Biochemical Pharmacology. Vol. 28. pp. 211–216.

0006-2952/79/0115-0211 $02.00

PHOSPHORYLATION, "AGING" AND POSSIBLE ALKYLATION REACTIONS OF SALIGENIN CYCLIC PHOSPHORUS ESTERS WITH α-CHYMOTRYPSIN

ROBERT F. TOIA* and JOHN E. CASIDA

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720, U.S.A.

(*Received* 20 *February* 1978; *accepted* 18 *May* 1978)

Abstract—Saligenin cyclic phosphonates, phosphates and *N*-alkylphosphoramidates readily phosphorylate α-chymotrypsin at the hydroxyl group of serine-195. The phosphoenzyme undergoes rapid further reaction (aging) both to release saligenin and form bound phenolic residues without regeneration of esteratic activity. The bound phenolics include about equal parts of trapped saligenin, possibly in the region of the active site, and material which may result from enzyme alkylation. The ratio between these types of bound phenolics is altered by pretreatment of the enzyme with ethoxyformic anhydride but not with diisopropylfluorophosphate. N- ε2 of the imidazole portion of histidine-57 may participate in the aging reaction by stabilizing a potential benzyl carbonium ion intermediate. This intermediate is subsequently trapped either by a hydroxyl nucleophile to produce saligenin or possibly by the stabilizing imidazole to yield *N*-alkylated enzyme. Trypsin with an esteratic site similar in configuration to chymotrypsin undergoes analogous phosphorylation and aging reactions.

An esterase phosphorylated with an organophosphorus inhibitor may undergo either spontaneous dephosphorylation to reactivate the esteratic site or "aging" via loss of a second substituent from the P atom to leave a charged group at the active site [1]. The aged phosphoenzyme is resistant to reactivation even by nucleophiles such as oximes.

Two "aging mechanisms" are proposed for phosphorylated cholinesterases (Fig. 1) [1]. One involves P—O bond cleavage from nucleophilic attack on the P atom and the other C—O bond cleavage implying acid-type catalysis and, therefore, carbonium ion character for the leaving group. The former process has been demonstrated with α-chymotrypsin and trypsin [2, 3] and, in phosphate and carbonate ester aging reactions with α-chymotrypsin, the histidine-57 residue participates in the reaction by acting as a nucleophile toward the tertiary enzyme-phosphate ester and as a general base in the carbonate ester case [4].

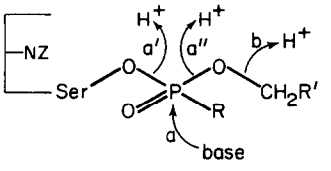


Fig. 1. Proposed mechanisms of dephosphorylation and aging for phosphorylated cholinesterase and other phosphorylated esterases. Nucleophilic attack at the phosphorus (a) is followed by spontaneous reactivation (a') or aging (a''). Acid-catalyzed attack (b) involving protonation at oxygen leading to carbonium ion formation also results in aging.

The saligenin cyclic class of phosphorus esters serves as a useful model for investigations of phosphorylation and aging reactions of α-chymotrypsin [5] (Fig. 2) since the enzyme-mediated hydrolysis (pathway II) should be similar to the nonenzymatic case [6] (pathway I) where the final products arise from a benzyl carbonium ion intermediate [7]. While both mechanisms are conveniently written with a discrete carbonium ion intermediate, it is also possible that these reactions have greater S_N2 character or, alternatively, that they proceed through an intermediate quinone methide resulting from nucleophilic attack on the phenolic proton. In any case, the implied intermediate has alkylating potential [8] and its fate is of interest.

The present study investigates the reaction of α-chymotrypsin with saligenin cyclic phosphorus esters with emphasis on the nature of the products formed from the *o*-hydroxybenzyl moiety released during the aging process.

