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A New Type of Esterase in Hog-Kidney Extract

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Among mammalian esterases, two different types have been distinguished in recent years: (1) esterases which are inhibited by certain organophosphorus compounds [the B-esterases of Aldridge (1953*a*)]; (2) hydrolytic enzymes which use the above inhibitors as substrates (the so-called A-esterases; Aldridge, 1953*a*). Activity of the first group of enzymes is based on the presence in the active centre of an imidazole group (Wilson & Bergmann, 1950; Bergmann, Segal, Shimoni & Wurzel, 1956) and probably serine (Schaffer, May & Summerson, 1953, 1954; Oosterbaan, Kunst & Cohen, 1955). The A-esterases, on the other hand, are believed to contain a sulphydryl group since they are sensitive to heavy metals, notably mercuric derivatives, to nickel and copper salts and are also inhibited to a certain degree by iodoacetate (Aldridge, 1953*b*). However, the pH–activity curves for the hydrolysis of diethyl *p*-nitrophenyl phosphate by A-esterase from rabbit's serum (Aldridge, 1953*b*) and of di*iso*propyl phosphorofluoridate (DFP) by the enzyme (DFPase) of hog kidney (Mounter, 1956) that hydrolyses DFP and other dialkyl phosphorofluoridates show a maximum at pH 7·6, similar to cholinesterases and aliesterases (Bergmann *et al.* 1956). These facts suggest that the active centres of A-esterases contain an imidazole ring as well as a sulphydryl group. The activity of both groups of esterases is thus based on the same fundamental principle, namely the presence of an imidazole ring, but is modified by the inclusion of either an alcoholic hydroxyl or a sulphydryl group.

It appeared of interest to test the A-esterases against carboxylic esters. Aldridge (1953*b*) has already reported that the enzyme from rabbit's serum acts on a special group, namely *p*-nitrophenyl esters. We have investigated systematically kidney DFPase against carboxylic esters as substrates, and observed that a new type of esterase is present in addition to the A-esterase. Since, according to Aldridge's nomenclature, A-esterases are not inhibited by organophosphorus compounds and do in fact hydrolyse them, and B-esterases are inhibited

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by very low concentrations of organophosphorus compounds, the new enzyme is termed 'C-esterase', since it is not inhibited by organophosphorus compounds and does not hydrolyse them.

MATERIALS AND METHODS

Preparation of enzyme. DFPase from fresh pig's kidney was purified according to Mounter, Floyd & Chanutin (1953). However, owing to the lack of a Spinco centrifuge the precipitates obtained at various stages of the procedure were separated in a Servall centrifuge at about 10 000 rev./min. This may be the reason why the fraction precipitated by 10% ethanol contained only about 10% of the total enzymic activity, whereas with between 10 and 30% ethanol about 40% of the DFPase separated from the solution. Mounter *et al.* (1953) recovered 90% of the total activity in their fraction *A*-1, obtained by addition of 10% ethanol. In our experiments it was found expedient to collect at once the precipitate between 0 and 30% ethanol by centrifuging. Extraction with 0·025 M-$NaHCO_3$ gave a dark-brown solution which, after centrifuging, served as enzyme source in all our experiments. The yield was 40–50% of the DFPase content of the original homogenate. The standard solution contained 20 mg. of protein/ml., as determined by precipitation with an equal volume of 20% (w/v) trichloroacetic acid. When diluted 1:60, the enzyme produced 17 ± 1 μmoles of CO_2/ml./hr. with 5×10^{-3} M-DFP as substrate.

For the determination of C-esterase, the enzyme was stored in a refrigerator for 12 hr. with 10^{-2} M-DFP, dissolved in the buffer described below. Activity was then measured against 0·4 M-diacetin. Our 'DFPase' preparation, in a final dilution of 1:20, produced $6·5 \pm 0·5$ μmoles of CO_2/ml./hr.

Hydrolysis was measured at 37° by a manometric method, a buffer (Warburg) of the following composition being used: 0·1 M-NaCl, 0·04 M-$MgCl_2$, 0·026 M-$NaHCO_3$ and 0·1% gelatin. The gas phase consisted of air–CO_2 (95:5). The pH of all solutions was adjusted to 7·4 before the experiment.

