removal of the solvent, the residue was chromatographed on 1 g of silica gel (using hexane–benzene for elution) to afford 30 mg (44.5%) of **38** as a colorless oil: IR ν_{max} ($CHCl_3$) 1690 cm^{-1}; NMR (CCl_4) δ 0.96 (3 H, s, $-CH_3$), 3.70 (3 H, s, OCH_3) 4.78–5.92 (3 H, m, $HC{=}CH_2$) 6.43–6.67 (2 H, m, C_3 H, C_5 H), and 6.83 (1 H, d, J = 8 Hz, C_6 H); *m/e* 298 (M^+). Anal. ($C_{20}H_{26}O_2 \cdot 0.5H_2O$) C, H.

Thermolysis of Compound 38. A solution of 30 mg of **38** in 4 mL of *o*-dichlorobenzene was stirred under an atmosphere of nitrogen for 4 h at 180 °C. After evaporation of the solvent, the residue was recrystallized from ethyl acetate to give 28.6 mg (95.3%) of *D*-homoestrone methyl ether (**1**); mp 160–162 °C (lit.[15,16] mp 155–157 °C, 158–160 °C, 162–163 °C), as colorless prisms: IR ν_{max} ($CHCl_3$) 1693 cm^{-1}; NMR (CCl_4) δ 1.08 (3 H, s, $-CH_3$), 3.69 (3 H, s, OCH_3), 6.39–6.72 (2 H, m, C_2H), 7.08 (1 H, d, J = 9 Hz, C_1 H); *m/e* 298 (M^+). Anal. ($C_{20}H_{26}O_2 \cdot \frac{1}{6}H_2O$) C, H. This was shown to be identical with the authentic sample in its IR ($CHCl_3$) and NMR (CCl_4) spectra.

***D*-Homoestrone (2).** A mixture of 10 mg of **1** and 500 mg of freshly prepared dry pyridine hydrochloride was heated at 200 °C for 40 min under an atmosphere of nitrogen. After cooling to room temperature, 2 mL of 5% HCl solution was added. The aqueous solution was extracted with ether and the ethereal extract was washed with water, saturated $NaHCO_3$ solution, and saturated NaCl solution, and finally dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue was recrystallized from hexane–ether to give 7.6 mg (80%) of **2** as colorless plates, mp 218–222 °C (lit.[6] 220–223 °C).

Acknowledgment. A part of this research was assisted financially by a grant from the Mitsubishi Foundation, which is gratefully acknowledged. We also thank Mrs. C. Koyanagi, Miss K. Mushiake, Mrs. R. Kobayashi, Miss R. Suenaga, Miss E. Nagaoka, Miss M. Tanno, and Mr. K. Kawamura, Pharmaceutical Institute, Tohoku University, for microanalyses and spectral measurements.

References and Notes

(1) A part of this work has been published as a communication in this journal; see T. Kametani, H. Nemoto, H. Ishikawa, K. Shiroyama, and K. Fukumoto, *J. Am. Chem. Soc.*, **98**, 3378 (1976).
(2) A. A. Akhrem and Y. A. Titov, "Total Steroid Synthesis", Plenum Press, New York, N.Y., 1970.
(3) R. Pappo in "The Chemistry and Biochemistry of Steroids", Vol. 3, No. 1, N. Kharasch Ed., Intra-Science Research Foundation, Santa Monica, Calif., 1969, pp 123–130.
(4) (a) S. Danishefsky and P. Cain, *J. Am. Chem. Soc.*, **98**, 4975 (1976); (b) N. Cohen, B. L. Banner, J. F. Blount, T. Tsai, and G. Saucy, *J. Org. Chem.*, **38**, 3229 (1973), and references cited herein; (c) U. Eder, G. Sauer, and R. Wiechert, *Angew. Chem., Int. Ed. Engl.*, **10**, 496 (1971); (d) N. Cohen, B. L. Banner, W. F. Eichel, D. R. Parrish, G. Saucy, J.-M. Cassal, W. Meier, and A. Fürst, *J. Org. Chem.*, **40**, 681 (1975).
(5) P. A. Bartlett and W. S. Johnson, *J. Am. Chem. Soc.*, **95**, 7501 (1973).
(6) S. Danishefsky, P. Cain, and A. Nagal, *J. Am. Chem. Soc.*, **97**, 380 (1975).
(7) T. Kametani and K. Fukumoto, *Heterocycles*, **3**, 29 (1975).
(8) T. Kametani, Y. Kato, T. Honda, and K. Fukumoto, *Heterocycles*, **4**, 241 (1976).
(9) T. Kametani, H. Nemoto, and K. Fukumoto, *J. Chem. Soc., Chem. Commun.*, 400 (1976).
(10) T. Kametani, Y. Kato, T. Honda, and K. Fukumoto, *J. Am. Chem. Soc.*, **98**, 8185 (1976).
(11) R. B. Woodward and R. Hoffmann, "The Conservation of Orbital Symmetry", Academic Press, New York, N.Y., 1970.
(12) I. L. Klundt, *Chem. Rev.*, **70**, 471 (1970).
(13) R. Huisgen and H. Seidel, *Tetrahedron Lett.*, 3381 (1964).
(14) (a) W. Oppolzer, *J. Am. Chem. Soc.*, **93**, 3833 (1971); (b) *ibid.*, **93**, 3834 (1971); (c) *Tetrahedron Lett.*, 1001 (1974); (d) W. Oppolzer and K. Keller, *J. Am. Chem. Soc.*, **93**, 3836 (1971).
(15) G. H. Douglas, J. M. H. Grares, D. Hartley, G. A. Hughes, B. J. McLoughlin, J. Siddall, and H. Smith, *J. Chem. Soc.*, 5072 (1963).
(16) S. N. Anachenko, V. Ye. Limanov, V. N. Leonov, V. N. Rzheznikov, and I. V. Torgov, *Tetrahedron*, **18**, 1355 (1962).
(17) W. S. Johnson, D. K. Banerjee, W. P. Schneider, C. D. Gutsche, W. E. Shelberg, and L. J. Chinn, *J. Am. Chem. Soc.*, **74**, 2832 (1952).
(18) (a) T. Kametani, M. Kajiwara, and K. Fukumoto, *Chem. Ind. (London)*, 1165 (1973); *Tetrahedron*, **30**, 1053 (1974); (b) M. P. Cava and M. J. Mitchell, *J. Org. Chem.*, **27**, 631 (1962).
(19) G. L. B. Carlson, F. H. Quina, B. M. Zarnegar, and D. G. Whitten, *J. Am. Chem. Soc.*, **97**, 347 (1975).
(20) W. E. Parham and E. L. Anderson, *J. Am. Chem. Soc.*, **70**, 4187 (1948).
(21) (a) T. Kametani, M. Kajiwara, T. Takahashi, and K. Fukumoto, *J. Chem. Soc., Perkin Trans. 1*, 737 (1975); (b) S. Yamada, K. Tomioka, and K. Koga, *Tetrahedron Lett.*, 61 (1976).
(22) G. Stork and G. L. Nelson, *J. Am. Chem. Soc.*, **93**, 3091 (1971).
(23) A. M. Gaddis and L. W. Butz, *J. Am. Chem. Soc.*, **69**, 1203 (1947).
(24) J. F. Norman, *Synthesis*, 63 (1972).
(25) R. E. Ireland and J. A. Marshall, *J. Org. Chem.*, **27**, 1615 (1962).
(26) E. W. Warnhoff, D. G. Martin, and W. S. Johnson, "Organic Syntheses", Collect. Vol. IV, Wiley, New York, N.Y., 1963, p 162.
(27) W. S. Johnson, *J. Am. Chem. Soc.*, **65**, 1317 (1943).
(28) G. A. Boswell, Jr., "Organic Reactions in Steroid Chemistry", Vol. II, J. Fried and J. A. Edwards, Ed., Van Nostrand-Reinhold, Princeton, N.J., 1972, p 388.

