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Nicholas D. K. Petraco,[1,2] *Ph.D.; Gloria Proni,*[1] *Ph.D.; Jennifer J. Jackiw,*[1] *Ph.D.; and Anne-Marie Sapse,*[1,2] *Ph.D.*

Amino Acid Alanine Reactivity with the Fingerprint Reagent Ninhydrin. A Detailed *Ab Initio* Computational Study

ABSTRACT: Ninhydrin is one of the most widely used reagents for chemical development of fingerprints on porous surfaces. The detection is based on the reaction of ninhydrin with a monoacidic component of the fingerprint to form an intensively colored compound named Ruhemann's Purple. A computational study of the mechanisms and reaction energetics of the formation of Ruhemann's Purple from ninhydrin and alanine is presented. Such a study is significant from a forensic science point of view because of the strong interest in the forensic chemistry and law enforcement communities in developing alternatives to the current generation of ninhydrin like chemicals for the detection and development of latent fingerprints. Information about the mechanism of reaction between ninhydrin and amino acids can ultimately help to design compounds with stronger chromo-fluorogenic properties in aid of detecting fingerprints at crime scenes. The three most accepted mechanisms of formation have been considered using *ab initio* quantum mechanical calculations. At relatively high temperature ($\sim 100^{\circ}C$) all three mechanisms are energetically feasible. However since it is recommended that forensic analyses be performed at room temperature, a revised mechanism is proposed for the formation of Ruhemann's Purple under this condition.

KEYWORDS: forensic science, computational, quantum chemistry, ninhydrin, mechanism, ruhemann's Purple, hartree-fock SCF calculations, forensic chemistry

Fingerprint comparison is one of the few ways to unequivocally identify unknown perpetrators and victims at a crime scene (1–3). Ninhydrin has been used to develop latent fingerprints at crime scenes for over half a century. The popularity of this crime-fighting tool stems from the production of Ruhemann's Purple upon reaction of ninhydrin with amino acids present in fingerprints (3,4). Ruhemann's Purple is a brightly colored compound which is easy to identify by eye and has the added advantages that it fluoresces slightly at the wavelengths 582 and 407 nm when treated with a zinc or cadmium salt (5,6). Other advantages of using ninhydrin are its low cost and relatively low toxicity (4).

In order to develop new investigative methods for crime fighting it would be ideal to synthesize new compounds that react with amino acids to produce species with properties that surpass those of Ruhemann's Purple. While a large literature exists for synthesis and characterization of alternative compounds to ninhydrin, so far none has been found which offers significant advantages in color development (7). In this regard we note the example of 1,8-diaza-9-fluorenone (DFO) which was synthesized in the late 1980s and found to have superior sensitivity compared to ninhydrin (8–10). However, the compound it produces upon reaction with amino acids in the fingerprint is not as strongly colored as Ruhemann's Purple. Certainly an important tool to improve the chromogenic and even fluorogenic properties of the current generation of fingerprint detection reagents is an unequivocal understanding of their mechanism of formation.

[1]Department of Science, John Jay College of Criminal Justice, City University of New York, 899 10th Avenue, New York, NY 10019.
[2]Graduate Center, Faculty of Chemistry City University of New York, 365 5th Avenue, New York, NY 10016.

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Since the discovery of ninhydrin as an indicator for the presence of amino acids, proteins and peptides, the mechanism of reaction has been under debate (4,11–16). One key piece of work missing from the literature however, is a detailed computational study of the mechanism and reaction energetics of the reaction of ninhydrin with amino acids to form Ruhemann's Purple. In this paper, we will attempt to understand the entire chemical system from a rigorous physical perspective using *ab initio* quantum chemical computations.

In this study we will compute the structures and energetics of the proposed mechanism in order to determine an approximate potential energy surface for the reaction of ninhydrin with amino acid. As this is our first set of computations on this system we have chosen alanine as a representative amino acid in order to keep our calculations within the means of our current computational power. We will also propose a room temperature mechanism of Ruhemann's Purple formation. Future studies will include alternative amino acids as well as ninhydrin analogues and indanediones along with electronic excitation energies of their products.

Computational Methods

All computations were performed using the Spartan suite of quantum chemistry computer programs (17). The structures of each molecule in the McCaldin, Lamothe, and Friedman mechanism were optimized at the spin-restricted Hartree–Fock self-consistent field (RHF-SCF) level of theory using analytic derivative methods. The 6-31G* basis set of Pople was employed in these Hartree–Fock electronic structure calculations (18,19). All stationary point geometrical structures were optimized with in their energetically most stable Abelian point group symmetry as is shown in Table 1. Cartesian coordinate gradients were optimized to less than 10^{-4} a.u. as convergence criteria. Harmonic vibra-

TABLE 1—*Hartree–Fock 6-31G* electronic energies of all structures.*

Structure (Abelian Point Group)	HF-SCF 6-31G* Energy (Hartree)
Ninhydrin (C_2)	− 643.756797
Alanine (C_1)	− 321.856559
1 (C_1)	− 889.591329
2 (C_s)	− 625.942873
3 (C_s)	− 549.052867
Acetaldehyde (C_s)	− 152.915966
4 (C_s)	− 625.949802
5 (C_1)	− 1116.812330
5′ (C_1)	− 1116.785179
6 (C_s)	− 568.873677
7 (C_1)	− 568.893213
8 (C_2)	− 1136.637100
9 (C_s)	− 547.889092
10 (C_{2v})	− 567.717445
11 (C_1)	− 813.565906
12 (C_s)	− 625.962849
Ruhemann's Purple, isomer 1 (C_1)*	− 1040.755468
Ruhemann's Purple, isomer 2 (C_1)	− 1040.770892
Ruhemann's Purple, isomer 3 (C_s)	− 1040.750919
H_2O (C_{2v})	− 76.010747
CO_2(D_{2h})	− 187.634176
NH_3(C_{3v})	− 56.184356
H^+ (K_h)	0.000000