Substrates. Diacetin, a gift of British Industrial Solvents Ltd., was stored in a desiccator over NaOH. *p*-Nitrophenyl acetate, m.p. 81°, was shaken with buffer for 10 min. and the undissolved ester filtered off. The saturated solution contained approx. 1·7 μmoles/ml., as determined by the hydroxamic acid method (Hestrin, 1949).

Inhibitors. Sodium *p*-chloromercuribenzoate was a commercial product of Sigma Chemical Co., St Louis, Mo., U.S.A.

Treatment with inhibitors (and activators) was carried out overnight in the cold unless stated otherwise. When a precipitate appeared during storage the mixture was centrifuged and activity determined with the supernatant only. In experiments with phenylmercuric acetate it was necessary to remove all Cl^- ion from the enzyme preparation before incubation, in order to prevent precipitation of the sparingly soluble phenylmercuric chloride. For such experiments the enzyme was dialysed in the cold against distilled water for 1 hr.

The I_{50} values, i.e. the concentrations of inhibitors producing 50% inhibition, were obtained graphically by plotting on semilogarithmic paper the percentage inhibition as a function of inhibitor concentration. The latter indicates final concentration whenever a reversible inhibitor was used. For irreversible inhibitors the figures refer to their concentrations in the incubation mixture with the enzyme.

RESULTS

Activity of hog-kidney extract against diisopropyl phosphorofluoridate and diacetin

In Table 1 (*a*) the activities of a tissue homogenate and the purified enzyme towards DFP and diacetin are compared both before and after treatment with DFP. It is apparent that in the homogenate the major part of the hydrolytic activity towards diacetin can be abolished by DFP whereas, after purification, 60–90% of the enzyme acting on this ester survives this treatment. Since the method of Mounter *et al.* (1953) was intended to concentrate DFPase, it seemed possible that the DFP-resistant activity towards diacetin was in fact due to DFPase itself. However, tests carried out with the dialysed enzyme [Table 1 (*b*)] showed that the DFPase lost a considerable part of its activity, in confirmation of the findings of Mounter (1956), whereas the rate of hydrolysis of diacetin remained unaltered.

These observations could be interpreted in two ways. Either the DFPase requires a cofactor for DFP hydrolysis, which is dispensable for the diacetin, or else two different enzymes are involved. Other means were therefore sought to differentiate, if possible, between these two alternatives.

Heat sensitivity of the enzymes in hog-kidney extract

The effect on kidney DFPase of short heating at 45–55° has been studied by Mounter & Dien (1956). Fig. 1 shows the heat inactivation at 37° and 53°

Table 1. *Comparison of hydrolytic activities of hog-kidney homogenate and purified enzyme*

Enzyme solutions were stored in the cold room for 24 hr. with 10^{-2} M-DFP. Dialysis was performed against Warburg buffer at +5° for 24 hr. The figures indicate μmoles of CO_2 liberated/hr./ml. of undiluted enzyme solutions.

(*a*) Before and after treatment with DFP

	Kidney homogenate		Purified enzyme	
Substrate	Untreated	After treatment with DFP	Untreated	After treatment with DFP
DFP	41·5	41·5	1060	1040
Diacetin	162·5	~1·5	185	125

(*b*) Before and after dialysis

	Purified enzyme	
Substrate	Untreated	Dialysed
DFP	1060	570
Diacetin	185	180

for the hydrolytic activities towards DFP and diacetin. In every case heat inactivation proceeded rapidly at the beginning but slowed down for longer heating times, so that the residual activity approached a more or less constant level. Nevertheless the amount of precipitate increased steadily. It is evident that the reaction with DFP is more sensitive than that with diacetin. It was therefore concluded that the kidney extract, prepared according to Mounter (1956), contains a second hydrolytic enzyme different from DFPase which henceforth will be called 'C-esterase'.

