N-Ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K) as a Reagent for Nucleophilic Side Chains of Proteins

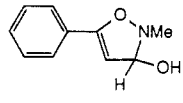
Kathryn Llamas, Mark Owens, Robert L. Blakeley,* and Burt Zerner*

Contribution from the Department of Biochemistry, University of Queensland, St Lucia, Queensland, Australia 4067. *Received September 23, 1985*

Abstract: N-Ethyl-5-phenylisoxazolium-3′-sulfonate (**1**) is converted to the corresponding keto ketenimine [ArC(O)CH=C=NEt, **2**] in a hydroxide-promoted reaction with $k_{^-OH} = 6.64 \times 10^5$ M^{-1} s^{-1} at 25 °C. The rate law for spontaneous hydration of **2** to form N-ethyl-3-oxo-3-(3-sulfonatophenyl)propanamide is $k^{sp}_{obsd} = k_{H^+}[H^+] + k_w + k_{^-OH}[^-OH]$, where $k_{H^+} = 442$ M^{-1} s^{-1}, $k_w = 0.0116$ s^{-1}, and $k_{^-OH} = 254$ M^{-1} s^{-1} at 25 °C ($\mu = 0.1$). In acetate buffers, $k_{obsd} = k^{sp}_{obsd} + k_{AcO^-}[AcO^-] + k_{AcOH}[AcOH]$, where $k_{AcO^-} = 0.245$ M^{-1} s^{-1} and $k_{AcOH} = 2.33$ M^{-1} s^{-1}. Both catalytic terms lead to the enol ester [3-acetyloxy-N-ethyl-3-(3-sulfonatophenyl)propenamide], and it is likely that the general acid catalysis term corresponds to specific acid catalysis of the attack of acetate ion on **2**. Imidazole, N-methylamine, the phenoxide ion of N-acetyl-L-tyrosinamide, and 2-hydroxyethanethiolate react nucleophilically with **2** with rate constants of 4.70, 125, 82, and 57 600 M^{-1} s^{-1}, respectively, at 25 °C, to form relatively stable enamine adducts with absorption maxima at 348.5 nm ($\epsilon = 23\,200$ M^{-1} cm^{-1}), 319 nm ($\epsilon = 16\,990$ M^{-1} cm^{-1}), 329 nm ($\epsilon = 19\,200$ M^{-1} cm^{-1}), and 349 nm ($\epsilon = 24\,200$ M^{-1} cm^{-1}), respectively. The results establish that **1** may be used as a spectrophotometric probe for imidazole, lysine, cysteine, and tyrosine residues under appropriate conditions. The selectivity of **1** for aspartate and glutamate residues may be maximized by adding it as an acetonitrile solution of the keto ketenimine **2**, to an enzyme solution maintained at low pH for the short time required for reaction.

The reactions of isoxazolium cations were first investigated in a series of theses from the University of Kiel beginning in 1902. In that year, Mumm recorded that N-methyl-5-phenylisoxazolium ion undergoes a very facile reaction with acetate ion.[1] There followed a number of papers which reported extensions of this observation to a wide variety of carboxylates and other nucleophiles.[2] The work found application in 1961, when Woodward et al. introduced N-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K, NEPIS, **1**, Ar = 3′-sulfonatophenyl in Scheme I) as a useful reagent for peptide synthesis with minimal racemization.[3a] Its rapid reaction with carboxylates, occurring under nonaqueous and exceptionally mild conditions, proved to be useful as the carboxyl-activating step in a simple and practical procedure.

Kohler[4] had earlier suggested that the reactions of 3-unsubstituted isoxazolium salts with nucleophiles proceeded through the intermediacy of a pseudo base, as for example in the case of attack by hydroxide ion (**6**). Woodward and Olofson[5] subsequently revealed that the high reactivity of these compounds could be rationalized in terms of the initial formation of an α-keto ketenime (**2** in Scheme I). The double bond system of **2** is a highly reactive center, dramatically illustrated by the rapid combination of $C_6H_5C(O)CH{=}C{=}NMe$ with azide ion in aqueous solution.[5b]



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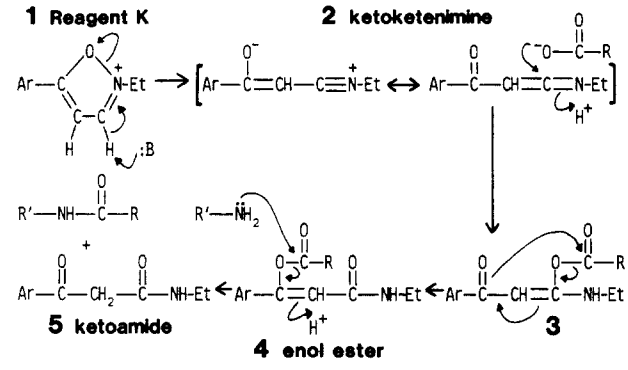
For peptide synthesis, triethylamine-promoted removal of a proton from **1** in acetonitrile produces the reactive keto ketenimine intermediate **2** which reacts with the carboxylate group of an N-protected amino acid salt to produce the unstable adduct **3** (Scheme I). Compound **3** rapidly rearranges to the enol ester **4**, with which an added amine reacts to form a peptide bond.[3,5]

Scheme I



In 1969, Reagent K was used in an attempt to identify active-site carboxylate side chains in trypsin,[6] and subsequently in carboxypeptidase A.[7] Since that time, Reagent K has been used

