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Ethoxyformylation of Proteins. Reaction of Ethoxyformic Anhydride with α-Chymotrypsin, Pepsin, and Pancreatic Ribonuclease at pH 4*

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ABSTRACT: The use of ^{14}C-labeled ethoxyformic anhydride has shown that this reagent rapidly acylates "accessible" imidazole and amino groups in proteins at pH 4. *N*-Ethoxyformylimidazole has a half-life of 55 hr at pH 7, 25°. At pH 4, one ethoxyformyl group is introduced into pepsin at all reagent concentrations tested. The site of modification is the α-amino group. There is no reaction with the single histidine residue and no change in enzymic activity. In ribonuclease, three of the four histidine residues react rapidly at about the same rate. The fourth histidine residue appears to be completely inaccessible.

The ethoxyformyl groups can be removed in minutes at pH 7 with 0.1 M hydroxylamine, with recovery of enzymic activity. At pH 4 several amino groups in ribonuclease are as reactive as the imidazole groups. At pH 4 and 10^{-4} M reagent, one ethoxyformyl group is introduced into α-chymotrypsin at the active-site serine residue. At higher concentrations amino groups are ethoxyformylated. The reactivity of the two histidine residues in chymotrypsin is very much depressed compared with that of the histidines in ribonuclease.

Ethoxyformic anhydride is at the same time an ester and an anhydride; because of resonance involving the ester function, it is less reactive than many anhydrides. Ethoxyformic anhydride has been introduced recently as a food preservative due to its bactericidal action. This anhydride is ideal for this purpose because in aqueous solution it hydrolyzes to ethanol and carbonic acid with a half-life of about 25 min near neutrality.

This paper presents results of an exploratory study of the reaction of ethoxyformic anhydride with bovine α-chymotrypsin, bovine pancreatic ribonuclease, and swine pepsin at pH 4. We were led to these experiments by the report of Fedorcsák and Ehrenberg (1966) that ethoxyformic anhydride inactivates ribonuclease and trypsin. Recently ethoxyformic anhydride has been employed to inactivate nucleases in the preparation of RNA (Solymosy *et al.*, 1968), to inactivate arginine and creatine kinases (Pradel and Kassab, 1968), and to modify actin (Mühlrad *et al.*, 1969). We have investigated the reaction of radioactive ethoxyformic anhydride with proteins in more detail and find that ethoxyformic anhydride has a specificity of reaction which, although similar, is significantly different from other acylating agents used in protein modification studies.

Experimental Section

[1-^{14}C-ethyl]Ethoxyformic Anhydride. Xylene and ether were dried over sodium wire. Small pieces of sodium were melted in dry refluxing xylene to remove the oxide coating. Sodium sand was prepared immediately before use by shaking the desired quantity of cleaned sodium in about 20 ml of hot xylene. Absolute ethanol was dried by reaction of water with magnesium ethoxide (Fieser, 1955).

Sodium sand (467 mg, 20.3 mmoles) was washed twice with dry ether and transferred in 15 ml of ether to a dry 50-ml flask placed in an ice bath and outfitted with a dropping funnel, gas inlet tube, and a Dewar condenser filled with ice and protected with a $CaCl_2$ drying tube. Dry ethanol (974 mg, 21.1 mmoles) was used in portions to transfer 1 mCi of [1-^{14}C]ethanol (ICN, 20 mCi/mmole) to the dropping funnel. Magnetic stirring and a stream of carbon dioxide through a concentrated sulfuric acid bath and then into the ether were started. The ethanol–ether was added over a period of 4 min and the dropping funnel was washed with two 2-ml portions of ether. The reaction mixture was then allowed to stand at room temperature for 4 hr. After the ether had been removed at reduced pressure, freshly

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[1] Abbreviations used are: *N*-Ac-L-TyrEt, *N*-acetyl-L-tyrosine ethyl ester; RNase, pancreatic ribonuclease. Although *Chemical Abstracts* lists ethoxyformic anhydride, European chemists tend to use the name diethyl pyrocarbonate for this compound. In view of the pattern of reactions with nucleophiles, *i.e.*, acylation of nucleophiles or ethoxyformylation, the name ethoxyformic anhydride is more appropriate.

distilled ethyl chloroformate (3.0 ml, 32 mmoles) was added, and the reaction mixture was kept at 58° for 17 hr. The white slurry was centrifuged and the supernatant was transferred to a Kontes short-path distillation apparatus. The precipitate was washed twice with 1 ml of ether and the wash was added to the distilling flask. The product was isolated by distillation *in vacuo*, the fraction boiling at 37° (0.25 mm) being collected. The overall yield of colorless product was 1.98 g or 59% based on ethanol. *Anal.* Calcd for $C_6H_{10}O_5$: C, 44.42; H, 6.22. Found: C, 44.56; H, 6.28. Density 1.12 g/ml at 25°. The [^{14}C]ethoxyformic anhydride had a molar activity of 5.9×10^{-2} mCi/mmole and was stored in sealed-glass ampoules at −15°. Unlabeled ethoxyformic anhydride was prepared in the same manner.

