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Stoichiometry of Formation of Ruhemann's Purple in the Ninhydrin Reaction[1]

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The course and mechanism of reaction of ninhydrin with amines has both bioanalytical and bioorganic significance since the reaction is widely used for analysis of amino groups and serves as a model for several biochemical reactions that occur in metabolism of phosphonic acid derivatives, deamination, transamination, and transpeptidation. In many cases, e.g., with lysine, cysteine, proteins, the yield of the ninhydrin product, Ruhemann's purple, does not correspond exactly to the expected 1 equiv. per amino group. Possible reasons for this apparent nonideal stoichiometry include slow formation, side reactions, hydrolytic, oxidative, and photolytic instability, and interfering color. The origin and contributions of each of these factors are examined.

INTRODUCTION

Reaction of amines with ninhydrin to form the colored reaction product, Ruhemann's purple (λ_{max} 570; $\varepsilon = 22\,000$), was discovered by Siergfried Ruhemann in 1910 at Cambridge University (*1*, *2*). Since that time, the reaction of amines, amino acids, peptides, proteins, and related model compounds with ninhydrin has found extensive use in qualitative and quantitative chemistry and biochemistry (*3*, *4*). The reaction is widely used to determine free amino acids and related ninhydrin-positive compounds in blood serum (*5*, *6*), and in urine (*7*), to detect and estimate optical purity of D- and L-amino acids, *N*-methylamino acids, and diastereoomeric L-D and L-L peptides after ion-exchange chromatography (*8–10*), and in paleobiochemical (*11*) and histochemical studies (*12*). The suggested mechanism of the reaction of 2-aminoethylphosphonic acid with ninhydrin is useful in explaining the mechanism by which phosphonic acids are metabolized and synthesized in living systems (*13*, *14*). The ninhydrin reaction also serves as a useful model for enzymatic and nonenzymatic decarboxylation, transamination, deamination, and transpeptidation reactions (*15*, *16*).

For several years we have been studying the factors which influence the course and mechanism of ninhydrin reactions for fundamental understanding and to improve their analytic utility (*17–22*). Although the ninhydrin reaction is used daily in thousands of laboratories throughout the world and is thus probably the most widely used organic reaction, several features associated with it appear anomolous. In many cases the amount of color formed varies with the product analyzed and does not always correspond exactly to theory. Causes for this nonideal behaviour are discussed. Experimental

[1] Paper VII in a series on the Ninhydrin Reaction.

data indicate the importance of some of these. As a result of this study, aqueous dimethyl sulfoxide is recommended as a reaction medium. Some factors that affect the stoichiometry of formation of Ruhemann's purple are now considered in detail.

I. *Slow reaction.* Some amines react with ninhydrin considerably more slowly than others (*17*) so a low color yield at one reaction time may sometimes be due simply to incomplete reaction. However, unless the reaction rate is impracticably small, measuring the color yield at several reaction times will show whether the maximum has been reached.

II. *Unfavorable equilibrium.* Because it has several steps, the ninhydrin reaction (Fig. 1) has several points at which amine may be diverted from Ruhemann's purple formation. However, loss of CO_2 and aldehyde formation are essentially irreversible (at least for low concentration of amine, and hence of aldehyde) so any equilibria before the last irreversible step (aldehyde formation) can only slow the rate of reaction, not lower the yield. This leaves the equilibria

$$\begin{array}{ccc} \text{hydrindantin} + NH_3 & \overset{K_{RP}}{\rightleftharpoons} & \underset{(RP)}{\text{Ruhemann's purple}} + H^+ \\ {\scriptstyle H^+}\downarrow\uparrow{\scriptstyle K_{NH_3}} & & \\ NH_4^+ & & \end{array} \qquad (1)$$

that may sometimes be established. Under the conditions of most analytical ninhydrin methods (pH ≥ 4, excess ninhydrin), the intermediate (2-amino-1,3-indaedione, VII) formed by loss of aldehyde seems to be trapped rapidly by excess ninhydrin to form Ruhemann's purple (*23*). At least this is the simplest explanation for the fact that the rate of formation and maximum yield of Ruhemann's purple from simple amino acids is usually greater than from ammonia. However, at low ninhydrin concentration, the 2-aminoindanedione has time before being trapped by ninhydrin to break down to ammonia and 2-hydroxy, 1,3-indandione, IX.

