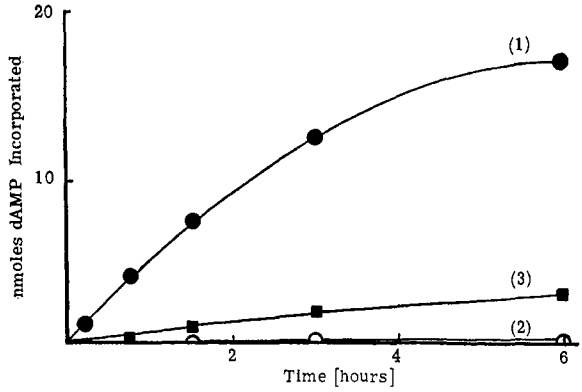

Figure 1. Synthesis of polynucleotides on ϕX174 DNA template in the presence of $MgCl_2$: curve 1 (●), complete system (+ 100 nmol of dTTP); curve 2 (○), incomplete system (−dTTP); curve 3 (■), test system (−dTTP, +d_NTTP from 1 μmol each of d_NT and sodium trimetaphosphate). Onefold synthesis corresponds to incorporation of 7.9 nmol of ^{3}H-dAMP fragments.

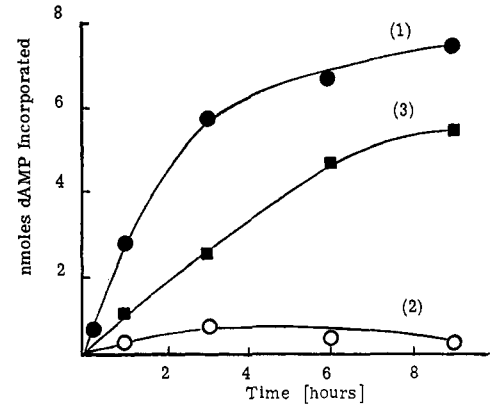

Figure 2. Synthesis of polynulceotides on ϕX DNA template in the presence of $MnCl_2$. The conditions were the same as for experiments in Figure 1, except $MnCl_2$ was used in place of $MgCl_2$.

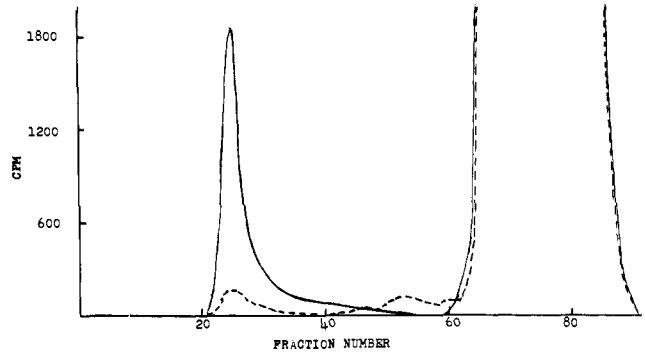

Figure 3. Separation on Sephadex from system containing d_NTTP, after treatment with 15% acetic acid for 5 min (——) and for 11 hr (------). The high CPM in fractions >62 stem primarily from residual, unreacted ^{3}H-dATP.

