A Kinetic Study of the Ninhydrin Reaction*

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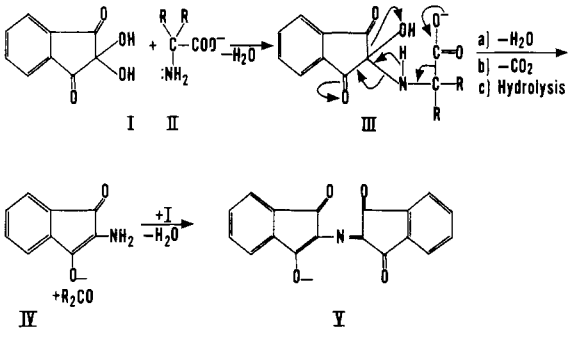
ABSTRACT: Reaction rates of α-amino acids with ninhydrin at 30 and 100° were studied as a function of basicities and steric environments of amino groups. Based on the observed reactivities of α-amino acids at 30°, a linear free-energy equation was derived that permits calculation of separate polar and steric parameters associated with the amino acids which influence rates.

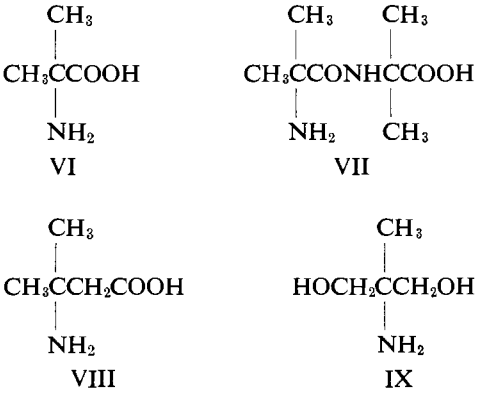
The rate-determining step in the ninhydrin reaction appears to involve a nucleophilic-type displacement of a hydroxy group of ninhydrin hydrate by a nonprotonated amino group. The time dependence of leucine color yields of several sterically hindered α-amino acids was determined. Reactivity studies with several model compounds are in accord with a general mechanism for the ninhydrin reaction. Evidence is presented supporting the view that the low color yields of Ruhemann's purple in the reaction of aminothiols with ninhydrin are caused by a competitive reaction of the sulfhydryl group with ninhydrin.

The reaction of ninhydrin with amines, amino acids, peptides, and proteins is used extensively in qualitative and quantitative biochemical investigations. Although the chemistry of this reaction has been widely studied and reviewed (McCaldin, 1960), a number of features associated with it appear anomalous. For example, no mechanistic explanations have been offered as to why primary aromatic amines do not give the expected color reaction with ninhydrin or why diamino and aminothiol acids do not yield stoichiometric amounts of diketohydrindylidenediketohydrindamine (also called Ruhemann's purple and represented by structure V in Scheme I). This chromophore is responsible for the ninhydrin color. The reaction mechanism of ninhydrin with enamines, such as β-aminocrotonic acid, also remains to be elucidated.



SCHEME I

A kinetic study was undertaken to obtain information on the effect of basicities and steric environments of amino groups on rates of reaction with ninhydrin. The results make it possible to calculate the separate polar and steric parameters that influence rates and permit conclusions as to which of several steps in the general mechanism for the ninhydrin reaction of α-amino acids is rate determining. A postulated mechanism of reaction of ω-amino acids is supported by the observed behavior



of model compounds VI–IX in the ninhydrin reaction, and an explanation for the behavior of aminothiol acids in the ninhydrin reaction is offered in terms of the transformations shown in Scheme II.

Experimental Section

Materials. The amino acids used were the best commercial grades available, the dipeptide VII was a gift of Dr. R. C. Shepard of the University of Liverpool, and the other compounds were synthesized as follows.

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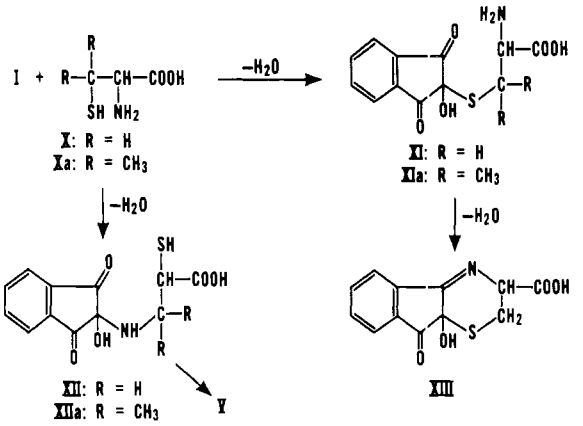

