that allylthiourea inhibition is due to chelation of the inhibitor with the essential metal. It now seems certain that the inhibition is not due to competition with ammonia, a theory adumbrated by Quastel & Scholefield (1951) since, as found previously by a different technique (Lees, 1952*a*) no competition between ammonia and allylthiourea could be detected (Table 2). Since the inhibition of nitrite formation by thiourea and allylthiourea is far more intense than the inhibition by any other of a wide range of chelating agents (Lees, 1952*a*) and since the thioureas are known to have a peculiarly high affinity for copper, there is good presumptive evidence for the theory already advanced (Lees, 1946) that the enzyme concerned in the primary ammonia oxidation by *Nitrosomonas* is a copper enzyme.

SUMMARY

1. The metabolism of cultures of *Nitrosomonas* has been investigated by the Warburg technique.

2. In contrast to results previously obtained by Meyerhof, the ammonia-oxidizing activities of *Nitrosomonas* in culture was found to be affected by change in pH in the same way as it is affected in soil.

3. Evidence is presented to show that the oxidation of ammonia to nitrite by *Nitrosomonas* proceeds via hydroxylamine. Hydrazine has been found to inhibit selectively the oxidation of hydroxylamine. In the presence of hydrazine, hydroxylamine accumulated, and was detected, during ammonia oxidation.

4. The oxidation of ammonia to hydroxylamine was completely inhibited by allylthiourea at concentrations which did not affect oxidation of the hydroxylamine.

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Return of Cholinesterase Activity in the Rat after Inhibition by Organophosphorus Compounds

1. DIETHYL *p*-NITROPHENYL PHOSPHATE (E600, PARAOXON)

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The inhibition of cholinesterase *in vitro* by organophosphorus compounds has generally been considered to be an irreversible process. However, many workers have shown that cholinesterase activity does return both *in vivo* and in isolated tissue preparations (Koelle & Gilman, 1946; Mazur & Bodansky, 1946; Grob, Lilienthal, Harvey & Jones, 1947; Dubois, Doull, Salerno & Coon, 1949; Freedman, Willis & Himwich, 1949; Hobbiger, 1951; Burgen & Hobbiger, 1951; Frawley, Hagan & Fitzhugh, 1952). Two alternative mechanisms of this return of activity have been considered. Koelle

& Gilman (1946), Mazur & Bodansky (1946) and Grob *et al.* (1947) have suggested that this return is due to the resynthesis of new enzyme, and that the original inhibition is irreversible. However, the difference between the rates of return of activity *in vivo* after inhibition by various organophosphorus compounds indicate that this view cannot be of general application (Dubois *et al.* 1949; Callaway, Davies & Risley, 1952). An alternative mechanism is based on the view that during inhibition the enzyme is phosphorylated. On the basis of experiments with electric-eel cholinesterase inhibited by

tetraethyl pyrophosphate (TEPP), Wilson (1951) has suggested that the phosphorylated enzyme is hydrolysed, thus giving the original enzyme and a substituted alkyl phosphoric acid. Aldridge (1953c) has shown that the inhibition of true cholinesterase of erythrocytes by dimethyl *p*-nitrophenyl phosphate reverses rapidly and has shown that the properties of this reversal are consistent with a dephosphorylation process.

In this paper the behaviour of true and pseudo cholinesterase in the rat has been studied. It is shown that the inhibition of the pseudo cholinesterase of the rat by diethyl *p*-nitrophenyl phosphate (E600) reverses rapidly *in vitro* and that the rate of return of activity *in vivo* is similar to that occurring *in vitro*. Rat-brain pseudo cholinesterase inhibited by E600 recovers activity at a much slower rate than other rat pseudo cholinesterases. The pseudo cholinesterase of the brain has the same substrate pattern as the other pseudo cholinesterases and the same sensitivity to inhibitors. *In vivo* and also *in vitro* the true cholinesterase recovers to about 60% activity, after which the recovery process is much slower.

