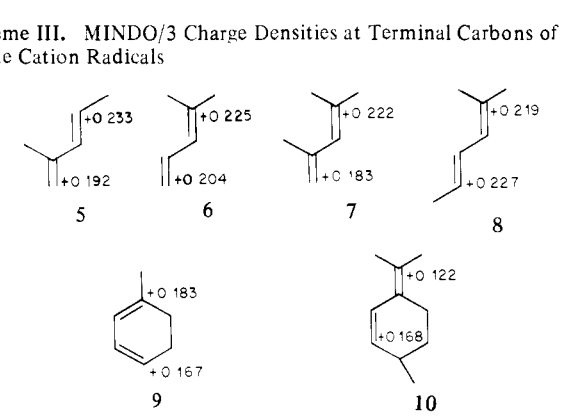
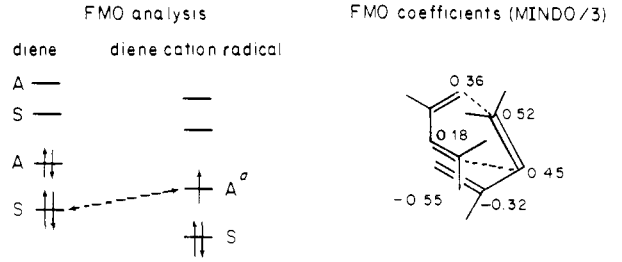
Scheme III. MINDO/3 Charge Densities at Terminal Carbons of Diene Cation Radicals



5 6 7 8 9 10

Scheme IV



[a] Locally symmetric (i.e., bonding at C_5–C_6).

substitution pattern). MINDO/3 charge densities are displayed in Scheme III for the six dienophilic cation radicals. In every case but **9**, the charge density is greatest at the terminal carbon with the highest reactivity. In this specific case the charge differential is especially minute and may reasonably be presumed to be dominated by the substantial steric repulsions involved in the bond formation process at the disubstituted dienic terminus. The existence of a charge density correlation appears theoretically plausible for a reaction having an early (reactant-like) transition state and especially if the cycloaddition reaction path is nonsynchronous, as suggested by MINDO/3. An FMO analysis[6] of the pericyclic transition state leads to essentially the same chemoselection predictions and in addition permits prediction of regioselection (Scheme IV). The appropriate FMO analysis focuses on the *locally* symmetric dienophile cation radical SOMO and the most bonding diene π MO, which are the most proximate in energy. The relevant MINDO/3 coefficients are illustrated for **7** in Scheme IV. Once again, the chemoselectivities of (1,2) dienes **8–10** requires recognition of the predominance of steric effects in these marginal cases.

In exploiting the CRDA for synthetic purposes, it should be remembered that these reactions are usually complete in 10–15 min at 0 °C and longer reaction times can lead to yield losses. Consequently, GC or other monitoring is recommended. It has been found that 3–5% of the aminium salt, which is now available from Aldrich Chemical, is usually sufficient to assure rapid and complete reaction.

Present research is concentrating on extending the CRDA and establishing new types of cation radical pericyclic processes.

Acknowledgment. We thank the National Science Foundation (CHE-8121700) for support of this research.

Registry No. 3β-(2-Methylpropanol)[2.2.2]bicyclooct-5-ene, 40572-29-0; 2,2-dimethyl-3α-ethenyl[2.2.2]bicyclooct-5-ene, 81277-87-4; 2α-(2-methylpropenyl)[2.2.2]bicyclooct-5-ene, 40600-18-8; 2,4,4,6,6-pentamethyl-5-isopropylidenecyclohexene, 68930-33-6; 2α-methyl-3β-(2-methylpropenyl)[2.2.2]bicyclooct-5-ene, 81277-88-5; 2α-methyl-3α-(2-methylpropenyl)[2.2.2]bicyclooct-5-ene, 81339-57-3; 2,2-dimethyl-3α-propenyl[2.2.2]bicyclooct-5-ene, 81277-89-6; (1β,4β,4aβ,8aβ)-1,7-dimethyl-1,4,4a,5,6,8a-hexahydro-1,4-ethanonaphthalene, 81277-90-9; (1β,4β,4aα,8aα)-1,7-dimethyl-1,4,4a,5,6,8a-hexahydro-1,4-ethanonaphthalene, 81339-58-4; 5-methyl-1,4,4a,5,6,7,8a-heptahydro-8-isopropylidene-1,4-ethanonapthalene, 81277-91-0; **1**, 592-57-4; *t,t*-**2**, 5194-51-4; **3**, 81277-83-0; **5**, 1118-58-7; **5** radical cation, 74111-61-8; **6**, 926-56-7; **6** radical cation, 74056-42-1; **7**, 1000-86-8; **7** radical cation, 81277-84-1; **8**, 28823-41-8; **8** radical cation, 81339-55-1; 9, 1489-56-1; **9** radical cation, 81277-85-2; **10**, 586-63-0; **10** radical cation, 81339-56-2; 1,3-butadiene, 106-99-0; ethene radical cation, 34470-02-5; cyclohexene radical cation, 34469-90-4; 2β-methyl-3α-(1-methylethenyl)[2.2.2]bicyclooct-5-ene, 81277-86-3.

