Studies on Esterases in the Chicken Central Nervous System

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Trityl phosphates have caused many cases of poisoning in humans involving damage to the nervous system which is characterized clinically by ataxia and paralysis of the limbs (Smith, Elvove, Valaer, Frazier & Malleroy, 1930; Henschler, 1958); also, in the hen a similar type of poisoning is produced and this species is therefore accepted as a suitable experimental animal for studying this type of neurotoxicity (Smith, Elvove & Frazier, 1930). Other organophosphorus compounds, e.g. di-isopropyl phosphorofluoridate and *NN'*-di-isopropyl phosphorodiamidic fluoride (Barnes & Denz, 1953) and a further series of phosphonofluoridates (Davies, Holland & Rumens, 1960), produce a similar effect in chickens. Although tri-*o*-tolyl phosphate is effective many other tri(alkylphenyl) phosphates are ineffective (Hine *et al.* 1956; Henschler, 1958, 1959), and in a more recent study out of 23 tri(alkylphenyl) phosphates only nine produced ataxia in chickens (Bondy, Field, Worden & Hughes, 1960; Aldridge & Barnes, 1961). From all of these studies no overall correlation between chemical structure and neurotoxicity has been established. Although many of these compounds produce inhibition of pseudo-cholinesterase of the chicken, this cannot be correlated with neurotoxicity (Davison, 1953; Aldridge & Barnes, 1961). Nevertheless, it is true that all of the compounds capable of inducing neurotoxicity have the property of inhibiting enzymes possessing carboxylic esterase activity *in vitro* or are converted *in vivo* into compounds that have this property (Aldridge, 1954*a*; Aldridge & Barnes, 1961; Casida, 1961). Therefore the possibility exists of a correlation between neurotoxicity and inhibition of esterases other than cholinesterases. This was the reason for the present study of esterases of the chicken central nervous system.

One of the problems in the study of esterases is that their physiological substrates are not usually known and the selection of substrates is largely guesswork. However, two facts suggested to us that, in this instance, a more logical approach might be made. Tri-*o*-tolyl phosphate is metabolized *in vivo* (Aldridge, 1954*a*) and a cyclic

phosphate (cf. Fig. 1) has been isolated; the neurotoxicity and anti-esterase activity of both the synthetic compound and the isolated material are very high and are identical (Casida, Eto & Baron, 1961; Baron, Bennett & Casida, 1962; Eto, Casida & Eto, 1962). Since inhibition of esterases by organophosphorus compounds and hydrolysis of substrates are in principle identical processes (see review by Aldridge, 1956), carboxylic esters analogous to the metabolite from tri-*o*-tolyl phosphate have been synthesized and used as substrates for the present studies. A preliminary account of this work has been presented (Poulsen & Aldridge, 1963).

METHODS AND MATERIALS

Abbreviation. DFP, Di-isopropyl phosphorofluoridate.

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: acetylcholine chloride was from Roche Products Ltd.; *n*-butyrylcholine perchlorate was prepared as described by Aldridge (1953*a*); phenyl benzoate and benzyl benzoate were from Hopkin and Williams Ltd.; neostigmine methyl sulphate was from L. Light and Co. Ltd.; DFP was from Allen and Hanbury Ltd.; *NN'*-di-isopropyl phosphorodiamidic fluoride (mipafox) was given by Dr G. S. Hartley (Fisons Pest Control

Fig. 1. Comparison of the structure of (I) a metabolite from tri-*o*-tolyl phosphate and (II) the carboxylic esters used. Phenyl saligenin phosphate which lacks the methyl group marked with an asterisk is an active neurotoxic compound (Casida, Baron, Eto & Engel, 1963) and would be produced as a metabolite from diphenyl mono-*o*-tolyl phosphate.

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Ltd.); diethyl *p*-nitrophenyl phosphate and tetraethyl pyrophosphate were given by Mr D. R. Davies; phenyl saligenin phosphate was given by Dr H. Coates (Albright and Wilson Ltd.); tri-*p*-tolyl phosphate and tri-*o*-tolyl phosphate were given by Dr H. Bondy (Coalite Products Ltd.).

