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The Reactivity of Affinity Labels: A Kinetic Study of the Reaction of Alkyl Halides with Thiolate Anions—a Model Reaction for Protein Alkylation

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Factors have been investigated which govern the electrophilic reactivity of alkyl halides with thiolate anions in aqueous solution. In the series of alkyl halides studied, some are potential metal-directed affinity labels, while others are frequently used in protein modification. Previous data on the kinetics of this type of alkylation are compared with the present results. The influence of electronic, polar, and steric factors on alkyl halide reactivity is seen. The following order of reactivity for alkyl halides bearing different α substituents was observed: $R-CH_2-CH(X)-COOCH_3 > R-CH_2-CH(X)-CONH_2 > R-CH_2-CH(X)-COOH > R-CH_2-CH_2X > R-CH_2-CH(X)-CH_2OH$. The metal-directed affinity labels are imidazole derivatives, some of which have substituents in their imidazole ring. The effect of the imidazole ring and of ring substitution on reactivity is seen. The nucleophilic reactivity of thiols is highly pH dependent since the thiolate anion (RS^-) is the reactive species, but only minor differences emerged between different free thiolates.

INTRODUCTION

Alkyl halides are used in protein modification (*1*), affinity labeling (*2*), and although often carcinogenic (*3*), they have widespread use in cancer chemotherapy (*4*). The basis for their action is their ability to alkylate nucleophilic groups, mediated through leaving of the halogen. A knowledge of the reactivity of alkyl halides is important for evaluating the usefulness of each alkylator.

The method most used for measuring alkylating reactivity, has been that of Baker and Jordaan (*5*) where alkylation of 4-(*p*-nitrobenzyl)pyridine gives a product which in alkaline solution absorbs in the 600-nm region. This is a fine method with highly reactive compounds, but was unsuitable in this work. Here the imidazole derivatives and several other alkyl halides examined were not reactive enough to alkylate the pyridinium nitrogen. In the present work, the second-order rate constant for the reaction of different alkyl halides with the thiolate of cysteine was used to measure their relative reactivity. Reaction with the thiolate anion is faster since this is a far better nucleophile than the pyridinium nitrogen (*6*). Using this method, alkylation with the least reactive compound could be established while the rate constant of the most reactive compounds could also be measured although a factor of 10^5 higher. Reaction was performed in a strongly buffered aqueous solution. It was followed by measuring unreacted thiol

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indirectly with DTNB, rather than by direct spectrophotometric determination of thiolate (*7*) or by titrimetric (*8, 9*) or chromatographic analysis (*9*). In addition to being more sensitive, reaction with thiolate also resembles the alkylation of cysteine residues, the most reactive nucleophilic groups in a protein.

Aliphatic nucleophilic substitution reactions are among the most studied reactions in organic chemistry (*6, 10*). Consequently the aim of this work has rather been to measure the relative reactivity of the alkyl halides to find out how steric hindrance and induction from neighbouring groups affect reactivity. Such knowledge is important for the design of alkylating affinity labels, as well as for the interpretation of affinity labeling results.

MATERIALS AND METHODS

Alkyl Halides

Compounds **I–X, XII,** and **XV–XXII** were generous gifts from Professor H. C. Beyerman, Technische Hogeschool, Delft, the Netherlands. Compounds XI and XIII were generous gifts from Dr. C. R. Ganellin, Smith Kline & French Laboratories, Welwyn Garden City, England. Compound XXV was a generous gift from Dr. D. J. Wilkins, Centre de Recherche Merrell International, Strasbourg, France. Compound XIV was from Sigma. XXIII and XXIV were synthesized by ourselves. References to the synthesis of the imidazole derivatives are given in Table 1.

Iodoacetate and iodoacetamide were from Sigma, 3-iodopropionate from Eastman, bromoacetate from Koch-Light, 2-bromoethanol from Merck, (*R, S*)-2-bromopropionate from Hopkins and Williams, and 2-chloroethanol from Fluka. Other reagents included L-cysteine and glutathione (reduced) from ICN Nutritional Biochemicals, *N*-acetyl cysteine from Sigma, 2-mercaptoethanol and cystamine from Fluka. Diethanolamine was from Merck and 5′, 5′-dithiobis(2-nitrobenzoic acid) (DTNB) from Sigma.

