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 p$K$ values of 4.7 and 6.3, and the enzyme-bromoacetate complex has p$K$ values of 5.8 and 7.4. The ratio of products [$N^{\pi}$-(carboxymethyl)histidine-119 RNase to $N^{\tau}$-(carboxymethyl)histidine-12 RNase] formed in the reaction was 4.4 and was independent of temperature. Calculations based on this ratio and the microscopic p$K$ 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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isolated active site of [the enzyme] in a bimolecular reaction with the same substrate". Unfortunately, the uncatalyzed reaction is usually too slow to be measurable and may proceed by a different mechanism.

We propose to use, instead of substrates, active-site-directed reagents since they resemble substrates in their behavior toward enzymes: they bind to the active site and their rates of reaction with the enzyme are facilitated, presumably by one or more catalytic factors. Furthermore, they usually react, albeit more slowly, with small molecules containing the same functional groups as those with which they react in the enzyme. Hence, active-site-directed reagents can be used to compare an enzymatically facilitated reaction with the same, uncatalyzed, chemical reaction.

We have studied the reaction of bovine pancreatic ribonuclease (RNase) and bromoacetate, which proceeds at a rate 2400 times that of the alkylation of free histidine by bromoacetate (Heinrikson et al., 1965) and results in the carboxymethylation of two neighboring histidine residues (12 and 119), both of which are involved in the catalytic mechanism of the enzyme (Richards & Wyckoff, 1971). Stark et al. (1961) explained the facilitation of the reaction by postulating that the protonated imidazole nitrogen of one histidine attracted the carboxylate anion of the haloacetate, orienting the reagent for nucleophilic attack by the unprotonated nitrogen of the other imidazole. Jencks (1975) has said that "this interaction [of RNase and bromoacetate] serves to decrease the free rotation and translation of the alkylating agent", implying a more favorable entropy of reaction. However, it is possible that enhanced reactivity of the histidines is due to other factors. Therefore, we have compared activation parameters for the pseudobimolecular alkylation of RNase by bromoacetate with those for the unfacilitated reaction with a model compound, histidine hydantoin. In the course of this work, we also determined the pH–rate profiles for the carboxymethylation, which are required to calculate the pH-independent rate constants for alkylation of the enzyme and which also define the macroscopic p$K$ values of the two histidines.

## Experimental Procedure

Bovine pancreatic ribonuclease A (RNase) was obtained from Worthington, either as the phosphate-free product and used without further treatment or as a crude preparation and purified according to the procedure of Taborsky (1959). After purification, the RNase was desalted and lyophilized from 5% acetic acid as described by Crestfield et al. (1963a). Yeast RNA was purchased from Worthington or was prepared by the method of Crestfield et al. (1955).

The kinetics of inactivation of RNase by bromoacetate were studied at 12, 25, 37, and 50 °C at pH values ranging from 3.90 to 7.73. The reaction mixtures contained 1 mg of RNase/mL and 2–200 mM sodium bromoacetate in 0.20 M sodium 2-($N$-morpholino)ethanesulfonate (Mes[1]–NaOH) buffer of the desired pH. This buffer was chosen because it did not affect the rate of inactivation of RNase by sodium bromoacetate (Plapp, 1973). Solutions of RNase and sodium bromoacetate were prepared immediately prior to use. Sodium bromoacetate solutions were prepared by dissolving recrystallized bromoacetic acid in a solution of NaOH of the equivalent concentration so that the final pH of the buffered solution was about the same as that of the initial pH of the buffer for each experiment. Aliquots of 10 μL of the reaction mixture were withdrawn at appropriate intervals and assayed for remaining RNase activity (Kunitz, 1946). The course of the reaction was followed until the enzyme activity had decreased to about 20% or less of its initial value. The pH of each reaction mixture was determined with a Radiometer Model PHM 26 pH meter at the conclusion of the experiment at the temperature of the reaction. A circulating water bath was used to maintain the reaction mixtures within ±0.1 °C of the stated temperatures. A control reaction without sodium bromoacetate was monitored periodically during the experiment as a check on possible denaturation of RNase.

[1] Abbreviation used: Mes, 2-($N$-morpholino)ethanesulfonate.

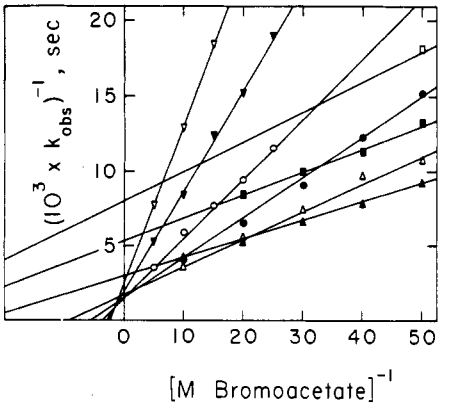



FIGURE 1: Dependence of the rate of carboxymethylation of RNase at 12 °C on bromoacetate concentration. The reciprocal of the pseudo-first-order rate constant for inactivation is plotted against the reciprocal of the concentration of bromoacetate. The lines are computed fits (Cleland, 1967) to eq 1 and correspond to the following values of pH: (□) 5.18; (■) 5.40; (▲) 5.95; (△) 6.26; (●) 6.57; (○) 6.88; (▼) 7.20; (▽) 7.51. Only the point corresponding to the highest concentration of bromoacetate used at pH 5.18 is shown; the remaining four points lie at 100, 150, 200, and 250 [M bromoacetate]$^{-1}$.

For the determination of the ratio of carboxymethylated histidines formed during reaction with bromoacetate, 3 mg of RNase in 0.5 mL of 0.2 M Mes–NaOH buffer, pH 6.6, was treated with 100 mM bromoacetate at 12, 25, 37, and 50 °C or at 25 °C with 5, 10, or 15% by weight dioxane. When about 20% of the RNase activity remained, 50 μL of 2-mercaptoethanol was added to the reaction mixture to destroy the remaining bromoacetate. Control reactions, in which 50 μL of 2-mercaptoethanol was added to the bromoacetate solution before dissolving the RNase, were also included at each temperature to correct for modification or destruction of histidine residues due to factors other than carboxymethylation. The RNase samples were desalted on a column of Sephadex G-25 equilibrated with 0.1 M HCOOH, oxidized with HCOOOH (Moore, 1963), hydrolyzed with 6 M HCl for 46 h (Moore & Stein, 1963), and analyzed as described previously (Lennette & Plapp, 1979). The ratio of His($\pi$Cm)/His($\tau$Cm) at the various temperatures gave a mean value of 4.4 ± 0.1, and with various concentrations of dioxane, it was 5.4 ± 0.6. Thus, temperature and dioxane did not appear to affect the ratio.

