Table I. The Desulfonylation of 2-Hydroxy-5-nitro-α-toluenesulfonyl-α-chymotrypsin (IV)[a]

E_0, M	S_0, M	k, sec^{-1}
7.77×10^{-5}	5.03×10^{-5}	6.78×10^{-4}
7.77×10^{-5}	5.03×10^{-5}	6.81×10^{-4}
7.77×10^{-5}	1.01×10^{-5}	6.63×10^{-4}
7.77×10^{-5}	1.01×10^{-5}	6.71×10^{-4}
1.55×10^{-5}	1.01×10^{-5}	6.77×10^{-4}

[a] At pH 7.58, 1.64% CH_3CN, and 25.0°.

We have examined the kinetics of the desulfonylation of IV at pH 7.58 and 25.0°. The results are indicated in Table I. Excess enzyme was used to eliminate the complications of spontaneous hydrolysis and turnover of the substrate. The kinetics were followed spectrophotometrically at 410 mμ, where $\Delta\epsilon$ is largest. All "runs" obeyed first-order kinetics, and the lack of any change in the rate constant, k, with fivefold variations in enzyme and sultone concentrations removes any doubts about the first-order nature of the observed reaction. Titration and dialysis experiments have also been performed, indicating: (1) that sultone-treated enzyme and native enzyme have identical activities after standing several hours at room temperature; (2) that CI and II attack the same active site,[7] and (3) that there is no detectably permanent incorporation of a chromophoric group. Preliminary results obtained with the unsubstituted sultone, I, show that it too reacts rapidly with chymotrypsin to form a sulfonyl-enzyme which then desulfonylates.[8]

Our observations that the sulfonyl-chymotrypsins produced by the reaction of CT with I and II desulfonylate at pH 7–8 and 25° are in remarkable contrast to the situation found when α-toluenesulfonyl fluoride reacts with the enzyme. In the latter case a fully inhibited species, α-toluenesulfonyl-chymotrypsin, is formed, and desulfonylation does not occur under conditions at which the enzyme is normally active.[9] An important question which arises is whether the phenolic hydroxyl group in IV must be in its protonated form in order for desulfonylation to occur. In an attempt to answer this question we have examined the pH dependence of the rate constant for desulfonylation. As indicated in Figure 1 the desulfonylation pH–rate profile is bell shaped, in contrast to the sigmoid profiles usually observed in the deacylation of acyl-CT,[10] and ionizing groups with pK's of 6.47 and 7.95 appear to be implicated in the desulfonylation reaction. The pK of 6.47 can be reasonably assigned to the ionization of a histidine residue.[11] However, the assignment of the

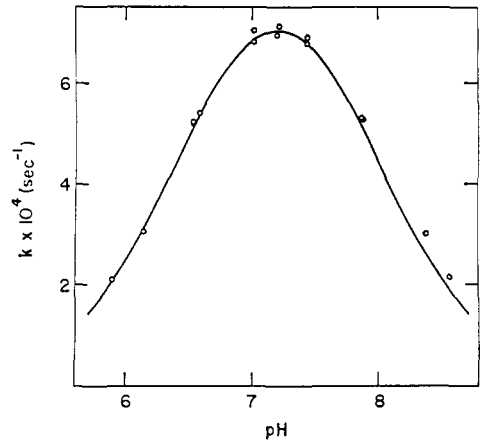

Figure 1.

pK of 7.95 is unfortunately very ambiguous since direct spectrophotometric measurements on the ionization constant of the phenolic hydroxyl in IV give a pK value of 7.2 for this group. One possible explanation for these observations is that the pK of 7.95 is attributable to the ionization of a group on the enzyme rather than to that of the substrate's hydroxyl. Another is that the pK of the phenolic hydroxyl in IV measured directly is different from the kinetically determined value because some change (*i.e.*, a conformational change) occurs in the desulfonylation step so that the pK of the hydroxyl group is perturbed during reaction.

We hope to elucidate the mechanism by which the sulfonylation and desulfonylation reactions of chymotrypsin take place.

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(7) In a typical experiment an excess of CI was added to a CT solution at pH 8. When II was added to this solution no "burst" whatsoever was observed at 390 mμ. The cinnamoyl-enzyme apparently will not form a sulfonyl-enzyme. In a complementary experiment a sixfold molar excess of the sultone I was added to a CT solution, and this solution was allowed to stand about 6 min to ensure the development of steady-state conditions. Although titrations are somewhat less accurate at this higher pH, addition of CI and II to separate samples of this solution showed that excess I cut the concentration of active sites accessible to reaction with *both* CI and II to the same extent. These experiments together with the titration results at pH 5 provide us with an excellent basis for concluding that CI and the sultones attack the same serine residue at the active site (see ref 4).

(8) Chymotrypsin does not appear to react with the acyclic compound *p*-nitrophenyl α-toluenesulfonate.

