylphosphonofluoridate. Although aging of inhibited

brain cholinesterase *in vivo* is known to occur (Hobbiger, 1957), it has not previously been investigated in relation to the recovery *in vivo* of inhibited cholinesterase in the brain.

Whilst this work was in progress suspicion was cast upon conventional methods of cholinesterase assay by Kewitz & Nachmansohn (1957), who claimed that, unless extracellular inhibitor was removed from poisoned brains before they were homogenized, falsely low cholinesterase levels would be obtained. The results of the present investigation do not support the idea that the inhibitors used are held at extracellular sites. The cholinesterase activities, which are reported here and which were measured by conventional methods of assay, are therefore considered to be reliable.

MATERIALS AND METHODS

Inhibitors. Diisopropyl phosphorofluoridate (DFP), tetraethyl pyrophosphate (TEPP), and isopropyl methylphosphonofluoridate (Sarin) were prepared in the Chemistry Section, C.D.E.E., by methods described by Blaber & Creasey (1960); dimethyl *p*-nitrophenyl phosphate (DMNP) was presented by Dr W. N. Aldridge, *N*-(2-diethylamino-*n*-propyl)phenothiazine hydrochloride (ethopropazine) was presented by May and Baker Ltd., and diethyl *p*-nitrophenyl phosphate (E 600) was obtained from Albright and Wilson Ltd.

Substrates. Acetylcholine chloride was supplied by Roche Products Ltd., and acetyl- β -methylcholine chloride by Savory and Moore Ltd.; butyrylcholine iodide was prepared in the Chemistry Section, C.D.E.E.

Preservatives. 8-Hydroxyquinoline was obtained from British Drug Houses Ltd., Merthiolate from Eli Lilly and Co. Ltd., and Nipasept (a mixture of the methyl, ethyl and propyl esters of *p*-hydroxybenzoic acid) from Nipa Laboratories Ltd.

Reactivators. Monoisonitrosoacetone (MINA) and 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate (P2S) were prepared in the Chemistry Section, C.D.E.E., as described by Blaber & Creasey (1960).

Animals. Rats were male albino 200–250 g.; mice were male albino 18–22 g.

Preparation of 'brain homogenate'. Brains were carefully removed, washed with distilled water to remove any adhering blood and then blotted dry. The brains were weighed and homogenized in an all-glass homogenizer with

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0.0312M-NaHCO₃-0.15M-NaCl, pH 7.8 (Davison, 1953), the final volume being adjusted so that 1.0 ml. was equivalent to 50 mg. of fresh brain.

Cholinesterase activity. This was estimated manometrically with the following reaction mixture: 0.5 ml. of 'brain homogenate', 0.5 ml. of 0.0312M-NaHCO₃-0.15M-NaCl pH 7.8, 0.5 ml. of 0.0828M-acetylcholine chloride (Davison, 1953), 1.5 ml. of 0.1 mM-ethopropazine hydrochloride, freshly prepared in 0.0312M-NaHCO₃-0.15M-NaCl, pH 7.8, gas phase N₂+CO₂ (95:5). Activity was expressed as μ l. of CO₂/30 min./25 mg. of fresh brain, allowance being made for non-enzymic hydrolysis.

Inhibition in vivo. Rats were given 50 mg. of atropine sulphate/kg. subcutaneously; 10 min. later solutions of inhibitors in 0.9% NaCl were injected as follows: DMNP 2.0 mg./kg. intraperitoneally, TEPP 0.25 mg./kg. subcutaneously, DFP 3.0 mg./kg. subcutaneously, and Sarin 0.012 mg./kg. subcutaneously.

Reactivation of inhibited brain cholinesterase and measurement of aging. 1.0 ml. of 0.1M-2-(hydroxyiminomethyl)-N-methylpyridinium methanesulphonate was mixed with 100 ml. of Sarin-, DMNP-, or TEPP-inhibited 'brain homogenate' and kept at room temperature for 1 hr. The DFP-inhibited 'brain homogenate', because of its low rate of reactivation, was kept overnight with this concentration of 2-(hydroxyiminomethyl)-N-methylpyridinium methanesulphonate. During the subsequent cholinesterase assay these mixtures were sufficiently diluted to prevent the occurrence of any cholinesterase inhibition by the oxime. The 1% error in the cholinesterase estimations, caused by the addition of oxime solution, was neglected. The percentage of the initial activity which could not be recovered by treatment with oxime was the percentage of aged enzyme.