*Ruhemann's Purple isomer used in reaction energetics analysis for Table 3.

tional frequencies at the SCF level were evaluated by numerical differences of analytic gradients. All RHF-SCF structures computed in this study are energetic minima. Benchmark structures for ninhydrin, alanine and Ruhemann's Purple were also computed using density functional theory (DFT) employing the Becke three parameter correlation functional combined with the Lee, Yang, and Parr exchange functional (B3LYP) and a 6-31G** basis set (18,19).

Results and Discussion

Qualitative mechanistic schemes for the reaction of ninhydrin with amino acids have been given by several authors (11–15,20–23). A composite of their ideas is shown in Fig. 1. Figures 2 and 3 show the DFT B3LYP 6-31G** optimized geometries of ninhydrin and an isomer of Ruhemann's Purple closest in structure to the postulated chromophore (24). We call this isomer, Ruhemann's Purple, isomer 1. Table 2 lists the corresponding energies of these two species. To the best of our knowledge, our DFT calculations are at the most sophisticated level of theory yet published in the chemical or forensic science literature for these two molecules.

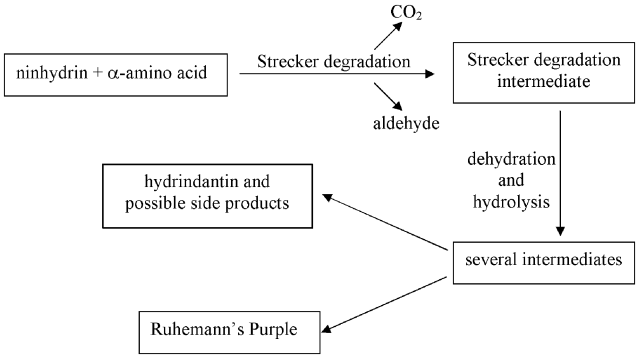

FIG. 1—*Qualitative schematic for the reaction of ninhydrin and an α-amino acid to form Ruhemann's Purple.*

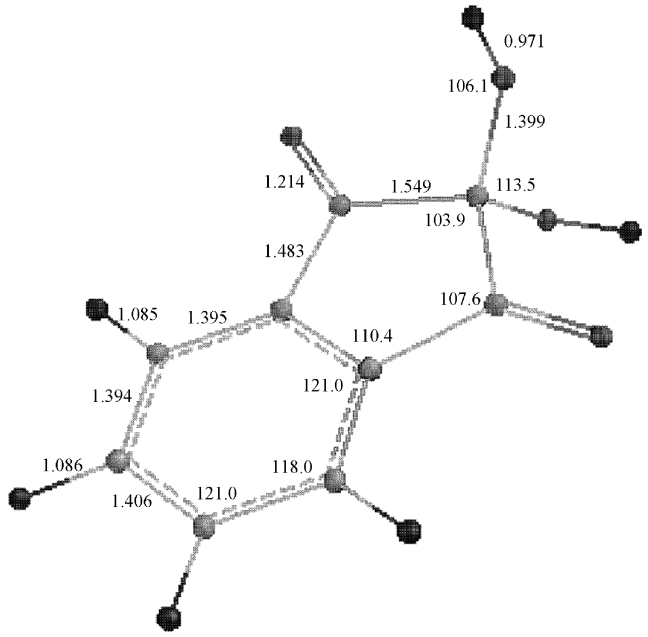

FIG. 2—*DFT B3LYP 6-31G** optimized geometry of ninhydrin. Bond lengths are in angstroms (shown to three decimal places) and bond angles are in degrees (shown to one decimal place).*

Figures 4–6 show the three schemes which are debated to be the actual mechanism of Ruhemann's Purple formation from ninhydrin. Bold numbers beneath each compound of Figs. 4–6 will be used to reference them through out the text. In the text the compound numbers are italicized. Table 1 lists the Hartree–Fock (HF) 6-31G* optimized energies of each of these compounds.

Note that Figs. 4–6 show three different isomers for Ruhemann's Purple which are labeled isomers 1–3. The exact structure which exists in solution or in solid phase (as in the case for fingerprint analysis) has been debated in the literature (24–28). Each of these different isomers shown have been found or inferred by using different spectroscopic techniques. It has been argued by both experiment (4,25) and theory (24) that the most brightly colored version of Ruhemann's Purple is an anionic version of isomer 1, where the proton is missing from the hydroxide group. According to the computational predictions of Dietz et al. (24) isomer 1 may very well be the protonated version of this colored anion, as their electronic structures and spectra are similar . Wigfield et al. however surmise that the protonated version of the anion is isomer 2. Grigg et al. carried out X-ray crystallography studies and found that the protonated version has the structure of isomer 3 (26,27). Elber, Frank, and Almog later confirmed the stability of the overall structure given by Grigg et al. using semi-empirical quantum calculations. Using their quantum chemical software they were in fact able to speculate on the forensic utility of new Ruhemann's Purple analogues based on isomer 3 (29).