Studies with model compounds have shown that the cleavage of internucleotide phosphoramidate links in dilute acid solution is rapid relative to depurination of purine nucleotides. One would therefore expect polynucleotides from the enzymatic reactions to be unusually sensitive to acid if they contained phosphoramidate groups. As a test of the incorporation of the aminodeoxythymidine phosphate into the polynucleotides, samples of polymerization mixtures (from systems with nucleotide template to primer ratio 23/1 and with $MnCl_2$) were acidified with acetic acid (to 5% in acetic acid), allowed to stand 16 hr at room temperature, and precipitated with trichloroacetic acid as before. The insoluble material from the complete system (+dTTP) exhibited only slightly fewer counts per minute (5–10% fewer) than the polymer that had not been subjected to the long acid treatment. In contrast, the product from the reaction with d_NTTP showed a reduction of 40–50% in counts per minute, indicative of appreciable breakdown to low molecular weight oligonucleotides during the acid treatment. Additional evidence for lability of the phosphoramidate polymer was obtained by gel filtration on Sephadex G-100 with 1.0 *M* aqueous sodium chloride, which separates polynucleotides according to size.[13] Controls with calf thymus DNA, *E. coli* tRNA, and thymidine showed these three substances to be separated in well-resolved bands with maxima at fractions 23 (void volume), 45, and 70, respectively (1.1-ml fractions were collected). The mixtures from the enzymatic polymerizations were brought to 15% acetic acid by addition of acetic acid and incubated for 5 min and for 11 hr; then they were neutralized with sodium hydroxide and separated on the Sephadex column and the counts per minute in the fractions were determined. For the system containing dTTP, ^{3}H-labeled polynucleotide material came out in a band near the void volume in both cases (5-min and 11-hr reactions). A similar pattern was found for the products from the reaction of d_NTTP after the 5-min acid treatment; however, the pattern for the products from the d_NTTP reaction which had been treated with acid for 11 hr was quite different (see Figure 3). The amount of high molecular weight material was greatly reduced and appreciable material was found in the region between the band for tRNA and the band containing short oligonucleotides and unreacted nucleoside triphosphates. These experiments demonstrate that the polymers obtained from the systems containing d_NTTP contain acid-labile bonds (*i.e.*, phosphoramidate bonds) not found in the polynucleotides derived from the four natural nucleoside triphosphates.

(13) Th. Hohn and H. Schaller, *Biochem. Biophys. Acta*, **138**, 466 (1967); C. A. Hutchison and M. H. Edgall, *J. Virol.*, **8**, 181 (1971).

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Mechanism of the α-Chymotrypsin-Catalyzed Hydrolysis of Specific Amide Substrates

Sir:

I wish to report further[1] evidence in support of an intermediate between the noncovalently bound enzyme–substrate complex and the acyl-enzyme in the chymotrypsin-catalyzed hydrolysis of specific amide substrates. This intermediate does not accumulate but exists in a low steady-state concentration. A change in rate-determining step from its formation to its breakdown with changing pH causes a kinetic pK_a in the pH–k_{cat} profile; k_{cat} varies with pH according to an ionization

(1) A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, **93**, 7079 (1971).

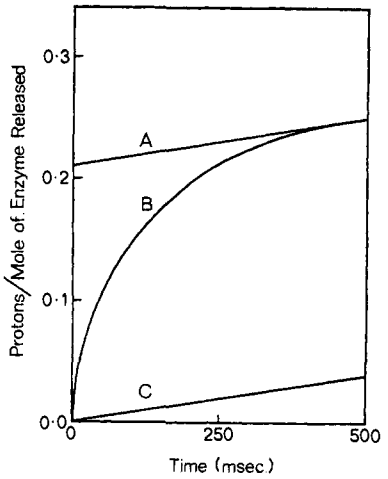

Figure 1. Theoretical and experimental curves for the proton release on mixing substrate (concentration = $1.08K_m$) and enzyme, both incubated at pH 6.10. The time axis is the base line found from mixing the enzyme, incubated in the *p*-nitrophenol buffer, with 0.1 *M* KCl solution. The absorbance changes are relative to this blank. Experimentally, C is found; a negligible burst of proton release followed by a slow, zero-order proton release as the substrate is hydrolyzed. A Michaelis complex or rapidly formed covalent intermediate of pK_a 6.08 would give A. An intermediate of pK_a 6.08 slowly formed from a Michaelis complex of $pK_a \sim 7$ would give B. The initial rate of proton release in B is greater than that expected from the steady-state hydrolysis of the substrate.

curve which does not represent the real ionization of any acid or base in the enzyme–substrate complex.

