Fractionation of C-Esterase from the Hog's Kidney Extract

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Evidence has been adduced for the presence of a new type of esterase in hog's kidney extract, in addition to B-esterase and an enzyme (DFPase) hydrolysing diisopropyl phosphorofluoridate (Bergmann, Segal & Rimon, 1957; Bergmann & Rimon, 1958). This so-called 'C-esterase' was characterized by the following properties: (a) It is not inhibited by diisopropyl phosphorofluoridate and related organic phosphates, in contrast with B-esterase (Aldridge, 1953). (b) It does not hydrolyse these phosphates, in contrast with A-esterase (Aldridge, 1953). (c) It is activated by concentrations of *p*-chloromercuribenzoate that fully inhibit DFPase (Bergmann *et al.* 1957).

Difficulties were, however, encountered in the complete characterization of the new enzyme because it could not be separated from the other hydrolytic enzymes present and because dialysis led to a partial loss of activity in some preparations whereas others, which had passed through exactly the same purification process, were insensitive to dialysis (Bergmann & Rimon, 1958).

Although the observations reported left little doubt about the definition of C-esterase as a new enzyme, final proof of its existence as an individual entity, and likewise clarification of its peculiar properties, awaited its separation from the other hydrolytic enzymes present in kidney extract. This has now been achieved by the use of diethylaminoethylcellulose as adsorbent.

MATERIALS AND METHODS

Enzyme preparations. Crude C-esterase was prepared as described by Bergmann *et al.* (1957), but the ethanol precipitate was dissolved in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) succinate buffer, pH 8.0, for passage through the diethylaminoethyl (DEAE)-cellulose column.

Chymotrypsin was a crystalline product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. The activity was 2000 μ moles/ml./hr./mg. of protein, when the hydrolysis of 25 mM-phenylalanine ethyl ester (Adkins & Billica, 1948) was measured by the manometric method of Park & Plaut (1953).

Enzymic activities. Activity of DFPase was measured at pH 7.4 against 5 mM-diisopropyl phosphorofluoridate (DFP) by the manometric method.

C-Esterase was determined at 28°, after incubation with 0.01 M-tetraethyl pyrophosphate for 5 min. (Bergmann & Rimon, 1958). Hydrolysis of the substrate, *mm*-*p*-nitro-

phenyl acetate, was measured at pH 7.6 by the spectrophotometric method described by Bergmann, Rimon & Segal (1958). In some experiments, 0.4 M-diacetin served as substrate, hydrolysis being measured by the manometric method.

B-Esterase activity was obtained as the difference between the total enzymic hydrolysis of *p*-nitrophenyl acetate minus the rate due to the catalytic effect of C-esterase.

For the experiments with chymotrypsin, both 0.4 mM-*p*-nitrophenyl acetate and mM-*p*-methoxyphenyl acetate served as substrates, hydrolysis being measured by the spectrophotometric method at 28°.

Buffers. For the manometric method, composition of buffer used was: 0.1 M-NaCl; 25 mM-NaHCO₃; 0.04 M-MgCl₂; 0.1% of gelatin. The gas phase consisted of air + CO₂ (95:5, v/v). The pH of all solutions was adjusted to 7.4 before the experiment, which was carried out at 37°.

For both B- and C-esterase, 5 mM-tris buffer, pH 7.6, was used, when the hydrolysis of phenyl acetates was measured.

For the experiments on pH-dependence the following buffers covered the range required: 0.1 M-sodium acetate for pH 5.0-6.5; 0.1 M-sodium phosphate for pH 6.5-8; 0.1 M-sodium pyrophosphate for pH 8-9.5; 0.1 M-sodium borate for values above pH 9.5.

Inhibitors. Sodium *p*-chloromercuribenzoate was a commercial product of Sigma Chemical Co., St Louis, Mo., U.S.A. Mercaptoethanol and β -mercaptopropionic acid were Eastman Kodak Co. products and were redistilled before use. Tetraethyl pyrophosphate was a gift of Dr A. D. Toy, Victor Chemical Works, Chicago, Ill., U.S.A.

Protein analysis. Protein was determined with Folin's reagent according to the procedure of Herriot (1942).