SCHEME II

Diketohydrindylidenediketohydrindamine Sodium Salt (*V*). The compound was prepared as described by Moore and Stein (1948) and was recrystallized from aqueous ethanol. The absorption spectrum of V was determined on a Cary 14 spectrophotometer[1] in a solvent system identical with that used to measure the same chromophore produced in the kinetic experiments.[2] The solvent mixture consisted of 25 ml of pH 5.5 acetate buffer, 75 ml of Methyl Cellosolve, 100 ml of water, and 500 ml of 50% ethanol. When V was subjected to the conditions of the kinetic studies at 30°, changes in its absorption maxima were negligible for periods up to 1 week.

β-Amino-β-methylbutyric Acid (*VIII*). The procedure described by Slimmer (1902) involving the reaction of ammonia solution with dimethylacrylic acid in an autoclave at 150° was modified and simplified as follows. To 20 g (0.2 mole) of dimethylacrylic acid (K and K Laboratories) in a Parr pressure reaction vessel, 1-l. capacity, equipped with a Series 4500 stirrer, was added 300 ml of liquid ammonia at around −50° (alcohol–solid carbon dioxide bath). The apparatus was sealed, warmed at room temperature, and then heated until the temperature of the reaction mixture reached 100°. The pressure gauge at this temperature read 860 psi. That temperature was maintained for 24 hr while the mixture was being vigorously stirred. The ammonia was then released from the cooled apparatus. The residual, brown syrupy liquid solidified after about 3 hr and was taken up in 150 ml of water. The water solvent was evaporated by means of an aspirator until all residual traces of ammonia were removed. The brown syrup was dissolved in 100 ml of water and decolorized by boiling for a few minutes with Darco activated carbon. The water was evaporated, and the colorless residue was recrystallized from ethanol–ether at 0–5°. The first crop weighed 11.3 g, and the final yield was nearly quantitative. The compound starts subliming at around 180° and melts at 220–222°, lit. (Slimmer, 1902) mp 217°.

Anal. Calcd for $C_5H_{11}NO_2$ (117): N, 11.96. Found: N, 11.83.

This modified Slimmer method might also be useful as a general procedure to prepare β-amino acids from acrylic acids and liquid ammonia.

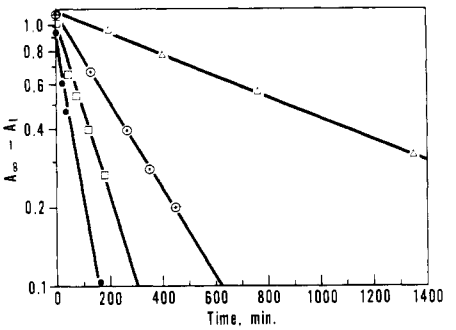

FIGURE 1: Plot of log $(A_\infty - A_t)$ *vs.* time for the reaction of amino acids (1×10^{-4} M) and ninhydrin (1.4×10^{-2} M) at pH 5.5 and 30°. ●, glycine; ⊡, DL-phenylalanine; ⊙, DL-norleucine; ◬, DL-norleucine with ninhydrin at a 3.5×10^{-3} M.

Kinetic Measurements at 30°. A typical experiment was carried out in the following manner. To 5 ml of a 2×10^{-4} M solution of the amino acid in a reusable vacuum hydrolysis tube with ball joints (Phoenix Precision Instrument Co.) was added 5 ml of the ninhydrin reagent solution prepared according to the procedure of Moore and Stein (1954). The reaction tube was evacuated by means of a vacuum pump for about 2 min and placed into a dark 30° water bath. Four to five such tubes were usually prepared in addition to a blank that contained everything except the amino acid. Deionized distilled water was used in all kinetic experiments. Periodically, each tube was removed and three 2-ml aliquots were diluted with 5 ml of 50% ethanol–water to quench the reaction. The absorption maximum at 570 mμ was read in a Beckman B spectrophotometer against a similarly diluted blank solution. These conditions gave consistently reproducible results.

Kinetic Studies at 100°. Five dilutions were prepared for each amino acid, ranging in concentration from 0.8 to 2.0×10^{-4} M. To 1-ml aliquots in triplicate and a water blank in matched test tubes was added 1 ml of ninhydrin reagent prepared according to Moore and Stein (1954). The tubes were covered with aluminum caps, placed in a boiling-water bath, and heated at 100° for specified periods of time. At the end of the heating period the tube rack was placed in a cold-water bath and to each tube was added 5 ml of a 50% ethanol solu-

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

[2] The reaction product V has two absorption maxima in the visible with nearly identical extinction coefficients: λ_{max} 570 and 409 mμ (ε 22,000 and 24,200, respectively) The absorption maximum at 570 mμ is usually utilized for qualitative and quantitative studies involving the ninhydrin reaction. However, it should be possible to use the absorption maximum at 409 mμ in an analogous manner although the blank is higher at this wavelength. This approach may prove advantageous when other substances in the reaction mixture interfere at the higher wavelength.