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Evidence by Gel Filtration at Subzero Temperatures for the Covalent Reaction Intermediate of Carboxypeptidase A in Ester Hydrolysis[1a]

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The primary advantage of cryoenzymologic methods[2-4] in the study of enzyme mechanisms derives from the potential to accumulate and stabilize reaction intermediates for structural and chemical characterization. With cryoenzymologic techniques, we have demonstrated temporal resolution of the esterolytic reaction catalyzed by carboxypeptidase A (CPA) and formation of a reaction intermediate that is best described as a mixed anhydride species.[5-7] X-ray diffraction studies[8,9] do not distinguish between the possible roles of the catalytically active residue glutamate-270 as a nucleophile in forming an acylenzyme (mixed anhydride) reaction intermediate or in positioning a hydrogen-bonded water molecule as the nucleophile in a general base mechanism. In addition, nucleophile trapping studies[10,11] designed to detect reaction intermediates of CPA have not been successful. In this communication we demonstrate that the reaction intermediate observed under subzero temperature conditions can be isolated by gel filtration at −60 °C and, therefore, is a covalent acylenzyme species.

The hydrolysis of the specific ester substrates *O*-(*trans*-*p*-chlorocinnamoyl)-L-β-phenyllactate[5,13] (ClCPL) and *O*-3-(2,2,5,5-tetramethyl-1-oxypyrrolinyl)propen-2-oyl-L-β-phenyllactate[14,15] (TEPOPL) catalyzed by CPA is governed by rate-limiting breakdown of the reaction intermediate that is detectable at subzero temperatures. The half-life of the reaction intermediate

(1) (a) This work was supported by NIH Grant GM 21900. (b) Predoctoral student supported by a training grant of the NIH (GM 07183).
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(11) Nucleophile trapping of a mixed-anhydride intermediate with, e.g., hydroxylamine, in principle is the most direct method to demonstrate a covalent reaction intermediate. In model reactions,[12] however, it has been shown that the interaction of the metal ion with the substrate renders this approach as unreliable. The results of recent EPR studies of the reaction intermediate of CPA formed during esterolysis show that the active-site metal ion coordinates the carbonyl oxygen of the scissile bond as well as a solvent molecule.[7] These factors are the likely origin for the lack of reproducible stoichiometries[12] in detecting reaction intermediates of CPA with hydroxylamine.
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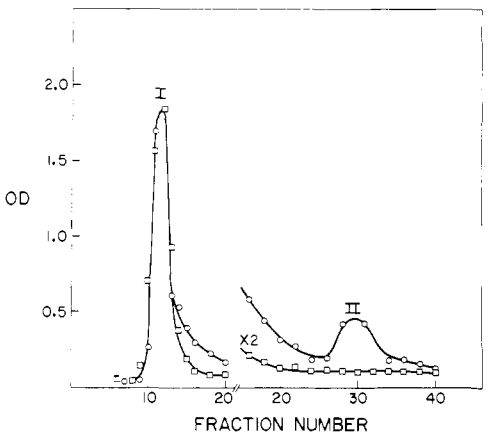

