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The Effect of pH Variations on the Activities of C-Esterase*

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In a recent paper, the occurrence in hog's kidney of a new type of esterase, called C-esterase, was demonstrated, which was characterized by the following distinctive properties (Bergmann, Segal & Rimon, 1957). (1) C-Esterase does not hydrolyse diisopropyl phosphorofluoridate. In this respect it differs from the A-esterases. (2) C-Esterase is not inhibited by DFP, a property which distinguishes the new enzyme from the B-esterases. (3) C-Esterase is strongly activated by aromatic mercurials.

The characteristics of the new enzyme raise the problem of the underlying hydrolytic mechanism. For the A- and B-esterases information about the active surface has been derived from studies on the

pH dependence of ester hydrolysis (Wilson & Bergmann, 1950; Bergmann & Wurzel, 1954; Bergmann, Segal, Shimoni & Wurzel, 1956; Mounter, Alexander, Tuck & Dien, 1957) and studies of inhibitors (Bergmann & Shimoni, 1952). Investigation of the influence of pH variations on the activities of C-esterase, described in the present paper, reveals a close relationship between C-esterase and other groups of hydrolytic enzymes, but points also to certain characteristic differences which so far have not been explained.

MATERIALS AND METHODS

Substrate hydrolysis. The derivatives of phenyl acetate used in this investigation and the spectrophotometric method of following the progress of their hydrolysis have been described by Bergmann, Rimon & Segal (1958). Enzymic rates throughout this paper represent the

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difference between total and non-enzymic rates. The curves, representing non-enzymic hydrolysis as function of pH, have also been given by Bergmann *et al.* (1958).

Inhibitors and activators. Tetraethyl pyrophosphate (TEPP) was a gift of Dr A. D. F. Toy, Victor Chemical Works, Chicago, Ill., U.S.A. As inhibition by TEPP of the B-esterase in the enzyme preparations is complete within a few minutes, and as this phosphate is hydrolysed rapidly by diisopropyl phosphorofluoridase (DFPase) but does not interact with C-esterase, the original method of elimination of B-esterase was modified by incubating the enzyme mixture at room temp. with 10 mM-TEPP for 5 min. before use.

Sodium *p*-chloromercuribenzoate (Sigma Chemical Co.), in concentrations of 10–50 μ M, was used as inhibitor of the DFPase present and as activator of C-esterase. In these concentrations the extinction of CMB, in the spectral range suitable for measuring the hydrolysis of phenyl acetates, is negligible.

Enzyme preparations. The C-esterase was purified from hog's-kidney extract as described previously (Bergmann *et al.* 1957). The final solution, representing the fraction precipitated by 30% ethanol, contained usually 45–60 mg. of protein/ml., as determined by addition of an equal volume of 20% (w/v) trichloroacetic acid. When diluted 1:200, the enzyme E-10, which was used in most of the present experiments, hydrolysed 0.4 μ mole of mm-*p*-nitrophenyl acetate/ml./hr. Among eight new enzyme preparations, again three were encountered which lost part of their activity upon dialysis during 48 hr. (to the extent of 40, 46 and 50% respectively), possibly owing to removal of a cofactor. The representative of the latter group of enzyme preparations used mostly in the present studies is designated E-9. It had a protein content of 44 mg./ml. and, under the conditions described above, it gave a hydrolytic rate of 0.35 μ mole/ml./hr.

Buffers. The pH range required for the present investigation was covered by the use of the following buffers: pH 5.0–6.5 by 0.1M-sodium acetate; pH 6.5–8.0 by 0.1M-sodium phosphate and pH >8.0 by 0.1M-sodium borate or pyrophosphate.

RESULTS

Properties of purified C-esterase

A linear relationship between enzyme concentration and rate of hydrolysis was established. The $pS [-\log (\text{concentration of substrate, M})]$ -activity curves of the three phenyl acetates used are also linear in the range 0.1–1.0 mM (Fig. 1), which is adequate for spectrophotometric measurements of rates. At pH 7.5 the descending order of relative velocities is as follows: *p*-nitrophenyl acetate, phenyl acetate, *p*-methoxyphenyl acetate. The order in this series is thus entirely different from the order reported earlier for true cholinesterase, but is similar to that for imidazole-catalysed hydrolysis (Bergmann *et al.* 1958). Accordingly, we may state that with C-esterase rates are the higher the smaller the electron density at the ethereal oxygen atom of the ester group.