N-Ethoxyformylimidazole. The published procedure of Staab (1957) was repeated. The reaction mixture was distilled and the fraction boiling between 45 and 50° at 0.3 mm was collected. This material was redistilled through a short Vigreux column, the fraction boiling from 48 to 51° (0.3 mm) being collected [lit. bp 99–100° (12 mm); however at this temperature the product slowly decomposes to *N*-ethylimidazole, which has the same boiling range]. *Anal.* Calcd for $C_6H_8N_2O_2$: C, 51.39; H, 5.76; N, 20.00. Found: C, 51.06; H, 6.22; N, 21.03. The ultraviolet spectra were measured in aqueous buffers over the pH range 1.0 to 7.5. Extinction coefficients of the protonated and basic species were obtained from Beer's law plots using standard solutions of ethoxyformylimidazole in 0.1 M HCl and 0.03 M phosphate buffer (pH 7.5). The pK_a value was determined spectrophotometrically at 230 nm by plotting log $[(A - A_A)/(A_B - A)]$ against pH.

O-Ethoxyformyl-N-acetyl-L-tyrosine Ethyl Ester. Freshly distilled ethyl chloroformate (0.28 ml, 3 mmoles) was added to a solution of 807 mg of *N*-Ac-L-TyrEt·H_2O (3 mmoles) in 4 ml of anhydrous, peroxide-free dioxane and 0.34 ml of 2,6-lutidine. After 3 hr the lutidine·HCl which precipitates was filtered and washed with 1 ml of dioxane. The filtrate was poured into 10 ml of ice water and the product which separates was recrystallized from 10 ml of ethanol and 10 ml of water; yield 34%, mp 101°. *Anal.* Calcd for $C_{16}H_{21}NO_6$: C, 59.43; H, 6.55. Found: C, 59.64; H, 6.30.

Kinetic Studies of Reactivity of N-Ethoxyformylimidazole. Buffers were prepared with glass-distilled water: 0.01 and 0.02 M citrate (pH 2.0 to 3.0), 0.04 and 0.10 M acetate (pH 4.0–5.7), 0.03 and 0.06 M phosphate (pH 6.7–7.7), and 0.02 and 0.04 M Tris (pH 6.3–9.3). The ionic strength was maintained at 0.10 M by addition of NaCl to all buffers except the acetate buffers. pH was adjusted with a Radiometer Automatic titrator (Model TTT-1) using a scale expander and a G202B glass electrode. Rate constants were obtained by plotting the extent of reaction, $A_\infty - A_t$, against time on semilogarithmic paper and by calculating the first-order rate constants from the equation $k = 0.693/t_{1/2}$. Rate constants were extrapolated to zero buffer concentrations.

Pepsin. Crystallized swine pepsin (Worthington Biochemical Corp.) was occasionally found to contain an impurity in which a histidine residue was reactive with ethoxyformic anhydride. Activation of crystalline pepsinogen (Worthington Biochemical Corp.) by the method of Rajagopalan *et al.* (1966) gave pepsin in 60% yield. The pepsin, judged to be pure by amino acid analysis, was stored as a frozen solution in sodium acetate buffer (0.1 M, pH 5.0) at 1.5 mg/ml. The specific activity of pepsin was determined using two substrates, hemoglobin and phenyl sulfite (Reid and Fahrney, 1967). For assay of sulfite esterase activity a 0.1 M stock solution of phenyl sulfite in dry acetonitrile was prepared and stored in a refrigerator. Freshly prepared 0.1 mM phenyl sulfite (3 ml) in sodium phosphate buffer (0.1 M, pH 2.0) was equilibrated at 25° in a 1-cm light path cuvet; 100 μl of enzyme solution was added with mixing and the increase in absorbance at 270 nm was recorded using a Gilford recording spectrophotometer. The concentration of pepsin was estimated from the absorbance at 280 nm (ϵ 5.1×10^4 cm^{-1} M^{-1}; Perlmann, 1966).

Pancreatic ribonuclease A was purified as described by Heinrikson *et al.* (1965) using commercial ribonuclease (Sigma Chemical Corp.). The ribonuclease fraction was concentrated to 10 ml by rotary evaporation, filtered through a 1.5 × 30 cm Sephadex G-25 column equilibrated with sodium acetate buffer (0.1 M, pH 5.5), and stored in a deep freeze. The concentration of ribonuclease was determined spectrophotometrically at 280 nm (ϵ 9.8×10^3 cm^{-1} M^{-1}). For assay of ribonuclease activity, 0.1 ml of enzyme solution was added to a cuvet containing 0.5 ml of 10 mM cytidine 2′,3′-cyclic phosphate and 2 ml of sodium acetate buffer (0.1 M, pH 5.0) thermostated at 25°. The hydrolysis of the cyclic phosphate ester was followed by the increase in absorbance at 300 nm. Initial rates were not measured because of marked product inhibition. Since the time required for the hydrolysis of a given fraction of substrate is inversely proportional to enzyme concentration, a standard curve of the time required for the middle phase (30–60%) of the reaction versus enzyme concentration was used.

Bovine α-chymotrypsin from Worthington Biochemical Corp. was used without further purification. The concentration of enzyme was estimated spectrophotometrically (ϵ 5.0×10^4 cm^{-1} M^{-1} at 280 nm); the concentration of active enzyme as measured by titration with phenylmethanesulfonyl fluoride (Gold and Fahrney, 1964) was generally 8–10% lower than the spectrophotometric values. Enzymic activity was assayed spectrophotometrically using *N*-Ac-L-TyrEt as substrate (Schwert and Takenaka, 1955).

