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The Conversion of Dimefox into an Anticholinesterase by Rat Liver *in vitro*

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The organic phosphorus insecticides schradan (bisdimethylaminophosphonous anhydride) and parathion (*OO*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) have been shown to be converted by mammals *in vivo*, and by liver tissue *in vitro*, into powerful anticholinesterases (see, e.g. Gardiner & Kilby, 1950*a*, *b*; Dubois, Doull & Coon, 1950; Diggle & Gage, 1951; Aldridge & Barnes, 1952; Casida, Allen & Stahmann, 1952, 1954; Gage, 1953; Metcalf & March, 1953; Davison, 1954, 1955).

Dimefox (bisdimethylaminofluorophosphine oxide), another insecticide, was claimed by Aldridge & Barnes (1952) to be converted into a more active inhibitor *in vivo*. High concentrations were required to inhibit rabbit-erythrocyte cholinesterase *in vitro* as compared with those calculated to occur *in vivo* after injection of dimefox. They reported that about 7×10^{-2} M dimefox caused 50% inhibition of rat-brain cholinesterase, and that rat-liver slices converted it into a more active but unstable anticholinesterase. Okinaka, Doull, Coon & Dubois (1954), however, found that 4×10^{-5} M dimefox *in vitro* caused 50% inhibition of rat-brain cholinesterase in 15 min., and that rat-liver slices reduced the anticholinesterase activity aerobically or anaerobically.

This paper presents further studies on the mode of action and metabolism of dimefox in mammals. The work was undertaken as part of a programme of investigation of the toxicology of dimefox, the systemic insecticidal properties of which are utilized to combat insect infestation on hops, sugar beet and other crops.

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MATERIALS AND METHODS

Dimefox, b.p. 205° at 760 mm., 96° at 18 mm., was prepared by fractional distillation of Hanane (Fisons Pest Control Ltd.) and was 98·6% pure by chemical assay. Diphosphopyridine nucleotide (DPN) (95/100%), adenosine triphosphate (ATP) and cytochrome *c* were obtained from L. Light and Co. Ltd. Triphosphopyridine nucleotide (TPN) was provided by Dr W. N. Aldridge and contained at least 70% of TPN (assayed with *iso*citric dehydrogenase) and no detectable DPN.

Glaxo–Wistar albino rats were killed by decapitation. Males were used unless otherwise stated.

Liver preparations. Liver slices about 0·5 mm. thick were cut and trimmed by hand to about 5 mm. square. They were washed twice in 0·9% (w/v) NaCl solution at room temp. before use.

For the preparation of homogenates, washed slices of liver were damp-dried on filter paper, chopped with scissors, weighed and homogenized in 2 ml. of ice-cold medium (isotonic sucrose or NaCl) in an ice-cold tube of the Potter–Elvehjem type. More liquid was then added to give a final volume of 4 ml./g. fresh weight of liver.

Cell-free suspensions were prepared by centrifuging the whole homogenate at 600 *g* for 5 min. at room temp., and withdrawing the supernatant. Chilled glassware was used throughout. Such suspensions had a cholinesterase activity, measured as described below, of about 5 μmoles of acetylcholine/ml./hr.

Further fractionation of homogenates in 0·25 M sucrose was carried out at 0° by the method of Schneider (1948). Cells and nuclei were removed by centrifuging at 600 *g* for 10 min., mitochondria by a further 10 min. at 8500 *g*, and microsomes by a further 60 min. at 18000 *g*. Each sediment was resuspended without washing in 0·25 M sucrose to the original volume of whole homogenate.

Boiled liver extract was made by dropping fresh chopped rat liver into boiling water (2 ml./g.) and boiling for 2 min.

The liver was homogenized and then centrifuged for 5 min. at 600 *g*. The supernatant liquid was used.

Preparation of rat-brain cholinesterase. A whole rat brain was washed free from blood with isotonic saline and homogenized in about 2 ml. of water. Further water was added to a final volume of 20 ml./g. fresh wt. of brain. This suspension was kept at room temp. for about 2 hr. and then centrifuged at 600 *g* for 10 min. The supernatant was withdrawn and stored at −5°. It maintained its cholinesterase activity for at least a month. The preparation hydrolysed acetyl-β-methylcholine readily, but butyrylcholine hardly at all. Its activity as measured by the method described below was about 20 μmoles of acetylcholine/ml. of preparation/hr.

Determination of cholinesterase. A manometric technique modified from that of Ammon (1933) was used. Each flask contained 2·0 ml. of Krebs–Ringer bicarbonate solution (see Umbreit, Burris & Stauffer, 1949), 0·5 ml. of the cholinesterase solution to be estimated, and 0·5 ml. of acetylcholine bromide (0·036 M) which was tipped from the side arm after the flasks had been gassed for 10 min. at 37° with CO_2+N_2 (1:19). After a further 10 min., manometer readings were taken at 10–20 min. intervals for calculation of CO_2 output.

Measurement of direct anticholinesterase effect. The inhibitor in isotonic saline (0·25 ml.) was added at zero time to a Warburg flask containing 2·0 ml. of Krebs–Ringer bicarbonate and 0·25 ml. of cholinesterase preparation, with 0·5 ml. of acetylcholine bromide (0·036 M) in the side arm. The flask was transferred at once to a water bath at 37° and shaken continuously. During the last 10 min. of the inhibition period (usually 30 min.) CO_2+N_2 (1:19) was passed through the flask, and the acetylcholine was finally tipped in to stop the inhibition reaction and measure the residual cholinesterase activity. Controls contained no inhibitor.