MATERIALS AND METHODS

Chemicals and spectroscopy. α-Chymotrypsin (type II, 3 × crystallized, salt free and lyophilized), trypsin and diisopropylphosphoryl-α-chymotrypsin from the Sigma Chemical Co. (St. Louis, MO) were used without further purification. Titration against saligenin cyclic phenylphosphonate (SCPP) showed that the chymotrypsin was ~70% (w/w) active enzyme assuming a 1:1 molar ratio of SCPP:enzyme for inhibition. Sephadex G-10 was obtained from Pharmacia Fine Chemicals Inc. (Piscataway, NJ) and silica gel thin-layer chromatography (T.L.C.) plates (60

* Present address: Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, CA 92502, U.S.A.

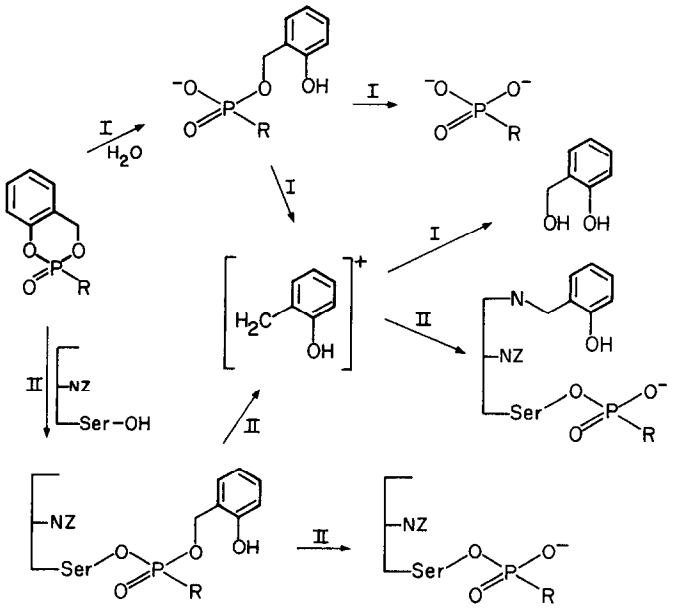


Fig. 2. Proposed mechanisms of hydrolysis of saligenin cyclic phosphorus esters (I) and of phosphorylation, dephosphorylation and aging of chymotrypsin reacted with saligenin cyclic phosphorus esters (II).

F-254, 0.25 mm) from EM Laboratories, Inc. (Elmsford, NY). The saligenin cyclic phosphorus esters, with the exception of SCPP which was synthesized [6] for this study, were obtained from Professor M. Eto (Kyushu University, Fukuoka, Japan). All other chemicals were obtained from commercial sources. Ultraviolet-visible data were obtained with either a Perkin–Elmer 576 ST or a Beckman DU spectrophotometer fitted with a Gilford absorbance meter, using 1 cm quartz cuvettes.

4-Aminoantipyrine (AAP) procedure for analysis of phenolic compounds. Aqueous AAP (0.2 ml, 0.2% w/v) was mixed with the reaction mixture in Na^+ phosphate buffer at pH 7.6 (4 ml); then aqueous $K_3Fe(CN)_6$ (0.2 ml, 0.8% w/v) was added and the mixture shaken again [9]. After 5 min at 25°, the absorbance (λ_{510}) was determined, then the solution was extracted with $CHCl_3$ (2 × 2.5 ml) and the absorbance redetermined. Centrifugation was used as required to achieve separation of the layers. The $CHCl_3$ extract was dried (Na_2SO_4) and its absorbance determined (λ_{460}). For quantitation of the saligenin-AAP adduct the following extinction coefficients were used [6]: λ_{510} aqueous $\varepsilon = 1.1 \times 10^4$; λ_{460} $CHCl_3$ $\varepsilon = 1.8 \times 10^4$. This adduct is sensitive to oxidation and pH changes so care should be taken in using this procedure beyond the conditions of studies described below.

Non-enzymatic hydrolysis of SCPP. SCPP (2 mg) in methoxytriglycol (MTG) (200 μl) was added to 50 ml of Na^+ phosphate buffer (0.01 M, pH 7.6). Aliquots (4 ml) withdrawn at various times were assayed for the $CHCl_3$/aqueous distribution of saligenin and other phenolic compounds using the AAP procedure.

