The Kinetics and Specificity of the Reaction of 2′(3′)-*O*-Bromoacetyluridine with Bovine Pancreatic Ribonuclease A†

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ABSTRACT: 2′(3′)-*O*-Bromoacetyluridine reacts rapidly and selectively with bovine pancreatic ribonuclease A at pH 5.5 and 25°. Under conditions of high molar ratios of nucleoside derivative to enzyme, the only derivative is *N*-3-carboxymethylhistidine-12 ribonuclease A. The reaction occurs almost exclusively with the histidine-12 residue at the active site and the inactivation of the enzyme is accompanied by the stoichiometric disappearance of unmodified ribonuclease A and appearance of the product, *N*-3-carboxymethylhistidine-12 ribonuclease A. Kinetic studies indicate a mechanism involving saturation of the enzyme by the nucleoside derivative. The inhibitor constant, K_b, is 0.087 *M* and k_3 is 35.1×10^{-4} sec^{-1}. The reaction of 2′(3′)-*O*-bromoacetyluridine with the enzyme occurs at a rate approximately 3100 times greater than that corresponding to the reaction with L-histidine. The alkylation reaction is inhibited competitively by uridine with a K_i of 0.013 *M*. 2′(3′)-*O*-Bromoacetyluridine inactivates ribonuclease A 4.5 times faster than bromoacetic acid and the specificity for alkylation of active-site histidine residues is different. 2′(3′)-*O*-Bromoacetyluridine reacts 1000 times more rapidly with ribonuclease A than iodoacetamide. The contribution of nucleoside binding to the overall rate of alkylation is discussed.

The reaction of the active-site histidine residues of RNase A[1] with alkylating agents has been extensively explored (Gundlach et al., 1959; Barnard and Stein, 1959; Heinrikson et al., 1965; Heinrikson, 1966; Goren and Barnard, 1970a). Studies with iodoacetic acid (Gundlach et al., 1959) and bromoacetic acid (Barnard and Stein, 1959) at pH 5.5 have shown that the major modification occurs at N-1 of the imidazole ring of the histidine-119 residue. Minor alkylations occur at the N-3 of the imidazole ring of histidine-12 (Fruchter and Crestfield, 1967) and at methionine residues (Fruchter and Crestfield, 1967; Goren and Barnard, 1970b). At pH 8.5, the dominant alkylated residue is the ϵ-amino group of lysine-41 (Heinrikson, 1966).

Reaction with iodoacetamide leads to a large reduction in rate and alkylation at the N-3 of the imidazole ring of histidine-12 (Fruchter and Crestfield, 1967).

Iodoacetamide and α-halo acids react more rapidly with active-site histidine residues than with free histidine. The order of reactivity, bromo > iodo > chloro, is the same for both the enzyme and for free histidine (Heinrikson et al., 1965; Korman and Clarke, 1956; Le Thi and Carty, unpublished results), while the specificity of reaction differs. In the early phases of the reaction each histidine residue of the enzyme undergoes modification at only one nitrogen (N-1 for histidine-119 and N-3 for histidine-12) while both nitrogen atoms of free histidine are alkylated. X-Ray crystallographic studies (Wyckoff et al., 1967) indicate that the steric inaccessibility of the N-1 of the imidazolyl moiety of histidine-12 can account for N-3 alkylation as the predominant product.

These observations indicate that the geometry of the active site of RNase A exerts steric control of the product distribution. Special steric and electronic effects give rise to increased nucleophilicities of active-site imidazolyl groups which account for large rate enhancements relative to model compounds. At present, there is no model capable of completely accounting for the kinetics and specificity of the alkylation reactions of α-halo acids and amides with RNase A.

The above chemical studies have examined the properties of groups at the active center of the free enzyme, unperturbed by the presence of ligands. To understand the relative steric disposition of alkylating groups at the time the enzyme is complexed to substrates or inhibitors, a study of the kinetics and specificity of the reactions of α-haloacyl nucleosides with RNase A was undertaken. In particular, it was of interest to determine what constraints the binding of nucleoside moieties places upon the spatial disposition of 2′- and 3′-*O*-haloacetyl substituents relative to active site residues.

In a preliminary study (Pincus and Carty, 1970), the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A was shown to result predominantly in the modification of a histidine residue, tentatively identified as histidine-12. The first-order rate constants for the alkylation process vary nonlinearly with the two concentrations of 2′(3′)-*O*-bromoacetyluridine employed, suggesting a preliminary binding step prior to covalent reaction. We now present a detailed study of this reaction which demonstrates that (1) the reaction of 2′(3′)-*O*-bromoacetyluridine exhibits almost total specificity for the histidine-12 residue; (2) the covalent reaction is preceded by an adsorption step which is characterized by a dissociation constant somewhat higher than that for uridine, a competitive inhibitor of RNase A; (3) the site of adsorption of the covalent modifier is identical with that of competitive inhibitors of RNase A; and (4) the rate

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[1] Abbreviations and nomenclature used are: RNase A, bovine pancreatic ribonuclease A; k'_{obsd}, observed first-order rate constant; k''_{calcd}, calculated second-order rate constant, equal to $k'_{obsd}/(B)$, where (B) is the concentration of alkylating agent, 2′(3′)-*O*-bromoacetyluridine; Cm, carboxymethyl. 2′(3′)-*O*-Bromoacetyluridine has been abbreviated BAU in a prior publication from this laboratory (Pincus and Carty, 1970).

of reaction of 2′(3′)-*O*-bromoacetyluridine with the enzyme is quite substantially enhanced over that of other alkylating agents such as bromoacetic acid and iodoacetamide. At least part of this rate enhancement may be attributed to a favorable orientation of the bromoacetyl residue imposed by nucleoside binding.

Materials and Methods

RNase A. RNase A (RASE OLB) was obtained commercially (Worthington). It was further purified as described previously (Carty and Hirs, 1968). Concentrations were determined from the extinction coefficient at 280 nm, 9350 M^{-1} cm^{-1}.

2′(3′)-O-Bromoacetyluridine. (A) 2′,3′-*O*-(1-Methoxy-2-bromoethylidene)uridine. Dried uridine (2 g, 8.2 mmol) was suspended in 6 ml of redistilled trimethyl orthobromoacetate, 0.4 g of *p*-toluenesulfonic acid added, and the suspension stirred at 20° in the dark. After 2 hr, the resulting solution was neutralized with solid $NaHCO_3$, filtered, and the excess orthoester removed by vacuum distillation (0.7 mm) at 35°. The remaining syrup was dissolved in $CHCl_3$ and applied to a 3.75 × 40 cm column of silicic acid. Chromatography was carried out using a linear gradient consisting of $CHCl_3$ (2 l.) and 8% MeOH–$CHCl_3$ (v/v) (2 l.) as the developing solution. Fractions corresponding to the dioxolane, appearing between 1820 and 2160 effluent ml, were pooled and evaporated to a foam which was coevaporated three times with 50-ml portions of ethanol. The amorphous powder was dried in vacuo over P_2O_5. The yield was 1.93 g (62%); uv_{max} (95% EtOH) 259 nm (ϵ 9660), uv_{min} 229 nm (ϵ 2440). Anal. Calcd for $C_{12}H_{15}N_2O_7Br$: C, 38.01; H, 4.00; N, 7.39; Br, 21.08. Found: C, 38.01; H, 4.20; N, 7.74; Br, 21.21.