Enzymic rates are linear with time at all pH values, at least for the first 10 min. Therefore the

pH dependence of hydrolytic activity can easily be derived from the initial portion of the curves, representing rates as function of time.

Recovery of enzyme activity after exposure to extreme pH values

In order to judge correctly the changes in enzymic activity which accompany pH variations, it is necessary to determine whether the enzyme protein has undergone irreversible change of structure. For this purpose the enzyme was kept for 30 min. at extreme pH values; thereafter the pH was readjusted to 8.0 and the recovered activity compared with that of the original preparation. At any pH value between 5.6 and 10.2 the reaction mixture remained perfectly clear and full activity could be restored by readjustment to

Fig. 1. Hydrolytic rates as function of substrate concentration. Enzyme E-9, after pretreatment with TEPP, was incubated at 0° with 20 μ M-CMB for 12 hr., before the addition of the substrate. Final enzyme dilution, 1:100; pH 7.5; temp. 27°. Simultaneously, blanks were run which contained all components except the enzyme, and the enzymic rates were calculated as the difference between total and non-enzymic rates. (The experiment with the *p*-nitro ester was carried out with an enzyme dilution of 1:600, however the rates were recalculated for the purpose of comparison.) All rates represent initial rates, obtained by extrapolation of rate curves to zero time. ●, Phenyl acetate; ○, *p*-methoxyphenyl acetate; ▲, *p*-nitrophenyl acetate.

pH 8.0 (see Table 1). At pH 5.6 the solution becomes just faintly turbid, but clears again when it is brought back to pH 8.0. However, this is not any more the case when the pH is lowered further. Thus the turbidity formed at pH 5.1 does not clear completely when the pH is raised again, and only four-fifths of the original activity can be recovered. In the extreme alkaline range, beyond pH 10.2, no precipitate is formed. Nevertheless, slow irreversible changes take place, and after exposure to pH 10.8 for 15 min. only about 55% of the activity was recoverable.

Enzyme E-9 proved to be more vulnerable (see Table 1). Thus at pH 5.6 one-third of the total activity was lost after exposure for 15 min., and at pH 10.2 almost two-thirds.

pH-Activity curves of C-esterase

As spectrophotometric measurements require at least a 1:100 dilution of the present coloured-enzyme preparations, it is often advantageous to increase the activity by the use of a mercurial. Therefore it was necessary to compare the pH dependence of hydrolysis both without and with activator. The curves in Fig. 2 show that for *p*-methoxyphenyl acetate practically identical results are obtained in the presence or absence of sodium *p*-chloromercuribenzoate (CMB). Similar observations were made with phenyl acetate as substrate.

The curves in Fig. 2 represent relative activities as function of pH, the maximum activity of a given preparation serving as 'internal' standard; for example, all rates in the presence of CMB were expressed as percentage of the maximum rate of the enzyme, activated with the same mercurial. If, however, the activation is calculated for each pH as the percentage increase in the non-activated

enzyme rate produced by activation, a different picture is obtained (Fig. 3). At low pH values the effect of CMB is practically nil, but it increases sharply when the pH rises to 7.3. Then the slope of the corresponding curve in Fig. 3 diminishes, and the activating effect remains on a nearly constant level up to pH 9.0. Beyond the latter value, the curve rises again.

The points in Fig. 3 represent measurements in which enzyme and activator were kept at pH 7.5 and 4° for 12 hr. Then this mixture was incubated with excess of buffer of the desired pH for 15 min. and finally the substrate added. However, identical results were obtained with the following procedure: enzyme and buffer were incubated at 28° for 15 min. before the mixture of mercurial and substrate was added. This shows that regardless of how the enzyme-activator complex was formed it readjusts itself immediately to a new environment with a different pH.