METHODS

The methods were those used by Aldridge (1950). Final substrate concentrations were: acetylcholine perchlorate (AcCh) 0.0138M, acetyl- β -methylcholine bromide (MeCh), propionyl- (PrCh), *n*-butyryl- (BuCh), *isobutyryl-* (*iso*BuCh), *n*-valeryl- (ValCh) and *isovaleryl-* (*iso*ValCh) choline perchlorates, all 0.03M, and benzoylcholine chloride (BzCh), 0.015M. MeCh was obtained from L. Light and Co. Ltd. and BzCh from British Drug Houses Ltd. The remaining substrates were prepared in this laboratory. Corrections were made for non-enzymic hydrolysis of the substrate and for tissue suspension and erythrocyte blanks, by taking manometer readings for 30 min. before tipping in the substrate. On addition of the substrate, recovery of the inhibited cholinesterase commences, but it is too small a change to be detected and the output of CO₂ remains sensibly linear. Male albino rats of about 200 g. were used throughout. The rats were killed by coal gas and the tissues were quickly removed, washed with saline, blotted and weighed. The heart was perfused from the aorta until the effluent was free from blood. After mincing with scissors the tissues were dispersed in buffer using an all-glass homogenizer (Potter-Elvehjem type). Approximate concentrations of suspensions were: brain, 50 mg./ml.; heart, 80 mg./ml.; jejunum, 40 mg./ml.; submaxillary gland, 200 mg./ml.; diaphragm, 400 mg./ml.; spinal cord, 70 mg./ml. and female-rat serum 0.5 ml./ml. One ml. of these suspensions gave suitable outputs of CO₂ with AcCh as substrate; for tissues containing small amounts of pseudo cholinesterase (such as brain) 3 ml. of suspension were taken with BuCh as substrate.

Reversal of inhibition in vitro. The method of studying reversal *in vitro* was essentially that of Aldridge (1953c). Excess E600 was removed by a preparation of rabbit serum (A-esterase) previously treated with 10⁻⁶M-diisopropylphosphorofluoridate (DFP) to remove cholinesterase

activity. After tissue suspensions had been incubated for 30 min. in bicarbonate saline (0.0312M-NaHCO₃, 0.15M-NaCl, pH 7.8) with suitable concentration of E600 at 37°, A-esterase preparation was added to them (approx. 1 ml. to not more than 8 ml. of suspension) and they were left for a further 30 min. to destroy all inhibitor. After this 60 min. incubation with inhibitor and serum, the first determination of cholinesterase activity was made, and the inhibition was defined as 100% inhibition at zero time. At intervals suitable volumes were taken with a syringe pipette and assayed for activity. Controls without E600 were treated similarly. In experiments lasting for 6 hr. bacterial growth was prevented by adding Merthiolate (Eli Lilly and Co. Ltd.) in 1:10 000 concentration. At this concentration Merthiolate does not interfere with cholinesterases. Suspensions of heart and, particularly, of jejunum often began to lose activity after 5 hr., but preparations of brain and spinal cord remained stable up to about 7 days. The pH remained approximately 7.8. In one experiment in which concentrated brain suspension was prepared in 0.067M-phosphate buffer (Sørensen's), pH 7.6, and penicillin was added in addition to the Merthiolate, the true cholinesterase remained stable up till 13 days.

Inhibition in vivo. White male rats were given E600 (0.25 mg./kg.) in saline into the tail vein or, for experiments on the intestine, by stomach tube (2.4 mg./kg.) in saline. These doses killed two to five out of every ten animals. All animals showed symptoms of salivation, fasciculations and tremors within 5 min. following intravenous injection but after an oral dose the symptoms were more delayed and less pronounced. Acute symptoms of poisoning disappear 1–2 hr. after injection. Pairs of animals were killed after 0.5 hr. and at stated times after that. Estimations were usually made at once, but occasionally samples were stored overnight at –5°. This caused no change in the cholinesterase level. Activity was measured using AcCh and BuCh as substrates and true and pseudo cholinesterase activities were calculated in terms of AcCh (see below). It proved difficult to reduce the pseudo cholinesterase activity level of brain *in vivo* by more than 50% although the activity of true cholinesterase may be as low as 5% of normal. Diaphragm cholinesterases behave similarly. This is due to the preferential inhibition of true cholinesterase by E600. Following intravenous injection of E600, heart pseudo cholinesterase is inhibited 25% at the most, submaxillary gland even less, while the jejunum is unaffected. If, however, E600 is given orally the jejunum pseudo cholinesterase is almost completely inhibited.