Molecular Orbital Studies of Enzyme Activity. 4. Hydrolysis of Peptides by Carboxypeptidase A

Steve Scheiner and William N. Lipscomb*

Contribution from the Gibbs Chemical Laboratory, Harvard University, Cambridge, Massachusetts 02138. *Received August 19, 1976*

Abstract: An approximate molecular orbital method is used to study the catalytic activity of carboxypeptidase A. A proton donor is positioned near the nitrogen atom of the scissile bond of a model substrate. Nucleophilic attack on the carbonyl carbon leads to hydrolysis of the peptide. An electrophile positioned proximate to the carbonyl oxygen is seen to greatly facilitate this hydrolysis. The electrophile first polarizes the carbonyl bond of the substrate, making the carbon more susceptible to nucleophilic attack. The tetrahedral adduct formed as a result of this attack is stabilized by the electrophile in several ways. Bonding between the electrophile and the carbonyl oxygen reduces the negative charge on the oxygen. The electrophile also acts to strengthen the bonding between the central carbon and its four substituents in the adduct. The metal electrophiles which model Zn^{2+} and its ligands are more effective at catalyzing the hydrolysis than are various hydrogen-bonding species. The effects of strain on the peptide bond as a result of binding to the enzyme are examined for each of the electrophiles. Finally, a comparison is made between several proposed modes of nucleophilic attack.

Carboxypeptidase A (CPA) catalyzes the hydrolysis of carboxy-terminal peptide bonds. CPA is a metalloenzyme in which the presence of Zn^{2+} is a cofactor for enzyme activity.[1] Peptidase activity has been observed when Zn^{2+} is replaced by Co^{2+}, Ni^{2+}, Mn^{2+}, and Fe^{2+}.[1-3] Substitution by Hg^{2+}, Cd^{2+}, and Pb^{2+} results in loss of peptidase activity, although these heavy metal derivatives retain esterase activity.[3,4] In Zn-CPA, the Zn^{2+} is coordinated to His-69, His-196, Glu-72, and one water molecule in a distorted tetrahedral configuration about the zinc.[5] On binding of glycyl-L-tyrosine[5] the zinc-coordinated water molecule is displaced by the carbonyl oxygen of the scissile peptide bond. The C-terminal carboxylate group

of the substrate forms a salt link with Arg-145 of the enzyme. The C-terminal side chain fits into the hydrophobic pocket of CPA. The only side chains of the enzyme which are near enough to the susceptible peptide bond to be directly involved in catalysis are Glu-270 and Tyr-248. There are reasons to believe that the arrangements mentioned above are essentially the same in complexes of CPA with reactive peptide substrates.[6] Chemical modification of Tyr-248 strongly affects the peptidase action of CPA.[6,7] The above information along with pH–rate profiles have led to the hypothesis[5] that Tyr-248 serves to donate a proton to the nitrogen of the scissile peptide bond. The pH–rate profiles also lead to the conclusion that Glu-270 is in its ionized form at the pH of optimum enzyme activity.[8] The carboxylate group of Glu-270 is hypothesized[5] to act as a nucleophile probably by attacking the substrate's carbonyl group directly or possibly by promoting the attack of a water molecule.

There is some question as to whether the Zn^{2+} is directly involved in catalysis or serves merely to hold the functional groups of the enzyme in a geometry suitable for productive binding and subsequent reaction.[9] In the "zinc–carbonyl" mechanism,[6] the metal may act to orient the carbonyl group of the substrate in a position suitable for nucleophilic attack. The metal may also polarize the carbonyl group, making the carbon more susceptible to nucleophilic attack. The carbonyl oxygen would acquire a formal negative charge in the tetrahedral adduct formed as a result of the attack. Interactions between the Zn^{2+} and the negatively charged oxygen would act to stabilize the adduct. In the alternate "zinc–hydroxide" proposal,[6] the zinc-coordinated water molecule, in its dissociated form, acts as the attacking nucleophile.

There is some evidence of an intermediate in the CPA-catalyzed hydrolysis of esters.[10] However, no intermediates have been detected as yet in the hydrolysis of peptides.[8] We concern ourselves in this study with peptidase activity only.