As shown previously (Bergmann *et al.* 1957), the bond between C-esterase and CMB is very stable, and even after dialysis for 72 hr. only about half of the 'bound' CMB is lost. We have now observed that the same applies to the whole pH range investigated. Accordingly, in short-range experiments dilution of a mixture of enzyme and activator does not influence the degree of activation reached, and it is not necessary (as was done in our early experiments with C-esterase) to add the mercurial to all solutions used subsequently. The changes in activating power as a function of pH, which are demonstrated in Fig. 3, are thus not due

Table 1. Recovery of enzymic activity after exposure of C-esterase to extreme pH values

The enzyme was kept at the pH indicated. The pH was then readjusted to 8.0 and activity against *p*-nitrophenyl acetate was measured spectrophotometrically. The rates so obtained were expressed as percentage of the rate of hydrolysis by the original enzyme preparation. The temp. was 28°. The same results were obtained, whether the enzyme was activated by CMB or not.

Enzyme	pH	Length of incubation period (min.)	Activity recovered (%)
C-Esterase E-10 (stable to dialysis)	5.1	30	83
	5.6	30	100
	10.2	30	100
	10.8	15	56
C-Esterase E-9 (partly unstable to dialysis)	5.6	15	62, 64
	9.5	15	76
	10.2	15	35, 40

Fig. 2. pH-dependence of the hydrolysis of *p*-methoxyphenyl acetate by C-esterase. O, Enzyme E-10, 1:50; ●, enzyme E-10, 1:200, after activation with 20 μM-CMB. Substrate was *p*-methoxyphenyl acetate (mM). All rates represent initial rates, calculated as the difference between total and non-enzymic hydrolysis and then expressed as percentage of the maximum rate.

to association or dissociation of enzyme and activator but express structural changes within the stable complex of C-esterase and mercurials.

pH-dependence of *p*-nitrophenyl acetate

Unexpected results were obtained with *p*-nitrophenyl acetate. Throughout the whole pH range, the rate increases steadily with increasing pH (Fig. 4), with or without activator. In most experiments it is possible to represent all points by one straight line. It was thought possible that *p*-

nitrophenol, one of the hydrolysis products, exerts an activating effect on the enzymic reaction, thus obscuring the expected decline of rates in the alkaline range. This could, however, be excluded by adding free *p*-nitrophenol to the system C-esterase-*p*-methoxyphenyl acetate in the alkaline range, e.g. at pH 9.0. No change of rate was observed. We are thus led to the assumption that OH⁻ ions themselves enhance the catalytic effect of C-esterase towards the *p*-nitro ester.

Fig. 3. Influence of pH on the activation of C-esterase by mercurials. Enzyme E-10, pretreated with TEPP, was incubated for 12 hr. at 4° with 20 μ M-CMB. The enzyme dilution in this mixture was 1:4. Thereafter 24 vol. of buffer were added and the mixture was left at 28° for 15 min. Finally, the substrate dissolved in an equal volume of water was added (at zero time) and spectrophotometric measurement started at once. Simultaneously, an identical control run was made without activator. Final enzyme dilution for experiments with CMB was 1:200, for control runs, 1:50 (the latter rates were divided by four for comparison). For activation with phenylmercuric acetate, the enzyme, pretreated with TEPP, was dialysed against distilled water for 2 hr. to remove all Cl⁻ ions, then 0.2 mm-activator was added. Enzyme dilution during incubation at 4° was 1:4. Subsequent procedure was as above, but dilution was effected with 5 vol. of buffer only, so that the final dilution of activated enzyme in this case was identical with that of the control runs (1:50). Final concn. of *p*-methoxyphenyl acetate, mM; temp. 28°. Non-enzymic hydrolysis rate was deducted from the rate measured. Percentage activation was calculated as:

$$\frac{100 \times \text{rate of activated enzyme} - \text{rate of non-activated enzyme}}{\text{rate of non-activated enzyme}},$$

and is plotted as a function of pH. ○, Phenylmercuric acetate (right ordinate); ●, CMB (left ordinate).