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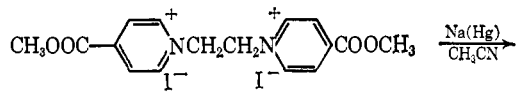
A Pyridinyl Diradical. Preparation and Association

Sir:

We report here the preparation of the stable diradical 1,1′-ethylenebis(4-carbomethoxypyridinyl) (**1**) which exists as a triplet (epr) at 77°K. Radical–radical association produces dimers or polymers without covalent bonds, but with reduced paramagnetism. The ends of the *n*-mer behave like monoradicals,[1] as in the case of Chichibabin's hydrocarbon.[2]



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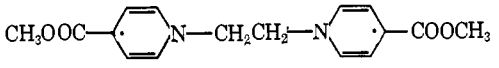

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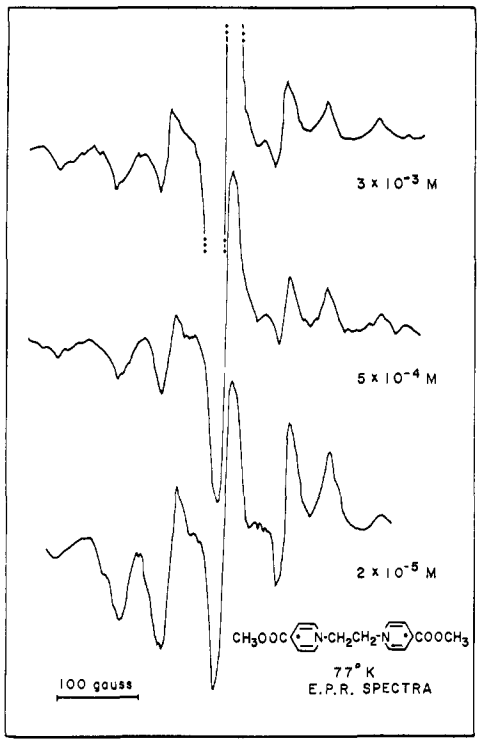


Figure 1. The epr spectrum of pyridinyl diradical **1** at 77°K at three different concentrations (measured with a Varian 4502 system).

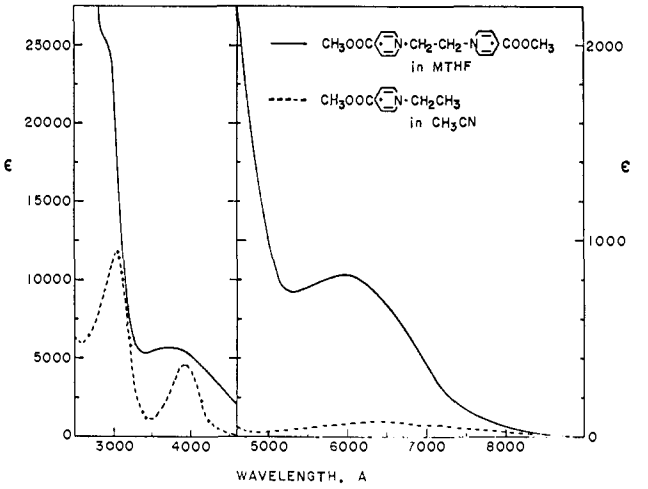
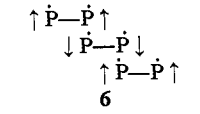


Figure 2. The ultraviolet and visible spectra of the pyridinyl diradical **1** in 2-methyltetrahydrofuran and 1-ethyl-4-carbomethoxypyridinyl (**4**) in acetonitrile. The latter spectrum is adapted from ref 1. Note that the intensity scales for the ultraviolet and visible bands are different.

The bis(pyridinium iodide) (**2**) (mp 208.5–209°)[3] was reduced by the general procedure of Kosower and Poziomek[1] as modified by Waits[4] with 3% sodium amalgam (200% excess) in acetonitrile at 10° for 40 hr. The solvent was removed from the pale green solution, leaving a residue which was extracted with 2-methyltetrahydrofuran (MTHF) and then, after filtration and evaporation, with benzene. The benzene was replaced with 2-methyltetrahydrofuran and spectroscopic, chemical, and epr studies were carried out on the MTHF solution. The concentration of **1** was determined by following spectroscopically the relatively slow formation of methylviologen radical cation from methylviologen dichloride.[5]

At room temperature, the epr spectrum has considerable fine structure for solutions of moderate concentration of **1**. Much less structure is noted in the epr spectrum (measured with the aid of a computer of average transients) of a very dilute solution (*ca.* 10^{-5} *M*). The relative strength of the epr signal *decreases* with increasing concentration, although there is little change in the ultraviolet and visible spectrum (between 2800 and 9000 A). Similar decreases in the epr signal strength have been noted with increasing concentration of the radicals, 1-methyl- (**3**), 1-ethyl- (**4**), and 1-isopropyl-4-carbomethoxypyridinyls (**5**),[6] in MTHF. The dimerization of **3** to diamagnetic diradical dimers as the temperature is decreased for an isopentane solution has been reported by Itoh and Nagakura.[7] We interpret the changes in the spectrum of **1** as due to association of the diradicals producing a species, as shown in **6**,



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which should have an epr resembling that of the monoradical.

At 77°K, the epr spectrum of **1** changes appreciably with the concentration, as shown in Figure 1. The monoradical signal that appears at high concentrations of **1** (due to **6**) disappears almost entirely at low concentrations. The other part of the epr spectrum is typical for a triplet, with three pairs of lines separated by $2D$, $D + 3E$, and $D - 3E$, respectively. The zero-field parameters[8] are $D = 0.0178$ cm^{-1} and $E = 0.0017$ cm^{-1}, with $D - 3E$ equal to 136 gauss. The D value is consistent with a spin–spin dipolar interaction[9] for an average separation of 5.4 A, implying that the pyridinyl radical moieties have either a *gauche* or an *s-trans* relationship.

The spectrum of the diradical **1** is compared to that of the monoradical **4** in Figure 2. It may be noted that the visible absorption is much more intense in the diradical but that there is a general similarity in the two absorption curves.

The diradical **1** should serve as a useful model for other diradicals and polyradicals.

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