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

2. A COMPARATIVE STUDY OF TRUE AND PSEUDO CHOLINESTERASE*

By A. N. DAVISON†

Medical Research Council Unit for Research in Toxicology, Serum Research Institute, Carshalton, Surrey

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There are two distinct types of enzyme capable of the hydrolysis of acetylcholine. True cholinesterase is predominantly associated with nervous tissue and erythrocytes, while pseudo cholinesterase is found in the serum, liver and pancreas. The two enzymes can be distinguished not only by their distribution, but also by their substrate specificity (Mendel, Mundell & Rudney, 1943) and their sensitivity to certain selective inhibitors (Aldridge, 1953*a*; Fulton & Moge, 1954). Furthermore, it has been shown that pseudo cholinesterase can be almost completely inactivated without the development of any significant signs of poisoning (Mazur & Bodansky, 1946; Hawkins & Gunter, 1946; Hawkins & Mendel, 1949), while complete inhibition of true cholinesterase is invariably fatal.

It has been shown in a previous paper (Davison, 1953) that the rates of reactivation of rat true and pseudo cholinesterases after inhibition by diethyl *p*-nitrophenyl phosphate (paraoxon) are markedly different. Indeed, it has been suggested that the rates of recovery are a characteristic of the inhibited enzyme. Recent work suggests that the organophosphorus compounds phosphorylate the enzyme's active centre (Aldridge, 1954) and that reactivation *in vitro* is the result of hydrolysis of the phosphorylated enzyme. The stability of the phosphorylated enzyme is, therefore, a measure of the strength of the link between the phosphorus of the inhibitor and the enzyme. The previous work on the reactivation of inhibited cholinesterase has been extended by the use of various organophosphorus compounds

and different cholinesterases. The results have been interpreted in terms of the phosphorylation theory.

Not only do the rates of recovery of phosphorylated rat true and pseudo cholinesterases differ, but while the reactivation of inhibited pseudo cholinesterase is complete and kinetically first order, that for inhibited true cholinesterase is partial only. One explanation of the results is that there are two types of true cholinesterase, differing in the stability of their phosphorylated active centres. It has also been found that the reactivation of phosphorylated rat, horse and chicken pseudo cholinesterase is acid catalysed, whereas the recovery of phosphorylated rat and rabbit true cholinesterase activity is not. This interesting finding may well indicate a fundamental dissimilarity between the active groups of the two enzymes and thus account for the obvious differences between them. This work is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Medicine.

MATERIALS AND METHODS

The inhibitors used in this work were: dimethyl, diethyl, di-*n*-propyl and diisopropyl *p*-nitrophenyl phosphates; di-*n*-propyl and diisopropyl phosphorofluoridates; tetraethylpyrophosphate; *NN'*-diisopropyl phosphorodiamidic fluoride and *NN'*-diisopropyl phosphorodiamidic anhydride. Diethyl and di-*n*-propyl *p*-nitrophenyl phosphates and di-*n*-propyl phosphorofluoridates were synthesized according to the methods of Fagerlind, Holmstedt & Wallen (1952) and McCombie, Saunders & Stacey (1948). The remaining compounds were obtained from Dr H. Coates, Albright and Wilson Ltd., and from Dr G. S. Hartley, Pest Control Ltd. Crystalline pepsin and trypsin were obtained from Armour Laboratories.

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† Present address: Laboratoire de Chimie Biologique, 96, Boulevard Raspail, Paris, 6^e.

Cholinesterase determinations and reversal technique. The methods used for the determination of tissue and blood cholinesterase have been given in full in a previous paper (Davison, 1953). The study of reversal of cholinesterase inhibition, depending upon the removal of excess inhibitor by rabbit serum A-esterase, was also as previously described (Aldridge, 1953*b*), except that thiomersalate (Eli Lilly and Co. Ltd., Basingstoke) 1:10 000 was added 4 hr. after the addition of A-esterase. The concentration of inhibitor used in these reversal experiments was about 10 times the concentration necessary for 50% inhibition.

