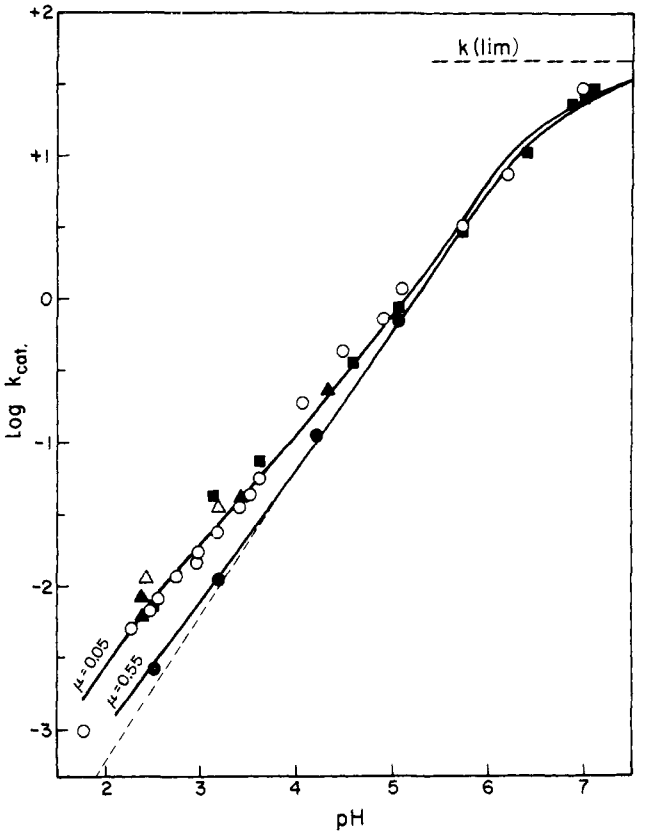

Fig. 1.—The α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan derivatives at 25.0°, 1.6% acetonitrile–water. μ = 0.05: ○, catalytic rate constant of *p*-nitrophenyl ester; ■, catalytic rate constant of ethyl ester; △, deacylation constant of acid with (E) > (S); ▲, deacylation constant of methyl ester with (E) > (S). All rate constants are sec.$^{-1}$; μ = 0.55: ●, catalytic rate constant of *p*-nitrophenyl ester. Lines are theoretical lines using pH − log $\alpha/(1-\alpha)$ = pK'_{int} − 0.868ωZ; ω = 0.062 for μ = 0.05 and 0.025 for μ = 0.55. Dashed line = slope of 1.

absorbance which is characteristic either of the anion of the acid and/or a mixture of the acid and acyl–enzyme.[10] The infinity absorbance of the ester hydrolysis is identical with the infinity absorbance of the reaction of the acid, N-acetyl-L-tryptophan, with α-chymotrypsin under comparable conditions,[10] indicating a true equilibrium between acyl–enzyme and acid under these conditions. The maximal amount of acyl–enzyme (position of the minimum) formed in this reaction is consistent with the calculation of two consecutive first-order reactions, the relative rate constants determined here, and the extinction coefficient of the acyl–enzyme determined later.[10] Other arguments indicating that the minimum is the acyl–enzyme include: (1) formation of the minimum is too slow to be a Michaelis complex since the latter form with rate constants approaching diffusion controlled reactions, and (2) the rate constant of the (equilibrium) decomposition of this minimum is equal to the rate constant of the (equilibrium) acylation of the enzyme by the acid.[10]

