Johnson, P. E., Abbott, S. J., Orr, G. A., Sêmêriva, M., & Knowles, J. R. (1976) *Biochemistry 15*, 2893.
Jones, S. R., Kindman, L. A., & Knowles, J. R. (1978) *Nature (London) 257*, 564.
Kaji, A., & Colowick, S. P. (1965) *J. Biol. Chem. 240*, 4454.
Kayne, F. J. (1973) *Enzymes, 3rd Ed. 8*, 353.
Li, T. M., Mildvan, A. S., & Switzer, R. L. (1978) *J. Biol. Chem. 253*, 3918.
Lowe, G., & Sproat, B. S. (1978) *J. Chem. Soc., Chem. Commun.*, 783.
MacFarlane, N., & Ainsworth, S. (1972) *Biochem. J. 129*, 1035.
MacFarlane, N., & Ainsworth, S. (1974) *Biochem. J. 139*, 499.
Midelfort, C. F., & Sarton-Miller, I. (1978) *J. Biol. Chem. 253*, 7127.
Morrison, J. F., & Heyde, E. (1972) *Annu. Rev. Biochem. 41*, 29.
Orr, G. A., Simon, J., Jones, S. R., Chin, G. J., & Knowles, J. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2230.
Parks, R. E., & Agarwal, R. P. (1973) *Enzymes, 3rd Ed. 8*, 307.
Purich, D. L., & Fromm, H. J. (1972) *Arch. Biochem. Biophys. 149*, 307.
Richard, J. P., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7757.
Rose, I. A., Grunberg-Manago, J., Korey, S. R., & Ochoa, S. (1954) *J. Biol. Chem. 211*, 737.
Sheu, K.-F. R., & Frey, P. A. (1978) *J. Biol. Chem. 253*, 3378.
Skarstedt, M. T., & Silverstein, E. (1976) *J. Biol. Chem. 251*, 6775.
Solomon, F., & Rose, I. A. (1971) *Arch. Biochem. Biophys. 147*, 349.
Spector, L. B. (1973) *Bioorg. Chem. 2*, 311.
Switzer, R. L., & Simcox, P. D. (1974) *J. Biol. Chem. 249*, 5304.
Todhunter, J. A., & Purich, D. L. (1974) *Biochem. Biophys. Res. Commun. 60*, 273.
Todhunter, J. A., Reichel, K. B., & Purich, D. L. (1976) *Arch. Biochem. Biophys. 174*, 120.
Usher, D. A., Richardson, D. I., & Eckstein, F. (1970) *Nature (London) 228*, 663.
Usher, D. A., Erenrich, E. S., & Eckstein, F. (1972) *Proc. Natl. Acad Sci. U.S.A. 69*, 115.
Walsh, C. T., & Spector, L. B. (1971) *Arch. Biochem. Biophys. 145*, 1.
Webb, B. C., Todhunter, J. A., & Purich, D. L. (1976) *Arch. Biochem. Biophys. 173*, 282.
Westheimer, F. H. (1968) *Acc. Chem. Res. 1*, 70.
Whitesides, G. M., Siegel, M., & Garrett, P. (1975) *J. Org. Chem. 40*, 2516.

Kinetics of Carboxymethylation of Histidine Hydantoin†

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ABSTRACT: The reaction of the imidazole group of histidine hydantoin with bromoacetate was studied as a model for carboxymethylation of histidine residues in proteins. pK values of 6.4 and 9.1 (25 °C) and apparent heats of ionization of 7.8 and 8.7 kcal/mol were determined for the imidazole and hydantoin rings, respectively. At pH values corresponding to the isoelectric points for histidine hydantoin, the rates of carboxymethylation at 12, 25, 37, and 50 °C were determined; the modified hydantoins were hydrolyzed to the corresponding histidine derivatives for quantitative amino acid analysis. At pH 7.72 and 25 °C, the imidazole *tele*-N was alkylated (k = 3.9×10^{-5} M^{-1} s^{-1}) twice as fast as the *pros*-N. The monocarboxymethyl derivatives were carboxymethylated at the same rate at the *pros*-N ($k = 2.1 \times 10^{-5}$ M^{-1} s^{-1}) but 3 times faster at the *tele*-N ($k = 11 \times 10^{-5}$ M^{-1} s^{-1}). The enthalpies of activation determined for carboxymethylation of the imidazole ring and its monocarboxymethyl derivatives were similar (15.9 ± 0.7 kcal/mol). $\Delta S^{\ddagger}$ for the four carboxymethylations was −25 ± 2 eu. The electrostatic component of $\Delta S^{\ddagger}$ ($\Delta S^{\ddagger}_{es}$) was calculated from the influence of the dielectric constant on the reaction rate at 25 °C. $\Delta S^{\ddagger}_{es}$ was slightly negative (−4 ± 1 eu) for mono- or dicarboxymethylations, indicating some charge separation in the transition state. The nonelectrostatic entropy of activation was −21 ± 2 eu for all four carboxymethylations.

