Gunsalus, C. F. & Tonzetich, J. (1952). *Nature, Lond.*, **170**, 162.
Gunsalus, I. C. & Hand, D. (1941). *J. biol. Chem.* **141**, 853.
Krebs, H. A. (1948). *Biochem. J.* **43**, 51.
Mardashev, S. R. & Pavlova, N. A. (1955). *Dokl. Akad. Nauk., S.S.S.R.* **101**, 135. (*Chem. Abstr.* **49**, 11039h.)
Meister, A. (1955). *Advanc. Enzymol.* **16**, 185.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Schmidt, G. (1932). *Hoppe-Seyl. Z.* **208**, 185.
Sprinson, D. B. & Rittenberg, D. (1951). *Nature, Lond.*, **167**, 484.
Stephenson, M. & Trim, A. R. (1938). *Biochem. J.* **32**, 1740.
Tannenbaum, S. W. (1956). *J. biol. Chem.* **218**, 733.
Wood, J. K. (1903). *J. chem. Soc.* **83**, 568.

The Hydrolysis of Indoxyl Esters by Rat Esterases

By ELUNED E. HOBBIGER
Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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It has been reported previously that human blood contains five esterases capable of hydrolysing unsubstituted indoxyl esters (Underhay, 1957). Three of these esterases, acetocholinesterase, an aliphatic esterase and an aromatic esterase, are found in the erythrocytes and the remaining two esterases, butyrocholinesterase and an aromatic esterase, which is not identical with the aromatic esterase of the erythrocytes, are present in plasma. When either human erythrocytes or human plasma are used as enzyme source, hydrolysis is mainly accomplished by the cholinesterases and the other esterases participate only to a small extent. In this paper, for reasons to be discussed below, the term 'aliphatic esterase' is replaced by 'organophosphate-sensitive esterase' and the term 'aromatic esterase' by 'organophosphate-resistant esterase'.

Most of the histochemical studies in which indoxyl esters have been used as substrates have so far been carried out on rat tissues (Holt, 1954, 1956). Experiments were therefore carried out to study the hydrolysis of unsubstituted indoxyl esters by erythrocyte preparations and plasma of the rat and by homogenates of rat liver from which the nuclei had been removed. The results obtained show that the hydrolysis of these esters by the three rat-enzyme preparations is mainly accomplished by organophosphate-sensitive esterases and that the cholinesterases and organophosphate-resistant esterases contribute to a lesser extent.

MATERIALS AND METHODS

Substrates. The substrates used were acetylcholine chloride, propionyl- and butyryl-choline iodides, indoxyl acetate, propionate and butyrate and α-naphthyl acetate. All were of analytical purity. Acetylcholine chloride was purchased from Roche Products Ltd. and the other substrates were kindly provided by Dr S. J. Holt.

Inhibitors. Inhibitors used were eserine sulphate, 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methyl sulphate (Ro 3–0422), diethyl *p*-nitrophenyl phosphate (E 600), di*iso*propyl *p*-nitrophenyl phosphate (DINP), di*iso*propyl phosphorofluoridate (DFP) and the selective anti-acetocholinesterase 3-oxo-1:5-diphenylpentane-*pp'*-bis(propyldimethylammonium) dibromide (B.W. 285C51).

Enzyme preparations. Male white Wistar rats were used for all experiments. Blood was rapidly withdrawn by cardiac puncture under ether anaesthesia and collected in a tube containing heparin. The plasma was pipetted off after the blood had been centrifuged for 10 min. at 3700 rev./min. in an International Centrifuge, size 1, Type SB, and the erythrocytes were twice washed with 0·9% NaCl. Since the erythrocytes agglutinate rapidly on standing they were immediately haemolysed with 9 vol. of 0·025 M-$NaHCO_3$. Throughout the text the terms 'erythrocyte preparation' and 'plasma' refer to preparations obtained in this way. Zajicek & Datta (1952, 1953) and Hines (1952) have shown that platelets and white cells of rats also contain acetocholinesterase. To investigate the effect of these components on the activity of the enzyme preparations described above, the following experiments were carried out. A cell count of one of the plasma samples showed that the number of platelets and white cells present was negligible; hydrolysis resulting from this contamination is too small to be recorded under the experimental conditions and more than 99% of the hydrolysis obtained with plasma as enzyme source is due to enzymes present in the plasma itself.

A cell count of two samples taken from one of the erythrocyte pellets showed that although both white cells and platelets were present they were far more numerous in the sample taken from the top than in that taken from the bottom of the pellet. Table 1 gives the rates of hydrolysis of acetylcholine and indoxyl acetate by the two samples together with a count of the cell types present. Also included are the rates of hydrolysis of both substrates by two different preparations of platelets. These results show that the platelets contribute approximately 30% to the hydrolysis of acetylcholine when erythrocyte preparations are used as enzyme source. With indoxyl acetate as substrate their contribution does not amount to more than 10% of the total hydrolysis. Inhibition studies with a 10^{-7} M-concentra-

Table 1. *Effect of platelets and white cells on the rate of hydrolysis by erythrocyte preparations*

A. Two samples were withdrawn from different levels of the erythrocyte pellet, one from the bottom and the other from near the top. After diluting with normal saline, the enzymic activity of each was determined manometrically and the cell counts were carried out.