Ethoxyformylation of proteins was performed at protein concentrations of 0.5–1.5 mg/ml in sodium acetate buffer (0.1 M, pH 4.0) unless otherwise indicated. For experiments at high ethoxyformic anhydride concentrations up to about 30 mM (saturation), the reagent was added directly to the enzyme solution with a microsyringe; a few seconds of agitation was sufficient to dissolve the ethoxyformic anhydride. Otherwise stock solutions of approximately 100 mM ethoxyformic anhydride were prepared in a vial fitted with a rubber septum by injecting 8 μl of [^{14}C]ethoxyformic anhydride into 0.5 ml of dry acetonitrile with a microsyringe. The concentration was checked by radioactivity assay or by a spectrophotometric assay. The latter is based on the reaction of ethoxyformic anhydride with imidazole to form *N*-ethoxyformylimidazole which has an absorption maximum at 230 nm (ϵ 3.0×10^3 cm^{-1} M^{-1}). An aliquot of ethoxyformic anhydride solution was added to 3 ml of 10 mM imidazole solution at pH 7.5 in a cuvet having a 1-cm light path; the reaction of ethoxyformic anhydride with imidazole is quantitative under these conditions ($t_{1/2} \approx 2$ sec). Stock solutions of ethoxyformic anhydride in acetonitrile are stable for several months, if refrigerated.

After the desired reaction time at 25°, the reaction mixture (0.5–2 ml) was applied to a column (0.6 × 12 or 1.1 × 25 cm) of Sephadex G-25 (fine) equilibrated in sodium acetate buffer (0.1 M, pH 6.0); this procedure afforded a good separation of

[^{14}C]ethoxyformyl protein from [^{14}C]ethanol and unreacted [^{14}C]ethoxyformic anhydride. The macromolecule fraction was diluted for spectrophotometric determination of protein concentration. Aliquots were taken for radioactivity and enzymic activity assays.

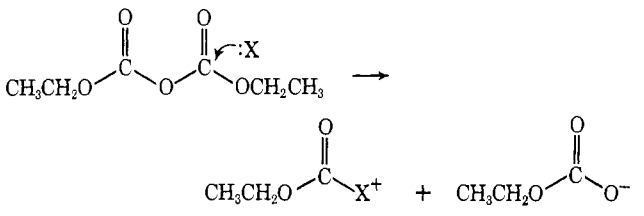
Radioactivity was measured by liquid scintillation counting. Protein solution (1 ml) was mixed with 10 ml of Bray's (1960) solution; corrections for quenching were obtained by adding [^{14}C]ethoxyformic anhydride internal standards.

Dansylation of Pepsin. Approximately 0.5 mg of pepsin or ethoxyformylpepsin in 0.35 ml was passed through a 0.9 × 13 cm column of Sephadex G-25 using 0.1 M NH_4HCO_3 as eluent. The protein fraction was lyophilized and the protein was placed in a 4 × 76 mm glass tube. Then 20 μl of a solution of dansyl chloride in acetone (10 mg/ml) was added. After 3 hr the protein was precipitated by adding 1 ml of acetone; the precipitate was washed twice with acetone and dried at 50°. After addition of 20 μl of 5.7 M HCl, the tube was sealed *in vacuo* and placed in a refluxing toluene bath for 18 hr. After HCl had been removed *in vacuo*, 20 μl of M NH_4OH was added and the suspension was centrifuged. The supernatent was spotted either on Eastman cellulose Chromagram sheet (No. 6065) for ascending chromatography in solvent A or in silica gel Chromagram sheet (No. 6061) for ascending chromatography in solvent B. The solvent systems were (A) petroleum ether–toluene–acetic acid–water (50:50:85:15, upper phase) and (B) ethyl acetate–2-propanol–acetic acid (45:35:20). Spots were located by their fluorescence and compared with standards run simultaneously.

Dinitrophenylation. The procedure of Fraenkel-Conrat *et al.* (1955) was used. After the protein had been hydrolyzed for 18 hr at 110° with 5.7 M HCl in sealed evacuated tubes, amino acid analyses were performed on a Beckman-Spinco Model 120C amino acid analyzer. A 3-cm column of PA 35 resin was used to establish the ε-DNP-lysine to arginine ratio, whereas the lysine to arginine ratio was determined with the normal 5-cm column. The area of ε-DNP-lysine peak, appearing soon after arginine, was calculated by Simpson's rule for integration with a precision of ±4% at the 25-nmole level (McCracken and Dorn, 1964).

Results

Model Studies. Ethoxyformic anhydride is susceptible to nucleophilic attack at one of the carbonyl carbon atoms. Studies on the formation and reactions of several ethoxyformyl derivatives provided information useful in interpreting results of the protein modification studies discussed below.



Imidazole reacts rapidly with ethoxyformic anhydride to form *N*-ethoxyformylimidazole. The rate of ethoxyformylation in aqueous solution can be followed spectrophotometrically at 230 nm, λ_{max} for *N*-ethoxyformylimidazole (ϵ 3.0 × 10^3 cm^{-1} M^{-1}). The second-order rate constant is 54 ± 2 M^{-1} sec^{-1} at 25° for attack by the free base. Ethoxyformic anhydride reacts quantitatively with a large concentration of imidazole as compared with the acylating agent, and the concentration of ethoxyformic anhydride solutions can be established spectrophotometrically. The reaction is complete in 30 sec at pH 7.0 and 0.10 M total imidazole.