Determination of true and pseudo cholinesterase. It was essential to have a method suitable for the determination of the low cholinesterase activity remaining in tissues *in vivo* after administration of inhibitors and also suitable for the determination of small amounts of pseudo cholinesterase in the presence of true cholinesterase and vice versa. The specific substrates, MeCh and BzCh (Mendel, Mundell & Rudney, 1943), are not hydrolysed at a high enough rate to give a method of sufficient sensitivity. The use of an inhibitor, *NN'*-diisopropylphosphorodiamidic fluoride (bis-monoisopropylamino fluorophosphine oxide; mipafox; Isopestox), which has a high selectivity for pseudo cholinesterase (Aldridge, 1953a) has enabled the cholinesterases of rat tissues to be separated. The activity remaining after incubation of a tissue suspension with 4 × 10⁻⁶M-mipafox for 30 min. at 37° has been taken as that of true cholinesterase. Then the difference between this and the original

uninhibited activity is the activity of pseudo cholinesterase. Fig. 1 shows that at this concentration of mipafox not more than 5% of the true enzyme is inhibited while pseudo cholinesterase activity is completely removed.

cholinesterase since there are only small differences in values for individual tissues. The percentage of true and pseudo cholinesterase has been determined by the following method. The ratio of the

Fig. 1. Inhibition curve for rat true and pseudo cholinesterase. Brain and heart suspensions were incubated for 30 min. at 37° with mipafox and true and pseudo cholinesterase activity determined with MeCh and BuCh respectively. Broken curve, heart pseudo cholinesterase; continuous curves, brain true and pseudo cholinesterase.

The results in Table 1 show that the activity of true and pseudo cholinesterases of different tissues against AcCh, PrCh and BuCh are similar. Thus the mean activities represent the substrate hydrolysis by true and pseudo

Fig. 2. Calibration curve for determining percentage of true and pseudo cholinesterase of the rat. The ratio AcCh/BuCh is determined as described in the text.

activity against AcCh to that against BuCh varies from 45.5 for true cholinesterase to 0.69 for pseudo cholinesterase. Fig. 2 shows the value of this ratio for mixtures of true and pseudo cholinesterase (expressed as percentage activities in terms of activity against one substrate). From the ratio determined for an unknown tissue its composition in terms of activity against AcCh can be obtained. The activity of each

Table 1. Relative rates of hydrolysis of substrates by various rat tissues

(The results were obtained by the method described in the text using AcCh, 0.0138M; BuCh and PrCh, 0.03M and mipafox, 4×10^{-6} M. AcCh rates in each case taken as 100.)

	True cholinesterase			Pseudo cholinesterase		
	No. of exps.	BuCh	PrCh	No. of exps.	BuCh	PrCh
Brain	3	2.3	57	1	141	260
Jejunum	3	2.3	61	3	150	247
Heart	2	3.7	86	1	148	230
Submaxillary gland	2	1.5	65	1	145	191
Diaphragm	1	3.8	65	1	150	244
Serum	2	0.5	56	3	140	237
Mean relative rate of hydrolysis		2.2	65		145	245

Table 2. Normal values for substrate hydrolysis by rat cholinesterases

(Values have been determined using 0.0138M-AcCh and 0.03M-BuCh expressed as μ l. CO₂/g. wet wt./hr.; the percentage of true cholinesterase (ChE) has been calculated as in the text, and corrected mean values for true (ACh) ChE and pseudo (ACh) ChE are recorded together with the standard deviation.)

