The Differentiation of the A-Type Esterases in Sheep Serum

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The differentiation of paraoxonase (an A-type esterase in serum) from related enzymes has been complicated by overlapping substrate specificities and by the complex nature of these enzymes. Rabbit-serum paraoxonase has been differentiated from hog-kidney diisopropyl phosphorofluoridate by Mounter (1954). He confirmed the observation of Aldridge (1953*b*) that one enzyme in rabbit serum hydrolyses *p*-nitrophenyl acetate and paraoxon (diethyl-*p*-nitrophenyl phosphate) and extended the observation to include tetraethyl pyrophosphate and diisopropyl phosphorofluoridate. The hog-kidney enzyme was activated by manganous ions (Mounter & Chanutin, 1953) but did not hydrolyse paraoxon (Mounter, 1956), whereas rabbit-serum paraoxonase was inhibited by manganese and hydrolysed paraoxon. Bergmann, Segal & Rimon (1957) have shown that diisopropyl phosphorofluoridate does not hydrolyse *p*-nitrophenyl acetate. In addition they have demonstrated a new C-esterase hydrolysing this substance in purified preparations of the hog-kidney enzyme. According to the usage of Aldridge (1954), C-esterase is an A-type esterase. The ability of rabbit-serum paraoxonase to hydrolyse *p*-nitrophenyl acetate could also be used to differentiate paraoxonase and diisopropyl phosphorofluoridate.

The paraoxonases of rabbit, rat and horse sera hydrolysed paraoxon and *p*-nitrophenyl acetate but the ratio of hydrolysis of *p*-nitrophenyl acetate to hydrolysis of paraoxon varied from 4 in rabbit to 18 in horse. From summation experiments Aldridge concluded that one enzyme in these sera was responsible for this hydrolysis and attributed the varying ratios to species differences. However, when the ratios for other sera are calculated, in many cases they are greater. For instance, from Aldridge's results the ratio for ferret serum was 130, for sheep 50, for goat 170 and for human 48. As observed in the present work, the ratio for hog serum was 5700. The possibility that A-type esterases, other than paraoxonase, hydrolysing *p*-nitrophenyl acetate, occur in some sera cannot be excluded. Mounter & Whittaker (1953) suggested that serum arylesterase and paraoxon-hydrolysing A-esterase were identical.

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Although hog-kidney diisopropyl phosphorofluoridate and rabbit-serum paraoxonase can be clearly differentiated, the situation becomes more confused when other tissues, sera and species are examined. Mounter (1955) studied the diisopropyl phosphorofluoridase activities in rat and hog liver and kidney fractions and observed that, unlike the enzyme of the soluble fraction, the enzymes of insoluble fractions of liver were inhibited by manganese and activated by calcium when di-*n*-butyl phosphorofluoridate was the substrate.

The distribution and nature of diisopropyl phosphorofluoridases have been studied in some detail by Mounter, Dien & Chanutin (1955). After comparing these enzymes in the tissues of various species they concluded that a number of diisopropyl phosphorofluoridases exist. Augustinsson & Heimbürger (1954) have reported evidence suggesting that serum and kidney tabunase are not identical and also that rabbit-serum tabunase and A-esterase are not identical. On the basis of summation experiments, Cohen & Warringa (1957) were unable to conclude that their highly purified hog-kidney B-fraction, derived from most purified A-2 fraction of Mounter, Floyd & Chanutin (1953), contained only one enzyme hydrolysing diisopropyl phosphorofluoridate and tabun (ethyl-*N*-dimethylphosphoramidocyanate).

In the present work a substrate-specificity study with purified paraoxonase preparations from sheep serum (Main, 1960) has been made for the purpose of further differentiating these various activities. Data from other experiments with rabbit, horse, sheep and pig sera have also been employed.

EXPERIMENTAL

Enzyme preparations. Various fractions from the purification of paraoxonase from sheep serum were used. The fractions are designated by their state of purification relative to the starting serum and, when relevant, by the fraction number in the flow scheme employed to illustrate the procedure (Main, 1960). The most pure paraoxonase fractions were purified 305- to 385-fold. Evidence from electrophoresis suggested that the product was 70-85% paraoxonase and contained two components.

Defibrinated sheep, horse, rabbit and rat sera were obtained from recently slaughtered animals and stored in the frozen state.

