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Effect of pH on the Activity of Eel Esterase towards Different Substrates

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Much fundamental information about the structure of the active centre in cholinesterases has been derived from studies on changes of activity with pH. These enzymes show a maximum activity at about pH 7–8. On the acid side, activity falls off in approximately the same manner for all substrates studied. However, in the alkaline range great differences were observed between different esters (Bergmann, Segal, Shimoni & Wurzel, 1956). The bell-shaped form of the pH–activity curves has been interpreted to indicate two different components of the esteratic site, one of which (the nucleophilic group G_1) is inactivated competitively by H^+ ions and the other (the electrophilic group G_2) by OH^- ions. Such an interpretation permits the calculation of the respective dissociation constants, pK_a and pK_b. The determination of the dissociation constants, in turn, leads to some speculation about the possible nature of these two groups (Bergmann *et al.* 1956).

A new argument about the influence of pH on esterase activity has been raised recently by Kistiakowsky & Mangelsdorf (1956). These authors observed that hydrolysis of methyl butyrate by horse-liver esterase varies according to reaction conditions. If reaction rates were measured immediately after addition of the enzyme to the alkaline buffer and for short periods, no decrease in rate in the alkaline range (beyond pH 9) was found. If, however, a pre-incubation period of about 20–60 min. preceded the addition of substrate to the enzyme–buffer mixture, the usual decrease in activity with increasing pH became apparent. The authors concluded from their results that the 'true' activity does not change, but that the enzyme protein undergoes slow changes in the alkaline range, which at first are reversible but progressively become irreversible. These changes are assumed to simulate the competition of substrate and OH^- ion for the active surface, which forms the basis of the above-mentioned interpretation of the pH–activity curve.

In the following experiments we have tried to elucidate this problem and to test our previous assumptions about the role of the electrophilic group G_2 in the hydrolytic mechanism.

MATERIALS AND METHODS

Substrates. Phenyl acetate, *p*-methoxyphenyl acetate and *p*-nitrophenyl acetate were prepared by refluxing the appropriate phenol with excess of acetic anhydride and subsequent distillation. Standard solutions of these esters were prepared in water, except for *p*-nitrophenyl acetate, which shows appreciable hydrolysis in aqueous solution. Therefore an M-solution in dioxan was diluted 1:2500 with water, immediately before use, to give a final concentration of 0·4 mM. The dioxan present was found to be devoid of any inhibitory effect in such dilutions.

Acetylthiocholine (AThCh) was the commercial product of Hoffmann-LaRoche, Basel, Switzerland.

Enzyme. Eel esterase was prepared from the electric organ of *Electrophorus electricus* according to the method of Nachmansohn & Rothenberg (1945). The standard preparation, diluted 1:7500, with 4 mM-acetylcholine (ACh) as substrate, hydrolysed 8 μmoles/hr./ml. at 37°.

Measurement of activity. The following buffers were used to cover the pH 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 8·0–9·5; aq. NH_3 soln.–ammonium acetate for pH values above 9·5.

For manometric measurements, a buffer of the following composition was used: NaCl, 0·1 M; $NaHCO_3$, 0·025 M; $MgCl_2$, 0·04 M. The pH was adjusted to 7·4. The gas phase was CO_2 + air (5:95, v/v).

Hydrolysis of AThCh was measured either by the Warburg manometric method (Ammon, 1933) or colorimetrically under conditions which are suitable for the determination of ACh (Hestrin, 1949). Non-enzymic hydrolysis of AThCh is negligible up to pH 10. Therefore this ester is the substrate of choice for the standardization of cholinesterase preparations. The p*S*–activity curve of AThCh, shown in Fig. 1, is different from that of ACh, although the maximum appears in the same concentration range.