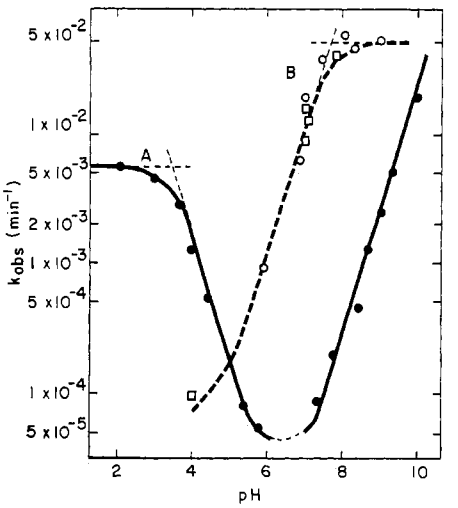


FIGURE 1: Comparison of the log k_{obs} − pH plots for hydrolysis of *N*-ethoxyformylimidazole (curve A) and ethoxyformyl-α-chymotrypsin (curve B), where k_{obs} is the observed pseudo-first-order rate constant at 25°. For curve B rates constants were determined by measuring loss of ^{14}C from [^{14}C]ethoxyformyl-α-chymotrypsin (□) and recovery of *N*-Ac-L-TyrEt esterase activity (○).

Several nucleophilic reactions of *N*-ethoxyformylimidazole are of interest. The pH profile for hydrolysis at 25° is shown in Figure 1, curve A. The half-life is about 2 hr at pH 2, 55 hr at pH 7, and 18 min at pH 10. The ultraviolet absorption spectrum of the protonated species shows a broad band at λ_{max} about 222 nm (ϵ 2.2 × 10^3 cm^{-1} M^{-1}, 0.1 M HCl). A spectrophotometric titration is possible in view of the relatively slow rates of hydrolysis of the acidic and basic forms and gives a value of 3.6 for the pK_a' of *N*-ethoxyformylimidazolium ion. The straight-line sections of the double logarithmic plot in Figure 1 have integral slopes of 0, −1, and +1 with increasing pH. The plateau and descending limb extrapolate to an intersection at pH 3.6 corresponding to a value of $pK_{app} = pK_a'$. This suggests that the marked increase in the rate of hydrolysis from pH 6 to 2 represents nucleophilic attack by water on the protonated species. The straight-line section above pH 7 represents attack by hydroxide ion on the neutral species.

Aqueous hydroxylamine (0.5 M, pH 7) removes the ethoxyformyl group completely in several minutes. The immediate product of the reaction of hydroxylamine with ethoxyformylimidazole is probably the hydroxamate of ethoxyformic acid. This compound was synthesized in this laboratory. It gives a deep blue color with acid–ferric chloride solutions, but the color fades too rapidly to be of analytical value. The same transient color test was observed for the reaction of hydroxylamine with ethoxyformylimidazole.

O-Ethoxyformyl-*N*-acetyltyrosine ethyl ester, synthesized in a nonaqueous solvent, is four-times less reactive toward hydroxylamine than *N*-ethoxyformylimidazole. The removal

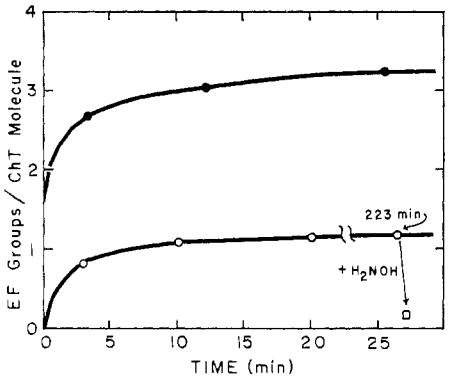


FIGURE 2: Time course for the reaction of α-chymotrypsin with 0.1 mM (○- - - -○) and 14 mM (●—●) [^{14}C] ethoxyformic anhydride at pH 4.0, 25°. □ represents ^{14}C [ethoxyformyl] groups remaining after treatment with 0.1 M hydroxylamine at pH 7.0 for 5 min.

of the *O*-ethoxyformyl group can be followed spectrophotometrically at 278 nm; the half-life in 1 M H_2NOH at pH 7 is about 45 sec at 25°. There is a 110-fold difference in the rate of alkaline hydrolysis of the two compounds. The rate constants for these reactions and for reactions of several related compounds are summarized in Table I.

Since ethoxyformic anhydride is water soluble and reacts rapidly with imidazole groups to give a relatively stable *N*-ethoxyformyl derivative, the reactivity of histidine residues in proteins toward this reagent was investigated. In view of the pH profile for hydrolysis of the imidazole derivative and reports in the literature that the reaction of ethoxyformic anhydride with amino groups in model compounds occurred only above pH 7 (Larrouquère, 1964; Mühlrad *et al.*, 1967), pH 6 might appear to be the optimal pH. However, for our exploratory studies ethoxyformic anhydride was allowed to react with proteins at pH 4 followed by gel filtration at pH 6 because experiments had shown that ethoxyformylation of amino groups in proteins is slow at pH 4 but becomes rapid at higher pH values. Larrouquère (1965) has reported that ionized sulfhydryl groups react rapidly with ethoxyformic anhydride, and proteins without SH groups were selected for initial studies.

Ethoxyformylation of α-Chymotrypsin. The reaction of chymotrypsin with 0.1 mM ethoxyformic anhydride at pH 4–7 results in reversible loss of enzymic activity with the introduction of 1.0 equiv of ethoxyformyl group/mole of active site. At pH 7 the loss of activity was complete within the time of mixing. Figure 2 presents the time course of ^{14}C labeling at pH 4 at two different ethoxyformic anhydride concentrations. At neutral pH ethoxyformylchymotrypsin recovers full enzymic activity with the concomitant loss of ^{14}C. The rates were followed by measuring the disappearance of protein-bound ^{14}C and the recovery of activity, starting with monoethoxyformylchymotrypsin prepared at pH 4 and then gel filtered into buffers at higher pH values. The reactions follow pseudo-first-order kinetics in each case; the log k_{obs} – pH profile is presented in Figure 1, curve B. The deacylation is dependent on the state of ionization of a group of pK_a' about 7.4, and the log k_{obs} – pH profile has a slope of 1.0.

