absolute configuration at C_6 has been determined,[11] the molecule is shown in the correct enantiomorphic form.

The conformation of the tetracycline ring system in the present structure differs markedly from that found in the hydrochloride salt structures.[3a,5a] As can be seen from the listing of dihedral angles in Table I, the major differences appear in the A and B rings; while there are significant variations in several structural parameters of the C and D rings, the basic conformations are very similar. In the A ring, the shift between the two conformers involves a drastic twist about the sequence C_4–C_{4a}–C_{12a}–C_1. The required rotations in the ring about the bonds C_4–C_{4a}, C_{4a}–C_{12a}, and C_{12a}–C_1 are 95.6, 109.4, and 94.0°, respectively. As would be expected, the associated rotations about C_1–C_2, C_2–C_3, and C_3–C_4 are relatively small. Similarly, in the B ring, the conformers differ by rotations of 88.1, 108.4, and 103.4° about C_{12}–C_{12a}, C_{12a}–C_{4a}, and C_{4a}–C_5, respectively.

Table I. Selected Dihedral Angles[a] in the Tetracycline Ring System

	Atoms	Angle, deg: 5-Hydroxytetracycline hydrochloride[b]	Angle, deg: 5,12a-Diacetyloxytetracycline[c]
Ring A	C_{12}–C_{12a}–C_1–C_2	−174.7	−80.7
	C_{12a}–C_1–C_2–C_3	19.2	−6.7
	C_1–C_2–C_3–C_4	17.1	−3.9
	C_2–C_3–C_4–C_{4a}	−17.0	−17.6
	C_3–C_4–C_{4a}–C_5	74.0	169.6
	C_4–C_{4a}–C_{12a}–C_1	49.1	−60.3
Ring B	C_{11}–C_{11a}–C_{12}–C_{12a}	−178.5	169.5
	C_{11a}–C_{12}–C_{12a}–C_1	80.7	168.8
	C_{12}–C_{12a}–C_{4a}–C_5	48.7	−59.7
	C_4–C_{4a}–C_5–C_{5a}	170.9	−85.7
	C_{4a}–C_5–C_{5a}–C_6	162.5	133.6
	C_5–C_{5a}–C_{11a}–C_{12}	−13.2	−24.9
Ring C	C_{10}–C_{10a}–C_{11}–C_{11a}	166.5	157.0
	C_{10a}–C_{11}–C_{11a}–C_{21}	178.9	−168.3
	C_{11}–C_{11a}–C_{5a}–C_6	43.0	34.9
	C_5–C_{5a}–C_6–C_{6a}	175.8	174.9
	C_{5a}–C_6–C_{6a}–C_7	−135.1	−139.0
	C_6–C_{6a}–C_{10a}–C_{11}	1.8	6.1
Ring D	C_8–C_9–C_{10}–C_{10a}	7.7	−1.8
	C_9–C_{10}–C_{10a}–C_{11}	177.0	−175.1
	C_{10}–C_{10a}–C_{6a}–C_7	11.4	0.0
	C_6–C_{6a}–C_7–C_8	−178.1	−178.4
	C_{6a}–C_7–C_8–C_9	2.0	1.3
	C_7–C_8–C_9–C_{10}	−2.7	0.4

[a] An arbitrary but self-consistent set defined for the sequence a–b–c–d as the positive clockwise rotation from a to d in the projection of the array down the line b–c. [b] The angles reported here have been calculated from the coordinates provided in ref 5a, with an estimated error given as ~0.01 Å. [c] The estimated error fhr the coordinates from which these angles were calculated is ~0.005 Å.

Despite the inevitable uncertainties in extrapolating from a crystal structure, it is useful to speculate upon the merits of this new conformation as a model for oxytetracycline or tetracycline derivatives in solution;[12] the issue is basic to the detailed understanding of important reaction mechanisms. Of particular interest is a comparison with the interpretation[13] of the nmr spectra of oxytetracycline derivatives in a variety of nonaqueous solvents. The bonding at C_4 and C_{4a} is precisely tetrahedral, the dihedral angle is 169.6°, the hydrogen atoms are in trans positions, and both are axial to the A ring; this conformation is in accord with the apparent coupling constants of 9–13 cps reported[13] for several oxytetracycline derivatives, including the present one. Similarly, in the same molecules, the small (0–2 cps) apparent coupling constants between the C_{4a} and C_5 protons indicate[13] an approximate right-angle relationship between the two protons. Indeed, in the present structure, the actual dihedral angle is 85.7°; the proton at C_{4a} is clearly equatorial with respect to the B ring and the proton at C_5 is, at best, pseudoaxial. The dihedral angle between the protons on C_5 and C_{5a} is 133.6°; this nearly eclipsed configuration about the C_5–C_{5a} bond is consistent with the relatively small coupling constant (<4 cps) observed.[13]