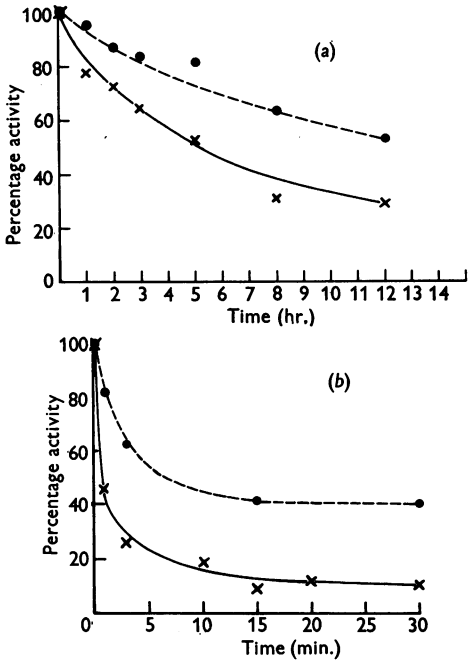

Fig. 1. Heat inactivation of kidney DFPase and C-esterase. Purified enzyme was first treated with DFP at +5° for 12 hr. and then placed in a thermostat at 37° or 53°. After various periods, samples were transferred rapidly to an ice bath and centrifuged. Only the supernatant was used for measurement of enzymic activity. Substrates: for DFPase, 5×10^{-3}M-DFP; for C-esterase, 0·4M-diacetin. Temp.: (*a*) 37°; (*b*) 53°. ×, DFPase; ●, C-esterase.

Table 2. *Effect of* Mn^{2+} *ions and histidine on DFPase and C-esterase*

The enzyme was first treated with DFP, then dialysed against distilled water and incubated with 10^{-3}M-$MnCl_2$ and 10^{-3}M-histidine for 30 min. at 37°. The figures indicate μmoles of CO_2/ml./hr.

Substrate	Enzyme alone	Enzyme + Mn^{2+} ion + histidine
DFP, 5×10^{-3}M	7·0	20·0
Diacetin, 0·4M	5·1	4·95

Activation by manganese

Manganous ions, especially in combination with heterocyclic compounds such as histidine, potentiate the activity of DFPase (Mounter *et al.* 1953). No effect on C-esterase could be detected (Table 2).

Effect of heavy metals on C-esterase

Additional support for the non-identity of DFPase and C-esterase was gained from inhibitor studies. Mercuric ions inhibit both enzymes within the same concentration range, but show a narrow zone of activation for DFPase only, in confirmation of the observations of Mazur (1946), as shown in Fig. 2. On the other hand, Cu^{2+} ion was about ten times as effective against DFPase (Table 3).

More striking differences were revealed by the organic mercurials. Phenylmercuric acetate (Fig. 3) and still more *p*-chloromercuribenzoate (Fig. 4) produce activation of C-esterase in a concentration range which is strongly inhibitory to DFPase. At higher concentrations the activating effect is progressively reduced and finally changes into inhibition. However, in the zone of decreasing activation cloudiness appeared, and with phenylmercuric acetate the protein gave increasing amounts of precipitate above $7{\cdot}5 \times 10^{-4}$M. It is thus possible that the decrease of C-esterase activity beyond the optimum activation range is not a true inhibitory phenomenon but represents inactivation due to protein denaturation.

Glutathione at high concentrations ($5–8 \times 10^{-2}$M) inhibited both enzymes, and this effect was completely reversible by dialysis. However, this compound also partially restored DFPase inhibited by *p*-chloromercuribenzoate and decreased the activating effect of this mercurial on C-esterase. Mounter *et al.* (1953) found 10^{-3}M-glutathione to activate their DFPase preparation, an effect shared by other sulphydryl compounds.

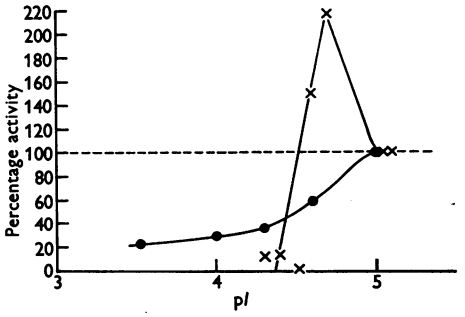

Fig. 2. Effect of Hg^{2+} ions on DFPase (×) and C-esterase (●). The horizontal, broken line at 100% represents the level of enzymic activity in the absence of Hg^{2+} ions. All points above this line indicate enzyme activation and all points below it, inhibition. $pI = -\log$ inhibitor concn. (M).

Table 3. *Effect of inhibitors on DFPase and C-esterase*

Enzyme, pretreated with DFP, was incubated with the inhibitor at +5° for 12 hr. Activity was tested against 5×10^{-3} M-DFP and 0·4 M-diacetin.