Hydrolysis of the phenyl acetates was measured spectrophotometrically, use being made of the large difference in $\lambda_{max.}$ between phenols and their acetates. As an example, Fig. 2 shows the ultraviolet-absorption spectra of *p*-nitrophenol, the corresponding phenoxide and the acetate. In order to make the calculation of rates at various pH values independent of the actual ratio phenol:phenoxide, the isosbestic point at 348 mμ was selected, although the sensitivity of the method is thus less than at the absorption maxima (320 and 400 mμ respectively). The relevant spectroscopic data for the three phenyl acetates used in this investigation are summarized in Table 1. As can be seen, for phenyl acetate and its *p*-methoxy derivative, which were used throughout at mM concentration, the extinction (*E*) of the ester is zero at the isosbestic point of phenol and phenoxide. The situation is somewhat different for the *p*-nitro series. Here, the acetate, which was used in a concentration of 0·4 mM, has an extinction of 0·152 at 348 mμ. Therefore the progress of hydrolysis in the time interval $t_2 - t_1$ is given by the formula

$$x_2 - x_1 = \frac{E_{t(2)} - E_{t(1)}}{E_2 - E_1},$$

where x designates the concentration of phenol, E_t the actual extinction readings and E_2 the extinction of 0·4 mM-*p*-nitrophenol at 348 mμ, whereas E_1 indicates the extinction of the corresponding acetate at the same wavelength.

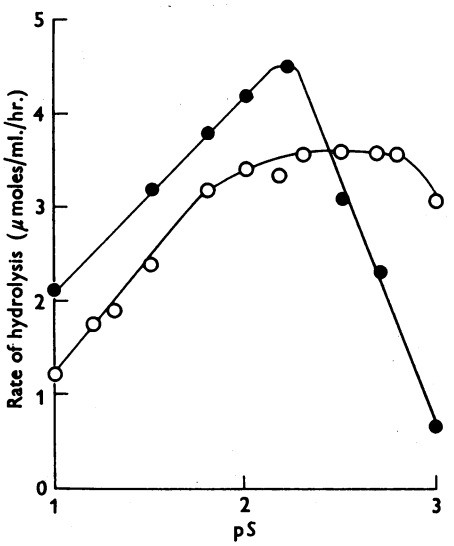


Fig. 1. p*S*-activity curves of eel esterase. Enzyme dilution 1:15 000, pH 7·4; temperature 37°. Rates of hydrolysis were measured by the Warburg manometric method. ●, Acetylcholine; ○, acetylthiocholine.

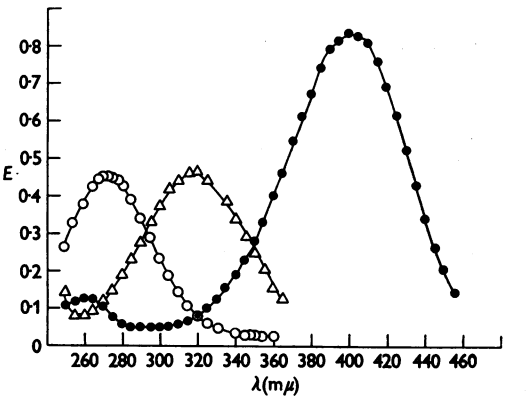


Fig. 2. Ultraviolet-absorption spectra. ●, *p*-Nitrophenoxide ion; △, *p*-nitrophenol; ○, *p*-nitrophenyl acetate. Concentrations, 0·05 mM.

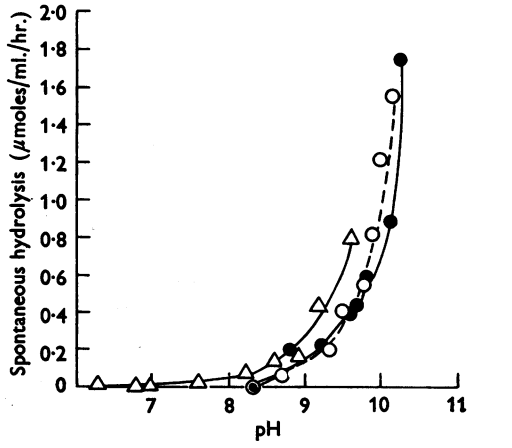


Fig. 3. Non-enzymic hydrolysis of phenyl acetates as a function of pH. △, *p*-Nitrophenyl acetate, 0·4 mM; ○, *p*-methoxyphenyl acetate, mM; ●, phenyl acetate, mM.

Table 1. *Spectroscopic data on phenols and their acetates*

Substituent	$\lambda_{max.}$ of Ester	$\lambda_{max.}$ of Phenol	$\lambda_{max.}$ of Phenoxide	Isosbestic point of phenol and phenoxide	E_2*	E_1*	p*K* of phenol
H	220	270	288	272·5	1·36	0	9·9†
p-OCH_3	275	288	308	295	2·12	0	10·2†
p-NO_2	271	320	400	348	5·20	0·38	7·0‡

* Extinction of mM solutions. E_2, Extinction of phenol or phenoxide at their isosbestic points; E_1, extinction of the ester at the isosbestic point of phenol and phenoxide.