Thus, the present structure fully supports the interpretation of the nmr spectra and provides an excellent detailed model for oxytetracyclines in solution. Nevertheless, in the case of tetracycline, the earlier conformation appears to be more appropriate.[13] The driving force between the two conformations is obscure, but in view of the results[5a,11] on 5-hydroxytetracycline, it cannot simply be the interaction of the –OH or –OAc at C_5 with the substituents at C_6.

Comparison with the two earlier structures reveals some significant differences in bond lengths and angles in the tetracycline ring system; these will be discussed in a detailed presentation of the structure.

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The Reactions of Nonspecific Ester Substrates with δ-Chymotrypsin

Sir:

The analysis of the pH dependence of chymotrypsin-catalyzed reactions remains a knotty problem. In the present communication we wish to report our findings with two nonspecific ester substrates, 4-nitrophenyl 3-nitrophenylacetate (I)[1] and 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone (II),[2] which show that the pH dependencies of the rate parameter k_2/K_s for the acylation or sulfonylation of δ-chymotrypsin by these compounds differ markedly from that previously observed for this enzyme with specific substrates.[3] In particular, we have found that the rates of acylation or sulfonylation of δ-chymotrypsin by the nonspecific substrates I and II, as reflected by the pH dependence of the rate parameter k_2/K_s, are strongly retarded at

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high pH. The acylation of δ-chymotrypsin by specific substrates as measured by the kinetic parameter k_{cat}/K_m (app) which is equal to k_2/K_s is still facile under these conditions.[3]

The rates of acylation of the active site of δ-chymotrypsin[4] by I and sulfonylation by II were measured used an Aminco-Morrow stopped-flow spectrometer at wavelengths of 400 and 390 nm, respectively, in the presence of excess enzyme. From the data good pseudo-first-order rate constants could be calculated (k_{exptl}) which obey the equation $k_{exptl} = k_2E_0/(E_0 + K_s)$. At pH values below 10 enzyme concentrations were employed such that $K_s > E_0$, and the data could be analyzed in terms of the equation $k_{exptl} = k_2E_0/K_s$. Above pH 10, higher enzyme concentrations were used in the case of II and values of the ratio k_2/K_s were determined from the slopes of plots of $1/k_{exptl}$ *vs.* $1/E_0$.

As seen in Figures 1a and 1b, the profile of the pH dependence of the quantity k_2/K_s is bell-shaped in the cases of the reaction of δ-chymotrypsin with both I and II, and in either instance the rate data can be analyzed with the assumption that two ionizing groups in the enzyme affect its acylation or sulfonylation behavior. A least-squares fit of eq 1, which is based on this assumption, to the data of Figures 1a and 1b yielded values of $pK_1 = 6.7$, $pK_2 = 10.1$, and k_2/K_s (lim) $= 4.0 \times 10^5\ M^{-1}\ sec^{-1}$ for the acylation of δ-

$$\frac{k_2}{K_s} = \frac{k_2/K_s(\text{lim})}{1 + ([H]/K_1) + (K_2/[H])} \qquad (1)$$

chymotrypsin by I and $pK_1 = 6.7$, $pK_2 = 9.2$, and k_2/K_s(lim) $= 3.3 \times 10^5\ M^{-1}\ sec^{-1}$ for the sulfonylation of the enzyme by II.[5] The effect of high pH on the acylation or sulfonylation of δ-chymotrypsin by our nonspecific substrates is most dramatically illustrated by our observation that the k_2/K_s value measured at pH 11.5 for I is $0.17 \times 10^5\ M^{-1}\ sec^{-1}$, less than $^1/_{20}$th the value of k_2/K_s(lim).

