

# THE CHEMISTRY OF NINHYDRIN

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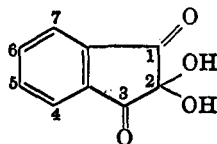
*Received August 31, 1959*

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## I. INTRODUCTION

Ninhydrin has been used for the detection of amino acids and amines for almost fifty years, and many suggestions have been made as to the mechanism of its reactions. In this review it is suggested that a consistent interpretation can be placed on the majority of the reactions of ninhydrin. The literature from 1910 to 1958 is discussed. The system of numbering the skeleton is shown below.



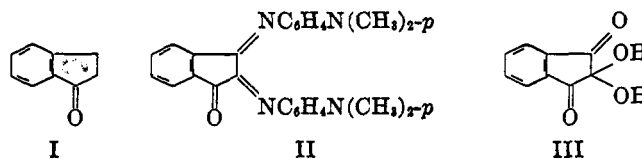
Ninhydrin is also known as triketohydrindene hydrate, Ruhemann's reagent, or more systematically as 2,2-dihydroxy-1,3-indandione.

## II. SYNTHESIS AND STRUCTURE OF NINHYDRIN

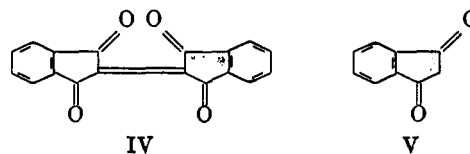
Ninhydrin was first prepared by Ruhemann (61) in an attempt to oxidize 1-hydrindone (I) to 1,2-diketo-

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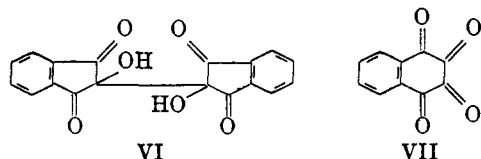
hydrindene with *p*-nitrosodimethylaniline. He did not obtain the expected monoanil but found that two molecules of the nitroso compound had reacted to produce the disubstituted hydrindone (II), which was hydro-



lyzed to ninhydrin (III). Ruhemann commented on the poor yield in this experiment and unsuccessfully attempted to improve it by using 2-hydrindone or 1,3-diketohydrindene as starting material (63). Kaufmann (34) had previously reported a synthesis of ninhydrin which involved oxidation of bis-1,3-diketoindanylidene (IV), but the compound that he obtained did not have the same properties as that isolated by Ruhemann. In 1933 Teeters and Shriner (80) published a synthesis

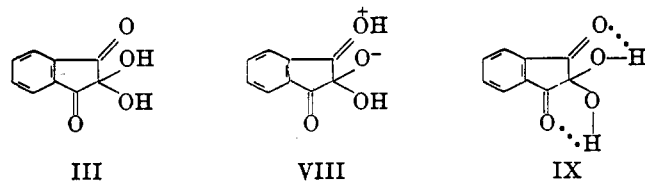


involving the oxidation of 1,3-diketohydrindene (V) by selenium dioxide. The yield in this case was only about 35 per cent; they attributed this to the formation of a bimolecular product and to the recombination of the starting material with the ninhydrin produced. The reaction failed when other oxidizing agents, such as ceric sulfate, were used in place of the selenium dioxide. Wanag and Lode (89) tried to prepare ninhydrin by decomposing 2-nitro-1,3-indandione under different conditions, but obtained only hydrindantin (VI), the



reduction product of ninhydrin. However, by refluxing 2-bromo-2-nitro-1,3-indandione in nitrobenzene, they obtained yields of ninhydrin comparable to those obtained by the previous method. In 1951, Gustowski (19) reviewed the published methods and suggested an improved preparation based on the work of Wanag and Lode. In the same year Khorkhlov, Shchukina, and Shemyakin (35) showed that ninhydrin and hydrindantin were formed when 1,4-naphthoquinone was subjected to long boiling in water at pH 7 in the presence of oxygen. A 48 per cent yield of ninhydrin was claimed, based on the intermediate 1,2,3,4-tetraketotetralin (VII). Although there are a number of side reactions, a method which requires such simple conditions might be useful for large-scale preparations.