Rat tissue	AcCh hydrolysis (μ l. CO ₂ /g./hr.)	BuCh hydrolysis (μ l. CO ₂ /g./hr.)	Percentage of true ChE as AcCh	True ChE activity as AcCh (μ l. CO ₂ /g./hr.)	Pseudo ChE activity as AcCh (μ l. CO ₂ /g./hr.)
Brain	10 883 $\pm$ 798	1 292 $\pm$ 115	94	10 168 $\pm$ 862	752.5 $\pm$ 86.5
Heart	5 940 $\pm$ 1 172	7 490 $\pm$ 1 110	12	856 $\pm$ 1 511	5 039 $\pm$ 514
Jejunum	12 940 $\pm$ 1 572	17 600 $\pm$ 1 625	5	751 $\pm$ 582	12 944 $\pm$ 1 352
Diaphragm	1 609 $\pm$ 343	859 $\pm$ 202	62	1 007 $\pm$ 337	602 $\pm$ 144.5
Submaxillary gland	2 865 $\pm$ 659	2 437 $\pm$ 530	42	1 210 $\pm$ 515	1 655 $\pm$ 342.5

enzyme against either substrate may be readily calculated. A similar procedure may be carried out with any pair of substrates and has been done for AcCh and PrCh. The results have always agreed well with those obtained using AcCh and BuCh. In Table 2 are the results obtained using these methods for the normal levels of true and pseudo cholinesterase for various tissues of the rat. Since all activity against choline esters is removed by 10^{-4} M eserine, it has been assumed that only cholinesterases hydrolyse these esters.

RESULTS

Inhibition of cholinesterase in vitro of rat by diethyl p-nitrophenyl phosphate

The concentrations of diethyl *p*-nitrophenyl phosphate (E 600) were determined which produced 50% inhibition of true and pseudo cholinesterase in 30 min. at 37° in suspensions of different tissues using MeCh and BuCh as substrates for true and pseudo cholinesterase respectively. The ratios of 50% inhibition values for pseudo and true cholinesterase (Table 3) show that true cholinesterase is selectively inhibited *in vitro*. For instance in brain when 90% of the true cholinesterase was inhibited only 50–60% of the pseudo was inhibited. It is probable that the differences in 50% inhibition values for the enzyme from various tissues are due to the varying levels of enzyme hydrolysing the inhibitor in each tissue, for rat blood, which contains the highest concentration of A-esterase (Aldridge, 1953b), requires the highest concentrations of E 600 for inhibition.

Table 3. *Inhibition of cholinesterases of various tissues of rat by diethyl p-nitrophenyl phosphate*

(The molar concentrations producing 50% inhibition were determined on tissues by incubating with E 600 for 30 min. at 37° in Warburg buffer. MeCh and BuCh, 0.03 M, have been used throughout for determination of true and pseudo cholinesterase respectively. Log percentage activity was plotted against concentration of E 600 and the best straight line drawn; concentrations producing 50% inhibition were read from the curve. The inhibitor ratio is the concentration producing 50% inhibition of pseudo cholinesterase over the concentration producing 50% inhibition of true cholinesterase.)

Tissue	Molar concentration for 50% inhibition		Inhibitor ratio
	True cholinesterase	Pseudo cholinesterase	
Blood	1.1×10^{-7}	2.8×10^{-7}	2.55
Brain	1.6×10^{-8}	3.7×10^{-8}	2.30
Heart	7.4×10^{-9}	2.6×10^{-8}	3.5
Jejunum	—	1.6×10^{-8}	—

Aldridge (1950) showed that straight lines were obtained when log percentage activity of washed sheep red cells inhibited with E 600 was plotted against time. If jejunum pseudo cholinesterase is

inhibited with E 600 straight lines do not result when $\log_{10}$ (percentage activity) is plotted against time (Fig. 3, broken line). Similar curves have been interpreted (Aldridge & Davison, 1952) as being due to

Fig. 3. Inhibition of rat-jejunum pseudo cholinesterase by E 600 (5×10^{-8} M in NaHCO_3 , NaCl buffer). The continuous line represents the effect of adding A-esterase preparation after 15 min. on the normal progressive inhibition curve shown by the broken line.