B. For the platelet preparations sodium citrate was used as anticoagulant. After centrifuging for 10 min. at 1500 rev./min. the platelets were obtained from the supernatant by centrifuging at 3000–4000 rev./min. for 15 min. The platelets were washed three times in normal saline solution and finally resuspended after breaking up the clumps. The error in the platelet count is estimated to be about ±20%.

	Cells/mm.^{3}			Hydrolysis (μl. of CO_2/10 min./0·1 ml. of enzyme soln.)	
	Erythrocytes	Platelets	White cells	Acetylcholine	Indoxyl acetate
A. Top sample	16 000 000	1 000 000	16 000	18·5	65·8
Bottom sample	16 000 000	12 000	1 000	12·7	59·4
B. Platelet preparation 1	—	500 000	—	4·9	6·6
Platelet preparation 2	6 000	510 000	2 000	5·0	6·8

tion of Ro 3–0422 (see below) showed that 85% of the hydrolysis of indoxyl acetate by platelets is accomplished by cholinesterase. From the results in Table 1 no conclusions can be drawn about the enzymic activity of white cells but it seems that any hydrolysis accomplished by them is not measurable under the experimental conditions.

The method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) was used to separate liver homogenates into two fractions, one of which contained the nuclei and the other the residual homogenate (fraction E). Both the nuclei and fraction E were made up as 10% (w/v) concentrates with 0·25M-sucrose, and 0·025M-$NaHCO_3$ was used for subsequent dilutions.

Measurement of esterase activity

Enzyme activities were determined manometrically in the Warburg apparatus at 37° in a medium of 0·025M-$NaHCO_3$, containing 0·1% (w/v) of crystallized bovine-plasma albumin (Armour Laboratories) and saturated with N_2+CO_2 (19:1) (pH 7·5) as described by Underhay (1957).

Substrate (0·3 ml.) dissolved in 50% (v/v) ethanol was used in each manometric vessel and the final substrate concentration was 6 mM in each case. The concentration of ethanol in the reaction mixture never exceeded 5% (v/v) (see Underhay, 1957) and control experiments showed that such a concentration of ethanol had no effect on the hydrolysis of acetylcholine by any of the enzyme preparations used.

In experiments involving the use of an inhibitor, enzyme and inhibitor were kept in contact for 20 min. at 37° before the addition of the substrate. The expression I_{50} denotes the concentration of a substance which inhibits hydrolysis by 50%.

The activities of enzyme preparations obtained from different rats were variable, especially with plasma, and the results given in Tables 2, 3 and 5 are means of several determinations. In a previous publication (Underhay, 1957) esterases which are inhibited by organophosphates, but not by eserine, were referred to as aliphatic esterases and those which are inhibited neither by organophosphates nor by eserine as aromatic esterases. Since it is known that the substrate specificity of these enzymes is not necessarily in accordance with these names (Mounter & Whittaker, 1953; Aldridge, 1954), and since it has been found in the experiments described in this paper that the indoxyl esters, which are aromatic compounds, are very rapidly hydrolysed by an organophosphate-sensitive enzyme in rat liver, the term 'aliphatic esterase' is replaced by 'organophosphate-sensitive esterase' and the term 'aromatic esterase' by 'organophosphate-resistant esterase'. The former should not be confused with the cholinesterases, which are also organophosphate-sensitive; the cholinesterases form an entirely separate group and are distinguished from other esterases by their high sensitivity to eserine and to Ro 3–0422 (see below). These terms are preferred to the Aldridge nomenclature of 'A' and 'B' esterase since the organophosphate-sensitive esterase does not, in this case, hydrolyse butyrates at the highest rate.

RESULTS

Hydrolysis of choline and indoxyl esters by esterases of rat-erythrocyte preparations

The rates of hydrolysis of the three indoxyl esters by erythrocyte preparations were compared with those of the corresponding choline esters. The results in Table 2 show that all the indoxyl esters are hydrolysed more rapidly than any of the choline esters. With both types of ester, the rate of hydrolysis is inversely related to the length of the acyl chain.

To determine the extent to which the different esterases contribute to the hydrolysis of indoxyl esters the effect of various inhibitors was investigated.

Eserine. Eserine (10^{-5}M) inhibits hydrolysis of acetylcholine almost completely, but reduces hydrolysis of indoxyl acetate by only 25%. Similarly, inhibition of the hydrolysis of indoxyl propionate and butyrate by 10^{-5}M-eserine amounts to 45 and 8% respectively (Table 3). Similar results are obtained with 10^{-4}M-eserine. Under the experimental conditions cholinesterase activity is completely inhibited by B.W. 285C51 and thus the eserine-sensitive hydrolysis of the indoxyl esters and the hydrolysis of the choline esters are accomplished by an acetocholinesterase.