Alternatively, at low pH acid-catalyzed hydrolysis of Ruhemann's purple becomes fast relative to the time of reaction. The equilibrium is shifted by increasing H^+ and removing NH_3. Once this equilibrium becomes established, the color yield is no longer kinetically controlled, but is given by the following expression derived from Eq. (1):

$$\text{Color yield} = \frac{(RP)}{(RP) + (NH_3) + (NH_4^+)} = \left[1 + \frac{\{(H^+)\,1 + (H^+)/K_{NH_3}\}}{(\text{hydrindantin})\,K_{RP}}\right] \qquad (2)$$

Equation (2) should also express the maximum possible color yield from ammonia under any particular conditions of pH and hydrindantin concentration.

Quantitatively, the yield of RP should approach 100% at high pH and high hydrindantin concentration, and approach zero at low hydrindantin concentration or in acid solution. In fact, however, ammonia and ninhydrin do react even in the absence of hydrindantin to form variable amounts of Ruhemann's purple (*24*, *25*, and our own observations). Since the reaction requires reduction at some step, it seems that excess ninhydrin itself acts as a reducing agent. Disproportionation of ninhydrin to give hydrindantin and phthalonic acid has been found to be catalyzed by nucleophiles such as hydroxide and cyanide ions (*26*). Thus, a similar oxidation reduction catalyzed by the nucleophile ammonia would be quite plausible. Note that for α-amino acids,

or primary amines, oxidation of the amino group to an aldehyde can provide the electron pair needed to reduce ninhydrin to hydrindantin.

III. *Side reactions.* Compounds that contain another nucleophilic group (amino, sulfhydryl, hydroxyl) close to the reacting amino group may form cyclic intermediates that are not converted to Ruhemann's purple, or converted only slowly. Thus, ornithine

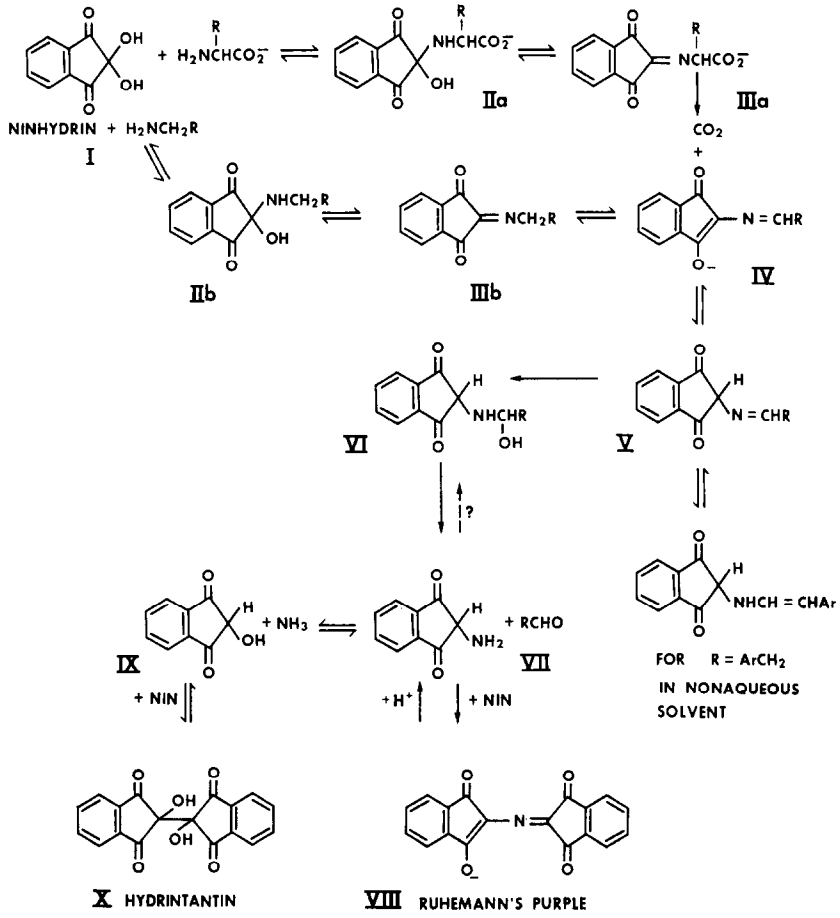

FIG. 1. Pathways in the ninhydrin reaction. Adapted from Refs. 1, 2, 17, 23.

(*27*, *28*), lysine (*29*), and penta- and hexamethylenediame (*28*) give color yields corresponding to only one of their two amino groups. We account for this as the result of rapid cyclization of the intermediate amino-aldehyde base (Fig. 2). Cysteine mainly cyclizes with ninhydrin (*17*, *30*, *31*) to form XII ($Y = S$).