Inhibitor	I_{50} (M) for DFPase	I_{50} (M) for C-esterase	Remarks
Copper sulphate	$3{\cdot}5 \times 10^{-5}$	$1{\cdot}1 \times 10^{-4}$	Cloudiness first observable at 3×10^{-4} M
Mercuric acetate	$2{\cdot}7 \times 10^{-5}$	$3{\cdot}2 \times 10^{-4}$	Cloudiness at 10^{-4} M; first precipitate at 5×10^{-3} M
Phenylmercuric acetate	$2{\cdot}6 \times 10^{-4}$	10^{-3}	Cloudiness at 5×10^{-4} M; precipitate at $7{\cdot}5 \times 10^{-4}$ M
p-Chloromercuribenzoate	$6{\cdot}6 \times 10^{-6}$	$1{\cdot}5 \times 10^{-3}$	Cloudiness at 10^{-3} M
Glutathione	4×10^{-2}	8×10^{-2}	—
Urea	No effect until 8 M	4·5	—

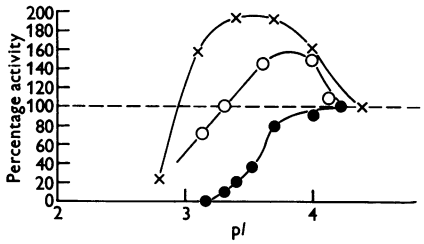

Fig. 3. Effect of phenylmercuric ion on DFPase and C-esterase. ×, C-esterase, as obtained in preparation VI only (not dialysed); ○, C-esterase, as obtained in preparations I–V (preparation VI, after dialysis, also follows this curve); ●, DFPase. The horizontal broken line indicates the level of standard activity for both enzymes.

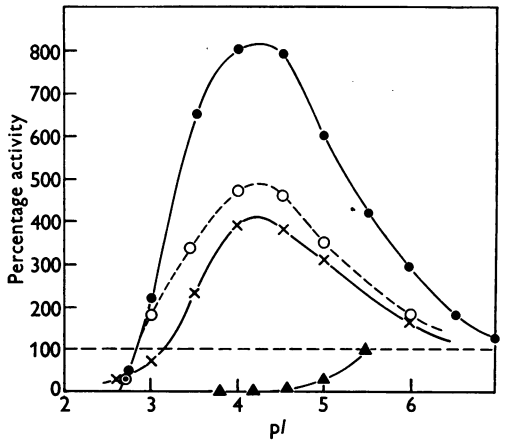

Fig. 4. Effect of *p*-chloromercuribenzoate on DFPase and C-esterase. The horizontal line indicates the standard level of activity for both enzymes. ●, C-esterase (enzyme preparation no. VI), undialysed; ○, same, after dialysis; ×, C-esterase (enzyme preparations I–V), after dialysis; ▲, DFPase.

Table 4. *Reversibility on dialysis of the activation of C-esterase by* p-*chloromercuribenzoate*

Enzyme, pretreated with DFP, was incubated at 5° with 2×10^{-5} M-chloromercuribenzoate. Part of the incubation mixture was dialysed in the cold against Warburg buffer for 72 hr. The control was an enzyme sample pretreated with DFP and then dialysed. Activity was tested against 0·4 M-diacetin.

	Activity (μmoles of CO_2/ml./hr.)
Enzyme control	6
Enzyme + chloromercuribenzoate	12·0
Enzyme + chloromercuribenzoate, after dialysis	9·2

Table 5. *Influence of histidine and glycine on the inhibition of C-esterase by* Cu^{2+} *ion*

Enzyme, pretreated with DFP, was incubated with $2{\cdot}5 \times 10^{-4}$ M-$CuSO_4$ and 10^{-3} M-histidine or glycine, at +5° under the conditions specified below. Activity was tested against 0·4 M-diacetin.

Experimental conditions	Activity (μmoles of CO_2/ml./hr.)
Enzyme alone	6·5
Enzyme + histidine	6·6
Enzyme + Cu^{2+} ion	3·8
Enzyme + Cu^{2+} ion; after 12 hr. incubation, histidine added	4·8
Enzyme, incubated for 12 hr. with mixture of Cu^{2+} ion and histidine	6·8
Enzyme + Cu^{2+} ion; after 12 hr. incubation, glycine added	3·8
Enzyme, incubated for 12 hr. with mixture of Cu^{2+} ion and glycine	4·1

High concentrations of urea were found to inhibit C-esterase, but had no effect on DFPase.

