CARBOXYLESTERASES (EC 3.1.1). THE SOURCE OF VARIATIONS IN SUBSTRATE SPECIFICITY AND PROPERTIES OF PIG LIVER CARBOXYLESTERASE

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Summary: During the final stage of purification of pig liver carboxylesterase on CM-Sephadex, several enzyme activities are present in addition to the pig liver carboxylesterase reported from this laboratory (Horgan *et al.*, 1969a). Variation in substrate specificity and specific activity of the material isolated from CM-Sephadex has been analysed with four substrates – methyl, ethyl and phenyl butyrates and butyrycholine. The sensitivity of various fractions to paraoxon, eserine and phenylmethanesulfonyl fluoride is also examined. The results support the conclusion that the variability of pig liver carboxylesterase reported from other laboratories is due to the heterogeneity here defined.

In a recent paper, Junge and Krisch (1973) discussed the four principal electrophoretic variants obtained in their modified purification of pig liver carboxylesterase (Heymann and Butenschön, unpublished results; Heymann *et al.*, 1974). They reported that the electrophoretic variants had differing substrate specificities and argue that these results support their earlier conclusion that the enzyme is a mixture of four trimers (AAA, AAB, ABB, BBB), where the monomeric species have identical molecular weights of 55,000-60,000 (Heymann *et al.*, 1972). At the same time they noted that they had no substantial evidence for the existence of two different subunits, that hybridization experiments had failed, and as a way out of their dilemma suggested the possibility of the existence of conformational isomers. (For a critical examination of conformers, see Dudman and Zerner, 1973.)

Farb and Jencks (1974) have also recently reported on the heterogeneity of their purified pig liver carboxylesterase (7 fractions on isoelectric focusing, pI 4.9-5.6). They noted varying substrate specificity and failed to achieve hybridization of fractions of low and high pI.

Since the first large scale purification procedure originally reported in detail from this laboratory (Horgan *et al.*, 1969a,b) has been called into question as unphysiological and unreproducible (Heymann *et al.*, 1974), it is the purpose of the present communication to comment critically on the electrophoretic variants obtained in Krisch's laboratory and on the variability of several preparations which have since been reported (Barker and Jencks, 1969; Junge and Krisch, 1973; Heymann *et al.*, 1974).

Experimental Section

All chemicals used were reagent grade, and all solutions made with distilled, deionized water. Measurements of pH were made at 25° on a Radiometer pH meter 4c. Methyl, ethyl and phenyl butyrates were

redistilled before use. Acetyl- and butyrylcholine perchlorates were recrystallised before use. Stock solutions of substrates were prepared in water. Solutions of eserine (British Drug Houses, Ltd.), phenylmethanesulfonyl fluoride (Cyclo Chemical Corp., m.p. 90-91°; lit. m.p. 90-91°, Davies and Dick, 1932)[1] and paraoxon (Koch Light, purified by molecular distillation) were made up in acetonitrile (Eastman Kodak, Spectro grade).

Enzyme Purification, Assay and Inhibition

Pig liver carboxylesterase was purified according to the method of Dudman and Zerner (1975). This procedure is a modification of that of Horgan *et al.* (1969a), but both procedures yield enzyme of very high purity and reproducible characteristics (*vide infra*).

In order to comment on the variability of other preparations of this enzyme, assays of enzyme fractions obtained from the final CM-Sephadex chromatography step were performed with four substrates – methyl butyrate, 2.0×10^{-2} M; ethyl butyrate, 1.25×10^{-2} M; phenyl butyrate, 2.1×10^{-3} M; and butyrylcholine, 1.94×10^{-2} M. Assays were performed at 38°, using the pH-stat technique described previously (Horgan *et al.*, 1969a).