Reaction of organophosphorus (OP) inhibitors with chymotrypsin and trypsin. Unless otherwise specified, chymotrypsin was dissolved in Na^+ phosphate buffer (0.01 M, pH 7.6) at 2 mg ml^{-1}. When necessary to determine the enzyme concentration, e.g. following chromatography, the absorbance at λ_{280} was compared with a standard curve for the same batch of enzyme. Enzyme activity was assayed as release of *p*-nitrophenoxide determined at λ_{400} by addition of ethanolic *p*-nitrophenyl acetate (2.5×10^{-2} M, 100 μl) to 0.5 of the chymotrypsin solution (2 mg ml^{-1}) plus 2.0 ml buffer.

The OP inhibitor was dissolved in MTG (10 mg ml^{-1}) and added to the enzyme solution (25°) as required to achieve 90 per cent inhibition unless specified otherwise. Enzyme activity and AAP assays were then carried out as above. Neither the enzyme activity nor the inhibition kinetics was altered by the MTG levels used. During the AAP assay, $CHCl_3$ extraction resulted in ~10 per cent precipitation of the enzyme (based on protein analysis), and the absorbance reading of the aqueous layer subsequent to this step was corrected accordingly. The AAP reagent does not give any color with chymotrypsin in either its native or partially denatured forms. The identity of the $CHCl_3$-extractable phenolics was verified as saligenin by thin-layer cochromatography with authentic material, both as the AAP adduct (ether–acetone, 2:1, R_f 0.32) and as free saligenin (hexane–ethyl acetate, 3:2, R_f 0.28). In the latter case, compounds were visualized using the $FeCl_3/K_3Fe(CN)_6$ spray reagent for phenols [10].

In an analogous manner, SCPP was reacted at a 1:1 molar ratio with diisopropylphosphoryl-chymotrypsin (esteratically inactive) and trypsin with similar AAP and esterase assays after 4 and 0.5 hr respectively.

Fractionation of phenolic derivatives bound to inhibited chymotrypsin. Enzyme–inhibitor products from chymotrypsin 90 per cent inhibited with SCPP

which form non-$CHCl_3$-extractable AAP color derivatives (referred to as bound phenolics) were subjected to three types of fractionation.

For acetone precipitation, the aqueous solution with enzyme at 2 mg ml^{-1} after AAP treatment was cooled to 5° and ice-cold acetone added (10 vol.) with gentle stirring. The precipitated, inhibited enzyme was recovered by centrifugation, dissolved in buffer and the process repeated. After lyophilization, the recovered protein ($\sim$79 per cent) was dissolved in buffer (2 mg ml^{-1}) and the absorbance at λ_{510} determined. Alternatively, the inhibited enzyme was subjected to the precipitation–washing–lyophilization procedure, dissolved in buffer and the protein concentration determined at λ_{280}; then the AAP assay was performed.

For $[NH_4]_2SO_4$ precipitation, enzyme at 2 mg ml^{-1} inhibited with SCPP as above was treated with AAP and then extracted with $CHCl_3$. The aqueous layer was saturated by slow addition of $[NH_4]_2SO_4$ and the precipitated protein recovered by centrifugation. After lyophilization, the protein was dissolved in buffer (2 mg ml^{-1}) and the adsorbance determined at λ_{510}.

For Sephadex chromatography, the enzyme level and buffer strength were increased (10 or 20 mg ml^{-1}, 0.05 M Na^+ phosphate). Enzyme 90 per cent inhibited with SCPP was assayed with AAP. A second aliquot (0.5 ml) was chromatographed on Sephadex G-10 (10 g, equilibrated with buffer) and the eluted fractions ($\sim$1 ml) were assayed for protein, esteratic activity and phenol residues. The process was repeated, using an aliquot first incubated for 1 hr with a 100-fold molar excess of $NH_2OH{\cdot}HCl$ before chromatography.