Carboxymethylation with haloacetates is commonly used to study protein structure and function (Gurd, 1967; Stark, 1970; Cohen, 1970). Histidine residues in several enzymes, such as human carbonic anhydrase (Bradbury, 1969), swine heart fumarase (Bradshaw et al., 1969), and bovine pancreatic ribonuclease (Gundlach et al., 1959), are unusually reactive toward alkylation, compared with denatured protein or lower molecular weight imidazole compounds. Carboxymethylation of free histidine (Heinrikson et al., 1965), N^{α}-acetylhistidine (Crestfield et al., 1963b), poly-L-histidine (Goren & Barnard, 1971), and the copper(II) complex of histidine (Wieghardt & Goren, 1975) have all been used as models for the spontaneous, or unfacilitated, reaction of haloacetates with histidine residues. However, the α-ammonium and carboxylate groups of free histidine can interact with the negatively charged haloacetate; N-acetylation can eliminate one ionic interaction, but the bulky substituent might interfere sterically with the reaction (Crestfield et al., 1963b). The copper(II)–histidine chelate may have several structures existing simultaneously

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Table I: Apparent pK Values of Histidine Hydantoin[a]

temp (°C)	pK_1	pK_2
12	6.6	9.4
25	6.4	9.1
37	6.1	8.8
50	5.9	8.6

[a] A 3.0-mL aliquot of 5.0 mM histidine hydantoin in 50 mM NaCl was titrated with 0.100 M NaOH, by using a Radiometer TTT1a Titrator, a SBR2c Titrigraph, and a SBU1a syringe burette, standardized at pH 7.0 and 10.0. A jacketed titration vessel connected to a thermostated water bath was used to maintain constant temperature. The titrant required for 3 mL of 50 mM NaCl was subtracted from the titration of histidine hydantoin to obtain the corrected titration curve. The pK values were estimated from a graph of the data according to the equation (milliequivalents of OH^-) = (milliequivalents of hydantoin) − (milliequivalents of OH^-) × $[H^+]/K_a$ (Lindley, 1960; Chaiken & Smith, 1969). As a control, histidine hydrochloride was also titrated at each temperature.

in solution (Kruck & Sarkar, 1973), including some in which the imidazole *pros*-N[1] is one of the ligands to the copper (Makinen et al., 1969; Kruck & Sarkar, 1973; Freeman et al., 1969). Poly-L-histidine has the α-amino and carboxyl groups in peptide linkages, but nearby imidazole groups may influence the reaction. We propose histidine hydantoin as a more suitable model since the amino and carboxyl groups are pinned back in the hydantoin ring, which reduces steric and ionic interactions.

We have determined the rates of carboxymethylation of histidine hydantoin at each of the imidazole nitrogens at varied temperatures and thus the activation parameters for the reactions. Effects of the dielectric constant on the rates of reaction were determined in order to evaluate the nature of electrostatic effects on the reaction. Comparison with similar data for alkylation of a histidyl residue in an enzyme should provide evidence on the effect of the protein on the reactivities of histidine residues. (See the following article for an example.)

Experimental Procedure

Bromoacetic acid was recrystallized from diethyl ether–petroleum ether; mp 49–50 °C (uncor). Histidine hydantoin hydrochloride was synthesized by the method of Stark & Smyth (1963), as modified by Stark (1967); mp 235–240 °C (uncor) with decomposition.

The concentrations of histidine hydantoin and its carboxymethylated derivatives were determined as free histidine or (carboxymethyl)histidines by amino acid analysis, after hydrolysis of the hydantoins in 6 M HCl at 110 °C for 96 h (Stark, 1967) in evacuated tubes (Crestfield et al., 1963a). After removal of the acid, the samples were analyzed (Spackman et al., 1958) by using the accelerated method of Spackman (1967) and a Beckman Spinco Model 120C amino acid analyzer as described previously (Crestfield et al., 1963b; Wieghart & Goren, 1975). When necessary, sensitivity was increased (Hamilton, 1963) to produce a full-scale absorbance response of 0.10; this allowed analysis of 1–10 nmol of an amino acid. Color values of 0.93, 0.97, and 0.93 times the average of the color values for the acidic and neutral amino acids (excluding proline and half-cystine) were used to calculate the amounts of N^π-(carboxymethyl)histidine, N^τ-(carboxymethyl)histidine, and N^π,N^τ-(dicarboxymethyl)-

[1] The imidazole N closest to the alanine side chain of histidine, formerly designated as N-1 by biochemists (or N-3 by other chemists), is termed *pros*, or π, and N-3 (biochemical) is termed *tele*, or τ, according to IUPAC–IUB.

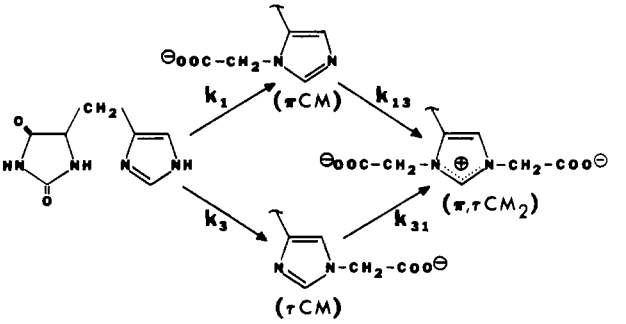

FIGURE 1: Reactions of histidine hydantoin with bromoacetate.

histidine, respectively (Hugli & Gurd, 1970).

