lated and may involve a vicinal dithiol grouping[14] on the coupling factor.

(14) A. Fluharty and D. R. Sanadi [*Proc. Natl. Acad. Sci. U. S.*, **46**, 608 (1960)], have implicated a vicinal dithiol–disulfide oxidation–reduction system in oxidative phosphorylation.

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EVIDENCE FOR A FUNCTIONAL CARBOXYL GROUP IN TRYPSIN AND CHYMOTRYPSIN

Sir:

We wish to report from this Laboratory experimental evidence which indicates that the enzymes trypsin and chymotrypsin possess a functional basic group with a pK in the vicinity of four. The existence of this group in chymotrypsin, concluded from an investigation of the hydrolysis of 2,4-dinitrophenyl acetate (DNPA), has been discussed previously.[1] This work has been verified under different experimental conditions and extended to trypsin using a specific substrate, N-benzoyl-L-arginine ethyl ester (BAEE), with the same result.

The hydrogen-ion inhibition constants, which may be used to help identify functional basic groups in trypsin and chymotrypsin, are given in Table I as pK values. They were determined by studying the effect of hydrogen-ion on the apparent rate constants related to the three-step mechanism presented in Fig. 1. The pK values are tabulated according to the rate constants from which they were evaluated and the enzyme or intermediate to which they correspond, *i.e.*, pK_1, the enzyme; pK_2, the Michaelis complex; and pK_3, the acyl-enzyme.

The pK_1 and pK_2 for chymotrypsin with DNPA were calculated by plotting the apparent values of K_m and I/k_2, respectively, against the hydrogen ion concentration. These apparent constants were obtained at several pH values between 3.6 and 7.1 by investigating the acetylation kinetics employing excess substrate in 10% isopropyl alcohol and using excess enzyme in water. At low pH, it was possible to use conventional mixing techniques, since the acetylation rate constant k_2 becomes small below pH 6.7.[6] The release of 2,4-dinitrophenoxide ion, which exists at low pH, was followed spectrophotometrically and a sensitivity of 1×10^{-7} mole/l. was attained. The results were treated theoretically by methods similar to those used for *p*-nitrophenyl acetate with chymotrypsin[5,7] and trypsin,[8] and DNPA with chymotrypsin.[4]

The apparent values of K'_m[2] and k_3 for trypsin with BAEE were determined by steady-state methods at several pH values, to values as low as 3.6. The pH dependence of these constants was used to evaluate pK_1 or pK_2, and pK_3, respectively. The rates of the hydrolyses were recorded by automatic titration using a Radiometer TTT1 control unit and a titration assembly, which was designed in this Laboratory, with a sensitivity of $>5 \times 10^{-5}$ ml. With this sensitivity, it was possible to follow the hydrolysis at low pH, even though the rate is diminished considerably below pH 5.[9] No abnormal behavior was found at low pH as indicated by

TABLE I

pK VALUES FOR TRYPSIN AND CHYMOTRYPSIN SYSTEMS

System	Related rate constants[a]	Hydrogen-ion inhibition constants pK_i values	Acid–base equilibrium assignments: Related pK_A values	Acid–base equilibrium assignments: Form ionizing
Trypsin–BAEE	k_3	6.25	pK_3	Acyl-enzyme
	K'_m	3.9	...	...
	$K'_m/k_3 = K_m/k_2$	3.9 and 6–6.5	pK_1 and pK_2	Enzyme and Michaelis complex
Chymotrypsin–DNPA	k_3	6.56[a]	pK_3	Acyl-enzyme
	k_2	6.76[b]	pK_2	Michaelis complex
	K_m	4.37[b]	pK_1	Enzyme
	K_m	3.8[c]	pK_1	Enzyme
Chymotrypsin–CI[3]	K_{eq}/k_2	4.4[b] and 6.8[b]	pK_1, pK_2 and pK_s	Enzyme, Michaelis complex and/or substrate

[a] 10% acetonitrile. [b] 1.6% acetonitrile or water, excess enzyme method.[4,5] [c] 10% isopropyl alcohol, excess substrate method.[1]