## Results

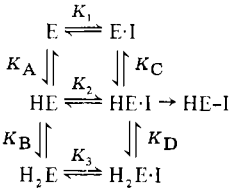
*Kinetics of Carboxymethylation.* The inactivation of RNase by bromoacetate followed pseudo-first-order kinetics with respect to enzyme concentration at all bromoacetate concentrations, pH values, and temperatures studied. As the concentration of bromoacetate was increased, the rate of inactivation increased with a hyperbolic dependence on the concentration, as is apparent from the linearity of the double-reciprocal plots in Figure 1. (At low pH, however, high concentrations of bromoacetate appeared to produce "substrate

Table I: Apparent Macroscopic p$K$ Values and Heats of Ionization for the pH Dependence of Carboxymethylation of RNase by Bromoacetate[a]

| group | p$K$ at 12 °C | 25 °C | 37 °C | 50 °C | $\Delta H_i$ (kcal/mol) |
|---|---|---|---|---|---|
| p$K_A$[b] | 5.49 ± 0.04 | 4.74 ± 0.12 | 4.49 ± 0.03 | 4.19 ± 0.22 | 14 ± 2 |
| p$K_B$[b] | 6.06 ± 0.06 | 6.27 ± 0.14 | 5.66 ± 0.04 | 5.75 ± 0.22 | 5 ± 4 |
| p$K_C$[c] | 6.18 ± 0.03 | 5.84 ± 0.08 | 5.34 ± 0.05 | 5.03 ± 0.10 | 13 ± 1 |
| p$K_D$[c] | 7.30 ± 0.04 | 7.41 ± 0.10 | 7.09 ± 0.06 | 6.76 ± 0.11 | 6 ± 3 |

[a] ±SD. [b] p$K$ values for free enzyme were determined from the variation of $k_3/K_I$ as a function of pH. [c] p$K$ values for the enzyme–bromoacetate complex were determined from the variation of $k_3$ as a function of pH.

Scheme I



inhibition"; these data were not used.) Such kinetic results are typical of Michaelis–Menten behavior in which a noncovalent complex of enzyme and inhibitor forms and reacts in a unimolecular manner to produce the irreversibly modified enzyme

$$E + I \overset{K_I}{\rightleftharpoons} E{\cdot}I \overset{k_3}{\rightarrow} E\text{–}I$$

and fits the equation

$$k_{obsd} = k_3[I]/(K_I + [I]) \quad (1)$$

The results in Figure 1 show that the intercepts ($1/k_3$ and $1/K_I$) and the slopes ($k_3/K_I$) are pH dependent. Rate constants for the pseudobimolecular reaction of free enzyme, $k_3/K_I$,[2] and for the unimolecular reaction of the enzyme–bromoacetate complex, $k_3$, are plotted as a function of pH in Figure 2, at different temperatures. The rate profiles are bell-shaped, suggesting the participation of two ionizing groups in the reaction, one of which must be unprotonated and the other of which must be protonated. At each temperature, the pH optimum for $k_3/K_I$ appears to be about 1 pH unit below that for $k_3$. Values of $K_I$, the dissociation constant for the enzyme–inhibitor complex, show that the affinity of the enzyme for bromoacetate is high at low pH and decreases with increasing pH. The binding of inhibitor appears to be dependent on a group which must be protonated and which has a p$K$ about equal to the pH optimum for the first-order alkylation of RNase.

The curves in Figure 2 represent nonlinear least-squares fits to the experimental data on the assumption of the mechanism shown in Scheme I for the pH dependency of alkylation. As described in various texts, e.g., Laidler & Bunting (1973), the pH dependencies for $k_3/K_I$ and $k_3$ fit the general equation for a bell-shaped pH dependency

$$k = \frac{\bar{k}}{1 + \frac{[H^+]}{K_x} + \frac{K_y}{[H^+]}} \quad (2)$$

in which $[H^+]$ is the hydrogen ion concentration and $K_x$ and $K_y$ are the macroscopic acid dissociation constants for the ionizable groups. The pH-independent rate constant, $\bar{k}$, is the theoretical value of $k$ at the optimum state of protonation, when every RNase molecule has one group unprotonated and the other group protonated. Approximate values for the pH-independent rate constants and the p$K$ values defined by the rate profiles were obtained by visual inspection of the data. More precise values for these estimates were then calculated by using an IBM 360/65 computer and a program for nonlinear least squares to fit the data for $k_3/K_I$ and $k_3$ separately to eq 2. The fits obtained by using this program were good; the coefficients of correlation were generally better than 0.960 and ranged from 0.926 to 0.994. Thus, there is no basis for trying to fit the data to more complicated schemes that include other ionizing groups.

Scheme II

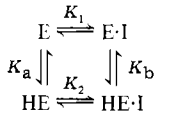


Table II: pH Dependence of Bromoacetate Binding[a]

| temp (°C) | mM ± SD $K_1$ | $K_2$ | p$K_b$ |
|---|---|---|---|
| 12 | 4.1 ± 0.1 | 550 ± 20 | 6.92 ± 0.04 |
| 25 | 4.8 ± 0.4 | 740 ± 40 | 6.76 ± 0.07 |
| 37 | 2.1 ± 0.2 | 460 ± 30 | 6.44 ± 0.10 |
| 50 | 6.4 ± 0.7 | 460 ± 40 | 6.29 ± 0.13 |

[a] Calculated from computer fits of the data shown in Figure 2 to eq 3. $K_1$ and $K_2$ are the limiting dissociation constants for the RNase–bromoacetate complex at low and high pH, respectively, and p$K_b$ is a macroscopic p$K$ for the complex bearing no simple relationship to the more useful p$K$ values in Table I.

Apparent p$K$ values, computed by using eq 2, for the free enzyme (p$K_A$ and p$K_B$) and the enzyme–bromoacetate complex (p$K_C$ and p$K_D$) at each temperature are given in Table I. The magnitudes of these p$K$ values suggest that they are related to ionizations of imidazole groups, probably those of histidines-12 and -119. However, it should be noted that these are macroscopic p$K$ values and do not correspond to microscopic p$K$ values of either histidine. Apparent heats of ionization of the four groups were calculated from the slopes of the plots by the van't Hoff relationship and are also given in Table I.

The data for the observed values of $K_I$ could be fitted to the overall equation derived for Scheme I, but the fits to this equation were no better than fits to the simpler model in Scheme II for which eq 3 applies. The fitted values are

$$K_I = \frac{K_2 + K_1[H^+]/K_b}{1 + [H^+]/K_b} \quad (3)$$

recorded in Table II.

Values computed from eq 2 for the pH-independent rate constants, $\bar{k}_3/K_I$ and $\bar{k}_3$, are given in Table III. These rate

[2] The pseudobimolecular rate constant, $k_3/K_I$, has units of moles$^{-1}$ liter second$^{-1}$ and is the second-order rate constant for the reaction E + I → E–I when the concentration of inactivator, I, is much lower than the dissociation constant, $K_I$ (Kitz & Wilson, 1962). Comparison of $k_3/K_I$ for various reagents (or $V_{max}/K_m$ for a substrate) gives a measure of the rate-enhancement specificity [see, e.g., Pincus et al. (1975)].