Results

pK Values of Histidine Hydantoin. In order to ensure that the alkylation of histidine hydantoin was studied at a pH at which the rate constants would be essentially independent of pH, the pK values of the imidazole and hydantoin groups were determined at each temperature at which the reaction would be run (Table I). The pK_1 values obtained for histidine hydantoin were assigned to the imidazole group, since they are similar in magnitude to pK_2 of histidine (6.0 at 25 °C). The pK_2 values were assigned to the hydantoin ring; the pK of hydantoin (glycine hydantoin) is given as 9.16 (Sober, 1970).

Apparent heats of ionization of 7.8 and 8.7 kcal/mol for the first and second ionizations, respectively, of histidine hydantoin were calculated by using the van't Hoff relationship. The heat of ionization of the imidazole group of histidine is 6.9 ± 1.0 kcal/mol (Cohn & Edsall, 1965) and is 8.6 kcal/mol for 4-methylimidazole (Sober, 1970).

Carboxymethylation of Histidine Hydantoin. Treatment of histidine hydantoin with bromoacetic acid leads to the formation of a dicarboxymethylated product via two carboxymethylated intermediates (Figure 1). Carboxymethylation was carried out at 12, 25, 37, and 50 °C, maintained within ±0.1 °C. Most rapid (and pH-independent) alkylation requires that the imidazole ring be unprotonated; however, it is also desirable to work at a pH below the pK of the hydantoin ring to avoid competing reactions. The pH of reaction was chosen to be at the isoelectric point of histidine hydantoin at each temperature. The concentration of bromoacetate used (0.1 M) was demonstrated to produce kinetics first order in this reagent.

Results of the carboxymethylation studies are shown in Figure 2. In each case, the amount of histidine hydantoin present decreases in an exponential manner, while N^π-(carboxymethyl)histidine hydantoin and, somewhat more rapidly, N^τ-(carboxymethyl)histidine hydantoin are formed. Eventually, N^π,N^τ-(dicarboxymethyl)histidine hydantoin appears. The concentrations of the N^π- and N^τ-(carboxymethyl)histidine hydantoins reach maxima and then decrease; the times of these maxima vary with temperature, but the concentration of N^π-(carboxymethyl)histidine hydantoin always reaches a maximum before that of N^τ-(carboxymethyl)histidine hydantoin. The curves in Figure 2 represent nonlinear least-squares fits to the experimental data, calculated as described below.

Analysis of Kinetic Data. The disappearance of histidine hydantoin and the formation of its carboxymethylated derivatives may be expressed as a function of time:

$$d[H]/dt = -(k_1 + k_3)[H] \quad (1)$$

$$d[\pi CmH]/dt = k_1[H] - k_{13}[\pi CmH] \quad (2)$$

$$d[\tau CmH]/dt = k_3[H] - k_{31}[\tau CmH] \quad (3)$$

$$d[DCmH]/dt = k_{13}[\pi CmH] + k_{31}[\tau CmH] \quad (4)$$

where k_1, k_3, k_{13}, and k_{31} are the pseudo-first-order rate constants for each reaction and [H], [πCmH], [τCmH], and [DCmH] refer to the concentrations of histidine hydantoin, N^{π}-(carboxymethyl)histidine hydantoin, N^{τ}-(carboxymethyl)histidine hydantoin, and N^{π},N^{τ}-(dicarboxymethyl)-histidine hydantoin, respectively.

These equations were integrated and approximate values for the rate constants were obtained by means of graphic solutions as described by Wang & Carpenter (1967). Provisional estimates of the rate constants at 12 °C (Figure 2A) were evaluated from an Arrhenius plot constructed by using the data at 25, 37, and 50 °C. These rate constants were used as initial estimates in a computer program (NONLIN; written by C. M. Metzler, The Upjohn Co.) that provides for parameter estimation of a nonlinear least-squares fit of all of the data at one temperature simultaneously to the four differential equations (eq 1–4).

The fits of the data obtained by using this program were very good; the coefficients of correlation were generally better than 0.990; the poorest was 0.973. The overall correlation coefficient, for all four sets of data at any temperature, was 0.999 in each case. Computed values for the pseudo-first-order rate constants were divided by the bromoacetate concentration (0.10 M) to obtain second-order rate constants, which are listed in Table II. Similar values were obtained from fits to the integrated equations.

Estimates of [H_0], the amount of histidine hydantoin initially present, were obtained from fits to the integrated form of eq 1. At each temperature, the average value of the amounts of histidine plus mono- and dicarboxymethylated histidines found was equal (±1 nmol) to the calculated value of [H_0]; thus, all of the products of the reaction could be accounted for.