Storage of half-brains. Half-brains were stored at 37° in stoppered tubes with 0.1 ml. of a 0.05% solution of 8-hydroxyquinoline in 0.9% NaCl soln.

RESULTS

Preliminary investigations

Before the behaviour of inhibited brain true cholinesterase was studied *in vivo* and *in vitro* the following preliminary investigations were performed.

Choice of method for measuring the true cholinesterase activity of rat brain. The cholinesterase activity of rat brain when measured against acetylcholine consists of approximately 90% of the true enzyme and 10% of the pseudo enzyme (Davison, 1953). The true cholinesterase activity was therefore measured in the presence of a specific inhibitor for the small proportion of pseudocholinesterase. *NN'*-Diisopropylphosphorodiamidic fluoride (mipaflox) (Davison, 1953), *NN'*-diisopropyl phosphorodiamidic anhydride (DPDA) (Austin & Berry, 1953), and *N*-(2-diethylamino-n-propyl)phenothiazine hydrochloride (ethopropazine) (Bayliss & Todrick, 1956) have previously been used for this purpose. In the present experiments ethopropazine was used (i) because of its higher specificity and (ii) because it is a reversible

inhibitor and the degree of inhibition would not, therefore, depend on the time of contact.

In agreement with the findings of Bayliss & Todrick (1956) 50 μ M-ethopropazine almost completely suppressed the hydrolysis of butyrylcholine by rat-brain homogenate but inhibited only 7% of the acetyl- β -methylcholine hydrolysis (Table 1). Because acetylcholine is hydrolysed faster than acetyl- β -methylcholine by true cholinesterase, acetylcholine was used as substrate throughout the present work. All cholinesterase measurements were made in the presence of 50 μ M-ethopropazine, and with acetylcholine as substrate this concentration of ethopropazine inhibited 13.7% of the brain cholinesterase activity; the residual 86.3% was assumed to be the normal level of true cholinesterase in the brain and all values obtained after organophosphorus poisoning were expressed as a percentage of this.

Possibility of reversal of ethopropazine inhibition of pseudocholinesterase by oximes. It was not convenient to remove the oxime from reactivated

Table 1. *Inhibition of brain true cholinesterase and pseudocholinesterase by ethopropazine*

The cholinesterase (ChE) activity of 0.5 ml. of 'brain homogenate' was measured in the presence and absence of 50 μ M-ethopropazine with 0.0138M-acetylcholine (ACh), 0.03M-acetyl- β -methylcholine (MCh) or 0.03M-butyrylcholine (BuCh) as substrate. Temp. 37°; gas phase N₂+CO₂ (95:5).

Substrate ...	ChE activity (μ l. of CO ₂ /30 min./25 mg. of brain)		
	0.0138M-ACh	0.03M-MCh	0.03M-BuCh
Control	121.0	66.5	18.0
+ ethopropazine	104.4	61.4	1.7
Inhibition (%)	13.7	7.7	91.5

Table 2. *Combined effect of oximes and ethopropazine on brain true cholinesterase (ChE) activity*

'Brain homogenate' (0.5 ml.) was kept at room temperature for 30 min. in the main compartment of a Warburg flask with: 50 μ M-ethopropazine, mM-2-(hydroxyiminomethyl)-N-methylpyridinium methanesulphonate (P2S) or 10 mM-monoisonitrosoacetone (MINA) and bicarbonate-saline buffer to 2.5 ml. The flasks were then equilibrated at 37° with N₂+CO₂ (95:5) and the ChE activity was measured manometrically after adding 0.5 ml. of 0.0828M-acetylcholine from the side arm. P2S, MINA and ethopropazine were omitted from the control.