Conversion of dimefox by liver slices. In a typical experiment, each Warburg flask contained 0·9 ml. of rat-brain cholinesterase preparation, 0·9 ml. of double-strength Krebs–Ringer solution, and 0·2 ml. of 0·1 M phosphate buffer (Umbreit *et al.* 1949) containing dimefox at the required concentration, in the side arm. Six liver slices were added (total dry wt. about 7 mg.) and the dimefox was tipped in after a 5 min. equilibrating period at 37°. Controls were run containing (*a*) liver slices but no dimefox, and (*b*) dimefox but no liver slices, to detect direct inhibition by dimefox. At the end of the incubation, the media were decanted from the slices, and 2 × 0·5 ml. of each was used for duplicate cholinesterase determinations.

Conversion of dimefox by liver homogenates. Inhibition of endogenous acetylcholinesterase activity of whole homogenates of liver or cell-free suspensions was used as a measure of the inhibitor produced. Generally, each flask contained 1·6 ml. of suspension in 0·25 M sucrose, 0·2 ml. of 0·25 M sucrose containing the activator or inhibitor being studied and 0·2 ml. of 0·1 M phosphate buffer pH 7·4 containing dimefox. The flasks were shaken in air at room temp. (20±2°) and at the end of the incubation period 2 × 0·5 ml. of the contents was taken for cholinesterase determination and gassed at once with CO_2+N_2 (1:19). Blank determinations (omitting acetylcholine) showed that the evolution of CO_2 due to the anaerobic production of lactic acid by the liver suspension was negligible. The direct inhibition of cholinesterase by unchanged dimefox was determined after gassing with N_2+CO_2 to prevent conversion occurring, or by using liver suspensions in water which contained cholinesterase but would not metabolize dimefox. In subsequent experiments, dimefox concentrations which had no direct effect on liver cholinesterase were used and dimefox was omitted from control flasks.

RESULTS

Direct inhibition of cholinesterase

The direct effects of dimefox on rat- and human-blood cholinesterase were determined (Table 1). Whereas rat-erythrocyte cholinesterase was inhibited 50 % by $2·2 \times 10^{-2}$ M dimefox in 30 min. at 37°, tetraethyl pyrophosphate produced the same effect at a concentration of about 10^{-8} M. This was taken as an indication of the freedom of the dimefox

Table 1. *Direct effect of dimefox on rat- and human-blood cholinesterase* in vitro

Dimefox was incubated with rat erythrocytes or plasma diluted with 3 vol. of water, or human erythrocytes or plasma diluted with 59 vol. of water, for 30 min. at 37° before adding acetylcholine bromide and measuring residual cholinesterase activity (see Methods section).

Cholinesterase preparation	Dimefox (M) concn. producing No inhibition	Dimefox (M) concn. producing 50% inhibition
Rat erythrocytes	10^{-4}	$2·2 \times 10^{-2}$
Rat plasma	10^{-4}	$3·3 \times 10^{-3}$
Human erythrocytes	10^{-3}	$1·1 \times 10^{-1}$
Human plasma	10^{-3}	$1·9 \times 10^{-2}$

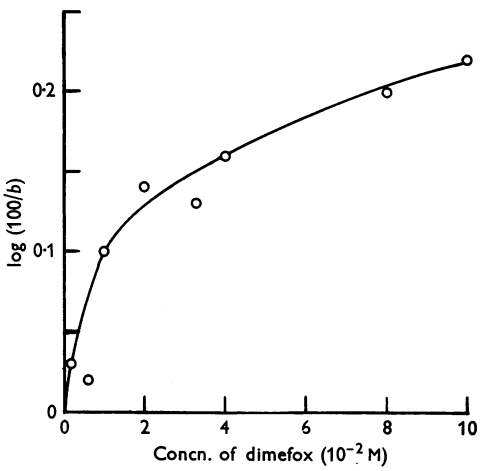


Fig. 1. Direct inhibition of rat-brain cholinesterase by dimefox. Dimefox was incubated with rat-brain cholinesterase for 15 min. at 37° before addition of acetylcholine bromide (see Methods section). *b* is the percentage of initial cholinesterase remaining.

sample from significant contamination by any direct anticholinesterase.

The nature of the direct inhibition of enzyme by high dimefox concentrations was examined briefly, although it probably has no practical significance *in vivo*. A straight line could not be obtained by plotting log (100/*b*) (where *b* = percentage of initial cholinesterase remaining) against dimefox concentration (Fig. 1), or against time with a fixed concentration of dimefox (see Aldridge, 1950). The rate of the inhibition reaction declined rapidly after about 10 min., possibly owing to the setting up of an equilibrium, since the inhibition of intact rat erythrocytes could be reversed by washing twice in three volumes of normal saline at room temperature.

Conversion of dimefox by liver slices

Incubation of dimefox (10^{-4}–10^{-1} M) with rat-liver slices for 1–2 hr., followed by application of the incubation medium to cholinesterase solutions (involving a 1/25 dilution) for 30 min., produced small and inconsistent inhibitions.

When the cholinesterase preparation was incubated with the slices and dimefox, more substantial and reproducible inhibitions were obtained. Dimefox (5×10^{-5} M) incubated in Krebs–Ringer phosphate (Umbreit *et al.* 1949) pH 7·4 at 37° in air for 15 min. with about 8 mg. (dry weight) of rat-liver slices and rat-brain cholinesterase caused about 50 % inhibition of cholinesterase. The dependence of the effects on incubation time and dimefox concentration is shown in Figs. 2 and 3. Log (100/*b*) was roughly proportional to dry weight of liver slices, although the consistency of results was not generally enhanced by correcting for small variations in liver weight between incubation flasks. This was presumed to be due to variations of slice thickness and, perhaps, of distribution of the enzyme system in them. The percentage inhibition was independent of the concentration of brain cholinesterase added to the system, and the effect on intact rat erythrocytes was not reversible by washing in normal saline at room temperature.

