A Kinetic Study of the Reaction of Thiols with *p*-Nitrophenyl Acetate*

JAMES W. OGILVIE, J. TYSON TILDON, AND BARRY S. STRAUCH

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore 5, Maryland

Received November 18, 1963

The second-order rate constants for the reaction of *p*-nitrophenyl acetate with L-cysteine, L-cysteine ethyl ester, DL-homocysteine, glutathione, 2-mercaptoethylamine, 2-mercaptoethanol, and mercaptoacetic acid were determined at 25.0° by following spectrophotometrically the release of *p*-nitrophenol. The reactive species of the thiols in this reaction was found to be the RS^- anion. Second-order rate constants for both RS^- species of cysteine ethyl ester [$R(NH_3^+)S^-$ and $R(NH_2)S^-$] were obtained. These second-order rate constants were correlated with the pK'_a values of the sulfhydryl groups via the Brönsted equation $\log k_r = \alpha pK'_a + C$, with α equal to 0.38 and C equal to -0.75.

The proteinases ficin (Hammond and Gutfreund, 1959) and papain (Kimmel and Smith, 1954) and the esterases cholesterol esterase (Hernandez and Chaikoff, 1957) and A-esterase (Aldridge, 1953) all possess sulfhydryl groups which appear to be essential for their esterase activity. Before beginning a study of the function or functions of the sulfhydryl group in enzyme-catalyzed ester hydrolyses, the present investigation of the reaction of thiols with *p*-nitrophenyl acetate (NPA)[1] was initiated in order to characterize the nucleophilicity of sulfhydryl compounds toward the ester sp^2 carbon. Prior to this investigation, Schonbaum and Bender (1960) reported the results of their study on the hydrolysis of NPA by *o*-mercaptobenzoic acid, and Jencks and Carriuolo (1960) reported second-order rate constants for the reactions of mercaptoethanol and sodium mercaptoacetate with NPA. While the present study was in progress, Whitaker (1962) reported the results of his study on the reaction of thiols with NPA. Whitaker found that the reactive species of the thiol was the RS^- anion and that the initial products of the reaction at *p*H 6.20 and at low thiol (cysteine) concentrations were the thiol ester and *p*-nitrophenol. Since Whitaker was able to demonstrate the formation of an intermediate thiol ester only at lower *p*H values and at low cysteine concentrations, he has suggested that at higher *p*H values the intermediate thiol ester is hydrolyzed as rapidly as it is formed.

In the present study the reaction of thiols with NPA has been investigated and the nucleophilicity of the thiols toward the ester sp^2 carbon has been correlated via the Brönsted equation by utilizing the microscopic and macroscopic ionization constants of the sulfhydryl groups of the thiols.

RESULTS

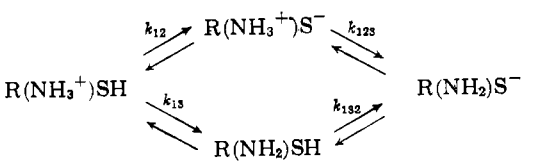
Reaction of L-Cysteine, L-Cysteine Ethyl Ester, S-Ethyl-L-cysteine, and 2-Mercaptoethylamine with p-Nitrophenyl Acetate.—The reaction between the thiol and NPA was followed at 25.0° in aqueous solution at constant *p*H (0.2 M potassium phosphate or 0.2 M potassium borate buffers) by measuring the rate of appearance of the *p*-nitrophenolate anion spectrophotometrically at 401 mμ. To simplify the kinetic treatment the thiol was present in large excess (50–100 ×) over NPA. Under these conditions and at constant *p*H the appearance of nitrophenol was found to follow first-order kinetics as can be seen in Figure 1. $d(\text{nitrophenol})/dt = k_{obs}(\text{NPA})$. The pseudo-first-order rate constant, k_1, for the reaction of the thiol with NPA at a given *p*H value was obtained by subtracting k_w, the solvolytic constant for NPA at the same *p*H, from k_{obs}. $k_1 = k_{obs} - k_w$. The values of k_w were determined independently for each *p*H value under the same conditions of buffer concentration and temperature as used in the determination of k_{obs}.