On the basis of his experimental work, Ruhemann (62) proposed 2,2-dihydroxy-1,3-indandione (III) as the structure of ninhydrin. Since ninhydrin was color-



less, the keto groups in the 1- and 3-positions must be separated. To explain the ready solubility of ninhydrin in water and its thermal stability, Schönberg and Moubasher (69) suggested an inner-salt or zwitter-ion formula (VIII) with the possibility of resonance between VII and VIII. A structure (IX) stabilized by hydrogen bonding is also possible, since although the O(keto)-O(hydroxyl) distance in ninhydrin is 3.18 Å. and the normal O—H—O distance in hydrogen-bonded molecules is 2.70 Å., other five-membered hydrogen-bonded systems are known (12).

### III. GENERAL PROPERTIES OF NINHYDRIN

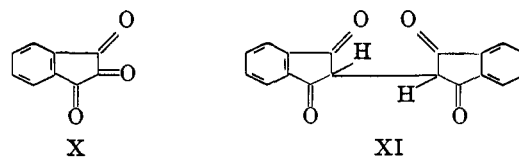
#### A. PHYSICAL PROPERTIES

Ninhydrin crystallizes as pale yellow prisms from ethanol; it gives a yellow aqueous solution and dissolves

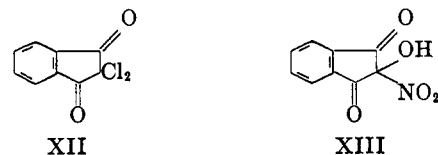
in other polar solvents. When the solid is heated, it changes to a pink, red, or reddish-brown color at 125–130°C., becomes a deep purple-red at 130–140°C., and melts sharply with decomposition at 241°C. (21, 69, 89). The compound becomes red when exposed to sunlight and should be stored in a cool dark place. The ultraviolet and visible absorption spectra of ninhydrin and many of its derivatives have been reported by several authors (43, 44, 56, 58, 67).

#### B. SIMPLE CHEMISTRY

When ninhydrin is treated with thionyl chloride or heated *in vacuo*, 1,2,3-indantrione (X) is obtained as dark red needles (69).

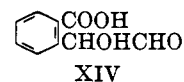


If the blue-green solution of the trione in benzene is shaken with water, the carbonyl group in the 2-position is hydrated, the color is lost from the benzene layer, and ninhydrin is produced. The trione is also produced in concentrated sulfuric acid solution, but if the temperature is raised, bisindandione (XI) is obtained (67). Heating ninhydrin in a current of air yields phthalic anhydride (69). Ninhydrin gives 2,2-dichloro-1,3-indandione (XII) with phosphorus pentachloride, and when treated with hydrogen cyanide yields the 2-substituted cyanohydrin (62). The 2-nitro compound (XIII),



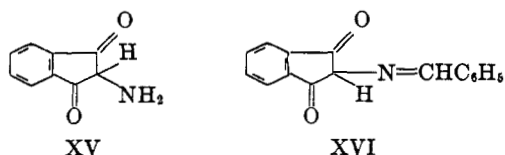
which is obtained with nitric acid, forms useful derivatives with most organic amines (7).

Ninhydrin shows not only ketonic properties but also reduces Fehling's solution and ammoniacal silver nitrate. This was explained by Ruhemann (61) as being due to the opening of the five-membered ring to give *o*-carboxyphenylglyoxylic aldehyde (XIV).