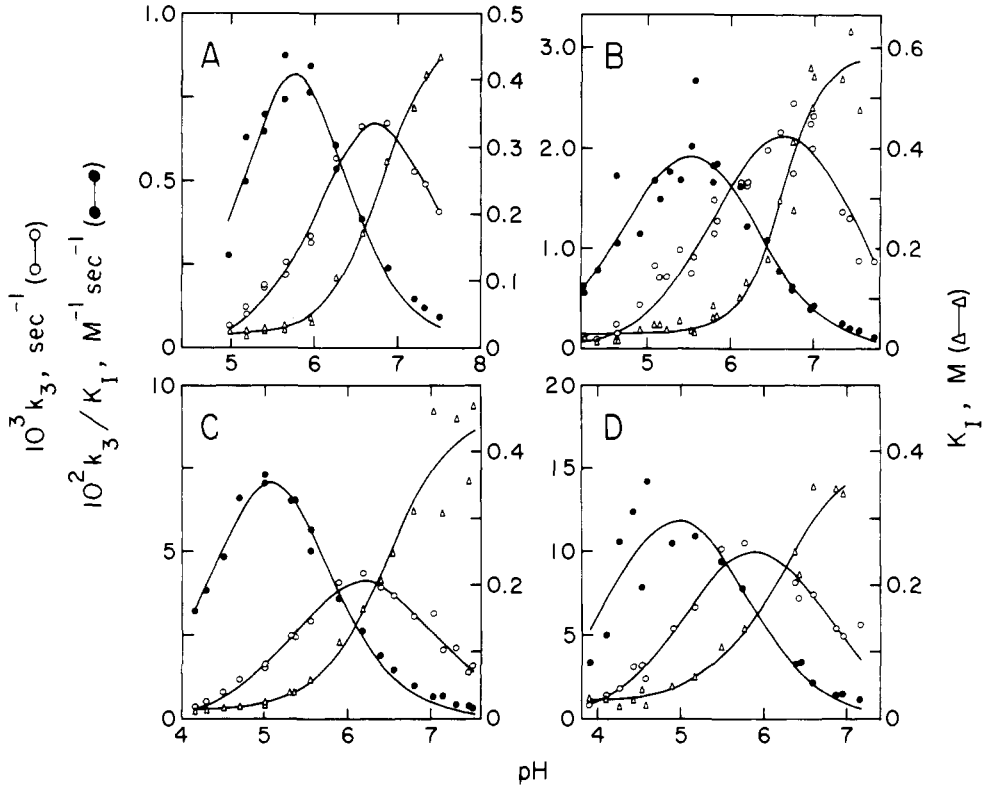



FIGURE 2: Dependence on pH of the rate of carboxymethylation of RNase by bromoacetate and of the binding of bromoacetate by RNase. (A) 12 °C. (B) 25 °C. (C) 37 °C. (D) 50 °C. Values of $k_3$, $k_3/K_I$, and $K_I$, obtained from computed fits of data such as those shown in Figure 1, are plotted as a function of pH. On each figure, the scales on the left refer to the rate constants, and the scales on the right refer to the dissociation constants. The lines for $k_3$ and $k_3/K_I$ are nonlinear least-squares fits to eq 2, and the lines for $K_I$ are fits to eq 3.

Table III: Effect of Temperature on the pH-Independent Rate Constants for the Carboxymethylation of RNase by Bromoacetate

| | $10^2$ × rate constant ($M^{-1}$ $s^{-1}$ ± SE) | | | $10^4$ × rate constant ($s^{-1}$ ± SE) | | |
|---|---|---|---|---|---|---|
| temp (°C) | $\bar{k}_3/K_I$[a] | $\bar{k}_{119}/K_I$[b] | $\bar{k}_{12}/K_I$[b] | $\bar{k}_3$[a] | $\bar{k}_{119}$[c] | $\bar{k}_{12}$[c] |
| 12 | 1.67 ± 0.09 | 1.36 ± 0.07 | 0.31 ± 0.02 | 10.4 ± 0.3 | 8.5 ± 0.2 | 1.91 ± 0.06 |
| 25 | 22.6 ± 0.3 | 2.1 ± 0.2 | 0.48 ± 0.06 | 28 ± 2 | 23 ± 2 | 5.2 ± 0.4 |
| 37 | 10.6 ± 0.3 | 8.6 ± 0.2 | 1.94 ± 0.06 | 52 ± 2 | 42 ± 2 | 9.5 ± 0.4 |
| 50 | 16 ± 3 | 13 ± 2 | 2.9 ± 0.5 | 127 ± 12 | 104 ± 10 | 23 ± 2 |

[a] Calculated from computer fits of the data shown in Figure 2 to eq 2. [b] Calculated from $k_3/K_I$, on the assumption that the ratio of His-119 to His-12 products is 4.4. [c] Calculated from $k_3$, on the assumption that the ratio of His-119 to His-12 products is 4.4.

constants represent the overall rate of alkylation of RNase; however, in order to examine the individual reactivities of histidines-12 and -119, it is necessary to determine the specific rate of alkylation of each histidine.

Since monocarboxymethylation of RNase yields only $N^{\tau}$-(carboxymethyl)histidine from His-12 and $N^{\pi}$-(carboxymethyl)histidine from His-119 (Crestfield et al., 1963b; Heinrikson et al., 1965), determination of the ratio of the two isomers of (carboxymethyl)histidine formed allows calculation of the specific rates of alkylation of either histidine from the observed overall rate. It should be noted that other alkylating agents, such as α-bromo acids, iodoacetamide, and 2′(3′)-*O*-(bromoacetyl)uridine, and other forms of RNase have also always given *tele* His-12 and *pros* His-119 derivatives, but the ratio of products varies (Heinrikson et al., 1965; Fruchter & Crestfield, 1965, 1967; Lin et al., 1968; Pincus et al., 1975). The ratio of forms of RNase with one histidine or the other protonated is theoretically pH independent if there are no other interacting groups (see Appendix), and the ratio of (carboxymethyl)histidine products actually found is also pH independent (Goren & Barnard, 1970). The results of our analyses gave a ratio of $N^{\pi}$- to $N^{\tau}$-(carboxymethyl)histidine of 4.4 ± 0.1, which is somewhat lower than the ratio of about 7:1 previously reported by Fruchter & Crestfield (1965) at pH 5.5 and 25 °C and by Goren & Barnard (1970) at pH 5.6 and 35 °C. The ratio was independent of temperature (12–50 °C), also in contrast to results observed by Fruchter & Crestfield (1965). The reason for these differences is not clear, but it may be noted that different buffer solutions have been used. On the assumption that the ratio of the amounts of $N^{\pi}$- to $N^{\tau}$-(carboxymethyl)histidine formed is proportional to the ratio of the rate constants for alkylation of histidines-119 and -12, respectively, the individual rate constants for carboxymethylation of each histidine were calculated (Table III).

The logarithms of the pH-independent rate constants given in Table III were plotted as a function of the reciprocal of the absolute temperature, and a weighted least-squares fit was made to the equation

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

where $k$ is Boltzmann's constant and $h$ is Planck's constant. Values for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the inactivation of RNase by bromoacetate are given in Table IV.