Theoretically, these cyclizations are reversible, so that all the amino nitrogen eventually react to form Ruhemann's purple. However, depending on the stability of the cyclized product, the rate of Ruhemann's purple formation may be slow, as with ethylenediamine (*28*), or practically zero, as with cysteine (*17*), so that the maximum color yield may be experimentally unobtainable. (Patil et al. (*32*) recently described a

method not involving RP formation for estimating cysteine and using an alkaline ninhydrin reagent and Gaitonde (*33*) an acid ninhydrin reagent.)

If a compound reagent contains some nitrogen-containing group such as an amine group that reacts under the experimental conditions to give free primary amine, then the yield of Ruhemann's purple will, of course, exceed that due to the original amine content. The stability of a compound under the reaction conditions, but in the absence of ninhydrin, can be checked easily enough by incubating the reaction mixture without

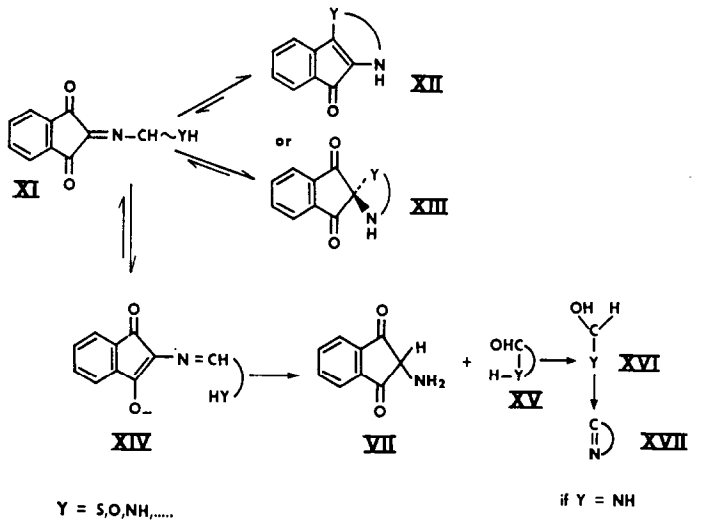

FIG. 2. Possible pathways in the ninhydrin reaction of trifunctional amino acids.

ninhydrin and then adding ninhydrin and measuring the color yield; however, ninhydrin catalysis of the hydrolysis or other reaction is hard to ascertain.

Although production of Ruhemann's purple is generally taken as diagnostic for aliphatic primary amino groups, several cases are known in which other nitrogen functionalities also give the dye. Yanari (*34*) has reported that the ninhydrin color yields of several leucine and alanine dipeptides approach 2 as reaction is prolonged. Also, several keratin proteins give a slow continued production of Ruhemann's purple long after all primary amino groups have reacted, and at a faster rate than can be explained by amide hydrolysis in the absence of ninhydrin and hydrindantin (*4*). Similarly, both *N*-methyl amino acids (*9*, *20*) and α-hydrazino amino acids (*35*) react slowly with ninhydrin to give Ruhemann's purple.

The dipeptides probably give 2 equiv of color not because ninhydrin reacts with groups other than amino groups but as a consequence of the rapid hydrolysis of intermediate α-acylamino acids.

IV. *Interfering color.* Since Ruhemann's purple is usually determined spectrophotometrically, any compounds present that absorb in the same region of the spectrum (especially 570–580 nm) will interfere. Color changes due to heating the reagent solution (*22*) can be determined by running a blank ninhydrin reaction without sample. This will also cancel any error to amine contamination of the solvent.

However, there are many compounds that produce color with ninhydrin without giving Ruhemann's purple. Amino acids such as proline, cyclic secondary amines such as piperidine (*4*), aromatic amines such as aniline (*18*), and the aromatic nucleus of tryptophan (*21, 36*), all condense with ninhydrin to form other color derivatives. Moreover, various non-nitrogenous compounds, chiefly hydroxy and keto aldehydes and acids, give a color with ninhydrin (*37, 38*). The porphyrin moieties of cytochromes and hemaglobin would also interfere but such sample absorbance is easily compensated for by running a blank.

Appreciable amounts of any such interfering compounds can, of course, be readily detected by comparing the sample spectrum with that of pure Ruhemann's purple, e.g., from a simple amino acid. If the ratio of the absorbance at 575 nm to that at 470 nm (a maximum and minimum in the spectrum of Ruhemann's purple), for example, is not the same in two cases, correction for interfering color is probably required. We have previously derived a general equation to correct for interfering color in the ninhydrin reaction (*22*).