Hayes and Kollman have used molecular orbital techniques to study the interaction between CPA and various substrates.[11] They modeled several catalytically important residues by fractional point charges and studied the effects of these charges on a model substrate. In this paper we use an approximate molecular orbital theory to study the effect of various electrophiles analogous to zinc on the hydrolysis of a peptide bond. We also examine the effect of variation of the basicity of the proton donor and the nucleophile. Finally, we compare direct nucleophilic attack by Glu-270 with glutamate-promoted attack of a water molecule. The "zinc–hydroxide" mechanism is also evaluated as an alternate reaction pathway. The method used, partial retention of diatomic differential overlap (PRDDO), closely reproduces minimum basis set ab initio results at relatively low cost.[12]

Results

The residues Glu-270 and Tyr-248 are partially exposed to solvent within the enzyme–substrate complex.[5] One would thus expect the acidities of these two species to be substantially different from those found in a completely hydrophobic environment. The side chains of the above two residues may be approximated by formic acid and phenol, respectively. The gas-phase proton affinities of formate and phenoxide anions are 342[13] and 348[14] kcal/mol, respectively. Interactions with solvent molecules and other atoms of the enzyme would be expected to stabilize the negatively charged anions substantially more than the neutral parent molecules. Within the environment of the enzyme–substrate complex, the proton affinities of the anions of Glu-270 and Tyr-248 should therefore be significantly lower than the above values. The proton affinities of formate and phenoxide anions are calculated by PRDDO to be 473 and 450 kcal/mol, respectively. These two species are thus far too basic for use in our model if we wish to approximate the protein environment. The proton affinity of H_2O is calculated by the PRDDO method to be 226 kcal/mol. Thus H_2O would appear to be a more suitable choice for the basic form of Glu-270 in our model. Similarly, we model the acidic form of Tyr-248, the proton donor, by H_3O^+. We do not imply that H_3O^+ is the actual proton donor in peptide cleavage.

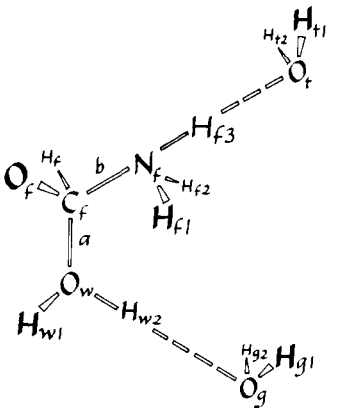


Figure 1. All OH bond lengths are 1.00 Å; NH, 1.04 Å. All bond angles to O_t and O_g are tetrahedral; $\angle N_f\text{–}H_{f3}\text{–}O_t = \angle O_w\text{–}H_{w2}\text{–}O_g = 180°$. If we exclude the atoms H_f, O_f, and H_{w1} a plane of symmetry passes through the atoms O_t, N_f, C_f, O_w, and O_g (when the dihedral angle $\phi(O_g\text{–}O_w\text{–}C_f\text{–}N_f) = 0°$).

The hydrolysis of a peptide bond, modeled by formamide, in the absence of an electrophile analogous to the zinc was studied first. The proton donor H_3O^+ was positioned proximate to the nitrogen atom of the peptide. Optimization of the geometry of the $HCONH_2\text{–}H_3O^+$ system yielded a planar configuration about the carbon and a very nearly tetrahedral one about nitrogen. One proposed mechanism involves the attack of a nucleophilic H_2O activated by Glu-270. A water molecule was therefore used as the attacking nucleophile. A second water molecule was placed so that it could accept a proton from the first.

The nucleophilic water dimer was allowed to approach the $HCONH_2\text{–}H_3O^+$ system from the side of the peptide plane opposite to the proton donor as shown in Figure 1. A similar relation between Glu-270 and Tyr-248 about the substrate has been found in the x-ray structure.[6] During the nucleophilic attack, a conformation was maintained in which O_g was nearly eclipsed by N_f (dihedral angle $\phi(O_g\text{–}O_w\text{–}C_f\text{–}N_f) \lesssim 30°$). This configuration conforms to the structural relation between the carboxylate group of Glu-270 and the nitrogen of the substrate.[6] Table I demonstrates that the carbonyl oxygen acquires the bulk of the negative charge from the attacking water dimer, while the carbon becomes a bit more positive.

As the C–N distance was increased, the proton donor was moved along with the nitrogen, yielding the systems $NH_3\text{–}OH_2$ and $HCOOH\text{–}H_3O^+$ as final products. In the latter system, O_g was in a position consistent with that of Glu-270 in the x-ray structure.[6] A potential energy surface is shown in Figure 2 as a function of the distances of both the attacking nucleophile and the nitrogen atom to the central carbon atom. For all points, the proton H_{f3} was allowed to bond to either O_t or N_f, the more stable configuration being chosen. Similarly, H_{w2} was allowed a choice of bonding to either O_w or O_g. The geometries were otherwise fully optimized.

In the preceding, we have modeled the proton donor by H_3O^+ and the nucleophilic Glu-270 by H_2O. In order to investigate the effect of increasing the basicity of these species, we have obtained an energy surface analogous to Figure 2, in which the above species were modeled by NH_4^+ and NH_3, respectively. This surface is quite similar to that shown in Figure 2. The only significant difference is that the reaction

Table I. Mulliken Charges During Nucleophilic Attack

$r(C_f-O_w)$, Å	O_f	C_f	H_f	$(N_fH_3)^a$	$(H_2O_t)^a$	$(H_2O_w)^a$	$(H_2O_g)^a$
∞	−0.030	0.066	0.185	0.585	0.194		
4.0	−0.034	0.072	0.185	0.587	0.190	−0.081	0.082
3.0	−0.043	0.083	0.181	0.586	0.188	−0.078	0.083
2.5	−0.065	0.095	0.177	0.579	0.186	−0.055	0.084
2.0	−0.161	0.144	0.136	0.528	0.185	0.076	0.093
1.75	−0.277	0.171	0.103	0.484	0.182	0.181	0.156
1.5	−0.386	0.173	0.074	0.445	0.181	0.339	0.172

[a] Group charges are sums of atomic charges.

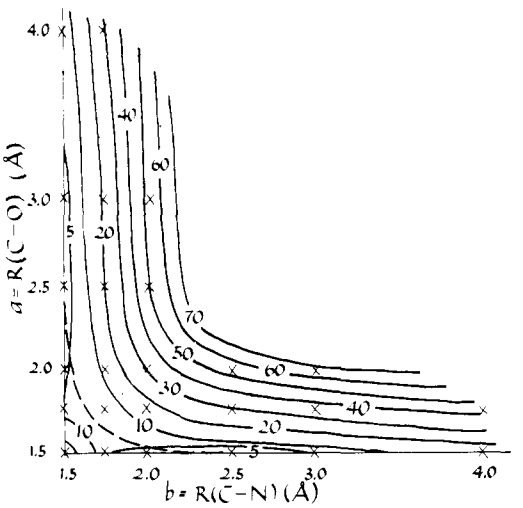


Figure 2. Potential energy surface for system $HCONH_2$–H_3O^+ + HOH–HOH. Energies are in kilocalories per mole. X's designate calculated points. Dashed line denotes optimum path between minima. H_{f3} is bonded to N_f for all points shown. H_{w2} is bonded to O_w for every point except $r(CO) = 1.5$, $r(CN) \geq 3.0$.