The esters were synthesized by a method described by Huggins & Lapidès (1947) as modified by Aldridge (1953*b*). The solid esters were recrystallized from ethanol, benzene or ethanol plus light petroleum (b.p. 30–40°). The esters had the following m.p. (uncorr.): *o*-tolyl phenylacetate, 44°; *m*-tolyl phenylacetate, 52°; *p*-tolyl phenylacetate, 76–77°; *p*-nitrophenyl phenylacetate, 64°; *p*-nitrophenyl benzoate, 140–141°; *p*-ethylphenyl phenylacetate, 27–28°.

Purity of phenyl phenylacetate, phenyl 3-phenylpropionate and phenyl 4-phenylbutyrate. For these three esters the purification procedure described by Huggins & Lapidès (1947) was modified; the benzene solution was shaken three times with 6*N*-NaOH instead of saturated NaHCO₃ solution and then washed with water to neutral pH. Phenyl phenylacetate had m.p. 44° (m.p. 42° according to Heilbron & Bunbury, 1946), and phenyl 4-phenylbutyrate 77–78° (Found: C, 79.6; H, 6.4. Calc. for C₁₈H₁₆O₂: C, 80.0; H, 6.7%). The hydrocinnamoyl chloride for the synthesis of phenyl 3-phenylpropionate was redistilled *in vacuo* (1–1.5 mm. Hg) and only the main fraction (b.p. 85°) used. The identity of hydrocinnamoyl chloride was checked by preparation of its amide and anilide. The synthesized phenyl 3-phenylpropionate was recrystallized three times from light petroleum (b.p. 30–40°) in a mixture of solid CO₂ and acetone and had m.p. 16–17° (Found: C, 79.7; H, 6.6. Calc. for C₁₈H₁₆O₂: C, 79.7; H, 6.2%).

The ultraviolet-absorption spectra of phenyl phenylacetate, phenyl 3-phenylpropionate and phenyl 4-phenylbutyrate were investigated in hexane solutions. The absorption maxima (m μ) were as follows: phenyl phenylacetate: 250, 256.5 and 262; phenyl 3-phenylbutyrate: 246.5, 251, 256.5, 262 and 265; phenyl 4-phenylbutyrate: 246.5, 251.5, 257, 259, 262, 265. Values of $E_{256.5}^{1\%1\text{cm}}$ were: phenyl phenylacetate: 4.38×10^3 ; phenyl 3-phenylpropionate: 4.66×10^3 ; phenyl 4-phenylbutyrate: 4.97×10^3 . The compounds were examined by the method of Hestrin (1949) for the determination of esters: equimolar concentrations of the esters gave the same extinction at 500 m μ .

Animals and their treatment. All chickens used in the present studies were of the cross-breed Light Sussex $\times$ Rhode Island Red of between 6 and 12 months of age. Tri-*o*-tolyl phosphate was given by a stomach tube.

Homogenizing and storage procedure. The chickens were killed by breaking their necks. The brain and spinal cord were dissected out. The brain stem was cut between the pons and medulla; the medulla and the cranial and haemorrhagic part of the spinal cord were discarded. The brain or spinal cord was immediately placed in ice-cold buffer [NaCl (150 mM) and NaHCO₃ (35.8 mM)], blotted free from blood and buffer, weighed and homogenized in buffer in a Nelco homogenizer (8000–10000 rev./min. for 2 min.). The homogenates were kept in ice until use later the same day. Storage for longer periods was at –5°. The esterase activity of brain was stable under these conditions for at least 7 days and of spinal cord for 4 days with not more than 10% loss. After storage of tissues intact at –18° for long periods they showed no difference in activity from fresh tissue. Other tissue homogenates were prepared in the same way.

