The great reactivity may well account for the fact that previous attempts to prepare and identify ruthenium hexafluoride have been unsuccessful.

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## THE FORMATION OF THE ACYL-ENZYME INTERMEDIATE, *TRANS*-CINNAMOYL-α-CHYMOTRYPSIN, IN THE HYDROLYSES OF NON-LABILE *TRANS*-CINNAMIC ACID ESTERS[1,2]

Sir:

The hydrolysis of labile acyl derivatives (*e.g.*, nitrophenyl esters) is catalyzed by α-chymotrypsin in a three-step process: (i) adsorption of the substrate on the enzyme; (ii) acylation of the enzyme with the release of the phenol; and (iii) deacylation of the acyl-enzyme giving the carboxylic acid product and regenerating the enzyme.[3] However, it has been maintained on the basis of kinetic arguments that the α-chymotrypsin-catalyzed hydrolysis of methyl hippurate[4,5] does not proceed through a hippuryl-enzyme intermediate and, by implication, that the α-chymotrypsin-catalyzed hydrolyses of other non-labile acyl derivatives also do not involve acyl-enzyme formation.

We have investigated the possible existence of *trans*-cinnamoyl-α-chymotrypsin (I) in the enzyme-catalyzed hydrolyses of a series of substrates, both labile and non-labile, derived from *trans*-cinnamic acid (see Table I). In each case, unequivocal evidence has been found for the involvement of I, both from rate measurements and absorption spectral characteristics. Therefore, we conclude

TABLE I

RATES OF α-CHYMOTRYPSIN CATALYZED HYDROLYSES

| Substrate | pH | $k_2 \times 10^3$ (sec.$^{-1}$) acylation | $k_3 \times 10^3$ (sec.$^{-1}$) deacylation |
|---|---|---|---|
| 1 N-Cinnamoylimidazole[a] | 9.0 | ... | 13 |
| 2 p-Nitrophenyl cinnamate[a] | 9.0 | ... | 13 |
| 3 m-Nitrophenyl cinnamate[a] | 9.0 | ... | 13 |
| 4 o-Nitrophenyl cinnamate[a] | 9.0 | ... | 13 |
| 5 p-Cresyl cinnamate[a] | 9.0 | ... | 13 |
| 6 Methyl cinnamate[b] | 7.80 | 2.66 | (11.1) |
| 7 Benzyl cinnamate[c] | 6.95 | ... | 5.31 |
| 8 N-Cinnamoylimidazole[c] | 6.95 | ... | 5.24 |

[a] $[E]_0 \cong 1.05[S]_0$[d]; $[S]_0 = 2-4 \times 10^{-5} M$; 25.6°; 1.6 to 3% CH$_3$CN. [b] For exact experimental conditions, see Fig. 1. [c] $[E]_0 = 8.68 \times 10^{-4} M$; $[S]_0 = 7.12 \times 10^{-5} M$; 25.0°; 3.2% CH$_3$CN. [d] $[E]_0$ = initial enzyme concentration; $[S]_0$ = initial substrate concentration.

(1) This research was supported by Grant H-5726 of the National Institutes of Health.

(2) Paper VII in the series "The Mechanism of Action of Proteolytic Enzymes," previous paper, M. L. Bender, G. R. Schonbaum, G. A. Hamilton and B. Zerner, *J. Am. Chem. Soc.*, **83**, 1255 (1961).

(3) For references, see G. R. Schonbaum, K. Nakamura and M. L. Bender, *ibid.*, **81**, 4746 (1959).

(4) S. A. Bernhard, W. C. Coles and J. F. Nowell, *ibid.*, **82**, 3043 (1960).

(5) See also M. L. Bender and W. A. Glasson, *ibid.*, **82**, 3336 (1960), for kinetic results in the hydrolysis of N-acetyl-L-phenylalanine methyl ester which cannot be explained on the basis of acyl-enzyme formation.
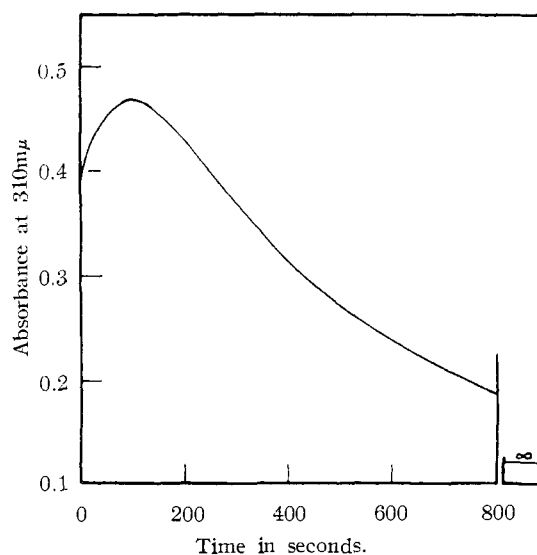
Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of methyl cinnamate: $[E]_0 = 1.33 \times 10^{-3} M$, $[S]_0 = 1.20 \times 10^{-4} M$, pH 7.80, 25.0°, 3.2% CH$_3$CN.

that the formation of an acyl-enzyme intermediate is not an artifact of α-chymotryptic catalyses resulting from the lability of the ester function, but is indeed part of the general mechanism of such catalyses.