TABLE I: Rates of Reaction of Norleucine with Ninhydrin as a Function of Concentration of Reactants at 30° and pH 5.5.

Norleucine (mole/l.)	Ninhydrin (mole/l.)	$t_{1/2}$ (min)	$k_1 \times 10^5$ (sec^{-1})	$k_2 \times 10^3$ (l./mole per sec)
1×10^{-4}	1.4×10^{-2}	180	6.40	1.14
1×10^{-4}	3.5×10^{-3}	730	1.58	1.13
1×10^{-4}	7.0×10^{-3}	350	3.50	1.18
2×10^{-4}	7.0×10^{-3}	345	3.34	1.19

TABLE II: Rates of Reaction of Amino Acids with Ninhydrin as a Function of pK_2 Values of Amino Groups at pH 5.5 and 30°.

No.	Amino Acid	$t_{1/2}$ (min)	$k_1 \times 10^5$ (sec^{-1})	k_{1A}- (sec^{-1})	pK Values of Amino Groups
1	Glycine	40	28.9	2.70	9.47[a]
2	L-3,5-Diiodotyrosine	30	38.4	0.064	7.72[b]
3	L-1-Methylhistidine	75	15.4	0.254	8.72[b]
4	L-Histidine	45	25.6	0.574	8.85[b]
5	L-Tyrosine	82	14.0	0.446	9.00[b]
6	DL-Phenylalanine	80	14.4	0.456	9.00[a]
7	DL-Methionine	85	13.6	0.479	9.08[a]
8	DL-Leucine	130	8.89	0.793	9.45[a]
9	DL-α-Alanine	165	7.00	0.826	9.57[a]
10	DL-Norleucine	180	6.42	0.867	9.63[a]
11	DL-α-Phenyl-α-alanine (2-amino-2-phenyl-propionic acid)	4,000	0.289	0.012	9.12[a]
12	DL-α-Methylmethionine (2-amino-2-methyl-4-methylthiobutyric acid)	4,800	0.240	0.0214	9.45[a]
13	α-Aminoisobutyric acid	10,500	0.110	0.0365	10.02[a]

[a] The pK values of the amino groups in these compounds were determined at 30° (Friedman and Wall, 1964). [b] The pK values of the amino groups in these amino acids given at several temperatures (Greenstein and Winitz, 1961; Perrin, 1965) were linearly extrapolated to 30°.

tion by means of a syringe. The solutions were stirred on a Vortex Jr. stirrer, and absorbance was read at 570 mμ against a similarly treated blank solution. Standard curves of concentration against absorbance were plotted. The color yields of the amino acids relative to leucine were determined from these plots.

Results

The reaction rates at 30° were followed by measuring the absorbance at 570 mμ of reaction product V. The fraction of amino acid left unreacted is given by $A_\infty - A_t$, where A_∞ is the absorbance at either infinite or 100% reaction time, and where A_t is the absorbance at time t.

Since the concentration of ninhydrin in the reaction mixture was always in large excess over that of the amino acid, the graph of log $(A_\infty - A_t)$ *vs.* time gave straight lines, establishing that the reaction followed pseudo-first-order kinetics, as illustrated in Figure 1. The half-lives ($t_{1/2}$) were read directly from these graphs, and the pseudo-first-order rate constants (k_1) and second-order rate constants (k_2) were calculated by means of formulas: $k_1 = 0.693/t_{1/2}$ and $k_2 = k_1$/concentration of ninhydrin. Table I summarizes kinetic data for the reaction of norleucine with ninhydrin. The second-order rate constants listed in the last column are essentially invariant over a range of concentrations, which proves that the ninhydrin reaction is second order (MacFadyen and Fowler, 1950).

