J. Biol. Chem. 235, 2801.
Setterfield, G., Neelin, J., Neelin, E., and Bayley, S. T. (1960), *J. Mol. Biol. 2*, 416.
Sjöquist, J. (1960), *Biochim. Biophys. Acta 41*, 20.
Stedman, E., and Stedman, E. (1950), *Nature 166*, 780'
Zubay, G. (1964), in The Nucleohistones, Bonner, J., and Ts'o, P. O. P., Ed., San Francisco, Calif., Holden-Day, p 95.

A Kinetic Study of the Reactions of Amino Acids and Peptides with Trinitrobenzenesulfonic Acid*

A. R. Goldfarb

ABSTRACT: The kinetics of the reaction of trinitrobenzenesulfonic acid with glycine, glycine peptides, and α-acetyllysine have been studied. The velocity constants are related to the pH of the reaction medium by the equation $\log k = a\text{pH} + b$. The values of a for glycine and α-acetyllysine are 0.92 and 1.0, respectively, and the value for both di- and triglycine is 0.56. The values of b for the first two compounds are -6.87 and -8.1, respectively, and the values for the last two are both -3.15. It is shown that trinitrophenylated (TNP) amino acids and peptides form complexes with sulfite ion giving association constants in the range of 10,000–21,000, corrected for the change with pH. The molar absorbance for the TNP-substituted ϵ-amino group is 1.15×10^4 and that for the α-amino group varies with chain length.

It was shown by Okuyama and Satake (1960) and Satake *et al.* (1960) that trinitrobenzenesulfonic acid[1] reacted with amino acids and peptides at relatively low temperatures. Since it was established that TNBS[1] reacted specifically with amino groups in a homogeneous aqueous system we have been studying the kinetics of the reaction of this compound with proteins. As part of this study we have investigated the reaction of TNBS with the model compounds glycine, di- and triglycine, and α-acetyllysine. All of the reactions followed a pseudo-first-order reaction when carried out in the presence of a large excess of TNBS. Evidence has been found that the trinitrophenylated (TNP) derivatives form a complex with sulfite ion.

Experimental Procedures[1]

Materials. Mono-, di-, and triglycine were obtained commercially and recrystallized from aqueous ethanol. α-Acetyllysine (*Anal.* Calcd: N, 14.9%. Found: N, 14.82%) was obtained from the Cyclo Chemical Co., Los Angeles, Calif., and on paper chromatography, showed a single component which differed from the ϵ-acetyl derivative and from lysine. The ϵ-acetyllysine was obtained from the same source (*Anal.* Calcd: N, 14.9%. Found: N, 14.8%); TNBS was obtained commercially and was recrystallized from 5 M HCl (mp 185°).

Trinitrophenyl derivatives of glycine (mp 162–163°), di- (mp 221°, dec) and triglycine (mp 235°), and α-acetyllysine (mp 119–220°) were made by the reaction of the amino acid or peptide with 100-fold molar excess of TNBS in 1% aqueous $NaHCO_3$ in the dark at room temperature for 24 hr. The products were recrystallized from aqueous methanol.

Methods. The reactions were carried out in stoppered 10-mm cuvets in a Bausch and Lomb 505 spectrophotometer connected to a Sargent recorder, Model SR. The absorbances were measured at 345 mμ unless otherwise stated.

Absorption spectra were obtained with a Bausch and Lomb 505 spectrophotometer and the absorbances were checked on a Beckman DU spectrophotometer. In all cases the agreement between these readings were within 0.005.

The reactions were run in 0.1 M sodium phosphate, 0.1 M sodium borate, and a mixture of 0.05 M phosphate plus 0.05 M borate buffers. All the studies were made at 16 ± 0.1°.

For the study of the interaction of sulfite with the trinitrophenyl derivatives, fresh solutions of Na_2SO_3 (chemically pure) were prepared in buffers and mixed with solutions of the pure trinitrophenyl derivatives after equilibrating both solutions at a temperature of 16°. The pH was determined with a Model G Beckman pH meter.

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* From the Division of Research of Sinai Hospital of Detroit, Michigan. *Received February 4, 1966; revised May 25, 1966.* Supported in part by Grant AT-05386, National Institutes of Allergy and Infectious Diseases, U. S. Public Health Service.