RESULTS AND DISCUSSION

Any destruction of Ruhemann's purple under the reaction conditions will lower the color yield. It is possible to measure the rate of destruction of Ruhemann's purple and extrapolate back to zero time. However, a true correction requires knowing the rate of color formation as well, since only dye already formed can be destroyed. Therefore, it is simpler and more certain to find reaction conditions such that the dye is stable. Three types of reaction can contribute to destruction of Ruhemann's purple; hydrolysis, oxidation, and photolysis. We have examined each of these as well as the reaction of proteins with ninhydrin hydrate experimentally.

Hydrolysis. The pH-bleaching profile of Ruhemann's purple was examined in neutral, basic, acidic, and mixed aqueous–DMSO solvents. Results are shown in Tables 1–4.

TABLE 1

STABILITY OF RUHEMANN'S PURPLE IN NEUTRAL AND BASIC SOLUTIONS AT ROOM TEMPERATURE EXCEPT AS SHOWN[a]

pH	Buffer	Remarks
7.0	Phosphate	Less than 2% change in 11 min
9.1	Borate	Less than 2% change in 13 min
13.1	0.1 *M* KOH	Less than 2% change in 12 min
14.0	1.0 *M* KOH	Spectrum decreased with $t_{1/2} = 127$ min; $k_{OH^-}^{25°C} = 0.9 \times 10^{-4}$ 1. M^{-1} sec^{-1}
	0.1 *M* KOH	At 100°C; $t_{1/2} = 17.4$ min

[a] Weighed 1.0 mg Ruhemann's purple in to a 6-ml vial and added 2.0 ml 2-propanol and 2.0 ml water. Heated gently until the solid dissolved. Added 1.0-ml protions of the dye solution 3.0 ml of various buffers in 1.0-cm uv cells and took spectra immediately and after 10–15 min.

Ruhemann's purple appears relatively stable in neutral and basic solution (Table 1). From the results in Table 1 it is possible to calculate an energy of activation in basic solution: $Ea = 6.15 \pm 0.25$ kcal/mole. This value is about one-half that in acid solution (see below).

Sattar and Chaturvedi (*39*) studied the stability of Ruhemann's purple in acid solution. They report that the reaction gives colorless products and is first order with respect to (H^+) and dye concentration. Extrapolating to 100°C at pH 5.5 (the usual analysis conditions), we estimate a hydrolysis rate of about 1%/min, which is not negligible.

To test this estimate, we measured decomposition rates in 0.1 *M* and 1.0 *M* HCl with a Perkin–Elmer 202 spectrophotometer by adding a few tenths of a milliliter of dye stock solution to 3 ml of HCl solution in the cell compartment, then rotating the drum back and forth about 10 nm at the 570-nm peak every 5 sec until reaction was complete. Plotting the OD's obtained at 22°C as a pseudo-first-order reaction (log OD vs time) gave a half-time of 9–10 sec in 0.1 *M* HCl. We estimated about 8 sec from Sattar and Chaturvedi's results. In 1 *N* HCl the reaction was over in 5 sec. Thus, we may tentatively conclude that the p*K* of Ruhemann's purple is near 0 since the hydrolysis rate increases with decreasing pH down to 0, where the rate appears to level off. More detailed studies are needed to prove this point.

The experiment described in the last paragraph does not really test extrapolation to 100°C. The results given in Table 4 and described more fully below are a better test. The half-time (8 min) for disappearance of Ruhemann's purple in 0.22 *M* LiOAc buffer at 100°C corresponds to 9% reaction/min, to be compared with the 1%/min estimated by extrapolation of Sattar and Chaturvedi's results. The significance of the 9-fold difference is difficult to determine, since the pH in the buffer at 100°C is not known; but hydrolysis of Ruhemann's purple under the usual analytical conditions is obviously potentially significant.

To look for acid protonation of Ruhemann's purple, we again measured the rate of hydrolysis, this time using a Cary 14 spectrophotometer at 22°C, recording the decrease in OD after adding several drops of a solution of 1.8 mg Ruhemann's purple (in 0.5 ml H_2O + 0.5 ml 95% EtOH) to 0.1, 1, 6, or 12 *N* HCl in a 1-cm cell and mixing with a disposable pipet. The OD change was followed at 575 nm for 0.1 *N* HCl. However, the dye changed color (from blue to red) in the stronger acid solutions, so the ΔOD^{575nm} was too small for accurate results in this case. Instead, we followed ΔOD^{475nm} and plotted the results as first-order reaction: $\log (OD_t - OD_\infty) = A - 0.303\ t/t_{1/2}$. The observed OD values and calculated half-lives are shown in Table 2. Visual observation of the solution confirms the general results: the blue Ruhemann's purple turns red in stronger acid solutions; the red color fades over a period of several seconds (almost instantly in 12 *N* HCl).