Determination of the apparent energy of activation. The rates of inhibition of rat pseudo cholinesterase (jejunum, 40 mg./ml. in bicarbonate buffer) were determined using *NN'*-diisopropyl phosphorodiamidic anhydride at final concentrations, of 1.4×10^{-6} M, 2.8×10^{-6} M, 8×10^{-7} M at 19.5, 28 and 37° respectively. Suitable volumes were withdrawn at intervals with a syringe pipette and after quickly gassing, the cholinesterase activity was determined in Warburg flasks at 19.5, 28 and 37° using butyrylcholine (0.03 M) as substrate.

Experiments in vivo. Male albino rats of about 250 g. were given the following doses of the different organophosphorus compounds: dimethyl, diethyl, di-*n*-propyl and diisopropyl *p*-nitrophenyl phosphates, 0.35, 0.25, 2 and 5 mg./kg. respectively by intravenous injection; di-*n*-propyl and diisopropyl phosphorofluoridate 2 and 0.5 mg./kg. by intravenous injection and *NN'*-diisopropyl phosphorodiamidic fluoride 40 mg./kg. by subcutaneous injection. These doses always produced acute signs of acetylcholine poisoning. Animals were killed in pairs at the stated times and cholinesterase activity was determined.

RESULTS

Evidence has been accumulating to support the view that inhibition of esterase activity by the organophosphorus compounds is the result of phosphorylation of the active centre (Aldridge, 1953*b*; Aldridge, 1954; Aldridge & Davison, 1953). The kinetics of inhibition, the reversal of inhibition, and the apparent energies of activation for these

two processes provide good evidence that true cholinesterase is readily phosphorylated by the organophosphorus compounds. The evidence for the phosphorylation of pseudo cholinesterase by this group of substances is less complete. It was therefore necessary to show that pseudo cholinesterase was phosphorylated by organophosphorus inhibitors before comparing the reactivation of inhibited true and pseudo cholinesterase.

The kinetics of inhibition of pseudo cholinesterase. Inhibition of rat jejunum suspension by *NN'*-diisopropyl phosphorodiamidic anhydride was studied at 37°, by the methods previously used (Aldridge & Davison, 1952). The results obtained indicated that the reaction was bimolecular and, since the inhibitor is present at a much higher concentration than the enzyme, obeyed the first-order equation with a rate constant of $K = 5.1 \times 10^4 \text{ min.}^{-1} \text{ l.mol.}^{-1}$. *NN'*-Diisopropyl phosphorodiamidic anhydride was chosen as a suitable inhibitor, since it is not enzymically hydrolysed by A-esterase (Aldridge, 1953*c*) and the inhibition is not easily reversed.

The apparent energy of activation of the inhibitory process was also determined by plotting the log rates of reaction at 37°, 28° and 19.5° against the reciprocal of the absolute temperature. The value obtained of 14 500 cal./mole, was consistent with the formation of a covalent bond between the phosphorus atom of the inhibitor and the enzyme.

The reactivation of phosphorylated true cholinesterase. Before studying the reactivation of phosphorylated cholinesterase *in vitro* it was necessary to determine the values for 50% inhibition of true and pseudo cholinesterase by the organophosphorus compounds (Table 1). It was then possible to calculate from these figures suitable inhibitor concentrations for the reversal experiments.

Table 1. *Inhibition values for rat true and pseudo cholinesterase*

The inhibitor was incubated with rat brain or heart suspension for 30 min. at 37°. True cholinesterase activity of the brain was measured by addition of 0.03 M acetyl- β -methylcholine, pseudo cholinesterase by addition of 0.03 M butyrylcholine. Log₁₀ percentage activity was plotted against concentration of inhibitor and the best straight line drawn; concentrations producing 50% inhibition were read from this curve. The inhibitor ratios were calculated by dividing the 50% inhibition value for true by that for pseudo cholinesterase.