The rates of enzyme-catalyzed hydrolysis of substrates 1 to 5, inclusive, are followed conveniently spectrophotometrically at 310 mμ; in each case one observes a very rapid increase in absorbance followed by a slower, first-order decay whose rate constant is $13 \pm 0.5 \times 10^{-3}$ sec.$^{-1}$. Further, the appearance of cinnamate ion at 260 mμ has the same rate as the decrease of absorbance at 310 mμ (loss of acyl-enzyme). Since the hydrolysis of o-nitrophenyl cinnamate has been shown[3] to proceed through I (whose deacylation is rate-controlling), the present data can best be interpreted in terms of the formation of the common intermediate, I, in the hydrolysis of all five substrates.[6] In each case, acylation is very fast compared with deacylation for these labile derivatives.

However, if the intermediacy of I is general, reactions in which either acylation or deacylation is rate-controlling may be expected to occur,[7] for example, in the hydrolysis of alkyl esters. Substrates 6 and 7 are two such esters. In order to make these systems experimentally accessible, high concentrations of the enzyme and ratios of $[E]/[S] > 1$ have been used. The hydrolysis of methyl cinnamate is shown in Fig. 1. The absorbance increases to a maximum in about 100 seconds and then decreases according to a strict first-order rate law (after approximately 450 sec.). The rate constant for this latter process is $2.66 \times 10^{-3}$ sec.$^{-1}$. Deacylation of I under identical conditions (see Fig. 1) has a rate constant of $11.1 \times 10^{-3}$ sec.$^{-1}$.[8] Consequently the decay process for

(6) Substrates 1, 2 and 3 have different rates of acylation; unpublished work with G. R. Schonbaum.

(7) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959).

(8) Since these reactions were carried out at pH 7.8 instead of pH 9.0, the deacylation is somewhat slower here than with substrates 1-5.

methyl cinnamate is a direct measure of the *acylation* reaction.[9] Application of the classical kinetic formulation for two consecutive first-order reactions[10] to this system leads to a quantitative fit of the absorbance *vs.* time curve. Using the independently determined rate constants for acylation $(2.6_6 \times 10^{-3}$ sec.$^{-1})$ and deacylation of the acyl-enzyme, I $(11._1 \times 10^{-3}$ sec.$^{-1})$, together with the independently determined molar absorptivities at 310 $m\mu$ of methyl cinnamate $(\epsilon = 3.27 \times 10^3)$, I $(\epsilon = 1.09_6 \times 10^4)$ and cinnamate ion $(\epsilon = 1.00 \times 10^3$ *under experimental conditions)*, one calculates $t_{max}$ for the absorbance *vs.* time curve as 105 seconds (observed, $100 \pm 10$ sec.), and $A_{max} = 0.49_6$ (observed, $0.47_0$). Thus, both qualitatively and quantitatively the increase in absorbance in the enzyme-catalyzed hydrolysis of methyl cinnamate can be accounted for on the basis of the formation of the highly absorbing intermediate, *trans*-cinnamoyl-$\alpha$-chymotrypsin.[11]

For benzyl cinnamate, the absorbance *vs.* time curve also shows a maximum, again indicating the formation of the acyl–enzyme intermediate. However, in this case, one obtains the rate of deacylation of I from the first-order portion of the curve. For example, the rate constant obtained under the conditions specified in Table I is $5.31 \times 10^{-3}$ sec.$^{-1}$. The rate constant for the deacylation of I under identical conditions is $5.24 \times 10^{-3}$ sec.$^{-1}$. Deacylation is therefore the slow step in the hydrolysis of this ester under the experimental conditions.

Thus the acyl–enzyme hypothesis achieves generality for $\alpha$-chymotrypsin-catalyzed hydrolyses of both labile and non-labile substrates. However, a discrepancy still exists between these results and those of Bernhard, *et al.*,[4] for the specific substrate, methyl hippurate and those of Bender and Glasson[5] for the specific substrate, N-acetyl-L-phenyl-alanine methyl ester.

(9) The values of the acylation and deacylation rate constants for methyl cinnamate are several orders of magnitude smaller than those of specific substrates of $\alpha$-chymotrypsin, but there is no reason to believe that the reactions reported here are qualitatively different from those of specific substrates.

(10) The acylation may be considered to be a pseudo first-order process since the enzyme is in great excess.

(11) Preliminary experiments with $\beta$-phenylethyl and cyclohexyl cinnamates show behavior very similar to that found for the methyl ester.

(12) Alfred P. Sloan Foundation Research Fellow.