When an ethanolic Ruhemann's purple solution is acidified with 0.1 *N* HCl, the purple color changes to red. This change is accompanied by disappearance of the 575-nm peak and appearance of peaks in the region 450–510 nm and 410–440 nm, the higher wavelength absorption being about twice as intense as the other. Addition of 0.5 *N* NaOH to the ethanolic–HCl solution results in the reappearance of a blue–purple color. These observations suggest that Ruhemann's purple does indeed participate in at least partly reversible acid–base equilibria. However, to demonstrate this fact more precisely, we need to use a flow cell to determine the kinetics of the forward and reverse

TABLE 2

STABILITY OF RUHEMANN'S PURPLE AS A FUNCTION OF HCl CONCENTRATION[a]

Concd HCl	$t_{1/2}$ (sec)	ΔOD_{max}^{475}
0.1 *N*	11.0	0.09
	12.0	0.19
1.0	2.6	~0.10
	2.9	~0.10
6.0	1.4	~0.10
	1.9	0.10
12.0		$\lesssim$ 0.01

[a] See text.

reactions under steady-state conditions. We hope to report such a study in the future.

The logical products of acid hydrolysis of Ruhemann's purple would be ammonia, and hydrindantin (Fig. 3). Spectral studies show that adding coned HCl to an aqueous Ruhemann's purple solution results in a solution with a uv spectrum quite similar to

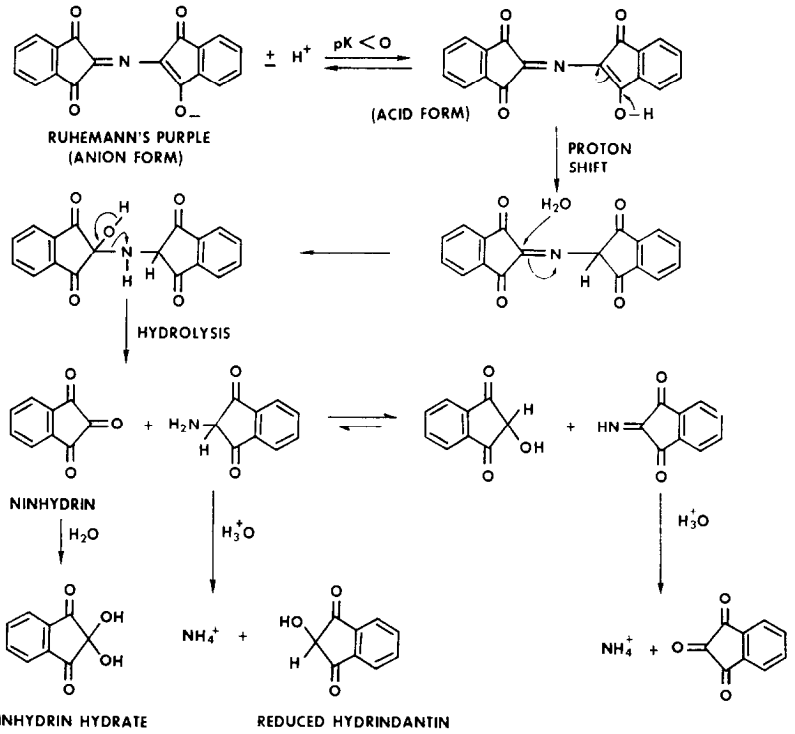

FIG. 3. Postulated mechanism for the acid-catalyzed hydrolysis of Ruhemann's purple.

the corresponding spectrum of an aqueous ninhydrin solution. The observed relative stability of the colored ninhydrin derivative in weakly acid, neutral, and alkaline media and its liability in strong acid can be rationalized as follows. The acid strength of Ruhemann's purple is comparable to that of sulfuric acid ($pK \leq 0$). For this reason, the dye exists entirely in the ionized (salt) form in the pH range 4–14. The molecule consequently resists attack by hydroxide ion because of charge repulsion. On the other hand, protonation of Ruhemann's purple in strongly acid media destroys electron delocalization in the molecule, generating an electrophilic site at the C=N bond, and in this way facilitates hydrolytic cleavage as suggested in Fig. 3.