Enzyme preparations used with most carboxylic ester substrates were first incubated with 1 mM-paraoxon for

30 min. at 37°. For some experiments with phenyl acetate and *p*-nitrophenyl acetate as substrates 10 mM-diisopropyl phosphorofluoridate (DFP) was employed. The activity of sheep serum was not affected by previous incubation with paraoxon or DFP.

Estimation of activity. The colorimetric method for paraoxonase previously described (Main, 1960) was frequently employed.

All comparisons of substrate specificity were made with a variation of the manometric method of Aldridge (1953*a*). Generally 0.5 ml. of enzyme preparation was placed in the side arm of the Warburg flask and 2.5 ml. of buffered substrate in the centre well. The final concentrations in the digestion medium, exclusive of substrates, were 0.025 M-NaHCO₃, 0.162 M-NaCl and 0.2% of gelatin. The flasks were gassed with CO₂+N₂ (5:95) at room temperature before being placed in a bath at 37°. Manometric readings were made at intervals of 5 min. over a period of at least 20 min. and where possible 30 min. after tipping. In addition, a reading was usually taken 2 or 3 min. after tipping.

The paraoxon K_m of paraoxonase preparations was 14 times higher than that for the sheep serum (Main, 1960). It was therefore desirable to calculate the activity ratios at the maximum velocities where possible, since paraoxon has a limited solubility and the paraoxonase K_m was almost equal to the saturation concentration of paraoxon.

Nitrophenyl esters were prepared by the method of Huggins & Lapides (1947). Stock solutions of the slightly soluble substrates, paraoxon, *o*-, *m*- and *p*-nitrophenyl acetate, *p*-nitrophenyl butyrate and phenyl acetate were prepared by dissolving the ester in methanol or in methanol-acetone (1:1, v/v). Appropriate volumes of the stock solutions were then diluted in buffer to obtain the desired concentration. Appropriate amounts of the more soluble substrates, DFP, tetraethyl pyrophosphate, tabun (ethyl-*N*-dimethylphosphoramidocyanate) and triacetin were dissolved directly in bicarbonate buffer to give a concentration of 12 mM.

Inactivation by heating. About 20 ml. of sheep serum in a thin-walled test tube containing a thermometer was placed in a suitably adjusted constant-temperature bath. Serum samples (2 ml.) were withdrawn at measured time intervals and immediately cooled. Paraoxon hydrolysis was measured colorimetrically with undiluted serum. Determinations of rates of hydrolysis of phenyl acetate, *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate were made by the manometric method with suitably diluted serum.

Inhibition with p-chloromercuribenzoate. Sheep serum was diluted with an equal volume of *p*-chloromercuribenzoate solution so that the final concentration of this compound was 0.4 mM. The inhibitor was incubated with the serum for 18 hr. at 4°. The serum hydrolysis rates for paraoxon and *p*-nitrophenyl acetate were then measured manometrically. The manometric buffer initially contained 0.04 M-MgCl₂ in addition to the NaCl, NaHCO₃ and gelatin. This concentration of magnesium inhibited hydrolysis of paraoxon by 42% and hydrolysis of *p*-nitrophenyl acetate by 65%.

RESULTS AND DISCUSSION

The substrate-specificity pattern of purified paraoxonase preparations is compared with that of the

original sheep serum in Table 1. Sheep serum had no measurable B-esterase activity, as defined by Aldridge (1953*a*), towards any of the substrates listed. Previous incubation of serum with 10 mM-DFP or 8 mM-paraoxon for at least 30 min. at 37° did not result in any significant change in the rates of hydrolysis of phenyl acetate, *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate. Sheep serum did not measurably catalyse the hydrolysis of such substrates as triacetin or ethyl butyrate. It was concluded that all the activities compared were of the A-esterase type.

Hydrolysis of p-nitrophenyl acetate. The ratio of hydrolysis of *p*-nitrophenyl acetate to hydrolysis of paraoxon at V_{max} decreased from 67 ± 7 for sheep serum to 9 ± 4 for purified paraoxonase preparation (Table 1). Aldridge (1953*b*) reported that the same ratios in the sera of rabbit, rat and horse were 4, 12 and 18 respectively. From summation experiments he concluded that one enzyme in these sera hydrolysed both *p*-nitrophenyl acetate and paraoxon. The hydrolysis ratios reported by Aldridge are in the same order as those found for the purified paraoxonase preparations examined. However, the sevenfold change in the ratio which resulted from purification suggested the presence in sheep serum of an enzyme, other than paraoxonase, which also hydrolysed *p*-nitrophenyl acetate.