(B) 2′(3′)-*O*-Bromoacetyluridine. 2′,3′-*O*-(1-Methoxy-2-bromoethylidene)uridine (1 g, 2.64 mmol) was added in portions to 100 ml of 5% acetic acid (v/v) at 4°. After 48 hr, the solution was lyophilized. The residue was dissolved in tetrahydrofuran, adsorbed onto 4 g of silicic acid, and dried. The resulting powder was applied to a 2.5 × 20 cm column of silicic acid equilibrated in $CHCl_3$. The column was eluted with a linear gradient of $CHCl_3$ (2 l.) and 10% MeOH–$CHCl_3$ (v/v) (2 l.) as the limiting solutions. Fractions appearing between 1465 and 1805 effluent ml were pooled and evaporated. The residue was dissolved in *p*-dioxane and lyophilized. The amorphous product was dried at room temperature over P_2O_5 in vacuo (2 × 10^{-3} mm), and is stable when stored dry at −15°. The yield was 0.96 g (92%): uv_{max} (1 *M* sodium acetate, pH 4.3) 260 nm (ϵ 9380); IR (KBr) 1739 cm^{-1} (C=O); NMR (CD_3COCD_3) δ 7.99 (d, 0.28, $J_{5,6}$ = 8 Hz, C_6H, 2′ isomer), 7.96 (d, 0.72, $J_{5,6}$ = 8 Hz, C_6H, 3′ isomer), 6.09 (d, 0.26, $J_{1',2'}$ = 5.1 Hz, $C_{1'}H$, 2′ isomer), 5.97 (d, 0.74, $J_{1',2'}$ = 6.7 Hz, $C_{1'}H$, 3′ isomer), 5.68 (d, 0.73, $J_{5,6}$ = 8 Hz, C_5H, 3′ isomer), 5.65 (d, 0.27, $J_{5,6}$ = 8 Hz, C_5H, 2′ isomer), 5.30 (m, 1, $C_{2'}H$ of 2′ isomer + $C_{3'}H$ of 3′ isomer), 4.54 (m, 1, $C_{2'}H$ of 3′ isomer + $C_{3'}H$ of 2′ isomer), 4.16 (m, $C_{4'}H$, 2′ + 3′ isomers), 4.13 (s, $COCH_2Br$) (combined weight of $COCH_2Br$ + $C_{4'}H$ protons equals 3), and 3.79 ppm (d, 2, $C_{5'}H$). Anal. Calcd for $C_{11}H_{13}N_2O_7Br$: C, 36.18; H, 3.60; N, 7.67; Br, 21.89. Found: C, 36.15; H, 3.88; N, 7.43; Br, 22.00.

Other Materials. Amberlite IRC-50 (BIO-REX 70, −400 mesh, sodium form) was a Bio-Rad product. It was processed for chromatography as previously described (Hirs, 1955). Sephadex G-25 medium (bead form) was purchased from Sigma and washed in 50% acetic acid (v/v) prior to use. Sodium 2′,3′-cyclic cytidylate was prepared from the barium salt (Schwarz Bio Research) and was prepared for use in the RNase A assay according to Murdock et al. (1966). All other chemicals were of the highest obtainable purity.

RNase A Assay. A difference spectrophotometric technique with cytidine 2′,3′-cyclic phosphate as substrate was used (Murdock et al., 1966).

Protein Chromatography. Amberlite IRC-50 (BIO-REX 70) columns were filled and operated as previously described (Murdock et al., 1966). Columns were loaded with 1-ml samples previously adjusted to pH 5.8 just prior to chromatography. Chromatograms were developed at a flow rate of 30 ml/hr, and the appearance of protein in the effluent was monitored spectrophotometrically at 220 nm using a flow cell with a 10-mm light path.

Preparative protein chromatography was performed on a column of Amberlite IRC-50 as previously described (Hirs et al., 1965).

Separation of C-Peptide and C-Protein. The purified RNase A derivative obtained from the reaction of 2′(3′)-*O*-bromoacetyluridine and the enzyme was subjected to cyanogen bromide cleavage according to the procedure of Gross and Witkop (1962).

Amino Acid Analysis. Amino acid analyses were performed by ion-exchange chromatography (Spackman et al., 1958). Analyses were carried out on a machine designed to operate in the 0.1-μmol range. Ninhydrin solutions in buffered aqueous Me_2SO were prepared and used according to Moore (1968).

Reaction of 2′(3′)-O-Bromoacetyluridine with RNase A—Analytical Experiments. Reaction mixtures contained from 1 to 50 mg of nucleoside dissolved in 0.2 ml of 0.5 *M* sodium acetate buffer (pH 5.5) and 0.15 ml of water. Reactions were initiated by the addition of 0.15 ml of 0.0078 *M* RNase A. Aliquots of 0.05 ml were removed at zero time and at suitable intervals thereafter and added to 4 ml of 0.02 *M* HCl solution. The pH of the inactivated samples was 2 and these were stored at 4° in the dark until analyzed for enzymatic activity and the yield of protein products. All reactions were conducted in the dark in thermostated cells.

Preparative Experiment. A reaction mixture containing 89.6 mg (245 μmol) of 2′(3′)-*O*-bromoacetyluridine in 2.0 ml of 0.5 *M* sodium acetate buffer (pH 5.5), 1.5 ml of water, and 1.5 ml of 0.0078 *M* RNase A (11.7 μmol) was stirred at 25° for 45 min. The contents were diluted to 7 ml with water and 0.7 ml of 1 *M* sodium phosphate buffer (pH 5.88) was added. The final pH was 5.81 and the entire sample was fractionated upon a preparative IRC-50 column as described above.

Analytical Reaction of RNase A with Bromoacetic Acid in the Presence of Uridine. A reaction mixture was prepared containing 0.016 *M* sodium bromoacetate, 0.016 *M* uridine, 0.0023 *M* RNase A, and 0.2 *M* sodium acetate buffer (pH 5.5), at 25° in a total volume of 0.5 ml. Aliquots were removed and processed for enzymatic assay and analytical chromatography as above.

Reaction of L-*Histidine with 2′(3′)-O-Bromoacetyluridine.* A reaction mixture was prepared containing 0.045 *M* L-histidine and 0.13 *M* 2′(3′)-*O*-bromoacetyluridine in 0.2 *M* sodium acetate (pH 5.5) at 25°. The rate of alkylation was obtained by following the disappearance of histidine on the basic column of the amino acid analyzer.