The inhibitory action of copper and mercury salts and of high concentrations of organic mercurials on C-esterase may lead to the assumption that this

enzyme, like DFPase, contains a sulphydryl group responsible for combination with heavy metals. However, additional experimental findings do not support this. First, the complex formed between C-esterase and 5×10^{-5} M-*p*-chloromercuribenzoate can be slowly dissociated by prolonged dialysis (Table 4). Furthermore, when C-esterase was incubated with Cu^{2+} ion and then treated with histidine, the latter only slightly modified the inhibition. If, however, enzyme was added to a mixture of cupric salt and histidine, subsequent incubation for 12 hr. did not produce any inhibition of C-esterase (Table 5). This effect is specific for histidine and is absent with other amino acids, e.g. glycine. The ability of the imidazole ring to form metal complexes is well known (Edsall, Felsenfeld, Goodman & Gurd, 1954; Li, White & Doody, 1954). The protective effect of histidine can thus be explained. However, if the C-esterase combined with the Cu^{2+} ion through a sulphur–metal bond, the enzyme should be able to abstract, during prolonged incubation, the metal from the histidine complex in solution.

A possible cofactor requirement for C-esterase

In five out of six kidney extracts the activity of C-esterase was not altered by dialysis. However, in one extract a very active preparation was obtained with the following characteristics: protein content, 45 mg./ml.; DFPase activity, 715 μmoles of CO_2/ml./hr.; C-esterase activity (after treatment with DFP), 320 μmoles of CO_2/ml./hr.

Dialysis against Warburg buffer reduced the C-esterase activity by about 50%. No method was found to restore the original activity. Organic mercurials activated this undialysed enzyme about twice as much as the other preparations (see Figs. 3 and 4). After dialysis of this particular preparation the activating effect of the mercurials became identical with that in the other cases. It is possible that these latter preparations had lost a cofactor in the purification procedure. Accordingly the enzyme containing the cofactor showed the same substrate specificity as all other preparations.

Substrate specificity of C-esterase

The p*S*–activity curves for a variety of carboxylic esters are shown in Fig. 5. *n*-Propyl chloroacetate has a higher affinity for C-esterase than *n*-propyl acetate. On the other hand, ethyl butyrate (not shown) was hardly attacked at all. Mono-, di- and tri-acetin in equivalent concentrations reacted at practically the same rate. *p*-Nitrophenyl acetate was attacked at about the same rate before and after inhibition by DFP. Special interest attaches to the question whether kidney DFPase attacks this substrate, as claimed for rabbit's serum by Aldridge (1953*b*) and Mounter (1954). This could be decided by using phenylmercuric acetate at a concentration which inhibits DFPase almost completely but is without influence on C-esterase. The results in Table 6 clearly demonstrate that all activity towards the *p*-nitrophenyl ester was due to C-esterase.

DISCUSSION

The new enzyme, which we have found in hog-kidney extract, differs from the known two types of esterases, the A- and B-esterases, in the following respects: (1) C-esterase is not inhibited by DFP. (2) C-esterase does not hydrolyse DFP, but attacks solely carboxylic esters. (3) C-esterase is activated by certain organic mercurials at concentrations in which no denaturating effect on the enzyme protein is visible. The activation can be abolished by prolonged dialysis. (4) C-esterase is inhibited by heavy metals such as mercury or copper. Metal-complexing agents such as histidine compete with the enzyme for the inhibitor and thus may protect

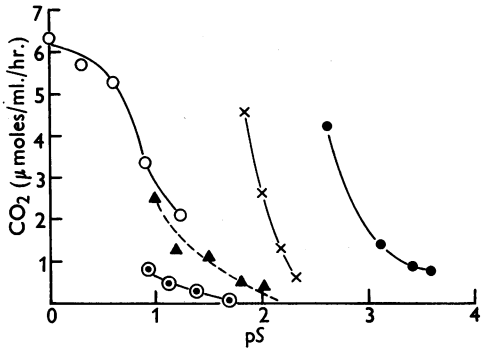

Fig. 5. p*S*–activity curves for C-esterase. p*S* = −log substrate concn.; ●, *p*-Nitrophenyl acetate; ×, *n*-propyl chloroacetate; ○, diacetin; ▲, *iso*propyl fluoroacetate; ⊙, *n*-propyl acetate.