Modification of chymotrypsin by reaction with diisopropylfluorophosphate (DFP) and ethoxyformic anhydride (EFA) prior to reaction with SCPP. Chymotrypsin was incubated for 20 min with various concentrations of DFP or EFA; then esterase activity was determined and SCPP was added in an amount equivalent to that giving 90 per cent inhibition of native enzyme. After 3 min, AAP assays were made as above.

RESULTS

Non-enzymatic hydrolysis of SCPP. SCPP hydrolyzes slowly in 0.01 M, pH 7.6, phosphate buffer to give two types of phenolic products analyzed as AAP derivatives, i.e. saligenin in the $CHCl_3$-extractable fraction and *o*-hydroxybenzyl phenylphosphonic acid in the aqueous fraction (Fig. 3A) [6]. *o*-Hydroxybenzyl phenylphosphonic acid is the major initial product and is an intermediate in the ultimate formation of saligenin and phenylphosphonic acid which occurs almost completely within 1350 min.

Cleavage of saligenin cyclic phosphorus esters by chymotrypsin and trypsin. SCPP is very rapidly cleaved by chymotrypsin (Fig. 3B) at a rate similar to that for inhibition of esteratic activity ($T_{1/2} \sim 0.4$ min) (Fig. 3B, Table 1). Comparison of panels A and B of Fig. 3 clearly establishes that SCPP undergoes enzyme-catalyzed cleavage. In confirmation, diisopropylphosphoryl-chymotrypsin does not cleave SCPP. The only $CHCl_3$-extractable product throughout the course of the cleavage reaction is saligenin

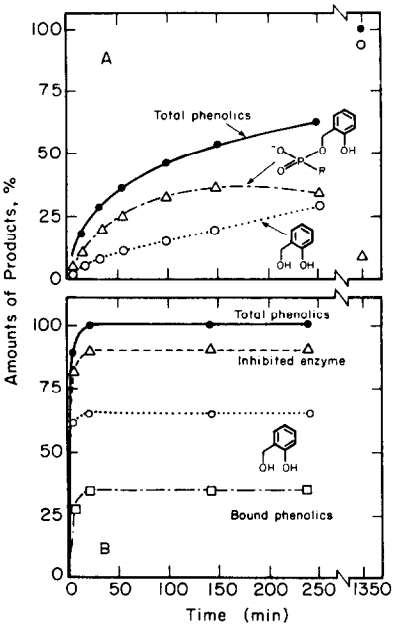
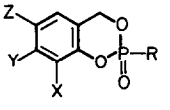


Fig. 3. Amounts and types of phenolic compounds released from saligenin cyclic phenylphosphonate on hydrolysis (A) or reaction with chymotrypsin (B). Inhibition of esterase activity is also shown (B). For non-enzymatic hydrolysis (A), the percentages of phenolics are relative to total saligenin equivalents added as specified in Materials and Methods. For reaction with chymotrypsin (B), percentages are relative to total saligenin equivalents added with a molar ratio of 0.9:1 for saligenin cyclic phosphorus ester to chymotrypsin.

Table 1. Rates of esterase inhibition and per cent of bound phenolics on reaction of various saligenin cyclic phosphorus esters with chymotrypsin



Substituents					Bound phenolics
R	X	Y	Z	$T_{\frac{1}{2}}$*	(%)†
C_6H_5‡	H	H	H	$\sim$0.4	41
OC_6H_5	H	H	H	$\sim$0.4	42
Cyclohexyl	H	H	H	2.5	40
OCH_3	H	H	H	6.9	39
$NHCH_3$	Cl	H	H	18.0	39
$NHCH_3$	H	CH_3	H	105	45
$NHCH_2CH_3$	H	H	Cl	26	43
$N(CH_3)_2$	H	H	H	>700	

* Min for 50 per cent inhibition of esteratic activity.