The rate constants of the reactions of the methyl ester at pH values of 2.3, 3.4, and 4.3 increase in that order, in agreement with the catalytic rate constants of the ethyl and *p*-nitrophenyl esters. It is not possible to determine precise rate constants for the formation of the intermediate, since the experimental data are too sketchy. However, the data for the decomposition of the acyl–enzyme lead to excellent first-order rate constants. The first-order rate constants from the "tail" of each reaction ((E)$_0$ = 1.8 to 3.8 × 10^{-3} *M* and (S)$_0$ = 6.8 to 7.4 × 10^{-4} *M*; (E)$_0$/(S)$_0$ = 3 to 5.2) are plotted in Fig. 1. These rate constants are similar to the catalytic rate constants (normal turnover constants when (S)$_0$ > (E)$_0$) of the corresponding ethyl and *p*-nitrophenyl esters, indicating that the "tail" of the present reactions is related to k_3, the rate-determining step of the ethyl and *p*-nitrophenyl ester reactions.[2] However, the rate constants from the "tails" of the reactions when (E)$_0$ > (S)$_0$ are slightly higher than the catalytic rate constants when (S)$_0$ > (E)$_0$, for example, k_{obsd}(tail) = 0.7 × 10^{-2} sec.$^{-1}$, whereas k_3 = 0.51 × 10^{-2} sec.$^{-1}$. This observation, however, is readily explained since k_{obsd}(tail) = k_3 + $k_5/(1 + K_m/(S))$ (a combination of deacylation and acylation of the acid) and thus k_{obsd}(tail) must be larger than k_3.[10]

Thus, the spectrophotometric observations of the hydrolysis of N-acetyl-L-tryptophan methyl ester, the pH dependence of the rate constants of the "tails" of these reactions, and the relationship of their rate constants to the catalytic rate constants of the ethyl and *p*-nitrophenyl esters indicate that direct observation indeed has been made of the formation and decomposition of an acyl–enzyme in the hydrolysis of the specific substrate, N-acetyl-L-tryptophan methyl ester.

Both the burst of *p*-nitrophenol from N-acetyl-L-tryptophan *p*-nitrophenyl ester and the time course of the reaction of N-acetyl-L-tryptophan methyl ester unequivocally point to the formation of an N-acetyl-L-tryptophanyl-α-chymotrypsin intermediate in the hydrolysis of these specific substrates. It is thus possible to generalize the acyl–enzyme mechanism represented by eq. 1.

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The Acylation of α-Chymotrypsin by N-Acetyl-L-tryptophan[1]

Sir:

α-Chymotrypsin can catalyze not only the hydrolysis of carboxylic acid derivatives but also the conversion of carboxylic acids to carboxylic acid derivatives.[2] One of these reactions, the α-chymotrypsin-catalyzed isotopic oxygen exchange of carboxylic acids and water,[3,4] has been measured kinetically and found to follow Michaelis–Menten kinetics.[5] More recently an acyl–enzyme has been postulated as an intermediate in α-chymotrypsin-catalyzed reactions,[6] which may be characterized as nucleophilic reactions.[6] One would expect that the α-chymotrypsin-catalyzed oxygen exchange of carboxylic acids would occur through the intermediacy of an acyl–enzyme, and that only the undissociated carboxylic acid would be the reactive species, even at pH 8 (since the carboxylate ion is unreactive toward nucleophiles). Thus one may write eq. 1, where E is enzyme, EAH and EA are the adsorption complexes of the protonated acid and anion, respectively, and EA′ is the acyl–enzyme. The

(1) This research was supported by grants from the National Institutes of Health, part XXVI in the series: The Mechanism of Action of Proteolytic Enzymes.

(2) M. L. Bender, *Chem. Rev.*, **60**, 95 (1960).

(3) D. B. Spriuson and D. Rittenberg, *Nature*, **167**, 484 (1951); F. Vaslow, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **30**, 45 (1956).

(4) M. L. Bender and K. C. Kemp, *J. Am. Chem. Soc.*, **79**, 116 (1957).

(5) The kinetics of the α-chymotrypsin-catalyzed synthesis of an amide from a carboxylic acid has also been investigated [O. Gawron, *et al.*, *Arch. Biochem. Biophys.*, **95**, 203 (1961)].

(6) See M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2582 (1962), for a summary of current information.

$$\begin{array}{ccccc} E + AH & \overset{K_m^{AH}}{\rightleftharpoons} & EAH & \overset{k_5}{\underset{k_3}{\rightleftharpoons}} & EA' + H_2O \\ K_I \updownarrow & & & & \updownarrow K_I' \\ E + H^+ + A^- & & \overset{K_I^A}{\rightleftharpoons} & & EA + H^+ \end{array} \qquad (1)$$

present communication presents evidence that eq. 1 does indeed describe the interaction of N-acetyl-L-tryptophan with α-chymotrypsin.