† Data of Fletcher (1946).

‡ Data of Clark (1928).

For evaluation of rates, only the initial linear part of the curve, representing E_t as a function of time, was used, i.e. that part where $x_2 - x_1$ is constant for a given time unit.

All measurements were carried out with a Beckman ultraviolet spectrophotometer, the temperature of 27–28° being maintained with the aid of a Beckman thermospacer.

Enzymic minus spontaneous hydrolysis was expressed as percentage of the maximum enzymic rate. The relative rates so obtained were then plotted as functions of pH, for comparative purposes. The pH dependence of the spontaneous hydrolysis of phenyl acetate and its *p*-nitro and *p*-methoxy derivatives is shown in Fig. 3.

RESULTS

Hydrolysis of acetylthiocholine by true cholinesterase as a function of pH

In Fig. 4 the pH–activity curve for the system eel esterase–AThCh is compared with that of ACh. All points above pH 8 were measured twice, (*a*) by adding the enzyme to the substrate–buffer mixture and taking readings at once, i.e. after incubation for only 0·5 min., and (*b*) by incubating enzyme and buffer for 20 min. before addition of the substrate. In each case identical results were obtained under these two conditions, so that the length of the incubation period had no effect on the enzymic rates. Only at the highest pH studied (10·5), when incubation was extended beyond 1 hr., was a decrease in activity observed under condition (*b*). Even such drastic treatment produced only a reversible change in the enzyme protein, since after readjustment of the pH to 9·5 the original activity was recovered. The results in Fig. 4 therefore are reliable evidence that AThCh, in contrast with ACh and all other *O*-alkyl esters studied previously (Bergmann, 1955), shows no decrease in rate of enzymic hydrolysis up to pH 10·6. This behaviour would be consistent with the assumption that a certain group in the enzyme, which forms a link with *O*-alkyl esters, cannot attach itself to the thiol ester. The most pronounced difference between the two atoms involved lies in their ability to form hydrogen bonds. The results of Fig. 4 thus suggest that a group is present in the active surface of eel esterase, which forms a hydrogen bond to the ethereal oxygen atom (i.e. attached to the alkyl radical) of the ester group in ACh and loses this ability progressively at pH values above 8.

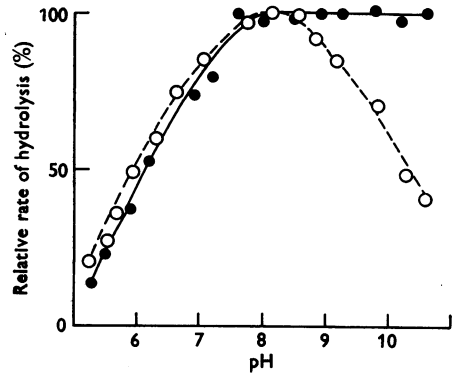
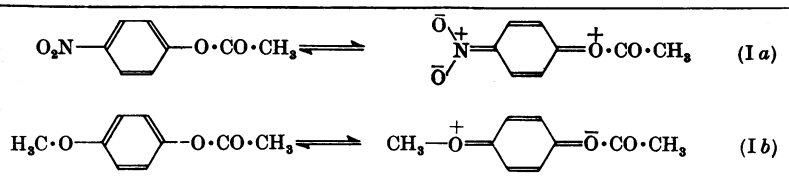


Fig. 4. Hydrolysis of choline esters by eel esterase as a function of pH. Enzyme, 1:1500; temp. 27°. ○, Acetylcholine, 5 mM; ●, acetylthiocholine, 6 mM.