The failure of *S*-ethylcysteine to significantly increase the rate of release of nitrophenol indicates, in agreement with the findings of Whitaker (1962), that the sulfhydryl group is essential for this reaction. The very small enhancement in the rate of nitrophenol release observed with *S*-ethylcysteine, which is indicated in Figure 1, may in part be associated with the other functional groups, the carboxyl and the amino group, present in the molecule.

The data on cysteine presented in Table I show that the apparent second-order rate constant, $k'_2 = k_1/(RS_T)$, where RS_T represents the total concentration of cysteine present in the reaction mixture, is *p*H dependent in the range investigated. The true second-order rate constant $k_2 = k_1/(RS^-)$ is *p*H independent, thus confirming the results obtained by Whitaker which indicated that the reactive species was the RS^- anion. The concentration of RS^- was calculated from equation (1) (Edsall and Wyman, 1958)

$$\alpha_{SH} = \frac{(RS^-)}{(RS^-) + (RSH)} = \frac{\frac{k_{12}}{(H^+)} + \frac{k_{12}k_{123}}{(H^+)^2}}{1 + \frac{k_{12} + k_{13}}{(H^+)} + \frac{k_{12}k_{123}}{(H^+)^2}} \quad (1)$$

where k_{12}, k_{123}, and k_{13} are the microscopic ionization constants for cysteine. The data on L-cysteine ethyl ester are also presented in Table I, and it is apparent that the second-order constant k_2 is in this case *p*H dependent. This is not unexpected when the individual microscopic ionization constants for L-cysteine ethyl ester are considered.



These ionization constants (Benesch and Benesch, 1955) for L-cysteine ethyl ester at 23° are $pk_{12} = 7.45$, $pk_{13} = 6.77$, $pk_{123} = 8.41$, and $pk_{132} = 9.09$.

Thus in the *p*H range in which these kinetic studies were carried out, there would exist significant concentrations of two RS^- species [$R(NH_3^+)S^-$ and $R(NH_2)$-

* This work was supported by a research grant from the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

[1] Abbreviation used in this work: NPA, *p*-nitrophenyl acetate.

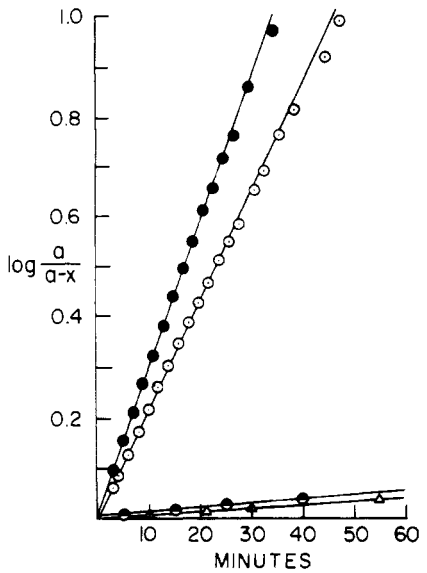


FIG. 1.—Release of nitrophenol from NPA at 25.0°. Cysteine (5.00×10^{-3} M, pH 7.00), ⊙–⊙; 4.97×10^{-3} M cysteine ethyl ester, pH 7.00, ●–●; 5.00×10^{-3} M S-ethylcysteine, pH 6.91, ⊖—⊖; H_2O, pH 6.91, △—△. All experiments carried out in 1% (v/v) dioxane-water solvent (0.2 M phosphate).