Stability of the metabolite. Since no difficulty has been reported by other workers in demonstrating an anticholinesterase in the medium after incubating schradan or parathion with liver slices, the stabilities of the three metabolites in Ringer phosphate, pH 7·4 (in which the slices had been incubated) were compared, as shown in Table 2.

Inhibition of conversion of dimefox by liver slices. The effects of various incubation conditions and inhibitors were investigated using rat-liver slices and brain cholinesterase. The presence or absence of 0·1 % (w/v) glucose in the medium was immaterial. Air was as effective as oxygen as gas phase provided the flasks were adequately shaken. Replacement of the air with $CO_2 + N_2$ (1 : 19) prevented all conversion, however. The effects of other inhibitors of

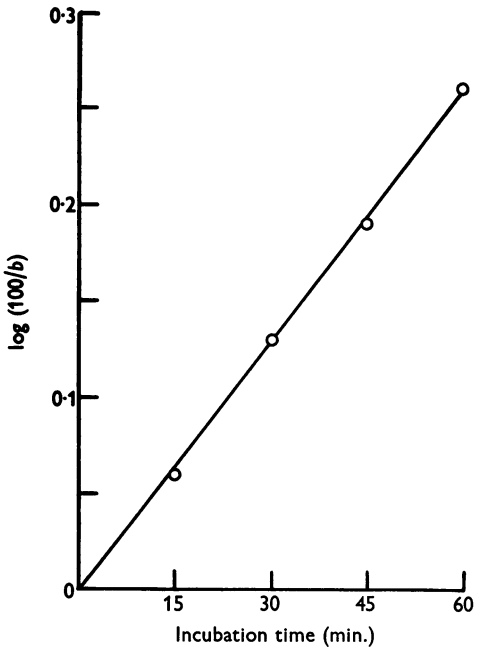


Fig. 2. Conversion of dimefox by rat-liver slices. A mean dry wt. of 6·1 mg. of liver slices was incubated with 5×10^{-5} M dimefox and rat-brain cholinesterase at 37° in a total volume of 2 ml. The gas phase was O_2 and the pH 7·4. Cholinesterase was determined in the medium at the end of the incubation. Controls contained slices but no dimefox.

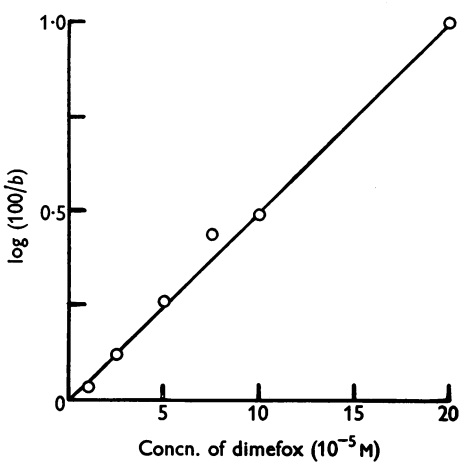


Fig. 3. Conversion of dimefox by rat-liver slices. A mean dry wt. of 8·1 mg. of liver slices was incubated with dimefox and rat-brain cholinesterase at 37° for 15 min. in O_2 at pH 7·4. The total volume of liquid was 2 ml. Cholinesterase was determined in the medium at the end of the incubation. Controls contained slices but no dimefox.

conversion are shown in Table 3, together with their effects on the overall oxygen uptake of the slices.

The addition of dimefox up to 0·1 M to liver slices with or without glucose in the medium did not increase the Q_{O_2} of the slices.

The concentration of cyanide in a flask with alkali in the centre well is uncertain. Therefore, the inhibitory effect was confirmed by using 10^{-4} M dimefox, incubated for 15 min. with liver slices, without KOH, and without gassing. Potassium cyanide (5×10^{-4} M) inhibited the conversion 38 and 45%, and 2×10^{-3} M-KCN 65 and 71% in two experiments.

Barbiturates disrupt oxidative phosphorylation *in vitro* at low concentrations (Johnson & Quastel, 1953; Brody & Bain, 1954), possibly inhibiting the oxidation of reduced DPN (Quastel, private communication). It was found, however, that the

Table 2. *Relative stabilities of liver-slice metabolites of dimefox, schradan and parathion*

Inhibitors were produced by incubating about 10 mg. dry wt. of rat-liver slices with the organophosphorus compound in 2 ml. of Krebs–Ringer phosphate for 15 min. at 37°. The medium was decanted and its direct inhibitory effect on rat-brain cholinesterase measured at the final equivalent concentration of original compound shown, and at the same time at half this concentration for subsequent determination of half-life. The last column shows the results of parallel experiments with simultaneous activation and inhibition.

	Concn. for production (M)	Equivalent concn. for detection of inhibitor (M)	Stability of inhibitor	Concn. to cause 50% inhibition of rat-brain cholinesterase, present simultaneously, in 15 min. (M)
Dimefox	10^{-2}	10^{-3}	None detectable 1 min. after removal of slices	5×10^{-5}
Schradan	5×10^{-4}	5×10^{-3}	Half-life at 20° 1·5 hr.	5×10^{-5}
Parathion	5×10^{-6}	10^{-7}	No loss of activity in 91 hr. at room temp.	10^{-6}

Table 3. *Inhibitors of dimefox conversion by rat-liver slices*

About 9 mg. (dry wt.) of male rat-liver slices was incubated with dimefox (2×10^{-5} M) and rat-brain cholinesterase for 1 hr. at 37° in Warburg flasks with 10% KOH in the centre wells, and gassed with O_2. Inhibitors were present initially except in the case of dinitrophenol, which was tipped from the side arm with the dimefox after 40 min. at 37°. O_2 uptake was measured over 45 min. during the incubation with dimefox and residual cholinesterase determined in the medium at the end of it. Inhibition of conversion is expressed as percentage decrease of log (100/b), where b is percentage of initial cholinesterase activity remaining. The mean control Q_{O_2} was 12·4, and the range 9·8–14·9 μl./mg. dry wt./hr.