By heat-inactivation and other experiments Mounter (1954) had confirmed the observation of Aldridge (1953*b*) that one enzyme in rabbit serum hydrolysed both *p*-nitrophenyl acetate and paraoxon.

Heat-inactivation studies with sheep serum, as shown in Figs. 1 and 2, presented a different picture. After incubation for 13 hr. at 48°, 31% of *p*-nitrophenyl butyrate-hydrolysing activity and 25% of the *p*-nitrophenyl acetate-hydrolysing activity remained, whereas 97% of the paraoxon-hydrolysing activity had been destroyed. After 19 hr. the paraoxonase activity was not measurable and 10% of the *p*-nitrophenyl acetate-hydrolysing activity and 16% of the *p*-nitrophenyl butyrate-hydrolysing activity remained. These results suggested that one of the enzymes hydrolysing *p*-nitrophenyl carboxylate esters does not hydrolyse paraoxon. This enzyme hydrolysing the acetate and butyrate esters, but not hydrolysing paraoxon, will be called D-esterase to differentiate it from other A-type esterases and from the C-esterase of Bergmann *et al.* (1957). From the results of heat-inactivation experiments of Mounter (1954) D-esterase is evidently absent in rabbit serum. The ratios of *p*-nitrophenyl acetate hydrolysis to paraoxon hydrolysis of sheep (59) and rabbit (4) are consistent with this view.

The ratio of hydrolysis of *p*-nitrophenyl acetate to hydrolysis of *p*-nitrophenyl butyrate changed

significantly from 8 in serum to 14 in preparation C, purified 305 times (Table 1). About 80% of the hydrolysis of *p*-nitrophenyl acetate by sheep serum may evidently be attributed to D-esterase. The ratios at 13 and 19 hr. (Fig. 1) were 6.0 and 5.7 respectively and probably approximated the ratio of D-esterase alone, since the paraoxonase activity at these heating times was negligible. The ratio in rabbit serum was 12 (Aldridge, 1953a) compared with 14 in the paraoxonase C preparation. These results suggest that, relative to hydrolysis of *p*-nitrophenyl acetate, D-esterase hydrolyses *p*-nitrophenyl butyrate about twice as readily as paraoxonase. The change in the hydrolysis ratio from 8 in serum to 5.7 in the absence of paraoxonase is consistent with the view that two enzymes in sheep serum hydrolyse *p*-nitrophenyl acetate.

With two purified samples (B and C, Table 1), it was observed that the paraoxon-hydrolysing activity decreased more rapidly during storage than the *p*-nitrophenyl acetate-hydrolysing activity. For example, after 56 days' storage at -15° the paraoxon-hydrolysing activity of sample B decreased from 86 to 56 μ l. of carbon dioxide/mg. of protein/min., and the *p*-nitrophenyl acetate-

hydrolysing activity had decreased from 1370 ± 37 to 1240 ± 21 μ l. of carbon dioxide/mg. of protein/min. Neither the K_m of paraoxon nor the K_m of *p*-nitrophenyl acetate changed significantly during storage. The DFP-hydrolysing activity decreased from 32 to 18 μ l. of carbon dioxide/mg. of protein/min., which compared with the loss of paraoxon-hydrolysing activity. As already noted, paraoxonase was more heat-labile than D-esterase. In addition, preliminary results show that the variation in the ratios of *p*-nitrophenyl acetate-hydrolysing to paraoxon-hydrolysing activities of the purified preparations may have resulted from the presence in varying amounts of two imperfectly separated *p*-nitrophenyl acetate-hydrolysing enzymes.

The K_m for hydrolysis of *p*-nitrophenyl acetate and the ratios of its hydrolysis to paraoxon hydrolysis for various samples of enzymes are given in Table 2.

The K_m of purified paraoxonase samples was significantly greater than that in serum and the ratio of hydrolysis of *p*-nitrophenyl acetate to that of paraoxon decreased. Both the K_m and the ratio for the purified paraoxonase samples approached the values for rabbit serum, suggesting a similarity in the paraoxonase of the two sera.