The mechanism of reaction of amino acids with ninhydrin, shown in Scheme I for α-amino acids, re-

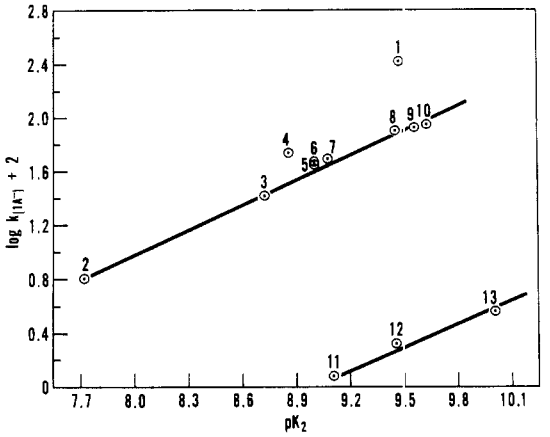

FIGURE 2: Variation of log k_{1A^-} with pK_2 values for the reaction of amino acids with ninhydrin at pH 5.5 and 30°. Numbers correspond to amino acids listed in Table II.

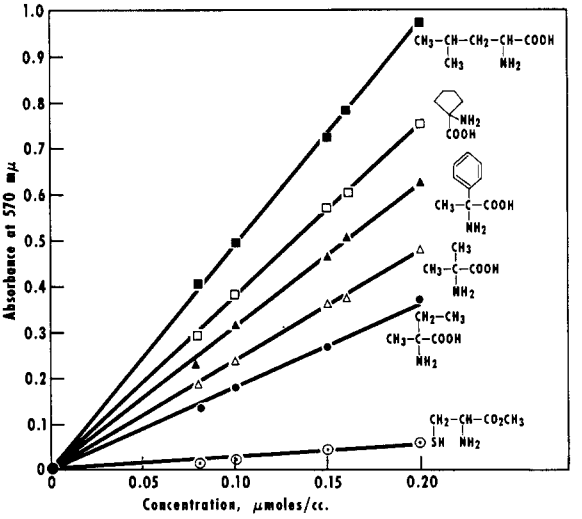

FIGURE 3: Plot of concentration against absorbance for reaction of amino acids with ninhydrin after a 20-min reaction time.

quires two molecules of ninhydrin for each molecule of amino acid to form V. For this reason pseudo-first-order rate constants were divided by one-half the actual ninhydrin concentration used to obtain the second-order rate constants listed in Table I. The small amount of hydrindantin present in the ninhydrin reagent solution was neglected in these calculations. Pseudo-first-order rate constants were used thereafter because they represent observed rates.

The kinetic results at 30° are summarized in Table II. The A_∞ for acid **1–10** in the table was estimated as follows. The absorbance of a solution of the authentic reaction product V, the concentration of which was identical with that of the amino acid solution used in the kinetic studies, was calculated from the extinction coefficient of V determined in an analogous solvent system.[2] The kinetic experiments were continued until the maximum absorbance was reached. The A_∞ for the sterically hindered amino acids **11–13** was taken as the leucine color yields of these amino acids after 2-hr reaction periods at 100°. The kinetic experiments for these amino acids were run to 50% completion, and the pseudo-first-order plots were extrapolated to complete reaction.

Brønsted-type plots of logarithms of pseudo-first-order anion rate constants (k_{1A^-}) *vs.* pK_2 values of amino groups for two steric series of amino acids in which the amino groups varied by being attached to secondary and tertiary carbon atoms, respectively, are shown in Figure 2. The k_{1A^-} values given in Table II and used in Figure 2 were calculated from the previously derived formula (Friedman and Wall, 1964)

$$k_{1A^-} = k_1(1 + H^+/K_2) \qquad (1)$$

where H^+ refers to the hydrogen ion concentration of the reaction medium and where K_2 refers to the ionization constant of the amino group.

Data for rates of reaction at 100° are summarized in Figures 3 and 4. Color yields relative to leucine were determined from plots of concentration against absorbance at 570 mμ, as illustrated in Figure 3. Color yields of several sterically hindered α-amino acids as a function of time are plotted in Figure 4.

Discussion

Effect of Polar and Steric Factors on Rates. To establish whether rates in the ninhydrin reaction are a function of basicities, and thus nucleophilicities (Edwards and Pearson, 1962), of amino groups, pseudo-first-order rate constants were determined for a series of α-amino acids with varying pK_2 values (Table II). The anionic form of an amino acid rather than the zwitterionic would be expected to react with ninhydrin because the latter form has a positive charge on the nitrogen atom and could not participate, therefore, in a nucleophilic displacement reaction. At any given pH the concentration of the amino acid is governed by the equation

$$pH = pK_2 + \log [A^-]/[HA]^{\pm} \qquad (2)$$

where A^- represents the amino acid anion and $HA^{\pm}$ the zwitterion. If the anion is the reactive species, eq 2 predicts that at any given pH the observed rates should decrease with increasing pK_2 values because the greater the pK_2 the lower the anion concentration.