Four oximes have been prepared from ninhydrin. The 2-oxime and the 1,2,3-trioxime were first obtained from diketohydrindene by Wislicenus (92, 93) and later by Ruhemann, who also showed that phenylhydrazine and *o*-phenylenediamine react in the expected manner. The reaction of phenylmagnesium bromide with ninhydrin 2-oxime (52) and the preparation of the 1,2- and 1,3-oximes have also been reported (28, 57). Reduction of ninhydrin 2-oxime with stannous chloride produces

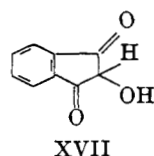
the diketohydrindamine (XV) (65), which is very unstable, but several derivatives are known, including the condensation product with benzaldehyde (XVI). Sev-



eral authors have reported reactions between ninhydrin and certain compounds containing the thiol group (79) and hydroxyl and carbonyl groups (20), but no general pattern can be discerned in these reactions.

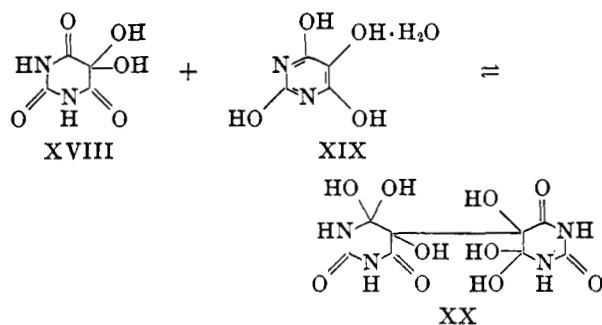
### C. REDUCTION OF NINHYDRIN

Ninhydrin may be reduced to 2-hydroxyindandione (XVII) with sodium amalgam (63), ascorbic acid (1), or



hydrogen sulfide. In the latter case the reaction proceeds further and hydrindantin (VI) is formed by combination with a molecule of ninhydrin (71). This last step is reversible (27).

The reactions of alloxan and alloxantin are strikingly similar. Alloxan (XVIII) can be reduced to alloxantin (XX) and, under appropriate conditions, this can dissociate into and be formed from alloxan and dialuric acid (XIX). It has been shown (51, 68, 70, 71) that hydrin-



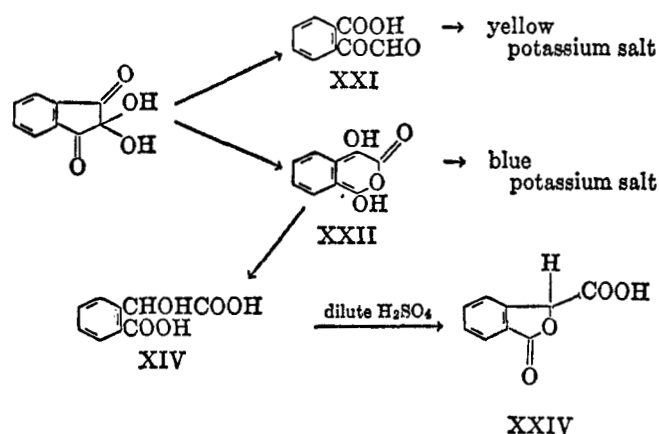
dantin and alloxantin are correctly represented by the pinacol structures XVII and XX, respectively, rather than by hydrogen-bonded formulas. Tipson and Cretcher (81) have confirmed these structures for alloxan and dialuric acid monohydrate from studies of the infrared and ultraviolet absorption spectra.

## IV. REACTIONS OF NINHYDRIN

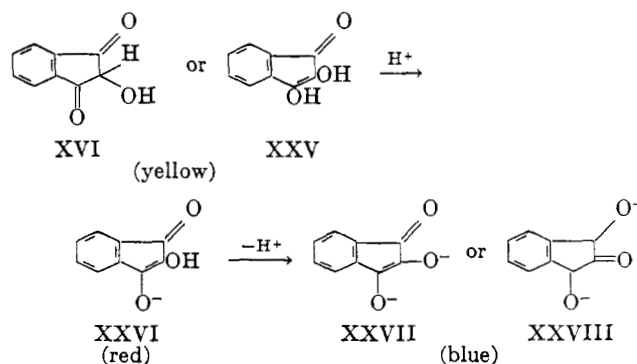
### A. REACTION WITH ALKALI

Ninhydrin exhibits many color reactions under different conditions. When the compound is dissolved in 4 *N* aqueous potassium hydroxide, it produces a yellow