Evaluation of the Activation Parameters. The transition-state theory was used to calculate the activation parameters for the reactions of histidine hydantoin with bromoacetate. According to the theory of absolute reaction rates (Glasstone et al., 1941)

$$\ln (k/T) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R + \ln (\boldsymbol{k}/h) \quad (5)$$

where k is the rate constant, R is the gas constant [1.987 cal/(deg mol)], $\boldsymbol{k}$ is Boltzmann's constant (1.380×10^{-16} erg/deg), and h is Planck's constant (6.624×10^{-27} erg s). Values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for each reaction were calculated from least-squares fits of the data in Table II to eq 5 and are presented in Table III.

Influence of the Dielectric Constant on the Reaction. In order to determine the contribution of electrostatic effects to the entropy of activation, we examined the variation of the rate constants with the dielectric strength of the medium at 25 °C. The reaction conditions were the same as those in Figure 2B, except that the reaction mixtures contained dioxane at concentrations ranging from 0 to 20% by weight. The time courses of the reactions were similar to that of the aqueous reaction. The system was adequately defined by eq 1–4, since the computed curves and the data agreed. The overall coefficients of correlation for each concentration of dioxane ranged from 0.989 to 0.999, but the correlation became poorer with increasing dioxane concentration. All four rate constants show a general overall decrease with increasing dioxane concentration; however, the changes are small, the largest, for k_{13}, being only about a twofold decrease (Figure 3). The uncertainty associated with the estimates of the rate constants, reflected in the relative errors, also increases with increasing dioxane concentration.

Table II: Rate Constants for Carboxymethylation of Histidine Hydantoin by Bromoacetate at Various Temperatures[a]

temp (°C)	$10^5 \times$ rate constant (M^{-1} s^{-1} ±SE)			
	k_1	k_3	k_{13}	k_{31}
12	0.61 ± 0.15	1.1 ± 0.2	3.3 ± 2.8	0.71 ± 1.4
25	2.0 ± 0.1	3.9 ± 0.1	11 ± 1	2.1 ± 0.1
37	6.5 ± 0.5	11.7 ± 0.5	29 ± 3	5.5 ± 0.7
50	21 ± 2	38 ± 2	90 ± 10	19 ± 3

[a] The values were obtained from the results in Figure 2, fitted by a nonlinear least-squares procedure to the appropriate differential equations. These rate constants are assumed to be pH independent, since each reaction was run at a pH value (see Figure 2) about 1.4 units greater than the pK of the imidazole group, where about 96% of the imidazole groups would be unprotonated.

Laidler & Bunting (1973) have extended the treatment of Scatchard (1932) to obtain a relationship between the dielectric constant of the medium and the rate of reaction:

$$\ln K^{\ddagger} = -\Delta G^{\ddagger}/RT = -\Delta G^{\ddagger}_{nes}/RT - z_A z_B e^2/\epsilon \boldsymbol{k} T r^{\ddagger}$$

where $\Delta G^{\ddagger}_{nes}$ is the nonelectrostatic component of the energy of activation, z_A and z_B are the charges on the molecules, e is the charge on the electron, ϵ is the dielectric constant, $\boldsymbol{k}$ is Boltzmann's constant, and $r^{\ddagger}$ is the separation between the charges in the transition state. Since k is proportional to $K^{\ddagger}$

$$\ln k = \ln k_0 - \frac{A}{T\epsilon} \quad (6)$$

where k_0 is the rate constant when ϵ is equal to infinity and A is equal to $z_A z_B e^2/\boldsymbol{k} r^{\ddagger}$. The logarithms of the rate constants were plotted as a function of $1/\epsilon$ (Figure 3), and a weighted least-squares computation to eq 6 fitted the data well. From the values for the slopes was calculated the electrostatic component of the entropies of activation:

$$\Delta S^{\ddagger}_{es} = (1.13 \times 10^{-4})A$$

The values obtained for $\Delta S^{\ddagger}_{es}$ and the nonelectrostatic component, $\Delta S^{\ddagger}_{nes}$, are listed in Table III.

Discussion

Suitability of Histidine Hydantoin as a Model Compound for Studies of Carboxymethylation. The overall rate of reaction of bromoacetate with histidine hydantoin at pH 7.72 and 25 °C ($k_1 + k_3 = 5.9 \times 10^{-5}$ M^{-1} s^{-1}) is about 7 times the rate of reaction with free histidine (8.6×10^{-6} M^{-1} s^{-1}) at 25 °C and pH 5.5 (Heinrikson et al., 1965). At 25 °C and pH 5.5, histidine is alkylated by iodoacetate more than 5 times faster than N^{α}-acetylhistidine (Crestfield et al., 1963b). The difference between the rate of carboxymethylation of histidine hydantoin and the observed rates of carboxymethylation of histidine, pK_2 = 6.0 (Sober, 1970), and N^{α}-acetylhistidine, pK_2 = 7.2 (Cruikshank & Kaplan, 1975), is most likely due to the differences in the state of protonation of the imidazole groups at pH 5.5. When the observed rates were corrected for differences in the pK values of the imidazole groups and for hydrogen ion activity [e.g., Holmquist & Bruice (1969)] according to the equation

$$k = k_{obsd}[(K_a + a_H)/K_a]$$

where k is the true bimolecular (pH-independent) rate constant, k_{obsd} is the observed pH-dependent rate constant, K_a is the acid dissociation constant of the nucleophile, and a_H is the hydrogen ion activity, the pH-independent rate constants for alkylation of N^{α}-acetylhistidine (8.5×10^{-5} M^{-1} s^{-1}, estimated),