The half-life of monoethoxyformylchymotrypsin in water is about 29 min at pH 7, which agrees well with that reported for the turnover of *p*-nitrophenyl ethyl carbonate (Hartley and Kilby, 1954; Shah and Conners, 1968). The rate is about 400 times faster than that of the model compound *N*-ethoxyformylimidazole, and the pH dependence of the two reactions is quite different (Figure 1). Dixon *et al.* (1956) have shown that the high reactivity of the acetyl group in acetylchymotrypsin is reversibly lost when acetylchymotrypsin is dissolved in 8 M urea. An examination of the reactivity of ethoxyformylchymotrypsin in 8 M urea gave similar results. The rate of hydrolysis was not detectable at an apparent pH value of 7.0. Although the reaction of ethoxyformylchymotrypsin with neutral hydroxylamine is too fast to measure conveniently in water, the rate of reaction with 1 M hydroxylamine in 8 M urea at pH 7.0 is very slow and is difficult to determine precisely. The upper limit for the rate constant was estimated to be 3×10^{-3} M^{-1} min^{-1}, based on total hydroxylamine concentration, which is three powers of ten less than the rate constant for the reaction of *N*-ethoxyformylimidazole with neutral hydroxylamine. These results appear to rule out acylation of a histidine residue.

Treatment of phenylmethanesulfonyl-α-chymotrypsin with 0.1 mM [^{14}C]ethoxyformic anhydride leads to the incorporation of less than 0.1 ethoxyformyl residue/molecule. Since phenylmethanesulfonyl fluoride reacts with the active-site serine residue of chymotrypsin (Gold and Fahrney, 1964), this result provides further evidence that site of ethoxyformylation is the hydroxyl group of the serine residue in the active site. Since recovery of activity and loss of the [^{14}C]ethyl moiety of the ethoxyformyl group proceed at the same rate, the reactivation of enzyme is probably a deacylation step analogous to that of ordinary substrates.

Experiments at higher concentrations of ethoxyformic

TABLE I: Rates of Reactions of Ethoxyformyl Derivatives and Related Compounds at 25°.

Compound	Alkaline Hydrolysis, k_{OH^-} (M^{-1} min^{-1})	Neutral Hydroxylamine, k_2[a] (M^{-1} min^{-1})
Ethyl *N*-methylcarbamate	3.4×10^{-4} [b]	
Diethyl carbonate	2.7[c]	
Ethyl acetate	6.8[d]	
O-Ethoxyformyl-*N*-acetyl-L-tyrosine ethyl ester	1.0×10^{2} [e]	0.91
N-Ethoxyformylimidazole	4.6×10^{2} [f]	4.0
N-Acetylimidazole	1.0×10^{4} [g]	*h*

[a] Based on the total concentration of hydroxylamine, pH 7.0, ionic strength 1.0. [b] Dittert and Higuchi (1963). [c] Estimated from data of Miller and Case (1935). [d] Kirsch and Jencks (1964). [e] Calculated from pseudo-first-order rate constant measured at pH 10.54, 0.1 and 0.2 M triethylamine. [f] Calculated from a plot of k_{obs} *vs.* [OH^-], pH 6.7–9.3, ionic strength 0.1. [g] Jencks and Carriuolo (1959). [h] Too fast to measure.

TABLE II: Amino Acid Analysis of Pepsin Derivatives.

Amino Acid	Theory	Found		
		Pepsin	DNP-pepsin	DNP-ethoxyformyl-pepsin
Lysine	1	1.03 ± 0.03	*c*	0.29 ± 0.02
Histidine	1	0.97 ± 0.05	*c*	*c*
Arginine[a]	2	2.00	2.00	2.00
DNP-lysine			0.83 ± 0.04	0.55 ± 0.02
Corrected DNP-lysine[b]			1.04	0.69
Total lysine species			1.04	0.98

[a] Arginine is used as a reference, at 2 moles/mole of enzyme. Each value represents average of duplicate experiments. [b] Assuming 80% recovery. [c] Not detectable.

anhydride proved that amino groups are ethoxyformylated and that little or no reaction takes place at histidine or tyrosine residues. Chymotrypsin (1 mg/ml) was allowed to react with varying concentrations of [^{14}C]ethoxyformic anhydride at pH 4.0 for 1 hr. The number of ethoxyformyl groups introduced per enzyme molecule was determined from the molar radioactivity after gel filtration at pH 4 and is plotted as a function of initial ethoxyformic anhydride concentration in Figure 3. Extrapolation of the linear portion of the curve for chymotrypsin indicates that ethoxyformic anhydride reacts preferentially with two groups; one is the serine-195 residue and the other probably an amino group. Chymotrypsin that has been allowed to react with 2 mM [^{14}C]ethoxyformic anhydride at pH 4 has no initial activity toward *N*-Ac-L-TyrEt and contains 2.1 ± 0.1 ethoxyformyl groups per molecule. Incubation of the inhibited enzyme at pH 10 for 45 min results in 95–98% recovery of enzymic activity with 1.0 ethoxyformyl group/molecule. None of this remaining ethoxyformyl label is lost after 2 days at pH 12; the stability of this ethoxyformyl group is indicative of an urethane derivative.