Table 1. Comparison of substrate specificities

Activities of three paraoxonase preparations purified from sheep serum are compared with sheep serum. All activities were measured manometrically. Where the K_m values of the substrates compared are known, the activities are compared at their respective V_{max} , as well as at the concentrations given. Paraoxonase preparation A was 385-fold purified, B was 330-fold purified and C was 305-fold purified. The letter after the figure in the purified paraoxonase column refers to the preparation used, as described above. The paraoxon activity was called 1 and the other activities were related to it. The specific activity of the 330-fold purified preparation was 86 μ l. of CO_2 /mg. of protein/min. at V_{max} , and the activity of the serum from which this preparation was derived was 0.26 μ l. of CO_2 /mg. of protein/min. Sera used with carboxylic esters were previously incubated with 1 mM-paraoxon for about 30 min. at 37° . Abbreviations used in this table and subsequent tables and figures are: PNPA, *p*-nitrophenyl acetate; MNPA, *m*-nitrophenyl acetate; ONPA, *o*-nitrophenyl acetate; PNPB, *p*-nitrophenyl butyrate; DFP, diisopropyl phosphorofluoridate; TEPP, tetraethyl pyrophosphate.

Substrate and concn. (mM)	Comparison at given concn. of substrate		Comparison at V_{max} .	
	Sheep serum	Purified paraoxonase	Sheep serum	Purified paraoxonase
Paraoxon (6.7)	1	1	1	1
PNPA (6.7)	68	16 (A)	68	12 (A)
	60	6.5 (B)	68	5 (B)
	49	14.5 (C)	56	11 (C)
DFP (10)	1.05	0.43 (A)	1.83	0.39 (A)
	1.10	0.45 (B)	1.90	0.41 (B)
	1.10	0.45 (C)	1.90	0.41 (C)
MNPA (6.7)	104	28 (C)	—	—
ONPA (6.7)	3.9	1.05 (C)	—	—
PNPB (3.4)	6.3	1.07 (C)	—	—
Phenyl acetate (3.4)	130	0 (C)	—	—
TEPP (10)	0.98	0 (C)	—	—
Tabun (10)	1.36	1.69 (C)	2.44	1.80 (C)
Sarin (10)	0.76	—	—	—
Triacetin (10)	Nil	Nil	—	—

The K_m (0.38 mM) for hydrolysis of *p*-nitrophenyl acetate of the discarded S2 fraction from sheep serum (0.3-fold purified) was 3.7 times less than the serum K_m , and the hydrolysis ratio for *p*-nitro-

phenyl acetate to paraoxon doubled. The comparable K_m of the A-type esterase in hog serum was 0.485 mM. The A-type esterase in hog serum had a negligible paraoxon-hydrolysing activity (one-fortieth of the activity of sheep serum) but a high *p*-nitrophenyl acetate-hydrolysing activity (three times the activity of sheep serum).

The *p*-nitrophenyl acetate-hydrolysing enzyme in hog serum appears on the basis of its high activity towards this compound and its K_m to be similar to sheep-serum D-esterase.

This evidence suggested that the K_m for hydrolysis of *p*-nitrophenyl acetate of D-esterase was about 0.4 mM, and of paraoxonase 2 mM, and was consistent with the view that two A-type esterases in serum contribute to the hydrolysis of this compound.

Differentiation of the enzyme hydrolysing phenyl acetate from paraoxonase. Sheep serum hydrolysed phenyl acetate 130 times more rapidly than paraoxon but purified paraoxonase did not hydrolyse it measurably. The amount of paraoxonase used with phenyl acetate was 20 times as great as that used to determine hydrolysis of *p*-nitrophenyl acetate and was twice that used to measure hydrolysis of paraoxon. Nevertheless, the hydrolysis rate remained negligible. The ability to hydrolyse phenyl acetate disappeared in fraction P4 (purified 30 times), suggesting that the phenyl acetate-hydrolysing enzyme may have remained with the cholinesterase in the supernatant S2, which on further precipitation would lead to the equivalent of Cohn fraction IV.4 (Cohn *et al.* 1946).

Fig. 2 shows the effect on the rates of hydrolysis of phenyl acetate, *p*-nitrophenyl acetate and paraoxon of preheating sheep serum for varying lengths of time at 53°. The activity hydrolysing the first

Fig. 1. Effect on the enzymic hydrolysis of *p*-nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB) and paraoxon of preheating sheep serum at 48° for varying times. Percentage of activity remaining: PNPA, Δ ; PNPB, $\circ$; paraoxon, $\bullet$. PNPA (6.7 mM) and PNPB (3.4 mM) hydrolysis rates were measured by the manometric method. Paraoxon (8 mM) hydrolysis rates were measured by the colorimetric method.