Although our observations on the high pH behavior of δ-chymotrypsin with our nonspecific substrates contrast markedly with those made in the case of this enzyme with specific substrates, drastic decreases in the rate parameter k_2/K_s, similar to those we have seen, have been observed in the acylation of α-chymotrypsin by specific substrates. It appears that the decreases found in the latter case are due primarily to the inability of α-chymotrypsin to bind specific substrates at high pH.[6-10] Originally, it had been suggested that there is a pH-dependent equilibrium between two major conformations of α-chymotrypsin which causes major

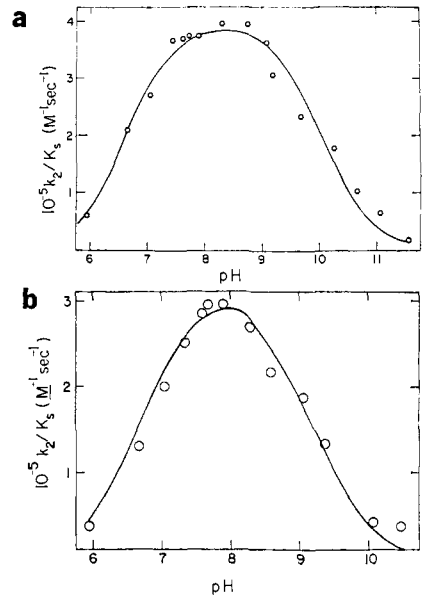

Figure 1. (a) pH–rate profile for the acylation of δ-chymotrypsin by 4-nitrophenyl 3-nitrophenylacetate at 25.0° in buffers of ionic strength 0.4. The curve is a theoretical one for $pK_1 = 6.7$, $pK_2 = 10.1$, and k_2/K_s(lim) $= 4.0 \times 10^5\ M^{-1}\ sec^{-1}$. (b) pH–rate profile for the sulfonylation of δ-chymotrypsin by 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone at 25.0° in buffers of ionic strength 0.4. The curve is a theoretical one for $pK_1 = 6.7$, $pK_2 = 9.2$, and k_2/K_s (lim) $= 3.3 \times 10^5\ M^{-1}\ sec^{-1}$.

changes in the ability of the enzyme to bind substrates and that the state of ionization of the α-amino group of the N-terminal residue, isoleucine-16, governs this equilibrium.[6-10] More recently, in view of the differences in behavior at high pH of α- and δ-chymotrypsin with specific substrates despite the presence of the N-terminal isoleucine in both enzymes, it has been proposed that in the inactivation of α-chymotrypsin at high pH, involvement of residues alanine-149 or tyrosine-146 takes place.[3] These residues are covalently bound in δ-chymotrypsin through the dipeptide threonyl–asparagine.

Our k_2/K_s measurements imply that the deprotonation of the isoleucine-16 amino group is a sufficient condition for the inactivation of δ-chymotrypsin with respect to the nonspecific substrates I and II. If we now consider the simple model for the alkaline pH dependence of the reaction of δ-chymotrypsin with I and II shown in eq 2 where the equilibria between the protonated and the unprotonated forms of the enzyme in the free state and its Michaelis complex with the substrate are represented, two possible explanations for the experimental results we have obtained emerge. One possibility is that binding of the substrate to the enzyme to give the ES species, the Michaelis complex which should predominate at high pH, does not occur readily. An alternative possibility is that the ES complex is formed readily but that it decomposes very slowly, if at all, to give ES′, the covalent acyl–enzyme or sulfonyl–enzyme species. To distinguish between these alternatives, it will be necessary to study the δ-chymotrypsin-catalyzed hydrolysis of nonspecific substrates for which the pH dependencies of the individual parameters k_2 and K_s can be determined.

(4) Worthington lot CDD 6032 δ-chymotrypsin was employed in this work. This is the same lot used in the studies reported in ref 3.

(5) A computer program written by Dr. P. L. Hall was used in these calculations. The parameters k_2 and K_s were not determined separately because this would have required very high enzyme concentrations, and the kinetic results would have been of low reliability because of the problem of enzyme dimerization and other complications. The difference in the pK_2 values measured for I and II with δ-chymotrypsin could be due either to differences in the pH dependence of K_s or that of k_2 for these substrates.

