protonation of A_1^- by the buffer acids which are different structural types except for chloroacetic and acetic acids. The Brønsted correlation is, therefore, not good, but its slope α is approximately 0.4.

A number of rate constants for similar reactions may be found. Using our notation and units, k_{-3}^{OH} for ionization of a methylene proton in nitroethane[13] (16.7) or in 1-*p*-methoxyphenyl-2-nitropropane[14] (**4**) (0.78 in 50% v/v H_2O–MeOH) and k_3^H for protonation of nitroethane anion[15] (15) are all considerably less than the corresponding constants for our nitroalcohol **2**, while the ionization constants K_{-3} for nitroethane, **4**, and **2** (2.5×10^{-9}, 6.3×10^{-10}, and 1.7×10^{-9}) are not very different. Evidently the 2-hydroxy group of **2** accelerates the loss or gain of a proton.

(13) J. E. Dixon and T. C. Bruice, *J. Amer. Chem. Soc.*, **92**, 905 (1970).
(14) F. G. Bordwell, W. J. Boyle, Jr., and K. C. Yee, *ibid.*, **92**, 5926 (1970).
(15) R G. Pearson and R. L. Dillon, *ibid.*, **75**, 2439 (1953).

Stewart's value[16] of k_1^{OH} (0.077) for the 3-methoxy-4-hydroxy analog of **1** is less than our value of 0.3 from Table IV, as expected for a reaction between two anions. His k_{345} (1.02×10^{-3} sec^{-1}) is the same order of magnitude as ours, but note that his solutions contained 1% ethanol.

Addition of thiosulfate to acrylonitrile[17] shows $k_1^{S_2O_3} = 1.5 \times 10^{-4}$. Other k_1 values are 0.74 and 0.0032 for the respective additions[18] of barbiturate and nitroformate ions to β-nitrostyrene in methanol at 40°, and 530 for methoxide ion with a cyclic β-nitrostyrene in methanol.[19]

Acknowledgment. We appreciate the support of Research Corporation and the National Science Foundation.

(16) R. Stewart, *ibid.*, **74**, 4531 (1952).
(17) R. Kerber and J. Starnick, *Tetrahedron Lett.*, **No. 26**, 3007 (1966).
(18) J. Hine and L. A. Kaplan, *J. Amer. Chem. Soc.*, **82**, 2915 (1960).
(19) F. A. Bordwell, K. C. Yee, and A. C. Knipe, *ibid.*, **92**, 5945 (1970).

Kinetics of the Reactions of Papain with Substituted α-Haloacetophenones

Richard W. Furlanetto[1] and E. T. Kaiser*[2]

Contribution from the Departments of Biochemistry and Chemistry, University of Chicago, Chicago, Illinois 60637. *Received May 8, 1973*

Abstract: The pH–rate profile for the reaction of papain with α-bromo-4-hydroxy-3-nitroacetophenone (I) has been determined. Under the conditions of enzyme in excess, analysis of the pH dependence of the quantity (k_{obsd}/$[E]_0$), where k_{obsd} is the observed first-order rate constant, suggests that papain can react with I by either of two pathways. One pathway involves catalysis by an enzymatic group ($pK_1 = 3.08$), presumably functioning as a general base, of the attack of the active site sulfhydryl group ($pK_2 = 9.06$) on I. In the pH region where this pathway predominates, the reaction of papain with I clearly depends on the integrity of the active site of the enzyme, as is required for an active site titrant. The other pathway which operates at high pH appears to involve the direct nucleophilic attack of the thiolate form of the enzymatic sulfhydryl group on I. α-Bromo-3-hydroxy-6-nitroacetophenone (VI) reacts with the papain's active site with 1:1 stoichiometry, leading to the inactivation of the enzyme. However, because VI reacts considerably slower than I does, it is not particularly suitable for use as an active site titrant. The spectrophotometric titration curves for the *o*-nitrophenol function in papain modified by I and the *p*-nitrophenol moiety introduced in the enzyme by the reaction of VI show sigmoidal dependencies, indicating that the ionization of these groups is not significantly perturbed by that of an enzymatic ionizing group having a comparable pK_a value. However, the pK_a shifts seen for the ionization of the nitrophenol groups due to their covalent attachment to the enzyme may be interpreted in terms of the less polar environment of the active site as compared with the aqueous solution.

Recently, we reported[3] that the phenacyl halide α-bromo-4-hydroxy-3-nitroacetophenone (I) reacts rapidly with the active site of papain to produce an inactive modified enzyme (II), containing an *o*-nitrophenol "reporter"[4] group covalently bound to the very center of the active site. This reaction allowed us to titrate the enzyme by two different experimental approaches. In one approach the titration procedure utilized differences in the ultraviolet absorption spectra of the reagent I and the bound species II. The other approach involved monitoring the extent of the inhibition of enzymatic activity due to the reaction of I by rate assays employing *p*-nitrophenyl *N*-benzyloxycarbonylglycinate (III)[5] and ethyl *N*-benzoylargininate (IV)[6,7] as the substrates. The extent of inhibition was found to correspond to the loss of sulfhydryl groups as measured by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (V),[8] and this provided strong evidence that the sulfhydryl group at the active site of papain is the function which reacts with I to

(1) Life Insurance Medical Scientist Fellow.
(2) Fellow of the Alfred P. Sloan Foundation.
(3) R. W. Furlanetto and E. T. Kaiser, *J. Amer. Chem. Soc.*, **92**, 6980 (1970).
(4) M. B. Hille and D. E. Koshland, Jr., *ibid.*, **89**, 5945 (1967).
(5) J. F. Kirsch and M. Igelstrom, *Biochemistry*, **5**, 783 (1966).
(6) M. L. Bender, M. L. Begué-Canton, R. L. Blakely, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, **88**, 5890 (1966).
(7) J. R. Whitaker and M. L. Bender, *ibid.*, **87**, 2728 (1965).
(8) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

give the inhibited enzyme. Also, since the results of direct spectrophotometric titration and the rate assay measurements agreed well, we concluded that the concentration of the same active species was being measured by the two experimental approaches.

To establish firmly the theoretical basis of an active site titration procedure, it is necessary to understand the kinetics of the reaction of the titrant with the active site of the enzyme. The study reported here deals with a kinetic analysis of the reaction of I with papain. In addition, the reaction of another "reporter" group-labeled titrant, α-bromo-3-hydroxy-6-nitroacetophenone (VI), with this enzyme has been briefly examined.