Manometric determination of esterase activity. Determinations were carried out in Warburg apparatus at 37° in a medium containing NaHCO₃ (35.8 mM), NaCl (150 mM) and gelatin (0.5%). The tissue homogenates were tipped in from the side arm of the Warburg flask after gassing and equilibration with CO₂ + N₂ (5:95). Soluble substrates (acetylcholine chloride and *n*-butyrylcholine perchlorate) were dissolved in, and liquid substrates (phenyl butyrate, tributyrin and phenyl 3-phenylpropionate) were emulsified with, the buffer–gelatin mixture in the main compartment of the Warburg flask. The other solid less-soluble substrates were, in the preliminary experiments (Table 1), dissolved in dimethylformamide and added to the buffer in the main compartment. When it was found that the concentration of dimethylformamide used (10%) decreased the activity by approx. 6% its use was stopped and the substrates were homogenized in the buffer–gelatin mixture with an all-glass homogenizer. The overall concentration of substrates was 10 mM except for acetylcholine chloride (14 mM), *n*-butyrylcholine perchlorate (30 mM), tributyrin (50 mM), phenylbutyrate (91 mM), benzyl benzoate (50 mM) and phenyl 3-phenylpropionate (20 mM). During the manometric determination, readings were taken at intervals of 5 min. for the first 30 min. The output was in most cases linear with respect to time; for the substrate phenyl phenylacetate a linear relationship was found up to 6 μ l. of CO₂/min. In all cases the rate of CO₂ evolution was linear with respect to enzyme concentration. Enzyme activities were obtained by calculating the best straight line by the method of Aldridge, Berry & Davies (1949) and expressed as μ l. of CO₂/g. wet.wt. of tissue/min.

Inhibition experiments. Solutions of water-soluble inhibitors were freshly prepared in the buffer used for homogenizing. The more insoluble organophosphorus compounds (tri-*o*-tolyl phosphate and tri-*p*-tolyl phosphate) were dissolved in dimethylformamide and the same amount of dimethylformamide was added to the control flasks. In all experiments *in vitro* the time of inhibition was 30 min. and the temperature 37°. The stated concentration of inhibitor was the concentration during incubation and before mixing with substrate, except in experiments with neostigmine where it was the concentration after mixing with the substrate. In the preliminary experiments the enzyme and the inhibitor were mixed and incubated in the side arm of the flask. For most experiments and always when two substrates were compared the following technique was used. A sample (1–5 ml.) of the suitably diluted tissue homogenate was placed in glass-stoppered tubes in an ice bath. To each tube was added a suitable volume of the inhibitor solution in buffer or in dimethylformamide (at 100 times the final concentration required) and as quickly as possible a suitable volume (up to 0.8 ml.) of the mixture in the tubes was placed in the side arm of the flask, and used for the determination of the residual enzymic activity. The time of inhibition (30 min.) was the time of incubation from the placing of the flask in the Warburg bath to mixing with the substrate by tipping in the content of the side arm. Gassing, equilibration etc., took place during the incubation.

Fractionation procedure. After decapitation of the chicken the brain was removed, rinsed in ice-cold 0.3*M*-sucrose, rough dried and weighed. One brain was homogenized in 15 ml. of ice-cold 0.3*M*-sucrose by using a smooth glass tube and Perspex pestle with an all-round clearance of

0.005 in. (Aldridge, Emery & Street, 1960). The homogenate was diluted to 25 ml. with sucrose and was divided by centrifuging at 145000g for 60 min. at 0° into two fractions, a particulate and a supernatant. The precipitate was re-homogenized in 15 ml. of 0.3M-sucrose and centrifuged as before, the supernatant discarded and the precipitate kept.

The protein content of chicken brain, determined by a modification (Aldridge, 1957) of the biuret method of Robinson & Hogden (1940), was found in two experiments to be 94 and 89.4 mg. of protein (as albumin)/g. wet wt. of brain; 71.9% and 25.0% was recovered in the particulate and supernatant fractions respectively.