[1] Abbreviations: TNBS, trinitrobenzenesulfonic acid; G, G_2, and G_3, glycine, glycylglycine, and triglycine; P, amino acid or peptide; ϵ_i, molar absorbance of compound "i;" SH^- and S^{2-}, bisulfite and sulfite ions.

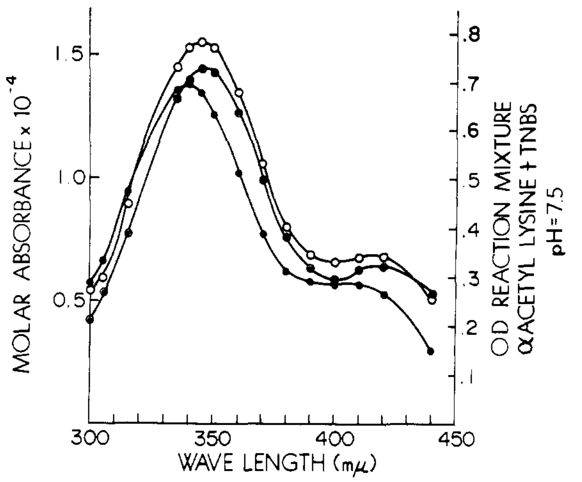

FIGURE 1: Absorption spectra of TNP-G (O), TNP-G_2 and -G_3 (●), and TNP-α-acetyllysine (⊙).

Results

Absorption Spectra. The absorption spectra of pure TNP-G, -G_2, -G_3 and α-acetyllysine are given in Figure 1. The curves showed no qualitative or quantitative changes between pH values of 4.0 and 10.0. They were all similar, showing a maximum near 345 mμ and a flat region at 410–420 mμ. On the other hand, the absorption spectra for the reaction mixtures showed bands with two maxima at about 345 and 425 mμ. (For an example, see Figure 2, curve D.) In attempting to explain the difference it was noted that 1 mole of sulfite was formed/mole of amino group reacted and it was considered possible that the sulfite formed a complex with the trinitrophenyl group, thus altering the absorption spectra. This was confirmed qualitatively by acidifying the reaction mixtures, in which case the absorption spectra which resulted were identical with those of the purified compounds.

Studies of the Complex Formation. On the assumption that the 1 mole of sulfite, released during the reaction, formed a complex with the TNP-amino group, equimolar mixtures of Na_2SO_3 and pure TNP-α-acetyllysine were prepared in buffers of varying pH. The absorbances were read within 15 sec after the mixtures were made and were found to be unchanged for 24 hr at 16°. The absorbances were divided by the molarity of the TNP derivative to give the molar absorbances recorded in Table I. At the lowest pH the molar absorbance approached the value for the pure TNP-α-acetyllysine (1.45×10^4). In the pH range 6.8–9.7 the molar absorbance was about 1.20×10^4 and this was in fair agreement with the value found by calculation from the kinetic data, as will be described below. At higher pH values the molar absorbances became smaller and it will be shown in the next section that a similar change also occurred at very large sulfite concentrations.

To determine the association constant, K, studies were made of the absorption spectra of mixtures of TNP-α-acetyllysine and sodium sulfite in molar ratios from 10:1 to 1:20 at pH values of 6.98 and 8.52. A family of curves was obtained (Figure 2) having an isosbestic point at 367 mμ. As the concentration of sodium sulfite increased the 345-mμ peaks became smaller and the flat portions of the curves in the region of 420 mμ became larger, giving distinct maxima. Indeed, the shape of the curves for the mixtures were very much like that of the curve for the products of the reaction of α-acetyllysine and TNBS. Using the data for the molar absorbances at 345 mμ, the association constants for the complexes of the TNP derivatives with sulfite were then calculated on the basis of the following derivation[2]

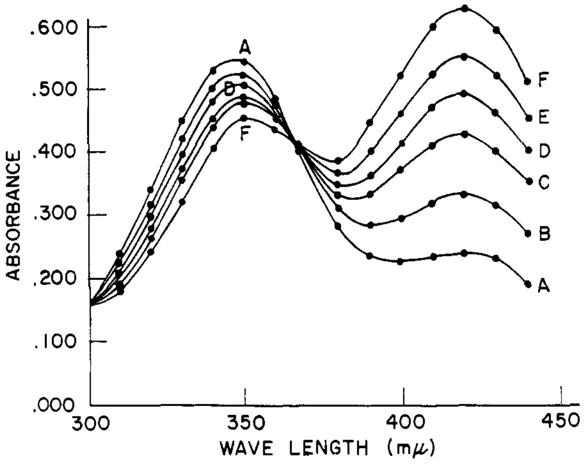

FIGURE 2: Absorption spectra of mixtures of TNP-α-acetyllysine (39.6×10^{-6} M) plus Na_2SO_3 in 0.1 M phosphate buffer, pH 6.98, at 16°. Curves A–F correspond to the presence of 0, 15.6, 31.3, 46.9, 62.9, and 92.9 $\times 10^{-6}$ M Na_2SO_3, respectively.