Additions	ChE activity (μ l. of CO ₂ /30 min.)	Inhibition (%)
Control	136.2	0
Ethopropazine	114.6	15.8
P2S	115.0	15.5
MINA	130.6	4.0
Ethopropazine + P2S	97.6	28.3
Ethopropazine + MINA	109.8	19.4

brain cholinesterase before measuring the enzyme activity, so that both oxime and ethopropazine were present during the activity determination. It was, therefore, important to be sure that pseudo-cholinesterase, inhibited by ethopropazine, would not be reactivated by the oxime. Table 2 shows that the inhibition of rat-brain cholinesterase by ethopropazine and 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate, or by ethopropazine and monoisonitrosoacetone, was additive; inhibition by ethopropazine was therefore assumed not to be reversed by oximes.

Inhibition of cholinesterase by oximes and their possible interference in the activity tests. Since cholinesterase is inhibited by oximes (Hobbiger, 1956) it was necessary to ensure that the oxime, added to the homogenate for the reactivation, did

not inhibit the cholinesterase in the subsequent activity test. The final concentrations of 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and monoisonitrosoacetone in the cholinesterase-activity determination were 0.167 and 1.67 mM respectively; reference to Fig. 1, in which percentage inhibition of true cholinesterase is plotted against the oxime concentration, shows that no correction was needed for these concentrations of 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and monoisonitrosoacetone.

Measurement of aging. As with erythrocyte cholinesterase (Blaber & Creasey, 1960) the percentage of aged cholinesterase was taken as the percentage of the initial activity which could not be recovered by oxime treatment; the ability to measure the proportion of aged brain cholinesterase, present at a particular instant, depends on the rate of the oxime-reativation reaction being much greater than that of the aging process; with TEPP- and Sarin-inhibited cholinesterase this appeared to be so, as oxime treatment resulted in complete reactivation of the freshly inhibited enzyme within 1 hr. (Table 3). With DFP- and DMNP-inhibited brain cholinesterase, however, only partial reactivation of the freshly inhibited cholinesterase by oximes was observed, indicating that some aging had occurred either during, or before, reactivation by oximes. Thus although it was possible to say whether or not the aging of the DFP- or DMNP-inhibited brain cholinesterase was complete it was not possible to observe it as accurately as with TEPP- or Sarin-inhibited brain cholinesterase. Because 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate was the faster reactivator this oxime was used in all the routine measurements of aging.

Storage of brain preparations at 37°. It was impossible to store rat brain without some loss of activity. Homogenates showed a high initial loss of activity, approx. 11% in the first 24 hr., even in

Fig. 1. Inhibition of brain true cholinesterase (ChE) activity by oximes. The reaction mixture contained: brain homogenate 0.5 ml., 0.028 M-NaHCO₃, 0.125 M-NaCl, 50 μM-ethopropazine, 0.0138 M-acetylcholine, and the stated concentrations of oxime, in a total volume of 3.0 ml.; pH 7.8, gas phase N₂+CO₂ (95:5). O, Monoisonitrosoacetone; ●, 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate.

Table 3. Reactivation of inhibited rat-brain cholinesterase by oximes

A homogenate of rat brain inhibited *in vivo* with DMNP, TEPP, Sarin or DFP was allowed to stand at room temperature with mM-2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate (P2S) or 10 mM-monoisonitrosoacetone (MINA). At suitable intervals the cholinesterase activity was determined and expressed as a percentage of the normal value.

Time of contact (hr.)	Percentage of normal cholinesterase activity							
	DMNP		TEPP		DFP		Sarin	
	P2S	MINA	P2S	MINA	P2S	MINA	P2S	MINA
0	17.2	17.2	24.9	12.4	31.6	31.6	31.8	31.8
0.5	87.0	36.6	98.5	93.5	65.5	56.4	97.3	96.1
1.0	88.0	42.6	100.0	98.4	77.6	68.0	100.0	100.0
2.0	88.1	63.3	100.0	100.0	80.9	77.2	100.0	100.0
3.5	88.0	70.6	—	100.0	89.0	86.5	—	—
4.5	93.7	80.4	—	—	—	—	—	—
5.0	—	—	—	—	85.4	85.6	—	—

Table 4. *Storage of half-brains*

Six freshly dissected normal rat brains were divided into *R* and *L* halves. The cholinesterase activity of the *L* halves was estimated immediately and the *R* halves stored at 37° in the presence of 8-hydroxyquinoline. The cholinesterase activity of the *R* halves was measured and expressed as a percentage of the corresponding *L* half at the intervals stated. Homogenate was stored at 37° in the presence of 8-hydroxyquinoline.