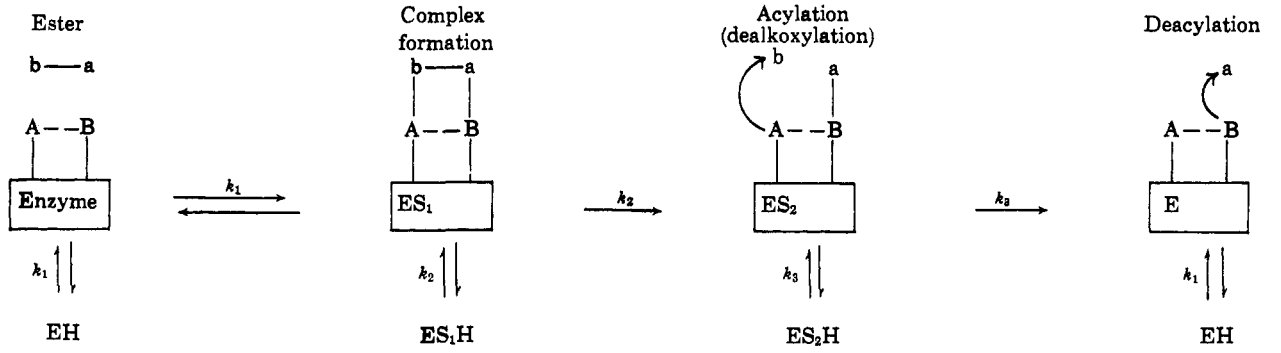

Fig. 1.—Interchange mechanism for esterases: A, carboxyl (aspartyl); B, hydroxyl (seryl); a, acyl, b, alkoxyl or aryloxy; E, enzyme; ES_1, Michaelis complex; and ES_2, acyl–enzyme.

(1) R. A. Dickie and J. A. Stewart, Abstracts of 140th National Meeting of the American Chemical Society, Chicago, Ill., Sept., 1961, p. 10C. In this abstract the reactive group was referred to as an acid; here, it will be referred to as a base, which is more explicit, since it is an electron donor in the pH region of interest.

(2) L. Ouellet and J. A. Stewart, *Can. J. Chem.* **37**, 737 (1959), these authors show that for deacylation $K'_m = [(k_3)/(k_2 + k_3)]\,[(k_{-1} + k_2)/(k_1)]$, which becomes $K'_m = (k_3/k_2)K_m$ when $k_2 > k_3$, so that $K'_m/k_3 = K_m/k_2$ where $K_m = (k_{-1} + k_2)/(k_1)$.

(3) M. L. Bender, G. R. Schonbaum and B. Zerner, *J. Am. Chem. Soc.*, **84**, 2562 (1962).

(4) G. W. Pepple, Senior Research Thesis, University of North Dakota, 1962.

(5) F. J. Kezdy and M. L. Bender, *Biochem.*, **1**, 1097 (1962).

(6) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956).

(7) H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956).

(8) J. A. Stewart and L. Ouellet, *Can. J. Chem.*, **37**, 751 (1959).

(9) H. Gutfreund, *Trans. Faraday Soc.*, **51**, 441 (1955).

the fact that pk_3 *vs.* pH gave a linear plot with a slope of unity as predicted by theory.[10] The K_m', which has been reported as probably being pH independent,[9] became dependent below pH 5. An appropriate plot of K_m/k_2[2] was used to establish the pK 3.9 for the trypsin–BAEE system. Since for this system the acylation kinetics were not studied, the K_m/k_2 ratio was not resolved.

Because the pK 3.9 for trypsin–BAEE is related to K_m/k_2, either the enzyme or the Michaelis complex is involved and this pK may be pK_1 or pK_2. However, the corresponding pK for chymotrypsin, determined by acetylation kinetics, is related solely to the Michaelis constant K_m. This suggests that it is pK_1, and the basic group in question is reactive in the enzyme but bound or non-functional in the intermediates,[11] *i.e.*, the Michaelis complex and the acyl-enzyme.

Also included in Table I are pK values for chymotrypsin with N-*trans*-cinnamoylimidazole (CI),[3] which parallel closely the values for the substrate DNPA. For the chymotrypsin–CI system, the pK = 4.4 was interpreted as being related to an ionizable group in the substrate with a pK_s = 3.65. The discrepancy between pK and pK_s was noted, but not explained. However, it is not necessary to associate this pK with the group in the substrate unless it aids or inhibits the binding of substrate to the enzyme surface. Since the other substrates, DNPA and BAEE, do not ionize in this pH region, at least their pK values may be considered as evidence for a basic group at the enzymic site.

The significance of pK 6–7, which may be indicative of a functional group such as imidazoyl, has been the subject of several publications,[12] but pK values of 3.8–4.4 have not been recognized to any extent as being concerned with esterase activity.

From two points of view the less basic pK values appear to correspond to a carboxyl group. In proteins, the pK range for a carboxyl of aspartyl or glutamyl is 3.0–4.7,[13] which is in agreement with the inhibition constants. Secondly, degradation studies have established that a dibasic acid, such as aspartic or glutamic, usually is condensed in sequence with serine, whose hydroxyl group is believed to constitute part of the esterase site.[12] The sequence at this site for trypsin and chymotrypsin is: aspartyl, seryl. The kinetic evidence, therefore, is in agreement with the possible physical existence of a carboxyl group at the site.

If a carboxyl (aspartic) and hydroxyl (serine) group are considered functional in trypsin and chymotrypsin, then the mode of esterase action becomes a simple interchange mechanism with the subsequent release of products.[14] The qualitative scheme shown in Fig. 1 is characterized by the interaction of complementary groups, and provides a kinetic pathway for both the alkoxyl and acyl moiety of the substrate. The release of products, and possibly the interchange, may be enhanced by nucleophilic attack and/or acid–base catalysis. The proposed scheme, therefore, does not contradict other mechanisms that utilize these features.