solution; if the solution is left to stand or is diluted, it becomes colorless; when the solution is boiled immediately after the addition of alkali, the yellow color changes rapidly to a deep blue and this color is not lost on dilution. The same blue color, which is not the color observed in the reaction with amino acids, etc., can be obtained by using cold concentrated alkali. Ruhemann (62) explained these changes as follows and the isolation of phthalidecarboxylic acid (XXIV) partially confirmed this.

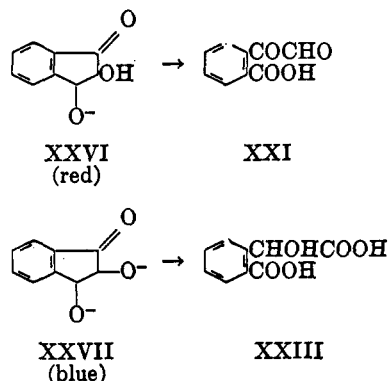


Hydrindantin gives a red color when dissolved in aqueous sodium carbonate, and a blue solution in sodium hydroxide (63). MacFadyen and Fowler (45) have shown that these colors are destroyed in the presence of oxygen. By strict control of the pH and oxygen content of their solutions, they were able to distinguish between the color reactions due to ninhydrin and those due to hydrindantin. This control was essential, since it had been shown that hydrindantin can be oxidized to give two molecules of ninhydrin and, at certain pH values, the latter can form hydrindantin by way of *o*-carboxyphenylglyoxal (XXI). The red and blue colors of hydrindantin were attributed to two anionic forms of 2-hydroxy-1,3-indandione, the monovalent ion (XXVII) of the enolic form (XXV) being responsible for the red color and the two possible divalent ions (XXVII and XXVIII) for the blue color.



Under anaerobic conditions the red and blue colors are interconvertible. The effect of oxidation is irreversible

for the blue color and is reversible for the red color only if ninhydrin is present in the solution. The most plausible explanation is that the red compound (XXVI) is oxidized to *o*-carboxyphenylglyoxal (XXI) and the blue compound (XXVII) to *o*-carboxymandelic acid (XXIII).



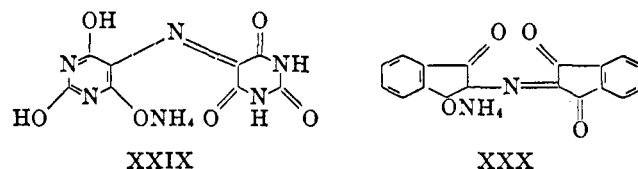
#### B. REACTION WITH AMINO ACIDS

This reaction has been used extensively for detecting and estimating amino acids. However, the mechanism of the reaction has given rise to a number of theories, and only recently has its nature been well understood. Ruhemann observed that ninhydrin, like alloxan, stained the skin, and that a warm aqueous solution of an amino acid gave a deep purple-blue color when treated with the reagent. He obtained the purple color with the amino acids that were available at that time, including  $\beta$ -alanine and  $\beta$ -aminopropionic acid, although aromatic amino acids, such as anthranilic acid, were shown to be the exception. There has been much confusion in later years concerning the type of amino acid which is reactive, and it has sometimes been stated (66) that only  $\alpha$ -amino acids undergo this reaction. It has not always been appreciated, when attempts have been made to explain the ninhydrin reaction, that  $\beta$ -,  $\gamma$ -,  $\delta$ -, and even  $\epsilon$ -amino acids do react under the appropriate conditions (11). This gives an indication of the probable course of the ninhydrin reaction since, if the reaction is as general as is stated above, it is likely that the purple color observed is the same in each case, and that only a fragment of the amino acid involved is contained in the colored compound.