The sensitivity of two fractions obtained from the final CM-Sephadex chromatography step towards inhibitors was also tested. Aliquots of eserine or paraoxon solutions were added to the enzyme sample in buffer (0.05 M Tris-HCl, 0.15 M in KCl) at 25°. The final concentration of acetonitrile was 1%. The paraoxon experiments were conducted at pH 7.5 and the eserine experiments at pH 7.5 and pH 8.5 (eserine, $pK'_a = 8.1$; Wilson and Bergmann, 1950). Assays to determine the extent of inhibition were carried out at 38°.

PMSF inhibition was tested as follows. Aliquots of enzyme were added at 25° to 0.1 M NaCl (pH 6.5); an aliquot of the inhibitor was added and the pH was maintained at 6.5 by a pH-stat. Samples of the inhibited enzyme were assayed at various times (pH 7.5).

Results and Discussion

The elution profile of the enzyme from the final CM-Sephadex chromatography step is shown in Figure 1. Also shown are the specific activities of the preparation towards the four substrates. It is at once clear that the final sample (fractions 66-75) is enzyme of high and constant specific activity towards methyl, ethyl and phenyl butyrates, but of very low specific activity towards butyrylcholine. This fraction is the pig liver carboxylesterase previously described by us (Horgan *et al.*, 1969a; Dudman and Zerner, 1975). The specific activity (Horgan *et al.*, 1969a) of this preparation towards ethyl butyrate was 590 ± 10. [*cf.* pooled peak specific activity, 580 (Dudman

[1] phenylmethanesulfonyl fluoride, PMSF.

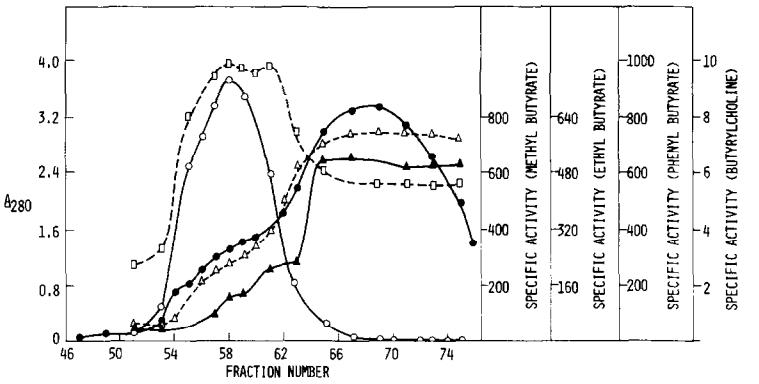

Figure 1. Chromatography of pig liver carboxylesterase on CM-Sephadex (column dimensions, 4 x 30 cm). A salt gradient (300 ml of 0.5 M ammonium sulfate and 300 ml of 0.1 M acetate buffer, pH 5 in each vessel of a linear gradient system) was commenced at fraction 1. Load, 340 mg; fraction size, 6 ml. A_{280}, ● . Specific activities: methyl butyrate, ▲ ; ethyl butyrate, △ ; phenyl butyrate, □ ; butyrylcholine, ○ .

and Zerner, 1975); pooled peak specific activity after Sephadex G-200 rechromatography, 570 (Horgan *et al.*, 1969a)].[2]

The earlier fractions (Figure 1, 52-64) contain the other enzyme species described by Junge and Krisch, as a comparison of substrate specificities and pIs is entirely consistent with their report (Junge and Krisch, 1973; their Figure 3). If our procedure, therefore, is unphysiological, the procedure of Heymann *et al.* (1974) must equally be. It is perhaps worth noting that their enzyme preparation contains only possibly 20% of the pig liver carboxylesterase described by us (very low activity towards butyrylcholine). Moreover, as Junge and Krisch (1973) have emphasized, rat liver and ox liver carboxylesterases do not measurably hydrolyse butyrylcholine. Further, the approx. two-fold lower specific activity (<0.5) towards ethyl butyrate of the enzyme prepared by Jencks's group using the procedure of Adler and Kistiakowsky (1961) is consistent with the proposition that this preparation contains a significant amount of the material which is present in the earlier fractions from our CM-Sephadex step (Barker and Jencks, 1969).