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Table I. Enzymes Inactivated by Woodward's Reagent K

no.	enzyme	source	new absrptn peak (λ_{max}, nm)	ref
1	β-trypsin	bovine pancreas		6
2	carboxypeptidase A	bovine pancreas	340[a]	7
3	staphylococcal nuclease	*Staphylococcus* sp	340[b]	8
4	ferredoxin-$NADP^+$ oxidoreductase	spinach chloroplast	340[c]	9
5	carboxylesterase	human pancreas	330 sh[d]	10
6	procarboxypeptidase A S_5	bovine pancreas		11
7	phosphoglycerate kinase	yeast		12
8	phospholipase C	*Bacillus cereus*		13
9	acid phosphatase	human liver		14
10	acid phosphatase	human prostate		15
11	α-mannosidase	*Phaseolus vulgaris*		16
12	L-asparaginase	*Erwinia carotovora*		17
13	BF_1-ATPase	*Escherichia coli*		18
14	carboxypeptidase B	goat pancreas		19
15	ATPase (coupling factor 1)	spinach chloroplast		20
16	phospholipase A_2	pig pancreas		21
17	Na, K-ATPase	pig kidney		22
18	lactoperoxidase	bovine milk		23
19	formate dehydrogenase	*Achromobacter parvulus*		24
20	transketolase	yeast		25

[a] In 5 mM phosphate buffer, pH 6.4 (1 mM in NaCl). [b] In 1 mM hydrochloric acid (10 mM in $CaCl_2$). [c] In 20 mM *N*-[tris(hydroxymethyl)-methyl]glycine buffer, pH 8. [d] In water.

as a probe for essential carboxylate groups in many other enzymes (Table I). However, spectral data are available for only four of the enzymes listed.

The enol ester **4** of a carboxylate in Scheme I would be expected to have a λ_{max} near 267 nm, by comparison with the enol ester derived from the keto ketenimine of *N*-methyl-5-phenylisoxazolium ion and acetic acid in acetonitrile, with λ_{max} (CH_2Cl_2) of 267 nm (ϵ = 18 700 M^{-1} cm^{-1}).[5] In contrast, several of the modified enzymes contain a major unidentified new absorption peak near 340 nm (Table I) as well as strongly enhanced and modified absorption below 300 nm.[7-10] These discrepancies have prompted an investigation of the reactivity of Reagent K in aqueous solution.

We now report the results of model studies which establish that Reagent K reacts via the keto ketenimine **2** with several nucleophilic groups found in protein side chains to give products which absorb in the 320–350-nm region.

Experimental Section

Materials. Reagent K (2 g, Aldrich Chemical Co.) was recrystallized from 8 mL of 0.1 M HCl at 4 °C by rapid addition of 40 mL of acetone. After thorough removal of residual acetone in vacuo, the white crystals were dried over P_2O_5 in vacuo at 61 °C for 5 h.[26] Acetonitrile (Eastman Kodak Spectro Grade) was fractionally distilled from fresh KOH: bp 81.6 °C/760 mmHg (lit.[27] bp 81.6 °C). *N*-Ethylmorpholine was fractionally distilled from KOH under nitrogen: bp 138–138.5 °C/760 mmHg (lit.[28] bp 138–139 °C). Triethylamine was fractionally distilled under nitrogen: bp 89.5 °C/760 mmHg (lit.[27] bp 89.35 °C). Imidazole was recrystallized from benzene: mp 88.5–89.5 °C (lit.[29] mp 90 °C). Methylamine hydrochloride (Eastman) was dried in vacuo over $CaCl_2$, and *N*-((benzyloxy)carbonyl)-L-lysine methyl ester hydrochloride (Cyclo Chemical Co.) was dried over concentrated H_2SO_4. *N*-Acetyl-L-tyrosinamide (Vega) and all other reagents (Analytical Grade) were used without further purification.

Buffer Solutions. Tertiary amine buffers were 2-(*N*-morpholino)-ethanesulfonic acid (MES), *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid, *N*-ethylmorpholinium, and triethylammonium. Solutions were prepared at 25 °C in deionized distilled water by titration with 1 M $HClO_4$ or 1 M NaOH and, where necessary, by adding solid sodium perchlorate to give the desired ionic strength (μ = 0.1). For the purpose of ionic strength calculations, a zwitterion is regarded as a uniunivalent electrolyte. The ion product of water was taken as 1.008×10^{-14} M^2 at 25 °C,[30] and values of pK'_a are taken from the literature.[31] Measurements of pH were made at 25 ± 0.1 °C with the use of a Radiometer PHM84 pH meter, fitted with a GK2401B glass electrode and standardized according to Bates.[30] The final pH of each assay mixture was measured in duplicate.

Stock Solutions. Stock solutions of Reagent K (~10 mM) were prepared daily at 4 °C, in 1 mM HCl. The concentration of **1** was determined spectrophotometrically.[26]

Solutions of the keto ketenimine **2** were prepared from recrystallized Reagent K suspended in dry acetonitrile at 25 °C by the addition of 1.5 equiv of triethylamine. The excess of triethylamine over stoichiometry assists solution and does not affect the kinetic results. The keto ketenimine decomposed slowly, as previously noted for PhC(O)CH=C=NMe in methylene chloride.[5b]

Stock solutions of the keto amide **5** were prepared by combining an equivalent of Reagent K with 1.1 equiv of $NaHCO_3$ in deionized distilled water. The keto amide was stable under all experimental conditions.