Experimental Section

Materials. Papain used throughout this work was purchased from Worthington Biochemical Corp. Various lots were employed and in most instances the enzyme was purified by the method of Blumberg, *et al.*[9] However, in some early work commercially available mercuripapain (lots 7KA and 9IA) was used without further purification.

The distilled water used in this work was demineralized by passing it through a mixed-bed, ion-exchange column (from Continental Demineralization Service). All organic solvents were commercially available reagent grades and were generally employed without further purification. An exception was acetonitrile which was distilled from phosphorus pentoxide before use. Acids and inorganic salts used throughout were reagent grade. Guanidine hydrochloride and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) were "ultra pure" and purchased from the Mann Research Laboratories. Sephadex G-25 (fine) and Sepharose 4-B were purchased from Pharmacia Fine Chemicals, Incorporated.

Ethyl *N*-benzoylargininate (mp 133–135°), purchased from Mann Research Laboratories, was top quality and used without further purification. *p*-Nitrophenyl *N*-benzyloxycarbonylglycinate (mp 127–128°), glutathione, L-cysteine (free base), and 2-mercaptoethanol were purchased from Sigma Chemical Co. and were not further purified. *p*-Toluenethiol, obtained from Eastman Organic Chemicals, was recrystallized twice from aqueous ethanol (mp 42.5–43.5°). *N*-Acetyl-L-cysteine (mp 99–101°) was obtained from Nutritional Biochemical Corp.

The following compounds, listed by supplier, were used in the preparation of the affinity chromatography column: *tert*-butyloxycarbonyl-*O*-benzyl-L-tyrosine *O*-hydroxysuccinimide ester and glycylglycine (Fox Chemical Co.); L-arginine monohydrochloride (Mann Research Laboratories); *tert*-butyloxycarbonyl azide and *N*-hydroxysuccinimide (Aldrich Chemical Co.); dicyclohexylcarbodiimide and cyanogen bromide (Eastman Organic Chemicals). All were of the highest grade and purity commercially available and were used without further purification.

Syntheses. 4-Hydroxy-3-nitroacetophenone was synthesized from 4-hydroxyacetophenone by the procedure of Bartlett and Trachtenberg.[10] Two recrystallizations of the crude product from ethanol gave yellow needles, mp 133° (lit.[10] mp 135°), in 70% yield.

α-Bromo-4-hydroxy-3-nitroacetophenone (I) was prepared from 4-hydroxy-3-nitroacetophenone by the method of Sipos and Szabo.[11] Two recrystallizations of the crude product from carbon tetrachloride gave yellow needles, mp 91.5–92.0° (lit.[11] mp 93°), in 74% yield.

3-Hydroxy-6-nitroacetophenone was prepared from 3-hydroxyacetophenone by the procedure of Klinke and Heinz.[12] The crude product was recrystallized twice from benzene giving yellow needles, mp 148–149° (lit.[12] mp 148–149°), in 15% yield.

α-Bromo-3-hydroxy-6-nitroacetophenone (VI). To 250 ml of a 1:1 $CHCl_3$–CCl_4 solution was added 0.906 g of 3-hydroxy-6-nitroacetophenone (0.005 mol). Sufficient ethyl acetate was added to dissolve the acetophenone and then 6.25 ml of a solution of 8×10^{-1} *M* Br_2 in $CHCl_3$ solution (0.005 mol) was added. The mixture was warmed to 61° where HBr gas evolved and the solution lost its color. The reaction mixture was held at this temperature for 5 min, The solvent was then removed under reduced pressure yielding an oily residue which was extracted with three portions of boiling CCl_4. The CCl_4 fractions were combined, the solvent was removed, and the remaining residue was crystallized from benzene–petroleum ether (30–60°). The yield was 200 mg of gray-white needles (15%), mp 112.5–113°.

Mass spectral analysis showed a parent peak consistent with the molecular weight of α-bromo-3-hydroxy-6-nitroacetophenone. The 60-MHz nmr spectrum showed two protons at τ 5.6 relative to tetramethylsilane (CD_3CN), whereas the starting material showed three protons at τ 7.15 under the same conditions. Comparison of the nmr spectra showed that there were no other changes.

Anal. Calcd for $C_8H_6BrNO_4$: C, 36.95; H, 2.33; N, 5.39. Found: C, 37.06; H, 2.37; N, 5.45.

Glycylglycyl-*O*-benzyl-L-tyrosyl-L-arginine dihydrochloride was synthesized starting from *tert*-butyloxycarbonylglycylglycine by the procedure of Blumberg, *et al.*[9]

Preparation of the Affinity Chromatography Column. Glycylglycyl-*O*-benzyl-L-tyrosyl-L-arginine was coupled to Sepharose 4-B by the method of Blumberg, *et al.*[9]

Methods. General Experimental Techniques. The kinetic experiments reported here were performed using spectrophotometric techniques. Routinely, a Cary Model 15 or Gilford Model 220 recording spectrophotometer was used. Both instruments were equipped with thermostattable cell compartments and cell jackets and were adjusted to 25.0 ± 0.1°.

Rapid reactions were followed using the stopped-flow technique. An Aminco-Morrow stopped-flow apparatus (thermostatted to 25.0 ± 0.1°) was used. In all stopped-flow experiments the total per cent transmittance change in the reaction observed was adjusted to be less than 5%. In the absence of a large background absorbance, transmittance changes of this magnitude are approximately proportional to absorbance changes, and, therefore, first-order plots could be constructed directly from the experimental traces.

Measurements of pH were made on a Radiometer type PHM 4C pH meter previously standardized with an appropriate Fisher Certified standard buffer solution.

Stock solutions of organic reagents were prepared in redistilled acetonitrile unless otherwise noted. Most reagent solutions were stable to prolonged storage when refrigerated at 4°. An exception was the stock solution of α-bromo-3-hydroxy-6-nitroacetophenone which acquired a brown tinge on standing over a few days, even when refrigerated. Consequently, solutions of this compound were prepared fresh daily.