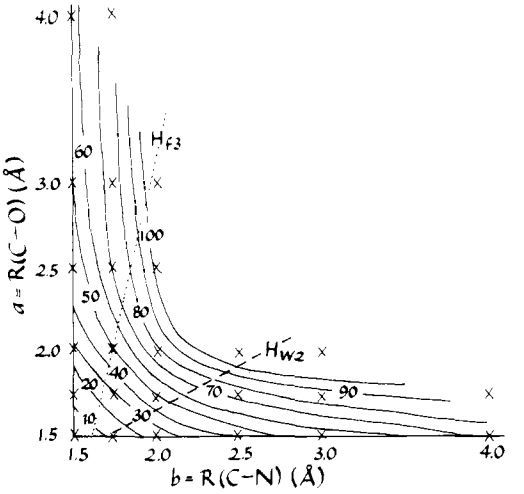


Figure 3. Potential energy surface for system Li^+–$HCONH_2$–H_3O^+ + HOH–HOH. H_{f3} (H_{w2}) is bonded to O_t (O_w) to left of dotted (dashed) line.

is calculated to be slightly exothermic when the nitrogenous species are used.

The electrophile in the proposed mechanism (Tyr 248) is a weaker proton donor than the conjugate acid of the nucleophilic Glu-270. This relative strength is true both in solution as measured by pK_a's and in the gas phase.[13,14] In order to simulate this pK_a difference, the proton donor was modeled by NH_4^+ and the nucleophile by the water dimer as above. Once again the energy surface obtained is quite similar to that shown in Figure 2. The only significant difference here is that the reaction becomes somewhat more endothermic when the electrophile of higher pK_a is used.

As a first approximation of the effect of zinc and its ligands on the hydrolysis, a lithium cation was added to the system near the carbonyl oxygen. The geometry of the entire system was then optimized. The potential energy surface for the hydrolysis of formamide in the presence of Li^+ is shown in Figure 3. The outstanding feature of this surface is the deep well whose bottom occurs at (1.5, 1.5).[15] Figure 2, on the other hand, reveals that exclusion of the Li^+ results in a rather steep hill with its crest at (1.5, 1.5). The minima in Figure 2 are at (2.5, 1.5) and (1.5, 2.5). We take these two points to be the initial and final points of the hydrolysis, respectively. The reaction path may be represented by the dashed line in Figure 2. The reaction is thus calculated to be nearly thermoneutral with an activation energy of 7 kcal/mol when the electrophile Li^+ is excluded. The reaction path is not nearly as constrained by energy considerations when Li^+ is added to the system. As shown in Figure 3, any path connecting the points (2.5, 1.5) and (1.5, 2.5) and lying within the 45-kcal contour will result in an exothermic reaction (−5 kcal/mol) with no activation barrier.

On the above energy surface the distance from the carbonyl oxygen to Li^+ varies between 1.6 and 1.8 Å. The analogous distance to the zinc in the enzyme–substrate complex is 2.1 Å.[6] To determine the effect on the reaction of variation of the distance of the Li^+, energy surfaces were obtained for larger values of $r(O_f$–Li). This distance was lengthened to 2.0 and 2.5 Å for each calculated point on the potential surface. No other geometry changes were made. It was found that the essential characteristics of the energy surfaces remained the same. The only significant change was a decrease in the steepness of the energy well as $r(O_f$–Li) was increased. The calculated energies of several important points on each surface are summarized in Table II.

In CPA, the Zn^{2+} is coordinated to two histidine residues, one glutamate (Glu-72), and a water molecule in a distorted tetrahedral arrangement.[5] As the glutamate bears a mononegative charge, the effective charge of Zn^{2+} and its ligands is +1. On binding, the substrate displaces the water molecule. In order to approximate this coordination, $[Be(OH)(NH_3)_2]^+$ was substituted for Li^+ in our calculations. The tetrahedrally coordinated Be atom was placed along the C_f–O_f axis at 2.0 Å from O_f. The geometry of the remainder of the system was not altered. The potential energy surface for this system is quite similar to those in which Li^+ was employed as the electrophile (see Table II). The depth and steepness of the well are greater than those for which Li^+ was maintained at 2.5 Å from O_f.

An important question to which we now turn our attention concerns the necessity of a metal electrophile for enzyme activity. If the metal's prime purposes are polarization of the carbonyl group of the substrate and stabilization of the ensuing tetrahedral adduct, we can envision several substitutes which

Table II.

Electrophile	Energy, kcal/mol				
	$(\infty, 1.5)^{15}$	(2.5, 1.5)	(1.5, 1.5)	(1.5, 2.5)	(1.5, ∞)
	17.0	0	17.1	2.2	24.2
$2(NH_3)^a$	24.8	4.1	15.5	0	24.1
$(NH_3)^b$	16.9	0	18.8	6.3	33.0
Li^+ opt	70.1	43.8	0	38.7	78.7
2.0	53.9	29.5	0	25.8	61.7
2.5	35.2	14.2	0	11.8	41.9
$Be(OH)(NH_3)_2^{+c}$	38.6	14.4	0	16.5	45.3
NH_4^{+d}	37.2	15.5	0	17.8	43.8
H_2O^e	17.4	0	10.8	7.1	26.9

[a] NH_3 substituted for H_2O_t and H_2O_g. All bond angles to N are tetrahedral; r(NH) = 1.04 Å. [b] NH_3 substituted for H_2O_t. [c] All angles in this species are tetrahedral, r(OH) = 1.00, r(NH) = 1.04, r(Be-N) = 1.69, r(Be-O) = 1.45 Å. [d] Tetrahedral geometry; r(NH) = 1.04 Å. [e] ∠HOH = 109.5°; r(OH) = 1.00 Å.

would serve the same purposes, not in carboxypeptidase A, but perhaps in other enzymes. For example, one might expect that the positively charged side chain of a lysine or arginine residue might produce effects similar to those of a metal ion. In order to investigate the effects of such a hydrogen-bonding species, a series of calculations was performed in which the electrophile Li^+ was replaced by NH_4^+. The position of NH_4^+ was optimized such that a linear hydrogen bond exists between the nitrogen atom and O_f. The energy surface obtained is quite similar to that in which $Be(OH)(NH_3)_2^+$ is employed as the electrophile (see Table II).