We however have chosen to use isomer 1 as the Ruhemann's Purple representative in our discussion of reaction energetics below, as it has both a similar geometric and electronic structure to the brightly colored anion. We have also carried out computational predictions of the structures and energetics of isomers 2 and 3 given by Wigfield et al. and Grigg et al. (25–27). The electronic energies of Ruhemann's Purple isomers 1–3 appear in Table 1. Because the total energies of isomers 2 and 3 differ from that of isomer 1 by only a few kcal/mol, we will not explicitly discuss them in our energetic analysis of the mechanism for Ruhemann's Purple formation. However, at any time the reader may include

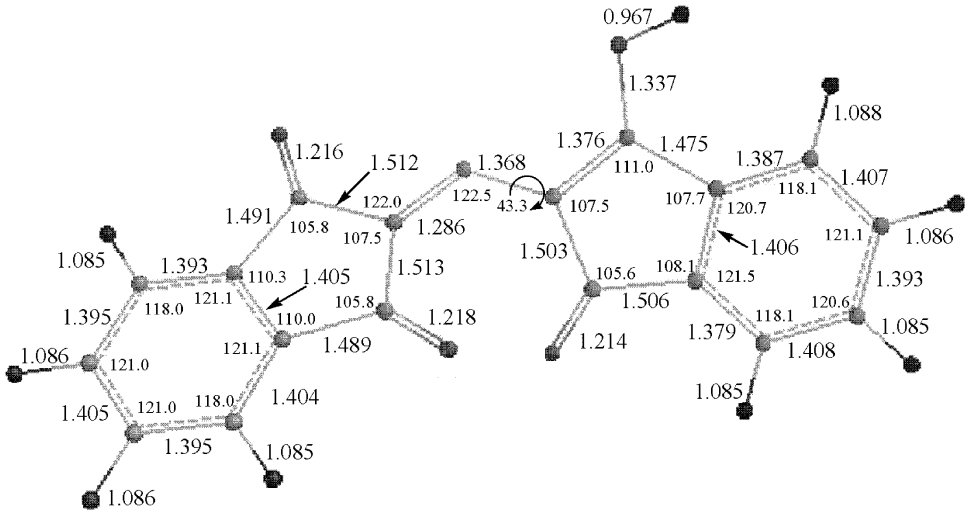

FIG. 3—*DFT B3LYP 6-31G** optimized geometry of Ruhemann's Purple, isomer 1. Bond lengths are in angstroms (shown to three decimal places) and bond angles are in degrees (shown to one decimal place).*

them in the energetic analysis by noting that isomer 2 is 9.7 kcal/mol lower in energy than isomer 1 and isomer 3 is 2.9 kcal/mol higher in energy than isomer 1, respectively (cf. Table 1).

Our reason for focusing on protonated versions anionic Ruhemann's Purple is due to the fact that the ninhydrin–alanine reaction generally takes place in solution or on paper (solid phase) in under somewhat acidic conditions. Thus we believe the protonated versions of Ruhemann's Purple are the most abundant under standard reaction conditions.

The mechanism of Ruhemann's Purple formation, shown in Fig. 4, is the earliest accepted scheme. It was put forth by Johnson and McCaldin and will be referred to as the *McCaldin mechanism* (11,12). The second, which we shall refer to as the *Lamothe mechanism*, is shown in Fig. 5 and represents essentially an extension of the McCaldin mechanism. It was given by Lamothe and McCormick in order to better account for the formation of hydrindantin (**8**) (13,14). Finally, Fig. 6 shows the mechanism suggested by Friedman and Williams (the *Friedman mechanism*) (15). In Figs. 4–6 the reactions are labeled alphabetically by letters (**a–w**) over the reaction arrows. Figure 7 our postulated mechanism for Ruhemann's Purple formation at about 25°C, will be discussed at the end of the text. Table 3 lists the reaction energetics (calculated as energy of the products minus energy of the reactants) for each of the labeled reactions in the mechanisms. These energies are arranged by mechanism and only those reactions unique to the particular mechanism are listed.

In his seminal work, Ruhemann theorized that the key intermediate **6** (2-hydroxy-1,3-indanedione) exists in solution upon reaction of α-amino acids with ninhydrin and goes on to form **8**, hydrindantin. He then postulated that **8** forms Ruhemann's Purple when combined with some unknown ammonium salt (30–35). Unfortunately however, he could not give more detail. What has been agreed upon since Ruhemann's studies is that the formation of Ruhemann's Purple can be understood as taking place in three general stages (4). The first, and rate determining, step is the amino acid attack on ninhydrin or its analogues (cf. Fig. 6) (4). As carbon dioxide and an aldehyde are ultimately produced, this stage is recognized as a special case of the Strecker degradation (21). Generally speaking the Strecker degradation is a mechanism which explains the formation of CO_2 and an aldehyde after hydrolysis of a conjugated carbonyl or carbonyl-nitro group. The second stage involves the production of several intermediates from principally dehydration. Finally, in the third stage, some of the intermediates become involved in side reactions and some go on to form Ruhemann's Purple (4).