† Percentages are relative to total saligenin equivalents added with a molar ratio of 0.9:1 for saligenin cyclic phosphorus ester to chymotrypsin. The remainder of the phenolics occur as $CHCl_3$-extractable saligenin or its chloro and methyl derivatives.

‡ SCPP. Similar values for $T_{\frac{1}{2}}$ and bound phenolics are obtained with chymotrypsin at pH 4.4 and tryspin at pH 7.6.

prior to AAP addition, and the saligenin-AAP adduct after AAP addition (thin-layer cochromatography). The saligenin-AAP adduct appears in an amount of ~0.6 mole/mole of inhibited enzyme. Products in the aqueous fraction undergo no subsequent reactions discernible with the AAP reagent and $CHCl_3$ extraction (Fig. 3B). The dye in the aqueous fraction does not originate from *o*-hydroxybenzyl phenylphosphonic acid because this compound would undergo cleavage on long-term incubations. It is, therefore, associated or bound in some way with the enzyme as discussed later.

Eight saligenin cyclic phosphorus esters vary > 1750-fold in their rates of chymotrypsin inhibition (Table 1). The reaction rate is rapid with SCPP and the phenylphosphate and cyclohexylphosphonate derivatives, intermediate with the methylphosphate and alkylphosphoramidate derivatives, and very slow with the dimethylphosphoramidate. The cleavage rate depends on both the stability of the P—O—aryl linkage (phosphonates < phosphates < alkylphosphoramidates < dialkylphosphoramidates [6] and the suitability as substrate analogs for the enzyme (preference for phenyl and cyclohexyl derivatives). With each compound, regardless of its reaction rate, about 40 per cent of the phenol residues released are not extractable into $CHCl_3$ after the AAP reaction.

Several variables were examined for their effects on the reaction of SCPP with chymotrypsin. Variation of the pH between 4.4 and 7.6 during the reaction has no discernible effect on the distribution of the phenol residues (Table 1) despite its influence on the hydrogen bonding configuration within the esteratic site of the enzyme [11]. The percentage of $CHCl_3$-recoverable saligenin is independent of the amount of SCPP until a 1:1 inhibitor:enzyme molar ratio is exceeded, after which the excess substrate is hydrolyzed at a rate approximating that in Fig. 3A. On varying the enzyme level with a constant molar ratio of SCPP:enzyme, no difference is found in the degree of esterase inhibition at chymotrypsin concentrations of 2–40 mg ml^{-1}. Even though this indicates no influence of enzyme concentration on the ease of phosphorylation, there is a marked effect on the released phenolics, i.e. a decrease in total AAP-detectable phenolics from ~100 per cent at ⩽ 2 mg ml^{-1} to ~40 per cent at 40 mg ml^{-1} (Fig. 4A), and an increase in the proportion of those phenolics that are bound (Fig. 4B). It appears that there is a decreased accessibility of the phenolic residues to the AAP reagent, a feature retained even on enzyme dilution before AAP addition. Depending on the enzyme concentration, there may be variation in the degree of self-association [12], conformation or degree of solvation of the enzyme molecules. The introduction of a new ionizable group into the enzyme after aging may be instrumental in the retention of the initially observed AAP reaction characteristics after dilution.

The inhibition of trypsin (2 mg ml^{-1}) with SCPP parallels that found with chymotrypsin in that only ~0.6 mole of $CHCl_3$-extractable saligenin is produced per mole of inhibited enzyme.

Nature of protein-bound derivatives on reaction of SCPP with chymotrypsin. Several methods were used to examine the nature of the non-$CHCl_3$-extractable derivatives, i.e. bound phenolics. No phenolics are bound on hydrolysis of SCPP in the presence of diisopropylphosphoryl-chymotrypsin or on incubation of saligenin with chymotrypsin. Thus, it appears that enzyme-catalyzed hydrolysis is a prerequisite for phenolic binding. Further, phenol released on the aging of enzyme phosphorylated with diphenylphosphorochloridate [2] is not bound, i.e. all the AAP adduct is $CHCl_3$-extractable, so the nature of the phenolic residue influences its binding characteristics.