Alkylation Rate Measurements

The addition of alkyl halide to thiolate anion leads to the formation of a thioether. This reaction can be measured directly, by taking advantage of the thiolate absorption at 250 nm (*11*). However, in this work the reaction was followed by measuring unreacted thiol with DTNB, which is a rapid and sensitive method (*12–14*). Unlike previous measurements, reaction was performed in a strongly buffered aqueous solution. Cysteine alkylations were generally carried out as follows: To 50 μmol alkyl halide was added 500 μl 0.1 *M* L-cysteine in 1 *M* diethanol amine–HCl buffer, pH 9.0, to start the reaction. With a pK_a of 8.90, diethanolamine strongly resists downward shifts of pH. This buffer is also advantageous due to its miscibility with water. This is because, when alkyl halides of low reactivity are measured, high concentrations of reactants are required, and thus high buffer concentrations. In most cases 1 *M* buffer was used, and found to

maintain constant pH. High buffer concentration means high ionic strength, but only minor effects on the reaction rate were observed. This agrees with the findings for the carboxymethylation of histidine hydantoin (*15*) and also for the reaction of iodoacetamide with thiols in 3 *M* guanidine–HCl (*16*). A possible disadvantage with diethanolamine as a buffer is that it may react as a nucleophile. However, it has not been observed to react with alkyl halides as such. Any reaction with buffer should result in deviation from second-order kinetics.

Reaction was carried out in a stoppered tube at 37°C, the alkyl halides being rapidly dissolved in the thiol-containing solution by rapid mixing. Direct dissolution has the advantage that the reactivity of alkyl halides of limited stability can be measured. Reaction was started with 0.1 *M* of both reactants. It was followed by withdrawing aliquots, diluting 20 times, and mixing 50 μl of this solution with 2.5 ml of 1 m*M* DTNB in 40 m*M* phosphate, pH 8.0, and measuring the absorbance at 412 nm. None of the alkyl halides were found to interfere with the thiol determination.

All solutions were purged with nitrogen to avoid oxidation of thiol to disulfide. The concentration of the cysteine solution was found to be 0.1 *M* with DTNB using a ϵ_{412} of $1.36 \cdot 10^4$ (*17*). The results were plotted in a second-order plot, and the apparent second-order rate constants calculated by linear regression are estimated to be within ±5%.

This procedure was followed for all the alkyl halides except iodoacetamide, iodoacetate, and bromoacetate, where 5 m*M* of both reactants were used in 0.25 *M* diethanolamine–HCl buffer, pH 9.0, and the chloromethyl imidazoles **XII** and **XIII** where 5 m*M* of both reactants were used in 0.1 *M* pyrophosphate buffer, pH 9.0. This procedure was also followed for the other thiols when they replaced cysteine.

The methyl esters **V–VIII** and **XVII–XX** were found to have a limited stability due to base-catalyzed hydrolysis to the respective acids. This agrees with lability previously found for haloacetate esters (*18*). The actual half times at pH 9.0 are estimated to be about 60 min. Alkylation with the methylesters was thus measured for less than 10 minutes. Over longer periods, deviation from second order-kinetics was observed.

Since the thiolate anion (RS^-) is the reactive form, the reaction rate is highly dependent on pH. In order to compare rate measurements at different pH values, and the nucleophilic reactivity of different thiolates, the pH-independent rate constant must be known. Following Lindley (*8*) the alkylation rate for a dissociable thiol, where the thiolate anion is the reactive species, depends upon pH according to

$$k_0 = k_{app}(1 + [H^+]/K),$$

where k_{app} is the apparent rate constant and K the dissociation constant for the thiol. The pK values for the different thiols used in this work are those given by Danehy *et al.* (*19*). They have been used uncorrected for temperature differences.