TABLE 2—*Density functional theory 6-31G** B3LYP energies of selected structures.*

Structure (Abelian Point Group)	DFT 6-31G** B3LYP Energy (hartree)
Ninhydrin (C_2)	− 647.460616
Alanine (C_1)	− 323.747976
Ruhemann's Purple isomer 1 (C_1)	− 1046.957475

McCaldin Mechanism

The McCaldin mechanism begins with an attack by the lone pair of electrons on the amino acid (we use alanine for this study) at the tertiary carbon of the five member ring on ninhydrin (11,12,36). By the loss of water, intermediate **1** is formed. We find this first reaction (reaction **a**) costs 7.08 kcal/mol in energy. The mechanism then indicates **1** looses CO_2 and H_2O in a Strecker like degradation to form the ylide, **2**. We find for this second step (reaction **b**) that the energy cost is very small, only 2.22 kcal/mol. The second stage of the McCaldin mechanism is shown as having two paths open to **2**. We find for the first path, labeled reaction **c** in Fig. 4, two protons can attach themselves exothermically by − 4.35 kcal/mol, followed by hydrolysis (reaction **e**) releasing − 5.20 kcal/mol. For the second path, labeled reaction **d**, **2** undergoes hydrolysis to form acetaldehyde and intermediate **3**, releasing − 9.55 kcal/mol. Intermediate **3** then has three routes of reaction open to it. It may loose ammonia via hydrolysis to form either **6** or **7** (by reactions **f** or **g** respectively). We have computed that it costs 3.50 kcal/mol for **6** to form from **3** while **7** results exothermically by − 8.76 kcal/mol. These two tautomers (**6** and **7**) are separated by 12.26 kcal/mol, with **7** being the lower in energy of the two. These two reaction paths end with the formation of **8** by the combination of **6** or **7** with ninhydrin and two protons followed by the loss of water. With reference to **7** reaction **j** costs only 1.36 kcal/mol. The third path **3** may take (reaction **i**) is to combine with ninhydrin to yield **5** and water. Reaction **i** is

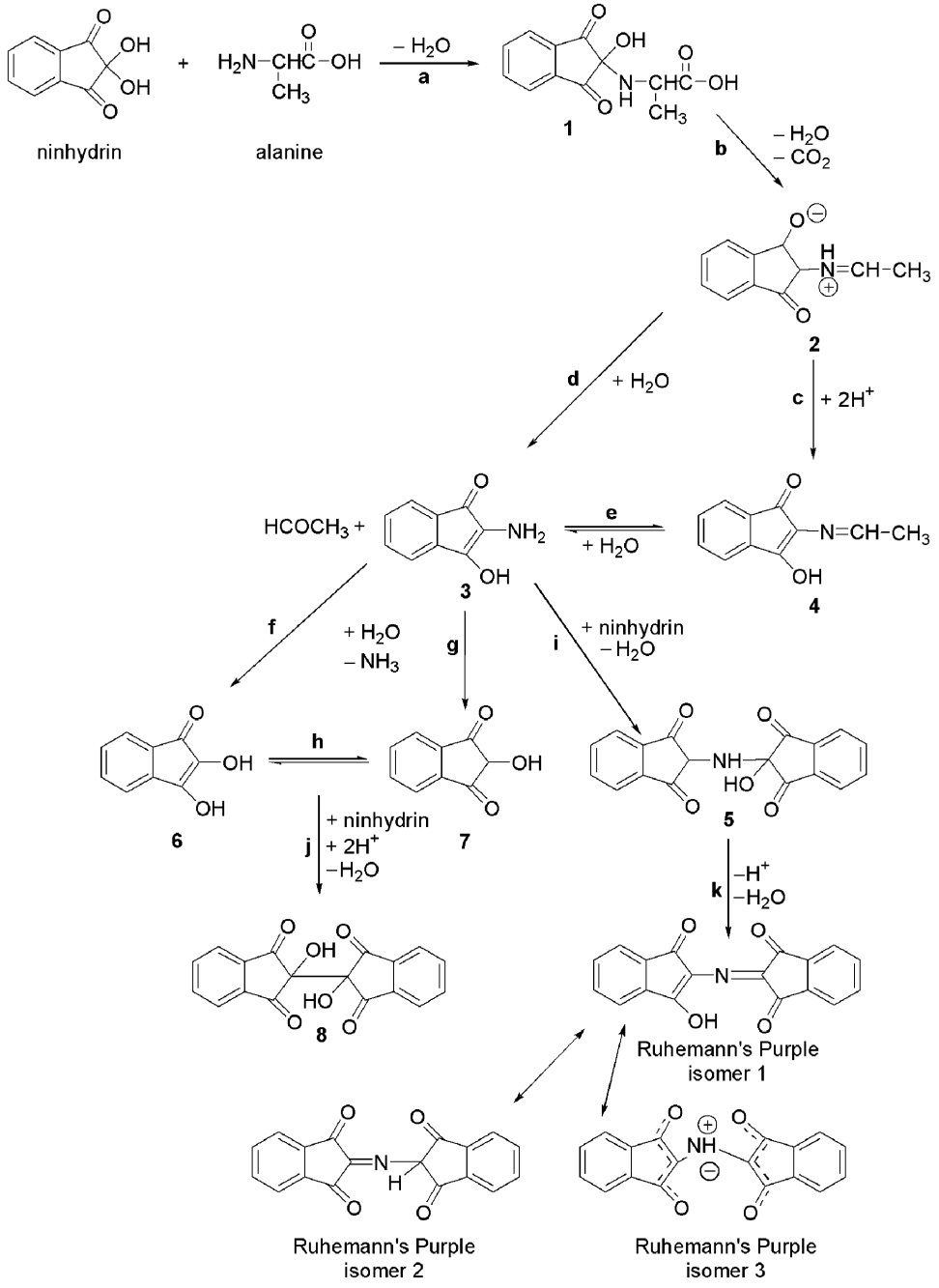

FIG. 4—*McCaldin mechanism.*

exothermic by − 8.42 kcal/mol; however, the reaction following it, which is the last step in the McCaldin mechanism (reaction **k**), has a surprisingly high cost: 28.94 kcal/mol.