Adducts of the keto ketenimine with acetic acid (1–1.5 equiv), imidazole (1–2.5 equiv), and 2-hydroxyethanethiol (1 equiv) were prepared at 25 °C from Reagent K (0.2–0.4 mmol, 1 equiv) suspended in 25 mL of an acetonitrile solution of the nucleophile, by addition of 1.0–1.5 equiv of triethylamine. For the reaction with *N*-benzyloxycarbonyl-L-lysine methyl ester hydrochloride (1.0 equiv) in acetonitrile, 2.0 equiv of triethylamine were used. The reaction with *N*-methylamine (1.0 equiv, as the hydrochloride) was carried out in acetonitrile which contained 0.25 M H_2O and 3.3 equiv of triethylamine. The heterogeneous reactions took up to an hour at 25 °C to reach completion, and the excess of nucleophile above stoichiometry improved the yield. In general, the yield of adduct in acetonitrile was ~90%, by comparison of spectra with those derived from a totally aqueous system in which the concentration of nucleophile ensured quantitative trapping.

Kinetic and Spectral Methods. Reactions were initiated and monitored within 7 s by the addition of an aliquot (25–50 μL) of a stock solution on a flat-ended glass stirring rod to 3 mL of buffer equilibrated at 25 ± 0.1 °C in the thermostatted sample compartment of a Cary 17 spectrophotometer. Since a first-order reaction is 99.9% complete at 10 half-lives, reactions were monitored between 10 and ~20 half-lives in order to evaluate the stability of the "infinity" spectrum. First-order rate constants (k_{obsd}) were evaluated from the appropriate logarithmic relationship by using data from the first 2 half-lives together with the method of linear least squares.

For determination of the effect of temperature on k_{obsd} for the formation of **2** from **1**, a 50-μL aliquot of stock Reagent K was mixed with 3 mL of a 0.1 M MES buffer (pH 5.55 at 25 °C) preequilibrated to the desired temperature. At various time intervals, 0.2-mL aliquots were quenched in 3 mL of 0.1 M HCl at 0 °C. Unreacted **1** was quantified at 283 nm.

The design of the experiments in which **2** reacts with nucleophiles depends on the following. A priori, it would be expected that the con-

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Table II. Characteristics of the Products of the Reaction of the Keto Ketenimine from Woodward's Reagent K with Nucleophiles in Aqueous Solution at 25 °C (μ = 0.1)

nucleophile	prod.	pK'_a	λ_{max}, nm (ϵ, M^{-1} cm^{-1}) conjugate acid		conjugate base	
water	**5**	9.75[a]	245	(11 340)	313	(13 470)
			283.5	(1 095)		
methylamine	**7a**	6.81[a]	246.5	(12 030)	319	(16 990)[b]
			~285	(1 180)		
imidazole	**7b**	~4.5[a]	342	(18 850)	348.5	(23 200)[a]
		~12.6[a]	348.5	(23 200)	~332	(~12 600)[c]
2-hydroxyethanethiolate	**7c**		349	(24 200)[d]		
phenoxide ion of *N*-Ac-L-Tyr-NH_2	**7d**		329	(19 200)[e]		

[a] Buffer contains 0.83% (v/v) acetonitrile. [b] In 1.0 M methylammonium chloride buffer (pH 10.55). [c] In 2 M NaOH. [d] The spectrum is independent of pH between pH 4.5 and 11.5. [e] No extensive investigation of the spectrum has been undertaken.

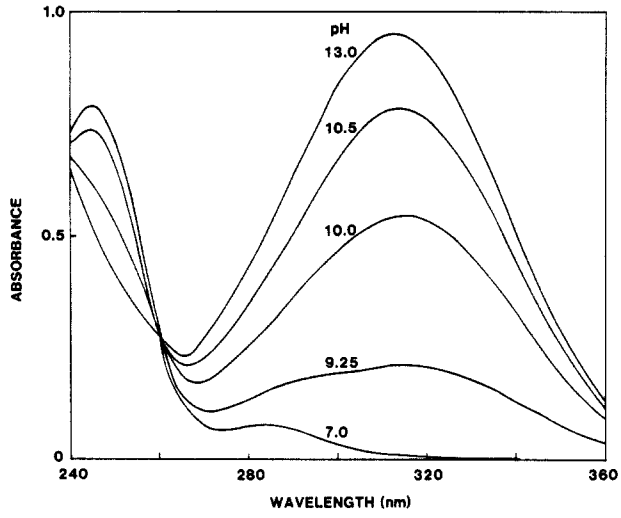


Figure 1. Effect of pH on the spectrum of the keto amide **5**.

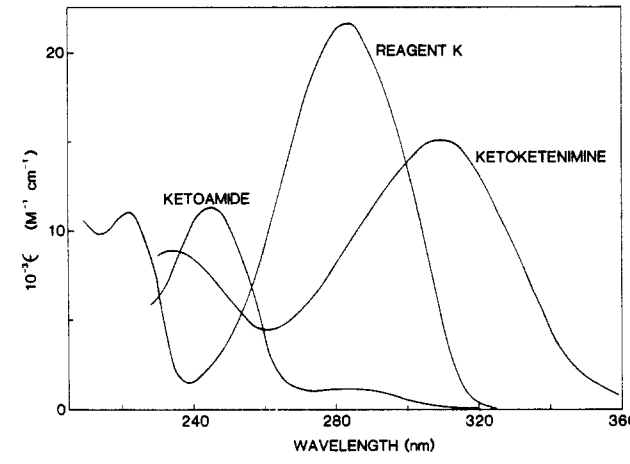


Figure 2. Absorption spectra of Reagent K (pH 1.0), the keto ketenimine **2** (pH 8.0) and the keto amide **5** (pH 7.0) at 25 °C and μ = 0.1.