The pK_a of the catalytically active histidine (His-57) of chymotrypsin is 7.0,[1,2] determined from the pH dependence of k_{cat}/K_m. This residue is slightly perturbed in enzyme–ligand complexes and the pK_a is usually in the range 6.7–7.0.[1] The pH dependence of k_{cat} for amide hydrolysis, where acylation of the enzyme is rate determining, usually gives the pK_a of the enzyme–substrate complex. However, for some anilide and hydrazide substrates this drops to about 6.[1,3,4] Formyl-L-phenylalanine formylhydrazide is hydrolyzed at 25° and ionic strength 0.1 with k_{cat} governed by a pK_a of 6.08 ± 0.06.[1]

There are three possibilities that could cause this anomalously low value: (a) the pK_a of His-57 is genuinely perturbed in the Michaelis complex; (b) a covalently bound intermediate of perturbed pK_a accumulates[3]; (c) an intermediate occurs, does not accumulate, and the rate-determining step of the reaction changes from its formation to its breakdown with changing pH.[1,5]

In cases a and b a genuine ionization occurs. In case c the ionization is not real but is composed of the real ionization constant of His-57 and the ratio of the formation and breakdown rate constants of the intermediate. This leads to a simple experimental test to distinguish between the three. If a genuinely low ionization constant occurs, then on mixing enzyme with substrate there should be a release of protons as the enzyme is converted to the more acidic enzyme–substrate complex.

(2) M. L. Bender, G. E. Clement, F. J. Kezdy, and H. d'A. Heck, *J. Amer. Chem. Soc.*, **86**, 3680 (1964).
(3) M. Caplow, *ibid.*, **91**, 3639 (1969).
(4) T. Inagami, A. Patchornik, and S. S. York, *J. Biochem. (Tokyo)*, **65**, 809 (1969).
(5) W. W. Cleland, "The Enzymes," 3rd ed, Vol. II, P. Boyer, Ed., Academic Press, New York, N. Y., 1970, p 1.

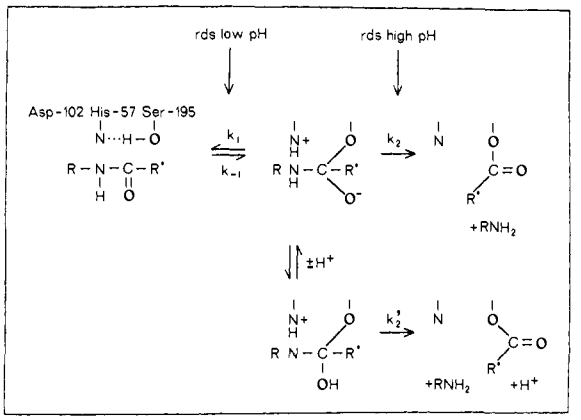

Figure 2. Proposed mechanism for the acylation of chymotrypsin by amides (A. R. Fersht and Y. Requena, *J. Amer. Chem. Soc.*, **93**, 7079 (1971)); $k_{-1} > k_1, k_2$.

For example, if enzyme of pK_a 7.0 is incubated at pH 6.08 and is the mixed with a saturating concentration of formyl-L-phenylalanine formylhydrazide to give a complex of pK_a 6.08, then 0.39 proton/mol of enzyme will be released. If the perturbed pK_a is that of the Michaelis complex there will be a rapid burst of proton release on mixing. If the pK_a is that of an accumulated covalently bound intermediate then there will be a burst of protons released as the intermediate builds up. If c is the case, the pK_a of the enzyme–substrate complex is 6.7–7.0 and a zero or small burst only is expected (see Figure 1).

I find on mixing 2×10^{-5} *M* α-chymotrypsin, incubated in 2×10^{-4} *M p*-nitrophenol as a chromophoric pH indicator, with formyl-L-phenylalanine formylhydrazide in a stopped-flow spectrophotometer (see Table I), that there is a negligible burst of proton release. The initial rate of proton release due to substrate hydrolysis is the same as that found from slow steady-state autotitration experiments (performed on a Radiometer TTTII apparatus) under the same conditions. Complications arising from conformational equilibria, dimerization, autolysis, or *p*-nitrophenol-induced perturbation of the enzyme that could cause a compensating proton uptake were shown to be insignif-

Table I. Initial Proton Release on Mixing α-Chymotrypsin with Formyl-L-phenylalanine Formylhydrazide at 25° and Ionic Strength 0.1[a]