Inhibitor	Concn. (M)	Inhibition of dimefox conversion (%)	Inhibition of Q_{O_2} (%)
$CO_2 + N_2$ (1:19)	—	100	—
KCN	2×10^{-3}	28	32
	4×10^{-3}	76	61
Nembutal	10^{-4}	60	0
	5×10^{-4}	79	42
Phenobarbital	10^{-4}	23	0
	5×10^{-4}	51	15
Thiopentone	10^{-4}	77	0
	5×10^{-4}	98	28
Chloretone	10^{-3}	20	0
	2×10^{-3}	53	18
2:4-Dinitrophenol	5×10^{-5}	5	(83% increase)
	5×10^{-4}	66	(16% increase)
SKF 525-A*	10^{-5}	49	0
	5×10^{-5}	100	0
Trimethylamine	10^{-3}	0	0
NN-Dimethylacetamide	10^{-3}	0	0
Dimethylnitrosamine	10^{-3}	0	0

* 2-(Diethylamino)ethyl diphenylpropylacetate hydrochloride.

addition of ATP (10^{-3} M) or DPN (10^{-3} M) did not reduce the effect of Nembutal [sodium 5-ethyl-5-(1-methylbutyl) barbiturate] on dimefox conversion. Subsequently it was noticed that the inhibitory effect of Nembutal (5×10^{-4} M) was greater at low concentrations of dimefox than at higher ones, e.g. 93 % inhibition of conversion of 2×10^{-5} M and 57 % of 10^{-4} M dimefox, indicating a competitive effect.

Measurement of liver efficiency. Units of anti-cholinesterase produced in a constant time with a constant dimefox concentration by a known weight of liver slices have been expressed as 100 log (100/*b*)/mg. dry wt. The results are shown in Table 4. Male rat liver was found to be about four times as efficient as female in converting dimefox. Guinea pigs were about twice and rabbits about five times as efficient as male rats. One slice of rabbit liver (less than 1 mg. dry wt.) was sufficient to cause 50 % inhibition of rat-brain cholinesterase in 15 min. with 5×10^{-5} M dimefox.

Table 4. *Relative efficiencies of slices of rat, guinea-pig and rabbit liver in converting dimefox*

Two different weights of slices from each animal were incubated for 15 min. at 37° with 5×10^{-5} M dimefox and rat-brain cholinesterase, before determination of residual cholinesterase activity in the medium.

	No. of animals	Liver efficiency* ± S.D.	Intra-peritoneal acute LD_{50} (mg./kg.)
Rat, female	3	1·6 ± 0·95	0·5
Rat, male	4	6·4 ± 1·8	0·5
Guinea pig, male	3	13 ± 2·1	2
Rabbit, male	2	31 ± 9·5	3

* Liver efficiency was calculated as 100 log (100/*b*)/mg. dry wt. of liver, where *b* is the percentage of initial cholinesterase activity remaining.

Liver homogenates

In accordance with reports on schradan and parathion (Metcalf & March, 1953; Casida *et al.* 1954; Davison, 1955; O'Brien & Spencer, 1955), 20 % homogenates of rat liver in 0·25 M sucrose or 0·154 M-NaCl showed no evidence of conversion of 10^{-4} M dimefox.

The addition of a boiled liver extract to an isotonic sucrose homogenate resulted in some conversion of dimefox, as indicated by inhibition of endogenous cholinesterase, and this was enhanced by the addition of 10^{-2} M nicotinamide and further by 10^{-2} M-$MgSO_4$.

When the liver was homogenized in isotonic sucrose or sodium chloride containing 10^{-2} M nicotinamide, significant conversion of added dimefox occurred. Centrifuging at 600 *g* for 5 min., to remove cell debris and probably most of the nuclei, did not result in loss of activity, although some of the cholinesterase was lost. Addition of nicotinamide about 10 min. after homogenizing had no effect, but if nicotinamide and DPN (10^{-3} M final concentration) were added together at this stage, conversion of dimefox again took place. TPN was equally effective in fortifying the homogenate in the presence of nicotinamide. The following experiment was carried out with the object of removing coenzymes and ATP from the homogenate and avoiding the conversion of DPN into TPN. A lightly centrifuged (5 min. at 600 *g*) liver homogenate (10 ml.) in 0·25 M sucrose containing 10^{-2} M-$MgSO_4$ was dialysed at 4° against two separate litres of isotonic sucrose–$MgSO_4$ solution for 7 hr. and a further 17 hr. respectively, with continuous stirring. It could still convert 10^{-3} M dimefox in the presence (but not in the absence) of added DPN and nicotinamide, but TPN could not now replace DPN.

Table 5. *Relative efficiencies of male rat-liver preparations in converting dimefox*

Liver preparations (1·6 ml.) were incubated with dimefox (10^{-4} M) in a total vol. of 2 ml. for 15 min. at 20° and pH 7·4 in air before measurement of residual endogenous cholinesterase activity. Slices were incubated in Krebs–Ringer phosphate and were homogenized and centrifuged for 5 min. at 600 *g* after incubation.