On the basis of eq. 1, the relationship between the observed rate constant of oxygen exchange and k_5 at $(H) << K_i$ is

$$k_{obsd} = \frac{k_5 K_I^A (H)}{2 K_m^{AH} K_i} \qquad (2)$$

Equation 2 takes into account the ionization of the carboxylic acid, AH, and the inhibitory properties of the carboxylate ion, A. At pH 7.9 and 25.0°, the oxygen exchange of N-acetyl-L-tryptophan had $k_{obsd} = 2.98 \times 10^{-2}$ sec.$^{-1}$ and K_m(apparent) $= 4.0 \times 10^{-3}$ M (equivalent to K_I^A) from the usual Lineweaver–Burk treatment.[4] Using these constants, $K_i = 2.34 \times 10^{-4}$ M, and a K_m^{AH} equal to the (real) K_m for the corresponding methyl ester, 1.18×10^{-3} M,[7] then $k_5 = 323$ sec.$^{-1}$. Thus at pH 7.9, acylation of α-chymotrypsin by N-acetyl-L-tryptophan (the undissociated acid), $k_5 = 323$ sec.$^{-1}$, is faster than deacylation of N-acetyl-L-tryptophanyl-α-chymotrypsin, $k_3 = 46.5$ sec.$^{-1}$.[8]

TABLE I

THE ACYLATION OF α-CHYMOTRYPSIN BY N-ACETYL-L-TRYPTOPHAN AS DETERMINED BY TITRATION WITH N-ACETYL-L-TRYPTOPHAN *p*-NITROPHENYL ESTER[a]

N-Acetyl-L-tryptophan × 10³ M	Time,[b] sec.	Free enzyme, %	$V/(E)_0$ of ester × 10² sec.$^{-1}$
0	0	100[c]	1.013[d]
5.94	0	100	0.886
5.94	10	91.6	.889
5.94	20	74.7	.912
5.94	30	59.9	.902
5.94	40	44.3	.882
5.94	70	41.3	.915
5.94	400	31.2	.873

[a] 25.3°, pH 2.3, citrate buffer, $\mu = 0.01$; burst of *p*-nitrophenol measured at 340 mμ; N-acetyl-DL-tryptophan *p*-nitrophenyl ester $= 5.46 \times 10^{-5}$ M; $(E)_0 = 4.82 \times 10^{-6}$ M; 0.8% acetonitrile–water. [b] Time elapsed between the addition of the enzyme and the *p*-nitrophenyl ester to the solution. [c] After 500 sec., the enzyme gave exactly the same titration value. [d] This value corresponds to k_{cat}; all others in this column are slightly (and equally) depressed due to inhibition.

If in fact the rate constant of acylation is larger than that of deacylation, at a low pH where an appreciable fraction of the carboxylic acid exists in the undissociated form, an equilibrium acylation of the enzyme by N-acetyl-L-tryptophan should be observable. This prediction was tested by determining the amount of N-acetyl-L-tryptophanyl-α-chymotrypsin formed from the acid, using as an analytical tool the titration of the active sites with N-acetyl-L-tryptophan *p*-nitrophenyl ester.[7] Since both the carboxylic acid and the *p*-nitrophenyl ester titrating reagent should give a common acyl–enzyme intermediate, the following should be found: (1) if the enzyme, the acid, and (a high concentration of) the *p*-nitrophenyl ester are mixed simultaneously, the presence of the acid should have no effect on either the initial burst of *p*-nitrophenol or on the steady-state rate constant; whereas (2) if the acid and the enzyme are premixed so that