RESULTS

Selection of substrates. In Table 1 the rates of hydrolysis of phenyl phenylacetate and other esters with a similar structure are compared with the rates of hydrolysis of acetylcholine, *n*-butyrylcholine, tributyrin and phenyl butyrate. Benzyl benzoate, phenyl benzoate and the more-soluble *p*-nitrophenyl benzoate were hydrolysed slowly. The activity against the tolyl and ethylphenyl esters of phenylacetic acid was about one-third of the rate of hydrolysis of phenyl phenylacetate, whereas *p*-nitrophenyl phenylacetate was hydrolysed at a higher rate than was phenyl phenylacetate. The relatively high rate of spontaneous hydrolysis and a considerable variation in results from the same homogenate made this *p*-nitrophenyl ester unsuitable for further experiments. Phenyl 3-phenylpropionate was hydrolysed five times as fast as phenyl phenylacetate and at a rate as high as that of acetylcholine, whereas phenyl

4-phenylbutyrate was hydrolysed at a rate less than half that of phenyl phenylacetate.

Since the identity of the compounds is beyond reasonable doubt (see the Methods and Materials section) the differences in the rates of hydrolysis of the such closely related members of an homologous series are noteworthy. It was therefore decided to use phenyl phenylacetate and phenyl 3-phenylpropionate for further experiments.

Hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by cholinesterases. To investigate whether phenyl phenylacetate and phenyl 3-phenylpropionate were hydrolysed by cholinesterases, experiments with the cholinesterase inhibitor neostigmine were performed (Table 2). With both spinal cord and brain preparation the hydrolysis of the two choline esters, acetyl- and *n*-butyryl-choline, was nearly completely inhibited by concentrations of neostigmine 10–100-fold less than those which had very little influence on the hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate. These two substrates are therefore apparently not hydrolysed at appreciable rates by cholinesterases in brain and spinal cord.

Inhibition of the hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by organophosphorus compounds. Organophosphorus compounds are known to be inhibitors of many enzymes with carboxylic esterase activity (Aldridge, 1956) and have been used to discriminate between the different enzymes with esterase activity which are present in tissue homogenates (Aldridge, 1953*b*, 1954*b*; Myers, Schotte, Boer & Borsje-Bakker, 1955; Myers, 1956). The hydrolysis of phenyl

Table 1. *Hydrolysis of various esters by homogenates of chicken brain and spinal cord*

All the solid substrates (except the two soluble ones, acetylcholine chloride and *n*-butyrylcholine perchlorate) were dissolved in dimethylformamide and added to the buffer-gelatin (final concn. of dimethylformamide 10%). The liquid substrates (tri-*n*-butyrin, phenylbutyrate and phenyl 3-phenylpropionate) were emulsified with buffer-gelatin. In parentheses are the numbers of homogenates examined.

Substrates	Activity (μ l. of CO ₂ /g. wet wt./min.)	
	In brain	In spinal cord
Acetylcholine	540–554 (2)	—
<i>n</i> -Butyrylcholine	47 (1)	24 (1)
Tri- <i>n</i> -butyrin	103 (1)	—
Phenyl butyrate	297 (1)	—
Phenyl benzoate	11–17 (4)	—
Benzyl benzoate	5 (1)	—
Phenyl phenylacetate	83–103 (6)	44–47 (2)
Phenyl 3-phenylpropionate	475–504 (3)	147–206 (3)
Phenyl 4-phenylbutyrate	40 (1)	22 (1)
<i>o</i> -Tolyl phenylacetate	36–44 (2)	—
<i>m</i> -Tolyl phenylacetate	36 (1)	—
<i>p</i> -Tolyl phenylacetate	36 (1)	—
<i>p</i> -Ethylphenyl phenylacetate	27 (1)	—
<i>p</i> -Nitrophenyl benzoate	6 (1)	—
<i>p</i> -Nitrophenyl phenylacetate*	157–202 (3)	—

* Spontaneous hydrolysis was 0.6 μ l. of CO₂/min. for this compound; it was negligible for all other substrates.

Table 2. *Inhibition by neostigmine of the hydrolysis of acetylcholine, n-butyrylcholine, phenyl phenylacetate and phenyl 3-phenylpropionate by homogenates of brain and spinal cord*

The homogenates were incubated for 30 min. at 37° with the neostigmine before the mixing with the substrate. The concentration of neostigmine given is the final concentration during the estimation of the residual activity.