Inhibitor	Molar concentration producing 50% inhibition		
	True cholinesterase (rat brain)	Pseudo cholinesterase (rat heart)	Inhibitor ratio
Dimethyl <i>p</i> -nitrophenyl phosphate	4.0×10^{-8}	6.5×10^{-6}	0.006
Diethyl <i>p</i> -nitrophenyl phosphate	1.6×10^{-8}	2.6×10^{-8}	0.6
Diisopropyl <i>p</i> -nitrophenyl phosphate	3.2×10^{-7}	7.1×10^{-8}	4.5
Diisopropyl phosphorofluoridate (DFP)	7.2×10^{-7}	1.6×10^{-8}	45
Di- <i>n</i> -propyl phosphorofluoridate	5.5×10^{-8}	1×10^{-8}	5.5
Tetraethyl pyrophosphate	1.4×10^{-8}	1.2×10^{-8}	1.2
<i>NN'</i> -Diisopropyl phosphorodiamidic fluoride	4.5×10^{-8}	1.7×10^{-7}	254
<i>NN'</i> -Diisopropyl phosphorodiamidic anhydride	2.7×10^{-4}	4.6×10^{-7}	590

The recovery of rat brain true cholinesterase activity after inhibition by dimethyl, diethyl, di-*n*-propyl and diisopropyl and diisopropylamino substituted phosphates (fluoridates and *p*-nitrophenates) has been examined *in vitro* and also *in vivo*. Inhibition by diisopropyl and diisopropylamino phosphorofluoridates was found to be irreversible *in vitro*, for on keeping inhibited rat brain suspension, treated with rabbit serum A-esterase and preservatives, at 37° for 10 days, no reactivation of the enzyme was detected. After injection of the diisopropyl or diisopropylamino phosphates into rats, however, a slow recovery of inhibited cholinesterase occurs (Fig. 1).

Fig. 1. The recovery of inhibited rat brain true cholinesterase activity *in vivo*. Each point represents the mean brain true cholinesterase activity of two rats. Dimethyl (x), diethyl (●), di-*n*-propyl (○), diisopropyl and diisopropylamino (Δ) substituted phosphates were used in these experiments.

This reactivation of the enzyme is, therefore, probably due to synthesis of new cholinesterase. However, the rate of recovery of inhibited true cholinesterase activity after inhibition by dimethyl, diethyl and di-*n*-propyl phosphates *in vitro* was similar to the recovery rate *in vivo* (Fig. 1). In this case it appears that recovery of true cholinesterase activity not only *in vitro*, but also *in vivo* is due to hydrolysis of the phosphorylated enzyme. Thus at least two factors are probably responsible for recovery of cholinesterase activity in the living animal (i) synthesis of fresh enzyme, (ii) hydrolysis of the phosphorylated cholinesterase.

It will be noted that the recovery curve for inhibited true cholinesterase (Fig. 1) is not exponential and shows a change of slope at about 55 % recovery. It seemed interesting to see if other mammalian true cholinesterases showed similar recovery curves

after inhibition by an organophosphate. Reactivation of human, rat, cat, dog and rabbit erythrocyte true cholinesterases inhibited by diethyl *p*-nitrophenyl phosphate was therefore studied by the reversal technique (Davison, 1954). Not only did all the recovery curves (with the possible exception of that of rabbit cholinesterase) show a distinct change of slope, but the rates of recovery were also similar. This, therefore, suggested that this change in slope of the recovery curve of phosphorylated true cholinesterase was a characteristic of mammalian true cholinesterases.

The possibility of two types of true cholinesterase. In view of the above results the effect of repeated

Fig. 2. The effect of repeated injections of dimethyl *p*-nitrophenyl phosphate on rat brain true cholinesterase. Injections of dimethyl *p*-nitrophenyl phosphate were given at zero, 1 and 2 days, and the brain true cholinesterase of pairs of animals determined 30 min., 24 hr. and 8 days after injection.

injections of an organophosphate on the rate of recovery of inhibited cholinesterase was examined. Dimethyl *p*-nitrophenyl phosphate (0.35 mg./kg.) was injected into a group of rats on 3 successive days. 30 min. after each injection between 80 and 90 % of the brain true cholinesterase activity was inhibited, while 23 hr. after each injection about 25 % of the true cholinesterase remained inhibited (Fig. 2). Thus there was no indication of a progressive decrease in recovery. Reactivation after the last dose of inhibitor was slow. This suggested that about 60 % of the true cholinesterase activity inhibited by dimethyl *p*-nitrophenyl phosphate is readily reversible while the remainder is more permanently inhibited.