For the aminothiols, this is, however, not valid due to overlapping pK values for the thiol and the amino group, which complicates the estimation of the amount of reactive thiol at different pH values. According to Benesch and Benesch (*7*),

the following scheme describes the equilibria among the various forms of an aminothiol:

$$\begin{array}{ccc} \text{(a)} & \mathrm{HSRNH_3^+} \overset{K_A}{\rightleftharpoons} \mathrm{^-SRNH_3^+} & \text{(b)} \\ & \Updownarrow K_B \qquad \Updownarrow K_C & \\ \text{(c)} & \mathrm{HSRNH_2} \overset{K_D}{\rightleftharpoons} \mathrm{^-SRNH_2} & \text{(d)} \end{array}$$

The pH-independent reaction rate for the aminothiols was calculated using the fraction present as reactive thiolate (b + d), at a particular pH.

$$\frac{\mathrm{RS^-}}{\mathrm{RS^-_{max}}} = \frac{\text{(b + d)}}{\text{(a + b + c + d)}} = \frac{K_A/K_B + K_D/[\mathrm{H^+}]}{[\mathrm{H^+}]/K_B + K_A/K_B + K_D/[\mathrm{H^+}] + 1}.$$

The microscopic dissociation constants used were those of Benesch and Benesch for cysteine (*7*) and of Martin and Edsall for glutathione (*20*). A buffer pH of 9.0 was chosen, since cysteine is mainly thiolate at this pH (56.4%). For cystamine, the pK values for the thiol and the amino group are so far apart that they can be considered as nonoverlapping, and form c can thus be neglected (*7*). Thus, the thiolate fraction for cystamine was calculated directly using the pK of the thiol group. At pH 9.0 it is 81.7%.

Most alkyl halides used in this work have a rather low reactivity. In order to increase this, rates have been measured at 37°C. To compare different literature values with each other and with the present results, it was desirable to correct some published rate constants to 37°C. The energy of activation is known for only a few of the alkylation reactions, but on the basis of these (*9, 12, 14, 21*), an activation energy of 18 kcal/mol has been used to estimate the reaction at 37°C.

RESULTS AND DISCUSSION

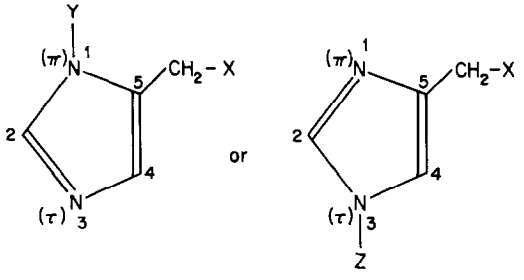
Reactivity of Alkyl Halides

Primary alkyl halides tend to undergo nucleophilic substitution by a S_N2 mechanism, as also do most of the secondary alkyl halides. The rate of reaction depends on the nature of the nucleophile and the alkyl halide. Nucleophilic reactivity generally follows the basicity of the nucleophile. The relative nucleophilicity of imidazole, pyridine, and thiolate ($C_6H_5S^-$) toward methyl iodide in methanol is 1 : 1.8 : 89.000 (*22*). Alkyl halide reactivity depends not only on the leaving potential of the halogen, but also on the nature of the alkyl group. The effect of the alkyl group on the reactivity is generally difficult to predict, since both bond-making and bond-breaking processes are important (*23*).

The rate constants listed in Tables 1 and 2 measure the reactivity of the alkyl halides toward the thiolate anions. A second-order reaction, characteristic of a S_N2 mechanism, has been observed with all. For the imidazole alkyl halides this is shown by the second-order reaction plots in Fig. 1. For analogous alkyl halides the present results fit in with those of Hellström (*39*), who found a relative reactivity for haloacetates toward thioglycolate of Cl : Br : I = 1 : 110 : 260 and for