*Influence of the Dielectric Constant on the Reaction.* In an attempt to determine the electrostatic contribution to the entropy of activation, the variation of the rate constants with the dielectric strength of the medium was examined at pH 6.11 and 25 °C, in reaction mixtures containing dioxane at con-

Table IV: Activation Parameters for the Carboxymethylation of RNase by Bromoacetate[a]

| | kcal/mol ± SE | | cal/(deg mol) ± SE | |
|---|---|---|---|---|
| rate constant | $\Delta G^{\ddagger}$ | $\Delta H^{\ddagger}$ | $\Delta S^{\ddagger}$ | $\Delta S^{\ddagger}_{es}$ |
| $\bar{k}_{119}/K_I$[b] | 20 ± 3 | 11 ± 2 | −28 ± 7 | +3.7 ± 0.1 |
| $\bar{k}_{12}/K_I$[b] | 20 ± 3 | 11 ± 2 | −31 ± 7 | +3.7 ± 0.1 |
| $\bar{k}_{119}$[c] | 21 ± 1 | 11 ± 1 | −33 ± 2 | 0 |
| $\bar{k}_{12}$[c] | 22 ± 1 | 11 ± 1 | −36 ± 2 | 0 |

[a] At 25 °C. [b] Pseudobimolecular rate constant for carboxymethylation of histidine-119 or -12 in the free enzyme. [c] First-order rate constant for carboxymethylation of histidine-119 or -12 in the enzyme–bromoacetate complex.

centrations ranging from 0 to 15% by weight. [Findlay et al. (1962) have shown that RNase retains almost full activity and the same pH optimum in 50% dioxane with cationic buffers.] Double-reciprocal plots of the rate of inactivation of RNase as a function of bromoacetate concentration were linear at all concentrations of dioxane, and the slopes, but not the intercepts, of these plots decreased slightly with dioxane concentration. The effect of dioxane on $k_3/K_I$ was consistent with a value for $\Delta S^{\ddagger}_{es}$ of 3.7 ± 0.1 cal/(deg mol), calculated as described before (Lennette & Plapp, 1979), and is associated with the bimolecular alkylation of the free enzyme, perhaps reflecting electrostatic binding of the negatively charged bromoacetate to an imidazolium group. However, Maurel & Douzou (1975) established the compensation temperature for cationic acids (including His-46 in trypsin) as 37 ± 5 °C. At the compensation temperature, the effect of organic solvent on the p$K$ of the ionizing group vanishes, regardless of the solvent system employed. Therefore, the preceding solvent studies were repeated at 37 °C (pH 5.12) in order to eliminate effects on the results due to possible changes in the p$K$ values of the Mes–NaOH buffer or histidines-12 and -119 at 25 °C. At 37 °C, no systematic effect of dioxane on $k_3$ or $k_3/K_I$ was observed. It is noteworthy that, at either temperature, $\Delta S^{\ddagger}_{es}$ = 0 for the unimolecular reaction of the enzyme–bromoacetate complex, $k_3$.

*Acetate Inhibition of the Carboxymethylation Reaction.* Investigations of RNase often employ sodium acetate or sodium chloride to control pH or adjust ionic strength of solutions of the enzyme. In particular, acetate buffers have been used in experiments designed to determine the rates of carboxymethylation of RNase by bromoacetate (Heinrikson et al., 1965) or iodoacetamide (Fruchter & Crestfield, 1967), the pH dependence of the carboxymethylation reaction (Goren & Barnard, 1970), and the p$K$ values of the histidines at the active site of the enzyme (Meadows et al., 1968; Markley, 1975; Markley & Finkenstadt, 1975; Cohen & Shindo, 1975; Shindo et al., 1976). Chloride ions bind to the histidines at the active site of RNase (Saroff & Carroll, 1962), and acetate probably binds to the active site of the enzyme and inhibits alkylation of RNase by haloacetates.

The effects of varying concentrations of sodium acetate on the rate of alkylation of RNase by bromoacetate are shown in Figure 3. No differences in the intercepts could be demonstrated by Student's $t$ test ($p \leq 0.05$), and a replot of the slopes against the inhibitor (sodium acetate) concentration was linear. Acetate thus appears to behave as a linear competitive inhibitor against bromoacetate in the carboxymethylation reaction. The data were fitted to the equation (eq 4) describing linear competitive inhibition by the method

$$k_{obsd} = \frac{k_3[I]}{K_I(1 + [\text{acetate}]/K_{is}) + [I]} \quad (4)$$

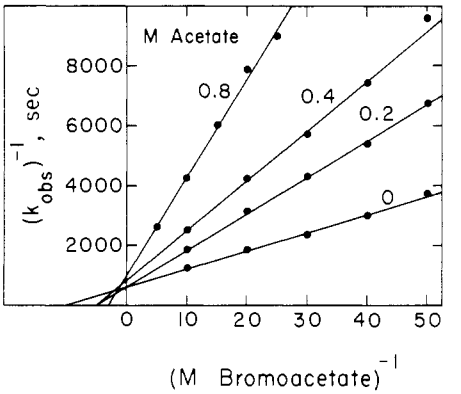



FIGURE 3: Inhibition by sodium acetate of the inactivation of RNase by bromoacetate. RNase (1 mg/mL) in 0.2 M Mes–NaOH buffer, pH 6.14, and sodium acetate at the indicated concentrations were allowed to react at 25 °C with sodium bromoacetate at various concentrations. The reciprocals of the pseudo-first-order rate constants for inactivation at each concentration of sodium acetate are plotted against the reciprocal of the bromoacetate concentration. The lines are least-squares fits to a hyperbola (Cleland, 1967).

of least squares (Cleland, 1967), and the following values for the kinetic constants were calculated: $k_3$ = 16 ± 1 $s^{-1}$, $K_I$ = 91 ± 10 mM, and $K_{is}$ = 167 ± 10 mM. The inhibition constant, $K_{is}$, is equivalent to the dissociation constant of the enzyme–acetate complex. Acetate probably inhibits the alkylation reaction by binding to the enzyme in the same manner as bromoacetate, and therefore it should be avoided in experiments designed to probe the active site of RNase and especially the properties of histidine residues 12 and 119.

## Discussion

*Explanation of Enhanced Reactivity of Histidine Residues in RNase.* The reaction of bromoacetate with RNase is facilitated: the pH-independent rate of the pseudobimolecular reaction at 25 °C (2.6 × $10^{-2}$ $M^{-1}$ $s^{-1}$) is 440 times greater than that with histidine hydantoin (5.9 × $10^{-5}$ $M^{-1}$ $s^{-1}$). Although the degree of facilitation is somewhat less than that reported previously (Heinrikson et al., 1965), the rates compared in this study are corrected for differences in the state of protonation of the reactants; they thus reflect the true reactivities of the imidazoles in the protein and in the model compound. His-12 in RNase is carboxymethylated at the *tele*-N 120 times more rapidly than is the *tele*-N of histidine hydantoin, and the *pros*-N of His-119 reacts 1000 times more rapidly than the *pros*-N of histidine hydantoin.

