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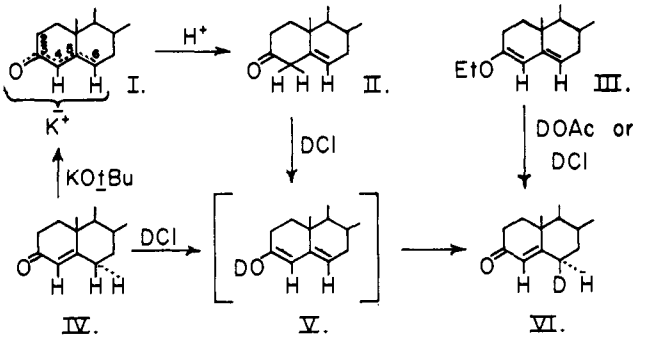
CHEMISTRY OF CONJUGATE ANIONS AND ENOLS. III. PROTONATION[1,2]

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.

under these conditions. The controlled hydrolysis of the enol ether with deuterium chloride in diglyme also gave VI free of C-4 deuterium.

Turning to direct sources of the neutral enol, cholest-5-en-3-one (II) was allowed to conjugate for 5 min. in diglyme–deuterium chloride–deuterium oxide solution[11] yielding the Δ^4-3-one (VI) containing only 1.06 atoms[8] of deuterium. The main C–D band in the infrared appeared at 2136 cm.$^{-1}$ while no more than 0.05–0.1 atom of deuterium was present at C-4.[12] An experiment terminated after 70% conjugation gave recovered II completely free of deuterium. If acid conjugation of the Δ^5-3-one proceeds *via* the $\Delta^{3,5}$-enol, which would appear to be the most likely path, then protonation of the enol, in contrast to the anion, occurs preferentially at C-6.

The deuterium incorporation pattern of the Δ^4-3-ketone in the presence of acid was also investigated. Although $\Delta^{2,4}$- as well as $\Delta^{3,5}$-enol formation is possible the more stable $\Delta^{3,5}$-enol (V) is the kinetically favored product in acid medium.[13] Thus cholest-4-en-3-one (IV) was treated with diglyme–deuterium chloride–deuterium oxide in the same concentrations[11] utilized to conjugate II. A reaction terminated after 7 hr. led to the incorporation of 1.5 atoms[8] of deuterium into the steroid with only 0.05–0.1 atom located at C-4[12] while 24 hr. exchange incorporated 2.4 atoms[8] with only 0.2 atom at C-4.[12] The 7-hr. product was shown to be almost completely deuterated at C-6β by the intensity and appearance of the 2136 cm.$^{-1}$ C–D band, by the shift of the C-4 proton out-of-plane deformation band[14] from 867 to 854 cm.$^{-1}$ and by the marked narrowing[15] of the C-4 proton peak in the n.m.r. spectrum. The deuterium chloride catalyzed exchange of another Δ^4-3-ketone, testosterone, in methanol-d_4, was followed by rapid scanning of the n.m.r. spectrum. After 7 hr. the C-4 proton had undergone little exchange although C-2 and C-6 proton exchange was extensive. A sample which after two hours had incorporated 3.2 atoms[16] (apart from O–D) of deuterium showed only about 0.05 atom[12] of C-4 deuterium. The greater specificity in his case appears to be solvent, rather than substrate-dependent.

These experiments almost certainly indicate preferential protonation of the enol at the γ-position (C-6)[17] in analogy to protonation of the $\Delta^{3,5}$-enol ether and to bromination of the enol.[13] When the enol has dissociated to the enolate anion, the highest negative charge density on carbon is at the C-4 position[3] and protonation[3] and alkylation[18] should occur at that position.[19] Product stability is unimportant in the latter case since there is little bond formation in the transition state[19] while protonation of the neutral enol must involve a greater degree of bond formation with some development of ketonic character. Under these conditions C-6 protonation with formation of the more stable Δ^4-3-ketone will be favored. The results reported herein strongly suggest that the anion protonates directly on carbon rather than on oxygen since formation of the neutral enol in acid medium leads to C-6 and not to C-4 deuteration.

(11) Fifty milligrams of steroid, 0.5 ml. of 0.5 *N* DCl, 5 ml. of diglyme.

(12) The amount of C-4 deuterium was determined by integration of the area of the C-4 proton peak in the n.m.r. spectrum and of the 2260 cm.$^{-1}$ C–D band in the infrared.