TABLE 1

IMIDAZOLE ALKYL HALIDES: SECOND-ORDER RATE CONSTANTS FOR REACTION WITH THE THIOLATE ANION OF L-CYSTEINE



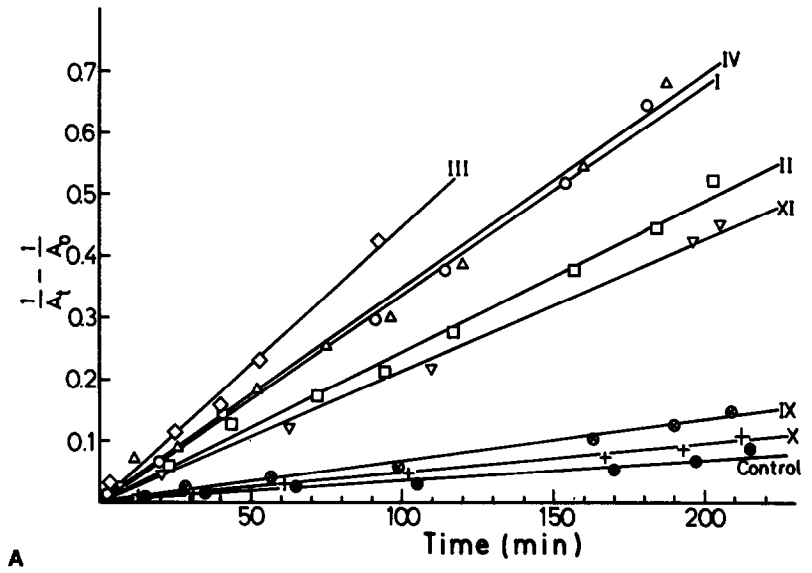
No.	Synthesis	Enantiomer	X	Y(π)	Z(τ)	$k_{app} \cdot 10^3$ (M^{-1} sec^{-1})	$k_0' \cdot 10^3$ (M^{-1} sec^{-1})
I	24	S	CHClCOOH	—	H	0.62	1.09
II	24	R	CHClCOOH	—	H	0.45	0.80
III	25	S	CHClCOOH	CH_3	—	1.13	2.00
IV	25	R	CHClCOOH	CH_3	—	0.64	1.12
V	24	S	$CHClCOOCH_3$	—	H	6.8	11.9
VI	24	R	$CHClCOOCH_3$	—	H	5.7	10.0
VII	25	S	$CHClCOOCH_3$	CH_3	—	7.0	12.4
VIII	25	R	$CHClCOOCH_3$	CH_3	—	5.8	10.3
IX	24	S	$CHClCH_2OH$	—	H	0.12	0.21
X	25	S	$CHClCH_2OH$	CH_3	—	0.09	0.15
XI		—	CH_2Cl	—	H	0.40	0.70
XII	26	—	Cl	—	H	55	100[a]
XIII		—	Cl (C-4,CH_3)	—	H	27	50[a]
IXV	27	RS	CHBrCOOH	—	H	18.6	32.6[b]
XV	27	RS	CHBrCOOH	CH_3	—	0.88	1.54
XVI	28	RS	CHBrCOOH	—	CH_3	2.10	3.68
XVII	27	RS	$CHBrCOOCH_3$	—	H	540	950
XVIII	27	RS	$CHBrCOOCH_3$	CH_3	—	930	1600
XIX	28	RS	$CHBrCOOCH_3$	—	CH_3	11.0	19.3
XX	28	RS	$CHBrCOOCH_3$	CH_2CH_3	—	1500	2600
XXI	27	RS	$CHBrCH_2OH$	—	H	0.76	1.33
XXII	27	RS	$CHBrCH_2OH$	CH_3	—	0.46	0.81
XXIII	29	—	CH_2Br	—	H	5.9	10.4
XXIV	30	RS	$CHNH_2CH_2Br$	—	H	12.3	21.6
XXV	31	RS	$CNH_2CH_2ClCOOH$	—	H	1.9	3.3

Note. Reaction conditions as in Fig. 1. The observed rate constant at pH 9.0 is k_{app}, while k_0' is the pH-independent rate constant. The general structure of the compounds is shown in the scheme, where X is the substitutent at position 5, and either Y is at 1 (π) or Z is at 3 (τ).

[a] Reaction condition: 5 mM of both reactants in 0.1 M pyrophosphate buffer, pH 9.0.

[b] Yankeelov and Jolley (*13*) found 17 M^{-1} sec^{-1} at 24.8°C.

haloacetamides of Cl : Br : I = 1 : 90 : 130. Acting through steric and electronic effects, the α substituent to the halogen is crucial for alkyl halide reactivity. The low reactivity of the imidazole alcohols **IX, X, XXI, XXII,** and of chloroethanol