Rates of enzymic hydrolysis of phenyl acetates at different pH *values*

In order to test this hypothesis, a group of esters has been taken in which the structure of the alcoholic portion would produce an effective change of the electron density at the ethereal oxygen atom and thus alter its hydrogen-bonding capacity. The benzene ring in phenyl acetate can function as an 'electron sink'. Furthermore, it would be expected that a *p*-nitro group, by placing a formal positive charge on the ethereal oxygen atom, as in the reaction sequence (I*a*), would enhance the general effect of the benzene ring. On the other hand, a *p*-methoxy substituent (I*b*) will produce a formal negative charge on the ethereal oxygen atom and thus diminish the effect of the aromatic ring. The phenyl acetates used have been tested previously as substrates of erythrocyte cholinesterase (Mounter & Whittaker, 1953). However, the influence of pH on their rates of hydrolysis has not been investigated. The pH–activity curves of these esters were measured both after 1 and 20 min. incubation, but no difference was found up to pH 10·5. The curves in Fig. 5 show that over the range from pH 7 to 9·5 the rates remain constant. Unfortunately, with *p*-nitrophenyl acetate the measurements could not be extended beyond this range, in view of the fast non-enzymic hydrolysis of this ester. With the other two esters, a rapid decrease of activity appears between pH 9·5 and 10·5. The question whether the *p*-nitro group delays the decreased rate of enzymic activity in the alkaline range has not been answered by these experiments.



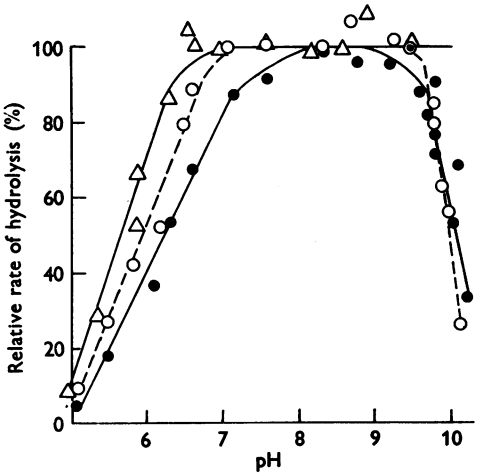


Fig. 5. pH–activity curves for the system eel esterase–phenyl acetates. Hydrolytic rates at 28° were determined by the spectrophotometric method. The maximum rates are designated as 100%. With *p*-nitrophenyl acetate, the plateau at 100% relative rate was calculated from the average value of eight experimental points covering the range pH 6·5–9·5. Therefore the points lying above or below this plateau express the experimental error of the method used. △, *p*-Nitrophenyl acetate; ○, *p*-methoxyphenyl acetate; ●, phenyl acetate; concentrations as in Fig. 3.

Dependence upon pH *of imidazole-catalysed hydrolysis of esters*

It has long been suspected that the imidazole ring of histidine represents the nucleophilic group G_1 of the esteratic site (Wilson & Bergmann, 1950). The catalytic effect of imidazole and some of its derivatives has been demonstrated for di*iso*propyl phosphorofluoridate (DFP) hydrolysis by Wagner-Jauregg & Hackley (1953) and for phenyl acetates by Bruice & Schmir (1956). Esters of aliphatic alcohols are not attacked.

The dependence of imidazole-catalysed hydrolysis upon pH has been determined recently by Bruice & Schmir (1957) in the range pH 6·3–7·9. We have extended these measurements as far as possible into the alkaline range to show that above pH 7 the reaction becomes independent of pH (Fig. 6).

The pH–activity curve of the system eel esterase–AThCh in Fig. 4 suggests that this ester can be split by interaction with G_1 only, without interference of G_2. If this explanation is correct, then imidazole alone should be able to split esters of the general structure R·CO·S·alkyl, although it is ineffective towards the corresponding *O*-alkyl esters. We have indeed found that AThCh is split by imidazole at about the same rate as *p*-methoxyphenyl acetate (Table 2).

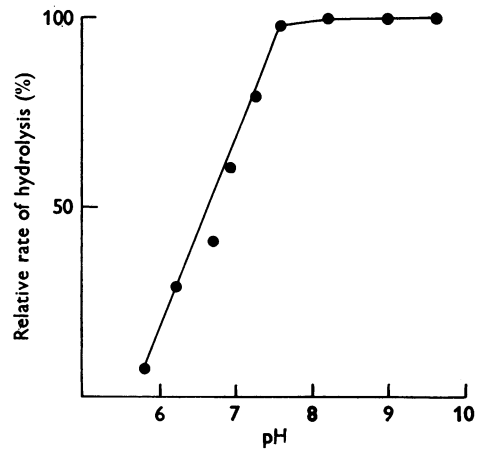


Fig. 6. Dependence upon pH of imidazole-catalysed hydrolysis of *p*-nitrophenyl acetate. Imidazole, mM; ester, 0·4 mM; temp. 28°. Concentration of dioxan in the reaction mixture, 5%. Hydrolysis was measured by the spectrophotometric method. The constant rate, observed between pH 7·5 and 9·5, is used as reference (=100%). All other rates were expressed as percentages of this maximum rate.