Column chromatography. DEAE-cellulose (Eimer and Amend, New York, N.Y., U.S.A.) was washed first with 0.1 N-NaOH and then with water until the effluent gave a neutral reaction. Subsequently the washing was continued with 0.1 N-HCl and water, and the adsorbent was finally equilibrated with tris buffer, pH 8.0, for 24 hr. The properties of this material change upon repeated use, although between two loadings the whole purification procedure was again applied. The results reported refer to the second and third runs. After the fourth run it was necessary to discard the adsorbent.

The column used had the following dimensions: length, 13 cm.; diameter, 13 mm.; wt. of adsorbent, 3.5 g.

RESULTS

Separation of C-esterase from B-esterase and DFPase. When a solution of crude C-esterase in tris buffer was passed through the DEAE column,

partial separation of the three hydrolytic enzymes was effected (Fig. 1). C-Esterase is the least adsorbable component and appears in two main fractions: (1) as a protein, which is not held at all by the column (the second peak of this enzyme is already mixed with that of B-esterase); (2) in the fraction obtained by elution with buffer + 0.02 M-NaCl. In fractions no. 23–25, only a small activity of C-esterase type was observed. The properties of these higher fractions are discussed below.

The fact that all three hydrolases in hog's kidney extract give at least two peaks may indicate that these enzymes are not homogeneous proteins.

The separation procedure was repeated many times with exactly the same results, thus providing final proof that C-esterase represents an independent enzymic entity. The high degree of purity of fraction 4, for example, is indicated by its specific activity (1.4 μ moles of *p*-nitrophenyl acetate/ml./hr./mg. of protein). For reasons which will become clear in the following, this preparation will

be called henceforth 'type I C-esterase'. Undoubtedly it still contains inactive protein as contaminant.

This purified enzyme is stable over long periods and can be freeze-dried without loss of activity. Since the colour of the crude kidney extract is retained in the upper part of the column, the freeze-dried form is almost colourless. It is now possible to prepare concentrated solutions with high hydrolytic power for use with the spectrophotometric method.

Properties of type I C-esterase. The availability of C-esterase free of other hydrolases made it possible to study in detail some of the properties that previously were not understood. Crude extracts, containing a 'dialysable cofactor' (Bergmann *et al.* 1957), always lost the latter during passage through the column (Table 1). Activation by sodium *p*-chloromercuribenzoate, the most curious feature of all crude enzyme preparations, was completely missing in type I C-esterase (Table 2). This observation suggested that on the column a component, which acted as 'native' inhibitor, had been removed. The latter probably is effective by virtue

Fig. 1. Separation of the hydrolases of hog's kidney extract by fractionation on a DEAE-cellulose column. In this experiment 16 ml. of a solution containing 31 mg. of protein/ml. in tris buffer, pH 8, and exhibiting the following enzymic activities was used: B-esterase, 0.67 μ mole of *p*-nitrophenyl acetate/ml./hr./mg. of protein; C-esterase, 0.48 μ mole of *p*-nitrophenyl acetate/ml./hr./mg. of protein; DFPase, 17.0 μ moles of diisopropyl phosphorofluoridate/ml./hr./mg. of protein. All fractions represent 10 ml. ○, B-esterase (left ordinate); ●, C-esterase (left ordinate); ▲, DFPase (right ordinate); Δ, protein (mg.) for total volume of each fraction. Graded elution: fraction nos. 1–7, tris buffer, pH 8.0; 8–12, tris buffer + 0.02 M-NaCl, pH 8.0; 13–15, tris buffer + 0.04 M-NaCl, pH 8.0; 16–20, tris buffer + 0.08 M-NaCl, pH 8.0; 21–25, tris buffer + 0.16 M-NaCl, pH 8.0; 26–32, tris buffer + 0.32 M-NaCl, pH 8.0.

Table 1. Influence of dialysis on the activity of crude C-esterase of type I and of type II

Fraction no. 24 was incubated with 0.2 mM-*p*-chloromercuribenzoate (CMB) for 10 min. at room temperature. Dialysis was carried out during 48 hr. against 5 mM-tris buffer, pH 8.0. Thereafter 0.01 M-tetraethyl pyrophosphate was added. After incubation for 5 min., activity was measured at pH 7.6, with mM-*p*-nitrophenyl acetate as substrate, by the spectrophotometric method.