If solutions of enzyme with one to six [^{14}C]ethoxyformyl groups are allowed to stand at pH 7.0 for 4 hr, then approximately 0.9 ethoxyformyl group is lost and full enzymic activity is recovered. If the solutions are subsequently made 0.5 M in hydroxylamine at pH 6.8 and allowed to stand for 45 min, then further loss of ^{14}C label occurs. The results indicate that an additional 0.4 labile group is introduced in ethoxyformic anhydride concentrations above 10 mM, as shown in Figure 3, curve B. The fact that this labeling does not approach 1 equiv/mole of enzyme is presumptive evidence that the reaction observed at histidine is due to the presence of partially unfolded conformations representing about 40% of the α-chymotrypsin used in these experiments. Complete retention of the remaining ^{14}C label after 2 days at pH 12 suggests that the active-site serine residue is the only aliphatic hydroxyl group reacting with ethoxyformic anhydride at pH 4, as would be expected. However, several amino groups are reactive under these conditions.

The reaction of ethoxyformic anhydride with pepsin at pH 4 results in the introduction of 1.0 ± 0.1 ethoxyformyl group per enzyme molecule (Figure 3, curve E). [^{14}C]Ethoxyformic anhydride (2–4 μl/ml) was added directly to pepsin solutions at 20-min intervals. The final degree of modification of the enzyme is dependent only on the total amount of reagent added, whether it is added in one aliquot or several. Monoethoxyformylpepsin has full peptidase and sulfite esterase activity, within experimental error. No loss of ^{14}C from ethoxyformyl$_{1.0}$-pepsin takes place in 0.5 M H_2NOH either at pH 7.0 or at pH 12. This stability is presumptive evidence that the ethoxyformyl group is bound to an amino group, either that of the N-terminal isoleucine or the ε-amino group of the single lysine residue.

Experiments to identify the reactive group were based on the ability of the ethoxyformyl group to offer protection against dansylation and dinitrophenylation. Pepsin and ethoxyformylpepsin were allowed to react with dansyl chloride and then analyzed for dansyl-isoleucine and ε-dansyl-lysine. After ethoxyformylation only a trace of dansyl-isoleucine could be found, although ε-dansyl-lysine was still present. This suggests that ethoxyformylation occurred at the α-amino group but not significantly at the ε-amino group. Quantitative results from dinitrophenylation of ethoxy-

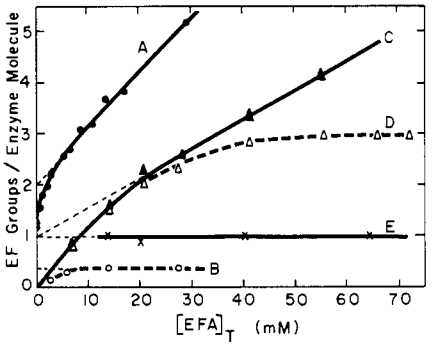


FIGURE 3: Comparison of the extent of ethoxyformylation of α-chymotrypsin, pepsin, and ribonuclease at pH 4.0 as a function of total concentration of ethoxyformic anhydride added. Curve A (●—●): chymotrypsin, total ethoxyformyl groups introduced. Curve B (○- - - -○): chymotrypsin, labile ethoxyformyl groups. Curve C (▲—▲): ribonuclease, ethoxyformyl groups introduced at amino groups. Curve D (△- - -△): ribonuclease, labile ethoxyformyl groups. Curve E (x—x): pepsin, total ethoxyformyl groups introduced (no labile ethoxyformyl groups observed).

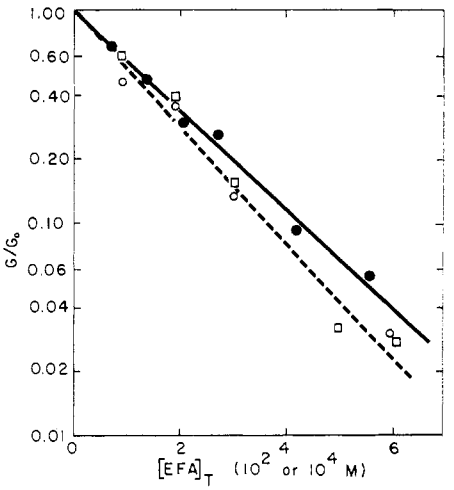


FIGURE 4: Semilog plot of G/G_0 as a function of total concentration of ethoxyformic anhydride added. For ribonuclease (●—●), G/G_0 = fraction of three histidine residues remaining; [ethoxyformic anhydride]$_T$ × 10^2 M, pH 4.0. For creatine kinase (○- - - -○) and arginine kinase (□- - - -□), G/G_0 = fraction of activity remaining; [ethoxyformic anhydride]$_T$ × 10^4 M, pH 6.1; data of Pradel and Kassab (1968).

formyl$_{1.0}$-pepsin support this conclusion. The results in Table II show that the ethoxyformyl group provided protection against dinitrophenylation of the ε-amino group. By inference, ethoxyformylation occurs preferentially at the α-amino group of the N-terminal isoleucine residue. The apparent partial protection of lysine has not yet been explained.

Reactivity of Ribonuclease toward Ethoxyformic Anhydride. The incorporation of ethoxyformyl groups into ribonuclease as a function of initial ethoxyformic anhydride concentration is also presented in Figure 3. In contrast to the results with chymotrypsin, there is reaction at a total of three histidine residues, as indicated by the results of the neutral hydroxylamine assay for labile ethoxyformyl groups (curve D). From the data in Figure 3, it is possible to arrive at the relative reactivity, k_{His}/k_{HOH}, where k_{His} is the second-order rate constant for the reaction of ethoxyformic anhydride with exposed histidine residues and k_{HOH} is the pseudo-first-order rate constant for attack by water. The linearity of the plot

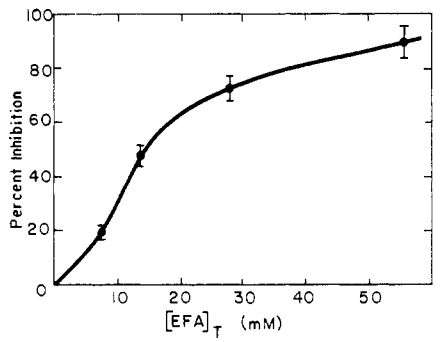


FIGURE 5: Inhibition of ribonuclease by ethoxyformic anhydride at pH 4.0, 25°. [Ethoxyformic anhydride]$_T$ is the total concentration of ethoxyformic anhydride added.