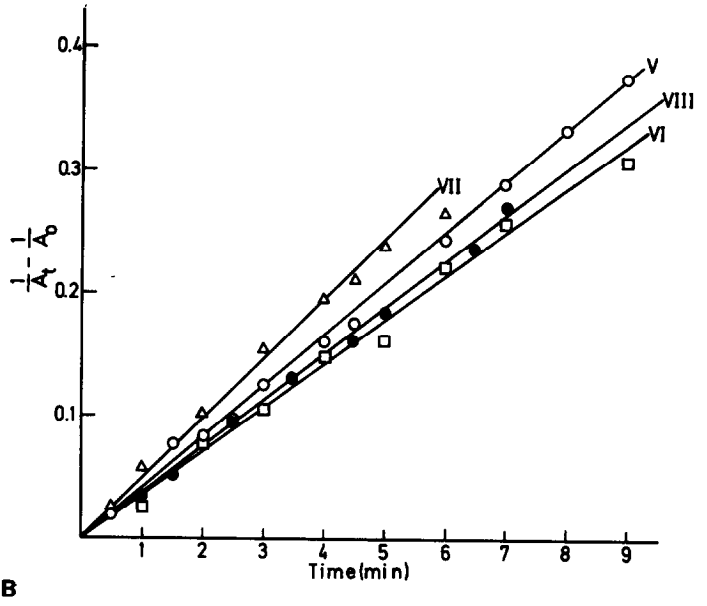
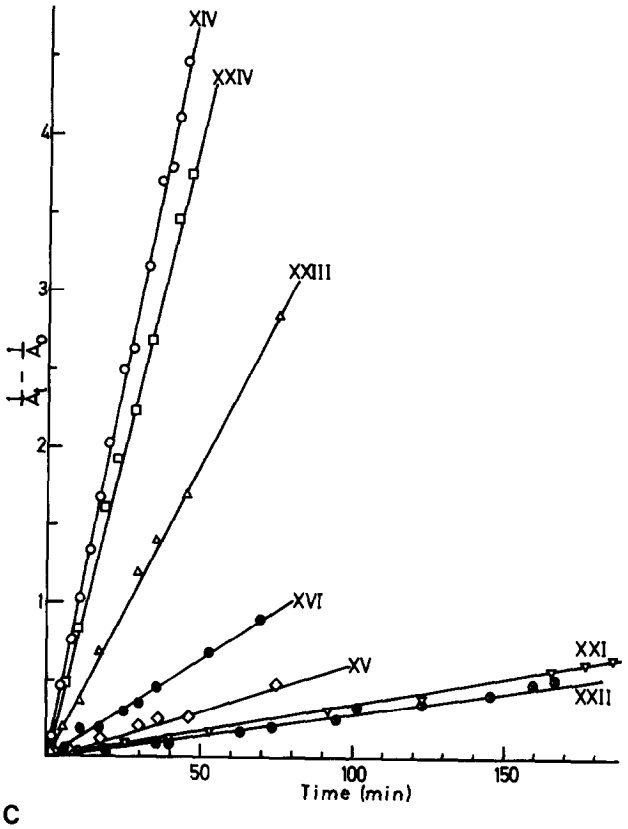
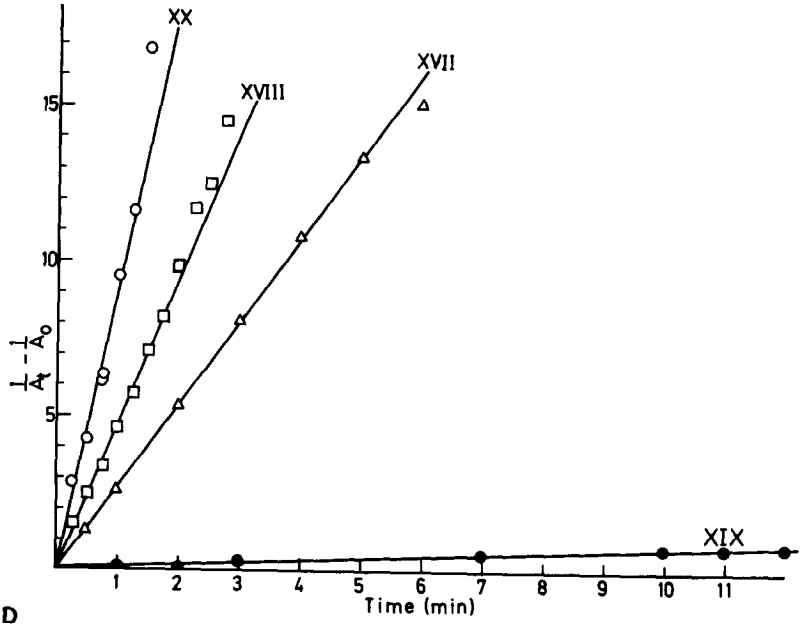