Fig. 4. Recovery of inhibited true cholinesterases of brain *in vivo* (●) and *in vitro* (×), spinal cord *in vitro* (○) and diaphragm *in vivo* (△). Each point represents the mean result for two animals for the *in vivo* experiments. Dose of E 600 was 0.25 mg./kg. by intravenous injection.

the hydrolysis of the inhibitor, but rat jejunum contains very little of the enzyme hydrolysing E 600 (Aldridge, 1953b). Fig. 3 also shows that, if A-esterase is added after 15 min. to jejunum treated with E 600, instead of progressive inhibition (broken curve), the inhibition is progressively reversed. This recovery is rapid. The shape of the

curve obtained when E 600 is not removed (Fig. 3) could be explained by an equilibrium being reached between the inhibitor and reversal processes. Using the reversal technique the cholinesterases of brain, spinal cord and blood have been found to recover slowly after inhibition by E 600 (Fig. 4). By 4 days, 50–60 % activity is reached; after that the recovery process is very slow. In a single experiment the recovery of inhibited true cholinesterase of rat erythrocytes was followed for 2 days. The rate of recovery was comparable to that of the true cholinesterase of brain.

Fig. 5. Recovery of pseudo cholinesterase *in vitro* after inhibition with E600. Total inhibition=inhibition at zero time. Pseudo cholinesterases of serum (○), jejunum (●), heart (×), diaphragm (△) and brain (□) are shown.

The pseudo cholinesterase of serum, diaphragm, submaxillary gland, heart and jejunum recovered rapidly (within about a day) and it was possible to examine the reversal directly in the Warburg flasks. The recovery process was exponential and has been treated as a first-order process and the approximate first-order constants and half-life periods for recovery determined (Table 4). By contrast, the recovery rates of pseudo cholinesterase of brain and spinal cord were slow. The recovery curve appeared to be exponential but as the pseudo cholinesterase was unstable it was impossible to study the recovery for more than 7 days (63 % reversal). In Fig. 5 all the results for the recovery of pseudo cholinesterases after inhibition with E 600 are recorded. The wide scatter of points is probably due to interaction of A-esterase preparation and the suspension since controls to which A-esterase preparation have been added also show a large variation under similar conditions. Inhibited jejunum pseudo cholinesterase appears to recover slightly more slowly than the pseudo cholinesterase of heart and serum, but this is probably due to

destruction of the recovered cholinesterase, for jejunum pseudo cholinesterase is the least stable of those examined. It may be said, however, that the rates of recovery of inhibited pseudo cholinesterase *in vitro* for heart, serum, submaxillary gland, diaphragm and jejunum are similar. The striking difference between rate of recovery of brain pseudo cholinesterase and that of other tissues is also seen in Fig. 5.

Table 4. Constants for rate of reversal of diethyl-p-nitrophenyl-phosphate-inhibited pseudo cholinesterase *in vivo* and *in vitro*

(The pseudo cholinesterase activity was determined with BuCh (0.03M). *In vitro* and *in vivo* experiments were as described in text. Half-life periods are the mean times required for 50 % recovery of the inhibited pseudo cholinesterase, determined directly from the recovery curves. First-order constants were the mean values for the constant determined from the slope of the best straight line, calculated by the method of least squares, when log percentage total inhibition was plotted against time.)

Pseudo cholinesterase	Half-life periods for recovery		First-order constant <i>in vitro</i> K (min. ⁻¹)
	<i>In vivo</i> (hr.)	<i>In vitro</i> (hr.)	
Brain	96	103	1×10^{-4}
Heart, jejunum, serum and submaxillary gland	4.9	5	2×10^{-3}

When the pseudo cholinesterases in horse and human plasma were inhibited with E 600, the reversal technique showed that the human-plasma enzyme recovered much more slowly than the horse-plasma enzyme. Horse plasma showed a slight recovery of activity in 5 days while no change could be detected in human plasma. Furthermore, no difference in curves of inhibition by E 600 of horse plasma was detected whether A-esterase was added after 15 min. or not. Hobbiger (1951) has shown that guinea-pig serum inhibited with TEPP recovers from 8.5 % of its original activity at zero time to 55 % in 72 hr. but that human serum similarly inhibited shows no recovery in the same period.