Time (days)	Cholinesterase activity (μ l. of CO ₂ /30 min./25 mg. of brain)					
	Intact half-brains			Homogenate		
	<i>L</i>	<i>R</i>	(<i>R</i> / <i>L</i>) $\times$ 100	(Units)	(% of initial activity)	
1	103.8	105.7	100	108	100	
2	97.7	97.3	100	96.5	89.4	
3	105.8	102.0	96.5	—	—	
4	108.4	100.8	93	79.4	73.5	
7	109.4	56.3	51.5	—	—	
8	97.5	82.6	84.8	—	—	
13	—	—	—	63.9	59.2	

the presence of preservatives such as 0.1% 8-hydroxyquinoline, 0.001% Merthiolate, or 0.08% Nipasept. However, when the half-brains were stored in tightly stoppered tubes in the presence of 0.1 ml. of 0.05% 8-hydroxyquinoline the cholinesterase activity remained unchanged for 48 hr.; during the following 6 days the activity gradually fell to approx. 85% of the initial value (Table 4). There was the occasional half-brain, e.g. Table 4, 7 days, which showed an abnormal loss of activity; in such cases the figures were rejected.

Interference from extracellular inhibitor. The assay procedures outlined so far were applied to homogenates prepared directly from the brains of poisoned animals. This practice has been criticized by Kewitz (1957) and by Kewitz & Nachmansohn (1957), who claimed that DFP and E600 could occur extracellularly in the brain, and that although they would not inhibit the cholinesterase *in vivo* they would do so *in vitro* as soon as the brains were homogenized in preparation for cholinesterase assay. Kewitz (1957) claimed that this interference could be prevented by vacuum-drying the brains and extracting with chloroform before homogenizing. Because of the doubt cast upon cholinesterase assays performed without Kewitz's extraction procedure it was necessary to find out whether the present cholinesterase assays would be invalidated in this way.

Some preliminary experiments showed that extraction of partially dried brains with chloroform resulted in complete loss of cholinesterase activity. In order to avoid this loss it was necessary to dry the brains for 3 days over P₂O₅ at 0.1 mm. Hg.

To investigate Kewitz's procedure further the behaviour of inhibited brain cholinesterase during the drying was studied in the following manner, where no chloroform extraction was performed. The brains from eight normal mice were divided

Table 5. *Change in cholinesterase activity during the vacuum-drying of mouse brain inhibited in vivo with E 600*

The dose of E 600 was 1 mg./kg. body wt. intraperitoneally. All percentages refer to the initial activity of the control brain.

	Cholinesterase activity (μ l. of CO ₂ /30 min./25 mg. wet brain)	
	Initial	After 3 days' drying
Control brain	112.5 (100%)	98 (87%)
Inhibited brain	45.9 (41%)	65.3 (58%)

into *R* and *L* halves; the *R* halves were pooled and weighed, and the mean cholinesterase activity (*a*) was estimated immediately. The *L* halves, after being weighed wet, were dried over P₂O₅ for 3 days at 0.1 mm. Hg and the mean cholinesterase activity after drying was expressed as a percentage of the initial activity of the *R* halves (*a*). Concurrently a similar experiment was performed on the brains from eight mice which had received an injection of 1 mg. of E 600/kg., the animals having been killed when signs of poisoning were well established. The mean cholinesterase activities of the pooled *R* half-brains before drying and of the pooled *L* half-brains after drying were again expressed as percentages of the initial cholinesterase activity (*a*) of the normal *R* half-brains.

The results (Table 5) show that during the drying the cholinesterase activity of the normal brains decreased to 87% of the control value whereas that of the poisoned brains increased by 17% of the control value, i.e. from 41 to 58%. This increase, obtained without chloroform extraction, indicates the spontaneous reactivation of 28.8% (i.e. 100 $\times$ 17/59) of the inhibited cholinesterase. The

increase in activity, observed by Kewitz & Nachmansohn (1957), after drying plus chloroform extraction, was equivalent to 32% of the inhibited enzyme. Thus spontaneous reactivation during the drying process could have accounted for the increase in cholinesterase activity which Kewitz & Nachmansohn attributed to the removal of extracellular inhibitor. Extracellular storage of inhibitor has, therefore, been assumed not to occur in the present experiments.