Table 2. K_m for hydrolysis of *p*-nitrophenyl acetate by various sera and sheep-serum fractions

K_m values were determined manometrically, typically with four concentrations of *p*-nitrophenyl acetate. The velocities were plotted according to the Dixon (1953) modification of the Lineweaver & Burk (1934) method. Sheep serum (0.02 ml.), 0.02 ml. of rabbit serum, 0.05 ml. of horse serum and 0.01 ml. of hog serum, diluted to 0.5 ml., were placed in the side arm of the manometer flask and 2.5 ml. of buffered solutions of *p*-nitrophenyl acetate in the centre well. Sera used with carboxylic esters were previously incubated for about 30 min. at 37° with paraoxon, usually 1 mM, but with rabbit serum higher concentrations were also used. For explanation of abbreviations see Table 1.

Sample	K_m (mM)	Activity ratio (PNPA/paraoxon)	
		6.7 mM	V_{max}
Sheep serum	1.42 ± 0.12	6.3	72
Purified paraoxonase (A, B, C; see Table 1)	1.87 ± 0.11	12 ± 5	9 ± 4
Rabbit serum	2.5	4.6	6.3
S2 discarded fraction from purification procedure	0.38	128	—
P2 retained fraction	—	40	44*
Hog serum	0.485	5700	—
Horse serum	1.39	20	—

* Calculated assuming that PNPA K_m was 1.4 mM.

two decreased at a significantly slower rate than that hydrolysing paraoxon. For example, serum heated for 15 min. retained only 29 % of the initial paraoxon-hydrolysing activity but 60 % of phenyl acetate-hydrolysing activity. After heating for 30 min., significant phenyl acetate- and *p*-nitrophenyl acetate-hydrolysing activity remained, whereas the paraoxon-hydrolysing activity had practically disappeared.

Fig. 2. Effect on the enzymic hydrolysis of *p*-nitrophenyl acetate (PNPA), phenyl acetate and paraoxon of pre-heating sheep serum at 53° for varying times. Percentage of hydrolysing activity remaining: phenyl acetate, Δ ; PNPA, $\circ$; paraoxon, $\bullet$. Phenyl acetate (3.4 mM) and PNPA (6.7 mM) hydrolysis rates were measured by the manometric method. Paraoxon (8 mM) hydrolysis rates were measured by the colorimetric method.

The disappearance of phenyl acetate-hydrolysing activity in a purified paraoxonase preparation, together with the different heat-inactivation rates of paraoxon-hydrolysing and phenyl acetate-hydrolysing activity, suggested that paraoxonase does not hydrolyse phenyl acetate and that the enzyme hydrolysing it does not hydrolyse paraoxon. The suggestion of Mounter & Whittaker (1953) that serum A-esterase, that is paraoxonase, is identical with the enzyme hydrolysing phenyl acetate would not appear on this evidence to be true. The enzyme hydrolysing phenyl acetate must be a distinct A-type arylesterase, since sheep serum does not hydrolyse common ali-esters such as triacetin and ethyl butyrate.

Evidence on the relationship between D-esterase and the enzyme hydrolysing phenyl acetate. The similar heat-inactivation curves for hydrolysis of phenyl acetate and of *p*-nitrophenyl acetate by sheep serum suggested the possibility that one enzyme might be responsible for both hydrolyses after the hydrolysis of the second compound by paraoxonase had been discontinued.

The hydrolysis rates of *p*-nitrophenyl acetate, phenyl acetate and paraoxon by the sera of rabbit, sheep, rat and hog are given in Table 3. If D-esterase was responsible for hydrolysis of phenyl acetate then one would expect a high rate of hydrolysis of this compound in hog serum. In fact, hog serum displayed the lowest hydrolysis of phenyl acetate of the sera examined. Rabbit serum, which apparently is free of D-esterase, had the highest phenyl acetate-hydrolysing activity and was 28 times that of hog serum. No consistent relationship between the hydrolysis activities of sera of these species for the three compounds could be detected.

The evidence suggesting that paraoxonase preparations may be contaminated with D-esterase is also not consistent with the idea that D-esterase is identical with phenyl acetate-hydrolysing enzyme, since these samples possessed no activity toward phenyl acetate.