Table III: Activation Parameters for the Carboxymethylation of Histidine Hydantoin by Bromoacetate

rate constant[a]	kcal/mol ± SE		cal/(deg mol) ± SE		
	$\Delta G^{\ddagger}$[b]	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta S^{\ddagger}_{es}$[c]	$\Delta S^{\ddagger}_{nes}$[c]
k_1	23.8 ± 0.6	16.6 ± 0.4	−24.2 ± 1.3	−3.5 ± 1.1	−20.7 ± 1.7
k_3	23.5 ± 0.3	16.5 ± 0.2	−23.3 ± 0.6	−3.5 ± 0.6	−19.8 ± 0.8
k_{13}	22.8 ± 0.4	15.3 ± 0.3	−25.3 ± 1.1	−4.3 ± 1.3	−21.0 ± 1.7
k_{31}	23.8 ± 1.1	15.3 ± 0.8	−28.5 ± 2.7	−6.5 ± 3.6	−22.0 ± 4.5

[a] These rate constants are defined in Figure 1. [b] At 25 °C. $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were determined with the data in Table II. [c] At 25 °C. The electrostatic and nonelectrostatic components of the entropy of activation were determined from the results in Figure 3. $\Delta S^{\ddagger}_{nes} = \Delta S^{\ddagger} - \Delta S^{\ddagger}_{es}$.

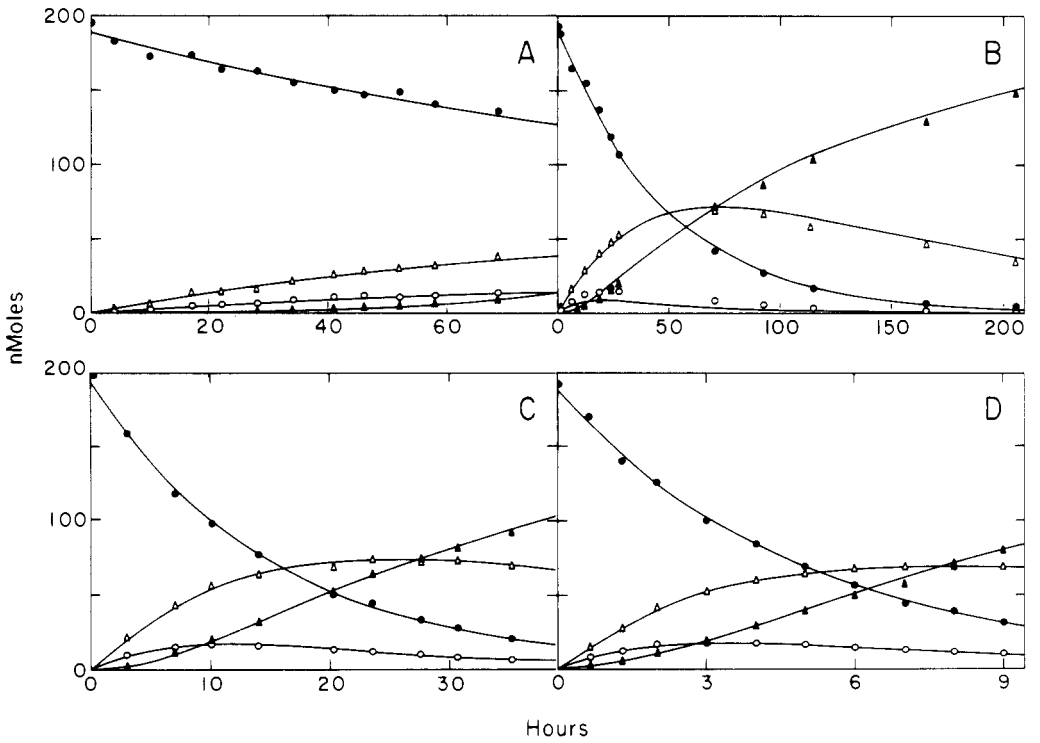

FIGURE 2: Time course of the reaction of histidine hydantoin with bromoacetate. A 1.0 mM solution of histidine hydantoin in 0.1 M sodium bromoacetate and 0.1 M triethanolamine hydrochloride buffer was allowed to react. Aliquots of 1.0 mL were withdrawn at the times indicated, added to 1.0 mL of 12 M HCl, and hydrolyzed, and the equivalent of 200 nmol of hydantoins was analyzed. The nanomoles of histidine (●), N^{α}-(carboxymethyl)histidine (○), N^{τ}-(carboxymethyl)histidine (△), and N^{π},N^{τ}-(dicarboxymethyl)histidine (▲) found are plotted against time. (A) 12 °C, pH 7.99. (B) 25 °C, pH 7.72. (C) 37 °C, pH 7.48. (D) 50 °C, pH 7.25.