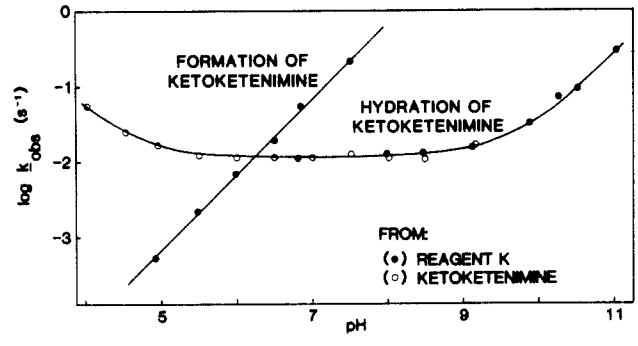


Figure 3. pH rate profiles for formation of the keto ketenimine **2** from reagent K and for its hydration at 25 °C (μ = 0.1): (○) **2** prepared in acetonitrile; (●) **2** prepared in situ in aqueous buffer.

jugate base of the nucleophile should be the reactive species. As will be seen, this is correct except for carboxylate buffers. The pH range for the reaction of all nucleophiles was therefore varied considerably.

For kinetic studies, 0.1 M acetate buffers (μ = 0.1) were prepared and diluted appropriately to hold this ionic strength. Similarly, 0.1 M imidazolium and *N*-methylammonium buffers (μ = 0.1) were prepared and diluted with a nonnucleophilic buffer of the same pH and ionic strength to give the desired concentration range of nucleophile. Solutions of 2-hydroxyethanethiol and *N*-acetyl-L-tyrosinamide were freshly prepared in tertiary amine buffers. A 25-μL aliquot of a stock keto ketenimine solution in acetonitrile was added to initiate the reaction.

Ultraviolet absorption spectra of each of the adducts formed with the keto ketenimine were obtained by mixing an aliquot of Reagent K with solutions of the nucleophile at a concentration high enough to ensure quantitative trapping of the keto ketenimine (based on $k_{Nuc}[Nuc]/(k^{sp}_{obsd} + k_{Nuc}[Nuc])$). Minor corrections were made for keto amide formation and, in the case of the enol ester, for slow formation and decomposition of the adduct at pH 5.5. The calculated fraction of enol ester was verified by using absorbance data at two wave lengths (269 and 290 nm).

Results

Reactions in Nonnucleophilic Buffers. The keto amide product **5** of spontaneous degradation of Reagent K in nonnucleophilic buffers has a pH-dependent spectrum from which a pK'_a of 9.75 was evaluated spectrophotometrically (Figure 1, Table II). The properties of the product obtained in the various buffers are identical with those observed when **5** was independently prepared in a stock solution with bicarbonate as the base. The spectrum of the conjugate acid of **5** is compared with that of the keto ketenimine **2** and Reagent K itself[26] in Figure 2.

The spontaneous hydration of the keto ketenimine **2** to form **5** has a half-life of about 1 min at 25 °C between pH 5 and pH 9. Spectral changes obeyed a simple first-order rate law, and rate constants for hydration were evaluated at μ = 0.1 in tertiary amine buffers between pH 4.5 and pH 11 and (by extrapolation to zero buffer concentration) in acetate and pivalate buffers at pH 4.0 (Figure 3). At pH 6.0 and 10.3, k_{obsd} was unchanged by decreasing the buffer concentration 10-fold at constant ionic strength. The rate constants were independent of whether **2** was preformed in acetonitrile or prepared in situ. The following rate law was obeyed for the hydration of **2**:

$$k^{sp}_{obsd} = k_{H^+}[H^+] + k_w + k_{^-OH}[^-OH] \quad (1)$$

where k_{H^+} = 442 M^{-1} s^{-1}, k_w = 0.0116 s^{-1}, and $k_{^-OH}$ = 254 M^{-1} s^{-1} at 25 °C.

Spectrophotometric traces during the spontaneous degradation of Reagent K in aqueous buffers (e.g., Figure 4) suggested a system of two sequential first-order reactions

$$A \xrightarrow{k'} B \xrightarrow{k''} C \quad (2)$$

The absorbance vs. time curve for this system will in general exhibit a maximum followed by an exponential decay if $\epsilon_A < \epsilon_B > \epsilon_C$.[32] It may be shown that

$$t_{max}k'' = (k'/k'' - 1)^{-1} \ln [(k'/k'')(\epsilon_B - \epsilon_A) + (\epsilon_A - \epsilon_C)]/(\epsilon_B - \epsilon_C) \quad (3)$$

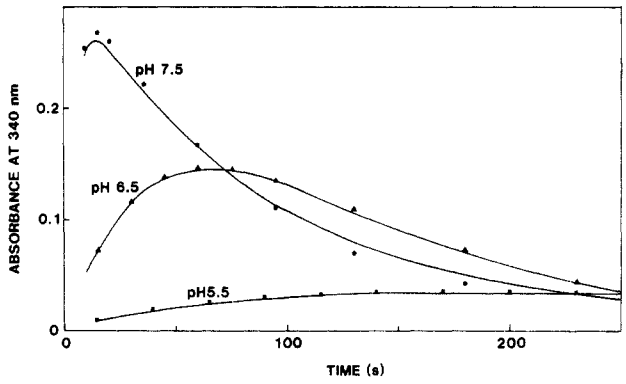


Figure 4. Spectrophotometric observation of the formation and hydration of the keto ketenimine **2** in nonnucleophilic buffers at 25 °C (μ = 0.1). Experimental values of λ_{340} when $[\mathbf{1}]_0 = 6.7 \times 10^{-5}$ M at pH 5.5 (■), pH 6.5 (▲), and pH 7.5 (●). Theoretical absorbance calculated as described in the text [$\epsilon_{340}(\mathbf{1})$ = 1.7, $\epsilon_{340}(\mathbf{2})$ = 4730, and $\epsilon_{340}(\mathbf{5})$ = 48 M^{-1} cm^{-1}] (—).