Purification of Papain. Commercially available papain was purified by the method of Blumberg, *et al.*,[9] using a glycylglycyl-*O*-benzyl-L-tyrosyl-L-arginine Sepharose 4-B affinity chromatography column. Routinely, a 250-mg sample of the commercially available enzyme was purified on a 1.5 × 22 cm column. During the purification, about 60% of the applied protein was not bound by the column. However, the 40% recovered by elution of the bound material with deionized water had approximately twice the specific activity of the initial sample and was 90–97% pure, based on active site titration.

In most cases the enzyme was then inhibited with approximately 1.5 mol of $HgCl_2$/mol of protein and the rather dilute protein solution obtained (about 100 mg in 130 ml) was concentrated to a final concentration of 10 mg/ml on an Amicon Model MC-8 protein concentrator fitted with a UM-10 membrane. This procedure required 5–6 hr at 45 psi N_2 and room temperature. The concentrated mercuripapain was stored at 4° under N_2 until needed. Mercuripapain is said to show no loss of potential activity after 30 days storage under these conditions.

When large quantities of active enzyme were required (for stopped-flow experiments), the enzyme was purified as described but was not inactivated with $HgCl_2$ prior to concentration. Concentration of the active enzyme (carried out at 45 psi N_2 and room temperature) resulted in approximately 5% loss of specific activity. The active concentrated enzyme was stored under N_2 at 4° and used the next day (active papain loses about 4% activity per day at 4°).

During initial purification studies it was found that good results were obtained only if the purification buffers were prepared using the disodium salt of EDTA (in contrast to the tetrasodium salt). The reason for this high sensitivity is quite likely related to the marked increase in ionic strength resulting from the use of the tetrasodium salt. In this regard it was also noted that the purification was more efficient when no excess of sodium chloride was present in the purification buffers. To avoid such an excess the

(9) S. Blumberg, I. Schechter, and A. Berger, *Eur. J. Biochem.*, **15**, 97 (1970).
(10) P. D. Bartlett and E. N. Trachtenberg, *J. Amer. Chem. Soc.*, **80**, 5808 (1958).
(11) G. Sipos and R. Szabo, *Acta Phys. Chem.*, 7, 126 (1961).
(12) P. Klinke and G. Heinz, *Chem. Ber.*, **94**, 26 (1961).

buffers were brought to the desired pH by very careful addition of either acid or base.

Activation of Mercuripapain. It is necessary to activate mercuripapain before use. The method chosen for activation was one which gives enzyme free of other thiol-containing compounds, the presence of which may complicate the experimental results.[13] A 1.3-ml solution of mercuripapain in deionized water (usually about 10 mg/ml) was gently shaken with two volumes of a toluene solution of *p*-toluenethiol (14 mg/ml, 0.113 *M*) for 7–8 min. The aqueous layer was removed and applied to a (1.2 × 14 cm) Sephadex G-25 fine column preequilibrated with the desired buffer. The column was eluted with the same buffer and a single fraction[14] was collected, the protein usually appearing between 8 and 16 ml of the eluate volume. The enzyme was stored under N_2 at 4° until needed (usually the same day).

Rate Assay Techniques. ***p*-Nitrophenyl *N*-benzyloxycarbonylglycinate (III) assays** were carried out in 0.02 *M* phosphate buffer, pH 6.80, 10^{-3} *M* EDTA, 6.8% CH_3CN with [III] ≫ $[E]_0$. Under the conditions employed,[15] the velocity of reaction was directly proportional to $[E]_0$, and it was possible to compare relative enzyme concentrations of two solutions by relative rate measurements.

Ethyl *N*-benzoyl-L-argininate (IV) assays were performed in 0.05 *M* acetate buffer, pH 5.2, 10^{-5} *M* EDTA, μ = 0.038, with $[IV]_0 \gg [E]_0$.[6] The average value of $(k_{cat}/K_{m_{app(IV)}})$ determined from measurements with three papain samples was $(1.41 \pm 0.04) \times 10^3$ M^{-1} sec^{-1}.

Determination of Free Sulfhydryl Groups. The sulfhydryl content of solutions was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (V).[8] Details of the assay depended on the thiol content in the stock solution of the species being analyzed. To determine the thiol content of papain solutions, 1.00 ml of the papain solution to be analyzed was mixed with 2.00 ml of 0.20 *M* phosphate buffer, pH 8.0, containing 3 × 10^{-3} *M* EDTA (final pH 7.90–8.0). A sufficient amount of V (2.0 × 10^{-2} *M* in 0.067 *M* phosphate buffer, pH 7.0) was added to give an initial reagent concentration of 3.28 × 10^{-4} *M* and the absorbance at 412.0 nm was measured after 6 min. The thiol content of glutathione and *N*-acetyl-L-cysteine solution was determined by adding an aliquot (usually 100 μl) of the solution to 3.00 ml of 0.067 *M* phosphate buffer, pH 8.06, 10^{-3} *M* EDTA. Again, the assay was initiated by addition of sufficient V to give an initial reagent concentration of 3.28 × 10^{-4} *M* and the absorbance was measured at 412.0 nm after 6 min.

Care was taken to have at least a sixfold excess of V present. An extinction coefficient of 13,600 M^{-1} cm^{-1} was used for 5-thio-2-nitrobenzoic acid (produced in the reaction of V with a free sulfhydryl group) at pH 8.

Inhibition Reactions. The inhibition of papain by the various reagents described in this paper was performed using the following standard procedure. To 1.00 ml of a buffered solution containing papain was added 25 μl of that concentration of reagent in acetonitrile necessary to give the desired final inhibitor concentration. After introducing a N_2 atmosphere and sealing with parafilm, the solutions were mixed by gently spinning and incubated at room temperature. When a larger volume of inhibited solution was required, the volume of both enzyme and inhibitor solutions was increased proportionately so as to maintain 2.4% CH_3CN in the final mixture. In all cases a control mixture of papain plus pure acetonitrile was simultaneously prepared.