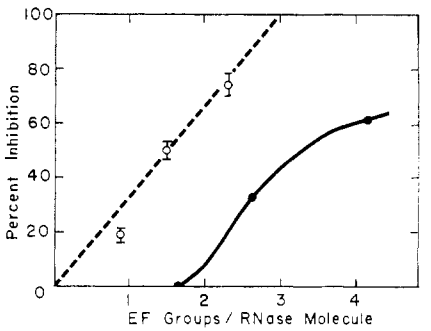


FIGURE 6: Inhibition of ribonuclease as a function of labile (○- - -○) and stable (●—●) ethoxyformyl groups introduced per enzyme molecule.

of log $(3 - x)/3$ as a function of [ethoxyformic anhydride]$_0$ shown in Figure 4 emphasizes that the three groups, probably histidines, have essentially the same reactivity toward ethoxyformic anhydride. The slope of this line is equal to the ratio, $-k_{His}:k_{HOH}$.

The original work of Fedorcsák and Ehrenberg (1966) demonstrated the rapid inhibition of ribonuclease by ethoxyformic anhydride. Figures 5 and 6 show the inactivation of the enzyme as a function of initial ethoxyformic anhydride concentration and degree of modification. Reaction of ribonuclease with 14 mM ethoxyformic anhydride modifies 3.2 groups, and enzymic activity decreases to 50%. A total of 1.5 ethoxyformyl groups was found labile in neutral and alkaline hydroxylamine; 98–100% recovery of enzymic activity was observed, provided freshly prepared solutions of recrystallized hydroxylamine hydrochloride were used. At this extent of modification the 50% inactivation is due to 50% reaction of three histidine residues, one of which is essential for activity. Clearly several amino groups react rapidly with ethoxyformic anhydride at pH 4. Figure 6 shows that two of the eleven amino groups in ribonuclease are readily acylated without affecting enzyme activity significantly. Modification of less reactive amino groups at ethoxyformic anhydride concentrations above 20 mM reduces enzymic activity.

Discussion

The work presented here demonstrates that ethoxyformic anhydride has a high degree of specificity for amino and imidazole groups at pH 4. The differences in reactivity among imidazole groups is interesting. The single histidine residue of pepsin and the two histidines of α-chymotrypsin do not appear to react at a measurable rate. Although a total of three histidine residues in ribonuclease react fairly rapidly at the same rate as the model imidazole, it is not yet known whether ethoxyformylation at histidine-12 and histidine-119 are mutually exclusive as observed for alkylation with iodoacetate (Crestfield *et al.*, 1963). If ethoxyformylation is mutually exclusive, then the other two histidines at positions 48 and 105 would have to be reactive toward ethoxyformic anhydride to account for the 3:1 stoichiometric quantity of labile ethoxyformyl groups introduced. Alternatively, ethoxyformylation may occur at histidine residues 12, 105, and 119 because X-ray structural studies have suggested that histidine-48 is rather inaccessible (Kartha *et al.*, 1967; Wyckoff *et al.*

1967). The question cannot be answered easily with ribonuclease because the lability of the *N*-ethoxyformylimidazole derivative precludes isolation of the modified peptides. Studies of the ethoxyformylation of the ribonuclease S-protein molecule in which the peptide containing histidine-12 is missing (Richards, 1958) may afford an indirect answer. While details of the reaction of ethoxyformic anhydride with the histidines of ribonuclease are not yet clear, the specificity is obvious.

The overall lack of reactivity of the histidine residues in chymotrypsin toward ethoxyformic anhydride compared with that observed in ribonuclease and the guanidine phosphotransferases merits comment. X-Ray diffraction studies of chymotrypsin have shown that one side of the imidazolium ion of histidine-57 is "freely available to the solvent" in both the native enzyme and its sulfonylserine-195 derivative at pH 4; histidine-40 is probably "inaccessible" to large reagents (Matthews *et al.*, 1967; Sigler *et al.*, 1968). Yet the reactivity of histidine-57 with ethoxyformic anhydride is greatly depressed both by sulfonylation and by the very rapid ethoxyformylation of the active-site serine. The reaction of only 0.4 histidine residue in α-chymotrypsin can be explained by assuming that partially unfolded species are present.

α-Chymotrypsin and ribonuclease each possess one amino group that is unusually reactive toward ethoxyformic anhydride at pH 4. Ethoxyformylation of other amino groups is extensive; that their reactivity is similar in both proteins is suggested by the ratio of the slopes of the linear section of curves A and C in Figure 3. This ratio is 1.8, and the ratio of amino groups, excluding the most reactive one, in α-chymotrypsin and ribonuclease is 16:10 or 1.6.