The details of the experimental work reported here are being prepared for publication.

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(15) National Institute of Health Predoctoral Fellow.

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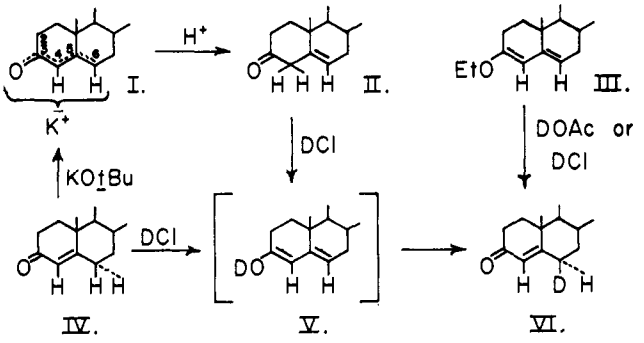
CHEMISTRY OF CONJUGATE ANIONS AND ENOLS. III. PROTONATION[1,2]

Sir:

It appears to be generally accepted[3-5] that protonation of an enolate anion with acid proceeds first on oxygen yielding the neutral enol and therefore it may be anticipated that kinetically controlled protonation of either the anion or the neutral enol will yield the same product.[5] We wish to present evidence demonstrating that although C-protonation of the mesomeric anion (I) of steroidal Δ^4-3-ketones proceeds principally at the α-position, the neutral enol (V) and the enol ether (III) undergo preferential γ-protonation.



Previously, we reported[6] that the addition of aqueous acetic acid to a solution of the potassium enolate (I) in *t*-butanol or diglyme led to the β,γ-unsaturated ketone (Δ^5-3-one) (II) in high yield demonstrating preferential C-4 protonation of the anion. When dilute sulfuric or hydrochloric acid was substituted for acetic acid, deconjugation of the order of 70–80% occurred, providing the solutions were cooled and work-up was rapid. Also, dropwise addition of a diglyme solution of the potassium enolate derived from cholest-4-en-3-one (IV) to a cold vigorously stirred solution of dilute hydrochloric acid (5 equiv.) gave 70% of II.

The acid-catalyzed hydrolysis of an enol ether was studied as a mechanistic parallel to protonation of an enol. 3-Ethoxycholest-3,5-diene (III), hydrolyzed in a mixture of diglyme, deuterium oxide and deuterioacetic acid[7] to about 80% completion, gave 6β-deuteriocholest-4-en-3-one [found[8]: 1.0 atom of deuterium, $\nu_{max}^{CHCl_3}$ 2136 cm.$^{-1}$ (6βD)[9]] free of deuterium at C-4 as demonstrated by complete absence in the infrared of a characteristic[10] C–D band at 2260 cm.$^{-1}$. No Δ^5-3-ketone could be detected although the rate of isomerization of II to VI was found to be much slower than enol ether hydrolysis

(1) Supported in part by grant T-185B, American Cancer Society, and grants CA-4550 and A-4044, U. S. Public Health Service.

(2) Previous paper in this series, H. J. Ringold and S. K. Malhotra, *J. Am. Chem. Soc.*, **84**, 3402 (1962).

(3) A. J. Birch, *J. Chem. Soc.*, 1551, 2325 (1950).

(4) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 568.

(5) H. E. Zimmerman, *J. Am. Chem. Soc.*, **78**, 1168 (1956).

(6) H. J. Ringold and S. K. Malhotra, *Tetrahedron Letters*, 669 (1962).

(7) One hundred milligrams of enol ether, 1 ml. of 50% deuterioacetic acid in 99.8% deuterium oxide, 0.5 ml. of diglyme, slight warming followed by 30 min. at 25°.

(8) Deuterium analyses by Mr. Josef Nemeth, 303 W. Washington St., Urbana, Illinois.

(9) We are very grateful to Mr. Neville Bacon for infrared analyses which were carried out on a Beckman IR-7 with Bausch and Lomb replica grating.

(10) In C-4-deuterated cholest-4-en-3-one, -testosterone and -17α-methyltestosterone, the C-D band appears between 2250 and 2260 cm.$^{-1}$.

(10) M. Dixon and E. C. Webb, "Enzymes," Academic Press, Inc., New York, N. Y., 1958, p. 143.

(11) One of the referees suggested that an ionization constant may be different in the various forms of the enzyme and its intermediates. However, the experimental work here did not detect any change.

(12) For a review see, J. A. Cohen, R. A. Oosterbaan, H. S. Jansz and F. Berends, *J. Cellular Comp. Physiol., Suppl. I*, **54**, 231 (1959).

(13) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N.Y., 1943, p. 445.

(14) J. A. Stewart, Abstracts of 140th National Meeting of the American Chemical Society, Chicago Ill., Sept., 1961, p. 7C.