Table 3. Comparison of the hydrolysis rates of phenyl acetate, *p*-nitrophenyl acetate and paraoxon by the sera of four species

Activities were determined by the manometric method. Substrate concentrations were: phenyl acetate, 5 mM; PNPA, 6.7 mM; paraoxon, 6.7 mM. Sera used with carboxylic esters were previously incubated for about 30 min. at 37° with paraoxon, usually 1 mM, but in a few instances (phenyl acetate, PNPA) higher concentrations were also used. For explanation of abbreviations see Table 1.

Serum	Activity (μ l. of CO ₂ /ml./min.)			Ratios of activities		
	Paraoxon	Phenyl acetate	PNPA	Phenyl acetate	Phenyl acetate	PNPA
				PNPA	Paraoxon	Paraoxon
Rabbit	148	4810	610	7.9	33	4
Sheep	15	2605	770	3.4	173	51
Rat	8	498	92	5.4	62	11
Hog	0.5	170	2830	0.06	340	5660

Table 4. *Effect of Mn²⁺ ions on the activity hydrolysing diisopropyl phosphorofluoridate, paraoxon and p-nitrophenyl acetate*

1 mM-Mn²⁺ ion was incubated for 20 min. with the enzyme preparation in the side arm of a manometric flask before tipping. For explanation of abbreviations see Table 1. +, Percentage activation; -, percentage inhibition.

Substrate and concn. (mM)	Effect of Mn ²⁺ ion on activity (%)				
	Sheep serum	P4 30-fold purified	Paraoxonase 385-fold purified	Horse serum	Rabbit serum
DFP (10)	+40	+14	-87	+42	-14*
Paraoxon (6.7)	-49	-60	-65	-34	—
PNPA (6.7)	-43	-73	-40	—	—

* From Mounter (1954).

The impression gained from this evidence is that D-esterase and the enzyme hydrolysing phenyl acetate are different. The phenyl acetate-hydrolysing A-type esterase may be the arylesterase of Mounter & Whittaker (1953).

Comparison of the hydrolysis rates of p-, m- and o-nitrophenyl acetates. It was of interest to determine whether the activity of *p*-nitrophenyl acetate-hydrolysing enzymes was dependent on the *para* orientation of the nitro group or whether esters with *ortho* and *meta* nitro groups could also be hydrolysed. For this purpose *o*- and *m*-nitrophenyl acetate, synthesized according to the method used for the *para* compound (Huggins & Lapides, 1947), were used as substrates. The results given in Table 1 show that the *meta* isomer is hydrolysed 2.14 times as fast as the *para* isomer with sheep serum, and 1.83 times as fast with a preparation purified 305 times. The *ortho* isomer was hydrolysed at about one-tenth of the rate for the *para* isomer. The lower hydrolysis rate of the *ortho* isomer may be due to the presence of a blocking ring structure between the adjacent acetate and nitro groups.

The non-enzymic blanks for the *meta* isomer indicated that this ester hydrolysed spontaneously at about half the rate of the *para* isomer. The greater enzymic hydrolysis rate of the *meta* isomer compared with that of the *para* isomer cannot therefore be attributed to the instability of the *meta* isomer. The nitrophenyl acetate-hydrolysing enzymes in sheep serum showed a decreasing activity in the order *meta*, *para*, *ortho*, with respect to the position of the nitro group on the benzene ring. The results also suggest that this may be true to the same extent for paraoxonase, but a definitive statement cannot be made until the possible contamination of the purified paraoxonase with D-esterase is re-examined.

Relationship between C- and D-esterases. Bergmann *et al.* (1957) reported a new C-esterase in the most pure (A-2) diisopropyl phosphorofluoridate fraction of Mounter *et al.* (1953). C-Esterase was not inhibited by DFP, hydrolysed carboxylic

esters such as diacetin, *n*-propyl acetate and *p*-nitrophenyl acetate and was relatively resistant to organo mercurials, such as *p*-chloromercuribenzoate. The *p*-chloromercuribenzoate *pI*₅₀ of C-esterase was 2.82.

The *p*-nitrophenyl acetate-hydrolysing activity of sheep serum was inhibited 53% by 0.4 mM-*p*-chloromercuribenzoate, a concentration which activated C-esterase. The paraoxon-hydrolysing activity of sheep serum was inhibited 27% by the compound.