Preliminary attempts have been made to separate brain true cholinesterase fractions *in vitro*. In the course of experiments on the effect of the addition of

proteinases to rat-brain suspensions, it was found that while pseudo cholinesterase activity was unaffected by the addition of pepsin, about 70 % of the true cholinesterase activity was more rapidly digested than the rest (Fig. 3). After inactivating the pepsin at pH 8.5 for 30 min. and bringing the pH to pH 7.6, the resistant true cholinesterase was inhibited by diethyl *p*-nitrophenyl phosphate and the reactivation studied by the reversal technique. Recovery of activity was about 6 % per day and thus was very slow when compared with the rate found for whole brain suspension. These experiments indicate the possibility that there are two types of true cholinesterase.

The reactivation of phosphorylated pseudo cholinesterase. The recovery curves for pseudo cholin-

Fig. 3. The effect of crystalline pepsin on true cholinesterase. Crystalline pepsin (2.5 mg./ml.) was added at pH 4.6 at 37°. After neutralizing suitable volumes the cholinesterase activity was determined in the Warburg apparatus using 0.03M acetyl- β -methylcholine as a substrate for true (x) cholinesterase.

esterase inhibited by different organophosphates have also been obtained *in vivo* and *in vitro* by the use of the reversal technique. In general, the rate of recovery of activity after inhibition of pseudo cholinesterase is much faster than for true cholinesterase inhibited by the same compound. As with true cholinesterase, inhibition by the diisopropyl and diisopropylamino phosphates is irreversible *in vitro*, although a slow exponential recovery occurs *in vivo* (Table 2). Inhibition of pseudo cholinesterase by dimethyl *p*-nitrophenyl phosphate is irreversible *in vitro*, although recovery of true cholinesterase activity after inhibition by the same compound is relatively rapid. There is also a difference between the rates of recovery *in vivo* of inhibited brain and other tissue pseudo cholinesterases. At present there is no explanation for this finding, for *in vitro* the rates of recovery of serum, jejunum, heart, salivary gland and brain pseudo cholinesterases are alike (Table 2, and Davison, 1953). Unlike the recovery curves for inhibited true cholinesterase, those for pseudo cholinesterase are exponential and the rates of recovery differ markedly with the species used (Table 3).

Myers (1953) has shown that there are large variations in the substrate patterns of mammalian and chicken pseudo cholinesterases. Thus the differences in rates of reactivation and in substrate patterns of pseudo cholinesterase suggest the enzyme for each species is quite distinctive.

The effect of pH on the reactivation of inhibited cholinesterase. The phosphorylated enzyme can be regarded as a special kind of phosphate ester. It, therefore, seemed interesting to examine the effect of acid and alkali on the hydrolysis of phosphorylated true and pseudo cholinesterase, since this may give useful information about the type of linkage between the inhibitor phosphorus and the enzyme active centre.

The stability of rat brain true and serum pseudo cholinesterases was first determined at various pH

Table 2. The rates of reversal of rat pseudo cholinesterase *in vivo* and *in vitro* after inhibition by organophosphorus compounds

The pseudo cholinesterase activity was determined with butyrylcholine (0.03M). *In vitro* and *in vivo* experiments were by techniques previously described (Davison, 1953). Half-life periods are the mean times required for 50% recovery of the inhibited pseudo cholinesterase determined directly from the recovery curves. Serum, jejunum, heart, salivary gland and brain pseudo cholinesterases have been used in these experiments.