Determination of Extinction Coefficients. The extinction coefficients of enzyme bound chromophores (*i.e.*, those resulting from reaction of the enzyme with inhibitors) were determined under conditions of enzyme in excess, thus assuring complete reaction of the inhibitor with the enzyme. In all cases the completion of reaction was confirmed using rate assay techniques. Under these circumstances eq 1 can be used to calculate $(\epsilon_{EI} - \epsilon_E)$ at any wavelength or pH where ΔA is the difference in absorption of the modified

$$(\epsilon_{EI} - \epsilon_E) = \frac{\Delta A}{[I]_0} \qquad (1)$$

vs. the unmodified protein, $[I]_0$ is the initial inhibitor concentration, and ϵ_{EI} and ϵ_E are the extinction coefficients of the modified and unmodified protein, respectively.

pK_a Determinations. The pK_a's of the enzyme-bound chromophores were determined under conditions of enzyme in excess, thus assuring complete reaction of the chromophoric species with the enzyme. Again, the completion of reaction was confirmed using rate assay techniques. To determine the absorbance *vs.* pH curves, 200 μl of the solution of the inhibited enzyme in dilute buffer was mixed with 1.00 ml of the buffer of the appropriate pH and ionic strength, and the absorbance of the resulting solution was recorded as a function of wavelength. An exactly equivalent papain solution, lacking inhibitor, was used as a blank. The pH of the inhibited solution was then carefully measured. The same pipets and cuvettes were used for each determination.

To determine the pK_a's of the free inhibitors, a much simpler procedure could be used. To 3.00 ml of the appropriate buffer was added 75 μl of a stock inhibitor solution (in acetonitrile). The increase in absorbance on addition of the inhibitor was recorded as a function of wavelength. The equivalent buffer was used as a blank.

Kinetics of the Reaction of I with Papain at Various pH Values. To study the reaction of I with papain at various pH values it was necessary to employ the stopped-flow kinetic technique under conditions of enzyme in excess over the substrate. Since this required large volumes of active papain solutions, the enzyme was not inhibited with Hg^{2+} before concentration. The following standard procedure was found suitable for studying the reaction over the entire pH range. To one syringe was added the active enzyme in deionized water. To the other syringe was added a solution of I in a buffer which, when diluted in half with water, would be of the desired ionic strength and pH. (It was previously determined that the inhibitor was stable for long periods of time in all the buffers employed.) The drive syringes were thoroughly rinsed with these solutions, then filled, and the solutions were allowed to equilibrate at 25.0 ± 0.1°. The reaction was initiated by the mixing of equal volumes of the above solutions. The course of the reaction was continuously recorded on the screen of a storage oscilloscope and photographed after the "infinity" value was reached. The effluent was collected to determine the exact pH of the reaction.

All reactions were followed at 322.8 nm, and the total change in per cent transmittance was always kept below 5%. The enzyme was maintained in at least a 9.7-fold excess. All reactions showed pseudo-first-order kinetics for a minimum of 4 half-lives.

Kinetics of the Reaction of I with Glutathione at Various pH Values. The reaction of I with glutathione (VII) was studied using two spectrophotometric methods. The first method, employed at pH values greater than 8.5, was essentially identical with that used in studying the reaction of I with papain. The only differences were the substitution of VII for papain and the observation of the reaction at 315.0 nm. The second method, used below pH 8.5 where the reaction was slower, employed the Cary 15 spectrophotometer to follow the reaction. These studies were performed as follows. To 2.00 ml of the appropriate buffer was added an aliquot of a stock solution of VII (in water) and the mixture was equilibrated at 25° for 10 min. The reaction was initiated by the addition of I from a stock acetonitrile solution, followed by a mixing period of approximately 10 sec. The reaction was followed to completion on the expanded scale slidewire at 315.0 nm. The large background absorbance (of the inhibitor) was blanked out by a 0.5 absorbance unit filter placed in the reference beam.

All reactions were run with at least a tenfold excess of VII and showed pseudo-first-order kinetics over a minimum of 5 half-lives.

Results

Kinetics of the Reaction of I with Papain. We found it most convenient to study the rate of reaction of I with papain with the enzyme in excess, since under these conditions simple pseudo-first-order kinetics were obtained.[16] The rapidity of the reactions observed in our experiments demanded that rate measurements be performed using the stopped-flow technique.

Since we were interested in comparing our results with those found for papain substrates and for other modifying agents, we investigated the rate behavior of our system over a wide pH range, pH 2.95 to 10.40. For this reason it was necessary to evaluate the stability of the enzyme below pH 3.5 and above pH 9.5, pH values at which papain is known to denature fairly

(13) M. Soejima and K. Shimura, *J. Biochem. (Tokyo)*, **49**, 260 (1961).
(14) The fraction which contained protein was predetermined by measuring the eluate volume for a dilute blue dextran solution.
(15) J. F. Kirsch and M. Igelström, *Biochemistry*, **5**, 783 (1966).
(16) G. Tomalin, M. Trifunac, and E. T. Kaiser, *J. Amer. Chem. Soc.*, **91**, 722 (1969).

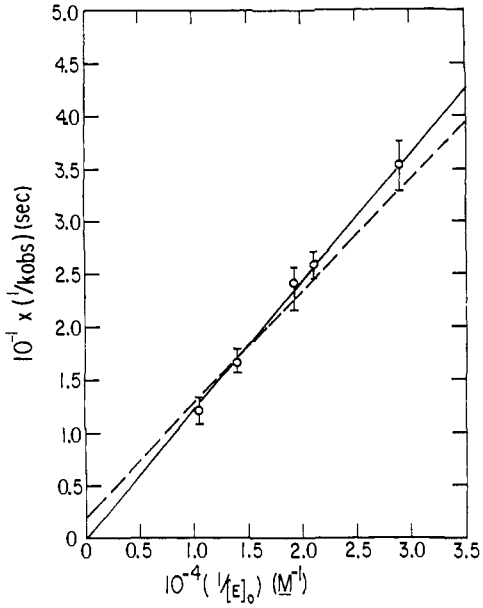


Figure 1. A plot of $(1/k_{obsd})$ *vs.* $(1/[E]_0)$ for the reaction of papain with I in phosphate buffer, pH 7.0, 10^{-4} *M* EDTA, μ = 0.05 at 25.0°. The solid line represents the best fit to the data while the broken line is drawn to give an estimate of the maximum positive ordinate intercept consistent with these results. The solid line gives an average value of $k_{obsd}/[E]_0$ = 838 M^{-1} sec^{-1}.