Estimation of the Uridine Liberated in the Reaction of RNase A with 2′(3′)-O-Bromoacetyluridine. A mixture

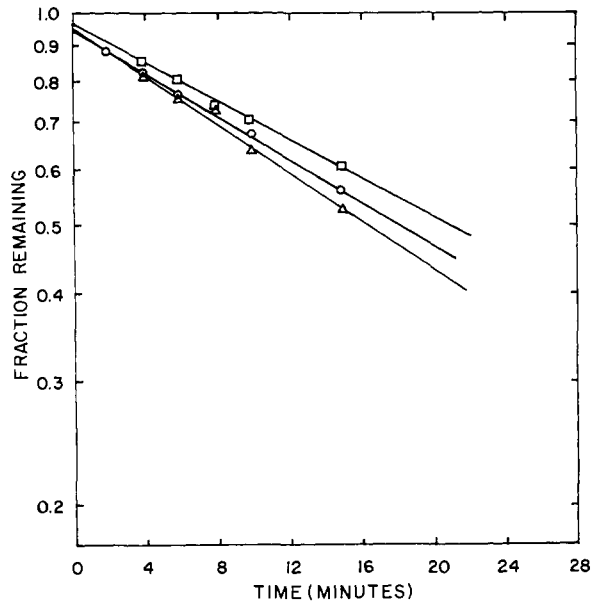

FIGURE 1: Semilogarithmic plots of the fraction of residual RNase A (△); enzymatic activity measured against 2′,3′-cyclic cytidylate (○); and the parameter, $A_0 - x$, where A_0 and x are the initial RNase A and *N*-3-Cm-His-12-RNase A concentrations, respectively (□); against time for the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A conducted at pH 5.5 and 30°. The initial concentrations of 2′(3′)-*O*-bromoacetyluridine and RNase A were 0.010 and 0.0023 *M*, respectively. Values for the fraction of RNase A remaining and the fraction of *N*-3-Cm-His-12-RNase A formed were estimated from chromatograms of the reaction products on Amberlite IRC-50 columns (cf. Figure 2). The lines were fitted by visual inspection of the data and k'_{obsd} values calculated from estimates of $t_{1/2}$, the half-time.

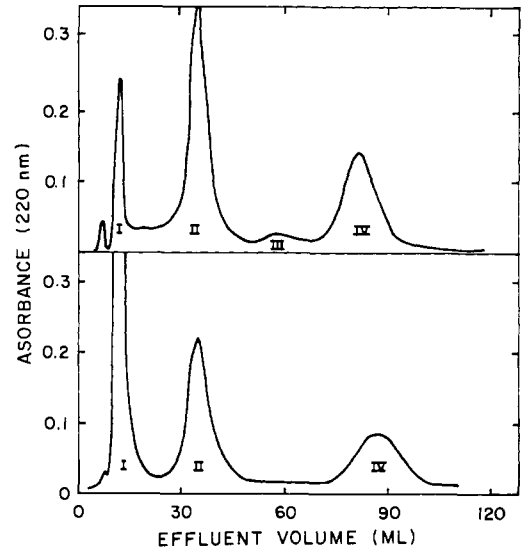

FIGURE 2: Representative elution curves obtained in the analysis of reaction mixtures formed from the covalent interaction of 2′(3′)-*O*-bromoacetyluridine withe RNase A. Top curve: The absorbance profile of the effluent obtained from chromatography on Amberlite IRC-50 of a 15-min sample of the reaction mixture which initially contained 0.010 *M* 2′(3′)-*O*-bromoacetyluridine and 0.0023 *M* RNase A. The experiment was conducted at pH 5.5 and 30°. Bottom curve: The absorbance profile from a similar chromatogram obtained from an analysis of a 2-hr sample of the reaction mixture initially charged with 0.062 *M* 2′(3′)-*O*-bromoacetyluridine and 0.0023 *M* RNase A at pH 5.5 and 0.5°. The chromatograms were selected so as to represent the same extent of reaction, 45% completion. The *zones* marked by Roman numerals are identified as follows: I, nucleoside material; II, native RNase A; III, unidentified minor reaction product; IV, *N*-3-Cm-His-12-RNase A.

was prepared containing 56.9 mg (156 μmol) of 2′(3′)-*O*-bromoacetyluridine dissolved in 0.67 ml of 0.5 *M* sodium acetate buffer (pH 5.5), 0.5 ml of water, and 0.5 ml of 0.0078 *M* RNase A (3.9 μmol). The reaction was carried out at 25° and aliquots of 0.5 ml were removed and analyzed for uridine content after 20 and 55 min by a modification of the method of Rammler and Rabinowitz (1962) using sodium periodate.

Inhibition of the Reaction of 2′(3′)-O-Bromoacetyluridine with RNase A by Uridine. A sample of 109.55 mg of 2′(3′)-*O*-bromoacetyluridine was dissolved in 0.4 *M* sodium acetate buffer (pH 5.5) up to a volume of 3 ml. Aliquots of 0.3 ml of this stock solution were frozen immediately and stored at −15° until used. Reaction mixtures were constituted by the admixture of 0.25 ml of the thawed solution plus 0.1 ml of stock uridine solution of appropriate concentration. Reactions were initiated by the addition of 0.15 ml of a stock solution of 0.0078 *M* RNase A. Reaction rates were determined from analysis of residual enzymatic activity found in 0.05-ml aliquots according to methods previously described.

Results

Analytical reactions of 2′(3′)-*O*-bromoacetyluridine were designed to establish the stoichiometric relationship between the disappearance of RNase A, the loss of enzymatic activity, and the appearance of the major product subsequently identified as *N*-3-Cm-His-12-RNase A. Figure 1 depicts the kinetic course of the reaction of 0.010 *M* 2′(3′)-*O*-bromoacetyluridine with 0.0023 *M* RNase A at pH 5.5 and 30°. All of the measured variables change via first-order processes through approximately one-half of the reaction. The first-order rate constant for the disappearance of RNase A is 6.5×10^{-4} sec^{-1}, that for the loss of enzymatic activity, 5.9×10^{-4} sec^{-1}, and that for the appearance of the major derivative, 5.2×10^{-4} sec^{-1}. The similar magnitude of the first two constants suggests that the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A is confined predominantly to the active site. A comparison of the rate constants for loss of enzyme activity and appearance of the major protein product indicates stereochemical control of the alkylation reaction at one of the several available substitution sites at the active center. This selectivity is illustrated in the top chromatogram of Figure 2. Examination of the absorbance profile reveals a single major product (peak IV), later identified as *N*-3-Cm-His-12-RNase A, preceded by a minor component (peak III). When the reaction is conducted at a lower temperature, 0.5°, the only observable protein product is found at a position in the elution profile corresponding to peak IV. This observation is indicated in the bottom trace of Figure 2.

The chromatographic mobility of the major protein derivative (peak IV) relative to the unmodified enzyme (peak II) is 2.0, the value found by Crestfield et al. (1963a) for *N*-3-Cm-His-12-RNase A. The relative chromatographic mobility of peak III to peak II is 1.67, somewhat different from the value of 1.45 found for *N*-1-Cm-His-119-RNase A (Crestfield et al., 1963a). The identity of this minor derivative remains unknown at this time.