	<i>In vivo</i> (hr.) (all tissues other than brain)	<i>In vivo</i> (hr.) (brain pseudo cholinesterase)	<i>In vitro</i> (hr.) (brain and other tissues)
Dimethyl <i>p</i> -nitrophenyl phosphate	72	—	> 200
Diethyl <i>p</i> -nitrophenyl phosphate	4.9	96	5
Di- <i>n</i> -propyl <i>p</i> -nitrophenyl phosphate	17	—	17
Diisopropyl <i>p</i> -nitrophenyl phosphate	72	108	> 250
Diisopropyl phosphorofluoridate			
<i>NN'</i> -Diisopropyl phosphorodiamidic fluoride			
<i>NN'</i> -Diisopropyl phosphorodiamidic anhydride			

Table 3. *The rates of reversal of different species pseudo cholinesterase after inhibition by diethyl p-nitrophenyl phosphate and tetraethyl pyrophosphate in vitro*

The serum pseudo cholinesterase activity was determined with butyrylcholine (0.03M). The reactivation was studied at 37° by techniques previously described (Davison, 1953). Half-life periods are the mean times required for 50% recovery of the inhibited pseudo cholinesterase activity determined directly from the recovery curves, except for the figures for horse and human cholinesterase which have been calculated by extrapolation. The figure for guinea pig serum has been obtained from the results of Hobbiger (1951). The rates of hydrolysis of butyrylcholine relative to acetylcholine (100) are inserted in the last column.

Inhibitor	Species	Half-life for reversal (hr.)	0.03M butyrylcholine hydrolysis
Tetraethyl pyrophosphate	Hen	2.2	110
Diethyl p-nitrophenyl phosphate	Rat	5	145
Tetraethyl pyrophosphate	Guinea pig	72	180
Diethyl p-nitrophenyl phosphate	Horse	200	216
Diethyl p-nitrophenyl phosphate	Man	730	250

Fig. 4. The stability of rat cholinesterases at different pH values. The stability of brain true (x) and brain and serum pseudo (●) cholinesterase have been determined by incubating at different pH values for 30 min., and then adjusting the pH to 7.6 before estimating the enzyme activity. Citric acid-sodium phosphate (McIlvaine), sodium acetate-hydrochloric acid (Walpole) and $\text{Na}_2\text{CO}_3\text{-NaHCO}_3\text{-NaCl}$ (Aldridge & Davison, 1952) buffers have been used. The substrate hydrolysis curve at different pH values was determined by use of the colorimetric method of Hestrin (1949), and is shown by the broken line. Acetylcholine perchlorate, 0.0138M and butyrylcholine perchlorate, 0.03M, were used as substrates for brain true (Δ) and serum pseudo (O) cholinesterase respectively.

values. Fig. 4 shows the effect on the two enzymes of 30 min. incubation at 37° in buffer solutions of different pH. The substrate hydrolysis curves for true and pseudo cholinesterase are also shown on the same figure. It will be noted that both enzymes are stable between pH 5 and 9 and this proved to be a suitable pH range for the following studies.

The effect of pH on the reactivation of phosphorylated pseudo cholinesterase. A standard preparation

of inhibited rat serum pseudo cholinesterase free from excess diethyl p-nitrophenyl phosphate was used throughout for the following experiments. First, the inhibited serum preparation was added to a suitable buffer and the pH determined, the serum was then incubated at 37° and at intervals samples withdrawn, neutralized and the cholinesterase activity determined. The percentage inhibition at zero time was defined as 100% inhibition of a control of uninhibited serum. The reactivation expressed as the log percentage of total inhibition was plotted at different times and for different pH values and the half-lives for reactivation calculated (Table 4). The results show that the hydrolysis of diethyl phosphorylated rat pseudo cholinesterase is acid catalysed. This seems to be a general property of inhibited pseudo cholinesterase, for the hydrolysis of horse and chicken serum pseudo cholinesterase inhibited by diethyl and di-n-propyl p-nitrophenyl phosphates respectively, is also acid catalysed.

The effect of pH on the reactivation of phosphorylated true cholinesterase. Since the recovery of true cholinesterase activity after inhibition by dimethyl p-nitrophenyl phosphate is fast, this compound has been used for the study of pH effects on the reactivation of inhibited true cholinesterases. In experiments on rat brain and rabbit erythrocyte true cholinesterase, it was found that reactivation of the enzyme inhibited by dimethyl p-nitrophenyl phosphate was optimum at pH 7.8. There was no evidence of either acid or base catalysis. In similar experiments on reactivation of rat brain true cholinesterase inhibited by diethyl p-nitrophenyl phosphate no acid or base catalysis was found.