Substrate	Brain		Spinal cord	
	Concn. of neostigmine (μM)	Inhibition (%)	Concn. of neostigmine (μM)	Inhibition (%)
Acetylcholine	1	76	—	—
	10	92	—	—
n-Butyrylcholine	0.65	100	0.1	100
	0.065	70	—	—
Phenyl phenylacetate	150	7	15	7
Phenyl 3-phenylpropionate	15	7	150	3

Table 3. *Inhibition by organophosphorus compounds of hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by homogenates of brain and spinal cord*

The tissue homogenates were incubated for 30 min. at 37° with a range of concentrations of inhibitor before the mixing with the substrate. The concentrations of inhibitors are those during the incubation period. The concentration of inhibitor to produce 50% inhibition was determined graphically.

Inhibitor	Concn. for 50% inhibition (μM)	
	Phenyl phenylacetate	Phenyl 3-phenylpropionate
Brain		
Mipafox	200	> 2000
DFP	1.3	6.0
Tetraethyl pyrophosphate	1.0	2.2
Diethyl p-nitrophenyl phosphate	0.16	0.63
Phenyl saligenin phosphate	0.10	0.09
Spinal cord		
Mipafox	25–200	1000
DFP	1.6	4.0

Table 4. *Inhibition by di-isopropyl phosphorofluoridate of the hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by homogenates of sciatic nerve*

Homogenates were incubated with inhibitor for 30 min. at 37° before the mixing with the substrate. The concentrations of inhibitors are those during the incubation period. Sciatic nerves from six chickens were pooled, stored at –18° and homogenized together.

Concn. of DFP (μM)	Inhibition (%)		Phenyl 3-phenylpropionate:phenyl phenylacetate hydrolysis ratio
	With phenyl phenylacetate	With phenyl 3-phenylpropionate	
0	0	0	5.8
1	49	15	9.3
10	73	56	9.7

phenylacetate and phenyl 3-phenylpropionate was inhibited by diethyl p-nitrophenyl phosphate, tetraethyl pyrophosphate, DFP, mipafox and phenyl saligenin phosphate (Table 3), the last-named being the most active (cf. Fig. 1). With DFP and mipafox a difference in the sensitivity of the hydrolysis of the two substrates was found with both brain and spinal cord (see below). In other experiments this was shown to be true also for sciatic nerve (Table 4).

Experiments with mipafox and di-isopropyl phosphorofluoridate. The hydrolysis of phenyl phenylacetate by brain homogenates was inhibited 15–20 % by 0.1 μM-mipafox, which had little influence on the hydrolysis of phenyl 3-phenylpropionate. The proportion of activity which was very sensitive to mipafox varied and in some homogenates was only 10 % of the total. In spinal cord a similar variability was found, the inhibition of the hydrolysis of phenyl phenylacetate varying between 20 and 40 % whereas the hydrolysis of phenyl 3-phenylpropionate was inhibited by not more than 10 %. This sensitive enzyme activity cannot be due to pseudo-cholinesterase, which does not hydrolyse phenyl phenylacetate (and phenyl 3-phenylpropionate) (Table 2). With higher concentrations of mipafox the difference between relative activities with the two substrates increased (Fig. 2). A similar but less pronounced discrimination was found in brain in experiments with 100-fold lower concentrations of DFP (Fig. 3).

There is evidence that mipafox or DFP can phosphorylate esterases (for review see Aldridge, 1956). Experiments with the esterases of chicken brain give no reason to doubt that the inhibition of these enzymes is of the same type. Inhibition is prevented by the substrate but is not reversed if the enzymes are preincubated with inhibitor. Inhibition is progressive and is much slowed down by a decrease in temperature. We conclude that these inhibitors are phosphorylating the enzyme; therefore the degree of inhibition of an enzyme for

a given time and temperature will be the same whatever substrate is used. That the degree of inhibition is not the same in this case shows that more than one enzyme is present, and they must have differing substrate specificity. The results in Figs. 2 and 3 have been regraphed in Fig. 4 to

Fig. 2. Inhibition by mipafox of the hydrolysis of phenyl phenylacetate (●) and phenyl 3-phenylpropionate (○) in brain homogenates. The inhibition was estimated by comparison with a control sample without inhibitor. The activities of the controls were: for phenyl phenylacetate, 110; for phenyl 3-phenylpropionate, 413 μ l. of CO_2 /g. of brain/min.