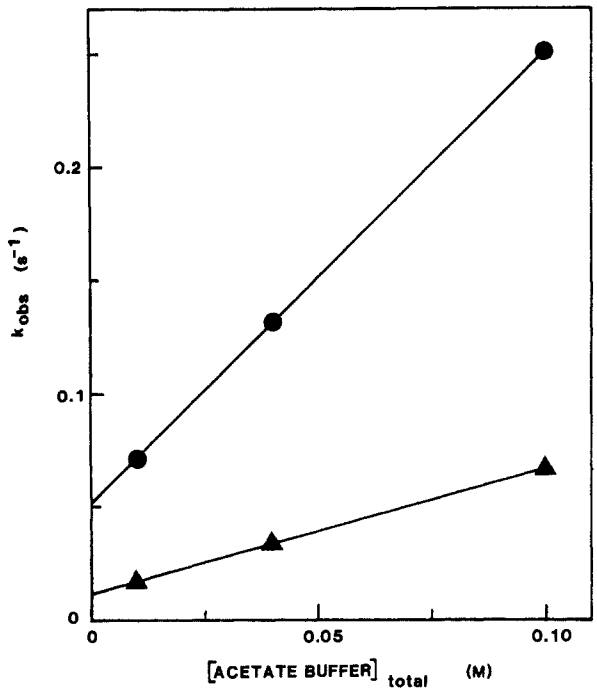


Figure 5. Kinetics of disappearance of the keto ketenimine **2** in acetate buffers (μ = 0.1) at 25 °C: (●) pH 4.04; (▲) pH 5.54.

where t_{max} is the time of maximum absorbance at a particular wavelength.[32a] If values of the molar absorption coefficients are known, then the right-hand side of eq 3 may be evaluated for a series of assumed values of k'/k''. For the system **1** → **2** → **5**, k'' (k^{sp}_{obsd} for spontaneous hydration of **2**) was known, and t_{max} was readily measured because of the large spectral differences (Figure 2). The best value of k'/k'' and hence k' at each pH was selected by comparing trial values of $t_{max}k''$ calculated as above from molar absorption coefficients with the product of the measured values of t_{max} and k''. Values of k' (k_{obsd} for formation of **2** from **1**) are plotted in Figure 3 and are described by $k' = k_{-OH}[^-OH]$ where $k_{-OH} = 6.64 \times 10^5$ M^{-1} s^{-1} at 25 °C. The validity of this analysis is established by the agreement of observed values of absorbance at 340 nm for the system **1** → **2** → **5** with the theoretical curves based on species concentrations calculated from the derived rate constants (Figure 4).

Relative values of k_{obsd} for formation of **2** from **1** in 0.1 M MES buffer (pH 5.55 at 25 °C) are as follows: 0.1 °C, 0.051; 10.0 °C, 0.19; 25.0 °C, 1.00; 38.0 °C, 4.4. A conventional Arrhenius treatment gives E_a = 19.7 kcal mol^{-1}.

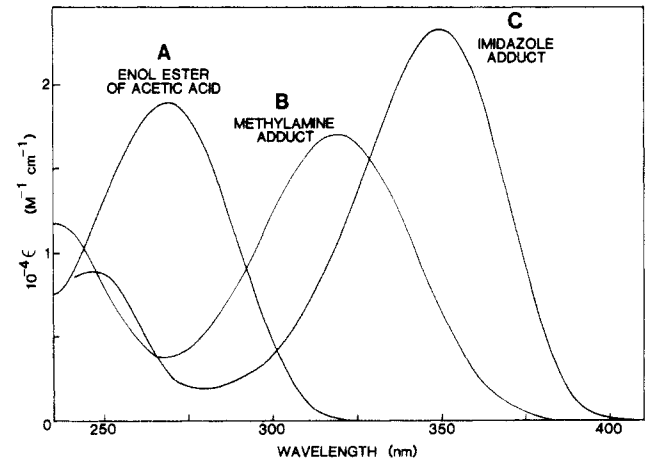


Figure 6. Absorption spectrum of adducts of the keto ketenimine **2** derived from reagent K: (A) enol ester **4** of acetic acid in 1.0 M acetate buffer, pH 5.5; (B) methylamine adduct **7a** in 1.0 M methylammonium chloride buffer, pH 10.55; (C) imidazole adduct **7b** in 1.0 M imidazolium chloride buffer, pH 7.0.

Table III. Reaction of Nucleophiles with the Keto Ketenimine from Woodward's Reagent K in Aqueous Solution at 25 °C (μ = 0.1)

prod.	nucleophile	pK'_a of conj acid	pH range	k_{nuc}, M^{-1} s^{-1}
7a	methylamine	10.62	7.2–9.8	125
7b	imidazole	7.05	6.0–7.5	4.70
7c	2-hydroxyethanethiolate	9.5	5.0–7.0	57000
7d	phenoxide ion of *N*-Ac-L-Tyr-NH_2	9.81[a]	7.0–10.1	82

[a] This work.

Since k' for **1** → **2** is much greater than k^{sp}_{obsd} for **2** → **5** at pH 8.0 (Figure 3), the spectrum of **2** was determined at 25 °C by first-order extrapolation of the absorbance at various wavelengths to the time of mixing Reagent K with a buffer at this pH. The keto ketenimine **2** has a λ_{max} of 310 nm (ϵ = 15080 M^{-1} cm^{-1}) (Figure 2).