Liver preparation	Fortification	*b**	Equivalent wet wt. of liver (mg./2 ml.)	Efficiency†
Slices	None	46	120	28
Whole homogenate in 0·25 M sucrose 4 ml./g. wet wt.	None	90	360	1·3
Supernatant from sucrose homogenate after 5 min. at 600 *g*	None	93	450	0·7
	Boiled liver extract	78	350	3·1
	$MgSO_4$, 10^{-2} M	80	450	2·2
	Boiled liver extract + $MgSO_4$	31	350	15
	Boiled liver extract + $MgSO_4$ + nicotinamide 10^{-2} M	17	350	22
	Nicotinamide + DPN 10^{-3} M	70	450	3·4
	Nicotinamide + DPN + $MgSO_4$	33	450	11

* *b* is the percentage of initial cholinesterase remaining. † 10^4 log (100/*b*)/mg. wet wt. of whole liver/2 ml.

The addition of 10^{-3} M ATP or 10^{-4} M cytochrome *c* to a homogenate in sucrose–nicotinamide solution did not enhance its activity.

The efficiencies of various liver preparations in converting dimefox are shown in Table 5. Slices were the most efficient converters, but the supernatant from a homogenate fortified with $MgSO_4$, nicotinamide and boiled liver extract was almost equally active, and one with magnesium sulphate, nicotinamide and DPN was about half as active as slices.

Homogenates of washed liver slices were more active than those of unwashed chopped liver, possibly because of the removal of blood. Greater inhibition of liver cholinesterase was obtained at 20° than at 37°, owing, probably, to the greater stability of the converting system at the lower temperature.

Stability of suspensions. The activity of a sucrose–nicotinamide suspension declined rapidly at room temperature, falling to zero in about 1 hr. If the suspension was stored at 4°, its activity could be preserved for at least 3 hr. but had disappeared within 24 hr. The rate of decline of dimefox-converting power was about the same as that of ability to reduce methylene blue, and the Q_{O_2} was maintained steadily for at least 2 hr.

DPN (10^{-3} M) and nicotinamide (10^{-2} M) were added to a suspension in sucrose–$MgSO_4$ which had stood for varying times at room temperature. The dimefox-converting capacity of the fortified suspension decreased as its age increased, falling almost to zero in 2 hr. Cooper & Brodie (1954) reported that reduced TPN was needed by the liver to oxidize barbiturates. We added ethanol (10^{-1} M) with DPN and nicotinamide to a fresh suspension and to one that had stood at room temperature for 1 hr., in order to generate DPNH via the liver-alcohol dehydrogenase. No further enhancement of dimefox conversion occurred in either case. The labile factor is therefore probably not a DPNH-generating system.

Effect of pH. The effect of pH on the converting system was investigated by using Clark and Lubs phosphate and borate buffers (see Vogel, 1951). The activity increased steadily from pH 6 to 7·5, when a plateau was reached, no significant decline occurring up to pH 9 (Fig. 4). At the extremes of pH used, the cholinesterase itself was not affected, nor was the performance of the converting system influenced by changing from one buffer to the other at pH 8·0.

Effect of salts. Phosphate buffer could not be used in investigating the effect of salts on the system, because of the formation of precipitates. Therefore, Clark and Lubs borate buffer (pH 8·0) was used. $CaCl_2$ was most effective in enhancing the conversion of dimefox, followed by $MgSO_4$ and $BaCl_2$ (Table 6). NaCl, $MnCl_2$ and $CuSO_4$ had no effect, so it is probable that the activating effects observed were due to the metal ions rather than to the associated anions. In subsequent experiments, $MgSO_4$ was used when an activator was required, as it conveniently formed no precipitate with phosphate buffer.

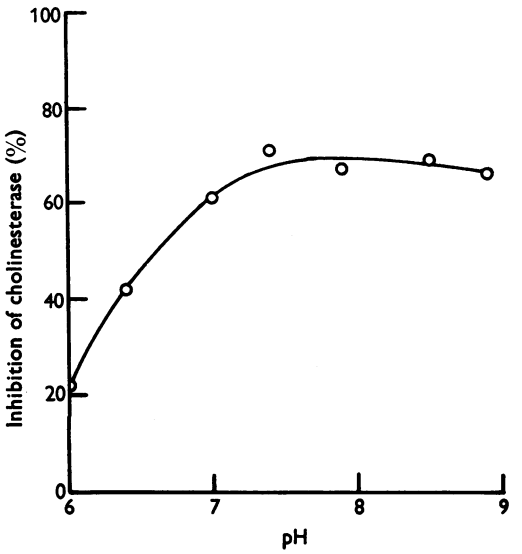


Fig. 4. Effect of pH on the conversion of dimefox. Rat-liver suspension in 0·25 M sucrose, 10^{-2} M nicotinamide and 10^{-2} M-$MgSO_4$ was buffered with Clark and Lubs phosphate and borate buffers (0·02 M). The suspension was incubated at 20° for 15 min. in air with 5×10^{-5} M dimefox, followed by determination of endogenous cholinesterase.

Table 6. *Effect of metal salts on dimefox conversion by rat-liver suspensions*

Male rat-liver homogenate in 0·25 M sucrose with 10^{-2} M nicotinamide (4 ml./g. wet wt.) was centrifuged for 5 min. at 600 *g*. Supernatant (1·6 ml.) was incubated for 15 min. at 20° in air with 5×10^{-5} M dimefox in borate buffer (0·02 M) pH 8·0 and salts as shown. Total vol., 2·0 ml. Cholinesterase activity was determined at the end of the incubation.