TABLE I: Equimolar Mixtures of TNP-α-acetyllysine and Na_2SO_3.

pH	$\epsilon_M{}^a$ (345 mμ)	$\epsilon_M{}^a$ (420 mμ)
4.5	1.42×10^4	0.646×10^4
6.81	1.21×10^4	1.230×10^4
7.21	1.20×10^4	1.31×10^4
8.5	1.19×10^4	1.37×10^4
9.7	1.19×10^4	1.38×10^4
10.6	1.15×10^4	1.39×10^4
11.03	1.12×10^4	1.39×10^4

[a] See footnote 2.

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[2] K is the association constant; K_a is the dissociation constant for HSO_3^-; $[S_t]$ is the total concentration of sulfite; [T] and [TS] are the concentrations of pure TNP derivative and of the complex, respectively; A_t is the total absorbance and A_i is the absorbance due to component "i;" ϵ_M is the molar absorbance for an equimolar mixture of TNP derivative and sulfite.

$$K = \frac{[\mathrm{TS}]}{[\mathrm{T}][\mathrm{S}^{2-}]} \qquad (1)$$

For the dissociation of bisulfite ion

$$K_a = \frac{[\mathrm{H}^+][\mathrm{S}^{2-}]}{[\mathrm{SH}^-]} = 1.02 \times 10^{-7} \qquad (2a)$$

Since $[\mathrm{S_t}] = [\mathrm{S}^{2-}] + [\mathrm{SH}^-] + [\mathrm{TS}]$ it can be shown that

$$[\mathrm{S}^{2-}] = \frac{[\mathrm{S_t}] - [\mathrm{TS}]}{1 - [\mathrm{H}^+]/K_a} \qquad (2b)$$

and it follows from eq 1 that

$$K = \frac{[\mathrm{TS}]}{([\mathrm{T_t}] - [\mathrm{TS}])([\mathrm{S_t}] - [\mathrm{TS}])}(1 + [\mathrm{H}^+]/K_a) \qquad (3)$$

The concentration of complex, [TS], is calculated from the experimental absorbances in the following manner: the absorbance, A_t, is the sum of $A_T + A_{TS} + A_{S^-} + A_{S^{2-}}$ and since, at 345 mμ, $A_{S^-} + A_{S^{2-}}$ is negligibly small then $A_t = A_T + A_{TS} = [\mathrm{T}]\epsilon_T + [\mathrm{TS}]\epsilon_{TS}$. Since $[\mathrm{T_t}] = [\mathrm{T}] + [\mathrm{TS}]$ it follows that

$$A_t = ([\mathrm{T_t}] - [\mathrm{TS}])\epsilon_T + [\mathrm{TS}]\epsilon_{TS} \qquad (4)$$

It can then be shown that

$$[\mathrm{TS}] = \frac{A_0 - A_t}{\epsilon_T - \epsilon_{TS}} \qquad (5)$$

where A_0 = absorbance, when $[\mathrm{S_t}] = 0$, of the pure TNP derivative. The values of [TS] were substituted into eq 3 to obtain the values of K.

In practice the values of $\epsilon_T - \epsilon_{TS}$ were not clearly given by the data. It should have been possible to obtain ϵ_{TS} by increasing the concentration of sulfite until the absorbance did not change. However, at comparatively high sulfite concentrations there continued to be a decrease in absorbance at 345 mμ. Further, the absorbance at 420 mμ, which had been increasing with increasing sulfite concentration, began to decrease. The changes at 420 mμ indicated that a second change occurred which was different from the formation of the first complex compound between the trinitrophenyl group and sulfite. It was, therefore, necessary to estimate $\epsilon_T - \epsilon_{TS}$ by a method of approximations in order to obtain a value that gave a constant K over the range of ratios $[\mathrm{S_t}]/[\mathrm{T_t}]$ from 0 to 10. Substituting the data obtained from the curves in Figure 2 into the equations described above, it was found that the association constant K for TNP-α-acetyllysine was 20,400 at pH 6.98 and 22,000 at pH 8.52. TNP-diglycine was studied as a prototype for the α-amino group and, in this case, the constants were 9980 at pH 6.98 and 8950 at pH 8.29.