TABLE I

THE EFFECT OF pH ON THE RATES OF REACTION OF p-NITROPHENYL ACETATE WITH THIOLS AT 25.0°

Thiol	pH[a]	k'_2[b] (liters/mole min^{-1})	k_2[c] (liters/mole min^{-1})
L-Cysteine (2.61×10^{-3}–5.02×10^{-3} M)	7.00	9.94	351
	7.17	14.8	360
	7.40	26.1	383
	7.83	57.6	373
	8.15	90.5	348
	9.50	257.	387
L-Cysteine ethyl ester (1.62×10^{-3}–$5.00 + 10^{-3}$ M)	6.25	4.95	106
	6.60	9.25	117
	7.00	16.0	133
	7.87	40.0	200
	8.17	58.2	242
	8.65	108.	298
	9.32	286.	436
	9.61	312.	401
2-Mercaptoethylamine (6.65×10^{-3} M)	7.00	11.5	225
	7.40	27.5	231
	7.88	67.0	231
	8.18	101.	225
	8.64	183.	262
	8.88	234.	292
	9.42	318.	341
Potassium mercapto-acetate (4.52×10^{-3} M)	7.04	2.69	5012
	7.38	5.45	4645
	7.80	13.1	4244
	8.72	56.0	2235
	9.24	131.	1674
	9.69	345.	1782

[a] Below pH 8.0, 0.2 M potassium phosphate buffer used; above pH 8.0, 0.2 M potassium borate buffer–0.2 M KCl used. [b] $k'_2 = k_1/(RS_T)$. [c] $k_2 = k_1/(RS^-)$.

$S^-]$, each species reacting with NPA at a different rate. Therefore

$$\frac{d\ (\text{nitrophenol})}{dt} = k_2[R(NH_3^+)S^- + R(NH_2)S^-][NPA]$$
$$= k_2^+[R(NH_3^+)S^-][NPA] + k_2^\circ[R(NH_2)S^-][NPA] \quad (2)$$

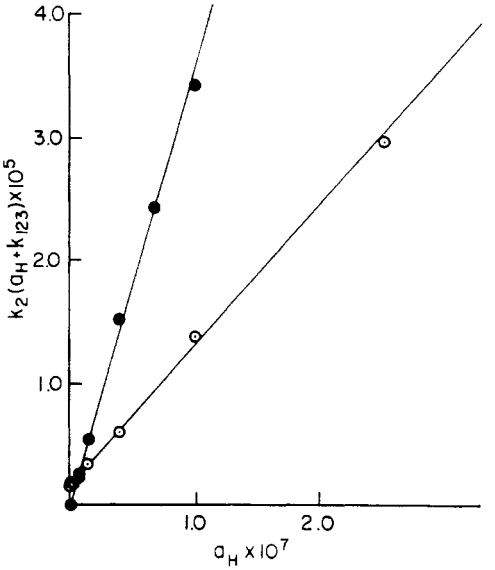


FIG. 2.—Plots of k_2 ($a_H + k_{123}$) versus a_H for the reactions of L-cysteine (●–●) and L-cysteine ethyl ester (⊙–⊙) with p-nitrophenyl acetate at 25.0°. Solvent, 1% (v/v) dioxane-water; 0.2 M phosphate or borate buffer).

and

$$k_2(a_H + k_{123}) = k_2^+a_H + k_2^\circ k_{123} \quad (3)$$

where a_H represents the hydrogen-ion activity determined by the glass electrode, k_{123} a microscopic ionization constant of cysteine ethyl ester, and k_2^+ and k_2° the true second-order rate constants for the reaction of NPA with $R(NH_3^+)S^-$ and $R(NH_2)S^-$, respectively. From equation (3) it follows that a plot of $k_2(a_H + k_{123})$ versus a_H should be linear with a slope equal to k_2^+ and an intercept at $a_H = 0$ of $k_2^\circ k_{123}$. In Figure 2 a plot of equation (3) is presented for L-cysteine ethyl ester. The values for k_2^+ and k_2° for cysteine ethyl ester calculated from Figure 2 are presented in Table II. A plot of equation (3) for cysteine is also presented in Figure 2. The value for k_2^+ for cysteine as determined from the slope of the line is shown in Table II. The microscopic ionization constants (Benesch and Benesch, 1955) for cysteine at 23° are $pk_{12} = 8.53$, $pk_{13} = 8.86$, $pk_{123} = 10.36$, and $pk_{132} = 10.03$. Therefore in the pH range investigated (7–9.5) k_2 appears to be pH independent and $k_2 = k_2^+$ within experimental error because the concentration of the $R(NH_2)S^-$ species is much lower than the concentration of the $R(NH_3^+)S^-$ species. Thus the contribution of the $R(NH_2)S^-$ species to the observed rates would be expected to be very small in the pH interval investigated. A value for k_2° for cysteine cannot be determined with any accuracy from this graph.