Paraoxonase contributed to the hydrolysis of *p*-nitrophenyl acetate by sheep serum, but since it was less inhibited than the overall *p*-nitrophenyl acetate-hydrolysing activity, the major portion of the inhibited hydrolysing activity must have been associated with D-esterase. The *p*-nitrophenyl acetate-hydrolysing activity of hog serum was inhibited 60% by 0.4 mM-*p*-chloromercuribenzoate. D-Esterase appears therefore to be significantly more sensitive to inhibition by this compound than does either C-esterase or paraoxonase.

Hydrolysis of ethyl butyrate and triacetin by sheep serum was negligible. C- and D-esterase thus appear to differ in their ability to hydrolyse short-chain aliphatic esters, although both hydrolyse *p*-nitrophenyl acetate. This preliminary evidence suggested that C- and D-esterase are not identical.

Evidence for the presence of diisopropyl phosphorofluoridase in sheep and other serum. This enzyme of hog-kidney purified fractions was activated by manganese (Mounter & Chanutin, 1953, 1954). Similar activation has been observed in other species and tissues, but not in serum. The DFP-hydrolysing activity of rabbit serum (Mounter, 1954) and of certain bacteria (Mounter, Baxter & Chanutin, 1955) was inhibited by manganese.

The effect of manganese on the hydrolysis of DFP, paraoxon and *p*-nitrophenyl acetate is shown in Table 4. With sheep and horse serum, the DFP-hydrolysing activity was activated by manganese, in contrast to its inhibiting effect on rabbit serum. The manganese had little effect unless first incubated

with the enzyme. Manganese concentrations in excess of 1 mM lowered the activation. Mounter & Chanutin (1954) reported that α -dipyridyl increased the activation effect of manganese with hog-kidney diisopropyl phosphorofluoridase. The addition of α -dipyridyl almost doubled the manganese activation in sheep serum. The DFP-hydrolysing activity of a paraoxonase preparation purified 385 times was strongly inhibited by manganese. The DFP-hydrolysing activity relative to paraoxon hydrolysis of this and other highly purified fractions decreased by 80 % compared with serum (Table 1). The relative disappearance of DFP-hydrolysing activity in the paraoxonase preparations, together with the change in the effect of manganese on the activity, suggested that sheep serum contained an enzyme similar to the hog-kidney enzyme and that the purification procedure (Main, 1960) separated this diisopropyl phosphorofluoridase from paraoxonase. The observation that the residual DFP-hydrolysing activity in the paraoxonase preparations was inhibited strongly by manganese suggested that this activity was not associated with diisopropyl phosphorofluoridase, but with paraoxonase.

The observation that manganese also activated the DFP-hydrolysing activity of horse serum suggested the possibility that diisopropyl phosphorofluoridase may occur, with paraoxonase, in other mammalian sera.

Aldridge (1953*b*) has observed that other tissues besides sera exhibit paraoxonase activity. The complex effect of manganese on the tissues of various species (Mounter, Dien & Chanutin, 1955) and in hog- and rat-liver fractions (Mounter, 1955) may be partly explained by the presence of varying amounts of diisopropyl phosphorofluoridase and DFP-hydrolysing paraoxonase in these tissues.

Loss of tetraethyl pyrophosphate-hydrolysing activity. The inability of the purified paraoxonase preparations to hydrolyse tetraethyl pyrophosphate suggested that the diisopropyl phosphorofluoridase in the starting sheep serum may have been entirely responsible for this hydrolysis. Mounter & Dien (1956) have reported that purified hog-kidney diisopropyl phosphorofluoridase hydrolysed the compound. At the substrate concentrations used, the hydrolysis rate of tetraethyl pyrophosphate was 80 % of that of DFP. Mounter (1954) reported that one enzyme (paraoxonase) in rabbit serum hydrolysed tetraethyl pyrophosphate, DFP and paraoxon. The hydrolysis rates of tetraethyl pyrophosphate and DFP were comparable and about one-third of the paraoxon-hydrolysis rate. Mounter (1954) based his conclusions on heat-inactivation, heavy-metal-inhibition and summation experiments. However, the preponderance of

paraoxonase in rabbit serum may have masked the other enzyme present.