	Activity (μ moles/ml./hr.)	
	Before dialysis	After dialysis
Crude C-esterase	15.0	8.5
Type I C-esterase (fraction no. 4)	6.8	6.8
Type II C-esterase after activation by CMB (fraction no. 24)	41.0	41.0

Table 2. Effect of *p*-chloromercuribenzoate on the activity of crude and type I C-esterase

The enzyme was incubated with 0.01 M-tetraethyl pyrophosphate for 5 min. and then with *p*-chloromercuribenzoate for 10 min. Activity was measured as described in Table 1.

	Rate of hydrolysis (μ moles/ml./hr.)		
	Without treatment with CMB	After incubation with	
		0.2 mM-CMB	0.02 mM-CMB
Crude C-esterase	15.0	—	42.0
Type I C-esterase	6.8	6.8	6.8

Table 3. Effect of glutathione on crude C-esterase before and after activation with p-chloromercuribenzoate

The enzyme was first incubated with 0.01 M-tetraethyl pyrophosphate for 5 min. and then with 0.08 mM p-chloromercuribenzoate (CMB) for 30 min. It was then treated with 0.03 M-glutathione for 30 min. This preparation was compared with an enzyme that had undergone the same treatment with the exception of treatment with CMB. Activity was measured against 0.4 M-diacetin by the manometric method.

	Rate of hydrolysis (μmoles/ml./hr.)
Crude C-esterase	84
Crude C-esterase + glutathione	71
Crude C-esterase + CMB	494
Crude C-esterase + CMB + glutathione	71

of an SH group, which becomes bound to the mercurial compound. Such an assumption is in accordance with previous observations on crude C-esterase that activation by p-chloromercuribenzoate can be reversed, wholly or partially, by glutathione, whereas the latter inhibits slightly (see Table 3).

Inhibitory action of other fractions from the column on type I C-esterase. It appeared possible that the native inhibitor was separated by adsorption on the column. Therefore a number of experiments was carried out to detect the inhibitor in the peak fractions of B-esterase and DFPase. However, addition of fractions nos. 12, 20 and 27, which after treatment with tetraethyl pyrophosphate had no catalytic effect on the hydrolysis of p-nitrophenyl acetate, to fraction no. 4 did not alter the effect of the latter on ester hydrolysis.

This result can be explained in two ways: either the native inhibitor was missing from all fractions examined (e.g. it may not be possible to elute the inhibitor from the column even by concentrated NaCl solutions) or it does not have an effect on pure type I C-esterase, in contrast with its influence on the crude enzyme.

Effect of sulphydryl compounds on type I C-esterase. In view of the above-mentioned results, the effect of sulphydryl compounds on the purified type I C-esterase was studied. Both mercaptoethanol and β-mercaptopropionic acid were entirely inactive. The properties of this enzyme were thus fundamentally different from those of crude C-esterase, which is strongly inhibited by both sulphydryl derivatives (Fig. 2).

Properties of C-esterase from higher fractions. Different results, however, were obtained with the C-esterase present in fractions nos. 23–25. Here the above-mentioned two mercapto derivatives were strongly inhibitory (Table 4). In addition, p-chloromercuribenzoate produced activation of

Fig. 2. pI-activity curve for the system crude C-esterase-sulphydryl compounds. Crude C-esterase was incubated at room temperature with varying concentrations of mercaptoethanol (●) or β-mercaptopropionic acid (○) for 15 min. Activity was measured against 0.4 M-diacetin by the manometric method. pI = - log concentration of inhibitor.

Table 4. Inhibition of the two types of free C-esterase by sulphydryl compounds

Fraction no. 24 was incubated for 5 min. with 0.01 M-tetraethyl pyrophosphate. Inhibition with mM concentrations of the mercapto derivatives was carried out for 10 min. and activity determined as described in Table 1.