To determine the stability of Ruhemann's purple in mixed solvents, rates of acid hydrolysis of the dye were examined in mixed HCl/DMSO/H_2O solutions (1.0 ml of either 1.0 *M* HCl or 0.1 *M* HCl + 2.5–9.0 ml DMSO/10.0 ml solution). The rate of hydrolysis was followed by noting the decrease in OD^{570} at room temperature with the Cary 14 Spectrometer. The results summarized in Table 3 are expressed in terms of calculated half-times ($t_{1/2}$) derived from Guggenheim plots.

TABLE 3

HALF-TIMES ($t_{1/2}$) FOR THE HYDROLYSIS OF RUHEMANN'S PURPLE IN MIXED DMSO/HCl SOLUTIONS AT ROOM TEMPERATURE

% DMSO	0.01 *M* HCl (sec)	0.1 *M* HCl (sec)
0	86	10.0
25	138	15.0
50	384	34.5
75	900	95.0
90	600	102.0

The trend is quite clear. Hydrolysis at room temperature in the presence of 80–90% DMSO is slower than in water by a factor of nearly 10.

To establish whether this rate difference exists at higher temperatures, we diluted the 4 *M* lithium acetate buffer 1 : 8 with H_2O and dissolved a little solid Ruhemann's purple in it. We then diluted 4 ml of this solution to 9 ml with (1) H_2O or (2) DMSO and placed three 3-ml samples of each in capped evacuated vials, heated these in the steam bath, and recorded the spectra (Table 4). Results in Table 4 show an even bigger (about 30-fold) rate difference at 100°C than at room temperature. (The nature and possible origin of the observed solvent effects in the absorption spectra of the ninhydrin chromophore were examined in a previous publication (*19*).)

We conclude that in a mixed solvent such as 90% DMSO in the presence of a reducing agent and large excess of ninhydrin as generally used in the ninhydrin reaction, Ruhemann's purple is not destroyed so fast as expected from the hydrolysis rate in the absence of DMSO. Two factors seem to account for this stabilization of color. First, hydrolysis is slower in mixed solvents (Tables 3 and 4). Also, as shown by Lamothe and McCromick (*23*), the acidity of acetate buffers in mixed solvents can be lower than in

TABLE 4

EFFECT OF DMSO ON STABILITY OF RUHEMANN'S PURPLE AT 100°C

0.22 *M* LiOAc buffer–H_2O			0.22 *M* LiOAc buffer–56% DMSO		
Heating time (min)	OD^{575}		Heating time (min)	OD^{585}	
10	0.276	$t_{1/2} \simeq 8$ min	10	0.610	$t_{1/2} \simeq 300$ min
30	0.061		45	0.551	
50	0.020		110	0.481	
			180	0.409	

purely aqueous solution by as much as a pH unit. Second, as discussed earlier, in the presence of hydrindantin, the ammonia formed from hydrolysis of Ruhemann's purple can react to re-form the dye.

Oxidation. Although the presence of oxidizing agents reduces the yield and stability of Ruhemann's purple in the ninhydrin reaction, no good evidence exists that this is due to oxidation of the dye itself. Oxidation of any of the intermediates (eg., 2-amino 1,3-indanedione) and oxidation of hydrindantin would have the same effect. The former would block Ruhemann's purple formation, while the latter would increase the apparent hydrolysis rate by preventing a re-formation of the dye from ammonia.

Photolysis. Since Ruhemann's purple absorbs both uv and visible light strongly, a light-catalyzed dye breakdown would not be surprising. A solution of the dye in pH 9 buffer (to minimize hydrolysis) is relatively stable in the dark at room temperature, but exposure to daylight, especially in the presence of oxygen, leads to considerable bleaching within 13 days (Table 5).

TABLE 5

EFFECT OF OXYGEN AND LIGHT ON STABILITY OF RUHEMANN'S PURPLE[a]

Conditions	OD^{575}	% Original	$t_{1/2}$ (hr)
(At start)	1.135	(100)	—
N_2, dark	1.069	94.0	3440
O_2, dark	1.029	90.5	2140
N_2, light	0.869	76·5	801
O_2, light	0.290	26.6	162

[a] Ruhemann's purple was dissolved in 0.01 *M* $Na_2B_4O_7$ (pH 9). The filtered solution was flushed with N_2 for 10 min. A 3-ml portion of solution was placed in each of four capped vials. These were evacuated. Two were then flushed with N_2 and two with O_2. One oxygen-saturated and one nitrogen-saturated vial were wrapped in aluminium foil and placed in the dark of 13 days in a 500-ml beaker containing distilled water to control the temperature. The matching pair of vials in a 500-ml beaker of distilled water was placed in direct sunlight for the same period.