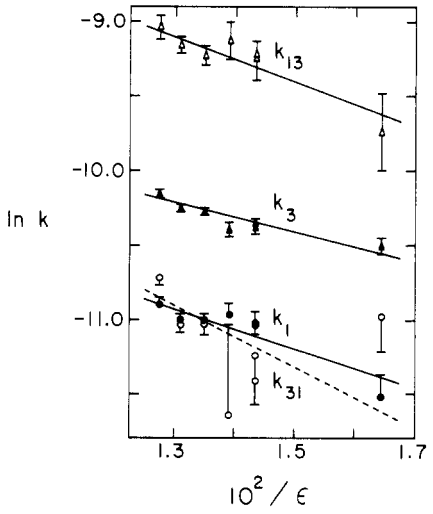

FIGURE 3: Influence of the dielectric constant on the rates of carboxymethylation of histidine hydantoin. The second-order rate constants, with error bars equal to ±SE, were determined at varied concentrations of dioxane, and ϵ, the dielectric constant, was calculated from the data of Åkerlöf & Short (1936). The lines represent linear least-squares fits of the data, weighted by the reciprocal of the variance at each point.

free histidine (3.6×10^{-5} M^{-1} s^{-1}), and histidine hydantoin (5.9×10^{-5} M^{-1} s^{-1}) are comparable. This indicates that the rates of carboxymethylation are not very sensitive to the presence of charged or uncharged substituents or to the various buffers used in the different experiments.

The copper(II) complex of histidine has been suggested as a model for an isolated histidyl residue in a protein by Wieghardt & Goren (1975). However, they found an overall rate constant for monocarboxymethylation of the complex (1.35×10^{-6} M^{-1} s^{-1}, at pH 6.1 and 22 °C) that is more than 10 times smaller than those for alkylation of the histidine compounds discussed above. Since the acid dissociation constant for the imidazole group was not determined by the authors, the pH-independent rate constant cannot be calculated. Nevertheless, the greatly decreased rate of alkylation raises the possibility that a major form of the copper(II)–histidine complex was one in which the *pros*-N was coordinated to the copper. The authors argue against the existence of forms of the complex in which the α-amino group is free, or in which the imidazole *pros*-N is bound to the copper, on the grounds that no N^{α}-(carboxymethyl)histidine was detected and that N^{π}-(carboxymethyl)histidine and (dicarboxymethyl)histidine were formed as well as the N^{τ}-(carboxymethyl)histidine.

However, at pH 6.1, an amino group would be a poor nucleophile and react slowly. Furthermore, alkylation could occur at the *pros*-N at a reduced rate. Histidine hydantoin and the copper(II)–histidine complex also differ in the relative rates for the four carboxymethylation reactions: k_3/k_1, k_{13}/k_3, k_{31}/k_1. Fitting the original data of Wieghardt and Goren (Table 1 in their article) to eq 1–4 as for histidine hydantoin gave values for the rate constants that differed less than 60% from the values calculated by these authors; thus, the method of calculation does not account for the differences. Because of the possible extraneous effects of the copper ion, histidine hydantoin should be a better model compound for studying the reactivity of the imidazole group of histidine.

Interpretation of Reaction Rates and Activation Parameters. The *tele*-N of histidine hydantoin is carboxymethylated twice as fast as the *pros*-N. This occurs despite the fact that the *tele*-H tautomer of the imidazole group in histidine and its derivatives predominates over the *pros*-H form by two- to fourfold (Reynolds et al., 1973). We assume that the tautomeric equilibrium of histidine hydantoin is similar. Since the pyrrole-like NH of the imidazole should be much less reactive than the pyridine-like N, the ratio of products would be proportional to the ratio of tautomers, *if* the tautomers were equally reactive.

Carboxymethylation of the *tele*-N of N^{π}-(carboxymethyl)histidine hydantoin (k_{13}) proceeds about 3 times faster than monocarboxymethylation at the *tele*-N (k_3). This decrease might reflect electron donating effects of the carboxymethyl group on the imidazole ring. However, carboxymethylation of histidine hydantoin at the *pros*-N (k_1 and k_{31}) proceeds at the same rate regardless of whether or not the opposite N is carboxymethylated. The enthalpies of activation determined for the four reactions are not significantly different and do not reveal differences in the intrinsic reactivities of the four nitrogens.

Examination of Corey-Pauling-Koltun space-filling models of N^{π}- and N^{τ}-(carboxymethyl)histidine hydantoin suggests that steric factors are unimportant in determining the reaction rates. The *tele*-N of the imidazole group of histidine hydantoin is directed outward toward the bulk of the medium regardless of whether the opposite N is alkylated or not. Access to the *pros*-N can be hindered slightly by the hydantoin ring, but carboxymethylation of the *tele*-N would not affect this access.