Results of product distribution studies on analytical reaction mixtures prompted us to prepare larger quantities of peak IV (cf. Figure 2) for the purpose of definitive structural identification. Figure 3 shows the chromatographic separation of the products formed in the reaction of 160 mg of

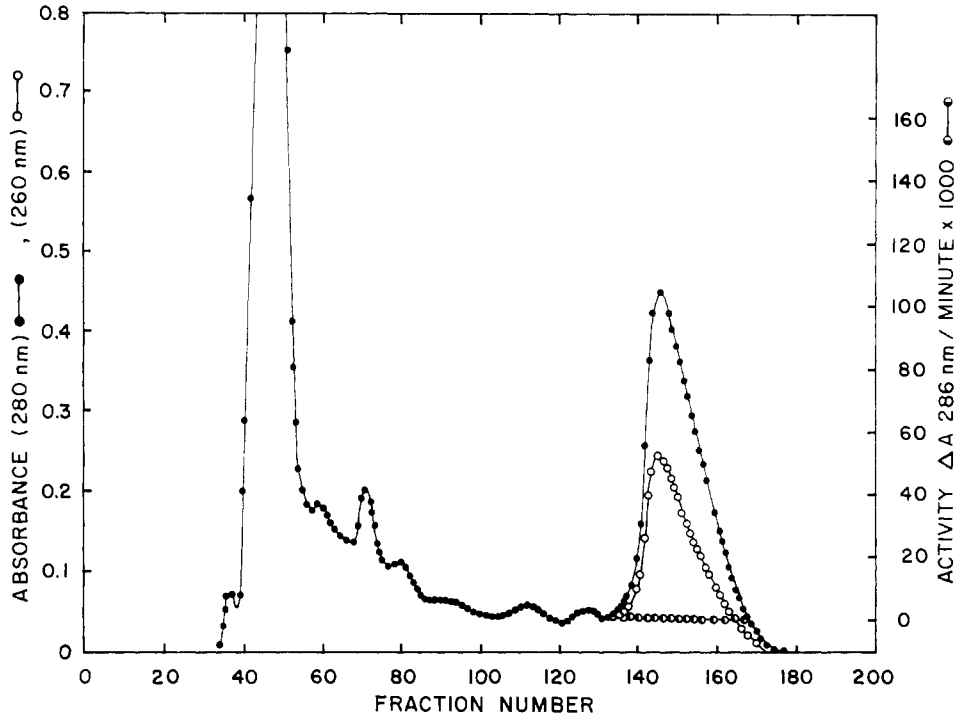

FIGURE 3: Chromatography on Amberlite IRC-50 of the reaction products in 5.0 ml of a mixture initially charged with 89.6 mg of 2′(3′)-*O*-bromoacetyluridine (245 μmol) and 160 mg of RNase A (11.7 μmol) in which the reaction was allowed to proceed at pH 5.5 and 25° for 0.75 hr (nine half-lives). The column, which measured 3.75 × 40 cm, was equilibrated with 0.2 *M* sodium phosphate buffer (pH 6.39) and was operated at 25° at a flow rate of 30.7 ml/hr. Fractions of 7.7 ml each were collected and the adsorbance of each fraction was determined at 280 and 260 nm. The enzyme activity of the main peak, localized between fractions 140 and 170, was measured by assaying every other fraction in the peak against cyclic 2′,3′-cytidylate. The major peak centered on fraction 47 is uridine. Unmodified RNase A was found as a narrow zone centered on tube 71.

RNase A with 89.6 mg of 2′(3′)-*O*-bromoacetyluridine at pH 5.5 and 25°. The major protein product, centered on fraction 146, is inactive against 2′,3′-cyclic cytidylate (cf. Discussion). The yield of the major component was 58%. A minor peak centering on tube 71 was identified as unreacted RNase A. The major 280-nm absorbing material found between fractions 40 and 56 is unreacted 2′(3′)-*O*-bromoacetyluridine and its hydrolysis product, uridine. Minor protein components appearing between fractions 75 and 130 have not been identified.

A 1.0-mg sample of the major protein product was oxidized with performic acid, hydrolyzed, and analyzed for amino acid content. The results are presented in Table I. The only differences noted are the loss of 1 mol of histidine and appearance of 1 mol of 3-Cm-histidine/mol of derivative relative to a similar analysis of unmodified RNase A. No modification of lysine, methionine, or other potentially alkylatable residues was found.

Fractions 140–170 (cf. Figure 3) were pooled, concentrated and desalted on a column of Sephadex G-25. A 95-mg sample of the desalted and lyophilized powder was treated with CNBr (Gross and Witkop, 1962). The separation of the cleavage products derived from 17 mg of the CNBr-treated protein was achieved on Sephadex G-25. The absorbance profile of the column effluent was identical in all respects with previously reported results (cf. Figure 2 in Gross and Witkop, 1962). Three major fractions are observed. In order of elution, they are the "C-protein" fraction, the "C-peptide" fraction, and homoserine lactone.

The fractions representing the "C-peptide" were pooled and concentrated and the entire sample was subjected to acid hydrolysis followed by amino acid analysis. The results are provided in Table I. Examination of Table I indicates the absence of histidine and the presence of 1 mol of 3-Cm-histidine in the modified "C-peptide" fragment. The "C-peptide" fragment derived from native RNase A contains 1 mol of histidine and this residue is the 12th in the peptide chain (Gross and Witkop, 1962). The recovery of modified "C-peptide" was 81%. Our results corroborate those of Heinrikson et al. (1965) who found that carboxyalkylation of the histidine-12 residue does not modify the CNBr cleavage of methionine-13.

The specificity of the reaction of 2′(3′)-*O*-bromoacetyluridine and RNase A characterized by alkylation of the histidine-12 residue contrasts directly with that observed for the reaction of bromoacetic acid with the enzyme wherein dominant active-site alkylation occurs at the histidine-119 residue. The fact that 2′(3′)-*O*-bromoacetyluridine is readily hydrolyzed to uridine and bromoacetic acid and the possibility that the presence of the nucleoside, uridine, at the active center might alter the specificity of alkylation of bromoacetic acid, prompted us to study the reaction of this α-halo acid with RNase A in the presence of uridine. Examination of absorbance profiles of column effluents from chromatograms of the reaction products formed at different times during the course of the alkylation of the enzyme by bromoacetic acid in the presence of uridine indicates that the distribution of carboxymethylated RNase A derivatives is qualitatively identical with previously published results obtained in the absence of uridine (cf. Figure 1 in Crestfield et al., 1963a). The major protein derivative formed is *N*-1-Cm-His-119-RNase A. In the presence of uridine, the ratio of products formed, *N*-1-Cm-His-119-RNase A to *N*-3-Cm-His-12-RNase A, is *ca.* 12, somewhat higher than the value of 8 obtained by Crestfield et al. (1963a). It is apparent, however, that the product distribution of carboxyalk-