Inhibition of rat cholinesterase by diethyl p-nitrophenyl phosphate in vivo

It was found that the pseudo cholinesterases of diaphragm, heart and jejunum rapidly recovered their activity *in vivo*, whereas true cholinesterases of brain and diaphragm, and pseudo cholinesterase of brain took much longer to do so.

It was not possible completely to inhibit all the pseudo cholinesterases *in vivo* (for example, heart and brain) without killing the animal. Therefore, on the basis of an exponential rate and to facilitate comparison, the activity of the tissue half an hour after the administration of E 600 has been taken as

Table 5. *Relative rates of hydrolysis of substrates by rat-brain and heart pseudo cholinesterases*

(Suspensions were incubated without and with 4×10^{-6} M-mipafox for 30 min. at 37° . The substrates were added immediately and the activities determined. The ratios of substrate activities inhibited by the mipafox were determined. AcCh values were taken as 100 in each case and the corresponding values for other substrates calculated.)

	BuCh	isoBuCh	PrCh	ValCh	isoValCh	BzCh
Rat brain	141	150	260	90	73	37
Rat heart	148	151	230	82	75	30

representing 100% inhibition and the percentage increase in activity (loss of inhibition) has been plotted against time (Fig. 6). As may be expected, there was a large scatter of results. The half-life values for reversal were calculated as previously (Table 4). Brain pseudo cholinesterase was found to recover much more slowly (half-life period about 96 hr.) than other pseudo cholinesterases (half-life period about 4.9 hr.). Results for true cholinesterase of brain, spinal cord and diaphragm (Fig. 4) show that recovery from inhibition with E 600 takes place slowly until on the fourth day, when 50–60% of normal brain cholinesterase activity has returned, reversal slows down considerably.

Fig. 6. Recovery of pseudo cholinesterases of the rat *in vivo*. Rats were given 0.25 mg./kg. E 600 intravenously or for the jejunum experiments 2.4 mg./kg. orally. Each point represents the pseudo cholinesterase activity of the tissue determined as described in the text. Zero activity = the activity after 30 min.; 100% activity = the normal activity of the tissue. Brain (○), heart (×), jejunum (●) and diaphragm (△).

Figs. 5 and 6 show that after inhibition by E 600 *in vitro* and *in vivo* the recoveries of heart, jejunum, diaphragm, serum and submaxillary gland pseudo cholinesterases are similar.

Pseudo cholinesterase of brain

Since a difference between rates of recovery of the pseudo cholinesterase of brain and that of other tissues had been demonstrated, it seemed important

to see if any difference between the two enzymes could be detected by more conventional methods. Accordingly, hydrolysis rates against seven substrates were determined for the pseudo cholinesterase of brain and heart (Table 5). Inhibition values for the pseudo cholinesterase of brain and heart using BuCh as substrate were also determined for four different inhibitors (Table 6). In all respects the behaviour of the pseudo cholinesterases was similar.

Table 6. *Comparison of inhibitor values for pseudo cholinesterase of brain and heart*

(The inhibitor was incubated with brain and heart suspension at 37° for 30 min. before adding the BuCh. Log percentage activity was plotted against concentration and the best straight line drawn. Concentrations producing 50% inhibition were read from the curve.)

Inhibitor	Molar concentration for 50% inhibition	
	Brain	Heart
DFP	2.6×10^{-8}	1.6×10^{-8}
DPDA*	5.2×10^{-7}	4.6×10^{-7}
Mipafox	1.7×10^{-7}	1.7×10^{-7}
E 600	3.7×10^{-8}	2.6×10^{-8}

* Diisopropylphosphorodiamidic anhydride.