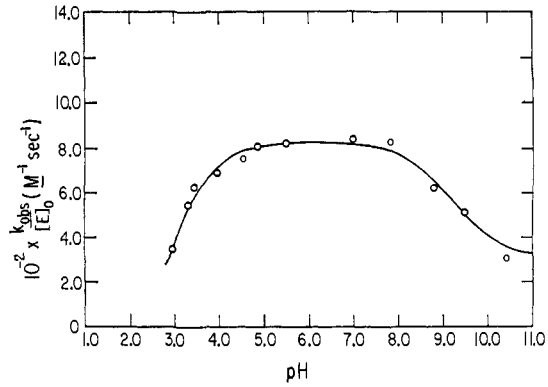


Figure 2. $-(k_{obsd}/[E]_0)$ *vs.* pH for the reaction of papain with I at μ = 0.05 and 25.0°. The circles are the experimental points while the curve is a theoretical one drawn for pK_1 = 3.08, pK_2 = 9.06, $(k_{i,EH}/K_{I,EH})$ = 820 M^{-1} sec^{-1}, and $k_{i,E}$ = 332 M^{-1} sec^{-1}. Data were gathered in formate buffer over the range pH 2.95 to 5.55, in acetate from pH 4.52 to 5.55, in phosphate at pH 7.01 and 7.84, in borate at pH 8.82 and 9.51, and in carbonate at pH 10.40. All buffers contained 10^{-4} *M* EDTA, and the $(k_{obsd}/[E]_0)$ values shown are the averages of three determinations. The buffers were prepared by the procedures given in C. Long, Ed., "Biochemists' Handbook," Van Nostrand, Princeton, N. J., 1961, pp 19–42.

rapidly.[17] Such a study was performed by incubating solutions of the enzyme under the conditions to be used in the stopped-flow experiments and assaying aliquots of these solutions at various time intervals with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate. The results obtained demonstrated that there was no detectable loss of enzymatic activity (no irreversible denaturation) after 1500 sec at pH values of 3.38 (formate buffer) and 10.41 (carbonate buffer) or after 100 sec at pH 2.97 (formate buffer). A 6% loss of activity did occur after 500 sec at pH 2.97. However, in our experiments the reaction of papain with I was essentially complete after 200 sec at this pH, and, therefore, the loss of activity due to denaturation was considered to be negligible over the course of the reaction.

The kinetic results for the reaction of I with papain performed in phosphate buffer, pH 7.01, 10^{-4} *M* EDTA, μ = 0.05, with variable (excess) enzyme concentrations, have been interpreted in terms of the scheme of eq 2 where E·I is a noncovalent complex of I with papain. The rate expression for the inhibition of the enzyme which corresponds to the formation of II is given by eq 3. Equation 4 shows the relationship of the observed first-order rate constant (k_{obsd}) to the parameters of eq 2, while eq 5 is the reciprocal form of eq 4 which can be used to evaluate these parameters.

$$E + I \underset{K_I}{\rightleftharpoons} E\cdot I \xrightarrow{k_i} II \tag{2}$$

$$v = \frac{d[II]}{dt} = \frac{k_i[E]_0([I]_0 - [II])}{K_I + [E]_0} \tag{3}$$

$$k_{obsd} = \frac{k_i[E]_0}{K_I + [E]_0} \tag{4}$$

(17) A. N. Glazer and E. L. Smith, "The Enzymes," Vol. III, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971, p 501.

$$\frac{1}{k_{obsd}} = \frac{K_I}{k_i[E]_0} + \frac{1}{k_i} \tag{5}$$

A plot of $(1/k_{obsd})$ *vs.* $(1/[E]_0)$ for the reactions run at pH 7.0 is shown in Figure 1. Error bars are included to show the maximum deviation of the points. Two straight lines are shown. One line (the solid line in Figure 1) is drawn to give the best fit to the data and passes through the origin. This implies that under the conditions employed the reaction was first order in enzyme (*i.e.*, $K_I \gg [E]_0$) and therefore that the second-order rate constant $(k_{obsd}/[E]_0)$ is equal to k_i/K_I.

The broken line in Figure 1 is drawn to give an estimate of the maximum possible value of $(1/k_{obsd})$ which is consistent with the data. From this it can be calculated that k_i has a minimum value of 0.50 sec^{-1} while the minimum value of K_I is 5.4 × 10^{-4} *M*. It should be stressed, however, that these are the extreme possible values and do not represent the best fit to the data.

Having established that at pH 7.0 and under conditions of enzyme in excess we were not observing saturation of the enzyme by the inhibitor (*i.e.*, $K_I \gg [E]_0$ and therefore, over the pH range where eq 2 holds, $k_{obsd}/[E]_0 = k_i/K_I$), it was assumed that this result was valid at all pH values, and the reaction was then studied as a function of pH. The data obtained at μ = 0.05 and 25.0° (Figure 2) can best be described by the relationship given in eq 6, which is derived from the scheme shown in eq 7a and 7b. Here EH_2, EH and E, and $EH_2\cdot I$, EH·I, and E·I symbolize, respectively, three forms of the enzyme and three forms of the enzyme–inhibitor complex, differing in the state of ionization of two ionizable groups, and I symbolizes the inhibitor, *independent* of its state of ionization.

$$\frac{k_{obsd}}{[E]_0} = \frac{k_{i,EH}/K_{I,EH}}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} + \frac{k_{i,E}}{1 + \frac{[H^+]}{K_2}} \tag{6}$$

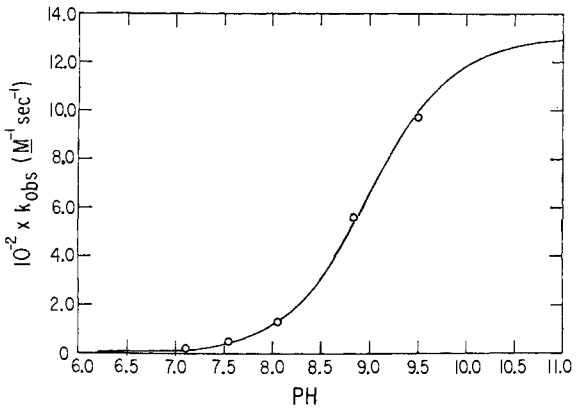


Figure 3. Plot of k_{obsd} *vs.* pH for the reaction of I with glutathione at μ = 0.05 and 25.0°, where k_{obsd} is the second-order rate constant obtained by dividing the measured pseudo-first-order rate constant by the glutathione concentration. The circles are the experimental points while the curve is a theoretical sigmoid calculated for pK_a = 9.0 and k_{RS^-} = 1300 M^{-1} sec^{-1}. Data were gathered in Tris buffer from 7.11 to 8.05 and in borate buffer at pH 8.84 and 9.51 in the presence of 10^{-4} M EDTA.