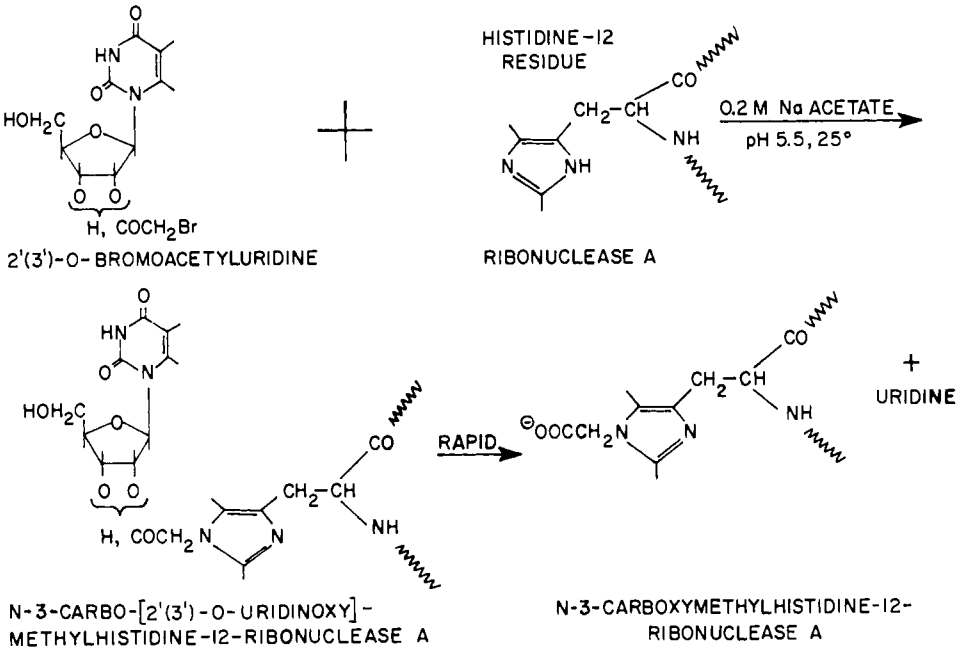

FIGURE 4: Reactants, products, and a postulated reaction sequence leading to the formation of *N*-3-Cm-His-12-RNase A from the covalent interaction of 2′(3′)-*O*-bromoacetyluridine and the native enzyme. The reaction conditions contributing to the production of the carboxyalkylated derivatives are shown.

ylated derivatives formed in the reaction of RNase A with bromoacetic acid is largely unaffected by the presence of uridine.

The major protein derivative in Figure 3 has a 280/260 absorbance ratio of 2.0, a value expected for native RNase A and its carboxymethyl derivatives. For a RNase A derivative to which a mole of uridine is covalently bound, the same ratio would be approximately 1.0 (Lan and Carty, 1972). The absence of uridine in the isolated protein derivative prompted us to search for alternative ways in which to demonstrate the transient existence of a protein to which the uridine moiety was attached in ester linkage.

Attempts to physically isolate *N*-3-carbo[2′(3′)-*O*-uridinoxy]methylhistidine-12 RNase A by rapid, low-temperature gel chromatography on Sephadex G-25 were unsuccessful. Ultraviolet spectral analysis of separated protein fractions failed to yield the expected 280/260 ratio for a RNase A derivative to which uridine is covalently bound.

An attempt was then made to infer the presence of an intermediate enzyme–nucleoside compound by measurement of the uridine liberated in the reaction of RNase A and 2′(3′)-*O*-bromoacetyluridine. Uridine may accumulate in the reaction medium as the result of three mechanisms. First, uridine is formed from the hydrolysis of the ester bond of 2′(3′)-*O*-bromoacetyluridine which proceeds at pH 5.5 and 25° with a rate constant of 8.6×10^{-6} sec^{-1} (M. Pincus and R. P. Carty, unpublished results). Second, RNase A may catalyze the hydrolysis of 2′(3′)-*O*-bromoacetyluridine, a process which would result in the accumulation of additional uridine above that observed for nonenzymatic hydrolysis. Third, esterolysis of a covalent enzyme–nucleoside compound may occur which would add more uridine to the medium above that expected from nonenzymatic hydrolysis of the nucleoside ester. For this last case, the amount of uridine in excess of that expected from hydrolysis should be no greater than the amount of enzyme which reacted with 2′(3′)-*O*-bromoacetyluridine.

In the periodate studies designed to determine the amount of excess uridine in the medium, after correction for the uridine liberated by nonenzymatic hydrolysis, the uridine concentration was less than that expected for complete destruction of an intermediate RNase A–nucleoside derivative. After 20 min of reaction time, only 70% of the expected uridine was recovered, while after 55 min, the value rose to 85%.

These results lead to the postulated reaction sequence depicted in Figure 4. The formation of a highly labile intermediate enzyme–nucleoside compound, *N*-3-carbo[2′(3′)-*O*-uridinoxy]methylhistidine-12 RNase A, is followed by a rapid hydrolytic step which gives rise to the observable products, uridine and *N*-3-Cm-His-12-RNase A.

The reactions of 2′(3′)-*O*-bromoacetyluridine with RNase A exhibit pseudo-first-order kinetics. Semi-logarithmic plots of residual enzymatic activity vs. time appear in Figure 5 for kinetic studies carried out at pH 5.5, 25°, at an initial RNase A concentration of 0.0023 *M* in which initial 2′(3′)-*O*-bromoacetyluridine concentrations were allowed to range from 0.012 to 0.30 *M*. Calculated first-order rate constants show a nonlinear dependence upon the 2′(3′)-*O*-bromoacetyluridine concentration characteristic of saturation kinetics. The kinetic behavior of the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A is considered in

$$E + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EB \xrightarrow{k_3} P \qquad (1)$$

in which the enzyme and nucleoside form a reversibly dissociable complex, EB, which precedes the alkylation (k_3 step). In the above model E represents free enzyme, B is 2′(3′)-*O*-bromoacetyluridine, and P is the carboxyalkylated product. The initial product is thought to be *N*-3-carbo[2′(3′)-*O*-uridinoxy]methylhistidine-12 RNase A which hydrolyzes rapidly to uridine and *N*-3-Cm-His-12-RNase A (cf. Figure 4). However, the proposed model is sufficient if it is assumed that the k_3 step, representing formation of the enzyme–nucleoside compound, is the rate-determining step, and all subsequent steps, such as hydrolysis, are rapid. As-

Table I: Amino Acid Analyses of Performic Acid Oxidized *N*-3-Cm-His-12-RNase A and the "C-Peptide" Fragment Derived from Its Treatment with CNBr.[a]

Amino Acid	Residues				
	RNase A	Theory	PO-*N*-3-Cm-His-12 RNase A	C-peptide	Theory
Lysine	10.1	10	9.98	2.00	2
Histidine	3.76	4	3.03		
Ammonia	18.9	17	24.7	1.77	1
Arginine	3.97	4	3.97	1.01	1
Cysteic acid			8.12		
Aspartic acid	15.2	15	14.6	0.17	
Threonine	9.76	10	9.23	0.97	1
Serine	14.3	15	13.3	0.29	
Glutamic acid	12.0	12	12.1	3.29	3
Proline	4.10	4	4.22		
Glycine	2.92	3	2.93	0.17	
Alanine	12.1	12	11.7	3.17	3
Half-cystine	7.08	8			
Valine	8.73	9	8.76		
Methionine	3.30	4			
Isoleucine	1.89	3	2.22		
Leucine	1.96	2	2.00		
Tyrosine	5.46	6	5.32		
Phenylalanine	2.99	3	3.14	0.99	1
3-Cm-Histidine			0.97	1.01	1
1-Cm-Histidine					
Methionine sulfone			3.59		
Homoserine + homoserine lactone				0.92	1

[a] The results represent single determinations with 22-hr hydrolysates and are expressed as molar ratios of the constituent amino acids. The first column is provided to permit comparison of the modified protein with an analysis of the native enzyme performed under the same conditions. All values are uncorrected for destruction during hydrolysis. In the analysis of the "C-peptide" fragment, the peak for homoserine partially overlapped with glutamic acid, thus causing the calculated molar ratio of glutamic acid to be slightly elevated. The color constant employed for the estimation of *N*-3-Cm-histidine is that found for glycine (Crestfield et al., 1963b). PO-*N*-3-Cm-His-12 RNase A is performic acid oxidized *N*-3-Cm-His-12-RNase A. Performic acid oxidations were carried out according to the method of Hirs (1956).