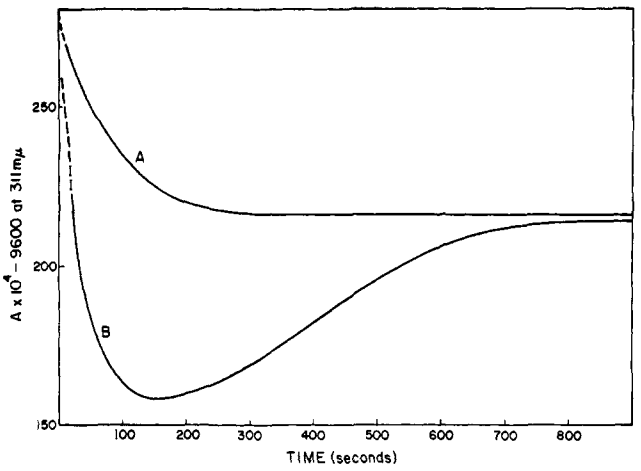

Fig. 1.—The reactions of N-acetyl-L-tryptophan (A) and N-acetyl-L-tryptophan methyl ester (B) with α-chymotrypsin at pH 2.42 and 25.0°, 0.05 M citrate buffer, 1.64% acetonitrile–water, $(E)_0 = 1.83 \times 10^{-3}$ M, $(acid)_0 = (ester)_0 = 6.8 \times 10^{-4}$ M. k_{obsd}(acid) $\leqq 1.1 \times 10^{-2}$ sec.$^{-1}$, k_{tail}(ester) $\leqq 0.7 \times 10^{-2}$ sec.$^{-1}$, k_3(NP ester) $= 0.51 \times 10^{-2}$ sec.$^{-1}$. The curves are Cary 14 PM spectrophotometer traces in which the noise level was $\pm 3 \times 10^{-4}$ absorbance unit.

reaction may take place to form the acyl–enzyme, the addition of *p*-nitrophenyl ester should result in a smaller burst as a consequence of the smaller number of unoccupied active sites, but the steady-state rate should remain identical with that found in (1).

The results of such experiments at pH 2.3 in the presence of N-acetyl-L-tryptophan (Table I) show that the amount of titratable enzyme active sites does decrease markedly with time.

These data, together with a value of k_3 from the *p*-nitrophenyl ester hydrolysis, can be used in eq. 3 and 4, derived from eq. 1 when $(H) > K_i$ and $(S) >> (E)$, to determine values of k_5 and the ratios k_5/k_3.

$$1/(k_{obsd} - k_3) = 1/k_5 + K_m/k_5(S)_0 \qquad (3)$$

$$((E)_0/EA') - 1 = (k_3/k_5) + K_m^{AH} k_3/k_5(S)_0 \qquad (4)$$

The k_5/k_3 ratios at pH 2.3 determined from eq. 3 and 4 are 3.77 and 2.86, respectively; in good agreement at pH 7.9 k_5/k_3 is 6.96. Furthermore, at the two pH values the absolute rate constants (k_5 is 3×10^{-2} and 323 sec.$^{-1}$) indicate that the pK_a on which this reaction depends is 6.86, quite similar to that found for the acylation of the corresponding ethyl ester, 6.77.[8]

The rate constant for acylation of α-chymotrypsin by N-acetyl-L-tryptophan ethyl ester at pH 7.8 is ~1000 sec.$^{-1}$,[9] while that for N-acetyl-L-tryptophan is 323 sec.$^{-1}$. In this nucleophilic reaction, the ratio of reactivities of acylation of ethyl ester–acid is therefore ~3. Under acid catalysis, the reactivity ratio of ethyl ester–acid has been found to be 2.5 to 3.4,[10] in good agreement. The *intracomplex* reaction of the acid with the serine hydroxyl of the enzyme to produce an ester of the enzyme may be compared with the *intramolecular* hydroxy acid–lactone equilibrium, the enzyme equilibrium, $k_5/k_3 = 6.96$ at pH 7.9, and the nonenzymatic equilibrium (lactone)/(hydroxy acid) = 2.66 for γ-hydroxybutyric acid and 13.86 for γ-hydroxyvaleric acid,[11] showing a striking similarity between the acyl–enzyme and five-membered lactones.