Kinetic Studies.[2] The kinetic studies for the reaction P + TNBS → TS were made with concentrations of $[\mathrm{P}] = 30\text{–}100 \times 10^{-6}$ M and $[\mathrm{TNBS}] = 1500\text{–}2500 \times 10^{-6}$ M, in which case the following pseudo-first-order eq 6 could be used

$$-kt = \frac{1}{[\mathrm{TNBS}]}\ln\left(1 - \frac{A}{A_L}\right) \qquad (6)$$

where A is the experimental absorbance and A_L is the theoretical absorbance at 100% reaction. Since 1 mole of TNBS is lost/every mole of amino group which has reacted, some absorbance units are lost from the reaction mixture due to the decrease in TNBS. Therefore, the observed absorbance, A, is equal to the absorbance due to the trinitrophenyl derivative which has been formed, $[\mathrm{TS}]\epsilon_M$, minus that due to the loss of TNBS ($[\mathrm{TS}]\epsilon_{TNBS}$). It can be shown that the observed absorbance $A = [\mathrm{TS}](\epsilon_M - \epsilon_{TNBS})$ and $A_L = [\mathrm{P}](\epsilon_M - \epsilon_{TNBS})$. When A_L was set equal to $[\mathrm{P}](\epsilon_T - \epsilon_{TNBS})$ no good value of k could be obtained since the calculated k decreased with time. In view of the findings in the studies of complex compound formation, a molar absorbance value of ϵ_M instead of ϵ_T should have been used. By a method of approximations we found only one value of A_L which gave a velocity constant that did not change with time.

A less laborious method for evaluating A_L was obtained using a method of equal time intervals. If A_a, A_b, and A_c are the absorbances at times t_a, t_b, and t_c and $t_b - t_a = t_c - t_b$ it can be shown that

$$A_L = \frac{A_aA_c - A_b^2}{(A_a + A_c) - 2A_b} \qquad (7)$$

From the estimated A_L we obtained the values of ϵ_M recorded in Table II along with ϵ_T for the pure trinitrophenyl derivatives. In all cases ϵ_M was less than ϵ_T.

Figure 3 shows a graph of log k *vs.* pH from which it was found that all compounds followed the general equation $\log k = a\mathrm{pH} + b$. For glycine $a = 0.92$, $b = -6.87$; for di- and triglycine $a = 0.56$, $b = -3.15$; and for α-acetyllysine $a = 1.00$, $b = -8.1$.

Discussion

In the pH range studied, the ionic species of sulfite consist of $\mathrm{HSO_3^-}$ and $\mathrm{SO_3^{2-}}$, either of which might form a complex with TNP derivatives. The parallel effects of increasing $\mathrm{Na_2SO_3}$ concentration and pH led us to assume that the $\mathrm{SO_3^{2-}}$ ion was the complexing species. This was confirmed by the good agreement between the association constants at pH values of 6.98 and 8.29 when the second dissociation constant for sulfite was assumed to be 1.02×10^{-7}.

If a single $\mathrm{SO_3^{2-}}$ ion combined with the compound, one would have expected that ultimately, at very large ratios of $\mathrm{SO_3^{2-}}$ to TNP derivative, a constant value of ϵ would have been reached and this would be ϵ_M. However, at a ratio as large as 200 no constant value

TABLE II: Molar Absorbances of TNP Derivatives (λ 345 mμ).

Compd	Pure compd (ϵ_T)	Calcd (ϵ_M)
TNP-glycine	$1.55\ (1.51)^a \times 10^4$	$1.239 \pm 0.13^b \times 10^4$
TNP-diglycine	$1.35\ (1.32)^a \times 10^4$	$1.144 \pm 0.05 \times 10^4$
TNP-triglycine	1.10×10^4	$0.994 \pm 0.03 \times 10^4$
TNP-acetyllysine	1.45×10^4	$1.164 \pm 0.07 \times 10^4$

[a] Values given by Satake *et al.* (1960) in $NaHCO_3$. [b] Standard deviation.

was obtained. The absorbance at 345 mμ, which had been decreasing, continued to decrease. The absorbance at 420 mμ, which had been increasing leveled off and started to decrease slowly. These further changes at large ratios of SO_3^{2-} to TNP derivative we attributed to the probable association with a second SO_3^{2-} ion.