Since ninhydrin, hydrindantin, and Ruhemann's purple are all polycarbonyl compounds, they may undergo similar degradative reactions. A large excess of ninhydrin and hydrindantin could, therefore, protect Ruhemann's purple from destruction (except possibly in a chain reaction) if their rates of destruction are similar or faster.

Model compounds and proteins. Slobodian et al. (*29*) reported that the yield from side chain lysine ε-amino groups of several proteins is only about two-thirds of the theoretical value. In attempting to determine the fate of the non-color-forming third of the lysine groups, we have concluded that some but not all of these residues that do not give color may react under conditions of the ninhydrin treatment to form a product that does not participate in the usual color reaction even though it still liberates lysine on acid hydrolysis of the ninhydrin-treated proteins.

Table 6 shows that for several model compounds, color yields are generally unchanged by time or temperature of reaction. In the case of polylysine the lack of dependence on

TABLE 6

NINHYDRIN COLOR YIELDS

Compound	Lysine content from color yield[a]	Conditions[b]
Polylysine HBr	324 mmoles/100 g	Room temp.; 65, 144 hr[c]
(*M* 2600)	330	78°C; 15, 45, 90, 180 min[c]
	308	127–132°C; 5, 15 min
	(478)	(expected from 100% yield)
Leucine (standard)	(1.0 mole/mole)	(2.14×10^4 OD ml/mmole-cm at 575 nm)
ε-Amino caproic acid	0.88	100°C; 10, 30, 60 min[c]
	0.91	100°C; 20 min; different sample
N-α-acetyl-lysine	0.98	100°C; 10, 20, 40 min[c]
N-ε-acetyl-lysine methyl ester	0.865	100°C; 10, 20 min[c]

[a] Ninhydrin color yields were measured as previously described (*17, 22*). The color yield (OD ml/mg) is found by multiplying the observed absorbance (optical density) at 575 nm, due to Ruhemann's purple, by the total volume of the solution (ml) and dividing by the corresponding sample weight (mg). The equivalent color yield for leucine, used as a standard, is 2.14×10^4 (OD ml/meq). From these data, the content of reactive amino groups in a protein is estimated as the color yield (OD ml/mg) divided by 2.14×10^4. This result must be multiplied by 10^5 to give mmoles per 100 g.

[b] Amine solution (0.5 ml) + 1.0 ml (2 % ninhydrin + 0.2% hydrindantin in 75% DMSO–25% pH 5.2 lithium acetate buffer).

[c] No variation in color yield was noted when separate aliquots were heated for stated time periods.

reaction temperature indicates that if the 60–70% yield were caused by two competing reaction pathways, only one of which leads to Ruhemann's purple, the two pathways must have similar energies of activation.

Of course, if the low color yield were due to a portion of the lysine side chains being sterically blocked from reaction with ninhydrin, the same lack of temperature dependence might appear. The results of Slobodian et al. seem to rule out this possibility,

since insulin, N^{α}-tosyl-lysine benzyl ester, and ε-aminocaproic acid all gave color yields per lysine and 0.67. If steric hindrance were the reason for the low color yields, the color yields should have been either zero (buried lysine) or one (unburied) or else have been time dependent. Determination of the color yields of some similar compounds (Table 6) indicates that the color yields reported by Slobodian et al. do not always show the full possible extent of reaction.

Several reasons might contribute to this discrepancy. In the case of ε-aminocaproic acid, any cyclization to caprolactam would block the free amino group and decrease color formation correspondingly. Also, Slobodian et al. ran the ninhydrin reaction for 3–5 min; this may be too short in some cases (see wool in Table 7), since primary

TABLE 7

COLOR YIELDS AND LYSINE CONTENT OF NINHYDRIN-TREATED PROTEINS[a]

Protein	Reaction time[b] (min)	A: Amino group content[c] (mmoles/100 g)	B: Lysine[d]	A + B
Polylysine HBr (*M.* 79,000)	30	269.0	128.0[e] 123.0[f] 3[g]	407.0
Lysozyme	0	27.9	30.3	30.3
	30	27.9	5.1	33.0
Wool	0		19.2	19.2
	5	8.7	10.6	19.3
	51	15.6	5.2	20.8
Bovine serum albumin (BSA)	0		69.8	69.8
	30	60.5	17.0	77.5
Ninhydrin-treated BSA control[h]			18.1	
Ninhydrin-treated BSA + methyl acrylate[i]			8.02	

[a] Samples hydrolyzed 24 h in 6 *N* HCl (except polylysine).