Lamothe Mechanism

Wittmann, Muller, and Ziegler later performed stoichiometric studies which led them to conclude that the McCaldin mechanism was basically correct however hydrindantin must somehow be connected to the formation of Ruhemann's Purple, directly or indirectly (22). Using kinetic studies Lamothe and McCormick modified the McCaldin mechanism noting that instead of **3** going on to form **8** (through **6** or **7**) and Ruhemann's Purple (through **5**), actually **3** is unstable in the presence of ninhydrin and primarily forms intermediates **9** and **6** (13,14). Compound **6** can then go on to dimerize and form an equilibrium with **8** or react with **9** to form Ruhemann's Purple directly (cf. Fig. 5). In this way the presence of hydrindantin (**8**) is more directly linked to color formation, consistent with Wittmann's et al. observations (4,22).

The first four steps proposed in the Lamothe mechanism are identical to steps **a–c** and **e** of the McCaldin mechanism. Notable is the absence of reaction **d** shown in the McCaldin mechanism connecting species **2** and **3**. We surmise from our computations that the path of **3** through reactions **c** and **e** (McCaldin mechanism) should not be eliminated from consideration. We find this reaction to be exothermic by the same amount as reaction **n** in the Lamothe mechanism and the experimental conditions of the synthesis are acidic (4).

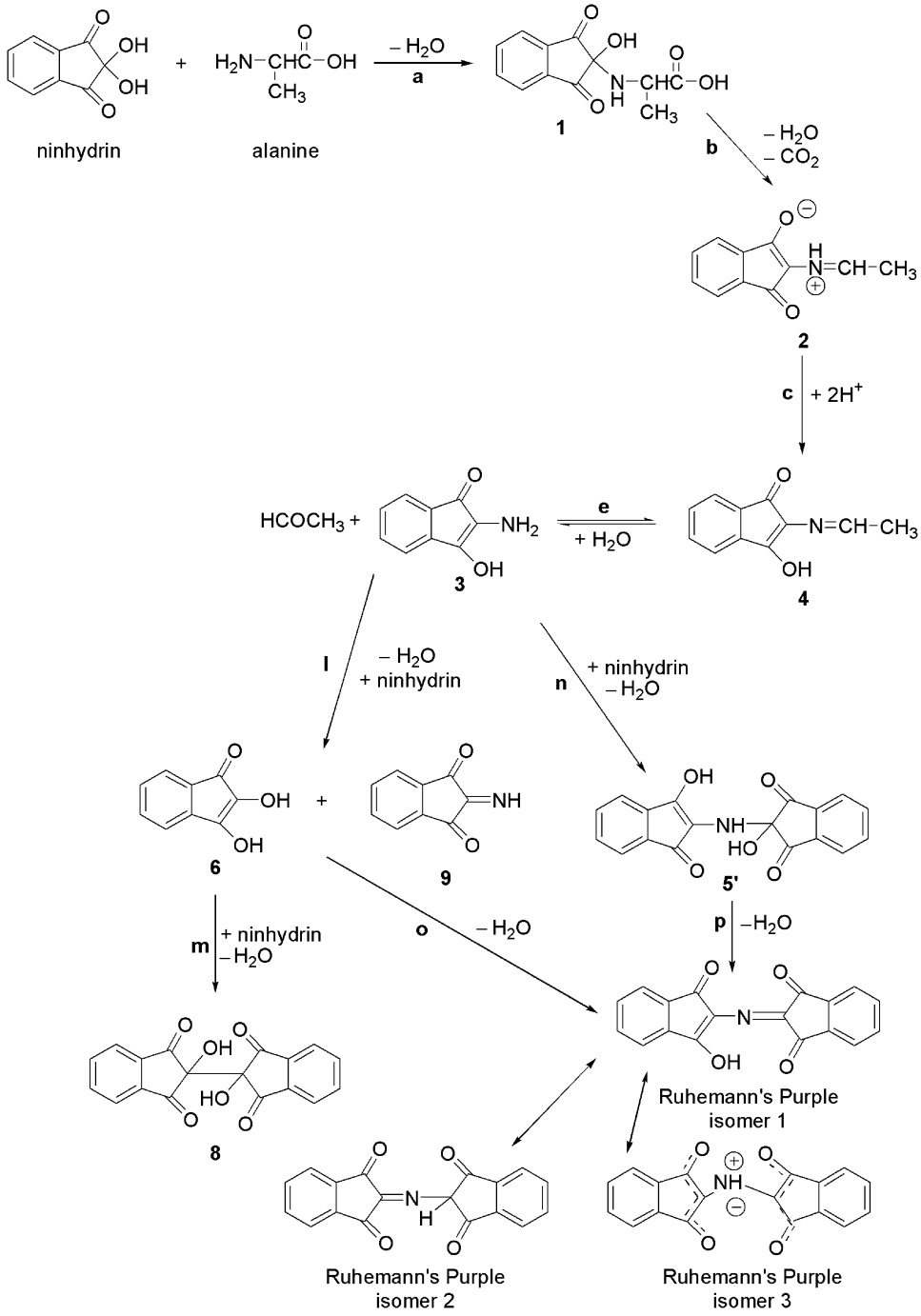

FIG. 5—*Lamothe mechanism.*

After the fourth step, the Lamothe and McCaldin mechanism part ways. Here Lamothe and McCormick first propose that **3** undergoes dehydration by reaction with ninhydrin to form **6** and **9** (13,14). We find this reaction (reaction **l**, Fig. 2) is energetically quite expensive, costing 22.68 kcal/mol. The intermediate **3** however can take another path via reaction **n**, by combining with ninhydrin followed by dehydration to form **5′**. Note that **5′** is the tautomer of **5** (cf. reaction **i** of the McCaldin mechanism) and the two species are separated by 17.04 kcal/mol, with **5** the lower in energy. In the next step, **5′** can dehydrate (reaction **5**) or **6** and **9** can combine losing water (reaction **o**), both paths producing Ruhemann's Purple. While reaction **p** costs 11.90 kcal/mol this is only about half as endothermic as the last step in the McCaldin mechanism. Also, reaction **o** is exothermic by -2.16 kcal/mol. Hydrindantin (**8**) is involved in the Lamothe mechanism via reaction **m** where **6** combines with ninhydrin to loose water. We find this process to be exothermic by -10.90 kcal/mol.