Fig. 4. pH-dependence of the hydrolysis of *p*-nitrophenyl acetate by C-esterase. ○, Enzyme E-10, pretreated with TEPP. Enzyme and buffer were kept at 28° for 15 min. before the substrate was added (at zero time). ●, Enzyme E-10, pretreated with TEPP, was incubated for 12 hr. at 4° with 20 μ M-CMB. Thereafter buffer was added and the mixture left for 15 min. at 28°. Finally the substrate was added (at zero time). Final enzyme dilution: without activator, 1:200; with CMB, 1:400. Substrate, mM-*p*-nitrophenyl acetate. All rates represent initial rates and are calculated as total minus non-enzymic hydrolysis.

DISCUSSION

Like other hydrolytic enzymes, C-esterase shows great stability when exposed to acid or alkaline media, which reduce the enzymic rates almost to zero. Within the range pH 5.6–10.2, activity can be recovered quantitatively by readjustment of the pH to its optimum value.

All known esterases possess a more or less well-defined pH optimum of activity, which serves as expression of the dual mechanism operative in enzymic hydrolysis (Bergmann *et al.* 1958), i.e. simultaneous catalysis by a nucleophilic and an electrophilic group, the latter acting probably through hydrogen bonding. Accordingly, the pH-activity curves in Fig. 2 may be interpreted as indicating the possible presence of an imidazol group, which may be responsible for the increase in rates when the pH rises from 5.5 to 8.0. Likewise, an electrophilic component of the esteratic site must be responsible for the decay of activity when the pH rises from 8 to 10. The active centre of C-esterase is thus similar, in principle, to that of A- and B-esterases. However, the curves in Fig. 2 differ in shape from the curves reported for eel esterase with the same substrates. With the latter enzyme, a plateau was found between pH 7.0 and 9.5. Only above pH 9.5 did the decay of enzymic activity become measurable (Bergmann *et al.* 1958). With C-esterase, on the other hand, a sharp optimum is apparent at pH 8.0 and above this value the rates decrease rapidly. The electrophilic group in the esteratic site is thus presumably different in the two enzymes.

C-Esterase differs markedly from other esterases in its susceptibility to activation by organic mercurials. For example, cholinesterases are very little influenced by CMB (Mounter & Whittaker, 1953), and DFPase is strongly inhibited (Mounter, Floyd & Chanutin, 1953). The variation of C-

esterase activation with pH changes sheds light on the character of the enzyme-activator complex. This complex is rather stable throughout the whole pH range of the present studies, as evidenced by the rapid return of the activating effect when the pH is changed and then restored, and also by dialysis experiments. Apparently the mercurials are firmly bound to a group which does not change its binding power markedly with pH. In addition, they must combine with a group in the active centre, which becomes 'inactivated' with decreasing pH. A reasonable assumption would be that an imidazol group is present, which in its basic form complexes the organic mercuric ion, but is unable to do so when converted into the imidazolium cation. The left-hand portion of both curves in Fig. 3 indicates indeed a group with pK 7.0–7.5. The carboxyl group in CMB enhances the activating effect above pH 8.0, as becomes evident by comparison of the curves in Fig. 3. Since in this pH range the carboxyl is completely ionized, the difference between CMB and phenylmercuric ion is probably to be ascribed to a change in the active centre itself.

The bond between C-esterase and the postulated dialysable cofactor (Bergmann *et al.* 1957) would be of a different character. The cofactor appears to be easily lost by pH changes in either direction. In addition, CMB and phenylmercuric ion activate also C-esterase with cofactor, so that the native activator would have to be bound to a group different from that to which the mercurials become attached.