The k_2 values for 2-mercaptoethylamine obtained in the pH range 7.0–9.42 are also indicated in Table I. These values show an increase at the higher pH values which again is probably a result of the contribution to the observed rate by the $R(NH_2)S^-$ species. Since k_{123} was not available for 2-mercaptoethylamine, this data could not be plotted by equation (3). In view of the results obtained with cysteine and cysteine ethyl ester, it was assumed that the k_2 value at pH 7.00 should be a close approximation to k_2^+ since the contribution of the $R(NH_2)S^-$ species to the observed rate should be exceedingly small at this pH.

Reaction of Other Thiols with p-Nitrophenyl Acetate.—A kinetic study of the reaction of NPA with 2-mercaptoethanol, glutathione, and potassium mercaptoacetate

TABLE II

SECOND-ORDER RATE CONSTANTS (25.0°) FOR THE REACTION OF *p*-NITROPHENYL ACETATE WITH SEVEN THIOLS

Thiol	pK_a	k_2 (liters/ mole min^{-1})	k_2^+ (liters/ mole min^{-1})	k_2° (liters/ mole min^{-1})	k_2^- (liters/ mole min^{-1})
L-Cysteine	8.53[a]	367	354		
L-Cysteine ethyl ester	7.45, 9.09[a]		116	437	
2-Mercaptoethylamine	8.27[b]	225[c]	225[d]		
DL-Homocysteine	9.14[e]	370			
Potassium mercaptoacetate	10.31[f]				1731
Glutathione	9.2[a]				383
2-Mercaptoethanol	9.4[g]				459

[a] Benesch and Benesch (1955). [b] Danehy and Noel (1960), corrected to 25° using ΔH_i = 6.08 kcal/mole. [c] k_2 at *p*H 7.00. [d] k_2^+ estimated (see text). [e] Ryklan and Schmidt (1944). [f] Danehy and Noel (1960), corrected to 25° using ΔH_i = 7.39 kcal/mole. [g] Danehy and Noel (1960), corrected to 25° using ΔH_i = 6.49 kcal/mole.

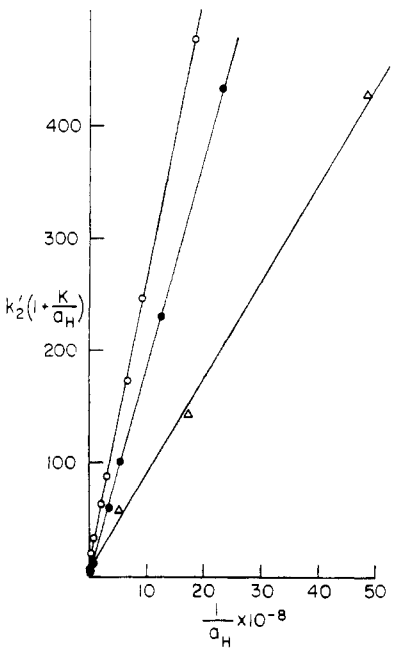


FIG. 3.—Plots of k_2' (1 + $[K/a_H]$) versus $1/a_H$ for the reactions of glutathione (○—○), 2-mercaptoethanol (●—●), and potassium mercaptoacetate (△—△) with *p*-nitrophenyl acetate at 25°. Solvent, 1% (v/v) dioxane-H_2O; 0.2 M phosphate or 0.2 M borate buffer).

was carried out in the *p*H range of 7–9.5. It was found that not only the k'_2 values, but also the k_2 values were *p*H dependent. As an example of this *p*H dependence of k_2, the data obtained for potassium mercaptoacetate are presented in Table I.