Recovery of brain cholinesterase activity in poisoned rats

In these experiments the rat-brain cholinesterase was inhibited *in vivo* by administering each inhibitor to a separate group of 72 animals. After inhibition the recovery *in vivo* and aging of the inhibited brain true cholinesterase was followed by performing cholinesterase assays before and after oxime treatment on a homogenate prepared from the pooled brains of batches of six rats taken at suitable intervals from each group. The cholinesterase activities were expressed as percentages of a normal value (α) previously determined in a group of 20 control rats. The percentage of aged enzyme was that percentage of the group normal (α) which could not be recovered by oxime treatment.

To follow the recovery *in vitro* of enzyme activity the brains of the first batch of six rats from each group were divided into R and L halves. The individual cholinesterase activities of the R halves, before and after oxime treatment, were measured immediately after removal from the animal. The L halves were stored at 37° in stoppered tubes with 8-hydroxyquinoline as preservative. For the first few days, whenever a batch of six rats was killed for brain cholinesterase measurements in the study *in vivo*, a stored L half-brain was also taken and its cholinesterase activity measured before and after oxime treatment. The activities so obtained were expressed as percentages of the group normal (α), and used to prepare the curve of recovery *in vitro*, a diagrammatic form of which is shown in Fig. 2.

Let the individual percentage cholinesterase activities of the R half-brains before oxime treatment = R_1 to R_6 , and the mean of these activities = R_m ; the individual percentage cholinesterase activities of the R half-brains after oxime treatment = $R_{0,1}$ to $R_{0,6}$, and the mean of these activities = $R_{0,m}$; the individual percentage cholinesterase activities of the stored L half-brains before oxime treatment = L_1 to L_6 ; the individual percentage cholinesterase activities of the stored L half-brains after oxime treatment = $L_{0,1}$ to $L_{0,6}$; corresponding R and L halves carry the same numerical subscript.

The mean values R_m and $R_{0,m}$ provided the first pair of points on both the *in vivo* and *in vitro* curves. Thus it was possible to follow the recoveries *in vivo* and *in vitro* from the same starting point and so achieve a fair comparison between the two. However, with Sarin, inhibition of the brain cholinesterase continued slightly after the first batch of rats was taken with the result that the curves of recovery *in vivo* and *in vitro* did not start from precisely the same cholinesterase level (Fig. 5).

The percentage of spontaneous reactivation which occurred during storage was given by the difference between the cholinesterase activities of the R and L halves of the same brain, i.e. $L_x - R_x$. The total cholinesterase activity after a particular period of storage, i.e. the uninhibited cholinesterase plus that produced by spontaneous reactivation, was given by $(L_x - R_x) + R_m$ and is the figure which is plotted on the spontaneous reactivation curve.

The percentage of non-aged cholinesterase present in the stored half-brains, i.e. the figure plotted on the aging curve, was given by the expression $(L_{0,x} - R_x) + R_m$, where $(L_{0,x} - R_x)$ is the difference between the cholinesterase activities of the oxime-treated stored L half-brain and the corresponding R half. $(L_{0,x} - R_x)$ includes the

Fig. 2. Diagram to illustrate the method of constructing the curves showing the changes *in vitro* of rat-brain true cholinesterase (ChE) activity after poisoning *in vivo* with organophosphorus cholinesterase inhibitors. For definition of letters see text.

increase in cholinesterase activity produced by spontaneous reactivation as well as that produced by oxime treatment.

The results of these experiments are shown in Figs. 3-6.

In vitro, significant spontaneous reactivation was observed only with the DMNP- and TEPP-

inhibited cholinesterase. Aging, on the other hand, was observed with all the inhibitors used, as has been described with inhibited erythrocyte cholinesterase (Blaber & Creasey, 1960). When all the oxime-reactivable cholinesterase had disappeared and only aged cholinesterase remained no further increase in DMNP-, DFP- or Sarin-inhibited cholinesterase activity was observed.