Pseudo-first-order rate constants (k_1) for two different steric series of amino acids are listed in Table II. In the first series (**2–10**), the amino groups are attached to secondary carbon atoms, and in the second series (**11–13**), to tertiary carbon atoms. The amino group has a similar steric environment within each series. Glycine (**1**) is the only example of an α-amino acid in which the

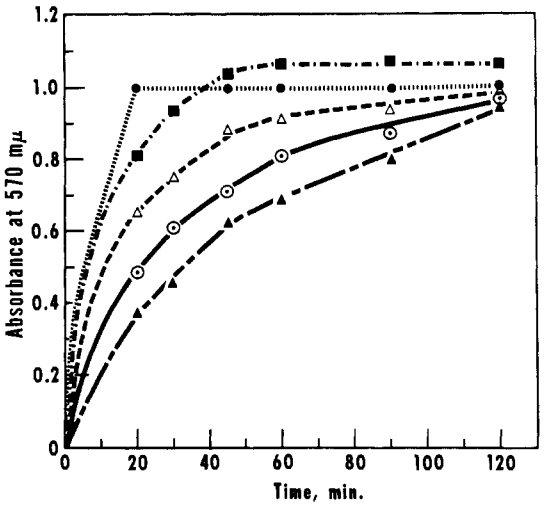

FIGURE 4: Time dependence of leucine color yields of sterically hindered α-amino acids. ●, DL-leucine; ■, 1-aminocyclopentane-1-carboxylic acid; △, DL-α-phenyl-α-alanine; ⊙, α-aminoisobutyric acid; ▲, DL-α-amino-α-methylbutyric acid (DL-isovaline).

amino group is attached to a primary carbon atom. Because the first-order rate constants within each series decrease with increasing pK_2 values, our predictions are confirmed that amino acid anions are the reactive species in the ninhydrin reaction.

To determine whether rates correlate with basicities of amino groups, pseudo-first-order anion rate constants (k_{1A^-}) were calculated by means of formula 1. Since anion rate constants measure rates at the same amino acid anion concentration, differences in these rate constants for a sterically similar series of amino acids should then be due to inherent differences in basicities of amino groups as measured by pK_2 values.

This point is strikingly illustrated by the observed k_{1A^-} values for L-tyrosine and L-3,5-diiodotyrosine (Table II). The anion rate constant for L-tyrosine is about seven times greater than the corresponding value for the diiodo derivative, although the steric environment of the amino groups in these two amino acids is nearly identical. This difference in rate constants must be due to the difference in pK_2 values of the amino groups in the two compounds.

No explanation can be offered why L-histidine reacts about twice as fast as predicted from the pK value of its amino group. The reason for the faster rate appears to be the NH group in the imidazole ring of this amino acid, because L-1-methylhistidine gives the expected rate constant.

Brønsted-type plots of log k_{1A^-} against pK_2 values for the two steric series of amino acids gave, with the exception of histidine, straight lines for each series (Figure 2). These linear relationships demonstrate that the calculated anion rate constants are a function of the basicities of the amino groups.

Each of the lines in Figure 2 may be described by

$$\log k_{1A^-} = \rho\,(\text{slope}) \times pK_2 + b\,(\text{intercept}) \quad (3)$$

Equation 3 is an extension of the Brønsted catalysis law, and since the slopes of the two lines in Figure 2 are about equal, it may be expressed as a Hammett–Taft-type free-energy relationship which correlates the logarithm of the ratio of the anion rate constants of any α-amino acid and that of glycine, the simplest amino acid, to differences in polar and steric factors.

$$\log \frac{k_{1A^-}\,(\text{any } \alpha\text{-amino acid})}{k_{1A^-}\,(\text{glycine})} = \rho\sigma^A + Es \quad (4)$$

The polar reaction parameter ρ gives a measure of sensitivities of rates to basicities of amino groups, and the polar substituent parameter σ^A, defined as the difference in pK_2 values between any α-amino acid and glycine, gives a quantitative measurement of the change in the basicity of an amino group due to the introduction of additional substituents into the α-carbon of glycine. The steric substituent constant Es depends on the size and steric requirements of the substituents in the reaction.

The average slope ρ of the two lines in Figure 2 is 0.60. The Es values for the individual amino acids were calculated by means of eq 4 from data in Table II and the results, together with the σ^A and $\rho\sigma^A$ values, are shown in Table III. The average Es value for the amino acids, except histidine, in which the amino groups are attached to secondary carbon atoms is -0.543 ± 0.039, and for those in which the amino groups are attached to tertiary carbon atoms, the corresponding value is -2.15 ± 0.06.