Ro 3–0422. This inhibitor is a far more potent anticholinesterase than eserine, and cholinesterase

Table 2. *Rate of hydrolysis by rat-erythrocyte preparations and plasma*

Esterase activity was determined manometrically at 37° with 0·025 M-$NaHCO_3$ as medium and a substrate concentration of 6 mM. Standard deviations are included and the number of determinations given in parentheses.

	Choline esters	Indoxyl esters	α-Naphthyl esters
	(μl. of CO_2/10 min./0·1 ml. of erythrocyte preparation)		
Acetate	15·6±2·5 (11)	60·7±5·1 (10)	57·4±11·4 (4)
Propionate	12·8±1·5 (2)	27·1±1·6 (4)	—
Butyrate	0·8±0·2 (3)	18·1±1·7 (4)	—
	(μl. of CO_2/10 min./0·2 ml. of plasma)		
Acetate	19·8±4·9 (11)	72·2±13·2 (10)	3660·0±61·6 (4)
Propionate	32·4±9·4 (2)	163·5±26·3 (7)	—
Butyrate	11·3±3·3 (8)	48·8±7·1 (4)	—

Table 3. *Inhibition of hydrolysis by erythrocyte preparations and plasma by eserine, Ro* 3–0422 *and diisopropyl phosphorofluoridate*

Enzyme and inhibitor were incubated together for 20 min. at 37° before the substrate was added from the side arm. Inhibition is expressed as a percentage of untreated controls. Standard deviations and the number of determinations (in parentheses) are given. A, Acetate; P, propionate; B, butyrate.

	Choline esters			Indoxyl esters			α-Naphthyl esters
Inhibitor	A	P	B	A	P	B	A
				Erythrocyte preparation			
10^{-5} M-Eserine	98	—	—	25±8·3 (7)	45±2·1 (3)	8±12·5 (3)	39±2·0 (4)
10^{-7} M-Ro 3–0422	100	100	—	27±6·8 (6)	47±5·0 (2)	6±8·6 (2)	41±1·6 (3)
10^{-5} M-DFP	100	—	—	43·5±13·7 (6)	83±2·0 (3)	98±1·4 (3)	77±5·5 (4)
				Plasma			
10^{-5} M-Eserine	95	—	92	28±6·4 (6)	7±9·7 (6)	0±3·9 (3)	7±7·9 (4)
10^{-8} M-Ro 3–0422	98	—	90	25±8·7 (7)	5±12·7 (5)	0±5·0 (2)	0±5·0 (2)
10^{-5} M-DFP	100	—	—	95±5·0 (3)	99±2·2 (2)	99±1·6 (2)	99±1·4 (2)

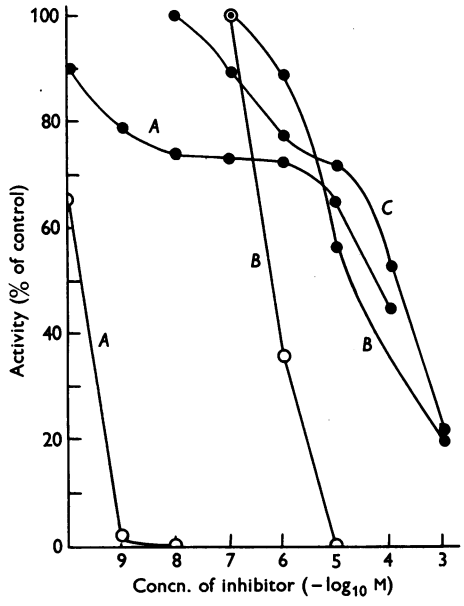

Fig. 1. Inhibition of hydrolysis by erythrocyte preparations after 20 min. pre-incubation of the enzyme with the inhibitor. Substrate concn. was 6 mM. ○, Acetylcholine; ●, indoxyl acetate. Curve *A*, Ro 3–0422; *B*, DFP; *C*, E 600.

inhibition obtained by this compound is not reversed by substrate (Hobbiger, 1954). From experiments in which various concentrations of Ro 3–0422 were used it can be calculated that the I_{50} for the acetocholinesterase of rat-erythrocyte preparations is $1·7 \times 10^{-10}$ M, and a 10^{-9} M-concentration inhibits the enzyme almost completely (Fig. 1).

If indoxyl acetate is used as substrate the inhibition curve shows two inflexions (Fig. 1). Twenty-seven per cent. of the hydrolysis is highly sensitive to Ro 3–0422 and the I_{50} of the enzyme involved is of the same order as that of acetocholinesterase. The inhibition curve shows a plateau between 10^{-8} and 10^{-6} M and the percentage inhibition achieved by these concentrations (27 %) is very similar to that obtained by 10^{-5} M-eserine. Thus 10^{-8}–10^{-6} M-Ro 3–0422 can be used instead of 10^{-5} M-eserine for selective inhibition of the acetocholinesterase of rat-erythrocyte preparations; experiments with 10^{-7} M-Ro 3–0422 and indoxyl propionate or *n*-butyrate as substrate confirm the results given by 10^{-5} M-eserine (Table 3). On increasing the concentration of Ro 3–0422 above 10^{-6} M further inhibition takes place.