Repetitive treatment of chromatographed pepsin obtained by activation of purified pepsinogen leads to the introduction of only one ethoxyformyl group. The reaction of the α-amino group of the N-terminal isoleucine predominates, with about 20% reaction at the ϵ-amino group of the single lysine. The intriguing possibility exists that pepsin conformations are present in which the α-amino group but not the ϵ-amino group is reactive toward ethoxyformic anhydride as well as conformations in which the reverse is true. This would account for the observations described in this paper. Partial proof of this assumption could come from quantitative analysis of the N-terminal residue in the monoethoxyformyl derivative now in progress.

The specific ethoxyformylation of one amino group in pepsin is consistent with the results of Lokshina and Orekhovich (1966), who found that *N*-acetylimidazole reacted with about one amino group at pH 5.6–5.8. Concomitant with this single N-acetylation, however, acetylimidazole caused acetylation of 11–12 tyrosine residues and 95% inhibition of pepsin's proteolytic activity. The failure of N-ethoxyformylation of pepsin to inhibit enzymic activity agrees with the findings of Herriott (1935) and Philpot and Small (1938) that neither N-acetylation with ketene nor destruction of amino groups with nitrous acid alters activity. None of the tyrosine, serine, or threonine residues in pepsin are modified by the less reactive ethoxyformic anhydride under conditions used.

Although blocking of protein imidazole groups by ethoxyformic anhydride cannot be conducted without reaction at several amino groups, ethoxyformic anhydride is a useful probe for accessible histidine residues in proteins. A recent interesting example is the study by Pradel and Kassab (1968) of the reaction of ethoxyformic anhydride with creatine kinase and arginine kinase. Difference ultraviolet spectra of the ethoxyformylated enzymes revealed a maximum at 240 nm which was attributed to the N^{im}-ethoxyformylhistidine residue. Following the suggestion of Ovádi *et al.* (1967), a value of 3000 cm^{-1} M^{-1} for the difference molar extinction coefficient was used to calculate the number of histidine residues ethoxyformylated. They concluded that one and two histidines are modified in arginine kinase and creatine kinase, respectively, leading to complete loss of activity. The essential roll of the histidines would be more firmly established, however, if it were shown that enzymic activity would be restored by treatment with dilute aqueous hydroxylamine at neutral pH, as in the case of ethoxyformyl ribonuclease. For comparison, the results of Pradel and Kassab (1968) have been recalculated and plotted in Figure 4 as log (fraction enzymic activity) against total ethoxyformic anhydride concentration. Assuming that the slope represents $-k_{His}/k_{HOH}$, then k_{His} for the guanidine phosphotransferases is about 100 times greater than k_{His} for the three reactive histidines in ribonuclease, since k_{HOH} is the same at pH 4 and 6. This 100-fold difference probably reflects a 100-fold change in the concentration of the free base at pH 6 compared with pH 4. Another important observation reported by Pradel and Kassab (1968) is that the accessible sulfhydryl groups in the two enzymes were not ethoxyformylated under the conditions used.

A variety of anhydrides have been used for protein modification studies (Cohen, 1968). Currently succinic and maleic anhydride are enjoying wide popularity for acylation of amino groups, particularly in studies of subunit structure. Acylation of the imidazole ring of histidine also occurs, but the products hydrolyze rapidly. The finding that *N*-ethoxyformylimidazole hydrolyzes about two orders of magnitude more slowly at neutral pH than *N*-acetylimidazole had raised the possibility that ethoxyformic anhydride might be useful as a specific acylating reagent for accessible histidine residues in proteins. Earlier model studies had suggested that amino groups do not undergo ethoxyformylation at pH values below 7 (Larrouquère, 1964; Mühlrad *et al.*, 1967). The unexpected finding that several amino groups in the proteins studied in this paper are very reactive toward ethoxyformic anhydride at pH 4 is a familiar story in protein chemistry. "Specific" reagents have looked promising in the beginning, but closer scrutiny usually discloses the fact that no reagent is truly selective, except perhaps those that make use of the unusual reactivity of a functional group at the active site of an enzyme. The search for a specific reagent for histidine residues will probably go on, even though as pointed out by Cohen (1968) the reactivity of all nucleophilic groups in a protein is a matter of degree.

In summary, ethoxyformic anhydride offers a greater degree of selectivity than other acylating agents. Amino groups can be modified under milder conditions than previously possible. Ethoxyformylation of "accessible" histidine residues is a facile reaction that can be readily reversed. Phenolic and aliphatic hydroxyl groups are untouched.

Appendix

If a reagent, *R*, reacts both with water and with a functional

group, G, on a protein, its total rate of disappearance is given by

$$-\mathrm{d}(R)/\mathrm{d}t = k_{HOH}(R) + k_G(G)(R) \quad (1)$$

where $k_{HOH} = k'_{HOH}$ (HOH). Assuming $k_{HOH} \gg k_G$

$$(R) = (R)_0 e^{-k_{HOH}t} \quad (2)$$

where $(R)_0 = (R)$ at time $t = 0$. The rate of reaction of the protein group G with R is

$$-\mathrm{d}(G)/\mathrm{d}t = k_G(G)(R) = k_G(G)(R)_0 e^{-k_{HOH}t} \quad (3)$$

Integration and setting $(G) = (G)_0$ at $t = 0$ and $(R) = 0$ at $t = \infty$ leads to

$$\log [(G)/(G)_0] = -k_G(R)_0/k_{HOH} \quad (4)$$

The same result is obtained no matter how the addition of reagent is distributed in time:

$$\log [(G)/(G)_0] = -[k_G/k_{HOH}][(R)_1 + (R)_2 + \ldots] \quad (5)$$

In other words, the final degree of substitution of a group is a simple function of the total amount of reagent added and of the ratio of the rate constants of the competing reactions.

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