The hyperbolic dependence of the rate of alkylation of RNase on bromoacetate concentration indicates that the reagent binds reversibly to the enzyme before reacting chemically. The competitive inhibition by acetate against bromoacetate is also consistent with the formation of a noncovalent complex. The existence of such a Michaelis complex, detected previously (Plapp, 1973), supports the proposal of Stark et al. (1961) for the mechanism of the facilitated reaction of RNase with iodoacetate, in which the anionic reagent is bound to the protonated imidazole of histidine-12 or -119 and is thereby oriented for nucleophilic attack by the other, unprotonated imidazole. The bell-shaped pH dependencies for $k_3$ and $k_3/K_I$ found in this study and in other studies (Gundlach et al., 1959; Lamden et al., 1962; Goren & Barnard, 1970), the mutually exclusive alkylation of one histidine or the other (Crestfield et al., 1963b,c), and the three-dimensional structure determined by X-ray crystallography (Richards & Wyckoff, 1971) also support the proposed mechanism.

Table V: Comparison of Rate and Activation Parameters for the Carboxymethylation of Histidine Hydantoin and RNase at 25 °C

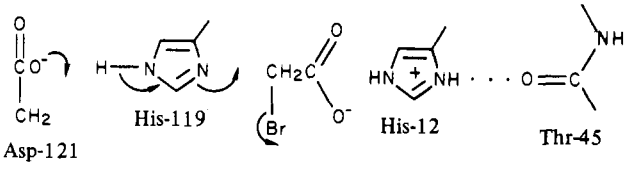
| | reaction | | | |
|---|---|---|---|---|
| | histidine hydantoin | | RNase | |
| parameter | $k_1$[a] | $k_3$[b] | $k_{119}/K_I$[a] | $k_{12}/K_I$[b] |
| rate ($M^{-1}$ $s^{-1}$) | $2.0 \times 10^{-5}$ | $3.9 \times 10^{-5}$ | $2.1 \times 10^{-2}$ | $0.48 \times 10^{-2}$ |
| $\Delta G^{\ddagger}$ (kcal/mol ± SE) | 23.8 ± 0.6 | 23.5 ± 0.3 | 20 ± 3 | 20 ± 3 |
| $\Delta H^{\ddagger}$ (kcal/mol ± SE) | 16.6 ± 0.4 | 16.5 ± 0.2 | 11 ± 2 | 11 ± 2 |
| $T\Delta S^{\ddagger}$ (kcal/mol ± SE) | −7.2 ± 0.4 | −7.0 ± 0.2 | −8 ± 2 | −9 ± 2 |

[a] Carboxymethylation at the *pros*-N of the imidazole. [b] Carboxymethylation at the *tele*-N of the imidazole.

If the rate enhancement seen in the reaction of bromoacetate with RNase is due to binding and orientation of the reagent, these factors should manifest themselves in the activation parameters of the reaction. The increased rate of alkylation by bromoacetate of RNase (Table V) as compared to the carboxymethylation of histidine hydantoin (Lennette & Plapp, 1979) is equivalent to a difference in $\Delta G^{\ddagger}$ of 3.2–4.2 kcal/mol. Interestingly, however, the principal contribution to this decrease in $\Delta G^{\ddagger}$ is not the entropy of activation as might be expected but is instead found in a decrease in the enthalpy of activation of 5.5 kcal/mol. This enhanced reactivity must derive from the structure of RNase, that is, of the microenvironments that might confer unusual properties on the histidines, or from the mechanism by which RNase catalyzes its self-carboxymethylation.

One possible explanation for the decrease in $\Delta H^{\ddagger}$ might be that the carbon–bromine bond of the bromoacetate is distorted or polarized. However, it should be noted that the leaving bromide ion is probably not attracted to a nearby group, such as the ammonium of Lys-41, since variation of the leaving group does not seem to affect the relative degree of facilitation of the reaction (Plapp, 1973). Furthermore, the fact that the specific and rapid alkylation of RNase also occurs in enzyme in which the ε-amino group of Lys-41 has been dinitrophenylated indicates that Lys-41 is not crucial for the reaction (Lin et al., 1968). Thus, there is no evidence for an effect on the carbon–bromine bond.

Another possible explanation for the decreased $\Delta H^{\ddagger}$ might be that the nucleophilicities of the imidazole nitrogens in RNase are increased. The ability of histidines-119 and -12 to abstract protons from water and the 2′-OH of ribose, respectively, in a "push–pull" mechanism of catalysis (Findlay et al., 1962; Roberts et al., 1969; Wang, 1968; Harris et al., 1969) suggests that these histidines could be abnormally good nucleophiles. Although it is speculative to try to explain the enhanced reactivity of histidines-12 and -119 in terms of the structure of RNase, we will do so with knowledge derived from X-ray crystallography and chemical modification studies. We propose that alkylation of His-119 may be facilitated by the formation of the structure




The carboxylate group of Asp-121 may contact the imidazole ring of histidine-119 (Richards & Wyckoff, 1971) and could remove the proton from the imidazole and greatly increase the nucleophilicity of the opposite nitrogen. The carboxylate might also stabilize the positive charge developing on the imidazole ring during formation of the transition state. This would be consistent with the near-zero value of the electrostatic component of $\Delta S^{\ddagger}$ associated with the unimolecular reaction of the enzyme–bromoacetate complex.

If Asp-121 does enhance the reactivity of His-119, His-119 might also be hyperreactive with another sample alkylating agent, such as iodoacetamide. However, His-119 appears to be unreactive toward iodoacetamide (Fruchter & Crestfield, 1967). We suggest that bromoacetate binds to a particular conformation of the enzyme and stabilizes the imidazole ring of His-119 in the position shown. The imidazole ring can occupy several positions in the crystalline enzyme (Richards & Wyckoff, 1971). A conformational change apparently occurs upon binding of 3′-UMP (del Rosario & Hammes, 1970) or 2′-deoxy-2′-fluorouridylyl-3′–5′-adenosine (Pavlovsky et al., 1978), and it has been postulated that the carboxylate of Asp-121 fixes the positively charged imidazole ring of His-119 in the catalytically active complex (Antonov, 1978). The role of Asp-121 is also indicated by the fact that His-119 in RNase lacking residues 121–124 is about (one assay point) 24 times less reactive with iodoacetate (Lin et al., 1968) than in native RNase. The p$K$ value for His-119 in this des-(121–124)-RNase is also increased by about 1 unit (Sacharovsky et al., 1973). Thus, Asp-121 could account for about half of the 1000-fold facilitation.

The reaction of His-12 could occur if bromoacetate bound in the opposite way between the two imidazoles. The 120-fold facilitation may be explained, in part, by the interaction of the *pros*-NH with the carbonyl oxygen of Thr-45. In addition, part of the facilitation may be due to electronic interactions. In contrast to His-119, His-12 is readily alkylated with iodoacetamide, albeit 7 times slower than with iodoacetic acid (Fruchter & Crestfield, 1967). Interestingly, the rate of inactivation by iodoacetamide shows a bell-shaped pH dependence with apparent p$K$ values of 3.8 and 6.2. Fruchter and Crestfield proposed that His-119 and an adjacent carboxyl group (e.g., Asp-121) may participate in a dipole–dipole interaction with the amide group of iodoacetamide. On the basis of recent NMR titration studies, it has been proposed that His-12 (and His-119) interact indirectly, through a conformational change, with Asp-14 (Markley, 1975; Cohen & Shindo, 1975).