Acetate Buffers. The effect of acetate buffer concentration on k_{obsd} for the disappearance of the keto ketenimine is shown in Figure 5. The observed rate law is $k_{obsd} = k^{sp}_{obsd} + k_{AcO^-}[AcO^-] + k_{AcOH}[AcOH]$, where k^{sp}_{obsd} for the spontaneous hydration of **2** is defined above, k_{AcO^-} = 0.245 M^{-1} s^{-1}, k_{AcOH} = 2.33 M^{-1} s^{-1} at 25 °C, and the pK'_a of acetic acid is 4.76 (μ = 0.1). Both catalytic terms lead to the enol ester **4** [R = CH_3, λ_{max} 269 nm (ϵ = 18950 ± 500 M^{-1} cm^{-1})] (Figure 6). This enol ester undergoes hydrolysis to **5** and/or rearrangement to the corresponding *N*-acetylketo amide[33] between pH 5 and 10 according to eq 1, where k_{H^+} = 1.06 × 10^{-3} M^{-1} s^{-1}, k_w = 2.5 × 10^{-5} s^{-1}, and k_{OH} = 4.8 × 10^3 M^{-1} s^{-1} at 25 °C.

Other Nucleophiles. In the presence of several nucleophiles (imidazole, *N*-methylamine, 2-hydroxyethanethiolate ion, and the phenoxide ion of *N*-acetyl-L-tyrosinamide) the rate of disappearance of keto ketenimine is markedly enhanced in accord with the following rate law

$$k_{obsd} = k^{sp}_{obsd} + k_{Nuc}[Nuc] \quad (4)$$

Kinetically, general acid catalysis was not observed even when the nucleophile was predominantly in the form of its conjugate acid. Values of k_{Nuc} are given in Table III. Some properties of the stable adducts are shown in Table II and Figure 6. Each adduct has a strong absorption peak in the 320–350-nm region. Spectra of the imidazole and *N*-methylamine adducts are dependent on pH, and the spectrophotometrically determined values of pK'_a are given in Table II. The adduct of the side chain of

(32) (a) Bender, M. L.; Zerner, B. *J. Am. Chem. Soc.* **1962**, *84*, 2550–2555. (b) Blakeley, R. L.; Hinds, J. A.; Kunze, H. E.; Webb, E. C.; Zerner, B. *Biochemistry* **1969**, *8*, 1991–2000.

(33) Hegarty, A. F.; Tuohey, P. J. *J. Chem. Soc., Perkin Trans. 2* **1980**, 1326–1330.

N-benzyloxycarbonyl-L-lysine methyl ester with the keto ketenimine (prepared in acetonitrile) has a λ_{max} of 322 nm and a pK'_a of 6.49, in reasonable agreement with that of the methylamine adduct.

The various adducts are generally quite stable near neutral pH. At 25 °C, λ_{max} of **7b** decays at an initial rate of ~3% per h at pH 4.5 and ~0.3% per h between pH 6 and pH 11 and with a k_{obsd} of 8.8×10^{-4} s^{-1} in 1.0 M NaOH. **7b** forms the keto amide **5** with a k_{H^+} of 0.066 M^{-1} s^{-1} in 0.01–1.0 M HCl. **7c** decomposes between pH 4.5 and pH 13 according to eq 1 where $k_w = 6 \times 10^{-5}$ s^{-1} and $k_{-OH} = 0.23$ M^{-1} s^{-1} at 25 °C. The methylamine adduct **7a** is very stable below pH 12.

Discussion

Properties of the Keto Amide 5. The final product of spontaneous degradation of Reagent K in aqueous solution is assigned the keto amide structure **5** on the basis of its absorption spectrum (Table II, Figure 1) in comparison to that of acetophenone [in alcohol, λ_{max} = 242 nm (ϵ = 12 300 M^{-1} cm^{-1}) and 280 nm (ϵ = 1050 M^{-1} cm^{-1})].[5b,34] The effect of pH on the spectrum of the keto amide (Figure 1) corresponds to a pK'_a of 9.75 for **5**. This value may be compared with that of acetophenone (18.24)[35] for loss of a CH proton and is consistent with the expected effect of an adjacent carboxamido group [cf. the pK'_a of ethyl acetoacetate (10.68) and that of acetone (19.16)[36]]. The spectrum of the conjugate base of **5** is consistent with that of the enolate anion of acetophenone [λ_{max} = 295 nm (ϵ = 10 000 M^{-1} cm^{-1})].[37]

Formation of the Keto Ketenimine 2. The pH-dependence in Figure 3 for formation of the keto ketenimine **2** from Reagent K in various aqueous tertiary amine buffers requires that the base which removes the proton with ring opening (Scheme I) be hydroxide ion in our experiments. Kemp previously established that the analogous ring opening of the *N*-ethylbenzisoxazolium cation has a k_{-OH} of 2.7×10^7 M^{-1} s^{-1} in aqueous solution at 30 °C and that significant catalysis by acetate and methoxyacetate ions occurs.[38]

Hydration of the Keto Ketenimine 2. The hydration of **2** to form the keto amide **5** displays a water reaction as well as hydrogen ion and hydroxide ion catalysis (eq 1). The flat region of the pH log k_{obsd} profile (Figure 3) is much broader than that observed in the hydrolysis of activated esters such as 4-nitrophenyl acetate.[39] Hegarty and Tuohey have previously shown that the spontaneous hydration of PhC(O)CH=C=N-*t*-Bu obeys the rate law $k_{obsd} = k_{H^+}[H^+] + k_w$ between pH 3.5 and pH 7.5, where k_{H^+} = 100 M^{-1} s^{-1} and $k_w = 3.9 \times 10^{-4}$ s^{-1} at 25 °C [μ = 1(KCl)].[33]

The mechanism of proton catalysis of the hydration of ArC(O)CH=C=NR could involve (a) rate-limiting proton transfer to carbon to form an unstable nitrilium ion [$ArC(O)CH_2C{\equiv}N^+R$] which subsequently reacts with water,[33] (b) nucleophilic attack of H_2O concerted with proton transfer from H_3O^+ (most likely to nitrogen or oxygen),[40] or (c) rate-limiting attack of H_2O on a conjugate acid of the keto ketenimine present at equilibrium. The critical solvent deuterium isotope effect has not been determined for keto ketenimine hydration, but mechanisms b and c for H_2O are suggested by the high susceptibility of the keto ketenimine to stronger nucleophiles without proton catalysis (see below).