Figure 1. Gel filtration of the reaction products of CPA and ClCPL. In a glass-jacketed column (1 cm × 30 cm) Sephadex LH-20 was packed to a height of 6 cm, with an ethylene glycol/water/methanol (40:40:20 v/v) cryosolvent mixture containing 0.25 M sodium chloride and 0.01 M sodium cacodylate at pH 7.5. Prior to initiation of the enzymic reaction, the temperature of the buffer was lowered to −60 °C. The flow rate of the solvent was regulated at approximately 15 mL/h with a peristaltic pump, as described by Fink.[16] Fractions of approximately 1.5-mL volume were collected. The optical density of the collected fractions was determined at 280 nm and 23 °C with a Cary 15 spectrophotometer. The identities of peaks I and II were verified individually in separate experiments by elution of the protein and the hydrolysis products under otherwise identical conditions. The following reaction conditions were used: A (○), in an aqueous ethylene glycol (50:50) mixture containing 0.25 M sodium chloride and buffered to pH 7.5, 1.0×10^{-7} mol of CPA and ClCPL was incubated for several hours at 0 °C to assure complete hydrolysis of the substrate. To the reaction mixture an appropriate aliquot of methanol was added so that the solvent was identical with the ternary cryosolvent in the column, and elution of the reaction products was carried out at −60 °C; B (□), enzyme and substrate were incubated for 15 min at −60 °C in the cryosolvent atop the Sephadex gel bed. These conditions are equivalent to those described for stabilization of the reaction intermediate for spectroscopic characterization.[5,7] Elution was then carried out as described in part A. There was no further detectable change in the optical density of the eluant up to fraction 70.

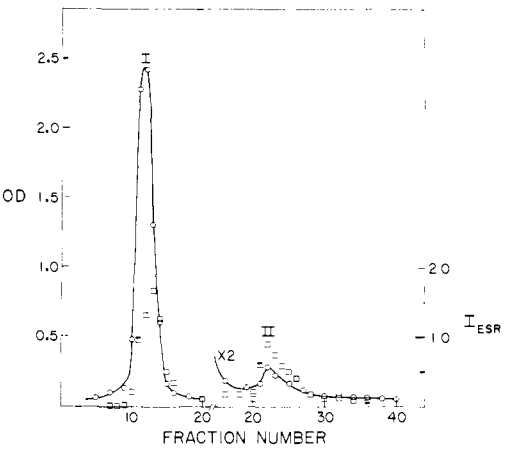

Figure 2. Gel filtration of the reaction products of CPA and the specific spin-label ester TEPOPL at −50 °C. Approximately 1.2×10^{-7} mol of CPA and TEPOPL were incubated as described in part B of Figure 1. In view of the greatly reduced rate of breakdown of the intermediate formed with TEPOPL[14,15] and the slow flow rate at −60 °C, elution was carried out instead at −50 °C. This change was necessary to overcome the tendency of the LH-20 medium to pack under prolonged peristaltic pumping of the viscous solvent in order to maintain conditions as for ClCPL. The optical density of the eluant was determined at 280 nm for fractions 1–20 and at 250 nm (peak absorbance of the hydrolyzed spin label[14]) for fractions 17–40: (○) optical density at 280 or 250 nm; (□) normalized EPR absorption intensity of the nitroxide free radical as determined with a Bruker ER200D spectrometer operated in the TE_{102} mode at 9.7-GHz and 100-kHz frequency modulation. No free-radical signal was detectable for fractions 40–70. The appearance of a small amount of the spin-lable nitroxide signal shown as peak II is due to hydrolyzed substrate because of the slightly higher temperature employed for elution. Migration of peak II relative to the protein peak is less retarded than in Figure 1. This difference in mobility is probably due to the π-bonding interactions of aromatic compounds with Sephadex LH-20 in the presence of organic solvents.[17] Elution of the reaction products of TEPOPL and CPA as in part A of Figure 1 results in a broad distribution of the spin label in fractions appearing after the elution of the protein (peak I).