That His-12 is in an unusual environment is also indicated by an NMR study of the p$K$ of His-12 as a function of temperature. The p$K$ values are a linear function of $1/T$, from 20 to 52 °C, but the enthalpy of ionization (2.9 kcal/mol) and entropy of ionization [−18.2 cal/(deg mol)] are unusually low (Westmoreland et al., 1975). In contrast, His-119 appears to be in a normal environment, with a $\Delta H_i$ of 6.4 kcal/mol.

Although most of the facilitation of carboxymethylation of histidines-12 and -119 is reflected in a more favorable $\Delta H^{\ddagger}$, it is possible that binding energy is used to orient the reagent and thereby decrease the loss of translational and rotational energy, as Jencks (1975) proposed, but that this is swamped out by solvation effects. Differential solvation effects on the carboxymethylation of histidine hydantoin as compared to ribonuclease could result in compensation between the changes

in enthalpy and the changes in entropy. Alterations in the conformation of RNase might have relatively large solvation terms, for instance (Jencks, 1975). However, since the reactive nitrogens of histidines-12 and -119 are exposed to solvent (Richards & Wyckoff, 1971) and dioxane had little effect on the rate of carboxymethylation of either histidine hydantoin or ribonuclease, differential solvent effects may not have been significant. Nevertheless, in the absence of quantitative information, we cannot exclude the interpretation that carboxymethylation of RNase is facilitated by intrinsic entropy effects.

*Use of Activation Parameters to Study Mechanisms of Enzyme Catalysis.* Interpretation of activation parameters is difficult (Jencks, 1969), but the problem may be simplified somewhat by ensuring that the conditions employed (such as the leaving group, type of reaction, other species present, etc.) in the reactions to be compared will isolate a single reaction and minimize effects that would alter the values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. Therefore, we think that data, such as those presented here, can provide an experimental basis for the sophisticated theories being developed to explain enzyme catalytic power. Although interpretation of such data must be provisional at this time, the results may at least allow one to classify reactions according to their driving forces. For instance, in the reaction of an active-site-directed reagent, it might be possible to differentiate facilitation due to binding of the reagent from that due to hyperreactive groups.

It may be noted that comparison of activation parameters for related reactions has afforded insight into several enzymatic mechanisms. Comparison of activation parameters for the spontaneous cleavage of the S–S bond in $SSO_3^{2-}$ by $CN^-$ with those for the reaction catalyzed by rhodanese revealed that the increased rate of the enzymatic reaction was due to a decrease in $\Delta H^{\ddagger}$ (Leininger & Westley, 1968); this decrease was interpreted as a stretching of the S–S bond by the $Zn^{2+}$ at the active site and attack on the bond by a nucleophile stronger than $CN^-$. In this case, enthalpy–entropy compensation did not appear to occur, and a contribution to the overall decrease in $\Delta G^{\ddagger}$ by changes in $\Delta S^{\ddagger}$ was also realized, since the electrostatic effects on $\Delta S^{\ddagger}$ due to the approach of the two anions $SSO_3^{2-}$ and $CN^-$ are eliminated in the transition state for the rhodanese-catalyzed reaction.

Baggott & Klapper (1976) have tried to determine the origin of the rate-enhancement specificity of chymotrypsin. They found that deacylation of $\alpha$-chymotrypsin appears to involve at least two reactions and, depending on the acyl group and temperature, the rate enhancement may be either enthalpy or entropy controlled. These observations rationalize some of the differing conclusions previously found for chymotrypsin and emphasize the difficulty in studying an enzyme-catalyzed reaction.

In chemical modification studies of enzymes, Pavlič (1973) found that tetraethylammonium increases the rate of methanesulfonylation of acetylcholinesterase by increasing $\Delta S^{\ddagger}$, which more than compensates for the higher $\Delta H^{\ddagger}$. The changes were attributed to structural alterations in the active site. Whitaker & Lee (1972) found that the reaction of 2-chloroacetamide with ficin proceeded with a more favorable $\Delta S^{\ddagger}$ and less favorable $\Delta H^{\ddagger}$ in the presence, as compared to the absence, of the inhibitor benzoyl-D-arginine ethyl ester. They also attributed the effect to a conformational change. Interestingly, both reactions of the enzyme were faster than the reaction of the model compound mercaptoethanol and were enthalpy driven. Halász & Polgár (1976) used methyl iodide and iodoacetamide to probe the microenvironments of the active sites of thiolsubtilisin and papain. They compared activation parameters and concluded that the –SH group of thiolsubtilisin is in a nonpolar pocket whereas the –SH group of papain is in a more polar environment. The facilitated reaction of iodoacetamide with the mercaptide ion form of papain was accompanied by a decrease in both $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ and was ascribed to hydrogen bonding of the reagent to the enzyme. Analysis of all of their results shows that increased rates of reactions are roughly correlated with smaller $\Delta H^{\ddagger}$ values and offsetting decreases in $\Delta S^{\ddagger}$.

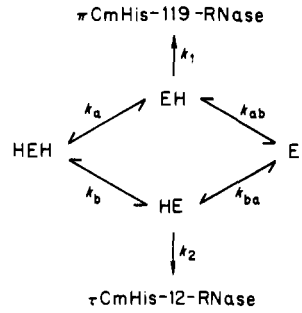
*pK Values for Histidine Residues.* At 25 °C, the free enzyme exhibits macroscopic p$K$ values of 4.7 and 6.3 (Table I). Upon formation of the complex with bromoacetate, the p$K$ values increase to 5.8 and 7.4. This significant shift probably results from the electrostatic effects of introducing the negatively charged acetate into the active site but may also reflect concomitant conformational changes. It should be noted that NMR titration studies of RNase are usually carried out with high concentrations of chloride or acetate. These anions certainly affect the apparent p$K$ values. The pH dependency for binding an anion, such as shown for bromoacetate in Figure 2, means that the effects will be pH dependent unless the concentration of anion is saturating at all pH values studied. The p$K$ values found from carboxymethylation of RNase are close to values for imidazole and its derivatives and to the p$K$ values determined by NMR titration: His-119, p$K$ 6.2; His-12, p$K$ 5.8, in $D_2O$ and in the presence of 0.3 M NaCl (Markley & Finkenstadt, 1975). Thus, the enhanced reactivities of the histidine residues in RNase do not seem to be accompanied by large perturbations in p$K$ values.