Table 4 shows that the acetocholinesterase of rat-erythrocyte preparations hydrolyses corresponding choline and indoxyl esters at very similar if not identical rates.

Table 4. *Rates of hydrolysis by cholinesterase of rat-erythrocyte preparations and plasma*

Figures are calculated from the data in Tables 2 and 3. The rate of hydrolysis of α-naphthyl acetate by organophosphate-sensitive esterase of plasma is too great to allow any conclusion to be drawn about its rate of hydrolysis by plasma cholinesterase. This also applies to the hydrolysis of the indoxyl esters and of α-naphthyl acetate by liver cholinesterase.

	CO_2 evolved (μl./10 min./0·1 ml. of erythrocyte preparation or 0·2 ml. of plasma)					
	Choline esters		Indoxyl esters		α-Naphthyl esters	
	Erythrocytes	Plasma	Erythrocytes	Plasma	Erythrocytes	Plasma
Acetate	15·6	19·8	15·8	19·2	23·0	—
Propionate	12·8	32·4	12·5	9·8	—	—
Butyrate	0·8	11·3	1·3	0·0	—	—

DFP and E 600. To determine whether the eserine-resistant enzymic hydrolysis of indoxyl esters is accomplished by an organophosphate-sensitive or an organophosphate-resistant esterase, or a mixture of both, the effect of DFP and E 600 on their hydrolysis was investigated. With indoxyl acetate as substrate the effect of increasing concentrations of either of the two organophosphates resembles that of higher concentrations of Ro 3–0422 (Fig. 1), but the inhibition curves for the eserine-resistant hydrolysis show a uniform slope and approx. 20 % of the total hydrolysis is unaffected by mM-DFP or -E 600. On the other hand, the eserine-resistant hydrolysis of indoxyl propionate and *n*-butyrate is inhibited 69 and 98 % respectively by 10^{-5}M-DFP. Since it has been shown that the inhibition of esterases by organophosphates is irreversible under the experimental conditions (Burgen & Hobbiger, 1951; Myers & Mendel, 1953) these results indicate that more than one enzyme is involved in the eserine-resistant hydrolysis of indoxyl esters by rat-erythrocyte preparations, but the participation of an organophosphate-resistant esterase could not be established with certainty.

Hydrolysis by esterases of rat plasma

Table 2 shows that each of the three indoxyl esters is hydrolysed more rapidly than any of the choline esters, but with both types of substrate the rate of hydrolysis decreases in the order propionate, acetate, butyrate.

Eserine. Eserine (10^{-5}M) inhibits the hydrolysis of the choline esters by more than 90 % (Table 3); the same concentration of eserine inhibits the hydrolysis of indoxyl acetate and propionate by 28 and 7 % respectively but has no effect on the hydrolysis of indoxyl butyrate.

Ro 3–0422. This inhibitor is only slightly less potent as an anticholinesterase if rat plasma is used as enzyme source instead of rat erythrocytes. The I_{50} for hydrolysis of acetylcholine by rat plasma is $3·5 \times 10^{-10}$M, and 10^{-9}M-Ro 3–0422 reduces cholinesterase activity by 95 % (Fig. 2). With indoxyl acetate as substrate the effect of Ro 3–0422 is similar to that previously found in experiments with

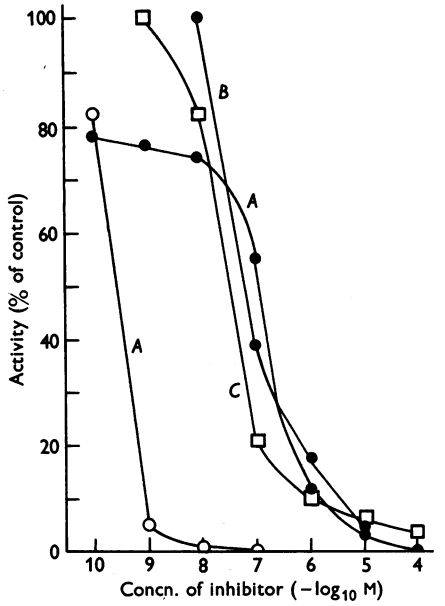

Fig. 2. Inhibition of hydrolysis by plasma after pre-incubation of the enzyme for 20 min. with the inhibitor. Substrate concn. was 6 mM. ○, Acetylcholine; ●, indoxyl acetate; □, α-naphthyl acetate. Curves *A*, Ro 3–0422; *B*, DFP; *C*, DINP.

rat-erythrocyte preparations. The inhibition curve shows a distinct plateau between 10^{-10} and 10^{-8}M and the inhibition achieved by these concentrations is of the same order as that obtained by 10^{-5}M-eserine (Table 3). Comparable results are also obtained when either 10^{-5}M-eserine or 10^{-8}M-Ro 3–0422 is used in experiments with indoxyl propionate and butyrate as substrate (Table 3).