Friedman Mechanism

Friedman and Williams did further stoichiometric studies and present what seems to be the most radical departure from the McCaldin mechanism (15). Their mechanism begins with a dehydration of ninhydrin followed by a Schiff base condensation of

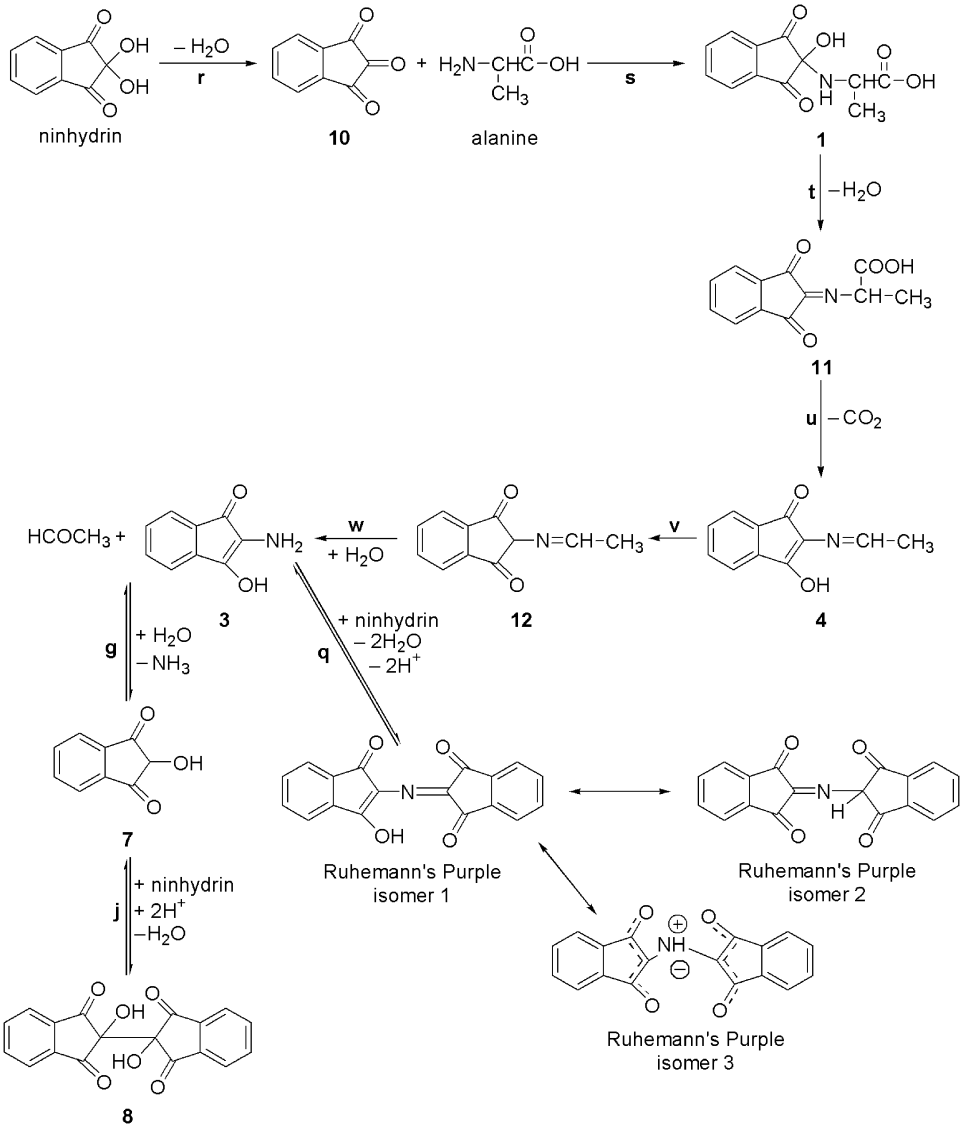

FIG. 6—*Friedman mechanism.*

the dehydration product (**10**) with alanine rather than an addition elimination type reaction (cf. Fig. 6). This is quite a clever scheme and our calculations show the Schiff base condensation yielding **1** (reaction **r**) is exothermic by − 10.87 kcal/mol. However, the initial dehydration of ninhydrin to the Schiff base adduct, **10**, costs 17.95 kcal/mol (reaction **q**) where as in the Lamothe and McCaldin mechanisms the initial addition elimination reaction to produce **1** costs the same amount as Friedman's two-step scheme, and occurs in one concerted way (12).

The next steps in the Friedman mechanism (reaction **s** and **t**, Fig. 6) show the formation of **4** by the loss of water and carbon dioxide via the intermediate **11**. In this way the formation of ylide **2** (cf. Fig. 4 or 5) shown in both the McCaldin and Lamothe mechanisms is avoided. The dehydration of **1** to form **11** costs 9.21 kcal/mol and the subsequent loss of CO_2 from **11** to form **4** is exothermic by − 11.34 kcal/mol.