The k_2 values for each of these thiols displayed an unexpected decrease in value with increasing *p*H and each appeared to approach its own limiting value at the higher *p*H values. The greatest change in k_2 with *p*H was observed with mercaptoacetate, the thiol with the highest pK_a. The k_2 values for 2-mercaptoethanol and glutathione decreased by 25% and 50%, respectively, in this *p*H interval. The results reported by Whitaker (1962) for glutathione, sodium mercaptoacetate, sodium β-mercaptopropionate, 2-mercaptoethanol, and *n*-propyl mercaptan at *p*H 6.90 and 7.80 also suggest that k_2 is *p*H dependent and decreases with increasing *p*H. A possible explanation of this observation may be that some other species present in the reaction mixture is able to facilitate the release of nitrophenol. Those thiols for which the k_2 was found to decrease with increasing *p*H also possess higher pK values than the α-amino thiols discussed above; therefore, at the lower end of the *p*H range investigated, a small contribution to the observed rate by another species could produce a marked change in k_2 since the concentration of RS^- at these lower *p*H values and its contribution to the observed rate would be very small. For example, if the RSH species were able to catalyze the release of nitrophenol, then

$$\frac{d\ (\text{nitrophenol})}{dt} = k'_2(RS^- + RSH)(NPA)$$
$$= k_2^-(RS^-)(NPA) + k_2^H(RSH)(NPA) \quad (4)$$

and

$$k'_2\left(1 + \frac{K}{a_H}\right) = k_2^- K\left(\frac{1}{a_H}\right) + k_2^H \quad (5)$$

where a_H represents the hydrogen-ion activity determined by the glass electrode, K the ionization constant of the sulfhydryl group, and k_2^- and k_2^H the true second-order rate constants for the reaction of NPA with RS^- and RSH, respectively. From equation (5) it follows that a plot of k'_2 (1 + $[K/a_H]$) vs $1/a_H$ should be linear with a slope equal to $k_2^- K$ and an intercept at $1/a_H = 0$ of k_2^H. In Figure 3 a plot of equation (5) is presented for 2-mercaptoethanol, glutathione, and sodium mercaptoacetate. As seen in Figure 3, the experimental data were found to be consistent with equations (4) and (5) and the k_2^- values reported in Table II were obtained from the slopes of these lines. It is apparent that k_2^H values as determined from the intercept at $1/a_H = 0$ are very small. Although the experimental data are consistent with equation (4), other explanations are readily apparent. For example, k_2^H could also represent a rate constant for contaminating materials in the thiols. It is unlikely that k_2^H represents only the rate constant for the reaction with the carboxyl anion since it is considerably larger than the 9.5 × 10^{-4} liter/mole min^{-1} reported by Bruice and Lapinski (1958) for the acetate anion. Furthermore, the k_2^H appears to be larger than the k'_2 of 0.0875 liter/mole min^{-1} calculated for *S*-ethylcysteine from Figure 1.

The k_2 values found for the reaction of homocysteine with NPA also decreased by approximately 30% with increasing *p*H in the *p*H interval 7–9.5. The homocysteine data cannot be plotted graphically by equation (5) because it is an amino thiol and its microscopic dissociation constants must be considered. Therefore the second-order rate constant, k_2, for homocysteine was obtained graphically as indicated in Figure 4 from equation (6)

$$k'_2 = k_2\alpha_{SH} \quad (6)$$

by plotting k'_2 versus α_{SH} where α_{SH} was calculated from equation (1). The k_2 value for homocysteine as determined in this manner appears in Table II.

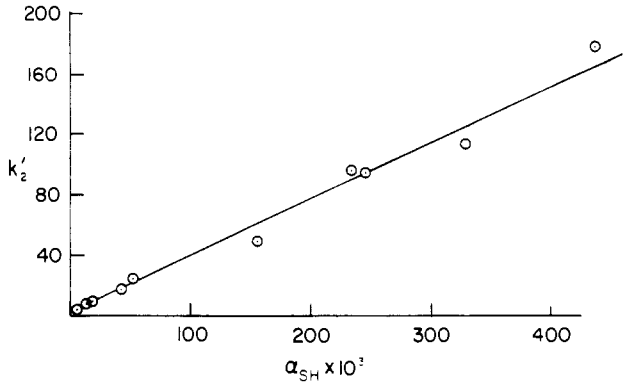


FIG. 4.—Plot of k_2' versus α_{SH} for the reaction of homocysteine with p-nitrophenyl acetate at 25°. Solvent, 1% (v/v) dioxane-water; 0.2 M phosphate or 0.2 M borate buffer).