	[H+]/[E] burst on mixing[c]		Initial rate of substrate hydrolysis, sec^{-1}	
pH_0[b]	Obsd	Calcd for pK_E 7.0 and pK_{ES} 6.08[d]	Obsd in stopped flow[e]	Obsd in steady state[f]
6.10	0.01 ± 0.02	0.21	0.08	0.09
6.39	0.00 ± 0.06	0.26	0.10	0.11

[a] Maintained with 0.1 *M* KCl. [b] 2×10^{-5} *M* enzyme, 2.1×10^{-4} *M p*-nitrophenol in 0.1 *M* KCl, and 20 m*M* of substrate in 0.1 *M* KCl; both incubated at this pH prior to mixing in the stopped flow spectrophotometer. [c] Calculated from change of absorbance at 400 nm of *p*-nitrophenolate ion. [d] Enzyme has pK_a of 7.0. k_{cat} follows a pK_a of 6.08. K_m is 8.3 m*M* at pH 6.39 and 9.3 m*M* at pH 6.10 so that the enzyme is 55 and 52% saturated at the respective values. [e] Calculated from the initial rate of change of absorbance at 400 nm. [f] Followed by autotitration in a Radiometer pH-stat.

icant by control experiments. Increasing the *p*-nitrophenol concentration sevenfold did not cause a decrease in observed proton release, nor did the substitution of indole for the substrate. We have previously shown that between pH 6.1 and 6.4 the fraction of α-chymotrypsin in the active conformation is pH independent at 83–84% so that the substrate-induced perturbation of the conformational equilibrium does not lead to pH changes.[6]

This is consistent with case c, and rules out a and b, as previously postulated from indirect evidence.[1] An additional intermediate occurs after the Michaelis complex and does not accumulate.

By analogy with nonenzymatic acyl transfer reactions[7] a reasonable interpretation[1] is that this intermediate is the tetrahedral adduct of Ser-195 with the carboxyl group of the amide. At low pH there is rate-determining formation of the intermediate, as this partitions favorably toward products by an acid-catalyzed route. At high pH its breakdown is rate determining; the intermediate reverts faster to the Michaelis complex than to the acyl–enzyme (see Figure 2).

(6) A. R. Fersht and Y. Requena, *J. Mol. Biol.*, 60, 279 (1971).
(7) A. R. Fersht, *J. Amer. Chem. Soc.*, 93, 3504 (1971).

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On the Mechanism of Epoxidation of Olefins by Covalent Peroxides of Molybdenum(VI)

Sir:

Mimoun, de Roch, and Sajus made the important discovery that molybdenum(VI) peroxo compounds such as I stoichiometrically epoxidize olefins under anhydrous conditions in organic solvents.[1a] These workers have also claimed[1b] that peroxo species of molybdenum, tungsten, and vanadium are regenerated *in situ* during epoxidations of olefins using organic hydroperoxides and catalytic quantities of salts of these metals,[2] strongly implicating these peroxo species as the key intermediates in this whole family of oxidation reactions. Mimoun and coworkers[1a] proposed the mechanism shown in Scheme I for reaction of olefins with complex I. We report here several new observations bearing on the mechanism of these unusual epoxidations.

Due to our interest in the mechanism of epoxidation of olefins by oxo transition metal compounds,[3] we felt it was important to exclude alternate mechanisms in which the oxo oxygen of I is transferred to the olefin. Complex I (L = hexamethylphosphoramide (HMPA), L′ = no ligand) was labeled with ^{18}O uniquely in the oxo oxygen by solution in dry methanol and exposure to 70 atom-% ^{18}O-enriched water for 3 hr at room temperature.[4] The ir spectrum of the exchanged substance

(1) (a) H. Mimoun, I. Seree de Roch, and L. Sajus, *Tetrahedron*, 26, 37 (1970); (b) H. Mimoun, I. Seree de Roch, L. Sajus, and P. Menguy, French Patent 1549184 (1968); *Chem. Abstr.*, 72, 3345*p* (1970); H. Mimoun, I. Seree de Roch, and L. Sajus, *Bull. Soc. Chim. Fr.*, 1481 (1969).
(2) For a leading reference to the numerous studies of these metal-catalyzed hydroperoxide oxidations, see G. R. Howe and R. R. Hiatt, *J. Org. Chem.*, 36, 2493 (1971).
(3) K. B. Sharpless and T. C. Flood, *J. Amer. Chem. Soc.*, 93, 2316 (1971).