Table 2. *Effect of imidazole on the rate of hydrolysis of* p-*methoxyphenyl acetate and acetylthiocholine*

Hydrolysis was measured by the Warburg manometric method, at pH 7·5 and 37°. Concentration of both imidazole and ester, 10 mM. The figures in col. 2 represent the difference between total and spontaneous rates.

Substrate	Rate of catalysed reaction (μmole/ml./hr.)	Spontaneous rate (μmole/ml./hr.)
p-Methoxyphenyl acetate	0·85	0·36
Acetylthiocholine	0·73	0·17

DISCUSSION

The experiments reported here demonstrate that eel esterase equilibrates with the OH^- ions of the medium so fast that the length of the incubation period has no influence on hydrolytic rates. The shape of the pH–activity curves, measured with eel esterase in the alkaline range, thus represents rapid reversible changes taking place in the active surface. It may well be that the different observations of Kistiakowsky & Mangelsdorf (1956) are due to a peculiar behaviour of their enzyme, which apparently equilibrates rather slowly with OH^- ions. Their findings in no way invalidate conclusions, drawn from the shape of pH–activity curves, about the structure of the active centres.

The dependence on pH in the system eel esterase–substrate varies markedly in the alkaline range,

according to the structure of the ester. Acetylthiocholine hydrolysis remains constant above pH 7, in sharp contrast with *O*-alkyl esters. This fact affords strong evidence that the decrease in reaction rate, observed with *O*-alkyl esters in the alkaline range, is real and leads to the conclusion that the active centre of the enzyme studied includes a group which (*a*) forms a hydrogen bond to the ethereal oxygen of the ester group, and (*b*) loses its catalytic activity with increasing OH^- ion concentration. This group is thus identical with the previously assumed 'electrophilic group G_2'. The greater the affinity of a substrate for this group G_2, the more pronounced is the enhancement of enzymic rate produced by G_2, and consequently the more rapid is the decrease in rate of enzymic hydrolysis at higher pH values. The pK values, derived previously for this component of the active surface, indicated as one possibility that the phenolic hydroxyl of tyrosine may be involved (Bergmann *et al.* 1956). Recently, it was found by Holland & Klein (1956) that diazonium compounds inactivate erythrocyte cholinesterase. This result again led to the assumption that a tyrosine unit participates in the active centre.

G_2 clearly exerts its effect, before the alcoholic component of the ester is split off, since, as seen in reaction sequence (II), the intermediate acyl-enzyme is identical for ACh, AThCh and the three phenyl acetates. Therefore the large differences in the hydrolytic rates of these five substrates, summarized in Table 3, must be due to differences in the velocity constants, characteristic for step (1) or (2) or both. This proves at the same time that step (3) cannot be the rate-determining step, at least not for acetates. G_2 has no effect on the hydrolysis of AThCh up to pH 10·6. Which role then does it play in the splitting of *O*-alkyl esters? In the acid-catalysed hydrolysis, the C–O–alkyl bond becomes polarized by attachment of a proton (Ingold, 1953). In a similar fashion, formation of a hydrogen bond polarizes the *O*–alkyl bond and thus facilitates both the removal of R′·OH and the nucleophilic attack of G_1 on the carbonyl carbon atom. Such an effect is missing or unimportant in the formation and splitting of the complex thiol ester–cholinesterase.

We have also observed that thiono esters, R·CS·O·alkyl, are not attacked at all by esterases (F. Bergmann & R. Segal, unpublished work). In all imidazole-catalysed hydrolyses, the nucleophilic nitrogen, which is similar to the OH^- ion, combines with the ester group to form the transition state (III). A negative charge is placed on the carbonyl oxygen atom, owing to the formation of the ortho ester-like intermediate. Such a charge distribution is still possible in the thiol esters, but cannot materialize in the thiono esters, because the electronegativity of sulphur is of the same magnitude as that of carbon (Pauling, 1948).

These considerations on the substrate specificity of eel esterase may be summarized as follows: (1) In a thiol ester the imidazole nitrogen attacks the carbonyl carbon atom, and produces an ortho ester-like intermediate. Here the intrinsic polarizability of sulphur is sufficient to break the C–S–alkyl bond. (2) In an *O*-alkyl ester, the ortho ester structure as such is stable and requires additional 'activation' by hydrogen bonding. (3) In a thiono ester the double-bonded sulphur atom is unable to acquire a negative charge. Therefore combination with the imidazole ring is impossible and hydrolysis does not take place.