formed with ClCPL is ~120 min at −60 °C in fluid, cryosolvent mixtures[5] and is sufficiently long for gel filtration chromatography. In Figure 1 are compared the elution profiles of the products of the reaction of CPA with ClCPL at either 0 or −60 °C. When ClCPL is reacted with the enzyme at 0 °C, the UV absorption upon chromatography at −60 °C arises from the enzyme (appearing at the void volume of the column), labeled peak I, and the hydrolyzed substrate, labeled peak II. When the reaction is initiated at −60 °C, peak II is absent, and the fractions belonging to peak I warmed to ambient temperature exhibit UV absorption spectra comparable to that of an equimolar mixture of the hydrolyzed substrate and enzyme. These observations indicate that the substrate comigrates with the enzyme only when reacted and eluted at −60 °C and that the enzyme has remained catalytically active. In Figure 2 is shown the corresponding elution profile of the reaction products of the spin-label ester substrate TEPOPL and CPA similarly reacted and eluted at subzero temperatures. The elution profile shows that the nitroxide-containing moiety, i.e., the acyl portion of the substrate, is predominantly associated with the enzyme.

The presence of the chromophore of both substrates in peak I as detected by UV absorption (for ClCPL) or EPR (for TEPOPL) demonstrates that the acyl portion comigrates with the enzyme. Since the elution volume of the free substrate is distinctly different from that of the enzyme, these data indicate that both ClCPL and TEPOPL form unusually tightly bound complexes or covalent reaction intermediates with CPA at temperatures near −60 °C. The length of time for gel filtration under the prevailing viscosity conditions[2] implies that, for noncovalently formed complexes, the dissociation equilibrium constants would have to be less than 10^{-7} M. This is significantly smaller than the K_s values of substrates of other well-characterized proteolytic enzymes that form covalent reaction intermediates and, additionally, is significantly smaller than the value (~10^{-4} M) of the K_m constants of ClCPL[5] and TEPOPL.[14] Since no hydrolysis products of ClCPL were detected for reaction conditions at −60 °C, as shown in Figure 1, the acyl moiety of the substrate could be expected to coelute stoichiometrically with CPA only if covalently attached. On this basis, the most direct interpretation of the data in Figures 1 and 2 is that the acyl moiety of each substrate has become covalently attached to the enzyme. These observations thus confirm our previous interpretations[5-7] that ester hydrolysis proceeds via formation of a covalent reaction intermediate. Since the rate of breakdown of the reaction intermediate formed with ClCPL is not attributable to hydrolysis of a cinnamoylated tyrosine side chain,[5,6] the intermediate must be formed by acylation of the γ-carboxylate group of glutamate-270, which is the only other available catalytic group in the active site.

In parallel cryoenzymologic studies we have been unable thus far to identify as single species reaction intermediates formed with peptide substrates. For instance, although the reaction with *N*-benzoylglycyl-L-phenylalanine near −60 °C exhibits negligible turnover, the EPR spectra of the low-temperature stabilized reaction products formed with Co^{2+}-substituted CPA show evidence of more than one enzyme–substrate species[15,18] in contrast to the single species demonstrated for the esterolytic reaction.[7] These observations are consistent with the suggestion of Cleland[19] that, in contrast to the esterolytic reaction catalyzed by CPA, formation of the mixed-anhydride intermediate with peptide substrates should

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be rate determining. Although Breslow and Wernick[10] have argued against the mixed anhydride mechanism for peptide hydrolysis, they point out that the results of their isotope-exchange studies would be accommodated by a mixed-anhydride model if not all of the water molecules in the active site are displaced by substrate binding. We have recently demonstrated[7] that the mixed-anhydride intermediate of the esterolytic reaction is a pentacoordinate metal ion species in which both the carbonyl oxygen of the substrate and a water molecule are coordinated to the metal ion. Since the stereochemical relationships for binding of specific ester and peptide substrates to the enzyme[9] and the pH profiles for their hydrolysis are similar,[5,13,20-22] all of the observations taken together are consistent with formation of a mixed-anhydride intermediate during the hydrolysis of both types of substrates.

Registry No. ClCPL, 61556-61-4; TEPOPL, 72250-35-2; CPA, 11075-17-5.

