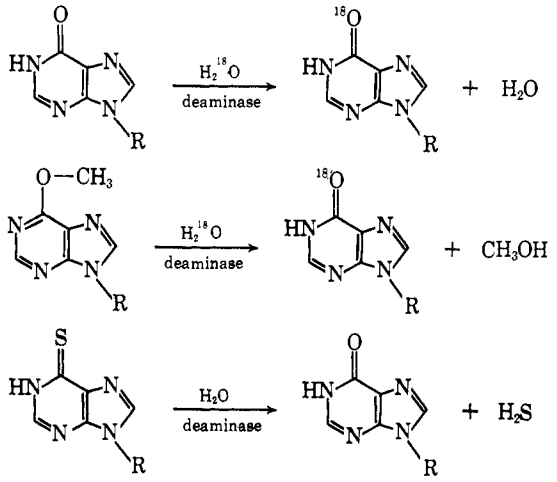

Figure 2. Reactions catalyzed by adenosine deaminase.

negligible exchange during the same period, as expected from the earlier results (Figure 1). Enzymatic cleavage of 6-methoxypurine ribonucleoside therefore occurs between C_6 and oxygen.

Table I. Rate Constants for Adenosine Deaminase Catalyzed Oxygen-18 Exchange with Inosine

Source of enzyme	k(ml/unit/hr) × 10^5
Calf intestine	1.76[a]
Takadiastase	0.41[b]

[a] Calculated from a first-order plot according to the equation

$$2.3 \log \frac{I}{I_\infty - I_E} = k[E]t$$

where I_∞ is the oxygen-18 excess in the completely exchanged product and I_E, the excess in product isolated after incubation with the corresponding concentration of enzyme, [E], for time, t = 24 hr. I_∞ and I_E were corrected for the small nonenzymatic exchange. [b] Calculated from the rate constant for the calf enzyme and the relative slopes of initial velocities shown in Figure 1.

Finally, 6-mercaptopurine ribonucleoside, itself completely stable in neutral solution, was found to be hydrolyzed to inosine and hydrogen sulfide in the presence of calf duodenal adenosine deaminase, as shown by spectrophotometry, paper chromatography, and analysis for sulfide. The binding constant of this substrate has not been determined, but the observed rate constant for this reaction, with 3.3×10^{-3} M substrate in 0.1 M potassium phosphate buffer, pH 6.5, was approximately 0.8 ml/unit hr, somewhat similar to the rate constant for oxygen exchange into inosine.

These reactions (Figure 2) illustrate the catalytic versatility of adenosine deaminases and are consistent with the purinyl–enzyme hypothesis for which independent kinetic support will be published elsewhere. The slowness of removal of the new leaving groups oxygen and sulfur presumably reflects, among other factors, the greater stability of C=O and C=S bonds than the C—N bond which is broken in the normal substrate adenosine.

Desulfuration of 6-mercaptopurine ribonucleoside is of interest for chemotherapy, since this is one of the most effective antitumor agents known,[17] and similar enzymes occur in mammalian serum, spleen, and intestine.[10] Oxygen exchange into inosine suggests a possible method of assay for the hypothetical deaminase which introduces inosinic acid residues at specific positions in yeast transfer RNA;[18–20] takadiastase adenosine deaminase has already been shown to deaminate adenylic acid residues in oligonucleotides.[14] It appears likely that any enzyme which catalyzes deamination of adenylic acid residues in a precursor of transfer RNA should also catalyze oxygen exchange into inosinic acid residues of the finished product.

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The Direct Observation of an Acyl-Enzyme Intermediate in the α-Chymotrypsin-Catalyzed Hydrolysis of a Specific Substrate at Neutral pH

Sir:

The hypothesis that chymotrypsin-catalyzed reactions involve an intermediate acyl-enzyme (eq 1) has proved very powerful in explaining the large body of data available for this enzyme.[1] The acyl-enzyme scheme predicts that the observed turnover kinetics should obey the equations derived from the Michaelis–Menten scheme (eq 2) and it also predicts quantitative

