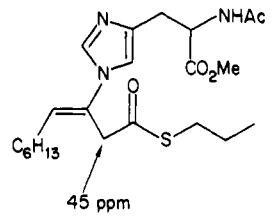
To a 50-mg (1.39 μmol) sample of dehydrase (determined gravimetrically) was added 1.0 subunit equivalent of 3-[2-^{13}C]decynoyl-NAC.[10] (Dehydrase is a dimer, MW = 36000.) Following virtually instantaneous[11] enzyme inactivation, a $^{13}C\{^1H\}$ NMR spectrum of the mixture (Figure 1c) showed the absence of free inhibitor. Instead, two new signals were evident, at 45.0 (LW = 36 Hz)[9] and 110.2 ppm (LW = 28 Hz),[9] in a ratio of ca. 2:1. Further insight came from a preliminary experiment in which aging of inactivated enzyme (2 weeks at 4 °C) caused the ratio of integrated areas of the peaks at 45 and 110 ppm to become *reversed*. This suggests that the 45 ppm signal is due to the initially formed adduct, which undergoes slow conversion to the species exhibiting the 110 ppm resonance.

Clearly, only C-2 of structure **1** (and *not* structure **2**) could produce the 45 ppm resonance. In fact, C-2 of the adduct formed



between *N*-acetylhistidine methyl ester and 2,3-decadienoic acid *n*-propyl thio ester (**3**,[12] C=C configuration unknown) resonates at 44.8 ppm ($CDCl_3$).[13,14]

Application of ^{13}C NMR spectroscopy[15] has also led to resolution of the question of whether one or two inactivator molecules are bound per dehydrase dimer.[16] Thus, to the sample giving the spectrum shown in Figure 1c was added a second equivalent of inactivator. While the proportions of the 45 and 110 ppm signals were changed (vide supra, Figure 1d), importantly, no free inhibitor was detected. Additionally, the total integrated area of the bound species was twice what had been observed in Figure 1c. With the addition of a third equivalent (Figure 1e), a sharp new resonance at 27.5 ppm (LW = 3 Hz, LB = 0)[9] appeared rapidly ($t_{1/2}$ = 10–15 min) at the expense of free thio ester (33.6 ppm). Control experiments showed that 3-[2-^{13}C]decynoyl-NAC is stable in buffer and that this 27.5 ppm resonance owes to the corresponding free acid, presumably formed by nonspecific, protein-catalyzed hydrolysis of the thio ester.[17] In addition, the small new signal at 45.4 ppm (LW = 13 Hz)[9] suggests that a small portion of the inactivator has become bound (as **1**) to a histidine on the periphery of the enzyme.[18]

In conclusion, inhibition of dehydrase by 3-decynoyl-NAC involves isomerization to 2,3-decadienoyl-NAC, followed rapidly by reaction of the latter with an active site histidine, forming species **1**. The stoichiometry of inactivation is clearly one molecule of inactivator for each dehydrase subunit, providing additional evidence that the subunits are identical and refuting the proposed "half-of-the-sites" reactivity.[16a,c,19]

Acknowledgment. Work at Catholic University was supported by the NIH, via Grant GM 26074 (to J.M.S.). We acknowledge instrumentation grants from the NSF (PCM 83-03176) and the NIH (RR 01934) and thank the A. P. Sloan Foundation for support of experiments conducted at Johns Hopkins. We thank Professors Bloch and Stein for conversations leading to simultaneous preparation of manuscripts.

(6) The isolation and purification of dehydrase from *E. coli* DM51 A will be described elsewhere.[7]

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(9) See legend to Figure 1.

(10) 3-[2-^{13}C]Decynoyl-NAC was made from 1-octyne by the following steps: (a) EtMgBr;[20] (b) $^{13}CH_2O$;[20] (c) PBr_3, pyridine;[20a] (d) CuCN, LiBr, DMF;[21] (e) MeOH, HCl;[16b] (f) dilute NaOH;[16b] (g) $PhOPOCl_2$, Et_3N;[22] (h) $TlSCH_2CH_2NHCOCH_3$.[23] $^{13}C\{^1H\}$ NMR δ 33.6 (see Figure 1a).

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Direct, ESR Observation of the Localized Biradical 1,3-Dimethyl-1,3-cyclobutadiyl

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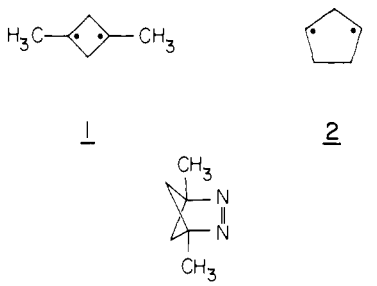
We describe herein the direct observation of the triplet state of 1,3-dimethyl-1,3-cyclobutadiyl (**1**) by ESR spectroscopy and a study of its thermal behavior. Buchwalter and Closs' landmark observation of triplet 1,3-cyclopentadiyl (**2**)[2] nearly 10 years ago



1 **2**

3

appeared to open the way for the direct spectroscopic study of localized biradicals, an important class of reactive intermediates.[3]