(13) This assumption which is supported by the observed (unpublished data, this Laboratory) acid-catalyzed monobromination of the Δ^4-3-ketone at C-6 and by the preferential formation of $\Delta^{3,5}$-enol ethers and -enol acetates is in accord with Hammond's postulate: G. S. Hammond, *J. Am. Chem. Soc.*, **77**, 334 (1955). In the presence of strong acid the transition state for enol formation should resemble the enol more than ketone and thus favor direct formation of the more stable $\Delta^{3,5}$-enol. See also H. J. Ringold and A. Turner, *Chem. Ind.* (London), 211 (1962).

(14) The out-of-plane deformation band of the C-4 proton occurs (KBr pellet) at 867 cm.$^{-1}$ in cholest-4-en-3-one. A 6β-deuterio substituent shifts this peak to 854 cm.$^{-1}$ while extensive deuteration leads to a doublet at 854 and 840 cm.$^{-1}$.

(15) It has been demonstrated (T. A. Wittstruck, S. K. Malhotra and H. J. Ringold, *J. Am. Chem. Soc.*, in press.) that substitution of deuterium, halogen or methyl at the 6β- but not 6α- position reduces the width of the C-4 proton peak in the n.m.r. by removal of long-range coupling with the 6β-proton.

(16) Determined by integration of the area of the methylenic protons (C-2 and C-6) in the n.m.r. We wish to thank Mr. Tom Wittstruck for the n.m.r. spectra which were obtained with a Varian 4300 spectrometer.

(17) An alternate but highly unlikely explanation may be posed for the virtual absence of deuterium at C-4: preferential deuteration of the enol occurs at C-4 yielding the 4-deuterio-Δ^5-3-one which then undergoes isomerization by addition of D^+ at C-6 followed by stereospecific loss of the C-4 deuterium atom despite an adverse isotope effect.

(18) *Cf.* ref. 2.

(19) See ref. 13 for a discussion of the protonation of non-enolic mesomeric carbanions.

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THE STRUCTURES OF THE CROCONATE IONS

Sir:

Recently, interest has been revived in the poly-keto cyclic acids, rhodizonic acid, croconic acid, squaric acid and related compounds.[1] In this connection a report of the structures of one of the members of the series seems appropriate.

The crystal structures of the mono- and diammonium salts of croconic acid have been determined by single crystal X-ray diffraction methods. Both salts crystallize with monoclinic cells: for $(NH_4)_2C_5O_5$, a = 7.45 Å., b = 13.32 Å., c = 3.56 Å., β = 97°, Z = 2, space group = Cm; for $(NH_4)HC_5O_5$, a = 7.71 Å., b = 10.50 Å., c = 7.82 Å., β = 102°, Z = 4, space group = $P2_1/c$. Weissenberg films, $(hk0)$–$(hk2)$ and $(0kl)$–$(5kl)$, were obtained at 80° K. for the diammonium salt. Weissenberg films, $(h0l)$–$(h7l)$, and precession films, $(0kl)$–$(3kl)$, $(hk0)$–$(hk3)$, were made at room temperature for the monohydrogen salt. Intensities were obtained by the visual method.

Structure of Diammonium Croconate.—Systematic absences indicated either space group C2 or Cm. Trial structures for both space groups were obtained from the assumed planar arrangement of the croconate ion and the short c dimension of the cell. Only the structure in Cm refined by least-squares methods to a low discrepancy factor. Approximate positions of the H atoms were obtained from a two dimensional electron density difference map and assumed N–H distances. Inclusion of the H atoms brought the discrepancy factor, R, to 10.6 % at the present stage of refinement (individual isotropic temperature factors).

The structure consists of NH_4^+ ions arranged in columns parallel to the c-axis at the corners of a roughly hexagonal net. The croconate ions, tilted 13° from the ab plane, are stacked above one another in the hexagonal column shaped cavities. The croconate ion is planar; the largest deviation of any atom in the ion from the least-square plane is 0.024 Å. The bond distances and estimated standard deviations for these distances are given in Fig. 1. The nitrogen–oxygen distances range from 2.80 to 3.10 Å.; the smallest intermolecular oxygen–oxygen distance is 2.79 Å. The carbon ring is a perfect pentagon within the precision of the determination.

Structure of Ammonium Hydrogen Croconate.—The structure was first obtained from rubidium hydrogen croconate crystals, which are isomorphous, by a

(1) K. Yamada, N. Mizuno and Y. Hirata, *Bull. Chem. Soc. Japan*, **31**, 543 (1958); M. Washino, K. Yamada and Y. Kurita, *ibid.*, **31**, 552 (1958); S. Cohen, J. R. Lacher and J. D. Park, *J. Am. Chem. Soc.*, **81**, 3480 (1959); **84**, 2919 (1962); R. West, H. Y. Niu, D. L. Powell and M. V. Evans, *ibid.*, **82**, 6204 (1960).