Fig. 3. Inhibition by DFP of the hydrolysis of phenyl phenylacetate (●) and phenyl 3-phenylpropionate (○) in brain homogenates. The inhibition was estimated by comparison with a control sample without inhibitor. The results are the mean of two experiments. The activities of the controls were: for phenyl phenylacetate, 109 and 110; for phenyl 3-phenylpropionate, 520 and 520 μ l. of CO_2 /g. of brain/min.

illustrate this point. As the concentration of inhibitor is increased the inhibition increases but not to the same degree with each substrate. The phenyl 3-phenylpropionate:phenyl phenylacetate ratio alters with increasing inhibition and increases rapidly from 4-5 to approx. 10 and then stays constant. Though this ratio stays constant with increasing inhibitor concentration the inhibition is increasing; the enzyme with a ratio of 10 therefore fulfils the criteria discussed above for a single enzyme hydrolysing both substrates.

Our experiments with DFP and mipafox give evidence for the presence in brain homogenates of three different enzymes hydrolysing phenyl 3-phenylpropionate and phenyl phenylacetate: (a) a variable amount of an enzyme which is sensitive to $0.1 \mu\text{M}$ -mipafox; (b) an enzyme which is inhibited by approx. $3 \mu\text{M}$ -DFP and 0.3 mM -mipafox and with a phenyl 3-phenylpropionate:phenyl phenylacetate ratio of less than 5 (Figs. 2, 3 and 4); (c) an enzyme inhibited by concentrations of DFP higher than $3 \mu\text{M}$ and of mipafox higher than 0.3 mM and with a phenyl 3-phenylpropionate:phenyl phenylacetate ratio of 10-11.

Experiments with tri-o-tolyl phosphate. Pure tri-o-tolyl phosphate had no inhibitory effect *in vitro* on human-serum cholinesterase and rat-serum 'tributyrinase' (Aldridge, 1954a). Similarly the hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by brain homogenate was inhibited by not more than 5% by concentrations of tri-o-tolyl phosphate up to $10 \mu\text{M}$.

Fig. 4. Influence of DFP and mipafox on the phenyl 3-phenylpropionate:phenyl phenylacetate hydrolysis ratio of brain homogenates. The phenyl 3-phenylpropionate:phenyl phenylacetate hydrolysis ratios after incubation with various concentrations of inhibitor were calculated from the results in Figs. 2 and 3. The inhibitors used were: ●, mipafox; ○ DFP.

After oral administration to chickens of 0.5–2.0 ml. of tri-*o*-tolyl phosphate/kg. body wt. the esterase activity against phenyl phenylacetate both in brain and spinal cord was inhibited 50 % or more, whereas the hydrolysis of phenyl 3-phenylpropionate was less inhibited (Table 5). As a result the phenyl 3-phenylpropionate:phenyl phenylacetate ratio was higher in treated chickens (approx. 7–8) than in the untreated ones (approx. 5). There was no apparent difference between the doses 0.5 and 2.0 ml./kg. even if the 2.0 ml./kg. was given as 0.5 ml. for 4 consecutive days. These results show that tri-*o*-tolyl phosphate in the chickens is metabolized to an inhibitor of the enzymes hydrolysing phenyl phenylacetate and phenyl 3-phenylpropionate; since there are several possible metabolites (Eto *et al.* 1962) it is not possible to say anything about the structure of the inhibiting metabolite(s). These results provide further indications of the presence of more than one enzyme hydrolysing phenyl 3-phenylpropionate and phenyl phenylacetate.