The electrostatic ($\Delta S^{\ddagger}_{es}$) and nonelectrostatic ($\Delta S^{\ddagger}_{nes}$) components of the entropy of activation are related respectively to solvent and structural effects. During the course of the reaction, shifts in electron density may occur, producing either increased or decreased solvent binding. An increase in electrostriction of solvent would result in a negative electrostatic entropy of activation. A $\Delta S^{\ddagger}_{es}$ of −3.5 cal/(deg mol) was calculated for carboxymethylation at either the *pros*-N (k_1) or *tele*-N (k_3) of histidine hydantoin (Table III) and is consistent with electrostriction of solvent due to charge separation in the transition state. The observed magnitude is only about one-third of the value to be expected on the basis of theoretical calculations (Laidler & Bunting, 1973). This difference may be due to the fact that the reaction was carried out at a finite ionic strength, without extrapolating the results to zero ionic strength. The values of $\Delta S^{\ddagger}_{es}$ observed for dicarboxymethylation of N^{τ}- and N^{π}-(carboxymethyl)histidine hydantoin are not significantly more negative than those found for carboxymethylation of histidine hydantoin.

The difference between $\Delta S^{\ddagger}$ and $\Delta S^{\ddagger}_{es}$ is the nonelectrostatic entropy of activation, $\Delta S^{\ddagger}_{nes}$, and is essentially the same for alkylation at either the *pros* or *tele* positions of both histidine hydantoin and (monocarboxymethyl)histidine hydantoin, −21 cal/(deg mol). $\Delta S^{\ddagger}_{nes}$ is related to structural effects and consists of an "unmixing" term of −8 cal/(deg mol), as well as probability factors associated with the frequency of collision and orientation of the two molecules necessary to produce the required effective encounters (Laidler & Bunting, 1973). The magnitudes of the entropies of activation for the carboxymethylation of histidine hydantoin fall within a range obtained for a number of nucleophilic displacement reactions (Bruice, 1970; Bruice & Benkovic, 1964).

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References

Åkerlöf, G., & Short, O. A. (1936) *J. Am. Chem. Soc. 58*, 1241–1243.
Bradbury, S. L. (1969) *J. Biol. Chem. 244*, 2002–2009.
Bradshaw, R. A., Robinson, G. W., Hass, G. M., & Hill, R. L. (1969) *J. Biol. Chem. 244*, 1755–1763.
Bruice, T. C. (1970) *Enzymes, 3rd Ed. 2*, 217–279.
Bruice, T. C., & Benkovic, S. J. (1964) *J. Am. Chem. Soc. 86*, 418–426.
Chaiken, I. M., & Smith, E. L. (1969) *J. Biol. Chem. 244*, 5087–5094.
Cohen, L. A. (1970) *Enzymes, 3rd Ed. 1*, 148–211.
Cohn, E. J., & Edsall, J. T. (1965) *Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions*, p 85, Hafner Publishing Co., New York.
Crestfield, A. M., Moore, S., & Stein, W. H. (1963a) *J. Biol. Chem. 238*, 622–627.
Crestfield, A. M., Stein, W. H., & Moore, S. (1963b) *J. Biol. Chem. 238*, 2413–2419.
Cruikshank, W. H., & Kaplan, H. (1975) *Biochem. J. 147*, 411–416.
Freeman, H. C., Guss, J. M., Healy, M. J., Martin, R.-P., Nockolds, C. E., & Sarkar, B. (1969) *J. Chem. Soc. D*, 225–226.
Glasstone, S., Laidler, K. J., & Eyring, H. (1941) *The Theory of Rate Processes*, McGraw-Hill, New York.
Goren, H. J., & Barnard, E. A. (1971) *Arch. Biochem. Biophys. 143*, 106–115.
Gundlach, H. G., Stein, W. H., & Moore, S. (1959) *J. Biol. Chem. 234*, 1754–1760.
Gurd, F. R. N. (1967) *Methods Enzymol. 11*, 532–541.
Hamilton, P. B. (1963) *Anal. Chem. 35*, 2055–2064.
Heinrikson, R. L., Stein, W. H., Crestfield, A. M., & Moore, S. (1965) *J. Biol. Chem. 240*, 2921–2934.
Holmquist, B., & Bruice, T. C. (1969) *J. Am. Chem. Soc. 91*, 2985–2993.
Hugli, T. E., & Gurd, F. R. N. (1970) *J. Biol. Chem. 245*, 1930–1938.
Kruck, T. P. A., & Sarkar, B. (1973) *Can. J. Chem. 51*, 3563–3571.
Laidler, K. J., & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, 2nd ed., pp 216–218, Clarendon Press, Oxford, England.
Lindley, H. (1960) *Biochem. J. 74*, 577–584.
Makinen, W. B., Pearlmutter, A. F., & Stuehr, J. E. (1969) *J. Am. Chem. Soc. 91*, 4083–4088.
Reynolds, W. F., Peat, I. R., Freedman, M. H., & Lyerla, J. R., Jr. (1973) *J. Am. Chem. Soc. 95*, 328–331.
Scatchard, G. (1932) *Chem. Rev. 10*, 229–240.
Sober, H. A. (1970) *Handbook of Biochemistry*, 2nd ed., Chemical Rubber Co., Cleveland, OH.

Spackman, D. H. (1967) *Methods Enzymol. 11*, 3–15.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Stark, G. R. (1967) *Methods Enzymol. 11*, 125–138.
Stark, G. R. (1970) *Adv. Protein Chem. 24*, 261–308.
Stark, G. R., & Smyth, D. G. (1963) *J. Biol. Chem. 238*, 214–226.
Wang, S., & Carpenter, F. H. (1967) *Biochemistry 6*, 215–224.
Wieghardt, T., & Goren, H. J. (1975) *Bioorg. Chem. 4*, 30–40.