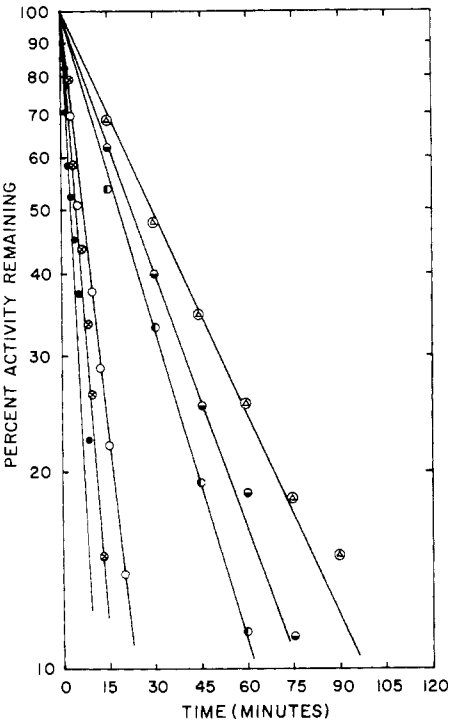

FIGURE 5: Semilogarithmic plots of the fraction of residual enzyme activity against the time for samples taken from reaction mixtures of 2′(3′)-*O*-bromoacetyluridine and RNase A conducted at pH 5.5 and 25°. The initial concentration of RNase A was 0.0023 *M* and each line represents the kinetics of the loss of enzyme activity at a different initial concentration of the alkylating agent, 2′(3′)-*O*-bromoacetyluridine. The six initial concentrations (*M*) used were: (△) 0.012; (◒) 0.016; (◐) 0.020; (⊗) 0.092; (■) 0.17; and (●) 0.30. The lines were fitted visually and k'_{obsd}, the pseudo-first-order rate constant, was calculated from $t_{1/2}$ values.

suming that E and B are in equilibrium with EB throughout the reaction, the rate law

$$\frac{d(P)}{dt} = \frac{k_3(B)(E_0 - P)}{[K_b + (B)]} \quad (2)$$

may be deduced (Gold and Fahrney, 1964). The constant, K_b, is the dissociation constant of the 2′(3′)-*O*-bromoacetyluridine–RNase A complex and is equal to k_2/k_1. If (B) is in excess of (E_0), a pseudo-first-order rate constant, k'_{obsd}, may be defined (Gold and Fahrney, 1964)

$$k'_{obsd} = \frac{k_3(B)}{[K_b + (B)]} \quad (3)$$

Values of K_b and k_3 may be obtained from the reciprocal of eq 3:

$$\frac{1}{k'_{obsd}} = \frac{K_b}{k_3(B)} + \frac{1}{k_3} \quad (4)$$

The plot of the reciprocal of the observed pseudo-first-order rate constant for loss of enzymatic activity against the reciprocal of the 2′(3′)-*O*-bromoacetyluridine concentration was found to be linear. From values of the slope and intercept of the line, K_b and k_3 were calculated to be 0.087 *M* and 35.1 × 10^{-4} sec^{-1}, respectively.

Reactions of 2′(3′)-*O*-bromoacetyluridine with RNase A show substantial rate reductions in the presence of uridine. Inhibition of the rate was examined over a range of uridine concentrations from 0.01 to 0.1 *M* at a constant 2′(3′)-*O*-bromoacetyluridine concentration of 0.049 *M*. The kinetic data were analyzed according to a competitive inhibition scheme

$$E + B \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EB \xrightarrow{k_3} P \quad (1)$$

$$E + I \rightleftharpoons EI \quad (5)$$

where I is uridine and EI is the reversibly dissociable enzyme–uridine complex. In all experimental situations (I) ≫ (EI) and may be taken as the total concentration of uridine. Assuming the absence of a reactive ternary complex between 2′(3′)-*O*-bromoacetyluridine, RNase A, and uridine, the rate law

$$\frac{d(P)}{dt} = \frac{k_3K_i(B)(E_0 - P)}{K_i[K_b + (B)] + K_b(I)} \quad (6)$$

is obtained where K_i is the dissociation constant of the EI complex. If (B) is held in excess of (E_0), then k'_{obsd}, the pseudo-first-order rate constant, is given by

$$k'_{obsd} = \frac{k_3K_i(B)}{K_i[K_b + (B)] + K_b(I)} \quad (7)$$

Inversion of eq 7 and separation of terms give

$$\frac{1}{k'_{obsd}} = \frac{K_b + (B)}{k_3(B)} + \frac{K_b(I)}{k_3K_i(B)} \quad (8)$$

A plot of $1/k'_{obsd}$ vs. inhibitor concentration is linear for such a mechanism with slope equal to $K_b/k_3K_i(B)$ and intercept equal to $[K_b + (B)]/k_3(B)$. A value of K_i was extracted from the value of the slope of a plot of the inhibition data according to eq 8 and is 0.013 *M*.

The rate of the reaction of a 3 molar excess of 2′(3′)-*O*-bromoacetyluridine with L-histidine was studied at pH 5.5 and 25°. The reaction was followed over a 25-hr period during which time slightly less than 20% of the histidine was consumed. The reaction exhibited pseudo-first-order kinetics from which a second-order rate constant of 1.3×10^{-5} M^{-1} sec^{-1} was calculated This value was compared to the ratio, k_3/K_b, for the reaction of RNase A with 2′(3′)-*O*-bromoacetyluridine. The ratio, k_3/K_b, has the units of a second-order rate constant, M^{-1} sec^{-1}, and its use for comparative purposes with rate constants derived from strictly bimolecular processes will be justified in the Discussion section. The value of k_3/K_b for the reaction of RNase A with 2′(3′)-*O*-bromoacetyluridine is 403×10^{-4} M^{-1} sec^{-1} which is ca. 3100 times greater than the value of the constant observed for the reaction of L-histidine with 2′(3′)-*O*-bromoacetyluridine.