The effect of trypsin on the reversal of inhibited pseudo cholinesterase. During the course of experiments on the reactivation of inhibited pseudo cholinesterase by different compounds (hydroxylamine, pyridine, etc.), it was found that addition of 2 mg./ml. trypsin almost doubled the rate of enzyme recovery. The pH of the preparation was

Table 4. *The effect of pH on the recovery of cholinesterase activity after inhibition by various organophosphorus compounds in vitro*

Methods are described in detail in the text. Citric acid-sodium phosphate (McIlvaine), sodium acetate-hydrochloric acid (Walpole) and Na_2CO_3 - NaHCO_3 - NaCl (Aldridge & Davison, 1952) buffers have been used, and the pH adjusted to pH 7.6 immediately before the cholinesterase estimations. Pseudo and true cholinesterases have been inhibited by diethyl and dimethyl *p*-nitrophenyl phosphates respectively and recovery studied by the reversal technique.

Approximate half-lives for reversal (min.)				
pH	5	6	7.8	9
Source of pseudo cholinesterase inhibited by diethyl <i>p</i> -nitrophenyl phosphate				
Rat serum	125	270	350	1000
Chicken serum	—	110	150	750
Horse serum	2 (days)	—	27 (days)	—
Source of true cholinesterase inhibited by dimethyl <i>p</i> -nitrophenyl phosphate				
Rat brain	1000	510	200	1000
Rabbit erythrocytes	—	275	100	140 (pH 8.7)

strictly controlled. It was unfortunately impossible to try the effect of trypsin on the reactivation of inhibited true cholinesterase since true cholinesterase is destroyed by trypsin.

DISCUSSION

The introduction of the organophosphorus compounds has made it possible to differentiate between true and pseudo cholinesterase, not only by means of selective inhibition, but also by the different stabilities of the inhibited enzyme. For the purposes of this work it was clearly important to demonstrate that the inhibition of both true and pseudo cholinesterase by the organophosphorus compounds was mediated by the same process. While there is good evidence to suggest that true cholinesterase is phosphorylated during inhibition (Aldridge, 1953b; Aldridge, 1954; Aldridge & Davison, 1953) the corresponding evidence for the phosphorylation of pseudo cholinesterase is less complete. Bournsnel & Webb (1949), and Jansen, Jang & Balls (1952) have shown that during inhibition of pseudo cholinesterase by DF^{32}P , the ^{32}P becomes bound to the enzyme. It has now been found that the high energy of activation for the inhibitory process and the kinetics of inhibition (see also Austin & Berry, 1953) support the view that phosphorylation occurs. Furthermore, rat pseudo cholinesterase inhibited by a series of different diethyl phosphates recovers its enzyme activity at a similar rate with the compounds examined (Aldridge & Davison, 1953). It is therefore concluded that inhibition of both true and pseudo cholinesterase by the organophosphorus compounds is the result of phosphorylation of the enzyme. As a result of the work reported hitherto (Davison, 1953, 1954) and in this paper, certain conclusions can be drawn about the mechanism of recovery of inhibited cholinesterase and the nature of the enzyme involved. Both substrate specificities and

reactivation rates of inhibited cholinesterase indicate that the true cholinesterases of different rat tissues are alike, and similarly the pseudo cholinesterases of different rat tissues are also alike, although it has been noted that there is a difference between the recovery rates of inhibited brain and tissue pseudo cholinesterases *in vivo*. It can also be concluded that at least two factors are responsible for recovery of cholinesterase activity after poisoning by organophosphorus compounds *in vivo*. If the inhibition is irreversible *in vitro* then recovery is due to resynthesis of fresh enzyme. If the inhibited enzyme is very unstable then the return of enzyme activity will be due to hydrolysis of the phosphorylated enzyme. Between these two extremes the return of cholinesterase activity will be due to both processes. There is no evidence that the hydrolysis of the phosphorylated enzyme is catalysed *in vivo*.