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Dilithium Semibullvalenide: An Unusual Organolithium Compound Extends the Scope of Homoaromaticity†,‡

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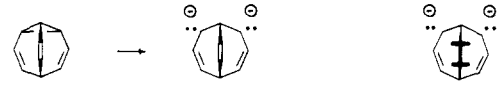
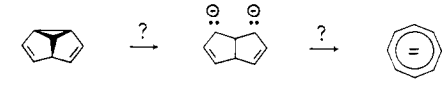
Bullvalene (**1**) is easily reduced to its dianion (**2**), whereas, under



1 **2** **3**

identical conditions, dihydrobullvalene is not.[1] Qualitative theory provides a simple explanation. The $C_{10}H_{10}$ dianion (**2**) is expected to be stabilized, because it is a mode (*2,0,0*) longicyclic.[2] The $C_{10}H_{12}$ dianion (**3**) is expected to be destabilized (i.e., bishomo antiaromatic), because it is an 8-π-electron pericyclic.[3]

The value of such predictions has increasingly been questioned in recent years,[4] and particularly as it applies to anions.[4a-e,h,j-l] Nevertheless, both bullvalene and dihydrobullvalene continue to behave as before, apparently oblivious to the current fashion.[5] Perhaps they do so for other reasons. The ethano bridge of dihydrobullvalene provides a rich source of alternative hypotheses. It might have prevented dianion formation by diminishing the loss of strain energy, by sterically inhibiting counter-ion stabilization, by transferring a hydrogen atom to a radical anion intermediate, etc. Whatever its role, excision of that bridge (as in semibullvalene, **4**) should then restore bullvalene-like behavior. In particular, the



4 **5** **6**

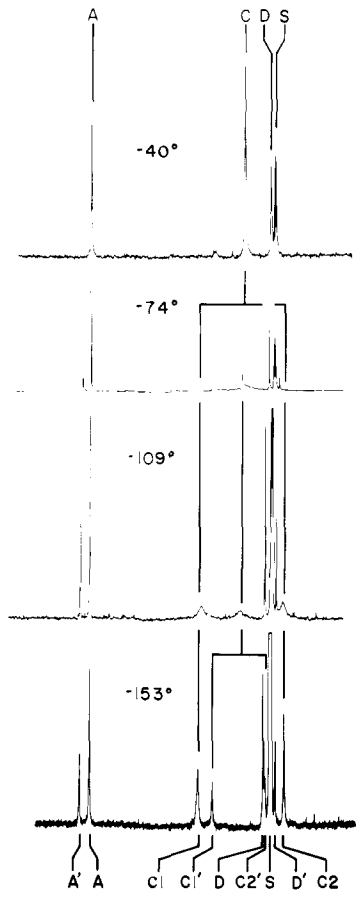


Figure 1. 75.47-MHz ^{13}C NMR spectra of dilithium semibullvalenide in dimethyl-d_6 ether at four temperatures. S is the solvent peak. Primed letters (e.g., A') denote peaks of the minor component (cf. Table II).

Table I. NMR Spectra of Dilithium Semibullvalenide and Bicyclo[3.3.2]decatrienide at "High Temperature"[a]

nucleus	$Li_2C_8H_8$ δ_H[b]	δ_C[c]	J_{CH}[d]	A_H[e]	$Li_2C_{10}H_{10}$ δ_H[f]	δ_C[c]	J_{CH}[d]	A_H[e]
A	6.40	146.49	143.3	1.91	6.14	131.5	135	1.90
B					4.63	106.3	151	1.94
C	2.56	73.73	144.3	4.12	3.06	75.3	159	4.22
D	3.78	61.51	128.5	1.96	2.31	36.3	122	1.94

[a] $Li_2C_8H_8$ at -38 °C in dimethyl-d_6 ether at 80 MHz; $Li_2C_{10}H_{10}$ at -20 °C in 1,2-dimethoxyethane-d_{10} at 90 MHz.[1] [b] Relative to benzene in dimethyl ether at $\delta_H = 7.30$. [c] Relative to (1:2) Me_4Si in the appropriate ether at δ_H 0.00. [d] In Hz. [e] Proton areas normalized to the appropriate sum. [f] Relative to $CHD_2O(CD_2)_2OCD_3$ at δ_H 3.31.

† Dedicated to Professor W. von E. Doering on the occasion of his 65th birthday.
‡ Presented in part at the Fourth International Symposium on the Chemistry of Novel Aromatic Compounds, Jerusalem, Sept 1, 1981.
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(5) The current fashion is equally oblivious to the dianion, **2**. Its synthesis and characterization apparently excaped the otherwise extensive literature surveys of ref 4b-l.

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