Hydrolysis of the indoxyl esters, which is inhibited by 10^{-5}M-eserine or 10^{-8}M-Ro 3–0422, can only represent cholinesterase activity. The hydrolysis of the choline esters is known to be accomplished by two different cholinesterases which differ in their substrate specificities; one is an acetocholinesterase and the other a propionocholinesterase (Mundell, 1944; Myers, 1953). B.W. 285C51 (10^{-4}M) was

found to inhibit completely the acetocholinesterase without affecting the propionocholinesterase. However, no attempt has been made to measure the relative rates of hydrolysis of the indoxyl esters by the two cholinesterases since their contribution to the total hydrolysis was too small to allow an accurate assessment of the effect of B.W. 285C51. Plasma cholinesterases hydrolyse indoxyl acetate and propionate but not indoxyl butyrate (Table 4), and thus it seems likely that the acetocholinesterase contributes significantly to their hydrolysis.

The hydrolysis of indoxyl acetate not attributable to cholinesterase(s) can be inhibited almost completely by DFP and concentrations of Ro 3–0422 greater than 10^{-8} M and is therefore due to an organophosphate-sensitive esterase. The inhibition curves obtained with these two organophosphates are continuous but do not entirely exclude the possibility that an organophosphate-resistant enzyme may contribute slightly to the hydrolysis (Fig. 2). With indoxyl propionate and butyrate as substrates 99 % inhibition is obtained by 10^{-5} M-DFP (Table 3).

Hydrolysis by esterase of rat liver

Since de Duve *et al.* (1955) have stated that measurements of enzyme activity of whole homogenates are unreliable, as a result of agglutination which usually occurs when nuclei are present, the nuclear fraction was separated from fraction E (see Methods) and the activity of the whole homogenate determined as the sum of the activities of each of these fractions. The hydrolysis of indoxyl and choline esters is mainly accomplished by fraction E (i.e. the cytoplasm; Table 5) and the activity of the nuclei is largely, if not entirely, due to contamination (Underhay, Holt, Beaufay & de Duve, 1956). This is especially noticeable with the indoxyl esters as substrates, and subsequent experiments in which the enzyme specificity of the esters was investigated were therefore only carried out with fraction E.

Fraction E. Ro 3–0422 in a 10^{-8} M-concentration inhibits the hydrolysis of acetylcholine by 95 %, but has no effect on the hydrolysis of indoxyl acetate (Fig. 3). Thus cholinesterase does not contribute to the hydrolysis of the latter under the experimental conditions used. The enzyme(s) responsible for the hydrolysis of indoxyl acetate is inhibited by E 600, DINP and concentrations of Ro 3–0422 above 10^{-8} M (Fig. 3). The shape of the inhibition curves shows that at least 90 % of the hydrolysis is accomplished by an organophosphate-sensitive enzyme. Since the inhibition curves show a tendency to flatten out at high concentrations of the organophosphate, there is a possibility that a second enzyme (i.e. an organophosphate-resistant esterase) contributes 10 % or less to the total hydrolysis.

With indoxyl propionate or butyrate as substrate the inhibition curves obtained with Ro3–0422 closely resemble those obtained with indoxyl acetate as substrate and these esters are therefore also mainly, if not entirely, hydrolysed by an organophosphate-sensitive esterase.

Eserine in concentrations above 10^{-6} M also inhibits the hydrolysis of indoxyl esters (Fig. 3), although cholinesterase plays no part in it. Thus concentrations of 10^{-4} or 10^{-5} M-eserine, which are widely used for selective inhibition of cholinesterase, do not act as such in this case.

Table 5. *Distribution of activity between the nuclei and cytoplasmic extract of rat-liver homogenates*

Nuclei (N) and cytoplasmic extract (E) of a sucrose–liver homogenate were separated according to the method of de Duve *et al.* (1955). Esterase activities of N and E were determined manometrically at 37° and pH 7·5 and the activity of the whole homogenate was obtained by combining these results in the correct proportion. Standard deviations and the number of determinations are given.

	CO_2 evolved (μl./10 min./100 mg. of liver)			Activity in N (%)
Acetylcholine	16·4	± 2·2	(5)	12·2
Propionylcholine	22·4	± 2·8	(3)	8·7
Butyrylcholine	15·9	± 0·9	(2)	7·9
Indoxyl acetate	4 570	± 440	(5)	5·1
Indoxyl propionate	10 000	±1 870	(3)	6·4
Indoxyl butyrate	3 900	± 670	(4)	5·6
α-Naphthyl acetate	4 350	± 700	(3)	5·2

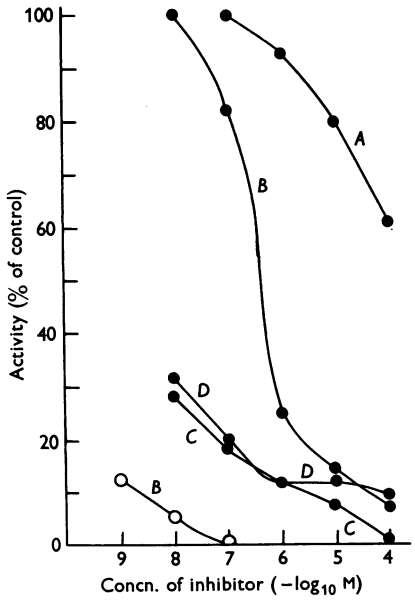

Fig. 3. Inhibition of hydrolysis by liver after pre-incubation of the inhibitor for 20 min. with the enzyme. Substrate concn. was 6 mM. ○, Acetylcholine; ●, indoxyl acetate. Curve *A*, eserine; *B*, Ro 3–0422; *C*, E 600; *D*, DINP.