All kinetic experiments were carried out at a constant buffer concentration; consequently, the ionic strength varied from a calculated value of approximately 0.2 M to 0.6 M. The effect of ionic strength changes of this magnitude on the rate of the reaction of thiols with NPA at a given pH was measured for four of the thiols by increasing the ionic strength of the reaction medium from a calculated 0.2 M to 0.7 M by the addition of KCl. No significant changes in the rates of the reaction of these thiols with NPA [$k_{2(\mu=0.7)}/k_{2(\mu=0.2)}$ = 0.97, 0.95, 1.0, 1.1 for cysteine, mercaptoethanol, mercaptoacetate, and glutathione, respectively] were observed as a result of increasing the ionic strength of the reaction medium.

Discussion

The nucleophilicity of oxygen and nitrogen bases toward the ester sp^2 carbon may be correlated by the Brönsted equation (equation 7).

$$\log k_r = \alpha pK'_a + C \qquad (7)$$

In Figure 5, a Brönsted-type plot is presented which was prepared by plotting the log of the true second-order rate constant versus the pK'_a of the sulfhydryl group for each of the thiols investigated. From Figure 5 it is apparent that bases characterized by $-S^-$ as the attacking nucleophile may be correlated by the Brönsted equation.

$$\log k_r = 0.38pK'_a - 0.75 \qquad (8)$$

One interesting feature of the Brönsted plot of the thiols is the low α value of 0.38. This is approximately one-half the α value of 0.8 reported by Bruice and Lapinski (1958) for oxygen and nitrogen bases. From a biochemical standpoint, the low α value of thiols could be of considerable importance since it implies that a sulfhydryl group with a pK_a low enough to be appreciably ionized in the physiological pH range would still be a very efficient nucleophile. The Brönsted plots for imidazoles and phenols drawn from the data of Bruice and Lapinski (1958) are also presented in Figure 5. It can be seen that a thiol with a pK_a of 7 would be approximately 4-fold more efficient as a nucleophile in this reaction with NPA than an imidazole with a pK_a of 7.

One possibility which was considered at the beginning of this study was that the polyfunctional thiols such as cysteine and β-mercaptoethylamine might utilize more than one functional group in a concerted mechanism. This seems very unlikely from the data since the thiols studied possess a variety of other functional groups but they all fit a single Brönsted plot. No conclusions can be drawn from these data on the possible role of the other functional groups in the breakdown of the postulated thiol ester intermediate.

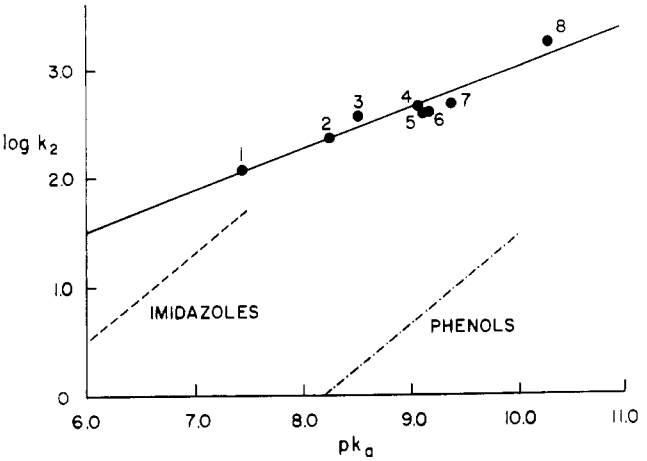


FIG. 5.—A Brönsted-type plot for the reaction of thiols with p-nitrophenyl acetate. (1) Cysteine ethyl ester [$R(NH_3^+)S^-$]; (2) 2-mercaptoethylamine [$R(NH_3^+)S^-$]; (3) cysteine [$R(NH_3^+)S^-$]; (4) cysteine ethyl ester [$R(NH_2)S^-$]; (5) homocysteine; (6) glutathione; (7) 2-mercaptoethanol; (8) potassium mercaptoacetate. Plots for imidazoles (---------) and phenols (—·—·—) from Bruice and Lapinski (1958).