FIG. 1. Second-order kinetic plots for the reaction of the imidazole-containing alkyl halides with L-cysteine. Both reactants were 0.1 *M* at the start of the reaction in 1.0 *M* diethanolamine–HCl buffer, pH 9.0, at 37°C. The rate was measured by withdrawing aliquots, and determining unreacted thiol with DTNB. A_0 and A_t represent absorbances at 412 nm in the assay with DTNB. The compounds were: (A) I (△), II(□), III(◇), IV(○), IX(⊗), X(+), XI(▽), and control with cysteine alone (●). (B) V(○), VI(□), VII(△), and VIII(●). (C) XIV(○), XV(◇), SVI(●), XXI(▽), XXII(⊗), XXIII(△), and XXIV(□). (D) XVII(△), XVIII(□), XIX(●), and XX(○).


XIV
XXIV
XXIII
XVI
XV
XXI
XXII
$\frac{1}{A_t} - \frac{1}{A_0}$
Time (min)

C


XX
XVIII
XVII
XIX
$\frac{1}{A_t} - \frac{1}{A_0}$
Time (min)

D

TABLE 2

RATE CONSTANTS FOR ALKYL HALIDE ADDITION TO THIOLS

Alkyl halide	Thiol	pH	Temperature (°C)	$k_{app} \cdot 10^3$ (M^{-1} sec^{-1})	$K_0 \cdot 10^3$ (M^{-1} sec^{-1})	$k_0' \cdot 10^3$ (M^{-1} sec^{-1})	Reference
Cl-acetate	GSH		25		25	80	32
Cl-acetate	Cys	6.0	25	0.088	30	97	33
Cl-acetamide	GSH	9.0	30	135	315	620	34
Cl-acetamide	Cys	9.0	30	97	170	330	8
Cl-acetamide	Cystamine	9.0	30	110	135	260	8
Cl-acetamide	ME		35		240	290	12
Br-acetate	Cys	9.0	37	2900	5200	5200	t.w.
Br-acetate	Cys	13.0	25	1141	1141	3700	35
2-Br-propionate	Cys	9.0	37	43	75	75	t.w.
3-Br-propionate	Cys	8.0	30	5.8	28	55	36
Br-acetamide	GSH	11.2	25	16900	17200	55000	14
2-Br-propionamide	GSH	11.2	25	150	155	490	14
2-Br-*n*-valeriamide	GSH	11.2	25	60	61	200	14
I-acetate	Cys	9.0	37	5500	9650	9650	t.w.
I-acetate	Nac-cys	9.0	37	1250	5400	5400	t.w.
I-acetate	Nac-cys		25		4350	14000	37
I-acetate	ME	9.0	37	1400	8700	8700	t.w.
I-acetate	Cystamine	9.0	37	6400	7800	7800	t.w.
I-acetate	GSH	9.0	37	4500	9600	9600	t.w.
I-acetate	GSH	11.2	25	2800	2850	9200	38
I-acetate	GSH		22		4000	17000	21
2-I-propionate (D)	Cys	7.0	20	1.65	59	320	9
2-I-propionate (L)	Cys	7.0	20	1.54	55	300	9
2-I-propionate	Cys	7.0	25	3.3	118	380	9
2-I-propionate	Cys		25		61	290	9
3-I-propionate	GSH	11.2	25	15	15	48	38
3-I-propionate	Cys	9.0	37	25	44	44	t.w.
I-acetamide	Cystamine	8.0	20	1280	4140	22000	16
I-acetamide	Cys	8.0	20	990	4800	26000	16
I-acetamide	Cys	9.0	37	17000	30200	30000	t.w.
I-acetamide	GSH	8.0	20	910	8800	48000	16
I-acetamide	GSH	11.2	25	27000	27500	89000	14
I-acetamide	GSH	7.0	23	460	38300	170000	21
I-acetamide	Nac-cys		25		33000	100000	37
2-I-propionamide (D)	Cys	7.0	20	3.8	136	740	9
2-I-propionamide (L)	Cys	7.0	20	5.1	182	990	9
2-I-propionamide	Cys	7.0	25	7.7	275	890	9
2-I-propionamide	Cys		25		144	470	9
2-Cl-ethanol	Cys	9.0	37	0.2	0.35	0.35	t.w.
2-Br-ethanol	Cys	9.0	37	2.5	4.4	4.4	t.w.