Moreover, some of the criteria used may not have been suitable for differentiating these two enzymes. For example, the rates of inactivation by heating appear to be almost identical for hog-kidney diisopropyl phosphorofluoridase (Mounter & Dien, 1956) and paraoxonase (Main, 1960). Heating for 5 min. at each temperature gave the following results: at 45°, 15 % of the former enzyme and 20 % of paraoxonase were inactivated; at 55°, 95 % and 98 % respectively were inactivated. Both enzymes appear to be inhibited by the same metal ions, with the notable exceptions of magnesium, manganese and cobalt. With most metal ions, similar concentrations give comparable inhibitions, especially when the difficulty of obtaining strictly comparable protein solutions is considered.

The use of summation experiments with two enzymes having overlapping substrate specificities must be regarded with caution, particularly when one enzyme appears to predominate. Tetraethyl pyrophosphate could conceivably act as a competitive inhibitor of paraoxonase. This point calls for further investigation. Although it is not possible at this time to accept without reservation the suggestion that rabbit-serum paraoxonase does not hydrolyse tetraethyl pyrophosphate, nor is it possible to accept the opposite conclusion.

Hydrolysis of tabun. From results on the rates of hydrolysis at $V_{\max}$, sheep-serum paraoxonase appeared to be responsible for about 74 % of the hydrolysis of tabun by sheep serum (Table 1). Cohen & Warringa (1957) reported that a diisopropyl phosphorofluoridase preparation purified from hog kidney hydrolysed tabun and DFP. However, they were unable to conclude that the same enzyme hydrolysed both substrates. It is probable that the diisopropyl phosphorofluoridase in sheep serum is responsible for a portion of the tabun hydrolysis.

SUMMARY

1. The substrate-specificity pattern of paraoxonase preparations purified approximately 300 times has been compared with that of sheep serum.
2. Paraoxonase did not hydrolyse phenyl acetate, whereas sheep serum hydrolysed the compound at a high rate.
3. The high phenyl acetate-hydrolysing activity of sheep serum and the results of heat-inactivation experiments suggested the presence of an A-type arylesterase in sheep serum distinct from paraoxonase.
4. The ratio of *p*-nitrophenyl acetate hydrolysis to paraoxon hydrolysis decreased from 67 ± 7 in serum to 9 ± 4 in purified preparations. Heat in-

activated the *p*-nitrophenyl acetate- and *p*-nitrophenyl butyrate-hydrolysing activity of sheep serum at a significantly slower rate than it did the paraoxon-hydrolysing activity. From this and supporting evidence involving the K_m for hydrolysis activity of sheep, rabbit and hog sera and of purified paraoxonase towards *p*-nitrophenyl acetate, it was concluded that a new esterase hydrolysing *p*-nitrophenyl acetate, but not paraoxon, existed in sheep and probably in hog serum. The esterase was called D-esterase.

5. Evidence was presented which suggested that D-esterase was not identical with the phenyl acetate-hydrolysing arylesterase or with the C-esterase of hog kidney.

6. The effect of manganese and the decrease in the diisopropyl phosphorofluoridate-hydrolysing to paraoxon-hydrolysing rate from 1.8 in serum to 0.4 in purified paraoxonase preparations suggested the presence in sheep serum of a diisopropyl phosphorofluoridase similar to that of hog kidney, in addition to paraoxonase.

7. The paraoxonase in sheep serum was responsible for most of the tabun hydrolysis.

8. Sheep-serum and purified paraoxonase preparations hydrolysed *m*-nitrophenyl acetate more rapidly than they hydrolysed *p*-nitrophenyl acetate, and *o*-nitrophenyl acetate was hydrolysed least rapidly.

9. Purified paraoxonase did not hydrolyse tetraethyl pyrophosphate.

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Methanol Lignin from *Eucalyptus regnans* F. Muell. and its Purification by Countercurrent Distribution

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It is not possible to define lignin satisfactorily from a chemical viewpoint. Brauns (1952) considers it to be a collective term for a group of high-molecular-weight amorphous compounds rather than a constitutionally defined compound. One of the major problems of lignin chemistry is that no simple criterion of purity of a lignin preparation is available.

In view of the above, differences between lignins from different sources and obtained by different

methods of preparation are to be expected. On the other hand, agreement between results on one lignin should be attainable if all substances extraneous to the lignin group were eliminated. The first step towards completing and reconciling apparently conflicting results ought to be the preparation of the substance in question in a satisfactory state of purity. The difficulties besetting the worker in the field of lignin chemistry are such that some compromise usually has to be made. It