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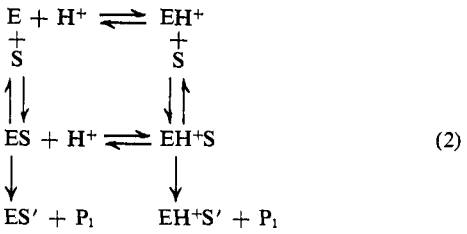

(2)

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The Problem with Carbon-13 Intensities in Proton-Decoupled Nuclear Magnetic Resonance Spectra. Undermining the Overhauser Effect with Free Radicals

Sir:

Although the low natural abundance (1.1%) of the ^{13}C isotope had earlier discouraged extensive use of ^{13}C nmr spectroscopy,[1] recent developments[2,3] in techniques and spectrometer design have now made possible the routine recording of single-sweep spectra of neat liquids and concentrated solutions. One major innovation has been the deployment of noise decoupling[3] the protons, thereby greatly simplifying the spectra by removing the proton multiplet structure. This technique can be considered to serve two functions with respect to circumventing the low signal-to-noise, S/N, in undecoupled spectra. On the one hand, S/N is increased by the collapse of the multiplet structure; however, this does not alter the integrated intensity for a given ^{13}C resonance. On the other hand, the S/N of even the collapsed resonance is further enhanced by the Overhauser effect[4] by up to a factor of 2.988 for dipolar C–H coupling.[5] However, the exact magnitude of the Overhauser enhancement factor, A, is critically dependent on the functionality of the carbon[6] and is thus highly variable even for carbons within the same molecule. Therefore, although the Overhauser effect can be considered advantageous if only S/N improvement is desired to locate resonance positions, its variable magnitude destroys the simple relationship between resonance intensity and the number of ^{13}C nuclei. Hence, the present deployment of noise-decoupled ^{13}C spectra unfortunately does not lend itself to *quantitative* analysis. The success of quantitative applications in proton nmr suggests that it would be highly desirable to extend such capabilities to ^{13}C nmr.

The maximum Overhauser enhancement of 2.988 for dipolar coupling[5] is not realized in most cases because of other relaxation mechanisms for ^{13}C. In the limit that relaxation mechanisms *other* than the C–H dipolar coupling are dominant,[4,5,7] A approaches zero. It therefore occurred to us that the Overhauser enhancement may be reduced upon introducing a *paramagnetic* substance into the solution, with the hope that the intensities would be affected at concentrations well below those where line broadening would become serious.[8] No theoretical justification for our experiments will be offered here. However, our qualitative reasoning was communicated to Natusch, during his visit to our laboratory, who will publish shortly a detailed theory of this effect.[9] We will be concerned here solely with some experimental findings which demonstrate that the addition of free radicals to a sample does indeed *reduce* the ^{13}C intensities of resonances which exhibit Overhauser enhancement.

The ^{13}C spectra were recorded on a Bruker HFX-90 spectrometer operating at 21,150 G, using 10-mm spinning tubes containing a 3-ml sample and a C_6F_6 capillary (^{19}F lock). The present lack of capabilities for integrating time-averaged spectra prevented us from obtaining the integrals for the undecoupled spectra, so that only *relative* intensities in single-sweep spectra could be obtained. The protons were decoupled by locating the CW decoupling frequency in each sample and then recording the spectra using noise modulation centered at the CW frequency.[10] Thus all spectra were recorded under identical conditions so that relative intensities could be easily obtained. Slow sweep rates (0.6–1.2 Hz/sec) were used and care was taken to operate at power levels well below saturation.

In Table I we list the relative ^{13}C integrated intensities (in arbitrary units) of *p*-dioxane as a function of added di-*tert*-butyl nitroxide,[11] DTBN. It is noted that the free radical rapidly reduces the ^{13}C intensity up to concentrations of ~0.025 M. At higher DTBN concentrations, only very slight changes occur, reaching a limiting value of ~10 (arbitrary units). This limiting value suggests that this may be the true intensity with no enhancement, making A in the neat sample ~1.4–1.5.

Table I. Relative ^{13}C Intensities of *p*-Dioxane as a Function of Added Free Radical

I^a	[c]^b	I^a	[c]^b
24.6 ± 1.0	0.000	14.5 ± 1.0	0.020
23.0 ± 1.0	0.004	13.0 ± 1.0	0.025
20.5 ± 1.0	0.007	13.5 ± 1.0	0.030
18.3 ± 1.0	0.010	10.3 ± 1.0	0.050
17.0 ± 1.0	0.015	10.0 ± 1.0	0.075

[a] Relative single sweep integrated intensities, in arbitrary units. [b] Molar concentration of di-*tert*-butyl nitroxide.

As another example, the single-trace integrated intensities of the two resonances in acetone were found

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(10) The minimum modulation was used to ensure a high power density at the CW decoupling frequency. The total decoupling power was maintained at 20 W.
(11) The di-*tert*-butyl nitroxide was purchased from Synvar Corp. The characteristic stability and versatile solubility of nitroxide free radicals makes DTBN an ideal agent for our purposes.