Thus far, we have been allowing both Li^+ and NH_4^+ to adopt optimal positions with respect to the carbonyl oxygen. However, one would expect any species in the interior of CPA, whether a tetrahedrally coordinated zinc ion or the side chain of a residue, to have some conformational constraints imposed on it. The effects that the various electrophiles have on the hydrolysis as a function of their distance from O_f thus become important. We first assume that in the bound enzyme–substrate complex, before nucleophilic attack has begun, the electrophile may adopt a very favorable position with respect to the carbonyl oxygen. As the nucleophile attacks and the carbonyl carbon becomes "tetrahedral," this oxygen will move away from the electrophile. The electrophile may follow the oxygen, but only to a limited extent. We thus allow the electrophile its optimum position at the initial point $(2.5, 1.5)^{15}$ and increase its distance from O_f in the adduct (1.5, 1.5). If the distance from Li^+ to the carbonyl oxygen is as much as 0.7 Å greater in the adduct than in the initial structure, the adduct remains more stable than the initial configuration. If the value of $r(Li\text{–}O_f)$ is chosen to be 2.0 Å in the initial structure, the above criterion is met for stretches of up to 0.5 Å in the adduct. In either case, the hydrolysis may be assumed to proceed with little or no activation energy, as shown in Figure 3. On the other hand, when NH_4^+ is pulled away from O_f by >0.3 Å from its optimized distance at (2.5, 1.5), the resulting adduct is less stable than the optimized initial structure. Smaller displacements are thus necessary in the case of NH_4^+ in order that the potential surface resemble Figure 2, thereby inducing an energy barrier along the reaction path.

Hydrogen bonding to neutral species such as water or the side chains of serine or threonine might also be expected to polarize the carbonyl group in certain enzymes. The NH_4^+ of the previous set of calculations was therefore replaced by H_2O, whose distance from O_f was then optimized. The potential surface for the hydrolysis of the formamide in the presence of H_2O is qualitatively similar to that in which there is no electrophile present (Figure 2). The maximum at (1.5, 1.5) is less pronounced as a result of the interaction of H_2O with the system (Table II), but an activation barrier is expected nonetheless. The effectiveness of a water molecule in facilitating the reaction is thus only a fraction of that of a metal electrophile. Several water molecules, all with rather stringent positional requirements, would be necessary to produce a potential surface qualitatively similar to that in Figure 3.

Table III. Mulliken Charges in Configuration $(\infty, 1.5)^{15}$

Electrophile (X)	C_f	O_f	X
	0.066	−0.030	
Li^+ opt	0.170	−0.108	0.751
2.0	0.157	−0.097	0.770
2.5	0.123	−0.094	0.838
$Be(OH)(NH_3)_2^+$	0.139	−0.067	0.801
NH_4^+	0.091	−0.138	0.936
H_2O	0.051	−0.072	−0.014
$(Li^+\text{-}H_2O)$	0.099	−0.125	0.924
$(Li^+\text{-}H_2O\cdot H_2O)$	0.086	−0.106	0.940

The polarizing effects of the various electrophiles on the carbonyl bond are shown in Table III. The transfer of negative charge to the metal electrophiles on binding of the substrate results in an increase in the positive charge on the carbonyl carbon. Here, NH_4^+ acquires less charge and produces less of an effect on the carbon than do the metals. The effect of H_2O is quite small and in fact results in a more electronegative carbon.[16] The charge transferred to $Be(OH)(NH_3)_2^+$ on binding of the substrate and the effect of this species on the charge of the carbon are intermediate between the effects of Li^+ at 2.0 and 2.5 Å. Of the charge acquired by $Be(OH)(NH_3)_2^+$, 40% is localized on Be, 30% on OH, and 15% on each of the NH_3 ligands. All of the electrophiles, particularly NH_4^+, increase the negative charge of the carbonyl oxygen.

The electron density rearrangements which occur during nucleophilic attack by $(H_2O)_2$ have already been shown in Table I. Inclusion of any of the electrophiles studied results in an increase in the positive charge acquired by the nucleophile as a result of its attack. In all cases, C_f becomes more positive during the attack, but the presence of any of the cationic electrophiles lessens this charge increase. All other groups, including the electrophile, become more negatively charged as a result of the attack.

In the tetrahedral species produced as a result of the nucleophilic attack, the carbonyl oxygen has substantial negative charge. By attracting additional electron density, the electrophile may reduce the negative charge on the oxygen and thereby stabilize the tetrahedral adduct. As shown in Table IV, the metal electrophiles substantially reduce the negative charge on O_f. H_2O produces only a small decrease, while NH_4^+ yields a small increase.[17] The hydrogen bonding species also extract significantly less electron density from the tetra-

Table IV. Interactions in Adduct (1.5, 1.5)[15]

Electrophile (X)	Charges O_f	Charges X	Overlap population $O_f - X$
	−0.386		
Li^+ opt	−0.288	0.478	0.561
2.0	−0.294	0.536	0.476
2.5	−0.318	0.673	0.326
$Be(OH)(NH_3)_2^+$	−0.312	0.680	0.364[a]
NH_4^+	−0.403	0.786	0.249[b]
H_2O	−0.375	−0.098	0.075[b]

[a] O_f–Be. [b] O_f–hydrogen bonding proton of electrophile.

Table V. Overlap Populations in Adduct (1.5, 1.5)[15]

Electrophile	C_f–O_w	C_f–N_f	C_f–O_f	C_f–H_f
	0.301	0.384	0.745	0.707
Li^+ opt	0.432	0.562	0.776	0.743
2.0	0.411	0.544	0.770	0.736
2.5	0.392	0.454	0.757	0.735
$Be(OH)(NH_3)_2^+$	0.394	0.453	0.777	0.731
NH_4^+	0.393	0.477	0.687	0.723
H_2O	0.347	0.409	0.680	0.721

Table VI. Overlap Populations

Electrophile	C_f–N_f	$(C_f$–$N_f)_\pi$[a]	C_f–O_f	$(C_f$–$O_f)_\pi$[a]
	0.549	−0.003	0.873	0.343
Li^+ opt	0.619	0.037	0.933	0.317
2.0	0.615	0.033	0.925	0.323
2.5	0.607	0.025	0.903	0.332
$Be(OH)(NH_3)_2^+$	0.611	0.027	0.912	0.330
NH_4^+	0.608	0.021	0.879	0.337
H_2O	0.590	0.010	0.867	0.341

[a] Overlap population over Slater atomic orbitals orthogonal to carbonyl plane.

hedral structure than do the metal electrophiles. The effect of $Be(OH)(NH_3)_2^+$ is intermediate between the effects of Li^+ at 2.0 and 2.5 Å. The overlap populations in Table IV show the same relative efficiencies of the various electrophiles as do the charges.