In a previous paper it was shown that there was a change in slope of the recovery curve for true cholinesterase inhibited by diethyl *p*-nitrophenyl phosphate *in vivo* and *in vitro*. Similar changes in the slope of the recovery curve have now been found after inhibition by various substituted phosphates and for different mammalian erythrocyte true cholinesterases (see also Frawley, Hagan & Fitzhugh, 1952; Augustinsson & Grahn, 1952). Possible explanations for this change in slope of the recovery curve have been discussed previously (Davison, 1953). It is, for example, possible that some of the phosphorylated true (but not pseudo) cholinesterase is removed *in vivo* as foreign protein and that the observed recovery curve represents the hydrolysis of the remaining phosphorylated enzyme followed by a slow synthesis of fresh cholinesterase. It may also be argued that the prolonged incubation of brain true cholinesterase *in vitro* results in a change of the enzyme resulting in the formation of a stable phosphorylated enzyme. However, in the shorter experiments with dimethyl *p*-nitrophenyl

phosphate and with hydroxylamine (see below) changes in the slope of the recovery curves are also noted. Furthermore, experiments *in vivo* indicate that following repeated injection of dimethyl *p*-nitrophenyl phosphate, recovery after each injection reaches the same level and does not progressively decrease as might be expected if the phosphorylated enzyme was removed as foreign protein. Another possibility is that there are two types of true cholinesterase differing in the stability of their phosphorylated active centres. In support of this hypothesis is the finding that about 30 % of brain true cholinesterase is more resistant to peptic digestion than the rest. The reactivation of this resistant fraction after inhibition by diethyl *p*-nitrophenyl phosphate is very slow. Recently Cunningham (1954) has examined the effect of hydroxylamine on the reactivation of diethyl phosphorylated α -chymotrypsin, finding that only 40 % of the original α -chymotrypsin activity could be recovered. It was thought that there may be two forms of chymotrypsin present, one forming a more stable phosphorylated enzyme than the other, since there was good evidence that an equilibrium between active and inactive chymotrypsin did not exist. Similarly using hydroxylamine for the reactivation of true cholinesterase inhibited by tetraethyl pyrophosphate, Hobbiger (private communication) has found that recovery readily occurs to about 60 % of the original activity, but thereafter reactivation is very slow. Thus the present evidence lends support to the hypothesis that there are two types of true cholinesterase, but it must be stressed that much more work is required before this is established.

Pseudo cholinesterase from different tissues of the rat after inhibition by diethyl *p*-nitrophenyl phosphate rapidly recovers activity, but unlike true cholinesterase the recovery rates of pseudo cholinesterase from one species to another varies a great deal. These differences, also reflected in the substrate patterns of the pseudo cholinesterase of mammalian species, indicate the large variations in the properties of pseudo cholinesterase compared to the similarities between true cholinesterases. A fundamental difference between the two enzymes is also illustrated by their susceptibility to proteinases. True cholinesterase activity is destroyed by trypsin and to a lesser extent by pepsin, whereas pseudo cholinesterase activity is not affected by these proteinases.

These results, while emphasizing the basic differences between true and pseudo cholinesterase, are extremely difficult to interpret in terms of the active centres of the two enzymes. However, the finding that the hydrolysis of phosphorylated pseudo cholinesterase is acid catalysed, whereas the hydrolysis of phosphorylated true cholinesterase is

fastest at about neutral pH, indicates a dissimilarity between the active groups of the two enzymes. Calculation of the dissociation constant of serum pseudo cholinesterase suggests that an amino group is involved in substrate binding (Lindley, 1954). The possibility that phosphorylation of pseudo cholinesterase occurs through an amino group, would explain the observed acid catalysed hydrolysis of the inhibited enzyme, since it is known that the N-P link in creatine phosphate, dimethylamides of phosphoric acid and dimethylamino ethoxyphosphoryl cyanide is largely hydrolysed by an acid catalysed reaction (Fiske & Subbarow, 1929; Winnick & Scott, 1947; Heath & Casapieri, 1951; Larsson, 1953). This hypothesis is also consistent with the finding that trypsin catalyses the splitting of phosphorylated pseudo cholinesterase for Holter & Li (1950) have shown that *N*-(*p*-chlorophenyl)-amidophosphoric acid is split by proteolytic enzymes at the N-P bond. The hydrolysis of phosphorylated true cholinesterase is optimum at neutral pH; it is therefore likely that the linkage of phosphorus to enzyme differs from that postulated for pseudo cholinesterase.