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (1)$$
$$\qquad\qquad + P_1$$

$$E + S \overset{K_M}{\rightleftharpoons} ES \xrightarrow{k_{cat}} E + P_1 + P_2 \quad (2)$$

relations between the macroscopic constants of this scheme and the microscopic constants of eq 1. Such

$$k_{cat} = k_2k_3/(k_2 + k_3) \quad (3)$$

predictions have been verified for some nonspecific substrates (*e.g.*, *p*-nitrophenyl acetate[2] and methyl cinnamate[3]) by direct measurement of the individual rate constants, but direct tests of the scheme's applicability to specific substrates are difficult because of the high rates and the low concentrations of intermediates present in the turnover reaction. However, since the step in which an enzyme–substrate complex (ES) is converted to a covalent compound (ES′) is a nucleophilic reaction and since free carboxylic acids are reactive toward nucleophiles, the hypothesis implies that at low pH the acid corresponding to a specific ester or amide substrate will react with the enzyme to form an equilibrium mixture in which acyl-enzyme will be present (eq 4). The discovery that mixing chymotrypsin

$$E + AH \overset{K_p}{\rightleftharpoons} E \cdot AH \underset{k_3}{\overset{k_{-s}}{\rightleftharpoons}} ES' \quad (4)$$

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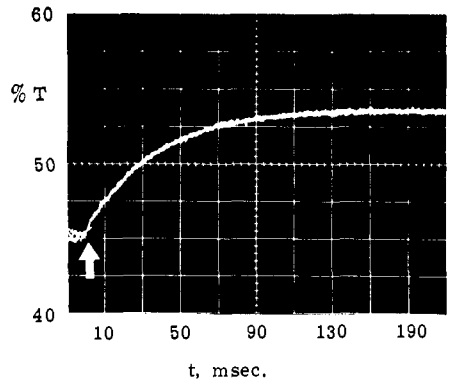

Figure 1. Deacylation of N-(2-furyl)acryloyl-L-tryptophanyl-α-chymotrypsin. Storage oscilloscope trace: per cent transmittance at 340 mμ *vs.* time. Arror indicates where the flow stops. Three consecutive runs are superimposed. After mixing: $[E_0] = 5.25 \times 10^{-5}$ *M*, $[S_0] = 5.00 \times 10^{-5}$ *M*, 0.05 *M* Tris, pH 7.90, $\mu = 0.15$, acetonitrile 0.5% (v/v); 25.0°.

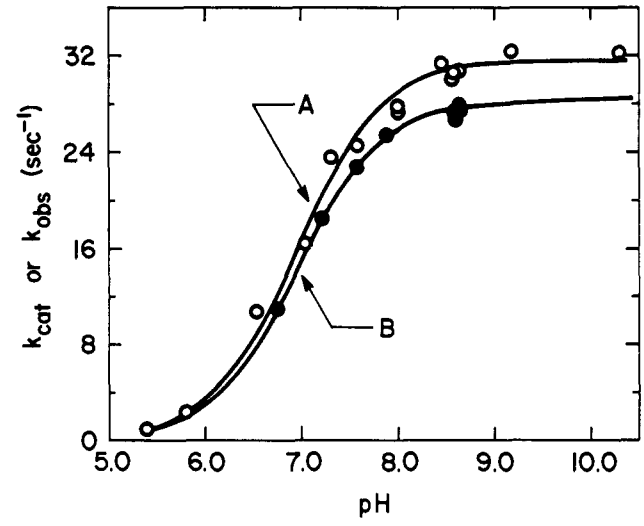

Figure 2. Deacylation of N-(2-furyl)acryloyl-L-tryptophanyl-α-chymotrypsin (k_{obsd}) and α-chymotrypsin-catalyzed hydrolysis of N-(2-furyl)acryloyl-L-tryptophan methyl ester (k_{cat}) at 25.0°: A (○): k_{obsd}, $\mu = 0.15$, 1.0% (v/v) acetonitrile; acetate, phosphate, Tris, or carbonate buffers; $[E_0] \cong [S_0] \cong 5 \times 10^{-5}$ *M*; B (●): k_{cat}, $\mu = 0.15$, 3.2% (v/v) acetonitrile; phosphate and Tris buffers. Both lines are theoretical using $k^{lim}(1/(1 + [H]/K_a))$, $pK_a = 6.95$, $k^{lim}_{cat} = 28.5\ sec^{-1}$, $k^{lim}_{obsd} = 32.0\ sec^{-1}$.

and N-acetyl-L-tryptophan at pH 2.4 results in spectral changes at 311 mμ and at the same time a decrease in the concentration of enzyme active sites[4] constituted one of the best pieces of evidence that at least at low pH an acyl-enzyme could be formed from a specific substrate. More recently, the applicability of eq 1 to specific substrates has been supported by the observation that the specific substrate N-acetyl-L-tryptophan ethyl ester displaces proflavin from the enzyme surface in a two-step process.[5] Using the proflavin-displacement technique, k_2 and K_S for this substrate could be measured up to pH 6.0.