Three procedures released 41–63 per cent of the chymotrypsin-bound phenolics derived from SCPP without affecting enzyme activity, i.e. acetone precipitation and washing, $[NH_4]_2SO_4$ precipitation and Sephadex chromatography (Table 2). In the latter case, recycling through the column does not appear to release additional amounts of saligenin. Further, NH_2OH produces no alteration in enzyme activity or quantity of bound phenolics, as determined following Sephadex chromatography.

On partial inhibition of the enzyme with DFP or EFA, a parallel reduction is found for total phenolics produced from subsequent addition of SCPP (Fig. 5A). With increasing DFP or EFA concentrations, the proportion of bound phenolics is not altered by DFP but is decreased by EFA (Fig. 5B).

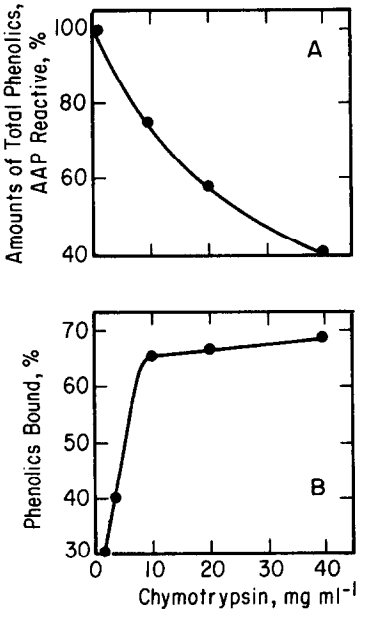


Fig. 4. Amounts and types of phenolic compounds released from saligenin cyclic phenylphosphonate on reaction with various concentrations of chymotrypsin. Percentages in portion A are relative to total saligenin equivalents added with a molar ratio of 0.9:1 for saligenin cyclic phosphorus ester to chymotrypsin. Percentages in portion B for bound phenolics are relative to total phenolics as given in A.

DISCUSSION

Saligenin cyclic phosphorus esters include potent insecticides (*O*-methylphosphorothionate), fungicides (*S*-methylphosphorothiolate) and delayed neurotoxic

Table 2. Ratio of bound phenolic types from reaction of saligenin cyclic phenylphosphonate with chymotrypsin as determined by various fractionation procedures

Chymotrypsin (mg ml^{-1})	Bound phenolics (%)*	Fractionation of bound phenolics	
		Method	Saligenin ÷ coval. deriv.
2	37	$(NH_4)_2SO_4$ ppt	1.1
2	40	Acetone ppt	1.7
10	58	Sephadex chrom	0.8
20	63	Sephadex chrom	0.7

* Percentages are relative to total saligenin equivalents added with a molar ratio of 0.9:1 for saligenin cyclic phosphorus ester to chymotrypsin. The remainder of the phenolics occur as $CHCl_3$-extractable saligenin or its chloro and methyl derivatives.

agents (phenylphosphate). These types of biological activity are attributed to their action as either phosphorylating or alkylating agents [7, 8].

Chymotrypsin reacts rapidly with saligenin cyclic phosphorus esters to yield a phosphoenzyme which undergoes rapid and quantitative aging (Fig. 2) as evident by electrophoretic studies [13], by analysis of phenolic residues formed, and by the failure of NH_2OH to regenerate enzyme activity. Free saligenin accounts for only ~ 60 per cent of the total phenolics released on aging of chymotrypsin phosphorylated with each of seven different saligenin cyclic phosphorus esters despite large differences in their inhibition rates.