Table 7. *Loss of cholinesterase activity on keeping suspensions at 37°*

Suspensions of rat brain were prepared in NaHCO_3 , NaCl buffer without a preservative. True and pseudo cholinesterase values, using 0.03 M MeCh and BuCh respectively, were measured on 1 ml. of suspension from the same tube. Each result is the mean of two observations.)

Time (days)	True cholinesterase ($\mu\text{l. CO}_2/\text{ml. min.}$)	Pseudo cholinesterase ($\mu\text{l. CO}_2/\text{ml. min.}$)
0	12.26	3.45
4	2.81	3.11
5	2.21	2.27
6	1.87	2.70

The recovery rate of brain pseudo cholinesterase, after inhibition with E 600, is closer to the recovery rate of true cholinesterase of brain than it is to that of the pseudo cholinesterase of other tissues. It is important therefore to show clearly that two discrete cholinesterases are in fact present. On keeping a rat-brain suspension at 37° without a preservative

for a number of days, the true cholinesterase of the suspension decreased quickly while the pseudo cholinesterase did so only slowly (Table 7). Indeed it was possible to destroy almost all the true cholinesterase before most of the pseudo cholinesterase had gone. In the presence of Merthiolate under similar conditions pseudo cholinesterase is the least stable. This must indicate that two enzymes of different stability are present. It will also be seen that the substrate patterns in the presence and absence of 4×10^{-6} M-mipafox (Table 1) indicate a very distinct difference in behaviour of the true and pseudo cholinesterase of brain and that in this respect they resemble the true and pseudo cholinesterases of all the rat tissues so far examined. This confirms the findings of Ord & Thompson (1950) who showed both true and pseudo cholinesterase were present in the rat brain. The two enzymes have also been identified in both human (Ord & Thompson, 1952) and dog brain (Burgen & Chipman, 1951).

DISCUSSION

The recovery rates of rat cholinesterases after inhibition by E 600 have been studied. It has been suggested that recovery from cholinesterase inhibition was due to resynthesis of new enzyme. However, varying rates of recovery after inhibition by organophosphorus inhibitors have been reported for man (Grob, Garlick & Harvey, 1950; Callaway *et al.* 1952), suggesting that regeneration of the enzyme is certainly not the sole factor concerned in the reversal of inhibition. Similarly, rat-serum cholinesterase (largely pseudo cholinesterase) has been found to recover in about 10 days after inhibition by DFP (Koelle & Gilman, 1946), whereas recovery after inhibition by E 600 has been shown in this paper to be much more rapid. Sprinson & Rittenberg (1949) have shown that the half-life period of rat-serum protein synthesis is about 7 days. It is therefore unlikely that resynthesis of the enzyme can play a significant part in the rapid recovery of pseudo cholinesterase after inhibition by E 600. This view is confirmed by the fact that the recovery rates *in vivo* are similar to rates *in vitro* when resynthesis presumably cannot occur.

Wilson (1951), Burgen & Hobbiger (1951) and Aldridge (1953c) have suggested that the enzyme is phosphorylated by the organophosphorus inhibitors and that reversal is in fact the hydrolysis of the enzyme phosphate. It may be expected that this phosphorylated enzyme would have a definite hydrolysis rate differing from one substituted phosphate (e.g. dimethyl, diethyl or diisopropyl) to another (Burgen & Hobbiger, 1951) and from one enzyme to another. Hydrolysis of the phosphorylated enzyme would then be expected to be an exponential process. This has been found for pseudo

cholinesterase reversal only. If the substituted phosphate is kept the same, in this case diethyl phosphate, different phosphorylated enzymes would be expected to have different hydrolysis rates. For example, reversal of human-plasma pseudo cholinesterase inhibited with E 600 was not detected in 5 days while recovery of horse-plasma pseudo cholinesterase was very slow when compared to that of rat plasma. Similarly, Hobbiger (1951) noted a difference between the rate of reversal of inhibition with TEPP of guinea pig and human-plasma pseudo cholinesterases. A difference between horse and human pseudo cholinesterase has been demonstrated by Kalow (1952) in spectrophotometric studies with procaine as substrate and also by Lévy & Denys (1951) with various substrates. It is, therefore, suggested that the reversal constants for inhibited enzymes may be a useful criterion of an enzyme's identity.