The experiments with N-acetyl-L-tryptophan suggest a method for the *direct* measurement of k_3 for a specific substrate at neutral pH. It should be possible to prepare a chromophoric acyl-enzyme in solution at low pH from a chromophoric specific substrate acid. By rapidly mixing this solution with a relatively concentrated buffer at high pH in a stopped-flow spectrophotometer the pH could then be jumped to well above the pK_a of the acid and the deacylation observed directly by the change in absorbance. Since the carboxylate anion is unreactive toward nucleophiles (in eq 4, $k_{-3} = 0$), the kinetics should be first order (assuming complex dissociation is fast relative to k_3). If the compound

$$ES' \xrightarrow{k_{obsd}} E + A^- \qquad (5)$$

generated at low pH really is the intermediate present in the turnover reaction of a corresponding specific substrate, k_{obsd} should be the k_3 of eq 1 and should be related to the turnover rate constant (k_{cat}) of the substrate by eq 3.

We have generated a chromophoric enzyme derivative from N-(2-furyl)acryloyl-L-tryptophan[6] (I) and chymotrypsin at low pH and studied the kinetics of its decomposition in the pH range of 5 to 9 by means of a "pH-jump–stopped-flow" technique. We have also studied the turnover kinetics of the chymotrypsin-catalyzed hydrolysis of the specific substrate N-(2-furyl)acryoyl-L-tryptophan methyl ester (II). This communication reports the results of these studies.

Figure 1 shows a picture of the per cent transmittance (340 mμ) *vs.* time trace which results when a solution in which chymotrypsin and I have been incubated for at least 2 min at pH 2.4 is mixed with a Tris buffer in a Durrum–Gibson stopped-flow spectrophotometer.[7] The final pH of the mixed solution was 7.90. Plots of log $(A_\infty - A)$ *vs.* time for such experiments were invariably linear to 90% reaction and were used to determine first-order rate constants (k_{obsd}) for this process. When I is omitted from the low pH solution no absorbance change is observed under the conditions described. Also, no comparable absorbance change is observed: (1) in mixing enzyme at low pH with substrate at high pH; (2) in mixing enzyme at high pH with substrate at low pH; or (3) if I and the enzyme are incubated at pH 8.0 and then mixed with the same (pH 8.0) buffer. The observed rate constant is independent of the concentrations of both enzyme and substrate as predicted by reactions 1 and 4. The rate constant is also insensitive to the exact pH of the low pH solution. By varying the pH of the high pH buffer solution k_{obsd} was measured as a function of the final pH. These results are plotted in Figure 2 (curve A). This plot is a sigmoid curve depending on a base of $pK_a = 6.95 \pm 0.05$.

The chymotrypsin-catalyzed hydrolysis of II was followed at 330 or 335 mμ on a Cary 14 spectrophotometer. At enzyme concentrations sufficiently high to avoid wall effects[8] ($>1 \times 10^{-7}$ *M*) the kinetics of this reaction are described by the Michaelis–Menten equation: $v = k_{cat}[E_0][S]/(K_m + [S])$. V_{max} was determined

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(5) K. G. Brandt, A. Himoe, and G. P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967).
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(7) Durrum Instrument Co., Palo Alto, Calif.
(8) R. L. Bixler and C. Niemann, *J. Am. Chem. Soc.*, **81**, 1412 (1959).

from the initial slope of experiments in which $[S_0] >> K_m$. The constant k_{cat} was then calculated using the equation $V_{max} = k_{cat}[E_0]$. The cinnamoylimidazole titration was used to determine $[E_0]$.[9] Figure 2 (curve B) shows a plot of k_{cat} as a function of pH.

We interpret these data as follows. (1) The results of Figure 2 show that k_{cat} and k_{obsd} have the same pH dependency and that k_{cat} and k_{obsd} are approximately equal. (As expected from eq 3, if $k_2/k_3 \neq \infty$, k_{cat} is slightly less than k_3.) This implies that the intermediate formed from the acid at low pH is the kinetically important intermediate in the turnover reaction of the ester (*i.e.*, $k_{obsd} = k_3$) and, furthermore, that this intermediate does not contain the alcohol moiety of the original substrate since it (the intermediate) can be prepared from a species (the acid) in which the leaving group is only water. (2) Only one process is observable in Figure 1. No other intermediate is seen. No nonrandom deviations from first-order kinetics can be observed in either the initial or the final stages of the deacylation reaction. This result argues against theories which involve a rate-determining transfer of the acyl group from one enzyme residue to another. (3) The constant k_3 applies to a first-order process, but k_{cat} is calculated by dividing a zero-order velocity by the enzyme concentration. The agreement of these two constants indicates that the cinnamoylimidazole titration and the other titration procedures[8] really do measure the absolute concentration of those active sites which are involved in the hydrolysis of specific substrates.