If the ratio of $N^{\pi}$-(carboxymethyl)histidine-119 to $N^{\tau}$-(carboxymethyl)histidine-12 formed in the reaction is assumed to be proportional to the ratio of unprotonated His-119 to unprotonated His-12 present in the monoprotonated enzyme, microscopic p$K$ values for histidines-12 and -119 may be calculated from the macroscopic p$K$ values. The derivation of the relevant equation is given in the Appendix. As would be expected, the microscopic p$K$ values for His-119 are less than those for histidine-12, by 0.65 pH units. However, Markley & Finkenstadt (1975) have calculated microscopic p$K$ values based on a mutual interaction model and find that p$K$ values for His-119 are 0.27 unit higher than the p$K$ values for His-12. This apparent discrepancy implies that the assumption that the ratio of alkylated products formed is simply a reflection of the relative amounts of unprotonated histidines-12 and -119 is incorrect. If one uses, however, the microscopic p$K$ values of Markley & Finkenstadt (1975), it is possible to calculate a value of 0.537 for the ratio of enzyme with unprotonated histidine-119 to enzyme with unprotonated histidine-12, where the opposite histidine is protonated. Since the ratio of carboxymethylated histidine-119 to carboxymethylated histidine-12 is 4.4, it appears that unprotonated His-119 is about 8.3 times more reactive than unprotonated His-12. This result supports again the proposal that the microenvironment of His-119 is unusual.

## Appendix

### *Relationship between Macroscopic pK Values, Determined from pH Dependence of Carboxymethylation of RNase, Microscopic pK Values, and Intrinsic Rates of Reaction of Histidines-12 and -119*

Assume that the carboxymethylation mechanism involves reaction of bromoacetate with two different monoprotonated species of the enzyme:




(1A)

where $k_a$ and $k_{ba}$ are the microscopic p$K$ values for the ionization of His-119, $k_b$ and $k_{ba}$ are the microscopic p$K$ values for His-12, and $k_1$ and $k_2$ are the pH-independent rate constants of reaction, such that $\bar{k}_{119} = k_1[EH]/[E_t]$ and $\bar{k}_{12} = k_2[HE]/[E_t]$.

If it is assumed the EH and HE react equally rapidly, then $k_1 = k_2 = \bar{k}$, and

$$k_{obsd} = \frac{\bar{k}([EH] + [HE])}{[HEH] + [EH] + [HE] + [E]}$$

Since $k_a = [EH][H^+]/[HEH]$, $k_b = [HE][H^+]/[HEH]$, and $k_{ab} = [E][H^+]/[EH]$

$$k_{obsd} = \frac{\bar{k}([EH] + k_b[EH]/k_a)}{\frac{[EH][H^+]}{k_a} + [EH] + \frac{k_b[EH]}{k_a} + \frac{k_{ab}[EH]}{[H^+]}}$$

Dividing by [EH]

$$k_{obsd} = \frac{\bar{k}(1 + k_b/k_a)}{1 + \frac{[H^+]}{k_a} + \frac{k_{ab}}{[H^+]} + \frac{k_b}{k_a}} \quad (2A)$$

Note that eq 2 and 2A are intrinsically the same, since $K_a = k_a + k_b$ and $1/K_b = 1/k_{ab} + 1/k_{ba}$ or $K_aK_b = k_ak_{ab} = k_bk_{ba}$. Since $[EH]/[HE] = k_a/k_b$, the macroscopic p$K$ values and the ratio of products (πCmHis-119-RNase/τCmHis-12-RNase) could be used to calculate microscopic p$K$ values.

However, if the rates of reaction of EH and HE are different, i.e., $k_1 \neq k_2$, eq 2A must be modified. If $k_2 = \alpha k_1$, it can be shown that

$$k_{obsd} = \frac{k_1(1 + \alpha k_b/k_a)}{1 + \frac{[H^+]}{k_a} + \frac{k_{ab}}{[H^+]} + \frac{k_b}{k_a}} = \frac{k_1\frac{1 + \alpha k_b/k_a}{1 + k_b/k_a}}{1 + \frac{[H^+]}{K_a} + \frac{K_b}{[H^+]}} \quad (3A)$$

In this case, the computed pH-independent rate constant is a complex function, and the ratio of carboxymethylated products cannot be used to estimate [EH]/[HE].

## References


Antonov, I. V., Gurevich, A. Z., Dudkin, S. M., Karpeisky, M. Y., Sakharovsky, V. G., & Yakovlev, G. I. (1978) *Eur. J. Biochem. 87*, 45–54.

Baggott, J. E., & Klapper, M. H. (1976) *Biochemistry 15*, 1473–1481.

Bender, M. L. (1971) *Mechanisms of Homogeneous Catalysis from Protons to Proteins*, Wiley-Interscience, New York.

Bender, M. L., Kezdy, F. J., & Gunter, C. (1964) *J. Am. Chem. Soc. 86*, 3714–3721.

Bruice, T. C. (1970) *Enzymes, 3rd Ed. 2*, 217–279.

Bruice, T. C., & Benkovic, S. J. (1966) *Bioorganic Mechanisms*, Vol. I, W. A. Benjamin, New York.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.

Cohen, J. S., & Shindo, H. (1975) *J. Biol. Chem. 250*, 8874–8881.

Crestfield, A. M., Smith, K. C., & Allen, F. W. (1955) *J. Biol. Chem. 216*, 185–193.

Crestfield, A. M., Stein, W. H., & Moore, S. (1963a) *J. Biol. Chem. 238*, 618–621.

Crestfield, A. M., Stein, W. H., & Moore, S. (1963b) *J. Biol. Chem. 238*, 2413–2419.

Crestfield, A. M., Stein, W. H., & Moore, S. (1963c) *J. Biol. Chem. 238*, 2421–2428.

del Rosario, E. J., & Hammes, G. G. (1970) *J. Am. Chem. Soc. 92*, 1750–1753.

Findlay, D., Mathias, A. P., & Rabin, B. R. (1962) *Biochem. J. 85*, 134–139, 139–144.

Fruchter, R. G., & Crestfield, A. M. (1965) *J. Biol. Chem. 240*, 3875–3882.

Fruchter, R. G., & Crestfield, A. M. (1967) *J. Biol. Chem. 242*, 5807–5812.

Goren, H. J., & Barnard, E. A. (1970) *Biochemistry 9*, 959–973.

Gundlach, H. G., Stein, W. H., & Moore, S. (1959) *J. Biol. Chem. 234*, 1754–1760.

Halász, P., & Polgár, L. (1976) *Eur. J. Biochem. 71*, 563–569, 571–575.

Harris, M. R., Usher, D. A., Albrecht, H. P., Jones, G. H., & Moffatt, J. G. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 246–252.

Heinrikson, R. L., Stein, W. H., Crestfield, A. M., & Moore, S. (1965) *J. Biol. Chem. 240*, 2921–2934.

Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, p 77, McGraw-Hill, New York.

Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219–410.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Koshland, D. E. (1962) *J. Theor. Biol. 2*, 75–86.

Koshland, D. E., & Neet, K. E. (1968) *Annu. Rev. Biochem. 37*, 359–410.

Kunitz, M. (1946) *J. Biol. Chem. 164*, 563–568.

Laidler, K. J., & Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, 2nd ed., pp 216–218, Clarendon Press, Oxford, England.

Lamden, M. P., Mathias, A. P., & Rabin, B. R. (1962) *Biochem. Biophys. Res. Commun. 8*, 209–214.