These conclusions are corroborated by analogous findings in the phosphate series. Thus it has been found by Diggle & Gage (1951) that the thiol

$$EH + R\cdot CO\cdot OR' \xrightarrow{(1)} [EH\ldots R\cdot CO\cdot OR']_{complex} \xrightarrow{(2)} R'\cdot OH + E\cdot CO\cdot R \xrightarrow{(3)} EH + R\cdot CO\cdot OH \quad (II)$$

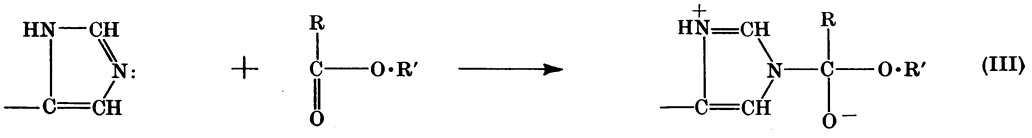
$$\xrightarrow{+H_2O}$$

(EH = enzyme)

Table 3. *Comparison of rates of hydrolysis of phenyl acetates*

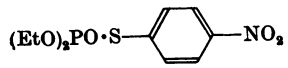
All determinations were carried out at pH 7·5 and 28°. With eel esterase, all substrates were studied at mM-concentration and the rates calculated for an enzyme dilution of 1:7500, a linear dependence on enzyme concentration being assumed. In the imidazole-catalysed reactions, k_2 represents the bimolecular velocity constant, based on the equation for initial rates: $v_2 = k_2[\text{imidazole}] \times [\text{ester}]$.

Substrate	Rate of hydrolysis by eel esterase (μmoles/ml./hr.)	Imidazole catalysis k_2 (l./mole/min.)
ACh	1·4	—
AThCh	7·0	0·07
Phenyl acetate	3·2	0·2
p-Nitrophenyl acetate	0·15	18·2
p-Methoxyphenyl acetate	1·2	0·08

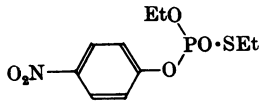


(III)

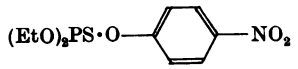
phosphates (IV) and (V) are active inhibitors of cholinesterases, whereas the thionophosphate (VI), i.e. pure parathion, is inactive.



(IV)



(V)



(VI)

In this series of esters, too, a substrate containing a double-bonded sulphur atom cannot be split by cholinesterases.

It is remarkable that the rates of enzymic hydrolysis of phenyl acetates do not follow the same order as the imidazole-catalysed reaction. Mounter & Whittaker (1953) found with erythrocyte cholinesterase the following series: phenyl acetate > *p*-methoxyphenyl acetate > *p*-nitrophenyl acetate. A similar relationship was observed by us with eel esterase (Table 3). In contrast with these findings, the following order results in imidazole-catalysed hydrolysis: *p*-nitrophenyl $\gg$ phenyl > *p*-methoxyphenyl.

With imidazole catalysis, the rate is evidently inversely proportional to the electron density at the ethereal oxygen, and thus the observed order finds a simple explanation. In cholinesterases, the electron distribution around this oxygen atom determines the affinity for hydrogen-bonding, which increases with increasing electron density. Therefore the same structural factor has a twofold influence on the enzymic hydrolysis, namely, increasing electron density at the ethereal oxygen atom (e.g. in the *p*-methoxy derivative) produces greater ability to form a hydrogen bond, but at the same time also increases the activation energy required to break the C–O linkage. In the *p*-nitro derivative both effects are reversed. These considerations make it at least qualitatively understandable why phenyl acetate itself is superior to both its *p*-nitro and *p*-methoxy derivatives.