Fig. 3. Behaviour *in vivo* (a) and *in vitro* (b) of the brain cholinesterase of rats poisoned with 0.25 mg. of TEPP/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

Fig. 4. Behaviour *in vivo* (a) and *in vitro* (b) of the brain cholinesterase of rats poisoned with 2.0 mg. of DMNP/kg. intraperitoneally. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

Fig. 5. Behaviour *in vivo* (a) and *in vitro* (b) of the brain cholinesterase of rats poisoned with 0.012 mg. of Sarin/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

Fig. 6. Behaviour *in vivo* (a) and *in vitro* (b) of the brain cholinesterase of rats poisoned with 3.0 mg. of DFP/kg. subcutaneously. Broken lines show the decrease in cholinesterase reactivatable by 2-(hydroxyiminomethyl)-*N*-methylpyridinium methanesulphonate and represent the aging of the inhibited enzyme. Full lines show the cholinesterase activity before oxime treatment and represent spontaneous recovery (*in vitro*) or spontaneous recovery plus new enzyme synthesis (*in vivo*).

With TEPP-inhibited cholinesterase, however, a slight and unexplained increase in activity was observed after all oxime-reactivable cholinesterase activity had disappeared (Fig. 3).

In vivo, spontaneous reactivation and aging of the inhibited cholinesterase occurred at rates similar to those observed *in vitro*. Spontaneous reactivation of the TEPP- and DMNP-inhibited cholinesterase occurred much faster than the simultaneous aging process; consequently very little aged enzyme was produced and it was impossible to measure the rate at which it was replaced. With Sarin and DFP, where spontaneous reactivation of the inhibited enzyme occurred to only a very slight extent, the recovery *in vivo* of inhibited brain cholinesterase activity proceeded at an exponential rate, and with DFP (Fig. 6) the half-life of the inhibited cholinesterase was approximately 14 days.

DISCUSSION

Kewitz & Nachmansohn (1957) suggested that the storage of injected E 600 at extracellular sites could seriously interfere with tissue cholinesterase estimations performed by orthodox methods. They based this suggestion on their observation that mouse brains or diaphragms poisoned *in vivo* by E 600 had a higher cholinesterase activity if they were dried *in vacuo* and extracted with chloroform before performing the cholinesterase assay than if this procedure were omitted. They considered that chloroform extraction removed extracellularly stored E 600 which would otherwise have caused further inhibition during homogenizing and so given a falsely low indication of the cholinesterase level present in the tissues of the intact animal. By using their chloroform-extraction technique Kewitz & Nachmansohn obtained an increase in cholinesterase activity over that of preparations not so treated equal to 32% of the inhibited cholinesterase activity.

The present work shows that mouse brains have to be dried *in vacuo* for 3 days before chloroform extraction can be used without risk of inactivating some of the cholinesterase. When only the drying procedure was applied to mouse brains poisoned *in vivo* with E 600 the inhibited cholinesterase underwent spontaneous reactivation to the extent of 28.8% of the inhibited enzyme activity. This increase in cholinesterase activity, almost equal in magnitude to that reported by Kewitz & Nachmansohn (1957), was obtained without chloroform extraction and was due solely to spontaneous reactivation of the inhibited enzyme during drying. It is possible therefore that this mechanism operated in Kewitz & Nachmansohn's experiments and could provide an alternative explanation of their

results. This view is consistent with the fact that in the present experiments DFP-inhibited cholinesterase did not undergo spontaneous reactivation and this could easily explain Kewitz & Nachmansohn's failure to detect extracellular DFP.

Although the possibility that some inhibitor occurs at extracellular sites cannot definitely be excluded on the basis of these experiments, in view of the alternative explanation offered for Kewitz & Nachmansohn's results, this possibility would appear to be slight and in the present work it has been discounted.

As for inhibited erythrocyte cholinesterase (Blaber & Creasey, 1960) rat-brain cholinesterase inhibited with organophosphorus compounds recovered its activity *in vivo* in one or two stages depending on whether or not the inhibited enzyme underwent spontaneous reactivation. Immediately after its formation the inhibited enzyme began to age, i.e. it changed spontaneously into a form which could no longer be reactivated by oximes or undergo spontaneous reactivation. With TEPP- or DMNP-inhibited cholinesterase the aging was accompanied by spontaneous reactivation of the inhibited enzyme, the two reactions proceeding simultaneously and their relative rates determining the extent of the spontaneous reactivation. With DFP- or Sarin-inhibited cholinesterase spontaneous reactivation of the inhibited enzyme occurred to only a very slight extent. During this stage of its recovery the inhibited cholinesterase behaved the same *in vivo* as *in vitro*. When the inhibited enzyme was present entirely in the aged form no further recovery occurred *in vitro*, and *in vivo* the second and slower stage of the recovery was reached.