Salt	Concn. of activator (M)	Inhibition of liver cholinesterase (%)
None	—	12
$CaCl_2$	10^{-4}	10
$CaCl_2$	10^{-3}	47
$CaCl_2$	10^{-2}	55
$MgSO_4$	10^{-2}	36
$BaCl_2$	10^{-2}	26
$MnCl_2$	10^{-2}	3
$CuSO_4$	10^{-2}	7
NaCl	10^{-2}	9
KCl	10^{-2}	15

Centrifugal fractionation of liver homogenates. After a liver homogenate in 0·25 M sucrose had been fractionated into cells and nuclei, mitochondria, microsomes and supernatant, most of the cholinesterase was found in the first two fractions, and the enzyme activity was low. Therefore, rat-brain cholinesterase was added to each fraction and used as a control, the endogenous liver cholinesterase being negligible by comparison. The results are shown in Table 7.

It was found, in one experiment, that TPN was as effective as DPN in activating the (microsomes + supernatant) system, and, in another, that the addition of a boiled liver extract to the microsome fraction did not enhance its activity materially.

Conversion of dimefox by other tissues

No evidence of activation of dimefox has been obtained with rat kidney, brain (cerebral cortex), duodenum, ileum, colon, leg muscle, heart muscle, thyroid or adrenal. With muscle, gut and brain, the inhibition of endogenous cholinesterase was measured by using 10^{-4} M dimefox and incubating for up to 3 hr. With kidney, thyroid and adrenal slices or bisects, brain cholinesterase was added. Homogenates of whole brain and ileum in 0·25 M sucrose, fortified with DPN, nicotinamide and $MgSO_4$, were also inactive.

Effect of plasma on dimefox conversion

Dimefox activated by liver slices was found to have considerably less effect on rat-plasma cholinesterase than on erythrocyte or brain cholinesterase. Also, the cholinesterase of rat erythrocytes suspended in plasma was less inhibited than that of erythrocytes in Krebs–Ringer phosphate, the magnitude of the effect varying with the concentration of plasma in the suspending medium. A similar phenomenon was noted with human erythrocytes and plasma (Table 8).

The following possible explanations of this effect were tested: (*a*) That dimefox was rapidly destroyed by an enzyme in plasma. Pre-incubation of 10^{-3} M dimefox in plasma for 15 min. at 37° did not reduce the subsequent effect on brain cholinesterase with liver slices at a 1/10 dilution. (*b*) That the inhibited erythrocyte cholinesterase was reactivated in the presence of plasma, as has been noted to occur with the insecticide dimethyl dichlorovinyl phosphate (M. L. Fenwick, unpublished observation). Erythrocytes, after inhibition by activated dimefox, were incubated for 15 min. at 37° in plasma. No recovery of activity occurred. (*c*) That plasma contained some material that had a higher affinity for activated dimefox than had erythrocyte cholinesterase, and competed with it. Plasma was pre-incubated for 15 min. with 10^{-3} M dimefox and liver slices to saturate all possible phosphorylatable sites. It subsequently still had the same effect as untreated plasma in reducing the inhibition of erythrocytes suspended in it.

These three possibilities having been eliminated, it was concluded that some material, possibly an enzyme, in plasma rapidly destroyed the activated dimefox before it could attack the erythrocyte cholinesterase. A similar effect on the inhibition of erythrocyte cholinesterase was noticed when plasma was replaced by lightly centrifuged aqueous homogenates of washed liver and kidney slices. Extracts of leg muscle, heart muscle, ileum, spleen or brain were inactive in this respect.

In view of the discovery of the inactivating material in liver tissue, the possibility was considered that magnesium sulphate, which fortified liver homogenates, might function by inhibiting the

Table 7. *Location of the dimefox-converting system in rat-liver cells*

Each precipitate was made up to volume of original 20% (w/v) homogenate in 0·25 M sucrose–10^{-2} M-$MgSO_4$. Each flask contained 0·8 ml. of brain-cholinesterase preparation, 0·4 ml. of liver fraction, 0·2 ml. of phosphate buffer (0·1 M, pH 7·4); DPN 10^{-3} M, nicotinamide 10^{-2} M, $MgSO_4$ 10^{-2} M, sucrose 0·25 M, dimefox 10^{-3} M final concentrations in a volume of 2 ml. Flasks were incubated 15 min. in air at room temp., followed by determination of cholinesterase on 0·5 ml. of contents.

Liver fraction	Inhibition of rat-brain cholinesterase (%)
Cells + nuclei	0
Mitochondria	12
Microsomes	33
Supernatant	0
Mitochondria + microsomes + supernatant	61
Mitochondria + supernatant	10
Microsomes + supernatant	53

Table 8. *Effect of plasma on the inhibition of erythrocyte cholinesterase*

Washed erythrocytes were suspended in plasma, or plasma diluted with Ringer solution and incubated for 15 min. at 37° with dimefox, and about 7 mg. dry wt. of male rat-liver slices. The slices were removed and the erythrocytes separated by centrifuging, washed and diluted with water (1/4 for rat; 1/20 for human) for residual cholinesterase determination.

Dimefox concn. (M)	Plasma concn. (%)	Inhibition of erythrocyte cholinesterase (%)	
		Rat	Human
10^{-3}	0	85	—
10^{-3}	100	12	—
10^{-4}	0	46	41
10^{-4}	12·5	35	30
10^{-4}	25	8	19
10^{-4}	50	0	0
10^{-4}	100	0	0

breakdown of converted dimefox. However, the addition of 10^{-2} M-$MgSO_4$ had no effect on the inhibition of erythrocyte cholinesterase, in the presence of a liver extract, with dimefox and liver slices.