The results reported here may shed light on the problem of enzymic ester hydrolysis in general. In contrast with acid- or base-catalysed hydrolysis, the enzymic reaction is inhibited by both H^+ and OH^- ions. The most likely interpretation of the enzymic splitting of *O*-alkyl esters involves a dual mechanism: nucleophilic attack of imidazole nitrogen at the carbonyl carbon atom and simultaneous electrophilic attack of an 'active' hydrogen atom on the ethereal oxygen atom. The peculiar combination of these two factors explains the pH optimum, characteristic for almost all animal esterases. The enzymic mechanism is equivalent partly to acid hydrolysis, since hydrogen-bonding acts similarly to the attachment of H^+ ion. It contains also certain features of general base catalysis, since addition of imidazole to the carbonyl carbon places a negative charge on the carbonyl oxygen atom, similar to the effect of OH^- ions or related agents.

SUMMARY

1. The dependence upon pH of the hydrolysis of acetylthiocholine, phenyl acetate, *p*-nitrophenyl acetate and *p*-methoxyphenyl acetate by true cholinesterase from the electric eel has been measured.

2. The pH–activity curves of these substrates above pH 7 become largely independent of pH, whatever the length of the incubation period.

3. These results confirm the existence of the second component in the esteratic site, postulated earlier, namely the electrophilic group G_2, and indicate that the latter functions via hydrogen-bonding with the oxygen atom attached to the alkyl radical of the ester group.

4. The implications of these findings for the mechanism of enzymic hydrolysis are discussed.

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Studies in Calcification*

THE REACTION OF SOME HARD- AND SOFT-TISSUE COLLAGENS WITH 1-FLUORO-2:4-DINITROBENZENE

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It has long been considered that those factors which determine whether mesodermal connective tissue will calcify or not are present in the organic fraction of the tissue, and much attention has been directed to the function of mucopolysaccharides as a local factor in mineralization (Sobel, 1955). However, little work has been done on the properties of the protein fraction of the organic matrix and its relation to calcification. This paper describes some of the chemical and structural properties of collagen in both hard and soft mammalian connective tissues, found by the use of Sanger's (1945) fluorodinitrobenzene method, and shows in which respects the reaction of the hard-tissue collagens with fluorodinitrobenzene differ from those of the soft-tissue collagens. These differences may well be significant in understanding why soft-tissue collagen does not calcify, whereas hard-tissue collagen does so despite the almost identical amino acid composition (Bowes, Elliott & Moss, 1955; Eastoe, 1955), appearance under the electron microscope and X-ray-diffraction pictures of both proteins (Bear, 1952).

Samples of collagen obtained from ox achilles tendon, ox hide, ox bone, ox dentine, rat-tail tendon and human dentine have been studied. The collagen was prepared with a minimum of chemical treatment, as it was thought that the small amounts of reticular and elastic fibres that might be present would lead to less error than the drastic treatments which are necessary for their complete removal. In most of the experiments demineralization of bone and dentine for structural studies with fluorodinitrobenzene was effected by shaking with ethylenediaminetetra-acetic acid at pH 8·0, but in some experiments demineralization was also carried out with hydrochloric acid or trichloroacetic acid, although it was recognized that treatment with acid may damage the protein. In some experiments the tissue was treated with ethylenediaminetetra-acetic acid and fluorodinitrobenzene simultaneously, thus avoiding any time lag between demineralization and subsequent dinitrophenylation.

Preliminary accounts of some of this work have already been published (Solomons & Irving, 1955, 1956*a*, *b*).

* Some of the results quoted in this paper were taken from a thesis presented by C. C. S. for the degree of Ph.D. of the University of the Witwatersrand.

MATERIALS

Ox-hide collagen. Hide from the thigh region of a freshly killed ox was washed first with water to remove blood, etc., then left to stand overnight in 0·9% NaCl soln. at 4° and then re-washed with water. The grain layer, which contains the greater part of the muscle and elastic tissue as well as hair roots, was split off and the remaining material, excluding adipose tissue, was cut into approx. 0·5 cm. cubes. The cubes were defatted with three changes of light petroleum (b.p. 40–60°).

Ox achilles tendon and rat-tail tendon. These were cut into thin strips, washed in 0·9% NaCl soln. and defatted with light petroleum.

Ox bone. The diaphysis of an ox tibia, soaked in 0·9% NaCl soln. overnight and then washed with water, was cut into sections about 1 in. thick with a hacksaw. These sections were then split into approx. 0·25 cm. cubes by means of root-splitting dental forceps. Care was taken here and in the preparation of the dentine to minimize the generation of heat, as this can lead to denaturation as well as solution of some of the protein fraction.

Human dentine. Approx. 0·25 cm. cubes of human root dentine from healthy incisor and canine teeth were