There is a very striking difference between the rates of recovery, following inhibition by E 600 of the pseudo cholinesterase of brain and spinal cord on the one hand and other pseudo cholinesterases on the other. In the central nervous system the pseudo cholinesterases recover at a similar rate to the true cholinesterase. Yet substrate patterns and inhibitory values have revealed no differences between the pseudo cholinesterase of heart and brain while the difference between the relative stabilities of the true and pseudo cholinesterase of brain, together with the different substrate patterns of the two enzymes (Table 1), emphasizes that there are two discrete cholinesterases in the brain. Thus, the reversal rates have shown a distinction between the pseudo cholinesterase of the central nervous system and that of other tissues which was undetected by conventional methods.

The brain differs from other tissues in having a high lipid content and it may be argued that this could act as reservoir of dissolved E 600 and thus prevent the cholinesterase from returning to normal at the usual rate. However, as A-esterase is present at least in the *in vitro* preparation, it is likely that any E 600 slowly liberated would be hydrolysed.* Different amounts of free amines or other compounds in the tissues might possibly exert a catalytic effect on the return of cholinesterase activity, but not in the brain. It seems more likely that the difference in reversal rates is due to a difference in stability of the phosphorylated enzyme, and that this, in turn, indicates some difference between brain and tissue pseudo cholinesterases, e.g. brain cholinesterase being a lipoprotein (Ord & Thompson, 1951).

* Note added in proof. Without Merthiolate and penicillin the rate of return *in vitro* of brain pseudo cholinesterase is the same as for other tissue preparations.

The true cholinesterase of the nervous system of the rat has been shown to recover quickly at first, thereafter only slowly. It is particularly interesting that the true cholinesterase of brain shows a similar recovery pattern *in vivo* and *in vitro*, for it would be expected that recovery would be exponential. There seem to be two possible explanations of the findings. It may be that part of the inhibited enzyme (about 40%) is removed as a foreign protein, and that all the remaining inhibited enzyme has recovered its activity after 4 days. The further slow rise in activity would then be due to enzyme resynthesis. Since the recovery rate is the same *in vitro* one must assume that the inhibited enzyme is also removed *in vitro*. A second possibility is that two enzymes are present in the true cholinesterase, one (60% of the whole) which recovers rapidly and a second enzyme whose rate of recovery is slow. In view of the demonstration of the difference between the

pseudo cholinesterase of brain and spinal cord and that of other tissues this must be considered.

SUMMARY

1. After inhibition of rat pseudo cholinesterase of heart, serum, jejunum and diaphragm by diethyl *p*-nitrophenyl phosphate (E 600) the total recovery of enzyme activity occurs in about a day both *in vivo* and *in vitro*.

2. A difference in recovery of activity (*in vitro* and *in vivo*) between the pseudo cholinesterase of brain and the other pseudo cholinesterases examined has been shown.

3. The recovery of true cholinesterase after inhibition with E 600 occurs slowly (60% in 4 days) both *in vivo* and *in vitro*.

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Studies of Cholesterol Biosynthesis

1. A NEW CHEMICAL DEGRADATION OF CHOLESTEROL

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The study of cholesterol biosynthesis from precursors labelled with isotopic carbon would be greatly assisted if methods were available for isolating each of the twenty-seven carbon atoms of the sterol in a form suitable for isotopic assay. The constituent atoms in the side chain can be separated (Wüersch, Huang & Bloch, 1952) by adapting known procedures originally designed to determine the structure of cholesterol, but known degradations

of the alicyclic ring structure do not easily lend themselves to the separation of individual atoms. New procedures were therefore devised; the first of these, aimed at rings A and B, has been worked out in detail.

Our point of departure was the observation of Achtermann (1934) and of Laucht (1935) that the keto aldehyde (I), from ergosterol, on pyrolysis gives the ketone (II) and the $\alpha\beta$ -unsaturated