Attempts to fractionate the enzymes. Brain homogenates were prepared in 0.3M-sucrose and these were separated by centrifuging into particulate and supernatant fractions. The phenyl 3-phenylpropionate:phenyl phenylacetate hydrolysis ratio in the particulate fraction was a little lower (3.5) than in the whole homogenate (4–5). In the supernatant a higher ratio (8–9) was found. The activity recovered in the supernatant was only 10–20 % of the total activity. The difference in ratios of activities shows the presence of more than one enzyme hydrolysing phenyl 3-phenylpropionate and phenyl phenylacetate. However, although the recovery of protein in the two fractions was 97 %, the re-

covery of activity against phenyl 3-phenylpropionate and phenyl phenylacetate was only 77.0 and 85.2 % respectively. We have not been able to improve on this recovery and therefore no conclusions about distribution may be made.

Hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by different tissues. The activity in the blood is very low, and that in pancreas, intestinal mucosa, kidney and liver high, compared with that in the central nervous system (Table 6). The phenyl 3-phenylpropionate:phenyl phenylacetate hydrolysis ratios are, in all tissues except pancreas, different from those obtained for the central nervous system. Experiments with tri-*p*-tolyl phosphate, which is a very active inhibitor of the hydrolysis of phenyl butyrate by the chicken liver (Aldridge & Barnes, 1961), showed (Table 7) that esterases hydrolysing phenyl 3-phenylpropionate and phenyl phenylacetate in the central nervous system (and pancreas) are resistant to that inhibitor in concentrations 10–100 times those giving a considerable inhibition with other tissues. Thus the enzymes hydrolysing phenyl 3-phenylpropionate and phenyl phenylacetate in the central nervous system are apparently different from those of most other tissues.

DISCUSSION

Compared with those on some tissues, and other than some histochemical work, there have been relatively few publications on the esterases, excluding cholinesterases, of the central nervous system (Cohn, Kaplan & Janota, 1941; Mendel & Myers, 1953; Mendel, Myers, Uyldert, Ruys & De Bruyn, 1953; Aldridge, 1954*b*; Myers, 1956; Myers,

Table 5. *Hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by homogenates of brain and spinal cord of chickens, 24 hr. after oral treatment with tri-*o*-tolyl phosphate*

Tri-*o*-tolyl phosphate was given by stomach tube and the chickens were killed 24 hr. later. In one chicken the dose (0.5 ml./kg.) was given for 4 consecutive days and the animal killed 24 hr. after the last dose. In parentheses are the numbers of homogenates examined and the results are given as means $\pm$ S.D.

Dose (ml./kg. body wt.)	Activity (μ l. of CO ₂ /g./min.)		Phenyl 3-phenyl- propionate:phenyl phenylacetate hydrolysis ratio
	With phenyl phenylacetate	With phenyl 3-phenylpropionate	
Brain			
0.5	43.4	368	8.5
	51.4	381	7.4
1.0	43.9	334	7.6
2.0	54.4	370	6.8
4 $\times$ 0.5	43.3	304	7.0
Untreated (13)	106.1 $\pm$ 10.4	536 $\pm$ 44.7	5.1 $\pm$ 0.19
Spinal cord			
0.5	12.0	114	9.5
	26.0	176	6.8
1.0	20.1	125	6.2
2.0	22.8	156	6.8
4 $\times$ 0.5	16.8	127	7.6
Untreated (10)	45.5 $\pm$ 7.8	205.3 $\pm$ 32.6	4.5 $\pm$ 0.26

Table 6. *Hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by various tissues*

Heparin-treated blood was separated into plasma and erythrocytes which were suspended in buffer to the original blood volume. In parentheses are the numbers of homogenates examined. Sciatic nerves from six chickens were pooled, stored at -18° and homogenized together.