$$\begin{array}{ccc} EH_2 & \overset{+I}{\rightleftharpoons} & EH_2 \cdot I \\ {\scriptstyle K_1}\uparrow\downarrow{\scriptstyle +H^+} & & \\ EH & \overset{K_{i,EH},\ +I}{\rightleftharpoons} & EH \cdot I \xrightarrow{k_{i,EH}} EHI \\ {\scriptstyle K_2}\uparrow\downarrow{\scriptstyle +H^+} & & \\ E & \overset{+I}{\rightleftharpoons} & E \cdot I \end{array} \qquad (7a)$$

$$E + I \xrightarrow{k_{i,E}} EI \qquad (7b)$$

This analysis implies that I reacts with papain by two distinct pathways: one (eq 7a) proceeds (as the reaction of a substrate would) *via* the formation of a noncovalent EH·I complex, and the other (eq 7b) goes *via* a simple bimolecular process.[18,19] The theoretical curve shown in Figure 2 was calculated using eq 6 and making the assumptions given in the legend.

Kinetics of the Reaction of I with Glutathione. As a model for the behavior of a nonenzymatic thiol group, the reaction of I with glutathione (VII) was examined. VII (1.7×10^{-3} M) was incubated in 0.067 M phosphate buffer, containing 10^{-4} M EDTA and 2.42% CH_3CN, with concentrations of I varying up to 1.27×10^{-3} M, and the resultant thiol content was measured using V. The results showed that the reaction occurred with 1:1 stoichiometry. In addition, the absorption spectrum of the product showed a shift in the absorption maximum from 320 (free I) to 315 nm and an increase in ϵ at 315 nm from 1.68×10^4 to 1.89×10^4 M^{-1} cm^{-1}. Similar results were obtained when I was reacted with *N*-acetyl-L-cysteine. Because of the spectral changes, it is easy to study the kinetics of the reaction of I with the model thiol compound, VII, over a broad pH range. In Figure 3 the kinetic data measured at μ = 0.05 and 25.0° are shown, along with a theoretical sigmoid curve calculated using values of pK_a = 9.0 and k_{RS^-} = 1300 M^{-1} sec^{-1}, defined by the scheme of eq 8.

$$RSH \overset{K_a}{\rightleftharpoons} RS^- + H^+$$

$$RS^- + R'X \xrightarrow{k_{RS^-}} RSR' + X^- \qquad (8)$$

The pK_a of 9.0 calculated for the ionizations of the thiol group of VII from our kinetic results appears to be somewhat higher than the pK_a's obtained by others using different (nonkinetic) methods. For example, Martin and Edsall[21] found a value of 8.64 at μ = 0.16 and 25.0°, while Lindley[22] obtained a value of 8.65 at μ = 0.1 and 30.0°. However, Lindley[22] also obtained a pK_a value of 8.9 at μ = 0.10 and 30.0° from kinetic measurements on the alkylation of VII by chloroacetamide, a value which agrees quite well with that calculated from our studies on the alkylation of VII by I.

In our earlier report[3] we showed that the fractional inhibition produced by the reaction of papain with I at pH 7.0 is identical when measured by any of three assay reagents, III, IV, and V. This result means either that all the reactive sulfhydryl groups in the assayed solutions are identical (*i.e.*, all are active site sulfhydryls) or, if different, all react with I at approximately the same rate under the conditions used. Our kinetic results with glutathione (VII) indicate, however, that model sulfhydryl groups do not react at the same rate as the active site sulfhydryl groups of papain at pH 7.0 (or at pH 5.2 in 0.05 M acetate buffer, conditions under which we have also found it convenient to titrate enzyme solutions). This allows us to exclude the second alternative and to conclude, therefore, that the sulfhydryl content, as measured by titration at pH 7.0 with I, is indeed equal to the active site molarity of papain solutions.

Our kinetic results also allow us to conclude that the reaction of I with papain is "enzymatic," *i.e.*, it depends on the integrity of the enzyme's active site. If simply a rate enhancement had been found for this reaction, it could be argued that this might just be an effect due to the nonspecific protein environment near the reacting sulfhydryl. However, the observation of a bell-shaped pH rate dependence for the papain reaction (Figure 2) in contrast to the sigmoidal pH dependence found in the model system reaction (Figure 3) strongly suggests that the papain reaction directly involves the catalytic apparatus of the active site.[23]

Determination of pK_a Values for the Ionization of the *o*-Nitrophenol Groups in I and II. Spectrophotometric titration of the *o*-nitrophenol groups in I and II at 322.8 nm at μ = 0.05 showed that the absorbances rose with increasing pH, following theoretical sigmoidal curves of pK_a 4.7 and 5.05, respectively. The absorption maximum occurring for the nitrophenolate

(18) The possibility that the reaction of eq 7b might occur through the formation of an enzyme–inhibitor complex having a high dissociation constant cannot be ruled out. However, the similarity of the results obtained with papain in the alkaline region where this pathway appears to be important to those seen with glutathione (*vide infra*) suggests that noncovalent enzyme–inhibitor complex formation is not required.

(19) A scheme very similar to that represented in eq 7a and 7b has been used to describe the pH dependency of the reactions of papain with haloacetic acids.[20]

(20) I. M. Chaiken and E. L. Smith, *J. Biol. Chem.*, **244**, 5095 (1969).

(21) R. B. Martin and J. T. Edsall, *Bull. Soc. Chim. Biol.*, 40, 1763 (1958).

(22) H. Lindley, *Biochem. J.*, **82**, 418 (1962).

(23) Since our discovery that I can be used to titrate the active site of papain was reported,[3] Folk and Gross[24] have demonstrated that I can be employed to titrate guinea pig liver transglutaminase and that it is a useful tool for probing the active site of the latter enzyme.