Reaction of 2 with AcO^-/AcOH. The enol ester **4** derived from the reaction of **2** with AcO^-/AcOH has a λ_{max} of 269 nm in aqueous solution (cf. ref 8). Kinetically, the disappearance of **2** in acetate buffers displays marked general acid catalysis as well as general base catalysis (k_{AcOH}/k_{AcO^-} = 9.5), and both terms lead to the enol ester as the exclusive product. While k_{AcO^-} presumably reflects simple nucleophilic attack of acetate ion on **2** without any assistance by H_3O^+, the general acid term could reflect either of two simple mechanisms—1,4-addition of the O–H of acetic acid across the diene system of **2**[5b] or the kinetically equivalent attack of acetate ion on **2H$^+$**. In the inaugural Kharasch lectures at the University of Chicago in 1961, Woodward positively favored the first possibility for reaction in CH_2Cl_2 ("Everything in this wallet says it's right!"), although the published work allows for the kinetically indistinguishable second in a footnote.[5b] However, it is very unlikely that molecular acetic acid is more nucleophilic than acetate ion in aqueous solution, and the most reasonable mechanism for the k_{AcOH} term is the kinetically equivalent attack of acetate ion on the small fraction of **2** that is protonated.[41] If k_{AcOH} and k_{H^+} reflect the attack of acetate and water, respectively, on protonated **2H$^+$**, then it may be shown that the dimensionless ratio of calculated second-order rate constants is

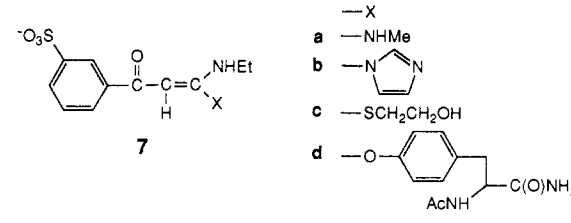
$$k_{AcO^-}(\text{on } \mathbf{2H^+})/k_{HOH}(\text{on } \mathbf{2H^+}) = 1.7 \times 10^4$$

This may be compared to the corresponding dimensionless reactivity ratio $k_{AcO^-}/k_{HOH} = 1.2 \times 10^3$ for attack of acetate and water on uncharged **2**. A priori, it might have been anticipated that the ratio for attack on **2H$^+$** would be less than that on **2**. However, the similarity of the two ratios may simply reflect an abnormally rapid hydration of the unprotonated keto ketenimine.

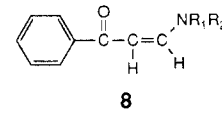
The rate of disappearance of the enol ester **4** (R = CH_3) is essentially independent of pH between pH 3 and 5 (half-life = 7.7 h at 25 °C) and is strongly promoted by hydroxide ion. Although the products were not characterized in this work, Hegarty and Tuohey showed that the corresponding water and hydroxide reactions of PhC(OAc)=CHC(O)NHMe are due to rearrangement to the corresponding *N*-acetyl keto amide [$PhC(O)CH_2C(O)NMeC(O)CH_3$] which subsequently undergoes a slower hydrolytic cleavage of the acetyl group. The corresponding specific acid catalysis reflected simple hydrolysis of the enol ester to release acetate.[33]

Other Nucleophiles. Simple models for several amino acid side chains undergo a facile reaction with keto ketenimine **2** in aqueous solution to form stable adducts. Values of k_{Nuc} in Table II show that imidazole, methylamine, 2-hydroxyethaneethiolate ion, and the phenoxide ion of *N*-acetyl-L-tyrosinamide are all more powerful nucleophiles toward **2** than is acetate ion. Kinetically, general acid catalysis by the conjugate acid of these stronger nucleophiles was undetectable in sharp contrast to the major catalysis displayed by AcOH.

The adducts **7a–d**, which absorb strongly in the 320–350-nm region (Table II), may be assigned the following enamine structures.



This assignment is based on comparison of the absorption spectra of the adducts with those of related enamines in ethanol solution.[42]



8

	R_1	R_2	λ_{max}, nm	ϵ, M^{-1} cm^{-1}
a	H	H	324	19 900
b	Et	H	338	23 200
c	Et	Et	343	26 000

Although **8a** and **8b** could exist in solution either with an intra-

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(35) Chiang, Y.; Kresge, A. J.; Wirz, J. *J. Am. Chem. Soc.* **1984**, *106*, 6392–6395.
(36) Chiang, Y.; Kresge, A. J.; Tang, Y. S. *J. Am. Chem. Soc.* **1984**, *106*, 460–462.
(37) pK'_a of enol of acetophenone, 10.34: Haspra, P.; Sutter, A.; Wirz, J. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 617–619.
(38) Kemp, D. S. *Tetrahedron* **1967**, *23*, 2001–2015.
(39) Bruce, T. C.; Lapinski, R. *J. Am. Chem. Soc.* **1958**, *80*, 2265–2267.
(40) McCarthy, D. G.; Hegarty, A. F. *J. Chem. Soc., Perkin Trans. 2* **1980**, 579–591.
(41) No kinetic evidence for protonation of a significant fraction of **2** was detected, and as a limit the pK'_a of **2H$^+$** must be no more than 3.
(42) Bowden, K.; Braude, E. A.; Jones, E. R. H. *J. Chem. Soc.* **1946**, 948–952.