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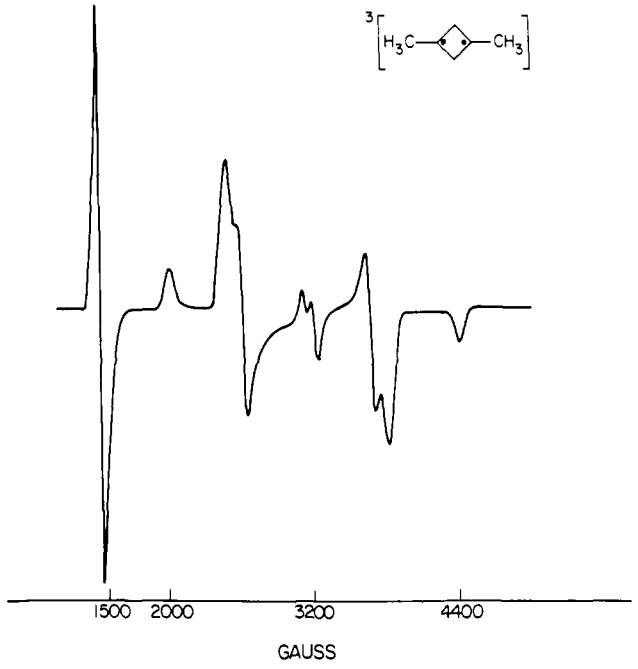

Figure 1. ESR spectrum of biradical **1** generated by irradiation of **3** in an MTHF matrix at 9.5 K. A weak monoradical signal is also present.

Additionally, Closs' study of **2** suggested heavy-atom tunneling[4] in the ring-closure reaction to give bicyclo[2.1.0]pentane. The significance and generality of these intriguing results, however, could not be explored because extension of such studies to other systems has not been successful. Despite our failure[5] to observe the parent 1,3-cyclobutadiyl, ab initio theoretical studies we had carried out[6] convinced us that cyclobutadiyls can possess triplet ground states, which may well be a necessary criterion[2,6] for success in such ESR studies. We therefore extended our studies to the dimethyl system, in the hope that radical-stabilizing substituents would facilitate observation of the biradical.

Photolysis of a frozen (<25 K) solution of azoalkane **3**[7] in 2-methyltetrahydrofuran (MTHF), propylene glycol, or isopropyl alcohol in the cavity of an ESR spectrometer produces a well-defined spectrum that is clearly ascribable to an organic triplet. Figure 1 shows a spectrum of **1** in MTHF, which is the superior solvent for obtaining high signal-to-noise ratios. That **1** is the carrier of the signal can be determined from the zero-field splitting (zfs) parameters of Table I. The D value (Table I) is a sensitive probe of the distance between the radical centers in such structures. This separation has been calculated by Schaefer[8] to be 2.37 Å in biradical **2**. Using the same level of theory (valence double-ζ basis set, ab initio HF), we find the analogous distance in triplet 1,3-cyclobutadiyl to be 2.10 Å.[9] If we apply our previously developed, semiempirical scheme for zfs calculations[10] to **1** and **2** and scale the results to precisely reproduce the experimental value for **2**, we calculate for **1** $|D/(hc)| = 0.114$ cm^{-1}. While the remarkable agreement between theory and experiment must be fortuitous to some extent, these results clearly indicate that both the direction and the magnitude of the change in D on going from **2** to **1** are fully consistent with our assignment of **1** as the carrier of the signal.

Table I. Zero-Field Splitting Parameters for Various Biradicals

biradical	$\|D/(hc)\|$, cm^{-1}	$\|E/(hc)\|$, cm^{-1}	ref
CH_2:	0.69	0.003	*a*
	0.027	0.0023	*b*
	0.084	0.0020	*c*
	0.112	0.005	*d*

[a] Bernheim, R. A.; Bernard, H. W.; Wang, P. S.; Wood, L. S.; Skell, P. S. *J. Chem. Phys.* 1970, *53*, 1280–1281. [b] Berson, J. A.; Bushby, R. J.; McBride, J. M.; Tremelling, M. *J. Am. Chem. Soc.* 1971, *93*, 1544–1546. [c] Reference 2. [d] This work.

When photolysis is discontinued, the signal due to **1** decays. Accurate determination of the kinetic parameters for the decay is frustrated by the fact that, as in other studies in rigid media,[2,11] the decay kinetics are nonexponential. Initial rates are relatively rapid, with half-lives on the order of minutes.[12] As the decay continues the rate slows considerably, such that after 1 h as much as 30–60% of the original signal intensity remains, and the decay rate is imperceptibly slow. These results indicate that matrix site effects play a significant role in the decay. We do note that, as in the study of **2**,[2] the initial rates of decay of **1** are quite insensitive to temperature, varying by a factor of less than 10 over the range 4–25 K.[12] To the extent that these initial rates are indicative of the decay process, such behavior requires either a very small activation energy (and, necessarily, an extremely low preexponential term to reproduce the rates observed)[13] or the operation of quantum-mechanical tunneling in the decay.[14]

Further studies on the effects of various substituents on the spectroscopy and chemistry of 1,3-cyclobutadiyls are under way.

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(12) Decay rates were measured after photolysis with a 1000-W Xe arc lamp by monitoring the intensity of the half-field transition. Typical initial rates obtained by using 15-s photolysis are 2.6 (±0.5) × 10^{-2} s^{-1} at 9.5 K and 4.2 (±0.5) × 10^{-2} s^{-1} at 25 K.

(13) For example, to reproduce the rates listed in ref 12 would require E_a = 0.015 kcal/mol and A = 0.063.

(14) Given that in all of our studies of the 2,3-diazabicyclohexene system[5,7,15] we have never seen hydrogen-shift (cyclobutene) products and that hydrogen abstraction from the matrix has been shown in other related systems[2,11a] to be much slower than the rates we observe, it seems probable that the decay process involves ring closure of **1**.

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