As Li^+ is moved further from the carbonyl oxygen, proportionately more of the increased electron density which Li^+ acquires as a result of the nucleophilic attack goes into its 2s orbital. When Li^+ is 1.6, 2.0, and 2.5 Å from O_f, 14, 24, and 32% of the additional electron density goes into the 2s orbital, respectively (none into the 1s orbital). Of the additional electron density acquired by the $Be(OH)(NH_3)_2^+$ complex, 30% is localized on Be, 30% on OH, and 20% on each of the NH_3 ligands. During the nucleophilic attack, the overlap populations between Be and its three ligands decrease, indicating a weakening of these bonds.

The strengthening effect on the bonding of the central carbon produced by each of the electrophiles is shown in Table V. This effect is most pronounced in the bonds to the nitrogen atom and to the attacking oxygen atom. It might be noted that the hydrogen-bonding electrophiles have a weakening effect on the carbonyl C–O bond. Similar effects are observed throughout the nucleophilic attack, but are most marked in the tetrahedral adduct.

We examine now the effects of the electrophiles on the π bonding within the substrate before the nucleophilic attack has begun. Overlap populations over atoms and over π orbitals are shown in Table VI. The electrophiles have a generally strengthening effect on both bonds shown. The strengthening of the C–N bond[18] is manifested in both the σ and π components in nearly equal amounts. The increase in total strength of the C=O bond masks a decrease in the π-bond strength. The electrophiles thus tend to strengthen the C–N π bond and weaken the C=O π bond. Once again, the effects of the hydrogen-bonding electrophiles are less than those of the metals. Be^{2+} and its ligands produce an effect greater than that of Li^+ at a distance of 2.5 Å.

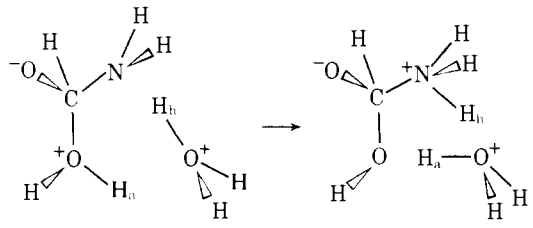
To test whether the results shown in Table VI are an artifact of either the presence of the proton donor or the nonplanarity of the nitrogen atom, the following calculations were performed. Formamide was held in the fully planar conformation and Li^+ was placed 2.0 Å from O_f along the C_f–O_f axis. No proton donor was included. The total overlap populations for both the C–N and C=O bonds increased as a result of the addition of Li^+. The overlap populations over π atomic orbitals were once again found to decrease for the C=O bond and increase for the C–N bond. The increased $(C–N)_\pi$ bond strength is manifested by the fact that the addition of Li^+ was found nearly to double the energy barrier to rotation about the C–N bond.[19] The bonding between the oxygen and lithium atoms was found to be nearly totally σ regardless of the planarity of the nitrogen or the presence of the proton donor.

An effort was made to distinguish between the nucleophilic attack on the substrate by water activated by Glu-270 and the direct attack of the latter residue. A geometry optimization was performed on the "tetrahedral" adduct formed by attack of formate anion on the carbonyl carbon of formamide. The adduct was found to be 30.6 kcal/mol more stable than the isolated optimized species. The attack on formamide by a water molecule which is coupled with a formate anion was also studied. The tetrahedral adduct in this case was calculated to be 15.8 kcal/mol more stable than the optimized reactants $HCOO^-$–HOH and $HCONH_2$. It must be pointed out that the nucleophiles are negatively charged in both cases. Hence, minimum basis set errors might be expected to have a significant effect.[20] However, since the negative charge is similarly distributed in the two cases, one might expect that the minimum basis set errors would approximately cancel in a comparison between the two. The direct attack by Glu-270 on the substrate would thus appear to be energetically favored. One must not neglect other important factors, however, such as accessibility of the carbonyl carbon to one or the other of the two potential nucleophiles.

The attacking nucleophile in the "zinc–hydroxide" mechanism[6] is proposed to be a hydroxyl group which is coordinated to zinc. The four ligands of Zn^{2+} are thus two uncharged histidine residues, one glutamate anion, and one hydroxide anion. We model this $[ZnL_4]$ moiety by $[Be(OH)_2(NH_3)_2]$.[23] This species was not predicted in this calculation to form a stable "tetrahedral adduct" with formamide. The interaction between BeL_4 and formamide is slightly attractive for large distances. For distances between the carbonyl carbon of formamide and the attacking oxygen atom of <2.7 Å, the potential becomes repulsive, however,[24] The zinc–hydroxide mechanism would thus appear to be a much less favorable reaction pathway than the zinc–carbonyl mechanism. One would also expect attack of an undissociated water molecule coordinated to Zn^{2+} to be even less likely, since the attacking nucleophile in this case is correspondingly weaker.

Breslow et al.[25] have proposed a mechanism in which, as above, the substrate is attacked by nucleophilic water which, in turn, is activated by Glu-270. A second proton from this water is then transferred to some other moiety, most likely Tyr-248. The tyrosine thereupon releases a proton to the nitrogen atom of the substrate, facilitating the breakdown of the tetrahedral species. In Breslow's mechanism Tyr-248 performs

the dual function of proton acceptor as well as donor. We model this function as below. It was found energetically fa-



vorable for the acidic H_3O^+ to release its proton, H_b, to the nitrogen regardless of the position of proton H_a. Tyr-OH might be expected to behave in a less acidic fashion than does H_3O^+. When the more basic H_2O was substituted for H_3O^+ the transfer of proton H_b to nitrogen was found to be contingent upon the transfer of H_a to the water. Transfer of only H_b results in the formation of the high-energy hydroxyl anion. As the acidity of tyrosine lies between these two extremes, it is uncertain as to whether proton donation to the nitrogen may occur prior to proton acceptance from the attacking nucleophilic water.