The intermediate **4** then proceeds to **3** through **12** which is not shown in either the Lamothe or McCaldin mechanisms. The first step in this process (reaction **u**) is the tautomerization of **4** to **12** and is exothermic by − 8.19 kcal/mol. Reaction **v** then shows the hydrolysis of **12** to form **3** and acetaldehyde which our computations show costs 2.99 kcal/mol. We believe however that it is more natural to show the formation of **3** directly from the hydrolysis of **4** (cf. McCaldin and Lamothe mechanisms) because it is a single exothermic process.

Another point of interest in the Friedman mechanism is that they concluded **9** and **6** do not form as suggested by Lamothe and McCormick (14,15). Instead **3** reacts with ninhydrin to form Ruhemann's Purple directly and does not pass through intermediate **5** (McCaldin mechanism) or **5′** (Lamothe mechanism) on the way (cf. Fig. 6). This reaction (reaction **w**) costs 20.52 kcal/mol.

Hydrindantin (**8**) is included in the Friedman mechanism in a similar way to the McCaldin mechanism, however differs in that **3** and **7** are shown in dynamic equilibrium while at the same time **7** and hydrindantin are also in dynamic equilibrium. In this way **8** can also be seen to play a role in the formation of Ruhemann's Purple.

Postulated Mechanism Under Forensic Field Conditions

The reaction of ninhydrin with α-amino acid is performed in the laboratory at temperatures anywhere from 30°C to 100°C. The

TABLE 3—*Hartree-Fock 6-31G* reaction energies for mechanisms shown in Figs. 4–6.*

Mechanism and steps	Reaction	ΔE (kcal/mol)*
McCaldin mechanism		
a	Ninhydrin+alanine → **1**+H_2O	7.08
b	**1** → **2**+H_2O+CO_2	2.22
c	**2**+2 H^+ → **4**	−4.35
d	**2**+H_2O → **3**+acetaldehyde	−9.55
e	**4**+ H_2O → **3**+acetaldehyde	−5.20
f	**3**+ H_2O → **6**+NH_3	3.50
g	**3**+ H_2O → **7**+NH_3	−8.76
h	**6** → **7**	−12.26
i	**3**+ninhydrin → **5**+H_2O	−8.42
j	**7**+ninhydrin+2 H^+ → **8**+H_2O	1.36
k	**5** → RP+H_2O+H^+	28.94
Lamothe mechanism		
l	**3**+ninhydrin → **6**+**9**+H_2O	22.68
m	**6**+ ninhydrin → **8**+H_2O	−10.90
n	**3**+ninhydrin → **5′**+H_2O	8.62
o	**6**+**9** → RP+H_2O	−2.16
p	**5′** → RP+H_2O	11.90
Friedman mechanism		
o	Ninhydrin → **10**+H_2O	17.95
r	**10**+alanine → **1**	−10.87
s	**1** → **11**+H_2O	9.21
t	**11** → **4**+CO_2	−11.34
u	**4** → **12**	−8.19
v	**12**+H_2O → **3**+acetaldehyde	2.99
w	**3**+ninhydrin → RP†+2 H_2O	20.52

*Calculated as energy of products minus energy of reactants. 1 hartree = 627.51 kcal/mol.

†Ruhemann's Purple isomer 1. Cf. Figs. 4–6.

highest yield of Ruhemann's Purple occurs at 100°C and at a pH of about 5 (4). We wish to emphasize that according to our data, all the reactions shown in the three mechanisms (McCaldin, Lamothe, and Friedman) are feasible from an energetic standpoint. At the relatively high temperature where the highest yield occurs, we surmise that all three mechanisms are valid and there are no less than five reaction pathways leading to the formation of Ruhemann's Purple. We are however interested in the development of forensic chemical methods applicable in the field and thus wish to understand the formation of Ruhemann's Purple under ambient temperatures.

When ninhydrin is applied to latent fingerprints in the field it is reasonable to assume that the ambient temperature is likely to be 25 ± 5°C. Figure 7 shows the mechanism of formation of Ruhemann's Purple from ninhydrin and alanine implied by our computations to be taking place in this relatively low-temperature domain. We chose the constituent reactions based on their exothermicity or relatively low (below 15 kcal/mol) endothermicity. We only use single headed reaction arrows (with the exception of tautomerization reactions) because our calculations cannot give equilibrium information. While our calculations are implicitly carried out in the gas phase and the actual reaction of ninhydrin with amino acids takes place on paper in the field, both reaction environments may be expected to yield similar results because we are not examining the relative mobility of the molecules in their respective environments only their relative reaction energetics.

Our postulated mechanism is basically the same as the McCaldin mechanism. The principal difference is that we have substituted reactions **n** and **p** of the Lamothe mechanism for reactions **i** and **k** of the McCaldin mechanism. This was done in consideration of the high energetic cost we found for **5** to form Ruhemann's Purple. We have not eliminated species **5** in favor of **5′** entirely in light of the fact that we find it forms from **3** and ninhydrin exothermically whereas **5′** does not (cf. Table 3). It is shown in tautomeric equilibrium with **5′** though we believe at 25°C very little **5** that forms goes on to form Ruhemann's Purple or tautomerize considering the high associated endothermicities.

It is tempting to also include reactions **m** and **o** from the Lamothe mechanism because of the exothemicity we found to accompany them (cf. Table 3). We chose to eliminate them as a possibility for the low-temperature mechanism however because of the high energetic cost (22.68 kcal/mol) involved with **3** and ninhydrin going on to form the reactants for these reactions (**6** and **9**).