Inhibitor added	Rate of hydrolysis (μmoles/ml./hr.) by C-esterase	
	Type I (fraction no. 4)	Type II (fraction no. 24)
None	6.8	1.3
Mercaptoethanol	6.8	0.7
β-Mercaptopropionic acid	6.8	0.65

Table 5. Unmasking of 'hidden' C-esterase by p-chloromercuribenzoate

B-Esterase activity was eliminated by treatment with 0.01 M-tetraethyl pyrophosphate for 5 min. p-Chloromercuribenzoate (CMB; 0.2 mM) was added to each fraction and the mixture was incubated at room temperature for 10 min. Thereafter activity was measured as described in Table 1. As control, the fractions untreated with CMB were used.

Fraction no.	C-Esterase activity (μmoles/ml./hr.)	
	Before activation with CMB	After activation with CMB
21	0	0
22	0	2.7
23	1	41.0
24	1.3	41.0
25	0.4	11.4
27	0	4.5
28	0	12.0
29	0	2.8
30	0	1.0
31	0	0

a much higher degree than that observed with the crude enzyme. This aroused the suspicion that these fractions contained, in addition to the small quantity of free C-esterase, a much larger proportion of hidden enzyme, i.e. enzyme bound to a native inhibitor. This assumption was confirmed by a systematic study of activation by *p*-chloromercuribenzoate, which revealed that, even in fractions completely devoid of C-esterase activity, the latter could be unmasked by treatment with the organic mercurial compound (Table 5). The enzyme in fractions nos. 22–30, which we designate as 'type II C-esterase', appears again concentrated in two peaks, like all other hydrolases in the kidney extract used.

These experiments led to the conclusion that most or all of type II C-esterase in the higher fractions was bound to a native inhibitor, the latter acting by virtue of an SH group. A search for this inhibitor in the free state was carried out among the higher fractions devoid of free C-esterase. However, fractions nos. 26–30 had no inhibitory effect on the free C-esterase in fractions nos. 23–25. Thus, in those fractions which contain neither free inhibitor nor free type II C-esterase, a stoichiometric enzyme-inhibitor complex must be present.

Non-identity of the native inhibitor with DFPase. The above-mentioned results support the assumption of an SH group as the essential part of the native inhibitor. It was shown by Mazur (1946) that DFPase possesses also a sulphhydryl group. This fact, combined with the observation that hidden C-esterase is present only in fractions exhibiting great DFPase activity, suggested that the latter enzyme may actually represent the native inhibitor of type II C-esterase. Since there is no free, i.e. excess of, inhibitor present in fractions nos. 22–30, the stoichiometric complex C-esterase-native inhibitor would have to be identical with a complex C-esterase-DFPase, which includes all of the DFPase available. This means that DFPase, when entering the complex, retains its enzymic activity whereas C-esterase does not. Such a possibility cannot be excluded. However, the following considerations contradict the assumption that DFPase represents the native inhibitor.

If DFPase participates as one component in the stoichiometric enzyme-inhibitor complex, then the ratio

$$\frac{\text{C-esterase recoverable by } p\text{-chloromercuribenzoate}}{\text{original DFPase}}$$

should be constant, since there is no excess of inhibitor available in any of the fractions tested. However, the data in Table 6 give evidence to the contrary. Therefore the native inhibitor is a separate, as yet unidentified, entity.

Further characterization of type II C-esterase. The fact that the hidden C-esterase appears only in higher fractions could be due to either of two factors: (i) this enzyme may be structurally similar to type I C-esterase, but by combination with the native inhibitor it is altered in such a way as to exhibit increased adsorbability on DEAE-cellulose and to require a high concentration of NaCl for elution; (ii) the structure of type II C-esterase itself may be essentially different. A decision between these two alternatives can be reached by rechromatography of the enzyme activated by *p*-chloromercuribenzoate on a DEAE-cellulose column. Pertinent experiments showed that the hidden enzyme, after its liberation from the native inhibitor, again is firmly adsorbed and requires 0.16 M NaCl for its elution, like the original enzyme-inhibitor complex. Therefore the behaviour of type II C-esterase is characteristic for the enzyme

Table 6. Evidence for the non-identity of DFPase with the 'native inhibitor' of C-esterase

Fraction no.	Hidden C-esterase
	DFPase
22	0.22
23	0.35
24	0.74
25	0.54
27	0.59

Fig. 3. pH-activity curves of chymotrypsin. For the hydrolysis of 0.4 mM *p*-nitrophenylacetate (○, left ordinate) 1 mg. of crystalline chymotrypsin/ml., and for *p*-methoxyphenyl acetate (●, right ordinate), 0.5 mg. of crystalline chymotrypsin/ml., was used.

itself and independent of the presence or absence of the native inhibitor.