In vivo the recovery of the DFP- or Sarin-inhibited cholinesterase occurred throughout at an exponential rate (Fig. 6) which was very close to that calculated for the turnover rate of brain protein by Gaitonde & Richter (1956) and based on the turnover of [³⁵S]methionine. In view of the similarity between the rates of cholinesterase recovery and brain-protein turnover it seems likely that the recovery of the DFP- or Sarin-inhibited brain cholinesterase activity was due entirely to new enzyme synthesis.

When the cholinesterase was inhibited with TEPP or DMNP two stages in its recovery *in vivo* could clearly be seen. Most of the enzyme activity returned during the first stage so that only a small residue of irreversibly inhibited cholinesterase was left as a result of the aging process. It was not possible to measure the rate at which this small quantity of inhibited cholinesterase was replaced during the second stage but it is reasonable to assume that replacement was by new enzyme synthesis and that the rate was exponential.

This exponential rate of new enzyme formation is in marked, though not unexpected, contrast to the linear rate of the second stage in the recovery *in vivo* of erythrocyte cholinesterase.

The two forms of inhibited brain cholinesterase of different stabilities originally postulated by Davison (1955) are probably the freshly inhibited enzyme which can undergo spontaneous reactivation and the aged enzyme which cannot.

These results show that the mechanisms by which the activity of the brain cholinesterase is restored to normal after organophosphorus poisoning are precisely analogous to those which operate for inhibited erythrocyte cholinesterase, namely spontaneous reactivation and aging of the inhibited enzyme, and new enzyme synthesis.

SUMMARY

1. Recovery of tetraethyl pyrophosphate- or dimethyl *p*-nitrophenyl phosphate-inhibited rat-brain true cholinesterase *in vivo* occurred in two stages, a rapid partial recovery being followed by a slower, and probably exponential, return to normal activity.

2. With diisopropyl phosphorofluoridate- or isopropyl methylphosphonofluoridate-inhibited rat-brain true cholinesterase only one stage was observed in the recovery *in vivo* which occurred throughout at an exponential rate.

3. The first stage in the recovery is explained in terms of spontaneous reactivation and aging of the inhibited enzyme, and the second stage is correlated with new enzyme synthesis associated with the normal turnover of brain protein.

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The Estimation of the Available Lysine in Animal-Protein Foods

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This paper describes the development of a procedure for the estimation in foods of the lysine units whose ϵ -amino groups will undergo the Sanger reaction with fluorodinitrobenzene. There is reason to think that those lysine units in the proteins of heat-processed foods whose ϵ -amino groups are bound to other groups, and so are unable to react, are likely also to be nutritionally 'unavailable' (Eldred & Rodney, 1946; Lea & Hannan, 1950; Henry & Kon, 1950). As a consequence of this, methods of analysis for lysine that begin with acid hydrolysis have been found to over-value foods as sources of this amino acid.

Our experiments have given results suggesting that chemical estimations of 'fluorodinitrobenzene-available' lysine can give a more reliable indication of the value of animal-protein foods (Carpenter, Ellinger, Munro & Rolfe, 1957; Lea, Parr & Carpenter, 1960).

Lysine is often the factor limiting the protein

quality of mixed diets for man or animals. Cereals, which are usually the staple energy source, also supply a large proportion of the protein and this cereal protein is seriously deficient in lysine. It is therefore of practical importance that the processing of high-protein foods should be controlled so that damage to the lysine is minimized. The procedure to be described has therefore been designed to be practicable for use in a routine analytical control laboratory.

Carpenter & Ellinger (1955*a, b*) used a simple procedure which proved useful for a range of animal materials, but it was affected significantly by interference from α -dinitrophenylarginine when applied to autolysed materials, and from dinitrophenol coming from breakdown of excess of dinitrofluorobenzene, if carbohydrate were present (Conkerton & Frampton, 1959). The interference was avoided by a modification (Bruno & Carpenter, 1957) in which methoxycarbonyl chloride was