Note. k_{app} is the observed rate constant with thiolate anion at the actual pH and temperature, k_0 is the pH-independent rate constant, and k_0' is the estimated pH independent rate constant at 37°C. The different thiols are: cysteine (cys), *N*-acetylated cysteine (Nac-cys), glutathione (GSH), cystamine, and 2-mercaptoethanol (ME). In the last column, "t.w." refers to this work.

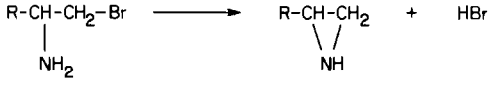
and bromoethanol show that compounds with a vicinal hydroxyl group are least reactive (Tables 1 and 2). The α-haloacids are far more reactive, and therefore widely used for protein modification and inhibition of metabolism (*38*). The α-haloamides are more reactive than the α-haloacids, and their esters even more so. Reactivity follows the expected order of electron attraction for the different substituents.

Comparing **XIV** with 2-bromopropionate, the latter being twice as reactive, shows that a β-imidazole ring has only a minor effect on reactivity. The effect of a α- or β-carboxylate group is illustrated by comparing the reactivities of 3-halopropionates with the haloacetates, which are for iodine 1 : 220 and for bromine 1 : 100 (Table 2) in agreement with previous findings (*32*). A similar comparison for an α- and β-amido group is difficult since β-halopropionamide cyclizes to form an imidoester (*40*). Steric hindrance is evident from the reactivities of the α-haloacids; **XIV**:2-bromopropionate:bromoacetate = 1:2:160. *N* substitution in the imidazole ring should increase the steric hindrance, the π substituent more than the τ substituent (Fig. 2). This agrees with the rates observed with **XIV, XV,** and **XVI** and also with **XXI** and **XXII.** The steric hindrance of the reaction is mediated through retardation of bond making, while electron withdrawing substituents accelerate the reaction by enhanced bond making. This indicates that bond making is the rate-determining process in these reactions.

Compounds **XI** and **XXIII** differ from the other imidazole derivatives in having a primary halogen and no electron-withdrawing substituents. The latter explains why the reactivity of **XXIII** is rather low for a primary alkyl halide and similar to that of 3-bromopropionate or bromoethanol. Reaction of **XXIII** with thiolate is, as indicated previously, significant (*29*). The following order of reactivity with thiolate summarizes the results presented in Tables 1 and 2:

$$\mathrm{R{-}CH_2{-}CH(X){-}COOH_3} > \mathrm{R{-}CH_2{-}CH(X){-}CONH_2} > \mathrm{R{-}CH_2{-}CH(X){-}COOH} > \mathrm{R{-}CH_2{-}CH_2(X)} > \mathrm{R{-}CH_2{-}CH(X){-}CH_2OH}.$$

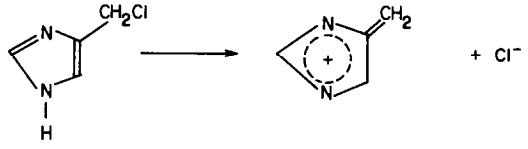
A few compounds need special comment. **XXIV** does not react in the same way as the other alkyl halides. Having an amino group β to the primary bromine, this compound will in an internal reaction immediately form an aziridine in aqueous solution at pH 9.0 (*30*).



As the aziridine, the alkylating reactivity of **XXIV** coincides with that of **XIV** (Table 1). The aziridine most frequently used in protein modification is ethylenimine. This forms from 2-bromoethylamine in aqueous solution and reacts preferentially with cysteine residues in proteins (*1*). A similar formation of an epoxide from alkyl halides with a carbonyl function in the β position is not significant (*9*).