Conclusion

This work is significant from a forensic science point of view because obtaining information about the energetics of the mechanism of reaction between ninhydrin and amino acids could lead to the design of better analogues of ninhydrin or even as yet undreamt of alternatives. Indeed, calculations are already underway for this purpose (D. Sapse, personal communication). The long-term aim of these computations is to help the forensic chemistry community to obtain products with stronger chromo-fluorogenic properties in aid of detecting fingerprints at crime scenes. This paper is a step in that process.

In this study we have computed the chemical structures and energetics for three proposed mechanisms of formation for Ruhemann's Purple from ninhydrin and an α-amino acid using high level *ab initio* quantum chemical calculations. We chose alanine as a representative amino acid due to its small size and uncomplicated structure. Our results imply that under relatively high temperatures ($\sim$ 100°C), where excess energy would be abundant, each chemical step in all three mechanisms is energetically feasible. The multiple reaction paths thus open to Ruhemann's Purple formation therefore help to explain the higher yields at these temperatures.

Using our computations as a guide, we have also given the mechanism for the formation of Ruhemann's Purple at a temperature of around 25°C. This is a reasonable temperature at which to assume most applications of ninhydrin to fingerprints would be preformed in the field. It was found that the scheme basically follows that of the McCaldin mechanism, with the principal difference being intermediate **5** of the McCaldin mechanism should be replaced with **5′** from the Lamothe mechanism.

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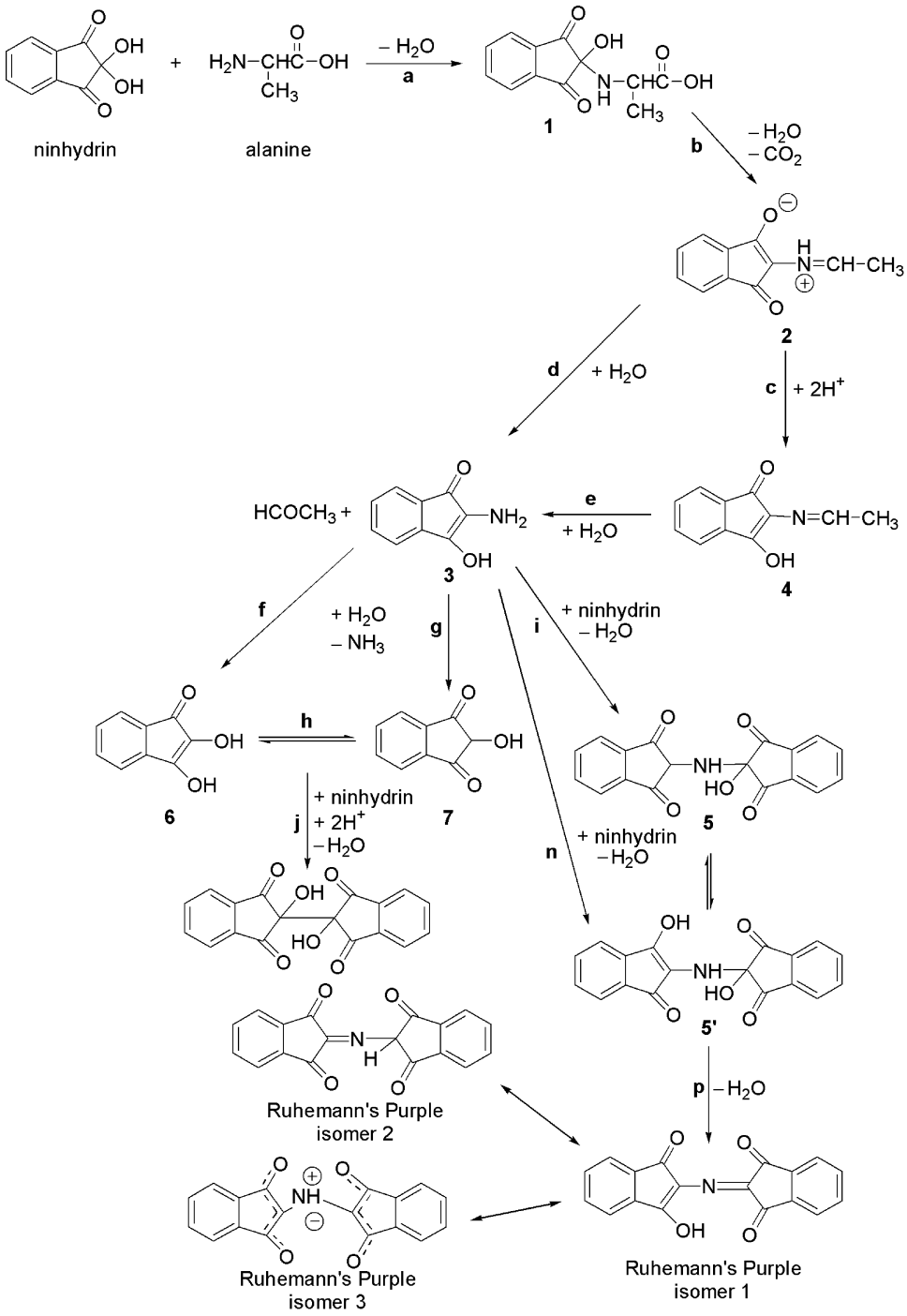

FIG. 7—*Predicted mechanism of Ruhemann's Purple formation in the field at room temperature (about 25°C).*

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Additional information and reprint requests:
Nicholas D. K. Petraco, Ph.D.
Department of Science
John Jay College of Criminal Justice
899 10th Avenue
New York
NY 10019
E-mail: npetraco@jjay.cuny.edu