(24) J. E. Folk and M. Gross, *J. Biol. Chem.*, **246**, 6683 (1971).

form of I at 320 nm (ϵ 1.74 × 10^4 M^{-1} cm^{-1})[25] was shifted to 322.8 nm in II. The large increase in the absorption at 322.8 nm resulting from the reaction of I with papain ($\Delta\epsilon = \epsilon_{II} - (\epsilon_I + \epsilon_{papain})$ = 6850) made the direct spectrophotometric titration of the enzyme's active site possible.

Reaction of VI with Papain. The results shown in Table I demonstrate that α-bromo-3-hydroxy-6-nitro-

Table I. Activity and Sulfhydryl Content of Papain Solutions Inhibited with Various Concentrations of VI[a]

10^5[VI]	Incubation time, hr	% of original enzymatic activity[b] remaining, Assay substrate III	IV	% original sulfydryl[b] content remaining
0.000	18.5	89.9	89.1	89.1
1.43	18.5	71.2	71.7	72.3
2.86	19.25	53.0	50.1	52.8
4.28	19.5	33.8	34.4	35.6
8.57	18.5	1.4	0	0

[a] The reaction mixtures contained acetate buffer, pH 4.46, 10^{-4} M EDTA, μ = 0.05, $[papain]_0$ = 6.95 × 10^{-5} M, $[sulfhydryl]_0$ = 6.51 × 10^{-5} M, 2.42% CH_3CN, and the temperature was 25.0°. [b] A value of 100% for the activity and sulfhydryl content corresponds to that of the control solution at incubation time zero. Over an 18.5-hr incubation period a 10% loss of activity occurred.

acetophenone (VI) reacts with the active site sulfhydryl group of papain with 1:1 stoichiometry, leading to the inhibition of enzymatic activity as measured by assays with both III and IV. However, VI inhibits papain at a much slower rate than I does, and for this reason VI is not a convenient active site titrant for the enzyme.

An absorption peak is seen at 406 nm (ϵ 18,700 M^{-1} cm^{-1}) for the ionized form of VI and at 324 nm (ϵ 8,000 M^{-1} cm^{-1}) for the neutral form. From measurements at 406 nm, the pK_a for the ionization of the nitrophenol group of VI was found to be 5.9 at μ = 0.05 and 25.0°. Spectra of the enzyme-bound chromophore exhibited a shift in the λ_{max} values to 413 at high pH (ϵ 17,000 M^{-1} cm^{-1}) and to 328 nm at low pH. Measurements at 413 nm of the pH dependency of the ionization of the bound chromophore closely fit a theoretical sigmoidal curve of pK_a 6.28. The 0.38 unit increase in the pK_a of the chromophore due to its presence near the active site of the enzyme is comparable with that seen in the case of I (0.35 unit).

Discussion

As discussed above, the pH–rate profile for the reaction of papain with I illustrated in Figure 2 can be analyzed in terms of the pathways shown in eq 7a and 7b. The nature of the groups responsible for the K_1 and K_2 ionizations is, of course, of interest. The ionizing group to which pK_1 = 3.08 is assigned cannot be the *o*-nitrophenol residue of I since this group is known to ionize with a pK_a of 4.7 when free in solution and 5.05 when bound to the enzyme (*vide ante*). A good possibility is that an enzyme-bound carboxylic acid group is the ionizing function involved. A similar assignment has been made in the interpretation of the kinetic data obtained in acidic solution for the alkylation of papain by α-halo acids.[20,26,27]

(25) The ionized form of I also has an absorption peak at 390 nm (ϵ 5.22 × 10^3 M^{-1} cm^{-1}).

From the descending part of the pH–rate profile for the reaction of papain with I at high pH, the pK_2 value obtained can be reasonably assigned to the sulfhydryl group of the enzyme. An analogous assignment has been made for the alkylation of papain with α-haloacetic acids.[20]

Turning now to the mechanism of the alkylation of papain by I, an explanation for our results (Figure 2) in the pH region where the pathway of eq 7a holds, which is consistent with the findings of others, is that attack of the active site sulfhydryl group on I is assisted by a group in the enzyme acting as a general base.

Normally, displacement reactions of the type under discussion would be expected to proceed more readily by a nucleophilic pathway than by the corresponding general base-catalyzed pathway. The reason for this may be that a displacement reaction occurring at a saturated carbon is relatively insensitive to the basicity of the attacking nucleophile, and, therefore, the transition state is not stabilized by the partial removal of the proton from the attacking nucleophile.[33] Nevertheless, general base catalysis of displacement reactions at the saturated carbon has been observed in some intramolecular cyclization reactions, the most notable of which is the cyclization of 4-chlorobutanol to tetrahydrofuran.[34,35] It therefore appears that under certain steric conditions the general base-catalyzed pathway is favored, and these conditions exist at the active site of the enzyme over the pH range where the pathway of eq 7a is dominant (where the reaction of I with papain is "enzymatic").

At high pH values nucleophilic attack by the thiolate form of papain predominates. The observation that the second-order rate constant, $k_{i,E}$ (eq 7b), seen at high pH (332 M^{-1} sec^{-1}) is similar to the limiting rate constant measured for the reaction of the model sulfhydryl compound glutathione with I in alkaline solution (1300 M^{-1} sec^{-1}) supports the hypothesis that at high pH the active site thiolate anion functions like a normal thiolate species in a bimolecular displacement process.

Our finding that the pH–rate profile for the reaction of I with papain was not perturbed by the state of ionization of the nitrophenol group in the reagent indicates that ionic interactions do not play an important role in the binding of this compound at the enzyme's active site. This conclusion is in sharp contrast to that reached for the α-halo acids where it has

(26) K. Wallenfels and B. Eisele, *Eur. J. Biochem.*, 3, 267 (1968).

(27) Whether the ionizing group responsible for the value of pK_1 we have measured in the alkylation of papain by I corresponds to the ionizing function important to the hydrolysis of substrates in acidic solution is not clear at this point. Our pK_1 value is somewhat lower than the ones usually found in the hydrolysis reactions.[7,28-30] In any event, while some workers have argued that the rising limbs of the k_2 *vs.* pH and k_3 *vs.* pH profiles in papain-catalyzed hydrolysis reactions are due to the ionization of a carboxyl group (Asp 158),[7,28,31] others have suggested that the deprotonation of an imidazolium function (His 159) is responsible.[29,32]

(28) M. L. Bender and L. J. Brubacher, *J. Amer. Chem. Soc.*, **88**, 5880 (1966).