[b] With ninhydrin/DMSO–H_2O at 100°C.

[c] Amino group content from ninhydrin color yield.

[d] Lysine content by amino acid analysis of hydrolysate.

[e] Hydrolyzed 144 hr.

[f] Hydrolyzed 72 hr.

[g] Solid residue from *f* hydrolyzed 72 hr.

[h] pH 9.1 buffer–DMSO, 20 hr.

[i] Ninhydrin-treated BSA was dialyzed for several days against distilled water to remove the purple color and then lyophilized. Reaction of ninhydrin-treated, ninhydrin-negative BSA with methyl acrylate was carried out with 20 mg protein in 20 ml 50% v/v pH 9.1 borate buffer-50% DMSO, 2% methyl acrylate, at room temperature for 20 hr. Under these conditions methyl acrylate completely alkylates lysine amino groups in BSA, lysozyme, ribonuclease, casein, and gluten (*42*). The proteins were then dialyzed against distilled water, and lyophilized. Samples were hydrolyzed in 6 *N* HCl for 24 hr in evacuated sealed tubes and were subjected to amino acid analysis. The results were automatically computed (*43*).

amines react more slowly than α-amino acids with ninhydrin (*18*) and accurate determination of the color yields from free amino groups requires measurement of color production at several reaction times and allowance for rates of color destruction and production by extrapolation to zero time (*22*).

Amino acid analyses of several ninhydrin-treated proteins (Table 7), turned up one novel fact: although the non-color-forming lysine is recovered unchanged after acid-catalyzed hydrolysis of the protein, nevertheless, only part of this recoverable lysine appears available to attack by some alkylating agents. For instance, treatment of ninhydrin-reacted BSA with methyl acrylate decreased but did not eliminate the lysine recovered after amino acid hydrolysis (Table 7). Thus, the conditions that limit reaction with ninhydrin do not necessarily prevent reaction with other reagents. The discovery that these ninhydrin-treated proteins contain lysine in a form that can be recovered after acid hydrolysis but is only partly reactive toward amino group reagents is not immediately apparent since factors causing reactivity differences of functional groups in proteins offer a special challenge for explanation. Differences in local geometrical or chemical environment are often invoked to account for such differences. We have also been attracted to the idea that ninhydrin may give alternate, non-color-forming products with some amino groups in proteins, and that these products yield the free amino acid by hydrolysis. We have no direct evidence for such product(s). If they are, in fact, formed, the results of subsequent reaction with methyl acrylate show that only a fraction of lysine residues reacts in this way. Two possible competing reactions may be Schiff's base formation between unreacted amino groups and amide formation between amino and carboxyl groups.

Conclusion. Hydrolysis of the starting reagents is not important, since ninhydrin and hydrindantin are not so acid labile as the dye. We recommend using mixed solvents, e.g., 0.22 *M* LiOAc buffer–DMSO, to stabilize the color. Oxidation and photolysis

TABLE 8

DIAMINO ACIDS COLOR YIELD

$H_2N(CH_2)_nCHCOOH$
|
NH_2

n	Color yield[a]
1	0.118
2	$0.71 + 1.5 \times 10^{-3} \times$ time (min)
3	1.00
4	0.99
$HO_2CCHCH_2CH_2CH_2CHCO_2H$	0.44
/ |	
NH_2 NH_2	
Leucine (reference)	1.00

[a] 3×10^{-4} *M* sample + 2/3% ninhydrin + 2/30% hydrindantin in 52% DMSO-48% H_2O, heated at 100°C for 5, 15, and 45 min. Except for *n*=2, all samples were completely reacted within 5 min.

seem to be important chiefly in storing the hydrindantin-containing reagent mixture. Solutions of ninhydrin alone are rather stable (*40*), so that the color yield is not affected by air, daylight, or oxidizing agents as long as the hydrindantin concentration is high enough (*41*). In any case, any photolytic or oxidative effects can be taken into account by excluding air and light, or by adding a standard of known color yield to the sample and measuring the increase in color yield.

Not only can oxidizing and reducing agents produce changes in the reagent concentrations but certain strong nucleophiles can catalyze destruction of ninhydrin. As mentioned earlier, hydroxide ion (*1*) and cyanide ion (*26*) both catalyze disproportionation of ninhydrin to hydrindantin and phthalonic acid. At high nucleophilic concentrations the reaction gives *o*-carboxymandelic acid instead.

Finally, the nonstoichiometric, low color formation in the ninhydrin reaction of some diamino acids (Table 8) and proteins remains to be explained.

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