When Tyr-248 was modeled by H_3O^+ the reaction step shown above was calculated to be endothermic by 7 kcal/mol. This reaction was exothermic by 4 kcal/mol when H_2O was used. In either case, when Li^+ was placed near the carbonyl oxygen the reaction became more *exothermic* by 13 kcal/mol. The presence of the electrophile may thus be seen to facilitate this reaction pathway as well.

Discussion

The presence of an electrophile near the carbonyl oxygen appears to facilitate the hydrolysis of a peptide bond in several ways. First, the electrophile makes the peptide more susceptible to nucleophilic attack by inducing a more positive charge on the carbon. The tetrahedral adduct produced as a result of this attack is stabilized to a greater extent by the electrophile than is either end point of the reaction. This stabilization of the adduct occurs through partial bonding between the electrophile and the carbonyl oxygen. By drawing some electron density from the tetrahedral species, the electrophile acts to reduce the negative charge on the carbonyl oxygen. The metal electrophiles which model zinc and its ligands are more efficient in performing all of the above functions than are the hydrogen-bonding electrophiles. A possible source of this difference in efficiency is the following. During formation of the tetrahedral adduct, the Mulliken charge of the hydrogen-bonding proton remains nearly constant. The charge acquired by the electrophile as a result of the nucleophilic attack must therefore bypass this hydrogen and localize itself on the more distant remainder of the electrophile. In contrast, the charge acquired by a metal electrophile may transfer directly to the empty orbitals of the metal with no intervening proton. The electrophiles further stabilize the tetrahedral adduct by causing a strengthening of the bonds between the carbon atom and its four substituents.

Model building[5] suggests that strain is induced in the susceptible bond on binding to the enzyme. This strain may be an important factor in activating the enzyme-substrate complex to catalysis by destabilizing this complex relative to the transition state. We have demonstrated above that electrophiles near the carbonyl oxygen act to strengthen the peptide bond. This effect, which is most pronounced with the metals, includes both the σ and π components of the C-N bond. Thus, the presence of a zinc-like electrophile will increase the energy required for a given angle of strain on the peptide bond and further destabilize the enzyme-substrate complex relative to the transition state.

In addition, the ability of NH_4^+ in place of Zn^{2+} to facilitate the hydrolysis is more dependent on its optimal position than is that of the metals. Water produces a much smaller effect. All of the above factors contribute to the greater efficiency of a metal ion such as Zn^{2+} in expediting the hydrolysis.

The zinc ligands histidine and glutamate were modeled by ammonia and hydroxide, respectively. The magnitudes of the effects of the electrophile were not found to be substantially altered by exclusion of the ligands (provided, of course, that the total charge of the electrophile is held constant). The zinc ion was modeled by the cations of first-row metals whose d orbitals were not included in our basis set. However, other calculations[26] suggest that the d orbitals of zinc are essentially uninvolved in forming a complex with a peptide bond. The electrophiles employed in this study may therefore serve as adequate models for the zinc and its ligands in CPA.

The electrophile has been suggested to act not directly on the substrate, but through an intervening hydration shell. It is thus important to consider the reduction in the effects of the electrophile when one or more water molecules separate it from the substrate. These effects are shown in Table III. Upon inserting one water molecule between Li^+ and the substrate, the positive charge of the carbonyl carbon is substantially reduced. The polarization of the carbonyl bond effected by ($Li^+ \cdot H_2O$) is similar to that produced by NH_4^+. Insertion of a second intervening water molecule results in a small additional decrease of the polarization.

The effect of partial solvation on the acidities of Tyr-248 and Glu-270 has already been discussed. In addition, the proximity of the phenolic hydrogen of Tyr-248 to both the sensitive peptide bond and the positively charged Arg-145 residue would be expected to further increase the acidity of this residue. This effect would be heightened by the proposed hydrogen bond of Tyr-248 with the second peptide nitrogen of the substrate.[5] The positive charge of zinc, by induction through the scissile peptide bond, may also contribute to the increased acidity of Tyr-248. These reasons have led to our modeling Tyr-248 and Glu-270 by H_3O^+ and H_2O, respectively. As described above, we have found that increasing the basicities of our models produces very little change in the potential surface for the hydrolysis.

After a substrate has been bound to the enzyme the attacking nucleophile (Glu-270 or a water molecule) is to be found at a finite distance from the susceptible peptide bond. We principally concern ourselves in this study with the hydrolysis which occurs subsequent to the binding. We have found that a local minimum occurs in the potential energy surface when the nucleophile is 2.5 Å from the peptide. The point (2.5, 1.5)[15] has thus been considered the initial point of the reaction. Similarly, we have made no attempt to study the dissociation of the hydrolysis products from the enzyme. After the scissile bond length has stretched past a certain point the dominant effects will become those involved in dissociation. A second minimum in the potential surface has been found when the C-N bond length is 2.5 Å. We thus consider (1.5, 2.5) the end point of the reaction.

The proposed reaction involves a proton transfer from Tyr-248 to the scissile peptide. Promotion of the nucleophilic attack of water by Glu-270 involves a second proton transfer from the former to the latter. When the hydrolysis is uncatalyzed by an electrophile the first proton is transferred to the nitrogen without an activation barrier in the initial state (Figure 2). The second proton is transferred from the attacking H_2O without a barrier after the peptide bond has been broken and the transition state passed. The transition state thus involves no proton transfer. When Li^+ is placed near the carbonyl oxygen the potential surface is such that the two proton transfers must occur along the reaction path (Figure 3). However, these transfers do not involve activation barriers. Regardless of the strength of the electrophile we would therefore not expect a proton transfer to contribute to the ac-

tivation energy of the transition state for the hydrolysis. The deuterium isotope effect observed in the hydrolysis of peptides by CPA is, in fact, close to unity.[8]