An interesting problem is posed by the curious pH-activity curve found for the system C-esterase-*p*-nitrophenyl acetate (Fig. 4). The following observations should be borne in mind. Eel esterase splits this substrate at a constant rate throughout the range pH 7.0–9.5 (Bergmann *et al.* 1958). On the other hand, the rate of chemical hydrolysis of the *p*-nitro ester by OH^- ions increases with pH. The increase is much greater than the corresponding rise in hydrolytic rate observed in the alkaline pH range for the system C-esterase-*p*-nitrophenyl acetate. This relationship is clearly demonstrated in Fig. 5, in which the two last-mentioned reaction rates are plotted as functions of OH^- ion concentration. It is suggested therefore that, in addition to the catalytic effect of OH^- ions, another factor comes into play which changes the reaction order with regard to OH^- ion and thus diminishes the increase in rate with increasing pH. We may, for example, assume that the change of rate of hydrolysis of the *p*-nitro ester with increasing pH is the resultant of two effects: (a) a decreasing catalytic influence of the enzyme analogous to the decline of activity in the alkaline range shown in Fig. 2; (b) an increasing catalytic effect of OH^- ion.

Fig. 5. Rate of hydrolysis of *p*-nitrophenyl acetate as a function of hydroxyl ion concentration. ○, Non-enzymic hydrolysis; ●, hydrolysis by enzyme E-9, 1:100 (total minus non-enzymic rate). Ester concn. mM; temp. 28°; all rates plotted represent initial rates.

SUMMARY

1. Purified C-esterase (from hog's kidney) is stable within the range pH 5.6–10.2. After exposure of the enzyme to these extremes of pH, its activity can be fully recovered by readjustment of the pH to 8.0.

2. The activating effect of organic mercurials is pH-dependent. The changes are immediate and completely reversible.

3. Some of the preparations of C-esterase appear to contain a dialysable cofactor, which is lost rapidly by exposure of the preparations to pH 5.6 or to pH 10.2.

4. The pH-activity curves for phenyl acetate and its *p*-methoxy derivative exhibit an optimum around pH 8. In this respect, C-esterase behaves similarly to esterases of the A- and B-type.

5. In contrast, the rate of the enzymic hydrolysis of *p*-nitrophenyl acetate increases steadily up to the experimental limit of pH 9.5.

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Condensed Tannins

2. BIOGENESIS OF CONDENSED TANNINS BASED ON LEUCO-ANTHOCYANINS*

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The previous work by Roux (1957*a, b*, 1958*a*) and by Roux & Evelyn (1958) showed the association of monomeric and complex leuco-anthocyanins of similar structural pattern in those typical 'condensed tannins' present in extractives of black-wattle bark and quebracho heartwood. Chromatographic evidence illustrated the apparent conversion of monomeric into the complex polymeric leuco-anthocyanins. The present study provides details of the chromatographic identification, isolation and characterization of some of these new monomeric leuco-anthocyanins. By means of a study of molecular weight evidence is also provided to support the concept of their conversion into complex tannins during radial translocation within the plant.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Analyses of C, H, methoxyl and acetyl are by Weiler and Strauss, Oxford. Tannin analyses by the hide-powder (shake) method are by Miss S. Whitelaw of these Laboratories.

* Part 1. Roux & Evelyn (1958).

Variations in quebracho woods. The wood of *Schinopsis balansae* from Vera, Santa Fe, and also from unknown areas in the Argentine, appeared uniformly dark-reddish brown in cross-section, without differentiation into heartwood and sapwood areas. A single specimen obtained recently from the same source was clearly divided into a white sapwood and a light-amber heartwood.

A cross-section of *Schinopsis quebracho-colorado* [syn. *S. lorentzii*; see Barkley & Meyer (1950) for discussion on nomenclature] collected from Atamisqui, Santiago del Estero, (specimen A) showed sharp subdivision into a yellow sapwood and a deep-amber, almost black, central-heartwood region (Roux, 1958*a*). Other samples obtained from the same source but from unknown regions of the Argentine, and also from the Union Department of Forestry plantations at Hanglip, Northern Transvaal (specimens B), differed in that a white sapwood 2 in. wide surrounded the light amber-brown central heartwood. These are almost identical in appearance with the divergent sample of *S. balansae*.

Examination of extractives of Schinopsis spp.

Isolation of a new leuco-fisetinidin. Dry wood (100 g.) of *S. quebracho-colorado* (specimen A, a sapling of 7–8 cm. diam.) was cut into chips and ground into a fine powder with a Wiley mill. The powder was extracted first with