This study had been undertaken partly to obtain reasonable values of ϵ_M for the TNP-α-amino groups and the TNP-ε-amino groups of amino acids in proteins. We could have followed the procedure of Okuyama and Satake (1960) and acidified aliquots of the reaction mixtures of proteins and TNBS and then used the known values for the TNP-ε-lysine and TNP-α-amino groups of amino acids and peptides. However, we found, early in the studies with proteins, that acidification of the reaction mixture of human serum albumin with TNBS often resulted in precipitation. It was also found that it was necessary, particularly at the lower pH values, to obtain data for 24–48-hr periods. We found that by automating the procedure we could obtain a continuous record of the absorbances. Therefore, it was necessary to obtain values of ϵ_M for the complexes which resulted at a ratio [T]/[S] = 1.0. The value of ϵ_M for TNP-α-acetyllysine was found to be 1.20×10^4 from the complex studies and 1.11×10^4 from the kinetic studies and we are assuming values of 1.15×10^4 for the TNP-ε-amino group of lysyl residues. The value of ϵ_M for the TNP-α-amino groups, as obtained by calculation from the kinetics, varied slightly with chain length and no precise value could be assumed although, as seen from Table II, they were about 1×10^4 for TNP-G_2 and -G_3.

In the kinetic studies the velocity constants varied with pH according to the equation $\log k = a\text{pH} + b$. If one postulates that the mechanism of the reaction involves only the free amino group it can be shown that $k = k_0K_2/([H^+] + K_2)$ where K_2 is the dissociation constant of the amino group, and k_0 is an intrinsic constant assumed not to vary with pH. On differentiating this expression with respect to pH it is found that $d \log k/d\text{pH} = a = [H^+]/([H^+] + K_2)$ and the slope varies with $[H^+]$. When $[H^+] \gg K_2 a = 1$ which agrees with the figures for α-acetyllysine and for glycine in the pH range which was studied. When $[H^+]$ approaches K_2 the slope approaches 0.5, which is the value obtained for di- and triglycine. The slope, therefore, changes in a manner dependent on the difference between K_2 and $[H^+]$. When the data was used to calculate k_0 for G, G_2, G_3 it was found that k_0 varied with pH in the pH range of this study. On the other hand, k_0 for α-acetyllysine remained constant. It was therefore concluded that a simple mechanism based solely on the reaction between TNBS and a free amino group could not be used exclusively to explain the findings. We have no satisfactory explanation for this fact at this time. It appears, however, that the presence of a peptide bond may have an effect on the reactivity of the amino group. In another report we shall show that a similar alteration of reactivity of the ε amino group of the lysine residue occurs for proteins (A. R. Goldfarb, unpublished data, 1966). It should be pointed out here that Goldfarb and Hoffman (1959), in a study of the

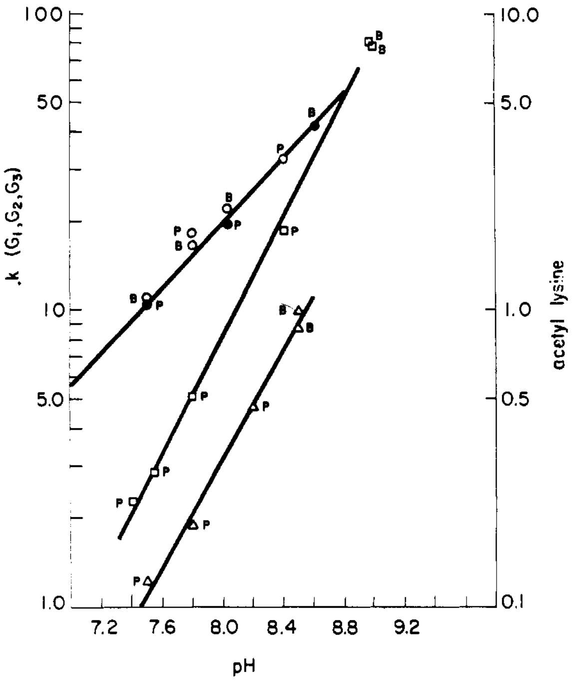

FIGURE 3: k *vs.* pH: (○) G_2; (●) G_3; (△) G; (□) α-acetyllysine; P, in phosphate buffer; B, in borate buffer; k, in liters $\text{mole}^{-1}\ \text{min}^{-1}$. The lines were calculated by the least-squares method.