Leininger, K. R., & Westley, J. (1968) *J. Biol. Chem. 243*, 1892–1899.

Lennette, E. P., & Plapp, B. V. (1979) *Biochemistry* (preceding paper in this issue).

Lin, M. C., Stein, W. H., & Moore, S. (1968) *J. Biol. Chem. 243*, 6167–6170.

Markley, J. L. (1975) *Biochemistry 14*, 3546–3554, 3554–3561.

Markley, J. L., & Finkenstadt, W. R. (1975) *Biochemistry 14*, 3562–3566.

Marshall, T. H., & Chen, V. (1973) *J. Am. Chem. Soc. 95*, 5400–5405.

Martinek, K., Dorovska, V. N., Varfolomeyev, S. D., & Berezin, I. V. (1972) *Biochim. Biophys. Acta 271*, 80–86.

Maurel, P., & Douzou, P. (1975) *J. Biol. Chem. 250*, 2678–2680.

Meadows, D. H., Jardetzky, O., Epand, R. M., Ruterjans, H. H., & Scheraga, H. A. (1968) *Proc. Natl. Acad. Sci. U.S.A. 60*, 766–772.

Moore, S. (1963) *J. Biol. Chem. 238*, 235–237.
Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–831.
Pavlič, M. R. (1973) *Biochim. Biophys. Acta 327*, 393–397.
Pavlovsky, A. G., Borisova, S. N., Borisov, V. V., Antonov, I. V., & Karpeisky, M. Y. (1978) *FEBS Lett. 92*, 258–262.
Pincus, M., Thi, L. L., & Carty, R. P. (1975) *Biochemistry 14*, 3653–3661.
Plapp, B. V. (1973) *J. Biol. Chem. 248*, 4896–4900.
Richards, F. M., & Wyckoff, H. W. (1971) *Enzymes, 3rd Ed. 4*, 647–806.
Roberts, G. C. K., Dennis, E. A., Meadows, D. H., Cohen, J. S., & Jardetzky, O. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1151–1158.
Sacharovsky, V. G., Cherrin, I. I., Yakovlev, G. I., Dudkin, S. M., Karpeisky, M. Y., & Shliapnikov, S. V. (1973) *FEBS Lett. 33*, 323–326.
Saroff, H. A., & Carroll, W. R. (1962) *J. Biol. Chem. 237*, 3384–3387.
Shaw, E. (1970) *Physiol. Rev. 50*, 244–296.
Shindo, H., Hayes, M. B., & Cohen, J. S. (1976) *J. Biol. Chem. 251*, 2644–2647.
Stark, G. R., Stein, W. H., & Moore, S. (1961) *J. Biol. Chem. 236*, 436–442.
Taborsky, G. (1959) *J. Biol. Chem. 234*, 2652–2656.
Wang, J. H. (1968) *Science 161*, 328–334.
Westmoreland, D. G., Matthews, R. C., Hayes, M. B., & Cohen, J. S. (1975) *J. Biol. Chem. 250*, 7456–7460.
Whitaker, J. R., & Lee, L.-S. (1972) *Arch. Biochem. Biophys. 148*, 208–216.

# *N*-Acetylglucosamine-1-phosphate Transferase from Hen Oviduct: Solubilization, Characterization, and Inhibition by Tunicamycin†

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ABSTRACT: UDP-*N*-acetylglucosamine (UDPGlcNAc): dolichyl phosphate, *N*-acetylglucosamine-1-phosphate transferase, the first enzyme of the dolichol cycle, has been solubilized from crude hen oviduct membranes by using 0.5% sodium deoxycholate. Accompanying solubilization was an 8- to 50-fold increase in the dependence of enzyme activity on the addition of dolichyl phosphate. The enzyme activity was stimulated by $MgCl_2$ (10 mM optimum) and KCl (0.4 M optimum) and exhibited a pH optimum around 8.0. Bisubstrate kinetic analysis indicated that the enzyme follows a sequential mechanism. The $K_m$ values for UDPGlcNAc and dolichyl phosphate were determined to be 4 and 9 $\mu$M, respectively. The inhibition of the enzyme by tunicamycin (TM) occurred at low concentrations of inhibitor ($10^{-8}$–$10^{-9}$ M) and was time dependent. These findings precluded conventional kinetic analysis to determine the mechanism of inhibition. By employing pseudo-first-order conditions, we determined a second-order rate constant of $7 \times 10^4$ $M^{-1}$ $s^{-1}$ for the association of the enzyme with TM at 23 °C. Plots of enzyme activity vs. inhibitor concentration were consistent with TM acting as a reversible tight-binding inhibitor. The $I_{50}$ for tunicamycin inhibition was found to vary with protein concentration. At a protein concentration of 11 mg/mL solubilized enzyme, the $I_{50}$ was $7 \times 10^{-9}$ M. The inhibition by tunicamycin is apparently competitive since high concentrations of UDPGlcNAc relative to TM protected the enzyme from inhibition. Based on the known structure of TM and its inhibitory properties, it is proposed that the antibiotic acts as a multisubstrate analogue in which the branched hydrocarbon side chain mimics dolichyl phosphate and the uracil–carbohydrate–GlcNAc backbone mimics UDPGlcNAc. This proposal explains why other GlcNAc transferases are not inhibited by TM. Since these transferases do not utilize dolichyl phosphate as an acceptor, it is unlikely that the bulky hydrocarbon side chain of TM would fit into the active site of these enzymes.

Work in the last decade has shown that glycosylation of asparagine to form N-linked glycoproteins proceeds via an en bloc mechanism in which a portion of the oligosaccharide chain is preassembled on a long-chain polyisoprenoid carrier, dolichyl phosphate, prior to transfer of the entire chain to a nascent polypeptide [for a review, see Waechter & Lennarz (1976)]. Since dolichyl phosphate is presumably regenerated subsequent to oligosaccharide transfer, the process of preassembly and transfer is often collectively referred to as the dolichol cycle.

Our laboratory has recently embarked on an investigation into the regulation of the dolichol cycle in hen oviduct. The oviduct is an excellent tissue for studying glycoprotein biosynthesis since it secretes copious quantities of glycoproteins and since synthesis can be induced in immature chicks by administration of estrogenic hormones (Kohler et al., 1969). We have begun our investigations by examining the first enzyme of the dolichol cycle, UDPGlcNAc:dolichyl phosphate (Dol-P), *N*-acetylglucosamine-1-phosphate transferase (GlcNAc-1-P transferase) (eq 1). This enzyme has previously

$$\text{UDPGlcNAc} + \text{Dol-P} \rightleftharpoons \text{Dol-PP-GlcNAc} + \text{UMP} \quad (1)$$

been studied by Heifetz & Elbein (1977), who solubilized it from porcine aorta membranes and demonstrated that the endogenous substrate is dolichyl phosphate. In the present study we report on the solubilization and partial characterization of the enzyme from oviduct membranes. We find that the enzyme carries out a sequential reaction; i.e., both substrates must be bound to the enzyme before product release.

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