The plasma inactivator was similarly unaffected by 10^{-3} M cupric or mercuric chlorides, in contrast to DFP-ase (Mounter & Chanutin, 1953) which hydrolyses di*iso*propyl phosphorofluoridate.

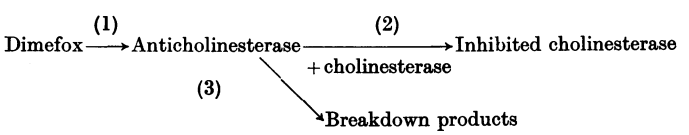
Conversion of dimefox by cockroach gut

Metcalf & March (1953) showed that the gut of the cockroach (*Periplaneta americana*) will convert parathion into an active anticholinesterase *in vitro*, and O'Brien & Spencer (1953, 1955) used cockroach gut to activate schradan. In both cases the homogenized tissue, variously fortified, was inactive.

Our experiments with dimefox are in accordance with these findings. The intact whole gut was active, causing inhibition of rat-brain cholinesterase when incubated with dimefox. But homogenates of the gut in 0·25 M sucrose, fortified with DPN (10^{-3} M), nicotinamide (10^{-2} M) and magnesium sulphate (10^{-2} M) effected no conversion of dimefox (10^{-4} M) in 15 min. at room temperature, when the endogenous acetylcholinesterase activity of the gut was used as an indicator of active inhibitor.

DISCUSSION

Dimefox, like schradan and parathion, has been shown to be converted into a powerful anticholinesterase in liver tissue. The results of our experiments with dimefox and rat-liver slices in the presence of rat-brain cholinesterase suggest that dimefox is metabolized by a relatively slow rate-limiting reaction (1) catalysed aerobically by a liver enzyme system; the product, a strong anticholinesterase, undergoes either an irreversible bimolecular reaction with cholinesterase (2) resulting in an inactive product or products, or a rapid spontaneous or (particularly *in vivo*) enzymic or chemical breakdown (3).



With a fixed liver concentration, the rate of reaction (1) is proportional to dimefox concentration, and does not decline appreciably within an hour under the conditions studied. The active anticholinesterase produced is present in small amount, as it is rapidly broken down by reaction (3), and its concentration is proportional to that of dimefox. If concentrations of dimefox, active inhibitor, and cholinesterase at time t are denoted by $[D]$, $[A]$ and $[C]$ respectively, and the percentage of initial cholinesterase activity remaining by b,

$$-\mathrm{d}[C]/\mathrm{d}t = k'[C][A] = k[C][D],$$

since $[A] \propto [D]$ (k', k are constants).

Integrating, $\ln(100/b) = k[D]t$. This relationship has been shown to be valid (Figs. 2 and 3). Similarly, since the rate of reaction (1) is proportional to concentration of liver enzyme, $\ln(100/b)$ is proportional to concentration of liver enzyme if $[D]$ and t are constant.

The quantity b is independent of $[C]$, other conditions remaining constant [unless the cholinesterase preparation also contains some factor that affects the rate of breakdown of A, reaction (3)].

The technique used to study this system, involving all three reactions, has given satisfactory consistency of results, and can be used as an assay method for either the liver-enzyme system, or low concentrations of dimefox.

Our results do not agree with those of Okinaka *et al.* (1954). The dimefox used by these workers had a high anticholinesterase activity, which was reduced by incubating with liver slices. A possible explanation is that it was contaminated with a powerful inhibitor which was destroyed by liver tissue, whereas the dimefox metabolite, being very unstable, remained undetected.

Brodie (1956) has reviewed work on a (liver microsomes + supernatant) system which accomplishes the oxidative metabolism of various drugs, including barbiturates, and Davison (1955) has compared this system with the one that oxidizes schradan and parathion.

Male rats are more resistant than female rats to anaesthetizing doses of barbiturates (Holck, Kanan, Mills & Smith, 1937), and we have noticed that male rat liver is a more efficient converter of dimefox, *in vitro*, than is female. SKF 525-A [2-(diethylamino)ethyl diphenylpropylacetate hydrochloride] inhibits both barbiturate metabolism and dimefox (and schradan and parathion) conversion. Furthermore, low concentrations of barbiturates inhibit the conversion of dimefox *in vitro*, and this effect cannot be reduced by addition of ATP or DPN. The barbiturates do in fact appear to compete with dimefox for the use of some part of the oxidizing system, which must have a very broad specificity. The reasons for the inhibitory effect of chloretone and dinitrophenol are not at present clear, although possibly they, too, undergo oxidation by the same system. Two compounds with dimethylamino

groups, *NN*-dimethylacetamide and dimethylnitrosamine did not inhibit dimefox conversion, however.

A difference between the dimefox-converting system and Brodie's drug-metabolism system is that DPN is an effective cofactor in the former, whereas DPN could not replace TPN in barbiturate metabolism.

The system that metabolizes dimefox is very unstable in liver homogenates, partly, but not wholly, owing to the rapid loss of DPN. Brodie and his colleagues suggest that a peroxide-like oxidizing agent is produced during the oxidation of TPNH. We considered the possibility that liver homogenates 'ran down', even in the presence of DPN, because of lack of DPNH. We found no increased dimefox conversion, however, on addition of ethanol to accelerate the production of DPNH via the alcohol dehydrogenase presumed to be present. On the other hand, cytochrome *c* also had no activating effect, although the system was inhibited by cyanide. The mechanism is evidently far from clear.

A few experiments with cockroach gut showed that, although the intact tissue will convert dimefox *in vitro*, homogenates fortified with nicotinamide, DPN and magnesium sulphate will not do so. It appears that there is a difference between the activating systems in rat liver and in cockroach gut—either a fundamental difference of mechanism or a difference in stability on homogenizing.