The steric substituent constants, Es, calculated from eq 4, are free-energy parameters that give a direct measure of the steric factor associated with the amino component. This point is illustrated best by comparing the reactivities of glycine, α-alanine, and α-aminoisobutyric acid. When compared to glycine, the structure of α-alanine has one of the hydrogens on the carbon atom to which the amino group is attached replaced by a methyl group. This methyl group causes an increase in the pK_2 value of the amino group (polar factor) as well as a change in the steric environment near the amino group (steric factor). Similarly, the structure of α-aminoisobutyric acid differs from that of glycine by having two methyl groups instead of two hydrogens on the carbon atom to which the amino group is attached. The introduction of these methyl groups unto the α-carbon atom of glycine changes both the pK_2 value and the steric environment of the amino group. The effect of the polar factor resulting from the change in the pK_2 value on rates is quantitatively given by $\rho\sigma^A$ and that of the steric factor by Es. The steric factor in α-aminoisobutyric acid is much greater than that in α-alanine because in the former, two methyl groups hinder the approach of a ninhydrin molecule to the amino group during the formation of the transition state, whereas in the latter, only one methyl group is involved in the steric hindrance. Equation 4 permits the

TABLE III: Polar and Steric Substituent Constants for the Rates of Reaction of Amino Acids with Ninhydrin at pH 5.5 and 30°.

Amino Acid	σ^*	$\rho\sigma^*$	*Es*	Rel Obsd Rates	Rel Anion Rates
Glycine	0	0	0	100	100
L-3,5-Diiodotyrosine	−1.75	−1.05	−0.575	133	2.37
L-1-Methylhistidine	−0.75	−0.450	−0.577	53.3	9.41
L-Histidine	−0.62	−0.372	−0.302	88.6	21.3
L-Tyrosine	−0.47	−0.282	−0.501	48.4	16.5
DL-Phenylalanine	−0.47	−0.282	−0.490	49.8	16.9
DL-Methionine	−0.39	−0.234	−0.520	47.0	17.7
DL-Leucine	−0.02	−0.012	−0.520	30.8	29.4
DL-α-Alanine	+0.10	+0.060	−0.574	24.2	30.6
DL-Norleucine	+0.16	+0.096	−0.589	22.2	32.1
DL-α-Phenyl-α-alanine	−0.35	−0.210	−2.14	1.0	0.444
DL-α-Methylmethionine	−0.02	−0.012	−2.09	0.83	0.793
α-Aminoisobutyric acid	+0.55	+0.33	−2.20	0.38	1.35

calculation of individual contributions of polar and steric factors to relative reactivities (Table III).

The magnitude of the steric factor for amino groups attached to tertiary carbon atoms compared to those attached to secondary ones becomes directly apparent from a comparison of relative rates of DL-leucine and DL-α-methylmethionine (Table II). Both of these amino acids have identical pK_2 values and therefore identical polar factors, yet the latter is 37 times less reactive. With this compound the slower rate is presumably the result of the greater steric factor.

The ρ values for the nucleophilic displacement reaction of amino groups with ninhydrin are around 1.5 and the *Es* values around two times greater than the corresponding values determined for the nucleophilic addition reaction of amino groups to α,β-unsaturated compounds (Friedman and Wall, 1964). This difference means that both polar and steric factors govern reactivities more in the ninhydrin than they do in the addition reaction. The larger *Es* value associated with the ninhydrin reaction is not unexpected because the aromatic ring system of the ninhydrin component is relatively rigid and nonflexible; consequently, the amino component is more limited in the number of orientations it can assume during the formation of the transition state than would otherwise be the case. Steric factors should therefore play a more significant part in governing reactivities of the ninhydrin reaction. Support for this hypothesis could possibly be obtained from a comparison of the entropies of activation for the two reactions.

A related observation was made that diastereoisomeric dipeptides, such as D-Leu-L-Tyr and L-Leu-L-Tyr, appear to react at different rates with ninhydrin at 100° depending on the concentration of Methyl Cellosolve or dioxane present in the reaction mixture, whereas enantiomorphic dipeptide pairs, such as D-Leu-Gly and L-Leu-Gly, react at equal rates (Yanari, 1956). The variation in reaction rates of the diastereoisomeric dipeptides might be due to the difference in the apparent pK_2 values of the amino groups in these compounds and to steric factors resulting from the different stereochemistry of the diastereoisomers.