These results indicate that in the steady-state hydrolysis of a specific ester substrate there is one kinetically important intermediate (other than an enzyme–substrate complex).[10] The fact that this intermediate is formed at low pH in equilibrium with the acid and the free enzyme strongly suggests that it is an acyl-enzyme although it does not directly identify the enzyme residue which is acylated. Our results do not, of course, exclude the possibility that other steps (such as changes in the conformation of the enzyme) may occur on the reaction pathway. They do indicate, however, that the rates of these steps, if they occur, must be considerably greater than that of the rate-determining step.

We believe that these experiments not only give strong support to the hypothesis that at neutral pH there is on the stopped-flow time scale (milliseconds) one kinetically important intermediate (other than an enzyme–substrate complex) in chymotrypsin-catalyzed hydrolyses of specific ester substrates; they also indicate that this intermediate is an acyl-enzyme.

(9) M. L. Bender, M. L. Begue-Canton, R. L. Blakeley, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Am. Chem. Soc.*, **88**, 5890 (1966).

(10) T. E. Barman and H. Gutfreund (*Biochem. J.*, **101**, 411 (1966)) have argued that the results of their study of the rate of ethanol release in the reaction of chymotrypsin and N-(furyl)acryloyl-L-tyrosine ethyl ester demand the inclusion of intermediates other than those indicated in eq 1. A. Himoe and G. P. Hess (*Biochem. Biophys. Res. Commum.*, **27**, 494 (1967)), however, have shown that these results can be satisfactorily accounted for by eq 1.

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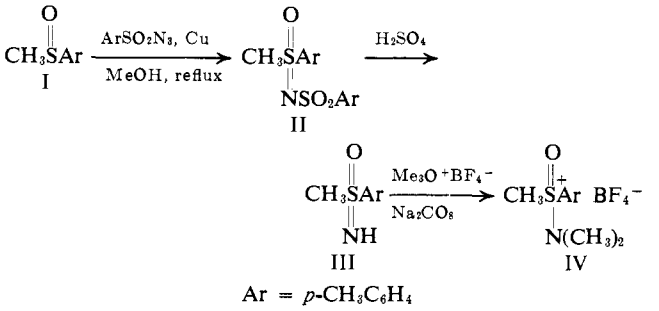
Synthesis of Optically Active Cyclopropanes and Oxiranes Using an Optically Active Oxosulfonium Methylide[1,2]

Sir:

Oxosulfonium ylides derived from salts of sulfoximines were shown in an earlier report to be convenient reagents for the synthesis of oxiranes and cyclopropanes. It was suggested that optically active ylides in this series might be useful in asymmetric synthesis.[3] We have found that optically active N,N-dimethylamino-*p*-tolyloxosulfonium methylide is capable of transferring its methylene in an asymmetric manner to suitably substituted electrophilic double bonds.

Beginning with the optically active sulfoxide I,[4] the salt, (*R*)-(−)-(N,N-dimethylamino)methyl-*p*-tolyloxosulfonium fluoroborate (IV), was prepared (Scheme I).

Scheme I



For the preparation of *dl*-sulfoximine III it was found convenient to react the *dl*-sulfoxide I with hydrazoic acid (sodium azide, sulfuric acid, chloroform); it was found, however, that these conditions lead to racemization of the optically active sulfoxide prior to conversion to sulfoximine. The problem was readily circumvented by the use of the copper-catalyzed decomposition of *p*-toluenesulfonyl azide[5] followed by acid hydrolysis.[6] The over-all yield for the sequence I → IV was 71%. The optical rotations for the various sulfur compounds are given in Table I.

Table I. Characteristics of Sulfur Compounds

Compd	Absolute configuration	$[\alpha]_D$, deg	Mp, °C
I	*R*	+149.1[a,c]	74–76
II	*R*	−144.0[a,d]	158–161
III	*R*	−33.8[a,e]	58–62
IV	*R*	−4.6[a]	65–67
VI	*R*	−165.9[b,f]	64–66

[a] Acetone. [b] Ethanol. [c] Optical purity 96% based on 155° reported by M. A. Sabol, R. W. Davenport, and K. K. Andersen, *Tetrahedron Letters*, 2159 (1968). [d] $[\alpha]_{546}$ −174.6° compared to −172.5° reported by D. R. Rayner, D. M. von Schriltz, J. Day, and D. J. Cram, *J. Am. Chem. Soc.*, **90**, 2721 (1968). [e] $[\alpha]_{546}$ −41.5° compared to −39.9° (reported by Cram)[d]. [f] Lit.[9] for *S* compound, +157°.

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(2) We gratefully acknowledge support by the National Science Foundation (Grant No. GP-5944).

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