Discussion

A significant feature of the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A at pH 5.5 is the specificity of alkylation for the histidine-12 residue. Modification of this residue produces a derivative with no detectable hydrolytic activity toward 2′,3′-cyclic cytidylate (cf. Figure 3). Several investigators have reported that *N*-3-Cm-His-12-RNase A has measurable activity toward this substrate (Crestfield et al., 1963b; Machuga and Klapper, 1974). In the present study, low concentrations of protein were used in the assay mixture and rate measurements were made over short time intervals so that the method may have been relatively insensitive to preparations of low specific activity. The assay system (Murdock et al., 1966), as employed in the present case, would therefore not appear to provide a definitive test of the possible existence of low residual hydrolytic activity in *N*-3-Cm-His-12-RNase A.

Modification of the N-3 atom of the imidazolyl ring of this residue was demonstrated by the chromatographic mobility of *N*-3-Cm-His-12-RNase A on IRC-50 columns, by the results obtained from amino acid analysis of the performic acid oxidized protein derivative and by the degradative studies with CNBr. Analysis of the kinetics of the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A indicates that there is an excellent correlation between the appearance of the carboxymethylhistidine-12 derivative and the loss of enzymatic activity. Both processes were observed to obey pseudo-first-order kinetics with the nucleoside in substantial excess over the enzyme. The marked specificity of the reaction for the histidine-12 residue is independent of the concentration of 2′(3′)-*O*-bromoacetyluridine at pH 5.5. The histidine-12 derivative is the major product at all temperatures studied between 0.5 and 30°.

Several lines of evidence suggest that the primary alkylation product is *N*-3-carbo[2′(3′)-*O*-uridinoxy]methylhistidine-12 RNase A and that this hydrolyzes in a rapid step to give the observed products, uridine and *N*-3-Cm-His-12-RNase A. First, from periodate studies, the amount of uridine liberated in the reaction of the enzyme with nucleoside derivative, after subtracting the amount due to nonenzymatic hydrolysis, is less than the amount of 2′(3′)-*O*-bromoacetyluridine which has reacted covalently with RNase A. The difference is a measure of the amount of covalent enzyme–nucleoside compound in the reaction mixture.

Greater support for the existence of *N*-3-carbo[2′(3′)-*O*-uridinoxy]methylhistidine-12 RNase A comes from studies of Lan and Carty (1972) in which the corresponding amide analog, *N*-3-carbo[2′-deoxy-2′-uridinamido]methylhistidine-12 RNase A, was isolated as a stable enzyme–nucleoside compound in good yield from the reaction of 2′-bromoacetamido-2′-deoxyuridine with RNase A. The structures of the ester and amide nucleoside derivatives are sufficiently similar to expect analogous reaction products and comparable alkylation rates with the enzyme. Values of K_b and k_3 for the reaction of 2′-bromoacetamido-2′-deoxyuridine with RNase A at pH 5.5 and 25° are 0.11 *M* and 57×10^{-4} sec^{-1}, respectively, similar to the values of K_b and k_3 for 2′(3′)-*O*-bromoacetyluridine.

The kinetic model used in this study assumes the existence of an equilibrium between a noncovalent enzyme–nucleoside complex, EB, and the products of its dissociation, free enzyme and 2′(3′)-*O*-bromoacetyluridine. This model is required because of the absence of enzyme turnover, a fact which is incompatible with the alternate postulate, the steady-state assumption. Also, the observation of saturation kinetics indicates that a steady state could not arise due to the persistence of very low concentrations of the EB complex throughout the entire time course of the reaction. Such a condition would require that the rate always be proportional to the 2′(3′)-*O*-bromoacetyluridine concentration.

The dissociation constant, K_b, for 2′(3′)-*O*-bromoacetyluridine is 0.087 *M*. The dissociation constant of the RNase A–uridine complex, K_I, determined from competitive inhibition studies of the enzyme-catalyzed hydrolysis of cytidine 2′,3′-cyclic phosphate (Ukita et al., 1961), is 0.013 *M*. Lindquist et al. (1973) have measured the dissociation constants of uridine, 0.009 *M*, and 2′-deoxyuridine, 0.045 *M*. The value of the ratio of K_i (2′-deoxyuridine)/K_i (uridine), 5.0, is quite similar to the value of our ratio of K_b/K_i (uridine), 6.7. The similarity of these ratios suggests that the decrease in affinity of 2′(3′)-*O*-bromoacetyluridine for RNase A relative to uridine might be attributed to the absence of a free hydroxyl group which could interact favorably with the enzyme rather than to the steric bulk of the bromoacetyl residue.

The inhibition of the 2′(3′)-*O*-bromoacetyluridine–RNase A reaction by uridine is strictly competitive. This finding reinforces the notion that 2′(3′)-*O*-bromoacetyluridine inactivates RNase A by stereospecific adsorption to the active site, followed by a covalent reaction, and not through alkylation of other potentially reactive groups which might coincidentally result in a structural unfolding and concomitant inactivation. The value of K_i for uridine at pH 5.5 and 25° is 0.013 *M*. It is identical with the previously reported value (Ukita et al., 1961) which was determined at pH 7.6 using measurements of enzyme activity against the substrate, cyclic 2′,3′-cytidylic acid.

An outstanding problem in enzyme kinetics is to find a method for the quantitative comparison of kinetic parameters derived from an enzyme model involving a unimolecular decomposition of the enzyme–substrate complex preceded by a reversible binding step with those derived from a model system which usually involves a bimolecular mechanism (Bender et al., 1964; Jencks, 1969, 1971). Such an evaluation may be made for systems involving the interaction of covalent active-site reagents with enzymes in which

Table II: Comparison of the Kinetic Constants for the Alkylation of RNase A and Free L-Histidine by 2′(3′)-*O*-Bromoacetyluridine, 2′-Bromoacetamido-2′-deoxyuridine, Bromoacetic Acid, and Iodoacetamide.[a]

Reaction	k_3/K_b or k''_{obsd} ($\times 10^4 M^{-1}$ sec^{-1})
2′(3′)-*O*-Bromoacetyluridine + RNase A	403
2′(3′)-*O*-Bromoacetyluridine + L-histidine	0.129
2′-Bromoacetamido-2′-deoxyuridine + RNase A	518
2′-Bromoacetamido-2′-deoxyuridine + L-histidine	0.113
Bromoacetic acid + RNase A	89.0
Bromoacetic acid + L-histidine	0.037
Iodoacetamide + RNase A	0.48
Iodoacetamide + L-histidine	0.0052

[a] Alkylation constants for the nucleoside derivatives are given as k_3/K_b. Second-order rate constants are included for reactions of the enzyme with bromoacetic acid and iodoacetamide, as well as for all reactions involving L-histidine as the nucleophile. All constants are expressed in units of M^{-1} sec^{-1}. The values reported are for constants obtained from experiments conducted at pH 5.5 and 25° except for the iodoacetamide reactions which were performed at pH 5.3 and 24°. The value of $205 \times 10^{-4} M^{-1}$ sec^{-1} obtained by Heinrikson et al. (1965) for the rate constant for the reaction of bromoacetic acid with RNase A was divided by 2.303 to convert the constant expressed in terms of common logarithms to one expressed in terms of natural logarithms. Similar conversions have been made for the second-order rate constants obtained for the reaction of bromoacetic acid with L-histidine (Heinrikson et al., 1965) and iodoacetamide with RNase A and L-histidine (Fruchter and Crestfield, 1967). Rate constants for the reactions of RNase A and L-histidine with 2′-bromoacetamido-2′-deoxyuridine are taken from the studies of Lan and Carty (1972).