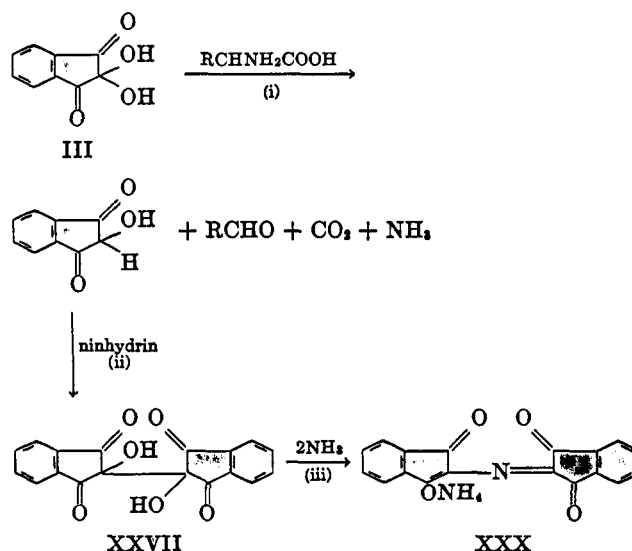
The early theories of the mechanism of this reaction will be considered in turn. For convenience, the name Ruhemann's Purple will be used to denote the substance responsible for the purple color. Any theory proposed must explain two experimental observations: (1) In the reaction of ninhydrin with  $\alpha$ -amino acids, ammonia, carbon dioxide, and an aldehyde with one carbon atom less than the  $\alpha$ -amino acid are produced, and under certain conditions may be obtained quantitatively. (2) Hydrindantin is formed when ninhydrin reacts with  $\alpha$ -amino acids (1, 50).

#### 1. The theory of Ruhemann

Ruhemann (61, 62, 63, 64, 65) was impressed with the close similarity between ninhydrin and alloxan. Strecker had shown that alloxan reacted with  $\alpha$ -amino acids to give carbon dioxide, an aldehyde, and a blue compound which appeared to be murexide (8, 77). Since alloxantin could be used to prepare murexide (55), Ruhemann was prompted to search for the ninhydrin analog of alloxantin, which he obtained and called hydrindantin. He found that it, like alloxantin, gave highly colored salts with alkalis and Ruhemann's Purple with amino acids. He also showed that Ruhemann's Purple was the analog of murexide, obtaining it from hydrindantin by following the method used to prepare murexide from alloxantin. This analogy was most striking and became the subject of later work (24). The structure of murexide has been accepted as XXIX (10, 55, 74), and Ruhemann naturally represented its nin-



hydrin analog as the ammonium salt of Ruhemann's Purple, the systematic name being diketohydrindylidene-diketohydrindamine (XXX). This was the substance believed to give rise to the purple color. His mechanism for the reaction is given below.

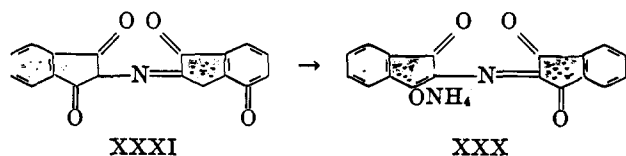
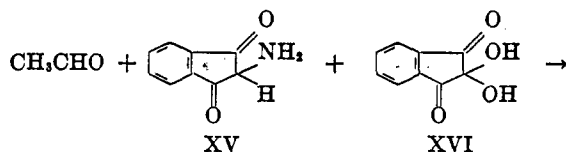
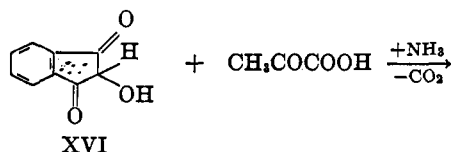
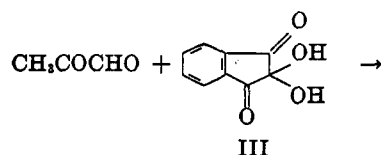
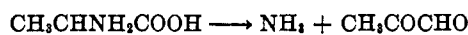