Table IV. Calculated Effect of pH on the Selectivity of Protein Side Chains toward Woodward's Reagent K

nucleophile	pK'_a of conj acid	k_{nuc}, M^{-1} s^{-1}	calculated k_{obsd}, M^{-1} s^{-1}			
			pH 3	pH 4	pH 5	pH 7
imidazole	6	4.70	0.005	0.047	0.43	4.3
RS^-	9	57000	0.057	0.57	5.7	570
RNH_2	10	125	10^{-5}	0.0001	0.0012	0.125
$RC_6H_4O^-$	10	82	10^{-5}	0.0001	0.0008	0.082
$RCOO^-$	4	0.245	0.022	0.12	0.22	0.245
RCOOH		2.33[a]	2.12	1.17	0.21	0.002

[a] k_{AcOH}.

molecular hydrogen bond[5b] or as the prototropic enol, **8c** necessarily exists as the enamine. Further, PhC(O)CH=C(OMe)-(NHEt) has a λ_{max} of 321 nm (ϵ = 19 600 M^{-1} cm^{-1}) in MeOH, and the initial azide adduct of the keto ketenimine derived from *N*-methyl-5-phenylisoxazolium ion has a λ_{max} (EtOH) of 348 nm.[5b] The prototropic enol structure of **7** is unlikely because it would be expected to have an absorption maximum near that of the enol ester **4** (269 nm) or methyl cinnamate [279.5 nm (ϵ = 22 100 M^{-1} cm^{-1})].[43]

The spectrum of the conjugate acid of the methylamine adduct (Table I) is essentially identical with that of the keto amide **5**, indicating that the conjugate acid must be $ArC(O)CH_2C(=N^+HEt)NHMe$ for which a pK'_a of 6.81 for dissociation of a CH proton is reasonable. (The nitrogen acid should have a pK'_a of greater than 12.[31])

Table IV lists the approximate pK'_a for a variety of nucleophilic protein side chains and the observed rate constants that would be expected for reaction of the conjugate base of the side chains with the keto ketenimine **2**. In addition the table lists values of k_{RCOOH} for the reaction of a carboxylic acid. It will be evident that reactions of tyrosine and lysine side chains should be of very little significance at pH 5, where however the reaction of *thiolate* is still dominant. Sufficient data are provided to assist in the design of discriminatory experiments. It should be borne in mind that these reactions operate in competition with the spontaneous hydration of the keto ketenimine at any of these pH's.

In 0.5 M hydroxylamine hydrochloride solution (pH 7.0), the characteristic 348.5-nm absorption peak of the imidazole adduct **7b** disappears with a half-life of 8.2 min at 25 °C, presumably owing to the release of imidazole. Such a reaction could partially account for the spectral effects of methoxyamine on carboxypeptidase A[7] and staphylococcal nuclease[8] previously modified by Reagent K.

(43) Bender, M. L.; Schonbaum, G. R.; Zerner, B. *J. Am. Chem. Soc.* **1962**, *84*, 2540–2550.

Summary

Our results establish that inactivation of an enzyme by Reagent K may not be taken alone as prima facie evidence for the presence of an essential carboxylate group. Several simple nucleophiles react with **2** to form stable adducts with a strong absorption peak in the 320–350-nm region (Table II). The absorption peak near 340 nm in the reaction of Reagent K with proteins (Table I) must arise from such adducts. The enhanced absorption below 300 nm in published spectra of protein adducts[7-10] is not well-defined, but it is presumably due to the enol esters of aspartate and glutamate as well as to their rearrangement product [*N*-acyl keto amides] and to low wavelength contributions from various side chain adducts.

Loss of enzymatic activity upon treatment of an enzyme with Reagent K may involve covalent blocking of any of several possible essential side chains. Reaction of an enol ester with hydroxylamine leads to a stable hydroxamic acid,[6] whereas reaction of the adducts **7a–d** with hydroxylamine may lead to regeneration of the group originally blocked by the keto ketenimine. In conjunction with further model studies, this difference in chemical reactivity may allow differentiation among possible types of side chains responsible for loss of enzymatic activity when an enzyme has been treated with Reagent K.

An important conclusion which derives from the present work is that the selectivity of Reagent K toward carboxylate groups may be improved by adding preformed keto ketenimine to an enzyme solution maintained at low pH for the very short time required for reaction. This is because as the pH is decreased, the rate of reaction of the keto ketenimine with carboxylate groups becomes independent of pH owing to acid catalysis, whereas that with the other nucleophilic side chains progressively decreases.

Adducts of keto ketenimines are themselves potentially susceptible to further reactions such as carbonyl additions, rearrangement, and C–C bond cleavage.[5] It should not be surprising therefore if secondary reactions take place in some protein adducts of Reagent K.

Finally, Reagent K is clearly a useful spectrophotometric probe for several different types of nucleophilic side chains found in polypeptides and proteins, and the interpretation of future experiments should be facilitated.

Acknowledgment. We are grateful to the Australian Research Grants Scheme and to the University of Queensland for financial support.

Registry No. 1, 4156-16-5; **2**, 103383-38-6; **5**, 103383-39-7; **7a**, 103383-40-0; **7b**, 103383-41-1; **7c**, 103383-42-2; **7d**, 103383-43-3; Ac-Tyr-NH_2, 1948-71-6; Z-Lys-OMe, 5591-93-5; $MeNH_2$, 74-89-5; imidazole, 288-32-4; 2-hydroxyethanethiolate, 57966-62-8; N^6[1-(ethylamino)-3-oxo-3-(3-sulfophenyl)-1-propenyl]-N^2-[(phenylmethoxy)-carbonyl]-L-lysine methyl ester, 103383-44-4.