saturation kinetics are observed. The comparison with model bimolecular reactions is possible precisely because the enzyme is a true reactant and does not turn over. The ratio, k_3/K_b, possesses the proper units for comparison with second-order processes and it remains to establish the physical significance of the ratio of the constants. Division of both sides of eq 3 by (B) gives

$$\frac{k'_{obsd}}{(B)} = k''_{calcd} = \frac{k_3}{[K_b + (B)]} \qquad (9)$$

The constant, k''_{calcd}, may be regarded as an apparent second-order rate constant obtained by dividing the value of the experimentally observed pseudo-first-order constant by initial (B). It is evident that as (B) approaches zero, k''_{calcd} approaches an upper limit defined by k_3/K_b. It follows that at very low values of 2′(3′)-*O*-bromoacetyluridine approaching zero, no limiting of the rate due to saturation will be observed. In effect, at infinitely low 2′(3′)-*O*-bromoacetyluridine levels, all collisions between alkylating agent and enzyme will, orientation factors permitting, produce an EB complex because at levels of (B) approaching zero, all enzyme molecules are present as free E. The value of k_3/K_b then becomes an effective estimate of the total rate enhancement due to reaction of the agent, B, with the enzyme, because it is free of saturation effects which serve to reduce the value of k''_{calcd}. This rate enhancement, compared to the bimolecular process for a model system, must necessarily include the sum of approximation effects in the binding step plus catalytic effects exerted in the unimolecular decomposition or k_3 step. In contrast, comparison of the contribution to the rate acceleration of the k_3 step alone with the kinetic parameter of an appropriately chosen model system would ignore that part of the contribution to the overall rate enhancement which is due to binding and its attendant orientation effects.

The above analysis permits a comparative examination of the specific rates of all known alkylating agents of RNase A, including 2′(3′)-*O*-bromoacetyluridine, 2′-bromoacetamido-2′-deoxyuridine, bromoacetic acid, and iodoacetamide as well as comparison of the reactions of these alkylating agents with RNase A and free histidine. The rate constants are summarized in Table II. The values of k_3/K_b for 2′(3′)-*O*-bromoacetyluridine and its amide analog, 2′-bromoacetamido-2′-deoxyuridine, are almost identical and approximately five times greater than the second-order constant for alkylation by bromoacetic acid. The same k_3/K_b values are roughly 1000 times greater than the rate constant for alkylation of RNase A by iodoacetamide.

It is possible to obtain a quantitative estimate of the contribution of nucleoside binding to the overall rate of alkylation by comparing the reactions of 2′(3′)-*O*-bromoacetyluridine and bromoacetamide with RNase A. This is possible mainly because each reagent reacts at the active site of the enzyme at the histidine-12 residue in contrast to what is found for bromoacetic acid. The value of k''_{obsd} for bromoacetamide alkylation at pH 5.5, 25°, is 1.9×10^{-4} M^{-1} sec^{-1} (L. Le Thi, M. Pincus, and R. P. Carty, unpublished results). This constant is less than the value of k_3/K_b for 2′(3′)-*O*-bromoacetyluridine by a factor of 210 and would represent the contribution of nucleoside binding to the overall alkylation rate if the rates of reaction of both alkylating agents with free histidine were identical. The calculated constant for the bromoacetamide–histidine reaction is 1.5×10^{-6} M^{-1} sec^{-1} (Fruchter and Crestfield, 1967; Korman and Clarke, 1956) which is 8.6 times slower than the value for the reaction of 2′(3′)-*O*-bromoacetyluridine with histidine. Therefore the effect of nucleoside binding on the overall reaction rate of 2′(3′)-*O*-bromoacetyluridine with RNase A must be revised downward to a factor of approximately 25 which represents the increment to the rate of alkylation provided for by specific active-site binding and orientation of the bromoacetyl residue. The overall rate enhancement of the reaction of 2′(3′)-*O*-bromoacetyluridine with RNase A relative to that with histidine is 3100, so that the rate acceleration due to the enhanced nucleophilicity of the imidazole ring of the histidine-12 residue is roughly 100-fold.

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The Specificity of Induced Conformational Changes. The Case of Yeast Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The specificity of induced conformational changes and of the probes used to detect them has been investigated in yeast glyceraldehyde-3-phosphate dehydrogenase. Cyanylation of the active-site SH groups in two of the four identical subunits of glyceraldehyde-3-phosphate dehydrogenase has no effect on reactivity of the unmodified SH groups toward the cyanylating reagent (2-nitro-5-thiocyanobenzoic acid, NTCB) but results in total loss of catalytic activity. Cyanylation of the dicarboxamidomethylated enzyme was four orders of magnitude slower than with the unmodified enzyme in contrast to cyanylation of the dicyanylated enzyme. Cyanylation by NTCB as well as alkylation by iodoacetate and acylation with β-(2-furyl)acryloyl phosphate are enhanced in the presence of NAD^+ while alkylation by iodoacetamide is inhibited by NAD^+. In the absence of NAD^+, hydrolysis of the acylated enzyme is faster than phosphorolysis while the reverse is true in the presence of NAD^+. NAD^+ accelerates hydrolysis of the 3-phosphoglyceroylated enzyme about 60-fold but decreases the rate of hydrolysis of the furylacryloylated enzyme by a factor of 17. Other examples of the specificity of the induced conformational changes and the probes are described. The conformational changes induced by NAD^+ make the protein specifically reactive toward its physiological substrates and less reactive toward extraneous competing compounds.

The role of conformational changes induced by substrates, inhibitors, and activators is now well established. These changes in protein structure play a role in enzyme specificity and enzyme regulation and in the cooperative interaction between subunits (Koshland and Neet, 1968). Nevertheless certain features of these conformational changes continue to raise questions. For example, a two-state model (Monod et al., 1965) suggests that a particular reactive group is either in one structural position or another and hence will exist in two different states of reactivity. On the other hand, a ligand induced model (Koshland et al., 1966) suggests that the position of the reactive groups will depend on the ligand inducing the conformational change and hence reactivity may depend specifically on the ligand which is binding to the protein. Secondly, different probes may give different signals when changes in structural alignments occur. For example, a probe which responds to hydrophobicity attached to a group which moves from one hydrophobic environment to another would signal "no net change in conformation", whereas one which measures accessibility to a chemical reagent would signal a significant change.

To throw further light on these questions, yeast glyceraldehyde-3-phosphate dehydrogenase has been examined. Extensive studies on the cooperativity properties of the enzyme (Kirschner et al., 1966, 1971; Chance and Park, 1967; Cook and Koshland, 1970; Kirschner, 1971; Sloan and Velick, 1973; Stallcup and Koshland, 1973a,c) and the mechanism of action of GPD from a variety of species (Krimsky and Racker, 1963; Trentham, 1971; Orsi and Cleland, 1972; Harrigan and Trentham, 1973, 1974) have been published.

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