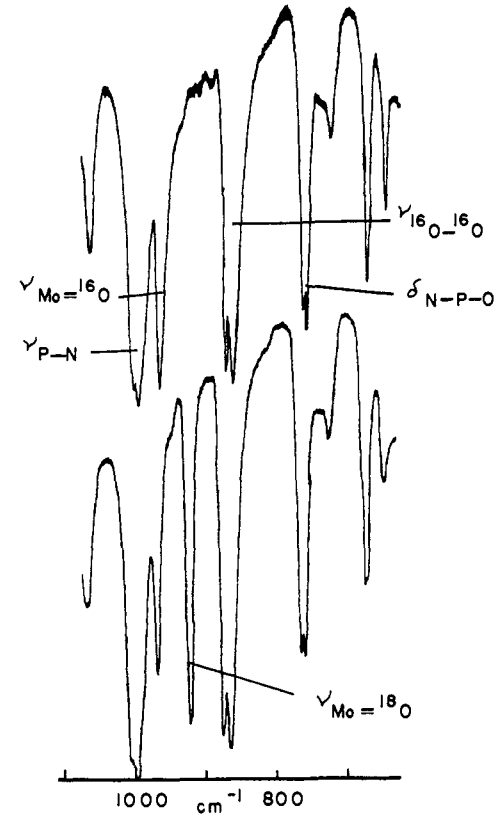
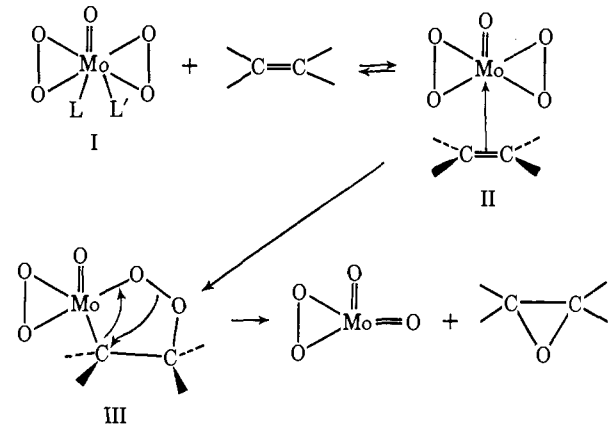


Figure 1. Infrared spectra (Nujol mulls) of $MoO_5 \cdot HMPA$, H_2O before (upper curve) and after (lower curve) exchange with 70 atom % ^{18}O-enriched water. Assignments follow ref 1c.

(Figure 1)[5] clearly indicates approximately statistical incorporation of ^{18}O into the oxo oxygen and no incorporation into the peroxo oxygens.[6] Two reactions of

Scheme I



labeled I (L = HMPA, L′ = no ligand) in dry methylene chloride with *trans*-cyclododecene, in mole ratios of

(4) No attempt was made to determine the minimum time required for exchange.
(5) The spectra shown are of the hydrated complex I (L = HMPA, L′ = H_2O). The labeling procedure and the oxygen-transfer reactions were performed with the anhydrous complex obtained by dehydration of I over P_2O_5 *in vacuo*. The spectra of the anhydrous complexes (labeled and unlabeled) are almost identical with the spectra of the corresponding hydrates in the region shown. Based on the assignments provided for the isotopically normal complex, a simple Hooke's law calculation predicts the $\nu_{Mo-^{18}O}$ band to appear at 920 cm^{-1} and $\nu_{^{18}O-O}$ bands to appear in the 810–850-cm^{-1} region.
(6) Molybdenum-catalyzed decomposition of hydrogen peroxide in ^{18}O-enriched water gave only isotopically normal oxygen; unpublished results of H. Taube, Stanford University. For similar results with other metal catalysts, see A. E. Cahill and H. Taube, *J. Amer. Chem. Soc.*, 74, 2312 (1952).