(29) E. C. Lucas and A. Williams, *Biochemistry*, **8**, 5125 (1969).

(30) G. Lowe and Y. Yuthavong, *Biochem. J.*, **124**, 117 (1971).

(31) E. L. Smith and M. J. Parker, *J. Biol. Chem.*, **233**, 1387 (1958).

(32) S. S. Husain and G. Lowe, *Biochem. J.*, **108**, 861 (1968).

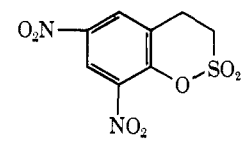
(33) W. P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, N. Y., 1969, p 169.

(34) C. G. Swain, D. A. Kuhn, and R. L. Schowen, *J. Amer. Chem. Soc.*, **87**, 1553 (1965).

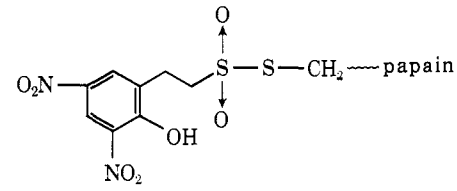
(35) Unpublished experiments of W. P. Jencks, I. Givot, and A. Satterthwait, cited in ref 33.

been postulated that an enzyme-bound imidazolium cation interacts with the carboxylate group of the reagents,[20,26] thereby orienting them for proper attack by the sulfhydryl function. If different factors are responsible for the binding of I than that of the α-halo acids, then the higher pK_2 value observed for the reaction of papain with I[36] may represent simply the ionization of the active site sulfhydryl group while, as suggested by others, the pK_2 values seen with the α-halo acids may reflect the dissociation of both the sulfhydryl and imidazole groups.

The pH dependencies for the ionization of the nitrophenol chromophores in papain alkylated by I and by VI are sigmoidal, demonstrating that the ionization of these groups is not detectably affected by that of an enzymatic ionizing group having a comparable pK_a value. This result differs from that obtained with papain sulfonylated by the reactive aromatic six-membered sultone β-(2-hydroxy-3,5-dinitrophenyl)-ethanesulfonic acid sultone (VIII) to give the enzymatic thiolsulfonate species IX.[37] Spectrophotometric titration of the dinitrophenol chromophore present in the thiolsulfonate species IX which is considerably more acidic than the nitrophenol functions present in papain alkylated by I and by VI indicates that the



VIII



IX

ionization of the dinitrophenolic hydroxyl is significantly perturbed by that of a group of similar pK_a at the active site of the enzyme. However, the identity of the perturbing enzyme-bound group has not been established yet.

Finally, a possible explanation for our observations that the nitrophenol groups in papain modified by I and by VI have significantly higher pK_a values than those in the reagents themselves is that the active site has a less polar environment than the aqueous solution. In accord with this interpretation, X-ray studies of crystalline papain indicate that the active sulfhydryl group is located in a nonpolar cleft.[38]

(36) For instance, the pK_2 value for the reaction of papain with L(−)-α-iodopropionic acid at 25.0° is 7.8[28] and with chloroacetic acid at 30.5° it is 8.1.[20]

(37) P. Campbell and E. T. Kaiser, *J. Amer. Chem. Soc.*, **95**, 3735 (1973).

(38) J. Drenth, J. Jansonius, R. Koekoek, and B. Wolthers, "The Enzymes," Vol. 3, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N. Y., 1971, p 484.

Synthesis of Saturated Isoimides. Reactions of *N*-Phenyl-2,2-dimethylsuccinisoimide with Aqueous Buffer Solutions

Carol K. Sauers,*[1a] Constance A. Marikakis,[1b] and Mary A. Lupton

Contribution from the Department of Chemistry, Douglass College, Rutgers—The State University of New Jersey, New Brunswick, New Jersey 08903.
Received April 16, 1973

Abstract: The synthesis of *N*-phenyl-2,2-dimethylsuccinisoimide, *N*-(*p*-anisyl)-2,2-dimethylsuccinisoimide, *N*-*n*-butyl-2,2-dimethylsuccinisoimide, *N*-phenyl-*endo*-*cis*-bicyclohept-5-ene-2,3-carboxisoimide, and *N*-(*p*-anisyl)-*endo*-*cis*-bicyclo[2.1.1]hept-5-ene-2,3-carboxisoimide by the dehydration of the corresponding amide acids with *N*,*N*′-dicyclohexylcarbodiimide or ethyl chloroformate–triethylamine reagents is reported. A detailed study of the kinetics of the reactions of *N*-phenyl-2,2-dimethylsuccinisoimide in aqueous buffers demonstrated that hydrolysis to the amide acid follows the rate law $k_{obsd} = 2 \times 10^{-4}\ sec^{-1} + 56\ M^{-1}\ sec^{-1}\ a_{H^+} + 510\ M^{-1}\ sec^{-1}\ a_{OH^-}$. The disappearance of isoimide is also catalyzed by the basic form of the buffers, carbonate, *N*-methylimidazole, acetate, and Dabco, as well as by acetic acid. In the presence of phosphate and tris(hydroxymethyl)aminomethane buffers, the buildup of intermediates was observed. Extensive rearrangement to the corresponding imide generally occurs in the presence of buffers. Saturated isoimides are more reactive than unsaturated isoimides; failure to isolate the former compounds by the direct dehydration of the corresponding amide acids can be attributed to their reactivity rather than to their failure to form.

Phthalisoimides were first synthesized in 1893[2] and the synthesis and chemical behavior of both phthalisoimides and malisoimides have received a considerable amount of more recent attention.[3] Only two reports of the isolation of cyclic isoimides derived

(1) (a) Visiting Associate Professor of Biochemistry, 1972–1973, Brandeis University, Waltham, Mass.; (b) Undergraduate Fellow, Research Corporation, 1970.

(2) S. Hoogewerff and W. A. van Dorp, *Recl. Trav. Chim. Pays-Bas*, **12**, 12 (1893).

(3) (a) See 3b and 3c and references contained therein: (b) M. L. Ernst and G. L. Schmir, *J. Amer. Chem. Soc.*, **88**, 5001 (1966); (c) C. K. Sauers, C. L. Gould, and E. S. Ioannou, *ibid.*, **94**, 8156 (1972).