	Activity (μ l. of CO_2 /g. or ml./min.)		Phenyl 3-phenylpropionate:phenyl phenylacetate hydrolysis ratio
	With phenyl phenylacetate	With phenyl 3-phenylpropionate	
Brain	88-121 (13)	459-623 (13)	5.1
Spinal cord	37-55 (10)	147-252 (10)	4.5
Sciatic nerve	16	76	4.8
Liver	1793 (3)	3676 (3)	2.1
Erythrocytes	4.0 (1)	2.3 (1)	0.6
Plasma	5.3 (1)	5.0 (1)	0.9
Kidney	959 (1)	2429 (1)	2.5
Heart	98 (1)	112 (1)	1.1
Intestinal mucosa	471 (1)	1103 (1)	2.3
Pancreas	227 (1)	815 (1)	3.7

Table 7. *Inhibition by tri-p-tolyl phosphate of the hydrolysis of phenyl phenylacetate and phenyl 3-phenylpropionate by various tissues*

The tissue homogenates etc. were incubated for 30 min. at 37° with the inhibitor before the mixing with the substrates. The concentration of inhibitor is that during the incubation period. Suitable volumes of the same incubation mixture were used for the estimation of the residual activity towards the two substrates.

Tissue	Phenyl phenylacetate		Phenyl 3-phenylpropionate	
	Concn. of inhibitor (μ M)	Inhibition (%)	Concn. of inhibitor (μ M)	Inhibition (%)
Brain	10	5	10	0
Spinal cord	10	10	10	6
Liver	0.1	71	0.1	79
Kidney	1	82	1	87
Heart	1	14	1	24
Intestinal mucosa	1	80	1	92
Pancreas	10	12	10	0

Kemp, Tol & De Jonge, 1957; Bernsohn, Possley & Liebert, 1959; Blaber & Cuthbert, 1962; Casida, Baron, Eto & Engel, 1963; Barron, Bernsohn & Hess, 1961; Erankö, Kokko & Söderholm, 1962; Aldridge & Johnson, 1959; Sellinger & De Balbian Verster, 1962). Many of these authors have emphasized the difficulties of establishing that single enzymes are involved in the hydrolysis of particular substrates. The present work indicates the magnitude of the problem. Two substrates, phenyl phenylacetate and phenyl 3-phenylpropionate, are each hydrolysed by several enzymes. There is some specificity, for one enzyme certainly hydrolyses phenyl 3-phenylpropionate approximately ten times as fast as it does the acetate. These findings make it impossible to predict whether the same enzymes are responsible for the hydrolysis of closely related substrates (Table 1).

It is now well established that the inhibition of esterases by organophosphorus compounds is in principle the same type of reaction as that involved in the hydrolysis of substrates (Aldridge, 1956). Thus the same structural features that, in a

substrate, enable rapid enzymic hydrolysis to take place will, in an organophosphorus compound, increase the inhibitory activity (Aldridge, 1953a; Heath, 1961; Becker, Fukuto, Boone, Canhan & Boger, 1963). In the present paper this concept has been used inversely and the structures of possible substrates have been derived from organophosphorus compounds. This logical approach has been successful in that the two esters chosen are hydrolysed rapidly by brain and, among the inhibitors tested, are most sensitive to inhibition by the organophosphorus analogue phenyl saligenin phosphate.

SUMMARY

1. The hydrolysis of various esters by chicken brain and spinal cord has been studied.

2. The hydrolyses of phenyl phenylacetate and phenyl 3-phenylpropionate have been studied in detail and the effects of various inhibitors determined.

3. By using the organophosphorus compounds di-isopropyl phosphorofluoridate and *NN'*-di-iso-

propyl phosphorodiamidic fluoride it has been established that phenyl phenylacetate and phenyl 3-phenylpropionate are hydrolysed by two or more esterases.

4. These esterases are present in brain, spinal cord and sciatic nerve.

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Changes in Glucose Utilization during Development of the Lamb

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The young lamb relies to a great extent for its energy requirements on exogenous carbohydrate and higher fatty acids, substances which are constituents of milk. The adult sheep, however, obtains the major proportion of its energy requirements from lower fatty acids which are absorbed from the rumen where they are produced by microbial fermentation of cellulose and related carbohydrates.

Annison & Lewis (1959) have discussed the importance of acetate in the metabolism of adult sheep, and Lindsay (1959) has reviewed various aspects of the role of glucose in ruminants. In adult sheep injected glucose is removed from the blood stream at a much slower rate than in non-ruminants or in very young lambs (McCandless & Dye, 1950; Jarrett & Potter, 1952). Lambs differ from adult