A direct spectrophotometric observation of the formation of N-acetyl-L-tryptophanyl-α-chymotrypsin from N-acetyl-L-tryptophan may be made similarly to the experiment described for the methyl ester.[7]

(7) F. J. Kezdy and M. L. Bender, *J. Am. Chem. Soc.*, in press.
(8) M. L. Bender, G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *ibid.*, in press.
(9) M. L. Bender and F. J. Kézdy, *ibid.*, in press.
(10) M. L. Bender, R. R. Stone, and R. S. Dewey, *ibid.*, **78**, 319 (1956).
(11) A. Kailan, *Z. physik. Chem.*, **101**, 86 (1922).

The infinity absorbance (per cent acyl–enzyme at equilibrium) and the rate of approach to infinity [$k_3 + k_5/(1 + K_m/(S))$] are identical for reactions starting with both the acid and the ester (Fig. 1).

Thus an acyl–enzyme is formed from a specific acid substrate of α-chymotrypsin. An excellent preparative method for specific acyl–enzymes may be based on the data of Table I.

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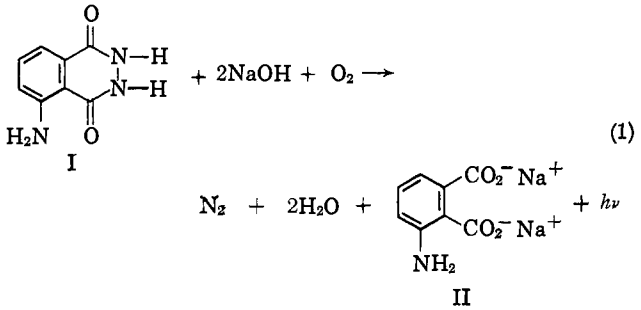
Chemiluminescence of Luminol: The Chemical Reaction[1]

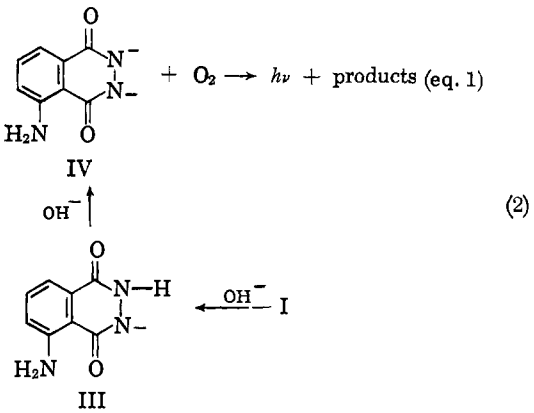
Sir:

Chemiluminescence, the production of light in excess of black body radiation by chemical reactions, is not well understood for systems in the condensed phase. A large number of chemiluminescent reactions have been discovered,[2] but virtually nothing is known about the chemistry involved. We report here an investigation of the chemiluminescence of luminol[3] (5-amino-2,3-dihydro-1,4-phthalazinedione, I), one of the most efficient and probably the best known of the chemiluminescent compounds. Light is produced when basic aqueous solutions of luminol containing oxygen are treated with an oxidizing agent such as potassium ferricyanide, although an increased amount of light is obtained if hydrogen peroxide is included in the system. The oxidizing agents used cause extensive degradation of the reaction products, however, and consequently the only product identified prior to our work was nitrogen.[4] The chemiluminescence in aprotic solvents, such as dimethyl sulfoxide and dimethylformamide, is considerably simpler,[2e] and in these solvents only oxygen and a base are required.