On the basis of present results, it may be concluded that both polar and steric factors influence rates in the ninhydrin reaction. An examination of the mechanism of this reaction for α-amino acids (Scheme I) reveals that both of these parameters are involved during the nucleophilic displacement of a hydroxy group of ninhydrin by an amino group in the first step of the depicted mechanism. Our results are therefore consistent with the view that the first step is rate determining.

It may be assumed that decarboxylation of III is not the rate-determining step because under the conditions of the ninhydrin reaction (pH 5.5) decarboxylation would be expected to be unimolecular and not subject to steric hindrance (Gould, 1959). A slight polar parameter might conceivably exist if the double bonds of the planar transition state are influenced by the R groups *via* hyperconjugation or π-orbital overlap. Neither the hydrolysis of III to IV nor the reaction of IV with I could be rate controlling because in the first place, rates should be governed by steric factors alone, and in the second place, all amino acids should have identical rates. Our results show that neither situation prevails.

The color yields relative to leucine at 100° of a series of sterically hindered α-amino acids in which the amino groups are attached to tertiary carbon atoms were found to increase with time and reached a maximum asymptotic value near 1.00 (Figure 4). At any given time relative color yields are determined by polar and steric factors as described. Low color yields usually reported for such sterically hindered amino acids at

15–20-min periods are therefore due to incomplete reaction.

Reaction Mechanism of ω-Amino Acids. The reaction mechanism of amino acids that do not have the amino α to the carboxyl group differs from that for α-amino acids shown in Scheme I in one major respect. The origin of the pair of electrons required for the transformation of III to a Schiff's base has to be a carbon–hydrogen bond and not a carboxyl group. Such a mechanism predicts that amino compounds which have the amino group attached to a tertiary carbon atom not α to a carboxyl group should be ninhydrin negative. Because the carbon atom that bears the amino group has no hydrogens or carboxyl group attached to it, the mechanism requires an energetically unfavorable carbon–carbon cleavage for these compounds.

These predictions were confirmed with several model compounds. Whereas α-aminoisobutyric acid (VI) has a molar color yield relative to leucine of around 1.00 after a 2-hr reaction period at 100° (Figure 4), the dipeptide VII of this amino acid, in which the amino group is attached to a tertiary carbon atom not α to a carboxyl group, gives no color. Moreover, no color reaction was observed when the homologous β-amino acid VIII and the tertiary amine IX were subjected to the conditions of the ninhydrin reaction for periods up to 2 hr. These observations support the postulated mechanism for ω-amino acids.

Reactions of Aminothiols with Ninhydrin. Another observation in the ninhydrin reaction that requires an explanation is the low color yield of aminothiols, such as cysteine. After a 20-min reaction period the color yield of cysteine relative to leucine was only 0.18 and of cysteine methyl ester, 0.07 (Figure 3). Color yields did not change significantly when the reaction time was increased to 1 hr. These results demonstrate that after a few minutes the amino groups in such aminothiols are unavailable for further reaction with ninhydrin. They also point against cysteine acting as a reducing agent toward ninhydrin because the expected oxidation products would either be cystine or oxygen-containing sulfur compounds such as cysteic acid. Both of these compounds give higher color yields than cysteine (Moore and Stein, 1954).

To establish that low color yields of cysteine and related compounds are due to the presence of the mercapto group in the molecule, ninhydrin color yields were determined for a series of *S*-alkylated cysteines, prepared in connection with another study (Friedman *et al.*, 1965) and consisting of *S*-cyanoethyl-, *S*-carbomethoxyethyl-, *S*-carboethoxyethyl-, and *S*-carbobutoxyethylcysteine. All these compounds had a color yield of 1.0 ± 0.03. These results indicate that the SH group in cysteine is responsible for its low color yield since the *S*-alkyl derivatives behaved normally in the ninhydrin reaction.

The low color yields of cysteine can be explained as being due to a competitive nucleophilic displacement of the sulfhydryl and amino groups of cysteine on ninhydrin to yield intermediates XI and XII in Scheme II. Only XII can proceed to give the ninhydrin chromophore V, whereas XI can cyclize to XIII and effectively prevent further reaction of the amino group to form V. Since the sulfhydryl group reacts about 300 times faster than the amino group in nucleophilic additions at comparable pK_2 values and steric environments (Friedman *et al.*, 1965), predominance in the reaction mixture of intermediate XI probably explains the low yield of V with cysteine and analogous compounds.