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## PREPARATION OF 1,2,4,4-TETRAMETHYL-3,5-DIMETHYLENECYCLOPENTENE; THE MAGENTA SPECIES DERIVED FROM IT BY PROTONATION AND FROM HEXAMETHYLCYCLOPENTADIENE BY HYDRIDE ABSTRACTION

*Sir:*

An intensely magenta solution is obtained from hexamethylcyclopentadiene[1] (I) upon treatment with trityl perchlorate[2] in acetonitrile; $\lambda_{max}$ 552

(1) L. de Vries, *J. Am. Chem. Soc.*, **82**, 5242 (1960); *J. Org. Chem.*, **25**, 1838 (1960).

(2) H. J. Dauben, *J. Am. Chem. Soc.*, **79**, 7557 (1957); *J. Org. Chem.*, **25**, 1442 (1960).

$m\mu$, $\epsilon_{max}$ $10^4$ (in 2 minutes). Hydride abstraction was proved by recovery of triphenylmethane from the pentane extracts.

It became desirable to attempt the preparation of 1,2,4,4-tetramethyl-3,5-dimethylenecyclopentene (II) so as to determine whether protonation of II also leads to the magenta species.

I with N-bromosuccinimide in carbon tetrachloride at 0° gave an unstable bromination product, which was dehydrobrominated readily with trimethylamine in 12 hours at 25°. Preparative vapor phase chromatography of the product yielded a new hydrocarbon, presumably 1,2,4,4-tetramethyl-3,5-dimethylenecyclopentene, (II) y., 29%, $n^{20}$D 1.5181, mass spec. mol. wt., 148. *Anal.* Calcd. for $C_{11}H_{16}$: C, 89.10; H, 10.90. Found: C, 88.96; H, 10.79. The structure assignment is based on these data: (a) infrared bands: $\bar{\nu}$ ($>$C $=CH_2$) 3090 cm.$^{-1}$ (m); 1726 cm.$^{-1}$ (w) (overtone of 860); 1395 cm.$^{-1}$ (m); 860 cm.$^{-1}$ (vs); $\bar{\nu}$ ($H_2C=C-C=C-C=CH_2$) 1618 cm.$^{-1}$ (vs). (b) $\lambda_{max}$ isoöctane 285.4 (sh), 275.0, 265.6, $\epsilon_{max}$ 16750, 25150, 21400. This agrees well with the spectrum of 3,6-dimethylenecyclohexene.[3] (c) n.m.r. spectrum (40 M.c.) in c.p.s. rel. to $Si(CH_3)_4$ solvent: $-43.5$ rel. a rea 6 (*gem*-dimethyl); $-72.3$, rel. area 6 (two vinyl methyl groups); $-185.8$ and $-190.0$ rel. area 2 each (two exocyclic methylene groups). The doublet is ascribed to unequal diamagnetic shielding of the a and b vinyl hydrogen atoms,[4] which do not split each others resonances further due to the low spin–spin coupling constants.[5]

An intensely magenta solution $(\lambda_{max}$ 552 $m\mu)$ could be obtained instantaneously: (1) upon addition of methanesulfonic acid (to conc. $5 \times 10^{-2}\,M$) to an initially colorless acetic acid solution of II $(6.4 \times 10^{-5}\,M)$, $\epsilon_{max}$ 23,500; (2) upon addition of a drop of the bromination solution of I to an ionizing solvent, such as acetic acid, formic acid or aq. HCl ($> 3\,M$). In both cases the color is discharged upon addition of sodium acetate in acetic acid, regenerated upon addition of methane sulfonic acid.
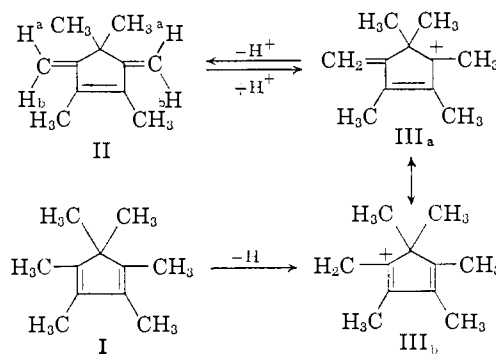


Protonation of II gives undoubtedly III ($III_a \longleftrightarrow III_b$). III is also the expected product from I by hydride abstraction. Since dehydrobromination of the bromination product of I gives II, it

(3) R. E. Benson and V. E. Lindsey, *J. Am. Chem. Soc.*, **81**, 4250 (1959); W. J. Bailey and R. Barclay, *ibid.*, **81**, 5393 (1959).

(4) See n.m.r. of longifolene, S. Dev, *Tetrahedron*, **9**, 1 (1960).

(5) J. A. Pople, W. G. Schneider and H. J. Bernstein, "High Resolution N.m.r.," McGraw-Hill Book Co. Inc., New York, N. Y., 1959, pp. 238-246.