Hydrolysis of α-naphthyl acetate

The rate of hydrolysis of α-naphthyl acetate is similar to that of indoxyl acetate when either rat-erythrocyte preparations (Table 2) or fraction E or the nuclei of liver homogenates (Table 5) were used as enzyme source. The hydrolysis by rat plasma, however, is approximately five times as great as that of indoxyl acetate (Table 2). α-Naphthyl acetate, like indoxyl acetate, is not a selective substrate for cholinesterases and, with rat-erythrocyte preparations or plasma as enzyme sources, only 40 % and less than 10 % respectively are attributable to these enzymes (Table 3). DFP (10^{-5} M) inhibits the eserine-resistant hydrolysis by plasma by 99 % but has far less effect if erythrocyte preparations are used as enzyme source (Table 3). The effect of organophosphates on the hydrolysis by fraction E is comparable with their effect on the hydrolysis of indoxyl acetate.

DISCUSSION

Previous work with human erythrocytes and human plasma has shown that indoxyl esters are substrates for several types of esterases (Underhay, 1957) and this is confirmed by the present results with rat-enzyme preparations. As the indoxyl esters have very low solubility in 0·025 M-$NaHCO_3$, the experiments had to be carried out in ethanolic solution (final concentration not more than 5 %). All the results, and the conclusions drawn from them, apply only to these conditions.

Acetocholinesterase of the rat hydrolyses choline and indoxyl esters at similar if not identical rates (Table 4). Human butyrocholinesterase hydrolyses indoxyl acetate, propionate and butyrate at 30, 29 and 9 % respectively of the rate of the corresponding choline esters (Underhay, 1957). No such studies could be carried out with rat propionocholinesterase, which is the equivalent of human butyrocholinesterase (Myers, 1953). The hydrolysis of indoxyl esters by rat plasma, an enzyme preparation which contains propionocholinesterase, is mainly accomplished by esterases other than cholinesterases. Since an acetocholinesterase is also present in rat plasma, selective inhibition of this enzyme would be necessary before the hydrolysis of the indoxyl esters by the propionocholinesterase could be measured. The accuracy required in such experiments cannot be achieved at present.

The major part of the hydrolysis of indoxyl esters by erythrocyte preparations, plasma or liver of the rat must be attributed to esterases which are not cholinesterases (Table 6). The latter have been classified by Aldridge (1953) and by Mounter & Whittaker (1953) according to their sensitivity to organophosphates.

Experiments designed to investigate the effect of different concentrations of organophosphates on the hydrolysis of the indoxyl esters showed that at least 90 % of the activity is arrested by 10^{-4} to 10^{-6} M-concentrations of Ro 3–0422, DFP, E 600 or DINP, when either plasma or liver is used as enzyme source, and the major part of the hydrolysis is accomplished by an organophosphate-sensitive esterase(s). The substrate-specificity pattern of this esterase is propionate > acetate ≥ butyrate (Table 6). The hydrolysis of α-naphthyl acetate by plasma or liver is also mainly accomplished by an organophosphate-sensitive esterase but its rate of hydrolysis differs from that of indoxyl acetate. The plasma enzyme hydrolyses α-naphthyl acetate very much faster than indoxyl acetate, whereas for the liver enzyme the rates are similar (Table 6). These results confirm the work of Aldridge (1954), who also found that the plasma and liver enzymes have different substrate specificities, but it was not possible to demonstrate the presence of either more than one organophosphate-sensitive esterase or of

Table 6. *Contributions made by cholinesterase and organophosphate-sensitive esterase towards the total hydrolysis by erythrocyte preparations, plasma and liver homogenates*

Organophosphate-resistant esterase activity could not be established with certainty and is therefore not included. Activity is expressed as μl. of CO_2 evolved/10 min./0·1 ml. of erythrocyte preparation, 0·2 ml. of plasma or 1 mg. of liver homogenate.

	Indoxyl acetate	Indoxyl propionate	Indoxyl butyrate	α-Naphthyl acetate
Erythrocyte preparation				
Cholinesterase	16	12	2	23
Organophosphate-sensitive esterase	45	15	16	34
Plasma				
Cholinesterase	19	10	0	0
Organophosphate-sensitive esterase	53	154	49	366
Liver homogenate				
Cholinesterase	0	0	0	0
Organophosphate-sensitive esterase	46	100	39	44

different esterases in either of these preparations on the basis of inhibition studies or by the use of mixed substrates. Only with indoxyl and α-naphthyl acetates as substrate does an organophosphate-resistant esterase seem to contribute to the hydrolysis, but the contribution by this enzyme cannot be more than 10% with liver and 5% with plasma.