In order to comment further on the several enzyme species present, inhibition of fractions 58 and 69 was carried out. The results of these experiments are shown in Table I. Paraoxon fails to discriminate between fractions 58 and 69 after 10 min. However, contrary to the report of Junge and Krisch (1973) that the enzyme is completely inhibited by 10^{-5} M eserine in 2 min (pH and temperature not specified), our results demonstrate that while the butyrylcholine activity (fraction 58) is reduced by 97% in 10 min by 10^{-4} M eserine at pH 7.5, the true carboxylesterase activity (fraction 69) is reduced by only 12% under the same conditions. At the same time, as the

[2] A specific activity of 580 (units/ml)/A_{280} is equal to 9670 (μkat/l)/A_{280}.

TABLE I

Inhibition of Fractions from CM-Sephadex Chromatography

Fraction Number[a]	Inhibitor	$10^5 [I]_o$ (M)	$10^5 [E]_o$[b] (M)	Percent Activity Remaining[c]	Inhibition Time (min)
58	Paraoxon	10.1	0.32	0[d,e]	10
69	"	15.1	1.23	0.2[d,e]	12
58	Eserine	0.98	0.32	24[d,e]	53
69	"	0.98	0.02	95[d,f]	45
58	"	9.8	0.32	3.4[d,e]	10
69	"	9.8	0.39	88[d,f]	10
58	"	9.8	0.32	7[g]	10
69	"	9.8	0.39	74[h]	10
58	PMSF	97.8	0.011	19[j]	2.3
69	"	97.8	0.03	1.7[j]	2.3

[a]See Figure 1. [b]Calculated as if all fractions were pure pig liver carboxylesterase. [c]Expressed as a percentage of the control (uninhibited) activity. [d]Inhibition performed at pH 7.5. [e]Residual activity determined against butyrylcholine at pH 7.5. [f]Residual activity determined against ethyl butyrate at pH 7.5. [g]Inhibition performed at pH 8.5; residual activity determined against butyrylcholine at pH 8.5. [h]Inhibition performed at pH 8.5; residual activity determined against ethyl butyrate at pH 8.5. [j]Residual activity determined against phenyl butyrate at pH 7.5 and 25°.

pH is raised, eserine becomes a less effective inhibitor of the butyrylcholine activity and a more effective inhibitor of the true carboxylesterase (*cf.* Wilson and Bergmann, 1950).

PMSF again clearly discriminates between the two principal activities. In 1972, Heymann *et al.* reported that the activity of their enzyme was reduced by 10^{-5} M PMSF to ~18% after 50 min (pH 6, 30°). Again their work provided clear evidence for the heterogeneity of their preparation and the result is consistent with our finding of decreased sensitivity of the butyrylcholine activity towards PMSF. Moreover, the *resolved* butyrylcholine activity may well be less sensitive to PMSF than indicated by our experiments, because of some contamination from other esterases (phenyl butyrate as substrate).

Butyrylcholine esterase (EC 3.1.1.8) is reported to hydrolyse butyrylcholine two to three times as rapidly as acetylcholine (Nachmansohn and Rothenberg, 1944). However, this ratio for fraction 58 is >260:1 (butyryl- and acetylcholine, 2 x 10^{-2} M, pH 7.5, 38°).

Conclusion

In a recent paper (Inkerman *et al.*, 1975), we noted that the major discrepancies in the work from various laboratories could arise from varying degrees of homogeneity of the several preparations. The present work provides adequate support for that conclusion. It may also comment on the different active-site sequences reported by Heymann *et al.* (1970).

None the less, the results of this and other laboratories reveal that we are dealing here apparently with at least four closely related enzymes with respect to size, pI and active-site sequence. Further, Heymann *et al.* (1971) have reported only one N-terminal (gly) for their preparation. Whether the butyrylcholine activity is indeed a distinct enzyme or whether it is an enzyme which may be converted to pig liver carboxylesterase (*e.g.* by the removal of lipid) continues under active investigation in this laboratory.

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