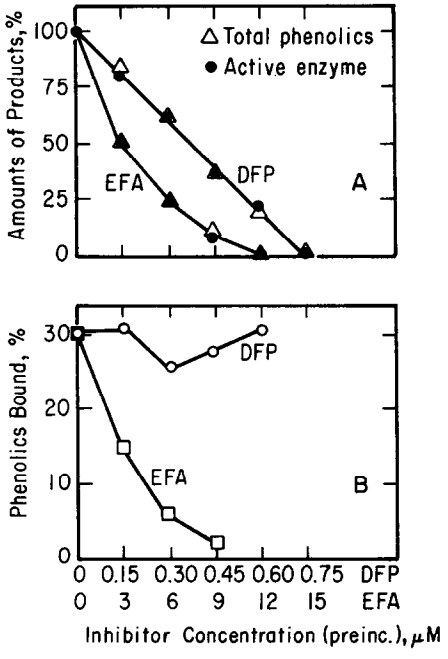


Fig. 5. Inhibition of esterase activity and amounts and types of phenolic compounds released from saligenin cyclic phenylphosphonate on reaction with chymotrypsin pretreated with various concentrations of diisopropylfluorophosphate (DFP) or ethoxyformic anhydride (EFA). Percentages are expressed as in Fig. 4.

The ratio of free saligenin to bound phenolics appears to be characteristic of the aging process for this class of organophosphorus ester and the conformation of the enzyme, in particular, of the esteratic site.

The bound phenolics consist of two forms. One portion is saligenin which is released on alteration of the physical environment of the protein, and may represent material trapped at the active site by a combination of hydrogen bonding and hydrophobic interactions. The remainder, equivalent to ~ 20 per cent of the amount of phosphoenzyme, is not released by the several techniques examined and may be covalently bound.

Sufficient information is available to speculate on the nature of the proposed covalently bound phenolics. These phenolics are not attached to P since the enzyme has undergone total aging based on its electrophoretic properties [13]. It appears therefore that, in addition to the phenylphosphonyl group at serine-195, there is also an *o*-hydroxybenzyl group at a proximal site. Although *o*-hydroxybenzyl phenylphosphonic acid is proposed as an alkylating agent in the inhibition of the —SH containing enzymes papain and alcohol dehydrogenase [8], this type of process is not involved with chymotrypsin since it lacks any free —SH groups [14]. While it is conceivable that the serine-195 hydroxyl group may attack the benzyl position of the substrate yielding an *o*-hydroxybenzyl ether derivative, this is not the case since attack of a nucleophile on the esters in question is normally directed to the P atom with cleavage of the more reactive P—O—aryl bond [6]. Moreover, this would yield an intermediate which releases phenylphosphonic acid on hydrolysis and, therefore, the modified enzyme would have no alteration in net charge, a result inconsistent with an electrophoretic study of this system [13].

The most feasible alternative is for alkylation to occur at an N atom, based on analogy with studies on model compounds [15] and the presence of histidine-57 in the active site [11]. Some support for *N*-alkylation comes from the finding that enzyme pretreatment with EFA markedly increases the free saligenin released on aging. EFA is commonly used for enzyme derivatization by *N*-ethoxyformylation of histidine [16, 17]. In the case of chymotrypsin at pH 4, EFA ethoxyformylates serine-195 in preference to histidine-57 while the other histidine residue is considered to be inaccessible to large reagents [18]. Therefore, after EFA treatment, only those chymotrypsin molecules with serine-195 free react with saligenin cyclic phosphorus esters so ethoxyformylation at a site other than serine-195 is responsible for the altered aging characteristics. It is reasonable to speculate that ethoxyformylation at N-ε2 of histidine-57 is responsible for altering the aging process but this point requires further study by different approaches. Preliminary results indicate that under the conditions of the present experiments (pH 7.6) there is significant EFA-induced absorption at λ_{242} normally associated with *N*-ethoxyformylation of an imidazole [16, 17].