absorption spectra of synthetic peptides, have presented evidence that peptide bonds could act as couplers between N- and C-terminal chromophore groups in a peptide chain. From this evidence they concluded that peptide bonds might have an effect on side-chain groups.

Acknowledgment

The author wishes to acknowledge the excellent technical assistance of Mrs. Judith Schmidt.

References

Goldfarb, A. R., and Hoffman, E. (1959), *Arch. Biochem. 81*, 493.

Okuyama, T., and Satake, K. (1960), *J. Biochem.* (*Tokyo*) *47*, 454.

Satake, K., Okuyama, T., Ohashi, M., and Shinoda, T. (1960), *J. Biochem.* (*Tokyo*) *47*, 654.

Heterogeneity of Amino Groups in Proteins. I. Human Serum Albumin*

A. R. Goldfarb

ABSTRACT: The reaction of human serum albumin with trinitrobenzenesulfonic acid has been studied in the pH range 6.98–8.40 at 16°. The reaction does not follow a simple second- or pseudo-first-order kinetics. A method is described for analyzing the data on the assumption that several classes of reacting amino groups exist, each having a specific velocity constant. This method of analysis reveals that human serum albumin contains 19 reactive ϵ-amino groups divided into three sets containing 14, 3.7, and 1.5 amino groups, respectively.

The structure of proteins depends, to a great extent, on the covalent bonds, hydrogen bonds, ionic bonds, apolar bonds, and van der Waals interactions. All of these are variously affected by the constituent amino acids and their associated functional groups. The proximity and interaction of these groups ultimately determine the shape and "fine structure" of the protein as well as the ability of the protein to function as an agent in biological systems (*e.g.*, as enzymes or as ion binding molecules). Conversely, the reactivity of a functional group is altered by its presence in a protein macromolecule. For example, studies of the titration of ionic groups and of the ultraviolet absorption characteristics of proteins (Foster, 1960) have shown that "masked" groups occur; *i.e.*, by various tests the number of groups that could be determined were fewer than the total number which were known to be present from amino acid analyses. "Masking" results either from the side chain being buried in the molecule or from some interaction with proximal groups or some combination of both. The older literature implied that masking was an "all or none" phenomenon. More recent evidence has demonstrated gradations of masking of the functional groups in native proteins which can be influenced by pH, temperature, ionic strength, and ionic composition (Shinoda, 1965; Klee and Richards, 1957; Scatchard *et al.*, 1957).

It should be relatively simple to determine whether a functional group in proteins is composed of subclasses by studying the kinetics of the reaction of the group with a specific reagent. If all of the unmasked groups are equally reactive the kinetic study should confirm this by revealing a simple second-order or pseudo-first-order curve. For example, M. Schlamowitz (unpublished observations, 1966), in an analysis of the data of Li (1945) on the iodination of pepsin, found that the reaction followed a simple second-order equation in which all ten iodinatable groups were equally reactive. On the other hand Klee and Richards (1957) reported that the reaction of ribonuclease with *O*-methylisourea did not give a pseudo-first-order curve and Boyer (1954) made a similar observation concerning the reaction of proteins with sulfhydryl reagents.

It had been reported by Okuyama and Satake (1960) that trinitrobenzenesulfonic acid (TNBS)[1] was a specific reagent for only the amino group of amino acids and peptides. Since TNBS is water soluble and reacts rapidly with amino groups under mild conditions it was chosen as a suitable reagent to investigate the reaction heterogeneity of the amino groups in proteins.

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* From the Biochemical Research Laboratories of the Division of Research, Sinai Hospital, Detroit, Michigan. *Received February 4, 1966; revised June 13, 1966.* Supported in part by Grant AI-085386 from the National Institutes of Health.

[1] Abbreviations: TNBS, 2,4,6-trinitrobenzenesulfonic acid; HSA, human serum albumin; ϵ, absorbance.