Experimental

Materials.—L-Cysteine ethyl ester, DL-homocysteine, 2-mercaptoethylamine, and S-ethylcysteine were obtained from Nutritional Biochemicals Corp. L-Cysteine was obtained from Mann Research Laboratories, Inc. Dioxane and 2-mercaptoethanol were Matheson Coleman and Bell products. The dioxane was passed over an alumina column before use to remove peroxides (Dasler and Bauer, 1946). Mercaptoacetic acid was an Eastman Kodak Co. White Label product. The 5,5'-dithio-bis-(2-nitrobenzoic acid) was obtained from Aldrich Chemical Co. p-Nitrophenyl acetate (mp 79.5–80°) was prepared by the method of Chattaway (1931).

Apparatus.—The spectrophotometric measurements were made with a Beckman Model DU spectrophotometer equipped with a thermostated cell compartment and a Sargent Model SRL recorder. The determination of pH values was carried out with a Leeds and Northrup Model 7764 pH meter.

Methods.—The release of p-nitrophenol was followed spectrophotometrically at 401 mμ (λ_{max}, p-nitrophenolate anion). All reactions were carried out at 25.0°. Below pH 8.0 a 0.2 M phosphate buffer in a 1% dioxane-water (v/v) solvent was used; above pH 8.0 a 0.2 M borate buffer–0.2 M KCl solution in 1% dioxane-water (v/v) was used. The NPA concentration was 3.33×10^{-5} M to 1×10^{-4} M and the thiol was present in large excess (50–100 ×) over the NPA. Under these conditions the appearance of nitrophenol was found to follow first-order kinetics (at constant pH).

All thiol solutions were freshly prepared immediately before use. The thiol concentration and the extent of oxidation of each thiol occurring in the course of a kinetic experiment were determined by titration with 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman, 1959). The rate of oxidation of each thiol was measured at pH 7.4 and 9.3 under conditions of temperature, thiol

concentration, and buffer concentration similar to those used in the kinetic experiments. Since it was found that less than 3% of the total thiol present was oxidized during the time required for a kinetic run, no corrections for the oxidation of the thiols were applied in the calculation of the rate constants.

References

Aldridge, W. N. (1953), *Biochem. J. 53*, 110.

Benesch, R. E., and Benesch, R. (1955), *J. Am. Chem. Soc. 77*, 5877.

Bruice, T. C., and Lapinski, R. (1958), *J. Am. Chem. Soc. 80*, 2265.

Chattaway, F. D. (1931), *J. Chem. Soc. 1931*, 2495.

Danehy, J. P., and Noel, C. J. (1960), *J. Am. Chem. Soc. 82*, 2511.

Dasler, W., and Bauer, C. D. (1946), *Ind. Eng. Chem. Anal. Ed.* (now *Anal. Chem.*) *18*, 52.

Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. I, New York, Academic, p. 500.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Hammond, B. R., and Gutfreund, H. (1959), *Biochem. J. 72*, 349.

Hernandez, H. H., and Chaikoff, I. L. (1957), *J. Biol. Chem. 228*, 447.

Jencks, W. P., and Carriuolo, J. (1960), *J. Am. Chem. Soc. 82*, 1778.

Kimmel, J. R., and Smith, E. L. (1954), *J. Biol. Chem. 207*, 515.

Ryklan, L. R., and Schmidt, C. L. A. (1944), *Arch. Biochem. 5*, 89.

Schonbaum, G. R., and Bender, M. L. (1960), *J. Am. Chem. Soc. 82*, 1900.