SUMMARY

1. Evidence has been presented to support the view that pseudo cholinesterase, like true cholinesterase, is phosphorylated by the organophosphorus compounds.

2. The stability of the phosphorylated enzyme depends upon the enzyme inhibited and the organophosphate used.

3. Some evidence has been presented to suggest that there may be two types of true cholinesterase, differing in the stability of their phosphorylated active centres.

4. The hydrolysis of phosphorylated pseudo cholinesterase is acid catalysed, the hydrolysis of phosphorylated true cholinesterase is optimum at neutral pH.

5. A working hypothesis has been put forward suggesting that the organophosphorus compounds combine with an amino group of pseudo cholinesterase, but some other group is present in true cholinesterase.

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Studies on Sulphatases

10. THE ISOLATION AND CHARACTERIZATION OF BIOSYNTHETIC ARYLSULPHATES*

By K. S. DODGSON, F. A. ROSE AND B. SPENCER
Physiology Institute, University of Wales, Newport Road, Cardiff

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Most phenols are partially excreted as the corresponding sulphate esters although, owing to the great solubility of the compounds and their sodium and potassium salts, few arylsulphates have been isolated from urine. The less soluble *p*-toluidine (Barton & Young, 1943) and *p*-bromoaniline (Laughland & Young, 1944) arylsulphate salts have been isolated after some preliminary treatment of the urine (Berenbom & Young, 1951) but isolation in this way is not very satisfactory when the arylsulphates are present in small amounts. Other naturally occurring sulphate esters are difficult to isolate from natural sources. They are usually precipitated from aqueous solution by organic solvents.

Egami (1939) noted that tryptaflavin (2:8-diamino-10-methylacridinium chloride), safranin (2:8-diamino-10-phenylphenazonium chloride) and rivanol (5:8-diamino-3-ethoxyacridine) gave insoluble salts with a number of different sulphate esters and suggested that these bases might be used to separate naturally occurring sulphates. In the present work a number of aminoacridines and related compounds have been examined as precipitating agents for organic sulphates, particularly arylsulphates. Many of these bases gave insoluble salts with sulphate esters and the most suitable, 5-aminoacridine, was used to isolate the sulphated phenols arising from the metabolism of

chlorobenzene, chlorophenol and 4-chlorocatechol. A preliminary account of this work has already appeared (Dodgson, Rose & Spencer, 1954).

MATERIALS

Eufavin (2:8-diamino-10-methylacridinium chloride), safranin, 5-aminoacridine hydrochloride, 2- and 3-aminoquinolines and 2-aminopyridine were commercial samples. 6- and 8-Aminoquinolines were obtained by reduction of the corresponding nitroquinolines by the methods of Linsker & Evans (1946) and Dikshoorn (1929), respectively. 5- and 7-Aminoquinolines were prepared from the corresponding 5- and 7-nitroquinolines (Bradford, Elliott & Rowe, 1947) by reduction, using the methods of Dikshoorn (1929) and Linsker & Evans (1946), respectively. 4-Amino-2-methylquinoline: 2-methylcinchonamide was obtained from isatin by the method of Bayer (1916) and was subsequently converted into 4-amino-2-methylquinoline according to the directions of Lions & Ritchie (1941). Arylsulphates were prepared by the method of Burkhardt & Lapworth (1926).

EXPERIMENTAL AND RESULTS

The precipitation of arylsulphates by aminoacridines and safranin

Three arylsulphates, phenyl sulphate (PS), *p*-nitrophenyl sulphate (NPS) and *p*-acetylphenyl sulphate (APS) were selected for study. Molar proportions (usually 0.002M) of the arylsulphate (potassium salt) and the precipitating reagent

* Part 9: Dodgson *et al.* (1955).