In diethanolamine buffer, the two chloromethylimidazoles **XII** and **XIII** were observed to react rapidly, but only part of the cysteine was consumed when reaction stopped. The same behavior resulted when **XII** was used to inactivate

yeast alcohol dehydrogenase (*41*). A rapid inactivation was followed by a plateau, the level of which depended on the concentration of the alkylating reagent. These reaction patterns result from the compounds reacting with both thiol and diethanolamine, while in the protein several residues are modified. Reaction ends when the alkylating reagent is used up, and a plateau is observed. Having the imidazole ring α to the halogen, **XII** and **XIII** are comparable to benzyl halides, and a similar carbonium ion stabilization can take place:



When **XII** and **XIII** were reacted with cysteine (5 m*M* of both reactants) in 0.1 *M* pyrophosphate buffer, pH 9.0, a second-order reaction was observed. Simple addition of the carbonium ion to the nucleophile explains reaction with diethanolamine, a reaction not observed with alkyl halides more reactive toward thiolate. This agrees with the findings of Fries *et al.* (*40*) for the corresponding chloromethyl pyrazoles. These proved very reactive toward 4-(*p*-nitrobenzyl)pyridine and rapidly and unspecifically modified an inactivated horse liver alcohol dehydrogenase.

A further aspect of thiolalkylation by the imidazole derivatives is that both steric and inductive effects are less important for the reactivity of the chloro compounds compared to the bromo compounds (Table 1). Steric hindrance retards, while electron-withdrawing substituens accelerate bond making. Since the reaction rate becomes more dependent on bond breaking when the poorer chloride leaving group replaces bromide, these effects are less important with the chloro compounds. This again indicates that at least with the bromo compounds, bond making is the rate-determining process.

For the imidazole derivatives available as enantiomers a slightly higher rate was observed with the *S* enantiomers in the reaction with L-cysteine (Table 1). The geometry of the transition state determines this stereoselectivity.

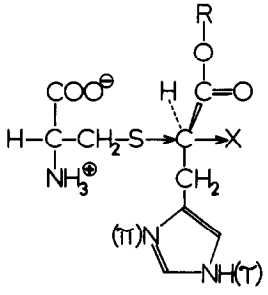


FIG. 2. Transition state model for nucleophilic substitution with the thiolate of cysteine of the halogen (X) of an imidazole derivative.

The free thiols used in this work (Table 2) show only small differences in reactivity when correction is made for the actual concentration of thiolate anions.

Metal-Directed Affinity Labels

Metal-directed affinity labeling with the compounds in Table 1, depends on the reversible binding of the imidazole ring to a protein metal prior to irreversible alkylation of a nearby amino acid residue (*41*). With these reagents, a sensitive method for measuring alkylating reactivity is of particular importance, because, in addition to being alkyl halides, they contain an imidazole ring with a nucleophilic reactivity about that of pyridine (*6*). To avoid self-alkylation and polymerization, these compounds cannot be too reactive as alkylating reagents.

To achieve labeling of different metalloenzymes, the alkylating side chain to the imidazole ring was varied (Table 1). However, as is evident from the present work, the neighboring groups to the halogen greatly influences the reactivity and the stability of the compounds. Thus in evaluating potential labels, not only metal affinity but also chemical reactivity has to be considered.

The acids have so far turned out to be the best labels (*41–44*), but synthesis of the corresponding amides should give stabile labels with a neutral side chain with similar alkylating reactivity. The methyl esters are interesting due to high reactivity, but their use as affinity labels is difficult since they are unstable, and the resulting acids are also reactive as alkylating reagents (*41, 43*). The alcohols seem to be too unreactive to produce any protein modification, and this agrees with previous findings for haloethanols (*45*). The haloethyl imidazoles **XI** and **XXIII** can be useful labels, but as primary alkyl halides their reactivity is low. The imidazole derivatives with a substituent on one of the imidazole nitrogens enable investigation of which nitrogen atom ligand to the metal atom. The chloromethyl imidazoles **XII** and **XIII** presumably react through carbonium ions. Enzyme modification with these is thus considered to be less specific, with the possibility of residues other than thiols being modified. As an aziridine-forming compound, **XXIV** is interesting for protein modification.

In this work, factors governing reactivity of alkyl halides in reaction with free thiolate were studied as a model reaction for alkylation of protein thiols. It seems that halogen type and electron-withdrawing substituents play about the same role in reaction with free thiols and protein thiols. However, the finding that iodoacetate is about 300 times more reactive than **XIV** toward free thiolate (Tables 1 and 2), contrasts with the similar reactivity observed in affinity labeling of liver alcohol dehydrogenase (*46*). This shows that in the reaction with **XIV** the enzyme facilitates its own alkylation.

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