A close and not surprising similarity has been observed between the behaviours of dimefox and the related compound schradan. It may be surmised that, by analogy with the metabolism of schradan (Heath, Lane & Llewellyn, 1952; Casida *et al.* 1954; Heath, Lane & Park, 1955), the anticholinesterase derived from dimefox is also either an amine oxide or a hydroxymethylamide. The most obvious difference lies in the relative instability of the dimefox liver metabolite *in vitro*. While 10^{-2}M dimefox, activated by liver slices, had no detectable inhibitory activity when transferred within 1 min. to cholinesterase at 1/10 concentration, 5×10^{-5}M dimefox, incubated with cholinesterase and the same weight of liver slices, produced 50 % inhibition in 15 min. The presence of an inactivator in liver no doubt reduces the survival time of the active material. (We have obtained a strong anticholinesterase in small yield, by oxidizing dimefox with hydrogen peroxide and extracting with chloroform. This was somewhat more stable and had a half-life of about 8 min. at pH 7·4 at 37°.) The additional presence of a similar material in plasma must further reduce the half-life of activated dimefox *in vivo*, as shown by the strong protective effect of plasma on erythrocyte cholinesterase *in vitro*.

Although concentrations and stabilities are very difficult to estimate with any accuracy in such a system under living conditions, some suspicion is cast on the ability of the liver metabolite of dimefox to reach a peripheral site in the blood in sufficient concentration to produce lethal effects. With this in mind we have tried to demonstrate conversion in various other organs and tissues, but without success. The effect may, of course, be more localized and not demonstrable by our macro-techniques. It can be seen from Table 4 that an efficient liver conversion cannot be correlated with a low LD_{50} in the rat, rabbit and guinea pig. On the other hand, there is indirect evidence that the liver is the critical site of production of an unstable toxic substance; symptoms of poisoning are slow to appear, often not starting for 2 or 3 hr. after an intraperitoneal LD_{50} dose, and death may only occur after 2 or 3 days; brain cholinesterase is inhibited to a much lesser extent than is erythrocyte cholinesterase after dimefox injection; previous treatment of rats with carbon tetrachloride reduces the rate of depression of plasma cholinesterase after injection of dimefox, and also delays the onset of external symptoms of poisoning (M. L. Fenwick, unpublished observation).

Work is continuing on these aspects of dimefox toxicology.

SUMMARY

1. Dimefox is a weak anticholinesterase, and its direct effect on rat-erythrocyte cholinesterase may be reversed by washing the intact cells.

2. Dimefox is converted into a powerful and unstable anticholinesterase in liver tissue. A technique for investigating the system *in vitro* has been described.

3. The stability of the metabolite is further decreased by the presence in liver, blood and kidney of a substance which inactivates it.

4. The converting system is present in the microsomes and soluble material of the liver cells, and bears certain resemblances to the system which metabolizes barbiturates and other drugs. It is probably the same as that which activates schradan.

5. The conversion in liver suspensions is accelerated by DPN and nicotinamide, and by Ca^{2+} or Mg^{2+} ions. It is inhibited in liver slices by lack of oxygen, by barbiturates, SKF 525-A [2-(diethylamino)ethyl diphenylpropylacetate hydrochloride], 2:4-dinitrophenol and potassium cyanide.

6. No site of conversion of dimefox in the rat other than liver could be demonstrated *in vitro*.

7. Conversion of dimefox also takes place in whole cockroach gut *in vitro*, but homogenates fortified with DPN, nicotinamide and magnesium were inactive.

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The Phosphatase and Metaphosphatase Activities of Pea Extracts

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Metaphosphates and inorganic polyphosphates are widespread in living organisms (see reviews by Ingelman, 1950; Schmidt, 1951; Winkler, 1953). Apart from the ubiquitous formation of pyrophosphate by various enzyme reactions, large amounts of these polyphosphates have been detected in moulds, bacteria, algae, the seeds of cotton plants and some insect tissues, although not so far in the higher animals or the leaves of higher plants. Enzymes which degrade the inorganic polyphosphates, with or without the liberation of orthophosphate, have also been detected in extracts of a wide range of organisms (see Ingelman, 1950; Schmidt, 1951). There have, however, been few studies on the purification and properties of these enzymes.

In the course of previous work on the purification of plant ribonuclease (Holden & Pirie, 1955*a*; Pierpoint, 1956), it was observed that pea-leaf extracts would liberate orthophosphate from commercial 'hexameta-phosphate'. In view of the increasing interest in the occurrence and metabolism of poly- and meta-phosphates, this metaphosphatase has been characterized further. In particular, some attention has been paid to its specificity, since very few of the metaphosphatases described have been tested against more than one or two inorganic polyphosphates.

Evidence is presented here that the metaphosphatase activity of the pea extracts is associated with an unspecific phosphoesterase, which liberates orthophosphate from trimetaphosphate and tripolyphosphate as well as a number of organic phosphates. During the purification of this enzyme by ion-exchange chromatography, it was resolved into two distinct components with similar but not identical properties.

EXPERIMENTAL

Enzyme preparations. Extracts of pea seedlings that had been grown in a glasshouse for two or three weeks were fractionated by the method of Holden & Pirie (1955*a*). The fractions are referred to by the letters that these workers used. Many of the experiments were performed with fraction E, since it contained most phosphatase activity on a protein-nitrogen basis. It was prepared by precipitation with ammonium sulphate between 50 and 85% saturation, from a citrate extract of pea seedlings that had been clarified