Whitaker, J. R. (1962), *J. Am. Chem. Soc. 84*, 1900.

Cleavage of Peptide Proline Bonds by Lithium Aluminum Hydride*

MICHAEL A. RUTTENBERG, TE PIAO KING, AND LYMAN C. CRAIG

From the Rockefeller Institute, New York

Received February 7, 1964

The effect of lithium aluminum hydride on peptides containing proline has been studied. The major reaction was a reductive cleavage at the acyl proline linkage to give an aldehyde and amino-terminal proline. The method was applied to the following peptides where the yields in the cleavage reactions are given in the parentheses: glycyl-L-proline (20% at 25°), L-leucyl-L-prolylglycine (23% at 0°; 98% at 25°), *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine (100% at 25°), gramicidin S (42% at 25°), and tyrocidine B (30% at 0°). The peptides were treated with lithium aluminum hydride for 1 hour in anhydrous tetrahydrofuran. Under these conditions reduction of amides besides those at proline nitrogen was slight; the overall reactivity of amides in the peptides studied was found to be tertiary amide ≫ primary amide > secondary amide.

Lithium aluminum hydride reduction of primary and secondary amides usually requires vigorous reaction conditions to yield the corresponding amines. However, similar reduction of tertiary amides proceeds with great ease; as a result of reductive cleavage, aldehydes or alcohols and secondary amines are often the products (Micovic and Mihailovic, 1953; Mousseron *et al.*, 1952; Mosettig, 1954; Brown and Tsukamoto, 1961). Knowledge of these reactivity differences suggests the use of $LiAlH_4$ treatment as a possible procedure for the specific cleavage of acyl proline bonds, as these bonds are the only tertiary amide linkages in peptides. Therefore reduction studies were carried out with five proline-containing peptides, and in each case the expected specific cleavage occurred. Several side reactions also took place, but they could be controlled by the proper choice of reaction conditions. These are the findings to be reported in this paper.

Materials and Methods

Materials.—Tetrahydrofuran was refluxed for 2 days over a sodium-potassium amalgam and then distilled in an atmosphere of nitrogen. It was stored under nitrogen in an amber glass bottle. All other solvents used were redistilled. $LiAlH_4$ (Metal Hydrides, Inc., Beverly, Mass.) was suspended in tetrahydrofuran and the mixture was refluxed for 6 hours, cooled, then filtered through a sintered glass disk. The solution was stored under nitrogen in an automatic buret with an amber glass reservoir. It was assayed from time to time by measuring the amount of hydrogen evolved when water was added to an aliquot. The concentration of $LiAlH_4$ remained constant at 2.3 M for several months.

Glycyl-L-proline was purchased from Nutritional Biochemicals, Inc. L-Leucyl-L-prolylglycine and *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine were generous gifts from Dr. Charlotte Ressler of the Institute for Muscle Disease. Crude gramicidin S (Sharp and Dohme) was purified by countercurrent distribution in the system chloroform–methanol–0.1 N HCl (2:2:1) (Craig *et al.*, 1950). Tyrocidine B was obtained from crude tyrocidine (Wallerstein No. ON 13554) by countercurrent distribution as described by King and Craig (1955).

All the glassware used for the reactions was heated to 110° in an oven for 24 hours and then allowed to cool in a dessicator.

$LiAlH_4$ *Reduction.*—In a typical experiment, a weighed amount of peptide, which had been dried in a vacuum dessicator for 4 days at room temperature over P_2O_5, was placed in a three-necked flask fitted with a reflux condenser, a dropping funnel, and a nitrogen inlet. A magnetic stirring bar was introduced and an appropriate amount of tetrahydrofuran was added (usually 1 ml per mg of peptide). The $LiAlH_4$ solution was then added dropwise and the mixture was stirred for the duration of the reaction. To stop the reaction, the mixture was cooled in an ice

* This study was made in partial fulfillment of the requirement for the Ph.D. degree by Michael A. Ruttenberg. It was partially supported by a research grant (AM 02493-06) from the U. S. Public Health Service.