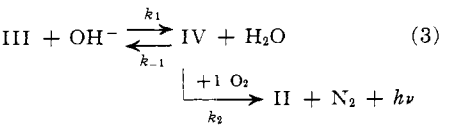
The products of the reaction are nitrogen and sodium aminophthalate (II) (plus traces of the aminobenzoate); these compounds were identified by gas–liquid and paper chromatography and by their spectra. The sodium aminophthalate was converted, furthermore, into acetamidophthalic anhydride and into dimethyl aminophthalate; yields of up to 90% of these compounds were obtained. As will be shown elsewhere,[5] a recovery of this magnitude, coupled with the quantum yields (which require that about 50% or more of the luminol molecules lead to an excited state of II) ensures that the aminophthalate ion is a product of the chemiluminescence proper, and that it does not stem from some accompanying "dark" reaction. Oxygen (1.04 moles) and 2.00 moles of NaOH were consumed in the reaction, and 0.99 mole of nitrogen was formed per mole of luminol, thus indicating the stoichiometry shown in eq. 1.

The reaction of 1 mole of a base with luminol (pK_a = 6)[4] yields a mononegative ion[6] (III), which does not react with oxygen at an appreciable rate. The addition of slightly more than 1 mole of base to solutions of luminol containing oxygen leads to chemiluminescence. The dinegative ion (IV) is undoubtedly the critical intermediate formed by the action of this second molecule of base; the amino group is not directly involved, since the same dependence of chemiluminescence on base was noted for phthalic hydrazide. In the absence of oxygen, basic solutions of luminol are stable indefinitely. The chemiluminescence of luminol, therefore, involves simply the reaction of oxygen with the dinegative ion of luminol (eq. 2).



To confirm this sequence of events, and also to show that the two oxygen atoms introduced into the aminophthalic acid framework came from the oxygen gas and not from hydroxide ion or from adventitious water (*e.g.*, in "anhydrous" dimethyl sulfoxide), the chemiluminescence was carried out with O^{18} enriched oxygen gas in a system of 70 mole % dimethyl sulfoxide, 30 mole % water, and an excess of sodium hydroxide. It was found that over 85% of the label ended up in aminophthalate ion II (the 15% loss of O^{18} was shown by suitable blanks to result from exchange reactions occurring during the analysis).

The kinetics of the chemiluminescence has also been measured in this system. The second ionization constant for luminol is estimated[4] to be about 10^{-13}, and only a low concentration of the dinegative ion (IV) is present in the reaction mixture. Substitution of amino or methoxy groups on the phthalic hydrazide skeleton increases the rate of the reaction, indicating that the oxidation is the slow step in the chemiluminescence. The reaction, therefore, involves an equilibrium with base leading to the dinegative ion, followed by a slow oxidation step (eq. 3).[7]



Using the steady-state approximation,[8] and with the logical assumption that $k_{-1}(H_2O) >> k_2(O_2)$, the ex-

(1) Abstracts, 134th National Meeting of the American Chemical Society, Chicago, Ill., Sept., 1958; p. 94P. This work was supported by the National Institutes of Health (Grant A-2399).

(2) (a) R. S. Anderson, *Ann. N. Y. Acad. Sci.*, **49**, 337 (1948); (b) W. Vaughan, *Chem. Rev.*, **43**, 447 (1948); (c) E. N. Harvey, "A History of Luminescence," The American Philosophical Society, Philadelphia, Pa., 1957; (d) A. Bernanose, *Bull. soc. chim. France*, 567 (1950); 39D (1952); (e) E. H. White, "Light and Life," W. D. McElroy and B. Glass, Ed., The Johns Hopkins Press, Baltimore, Md., 1961, p. 183; E. H. White, *J. Chem. Educ.*, **34**, 275 (1957); (f) H. Stork, *Chemiker Ztg.*, **85**, 467 (1961).

(3) H. O. Albrecht, *Z. Physik. Chem.* (Leipzig), **136**, 321 (1928).

(4) F. H. Stross and G. E. K. Branch, *J. Org. Chem.*, **3**, 385 (1938).

(5) E. H. White and M. M. Bursey, *J. Am. Chem. Soc.*, **86**, 941 (1964).

(6) The sodium salt of luminol has been isolated by E. H. Huntress, L. N. Stanley, and A. S. Parker [*ibid.*, **56**, 241 (1934)].

(7) For the reaction in protic solvents see ref. 2e.

(8) J. Hine, "Physical Organic Chemistry," 2nd Ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 66.