Inclusion of a cationic electrophile into our model system results in a tetrahedral adduct which is considerably more stable than either end point of the reaction. The two end points are calculated to be approximately equal in energy for each of these electrophiles. A reaction path may thus be followed which leads directly from reactants to products and avoids the tetrahedral adduct (see Figure 3). A path which passes through or close to the adduct will not necessarily lead to a long-lived intermediate, as the potential energy provided by the initial point of the reaction should be sufficient to carry this reaction step to completion. A subsequent step of catalysis may occur rapidly enough to prevent the complex from sliding back into the potential well. In either case, the hydrolysis can proceed with little or no activation barrier. There may also be interactions between the enzyme and substrate which preferentially destabilize the adduct. For example, a tetrahedral configuration about the carbonyl carbon may result in a less stable salt link between Arg-145 and the C-terminal carboxyl group of the substrate. Also, the substrate's C-terminal side chain may not fit well into the CPA pocket in the tetrahedral conformation. Our results do not rule out the possibility of a detectable intermediate. Such an intermediate in the CPA-catalyzed hydrolysis of peptides has not been observed to date.[5]

Some important limitations of this paper include our study of the reaction in vacuo. This feature was necessitated by the large increase in the cost of quantum mechanical calculations caused by only moderate increases in the size of the system studied. An attempt was made to account for the effects of neighboring atoms within the enzyme by modeling Tyr-248 and Glu-270 by water as described above. The relation between the pK_a's of the two residues within the enzyme's interior and the gas-phase value for water is only an approximate one, however. Use of a larger basis set and inclusion of electron correlation might improve some of our results. Correlation effects would be expected to make important contributions in the bond formation and breakdown involved in the amide hydrolysis studied here. However, we are primarily interested in the effects of various electrophiles on the hydrolysis. It is expected that the correlation effects will approximately cancel in a comparison of the hydrolysis with and without the electrophiles.

The system which we have studied is an idealized model of the geometry within the active site. As such, it neglects some aspects of strain which may be important to catalysis. Our system may only serve as a model for peptide cleavage by CPA subsequent to the initial association of the substrate with the enzyme. The dissociation of the hydrolyzed products from the enzyme has also been left untreated here. We have thus neglected such important effects as desolvation, loss of degrees of freedom, and other entropy-related processes. Of course, these free energetic contributions may be very important to a final understanding of the mechanism of CPA.

Acknowledgment. We are grateful to the National Institutes of Health Grant GM 06920 for support. S.S. wishes to thank the National Science Foundation for a predoctoral fellowship. We would also like to thank Dr. P. A. Kollman for sending us a copy of his manuscript prior to publication. The figures were drawn by Jean Evans.

References and Notes

(1) B. L. Vallee, J. A. Rupley, T. L. Coombs, and H. Neurath, *J. Am. Chem. Soc.*, **80**, 4750 (1958).
(2) J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, **235**, 390 (1960).
(3) J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, **236**, 2244 (1961).
(4) B. L. Vallee and J. F. Riordan, *Proc. Natl. Acad. Sci. U.S.A.*, **49**, 109 (1963).
(5) F. A. Quiocho and W. N. Lipscomb, *Adv. Protein Chem.*, **25**, 1 (1971).
(6) W. N. Lipscomb, J. A. Hartsuck, G. N. Reeke, F. A. Quiocho, P. H. Bethge, M. L. Ludwig, T. A. Steitz, H. Muirhead, and J. C. Coppola, *Brookhaven Symp. Biol.*, **21**, 24 (1968).
(7) G. Muszynska and J. F. Riordan, *Biochemistry*, **15**, 46 (1976).
(8) E. T. Kaiser and B. L. Kaiser, *Acc. Chem. Res.*, **5**, 219 (1972).
(9) E. T. Kaiser, T. W. Chan, and J. Suh, "Protein–Metal Interactions", M. Friedman, Ed., Plenum Publishing Co., New York, N.Y., 1974, pp 59–80.
(10) J. Suh and E. T. Kaiser, *Biochem. Biophys. Res. Commun.*, **64**, 863 (1975).
(11) (a) D. M. Hayes and P. A. Kollman, *J. Am. Chem. Soc.*, **98**, 3335 (1976); (b) D. M. Hayes and P. A. Kollman, *ibid.*, **98**, 7811 (1976).
(12) T. A. Halgren and W. N. Lipscomb, *J. Chem. Phys.*, **58**, 1569 (1973).
(13) R. Yamdagni and P. Kebarle, *J. Am. Chem. Soc.*, **95**, 4050 (1973).
(14) J. H. Richardson, J. M. Stephenson, and J. I. Brauman, *J. Am. Chem. Soc.*, **97**, 2967 (1975).
(15) Notation (*a*, *b*) refers to the distances ($r(C_I–O_w)$, $r(C_I–N_I)$) (see Figure 1).
(16) The increased electron density on carbon is a result of charge transfer from the proton-donating water H_2O_t. The presence of the water hydrogen bonding to the carbonyl oxygen triggers a release of electrons from H_2O_t to the amide system. However, unlike the charged electrophiles (e.g., Li^+, NH_4^+) the neutral water can only accommodate a very small fraction of this electron density. The result is an increase of negative charge on all groups of the amide, including the carbon atom.
(17) This increase is a reflection of the very large increase in the negative charge of the carbonyl oxygen caused by the interaction of the NH_4^+ electrophile with the amide in its planar configuration (see Table III).
(18) Hayes and Kollman have found (ref 11b) that an electropositive point charge (which represents Zn^{2+}) positioned near the carbonyl oxygen of *N*-methylacetamide has a strengthening effect on the C–N bond of that molecule.
(19) An ab initio technique has found a similar increase in the rotation barrier in formamide as a result of binding of Li^+: A. M. Armbruster and A. Pullman, *FEBS Lett.*, **49**, 18 (1974).
(20) For a more detailed explanation, see Papers 2 and 3 in this series (ref 21 and 22).
(21) S. Scheiner, W. N. Lipscomb, and D. A. Kleier, *J. Am. Chem. Soc.*, **98**, 4770 (1976).
(22) S. Scheiner and W. N. Lipscomb, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 432 (1976).
(23) $r(Be–O) = 1.55$ Å. The remainder of the geometry is similar to that of $[Be(NH_3)_2(OH)]^+$ (see footnote *b* of Table II).
(24) Similar interactions have been found between carbonyl systems and other uncharged nucleophiles in ref 21.
(25) R. Breslow and D. E. McClure, *J. Am. Chem. Soc.*, **98**, 258 (1976); R. Breslow and D. Wernick, *ibid.*, **98**, 259 (1976).
(26) W. N. Lipscomb, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **30**, 1042 (1971).