Transition-State Analysis of the Facilitated Alkylation of Ribonuclease A by Bromoacetate†

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ABSTRACT: Bromoacetate reacts with histidine residues 12 and 119 at the active site of bovine pancreatic ribonuclease (RNase) much more rapidly than with free histidine. The mechanism of this facilitated alkylation was investigated by studying the dependence of the reaction on temperature and pH. RNase was treated with bromoacetate under pseudo-first-order conditions at 12, 25, 37, and 50 °C. The rate of inactivation of the enzyme showed a hyperbolic dependence on bromoacetate concentration, indicating formation of an enzyme–bromoacetate complex (K_I = 41 mM at pH 5.5 and 25 °C). Two groups, one of which must be unprotonated and the other protonated, are required for carboxymethylation of RNase by bromoacetate. At 25 °C, the free enzyme exhibits macroscopic pK values of 4.7 and 6.3, and the enzyme-bromoacetate complex has pK values of 5.8 and 7.4. The ratio of products [N^{π}-(carboxymethyl)histidine-119 RNase to N^{τ}-(carboxymethyl)histidine-12 RNase] formed in the reaction was 4.4 and was independent of temperature. Calculations based on this ratio and the microscopic pK values of histidines-119 and -12 determined by NMR titration suggest that the pH-independent alkylation of histidine-119 is about 8 times faster than that of histidine-12. The pH-independent rate of alkylation of RNase is 440 times that of histidine hydantoin at 25 °C. Significantly, $\Delta H^{\ddagger}$ for carboxymethylation of either histidine residue was 11 ± 2 kcal/mol, compared to 16.5 ± 0.4 kcal/mol for alkylation of histidine hydantoin. Values of $T\Delta S^{\ddagger}$ for the pseudobimolecular reaction of RNase and bromoacetate (−9 ± 2 kcal/mol) and for histidine hydantoin (−7.1 ± 0.3 kcal/mol) were similar. The carboxymethylation of RNase appears to be greatly facilitated by enthalpic factors, which increase the inherent reactivities of the histidine residues, rather than by entropic factors, such as binding and orientation. (However, differential solvation effects may have masked the intrinsic entropy changes that can facilitate the enzymatic reaction by reducing the loss of translational and rotational entropy in forming the transition state.) There is little or no electrostatic component of $\Delta S^{\ddagger}$ associated with the unimolecular reaction of the enzyme–bromoacetate complex, suggesting that charge separation is stabilized in the transition state. The microenvironments of the histidine residues in the RNase–bromoacetate complex apparently confer unusual properties on the imidazole groups.

Enzymes are enormously efficient catalysts, which accelerate reactions by proximity, general acid or base catalysis, use of alternate pathways or covalent intermediates, orientation, strain, and microenvironment, ion-pair, and solvent effects (Koshland & Neet, 1968; Jencks, 1969; Bruice, 1970; Bender, 1971). An important factor in catalysis is probably the juxtaposition, in a precise stereochemical manner, of the substrates and catalytic groups (Bruice, 1970). This concept has been variously described as the propinquity effect (Bruice & Benkovic, 1966), proximity and orientation (Koshland, 1962), and the Circe effect (Jencks, 1975). Another factor may be hyperreactive groups at the active sites of enzymes, which are especially reactive with chemical reagents (Shaw, 1970). Hyperreactivity may be induced by local charge effects or side-chain interactions.

A problem in studies on enzyme catalysis is evaluation of the relative importance of the various factors. Since a catalyst effectively lowers the free energy of activation ($\Delta G^{\ddagger}$) for a reaction and since $\Delta G^{\ddagger}$ is a function of the enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of activation, which are measures of the catalytic factors, transition-state analysis should be useful and has been applied in the investigation of enzymatic mechanisms (Bender et al., 1964; Leininger & Westley, 1968; Whitaker & Lee, 1972; Martinek et al., 1972; Marshall & Chen, 1973; Halâsz & Polgâr, 1976; Baggott & Klapper, 1976).

$\Delta H^{\ddagger}$ is related to the strength of the bonds to be broken or formed during the reaction; thus, bending, distortion, and polarization of bonds would be reflected in $\Delta H^{\ddagger}$, which we suggest is a measure of hyperreactivity or intrinsic reactivity of functional groups. On the other hand, $\Delta S^{\ddagger}$ is a measure of the randomness of the system and includes such effects as approximation of reactants, orientation, ion-pair formation, solvent effects, and stabilization of charge separation in the transition state. Thus, it becomes of interest to determine whether an enzyme lowers $\Delta H^{\ddagger}$ or raises $\Delta S^{\ddagger}$ for a reaction, compared to a nonenzymatic reaction. This is difficult in practice, however, for, as Bruice & Benkovic (1966) point out, "the only valid means of ascertaining the importance of bringing groups together in the enzymatic process would be to compare the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for [the enzyme], reacting with a particular substrate, to the reaction of the

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