Considering the known structure of the active site of the enzyme [11], a feasible explanation for the rapid aging involves stabilization by N-ε2 of the imidazole group of the appropriately positioned histidine-57

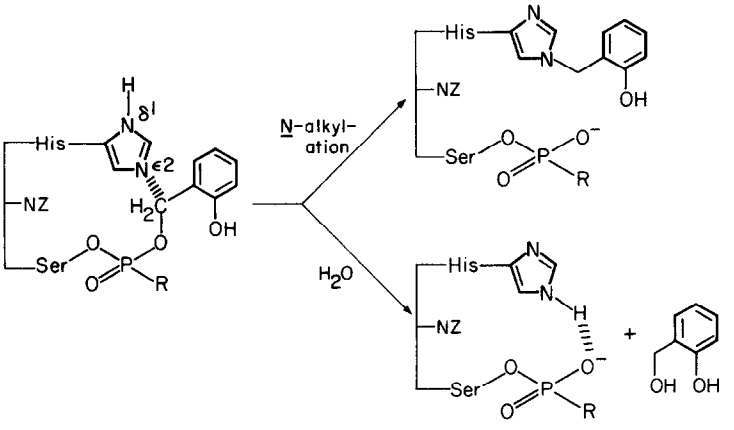


Fig. 6. Possible involvement of histidine-57 in the rapid aging of chymotrypsin phosphorylated at serine-195 with saligenin cyclic phenylphosphonate.

residue of the potential carbonium ion intermediate (Fig. 6). This moiety is trapped either by a hydroxyl nucleophile to produce saligenin or by the histidine-57 residue to give *N*-(*o*-hydroxybenzyl)-derivatized phenylphosphonylchymotrypsin (Fig. 6).

The aging reaction for chymotrypsin reacted with saligenin cyclic phosphorus esters also appears to be applicable to trypsin which contains a similar active site [19].

Acknowledgements—Supported in part by the National Institute of Environmental Health Sciences Grant 5 PO1 ES00049.

REFERENCES

1. W. N. Aldridge, *Croat. chem. Acta* **47** (3), 225 (1975).
2. W. Lee and J. H. Turnbull, *Experientia* **17**, 360 (1961).
3. S. E. Hamilton, N. P. B. Dudman, J. deJersey, J. K. Stoops and B. Zesner, *Biochim. biophys. Acta* **377**, 282 (1975).
4. M. L. Bender and F. C. Wedler, *J. Am. chem. Soc.* **94**, 2101 (1972).
5. M. Eto, J. E. Casida and T. Eto, *Biochem. Pharmac.* **11**, 337 (1962).
6. M. Eto and Y. Oshima, *Bull. agric. chem. Soc. Japan* **26**, 452 (1962).
7. H. Ohkawa and M. Eto, *Bull. agric. chem. Soc. Japan* **33**, 443 (1969).
8. M. Eto, *Rev. Plant Prot. Res.* **9**, 1 (1976).
9. J. E. Casida and K.-B. Augustinsson, *Biochim. biophys. Acta* **36**, 411 (1959).
10. E. Stahl (Ed.), *Thin-Layer Chromatography. A Laboratory Handbook*, 2nd Edn, p. 876. Springer, New York (1969).
11. D. M. Blow, *Accts Chem. Res.* **9**, 145 (1976).
12. R. Tellam and D. J. Winzor, *Biochem. J.* **161**, 687 (1977).
13. R. F. Toia and J. E. Casida, Abstr. 37, Division of Pesticide Chemistry, *One Hundred Seventy-fifth ACS National Meeting*, Anaheim, CA, March, 1978.
14. D. M. Blow, in *The Enzymes* (Ed. P. D. Boyer), Vol. III, Chap. 6, pp. 185–212. Academic Press, New York (1971).
15. M. Iio, M. Eto, M. Eto and H. Omura, *Agric. biol. Chem., Tokyo* **37**, 115 (1973).
16. L.-A. Pradel and R. Kassab, *Biochim. biophys. Acta* **167**, 317 (1968).
17. S. Tsurushin, A. Hiramatsu, M. Inamasu and K. T. Yasunobu, *Biochim. biophys. Acta* **400**, 451 (1975).
18. W. B. Melchior Jr. and D. Fahrney, *Biochemistry* **9**, 251 (1970).
19. J. Kraut, *A. Rev. Biochem.* **46**, 331 (1977).