The preparation of XIII from 1 mole of cysteine and 1 mole of ninhydrin in aqueous solution (Kuhn and Hammer, 1951) was repeated. This reaction supports our hypothesis. The structure of XIII was confirmed by means of a nuclear magnetic resonance spectrum taken in trifluoroacetic acid with tetramethylsilane as an internal reference (Bovey and Tiers, 1959). The aromatic hydrogens appear at 491 cps (τ 1.82), the CH hydrogen as a triplet at 345 cps (τ 4.25), and the CH_2 hydrogens as a doublet at 248 cps (τ 5.87). The aliphatic hydrogens have nearly identical coupling constants of 5.7 cps.

The color yield of β,β-dimethylcysteine (penicillamine, Xa), moreover, was 0.21 after a 20-min reaction period and 0.42 after 2 hr. This result is consistent with the observation that the sterically hindered sulfhydryl group in penicillamine is only about seven times more reactive as a nucleophile than is the amino group (Friedman *et al.*, 1965). The higher maximum color yield of penicillamine as compared to that for cysteine must therefore be due to a lesser predominance in the reaction mixture of intermediate XIa over XIIa for penicillamine as compared to XI over XII for cysteine.

In conclusion, our results show that the kinetics of the ninhydrin reaction are influenced by both polar and steric factors associated with the amino component and that it is possible to calculate the separate contributions of each of these parameters to rates by means of a free-energy equation. This equation may be used to calculate predicted rates for new amino acids.

The low color yields of aminothiols appear to be due to a competitive reaction of the sulfhydryl group with ninhydrin during which the amino group is effectively prevented from further reaction. Future publications will be concerned with other aspects of the ninhydrin reaction.

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Poly Vinyl(*N*-phenylenemaleimide). A New Selective Binding Agent for Thiols*

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ABSTRACT: The preparation of poly vinyl(*N*-phenylenemaleimide) from polyaminostyrene and maleic anhydride is described. This material was found to react rapidly and irreversibly with sulfhydryl compounds but not with nucleotides, soluble ribonucleic acid, and other nonsulfhydryl compounds.

It is frequently desirable to remove thiols from solutions. Although an organomercurial polysaccharide has been described (Eldjarn and Jellum, 1963) and used to fractionate proteins into SH proteins and non-SH proteins, it suffers from the disadvantage that several substances such as chelating agents interfere with its mode of action. Furthermore the preparation of the material is somewhat complicated. We have therefore developed an alternative material involving the use of a maleimide derivate of polyaminostyrene. The purpose of this paper is to describe the preparation and some properties of this material.

Experimental Section

Materials. High molecular weight polyaminostyrene in the form of a 400 mesh powder was obtained from Kodak Ltd., London, technical grade maleic anhydride was obtained from Hopkins and Williams Ltd., London, *N*-ethylmaleimide, L-cysteine, and glutathione from B.D.H. Ltd., London, thioglycolic acid, thiosalicylic acid, and β-mercaptoethanol from Koch-Light Laboratories Ltd., London, nucleotides from Sigma Chemical Co., St. Louis, and yeast and *Escherichia coli* soluble RNA from General Biochemicals Inc., New York. All other reagents were Analar grade, supplied by B.D.H. Ltd.

Analytical Methods. Sulfhydryl groups in solution were estimated by a method similar to that described by Roberts and Rouser (1958) and Alexander (1958). Aliquots of the solution to be analyzed were mixed with aliquots of 2 mM *N*-ethylmaleimide in phosphate buffer, pH 7, and allowed to stand for 20 min. The absorbance at 302 mμ was measured using a Zeiss PMQ II spectrophotometer, and the change of absorbance with respect to a control was used to calculate the thiol content. A value of 0.61×10^3 (Gregory, 1955) for the change in absorptivity was assumed. Suspensions of resin were filtered using a 0.22 μ millipore filter.

Electrometric titrations were carried out with the aid of an E.I.L. Model 23A direct reading pH meter in conjunction with a GM 23 micro glass electrode supplied by the same firm.

Preparation of Poly Vinyl(N-phenylenemaleimide) (PVM[1]). The preparation of this material was based on the well-known synthesis of *N*-phenylmaleimide (Cava *et al.*, 1961) from aniline and maleic anhydride according to the reaction shown in Scheme I.

The polyaminostyrene was washed with 1 N HCl to remove soluble material and allowed to stand overnight with 1 N NaOH. The resin in the unprotonated form was washed extensively with distilled water until the filtrate was neutral and then dried *in vacuo* over P_2O_5.

Maleic anhydride (80 g) was placed in a 500-ml flask fitted with a reflux condenser surmounted by a $CaCl_2$ drying tube. The flask was gently heated on a sand bath

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[1] Abbreviations used in this work: PVM, poly vinyl (*N*-phenylenemaleimide); NEM, *N*-ethylmaleimide.