Finally, type II C-esterase, after activation by *p*-chloromercuribenzoate, does not suffer any loss of activity during dialysis, a property shared with the type I enzyme, but contrasting with the behaviour of the crude enzyme used in the present investigation (Table 1).

Effect of pH on the hydrolytic activities of either type of C-esterase. In view of the isolation of two different types of C-esterase from hog's kidney extract, it was of interest to determine their pH-activity curves. Both forms of the enzyme revealed again a curious property, observed previously with the crude C-esterase (Bergmann & Rimón, 1958). The pH-activity curve for *p*-methoxyphenyl acetate is bell-shaped, whereas the hydrolysis of *p*-nitrophenyl acetate accelerates steadily with increasing pH. This behaviour is not unique for C-esterases. We have found that chymotrypsin exhibits similar pH-activity curves for the above-mentioned two substrates (Fig. 3). The effect of pH on various hydrolytic reactions catalysed by C-esterases apparently is independent of the degree of purity of the enzyme or the presence or absence of a mixture of different types.

DISCUSSION

The present experiments establish beyond doubt the existence of C-esterase as a separate enzyme. In addition they reveal that in hog's kidney extract two different types of this esterase are present, the properties of which are summarized in Table 7.

These observations provide a rational explanation for the varying degrees of activation of crude C-esterase by *p*-chloromercuribenzoate (between 50 and 800 %). The crude preparation contains not only a mixture of type I and II esterase in various proportions, but the latter may be masked to different extents by bonding to the native inhibitor.

Since the discovery of C-esterase, many unsuccessful attempts were made to purify this enzyme, i.e. to separate it from the accompanying hydrolases. Neither the use of various salts nor the use of

organic solvents effected a separation, although the ratio between DFPase, B-esterase and C-esterase differed from one fraction to the other. It is now apparent that the crude C-esterase contains at least four components, differing in their structure, as indicated by the double peak for type I (Fig. 1) and type II (Table 5) C-esterase. Only the much more delicate selectivity of DEAE-cellulose makes this separation possible. However, there is no evidence that the peak fractions isolated in the present investigation represent already homogeneous enzyme proteins.

Table 7 and likewise the pH-activity curves demonstrate a close similarity between the two types of C-esterase. This raises the question whether type I may be derived from type II by partial degradation.

Inhibition by sulphydryl compounds is a common phenomenon among enzymes bearing a metal atom in the active centre. However, no information on this point will be available for type II C-esterase until this enzyme can be obtained free of the other enzymes and of its native inhibitor. In this connexion it should be recalled that, in a large percentage of our crude enzyme preparations, C-esterase lost part (40–50 %) of its activity during dialysis. However, this was no longer the case with the enzymes recovered from the DEAE-cellulose column (type II being dialysed after activation by *p*-chloromercuribenzoate). Therefore inhibition by sulphydryl compounds is in no way connected with the presence of the dialysable component of C-esterase.

The similarity of the pH-activity curves of C-esterase and chymotrypsin, when phenyl acetates serve as substrates, indicates that the hydrolytic mechanisms of these enzymes cannot be fundamentally different. In view of the evidence for the presence of imidazol in the active centre of chymotrypsin (Dixon & Neurath, 1957), it may be assumed that this group also represents the essential component in the active surface of C-esterases.

Thus there exist two groups of esterases in animal tissues, both of which make use of imidazol for their catalytic mechanism: one group that requires, in addition, serine and therefore is

Table 7. *Survey of the characteristics of the two types of C-esterase*

	Type I C-esterase	Type II C-esterase
Inhibition by organophosphorus compounds	Nil	Nil
Hydrolysis of organophosphorus compounds	Nil	Nil
Inhibition by sulphydryl compounds	Nil	Strong
Adsorption on DEAE-cellulose at pH 8.0	Nil	Strong
Dialysis	No effect	No effect
Native appearance	Free	Predominantly in combination with native inhibitor

sensitive to phosphate inhibition (B-esterases); a second group which supposedly works without serine (A- and C-esterases), although no positive evidence is available on this point.