The hydrolysis, not attributable to acetocholinesterase, of indoxyl acetate by erythrocyte preparations is about 100 times less sensitive to organophosphates than is that of either plasma or liver. This difference is not the result of enzymic hydrolysis of the organophosphates, as was shown by appropriate control experiments. Since progressive enzyme inhibition was obtained with increasing concentrations of the organophosphates up to mM, it was not possible to establish the extent to which an organophosphate-resistant esterase contributes to the hydrolysis of indoxyl acetate. The inhibition of the hydrolysis of indoxyl propionate and butyrate by organophosphates is even more complete than the inhibition of hydrolysis of indoxyl acetate. Since Myers & Mendel (1953) have shown that the inhibition of esterases by organophosphates is irreversible under the experimental conditions used, this difference in the sensitivity to organophosphates of the organophosphate-sensitive esterase in erythrocyte preparations, when different substrates are used, seems to indicate that more than one enzyme is involved.

All the results as well as their interpretation only apply to the experimental conditions used. For instance, Davies & Rutland (1956) have recently reported that acetylcholine and butyrylcholine are hydrolysed at the same rate by rat-erythrocyte preparations whereas the present investigation shows that butyrylcholine is hydrolysed at only 5% of the rate of acetylcholine. The reason for this discrepancy is that Davies & Rutland used higher substrate concentrations (0·015M for acetylcholine and 0·03M for butyrylcholine; Dr D. R. Davies, personal communication) and with these concentrations identical results were obtained in this Laboratory. In the present work it was also found that the presence of 5% of ethanol inhibits the hydrolysis of 0·03M-butyrylcholine by rat-erythrocyte preparations by 80%, although the same concentration of ethanol has no effect on the hydrolysis of acetylcholine by erythrocyte preparations or, incidentally, of butyrylcholine by plasma.

During this work Ro 3–0422 has been successfully used as a selective inhibitor for cholinesterases. Hobbiger (1954) reported that this compound is more potent than any other organophosphate in inhibiting human cholinesterases and, furthermore, that its effect, like that of other organophosphates with a diethyl- or di*iso*propyl-phosphoryl group, is not reversed by substrate. Hobbiger, Simpson & Tait (1954) found that Ro 3–0422 is a more selective anticholinesterase than tetraethyl pyrophosphate (TEPP) or DFP. It has now been found that this also applies to rat cholinesterases and that these enzymes have a 100–1000-fold greater sensitivity to Ro 3–0422 than other esterases in the rat. Ro 3–0422, unlike other organophosphate inhibitors, can thus be used instead of eserine as a selective inhibitor of cholinesterases over a wide range of concentrations. The experiments with rat liver show that this substance is an even more selective anticholinesterase than eserine. Ro 3–0422 inhibits the cholinesterases of rat liver in concentrations which have no effect on other esterases, whereas eserine does not act as a selective inhibitor in this case although it can be used as such with rat-erythrocyte preparations or plasma. Similarly, Myers (1953) reported that the hydrolysis of butyrylcholine by Harderian gland of the rat could be inhibited by 10^{-6}–10^{-4}M-concentrations of eserine although another potent anticholinesterase Nu 683 [dimethyl carbamate of (2-hydroxy-5-phenyl)benzyltrimethylammonium bromide] had no effect on it.

SUMMARY

1. Unsubstituted indoxyl esters are hydrolysed by rat-erythrocyte preparations, plasma and liver homogenates at a considerably greater rate than any of the corresponding choline esters.

2. The enzymes chiefly responsible for the hydrolysis of the indoxyl esters are organophosphate-sensitive esterases. Under the experimental conditions described, cholinesterases of erythrocyte preparations and plasma contributed only a fraction of the total hydrolysis, and any contribution by liver cholinesterase was too small to be detected. An organophosphate-resistant esterase may contribute to an extent of not more than 10% in each case.

3. The organophosphate Ro 3–0422 is a more selective anticholinesterase than eserine and has been successfully used as such with all three enzyme preparations.

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REFERENCES

Aldridge, W. N. (1953). *Biochem. J.* **53**, 110.
Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
Burgen, A. S. V. & Hobbiger, F. (1951). *Brit. J. Pharmacol.* **6**, 593.
Davies, D. R. & Rutland, J. P. (1956). *Nature, Lond.*, **178**, 697.

de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
Hines, B. E. (1952). *Nature, Lond.*, **170**, 78.
Hobbiger, F. (1954). *Brit. J. Pharmacol.* **9**, 159.
Hobbiger, F., Simpson, S. A. & Tait, J. F. (1954). *Biochem. J.* **58**, x.
Holt, S. J. (1954). *Proc. Roy. Soc.* B. **142**, 160.
Holt, S. J. (1956). *J. Histochem. Cytochem.* **4**, 541.
Mounter, L. A. & Whittaker, V. P. (1953). *Biochem. J.* **54**, 551.
Mundell, D. B. (1944). *Nature, Lond.*, **153**, 557.
Myers, D. K. (1953). *Biochem. J.* **55**, 67.
Myers, D. K. & Mendel, B. (1953). *Biochem. J.* **53**, 16.
Underhay, E. E. (1957). *Biochem. J.* **66**, 383.
Underhay, E., Holt, S. J., Beaufay, H. & de Duve, C. (1956). *J. biophys. biochem. Cytol.* **2**, 635.
Zajicek, J. & Datta, N. (1952). *Acta haemat.* **7**, 39.
Zajicek, J. & Datta, N. (1953). *Acta haemat.* **9**, 115.