Table 6. *Evidence for the exclusive hydrolysis of* p*-nitrophenyl acetate by C-esterase*

Enzyme, pretreated with DFP, was dialysed against distilled water for 1 hr. and a sample was incubated at +5° with 4×10^{-4} M-phenylmercuric acetate for 12 hr. The original enzyme, before and after treatment with DFP, served as controls. All enzyme samples were tested against both DFP and *p*-nitrophenyl acetate as substrates.

Experimental conditions	Activity (μmoles of CO_2/ml./hr.) with *p*-Nitrophenyl acetate ($1{\cdot}2 \times 10^{-3}$ M)	Activity (μmoles of CO_2/ml./hr.) with DFP (5×10^{-3} M)
Original enzyme	8·4	13·5
Enzyme + DFP	8·4	13·5
Enzyme + DFP + phenylmercuric acetate	8·3	Nil

C-esterase against metal inhibition. (5) C-esterase is not activated by Mn^{2+} ions, with or without heterocyclic compounds.

In addition, C-esterase may contain cofactors which are removable by dialysis. Lack of the cofactor(s) diminishes enzymic activity but does not abolish it or change the substrate specificity. These features are sufficient to differentiate C-esterase from the DFPase also present in kidney extract.

The only carboxylic esters studied previously as substrates of A-esterase are the derivatives of *p*-nitrophenol. Mounter (1954) has produced evidence that hydrolysis of DFP and *p*-nitrophenyl acetate is catalyzed by the same A-esterase of rabbit's serum. He observed identical heat inactivation and identical inhibition by heavy metals for both substrates. Mounter's observations are thus fundamentally different from our own results on hog-kidney extract, since we have excluded any participation of kidney DFPase in the hydrolysis of *p*-nitrophenyl acetate (see Table 6).

From the information available so far it is difficult to draw definite conclusions as to the structure of the active surface of C-esterase and the hydrolytic mechanism. The behaviour towards a variety of inhibitors suggests that the enzyme forms rather stable metal complexes, which can, however, dissociate if another complexing agent competes with the active surface (Table 5) or if dissociation of the enzyme–metal combination is promoted by continuous removal of the metal by dialysis (Table 4). It has been shown by Tanford (1952) that metal binding by proteins such as serum albumin takes place preferentially through the imidazole ring of histidine. It is therefore conceivable that the active centre of C-esterase may include histidine as a specific component. The assumption that an imidazole group participates in the active surface does not, however, explain why some metal derivatives produce such pronounced activation of C-esterase. If the few tests carried out permit generalizations, the impression is gained that univalent ions of heavy metals in the form of organic derivatives form activated complexes, whereas combination with bivalent-metal ions produces inhibition.

These conceptions are also in agreement with present knowledge about the hydrolytic activity of imidazole. Among all substrates which we have tested against C-esterase, *p*-nitrophenyl acetate shows the highest affinity (see Fig. 5). Bender & Turnquest (1957) found in their experiments on imidazole-catalysed hydrolysis that this same ester is split at the highest rate.

At present, the assumption that imidazole represents an essential part of the active surface of C-esterase can only be considered as a working hypothesis. A more thorough attack on this problem will become possible when the enzyme has been separated from DFPase and purified sufficiently to determine other properties, e.g. its pH–activity curve.

SUMMARY

1. Hog-kidney extract contains, in addition to the known enzyme (DFPase) that hydrolyses di*iso*propyl and other dialkyl phosphorofluoridates, an enzyme that hydrolyses carboxylic esters and that has been called 'C-esterase'.

2. C-esterase does not use di*iso*propyl phosphorofluoridate as either substrate or inhibitor.

3. C-esterase is inhibited by certain heavy-metal ions and is activated strongly by certain organic mercurials. Manganese ions, on the other hand, have no effect.

4. The relationship of C-esterase to A- and B-esterases is discussed. It appears possible that C-esterase too contains an imidazole ring in the active surface, similar to the other esterases of animal origin.

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