SUMMARY

1. C-Esterase was separated from other hydrolytic enzymes in hog's kidney extract by fractionation on a diethylaminoethylcellulose column. The non-adsorbable protein contained type I C-esterase, free of B-esterase and an enzyme (DFPase) hydrolysing diisopropyl phosphorofluoridate.

2. In addition, most of the fractions with DFPase activity contained a second type of C-esterase, predominantly in hidden form, i.e. linked to a native inhibitor.

3. Type II C-esterase is liberated from this inhibitor by treatment with *p*-chloromercuribenzoate. It differs from type I C-esterase by its sensitivity towards sulphhydryl inhibitors and by its stronger adsorbability on diethylaminoethylcellulose.

4. Both C-esterases show an abnormal pH-activity curve towards *p*-nitrophenyl acetate. With increasing pH the hydrolytic rate rises steadily. This property is also shared by chymotrypsin.

5. The properties of the two types of purified C-esterase can explain most of the observations made on crude enzyme mixtures.

REFERENCES

- Adkins, H. & Billica, H. R. (1948). *J. Amer. chem. Soc.* **70**, 3121.
 Aldridge, W. N. (1953). *Biochem. J.* **53**, 110.
 Bergmann, F. & Rimon, S. (1958). *Biochem. J.* **70**, 339.
 Bergmann, F., Rimon, S. & Segal, R. (1958). *Biochem. J.* **68**, 493.
 Bergmann, F., Segal, R. & Rimon, S. (1957). *Biochem. J.* **67**, 481.
 Dixon, G. H. & Neurath, H. (1957). *J. Amer. chem. Soc.* **79**, 4558.
 Herriot, R. M. (1942). *Proc. Soc. exp. Biol., N.Y.*, **46**, 642.
 Mazur, H. (1946). *J. biol. Chem.* **164**, 271.
 Park, R. E., jun. & Plaut, G. W. E. (1953). *J. biol. Chem.* **203**, 755.

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The Partial Purification and Properties of Thyroxine Dehalogenase

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In most mammals, dehalogenation is one of the most important metabolic transformations of L-thyroxine, 3:5:3'-tri-iodo-L-thyronine and related substances (Pitt-Rivers & Tata, 1959). The physiological significance of dehalogenation of thyroid hormones remains doubtful although numerous reports have appeared in recent years on the deiodination of thyroid hormones by incubation with slices, homogenates or extracts of various tissues (Albright, Larson & Tust, 1954; Larson, Tomita & Albright, 1955; Sprott & MacLagan, 1955; Béraud & Vannotti, 1956; Tata, Rall & Rawson, 1957; Tata, 1957, 1958*b*; MacLagan & Reid, 1957; Plaskett, 1958; Albright & Larson, 1959; Yamazaki & Slingerland, 1959; Etling & Barker, 1959*a*; Lissitzky, Bénévent, Roques & Roche, 1959). Unfortunately there is little agreement on the nature and products of enzymic deiodination. The major causes of this confusion are: (a) the predominant interest of some investigators in demon-

strating partial deiodination of thyroxine to tri-iodothyronine, (b) variability in techniques of detection and measurement of deiodination, (c) failure to realize the influence of tissue source and method of enzyme preparation used and (d) neglect of non-enzymic processes which can occur simultaneously (Lissitzky, Michel, Roche & Roques, 1956; Tata, 1959*a*; Etling & Barker, 1959*b*; Lein & Michel, 1959).

A series of preliminary investigations were undertaken by the author to overcome the above objections before studying the enzyme system(s) involved in deiodination of the thyroid hormones. These investigations have shown that direct chromatographic analysis with multiple solvent systems is the most reliable method for measurement of deiodination (Tata *et al.* 1957; Tata, 1957, 1958*b*); direct chromatography has also been used by others (Lissitzky, Bénévent, Roques & Roche, 1959; Yamazaki & Slingerland, 1959; Etling & Barker, 1959*a*). It is, also, now possible to distinguish between true enzymic dehalogenation and one

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