The Metabolism of 2:3-, 2:6- and 3:5-Dichloronitrobenzene and the Formation of a Mercapturic Acid from 2:3:4:5-Tetrachloronitrobenzene in the Rabbit

By H. G. BRAY, SYBIL P. JAMES and W. V. THORPE
Department of Physiology, The Medical School, University of Birmingham

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Previous papers (Bray, Hybs, James & Thorpe, 1953; Betts, James & Thorpe, 1955; Bray, James & Thorpe, 1956, 1957; Betts, Bray, James & Thorpe, 1957) have described the metabolism in the rabbit of all members of the chloromononitrobenzene series except 2:3-, 2:6- and 3:5-dichloronitrobenzene. One aspect of the metabolism of these compounds of particular interest is the readiness with which mercapturic acids are formed by most members of the series. Furthermore, these mercapturic acids are all formed, in effect, either by acetylcysteyldenitration or by acetylcysteyldechlorination, and not by acetylcysteyldeprotonation as had been observed in all previous instances of the formation of nuclear mercapturic acids (for references see Bray *et al.* 1956). The present paper describes the metabolic fate of 2:3-, 2:6- and 3:5-dichloronitrobenzene. No conclusive evidence has been obtained for the formation of mercapturic acid by these three compounds.

The early work on tetrachloronitrobenzenes (Bray *et al.* 1953) was published before an example of nuclear acetylcysteyldechlorination had been discovered. When this type of mercapturic acid formation was observed (Bray *et al.* 1956, 1957; Betts *et al.* 1957) it became necessary to re-examine the tetrachloronitrobenzenes to see whether a product of acetylcysteyldechlorination had been overlooked, especially with 2:3:4:5-tetrachloronitrobenzene, from which no mercapturic acid had been obtained. The search for a mercapturic acid formed by elimination of chlorine was facilitated by the fact that the resulting mercapturic acid retained the nitro group and, therefore, could be more readily detected by its colour in alkaline solution if only small amounts were present. It has, in fact, now been shown that 2:3:4:5-tetrachloronitrobenzene does form a small amount of mercapturic acid by acetylcysteyldechlorination. The previous finding that no *N*-acetyl-*S*-(2:3:4:5-tetrachlorophenyl)-L-cysteine is formed has been confirmed.

MATERIALS

All melting points recorded are uncorrected. Elementary microanalyses were carried out by F. and E. Pascher, Bonn.

2:3- and 3:5-Dichloronitrobenzene were the gift of Bayer Products Ltd. Some 3:5-dichloronitrobenzene as well as 2:6-dichloronitrobenzene was obtained by the method of Holleman (1904).

2:3-Dichloroaniline was purchased; 2:6- and 3:5-dichloroaniline, m.p. 49° and 39° respectively, were prepared by reduction of the nitro compounds. The three bases were converted into the corresponding dichloroacetanilides, m.p. 159°, 174° and 187° respectively. The *picrate* of 2:6-dichloroaniline formed orange needles from water, m.p. 89° (Found: 2:6-dichloroaniline, 42·5. $C_{12}H_9O_7N_4Cl_3$ requires 2:6-dichloroaniline, 41·2%).

3:5:3':5'-*Tetrachloroazoxybenzene* was prepared by boiling 3:5-dichlorophenylhydroxylamine in 96% (w/v) ethanol for 15 min. according to the method of Haworth & Lapworth (1921). It formed fine colourless threads, m.p. 168°, from aqueous ethanol (Found: C, 42·7; H, 1·7; N, 8·3; Cl, 42·7. $C_{12}H_6ON_2Cl_4$ requires C, 42·9; H, 1·8; N, 8·3; Cl, 42·2%).

N-*Acetyl*-S-(2-*chloro*-6-*nitrophenyl*)-L-*cysteine*. This was prepared from 2-chloro-6-nitroaniline by the method of Parke & Williams (1951) as modified by Bray *et al.* (1956). 2-Chloro-6-nitroaniline was obtained by hydrolysis of 2-chloro-6-nitroacetanilide prepared as described by Franzen & Engel (1921). The mercapturic acid was also made by acetylation (cf. Zbarsky & Young, 1943) of *S*-(2-chloro-6-nitrophenyl)-L-cysteine obtained by interaction of 2:3-dichloronitrobenzene and cysteine in alkaline solution (Suter, 1895). N-*Acetyl*-S-(2-*chloro*-6-*nitrophenyl*)-L-*cysteine* formed pale-yellow rectangular plates from water, m.p. 185°