In modern terms the purple color would be associated with the anion rather than the ammonium salt (see below). The first stage of the reaction (i) and the last (iii), in which hydrindantin reacts with two molecules of ammonia in a most unlikely manner, are not discussed. Although Ruhemann was unaware of the fact, ninhydrin also gives a purple color with ammonium

salts (53) and his mechanism cannot account for this. Moreover, the intensity of the purple color formed from ninhydrin and ammonium salts is reduced in the presence of hydrindantin (86). The theory cannot account for the more rapid chromogenic reaction of  $\alpha$ -amino acids with ninhydrin and with hydrindantin, as compared with amines and ammonium salts (24, 66, 86). Nevertheless, his interpretation laid the foundation for future work and his suggestion as to the origin of Ruhemann's Purple is substantially correct.

### 2. The theory of Harding and MacLean

New experimental evidence led these workers (23, 24) to attempt to modify Ruhemann's theory, whilst retaining the formula for the purple compound. They showed that the ultraviolet and visible absorption spectra of murexide (26) and Ruhemann's Purple (20) were similar, that pyridine greatly increased the rate of color formation, and that ninhydrin reacted with ammonium salts, preferably in alkaline solution. The complete reaction with alanine was interpreted as shown below:

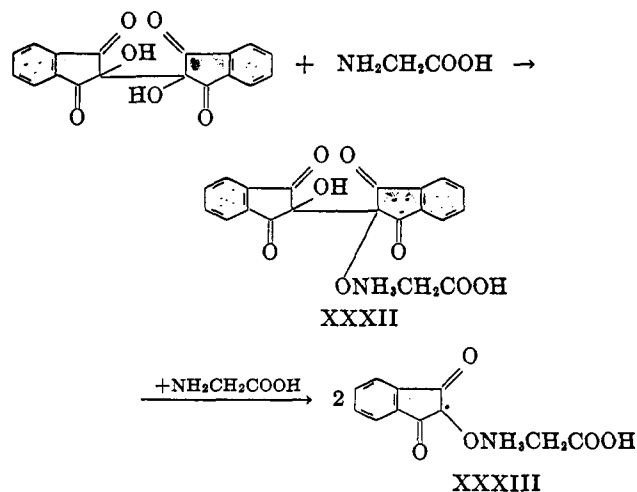


They utilized the theory of Dakin and Dudley (9), which postulated that  $\alpha$ -amino acids can undergo a dissociation or decomposition into ammonia and the corresponding glyoxal. Methylglyoxal is a powerful reducing agent and possibly could reduce ninhydrin to 2-hydroxy-1,3-indandione (XVI) which, with ammonia obtained from the degraded amino acid, would form the amine (XV). This amine is known to condense readily and would be expected to yield diketo-hydrindylidene-diketo-hydrindamine (XXXI) with ninhydrin. The ammonium salt of the product (XXX) would produce the purple color on ionization.

The immediate advantage of this interpretation is that it explains why ammonium salts are able to produce a purple color. However, since ammonia is postulated as an intermediate, the theory does not explain why amino acids react faster with hydrindantin than does ammonia (45). Other criticisms of the theory are that a negligible amount of ammonia is evolved from amino acids in the absence of ninhydrin (42), and the source of carbon dioxide, which is evolved, cannot be the  $\alpha$ -keto acid. It has been shown that the evolution of carbon dioxide from  $\alpha$ -keto acids is much slower than from amino acids in the presence of ninhydrin (86, 87).

### 3. The theory of Retinger

On the basis of experimental work which has remained unpublished, Retinger rejected the previous theories of the ninhydrin reaction and proposed another (59, 60). In this interpretation the amino acid or amine yielded a monobasic colorless salt (XXXII) with hydrindantin formed during the reaction. This salt then produced an unstable dibasic compound which immediately split into two equal radicals (XXXIII), which were supposed to account for the characteristic purple color.

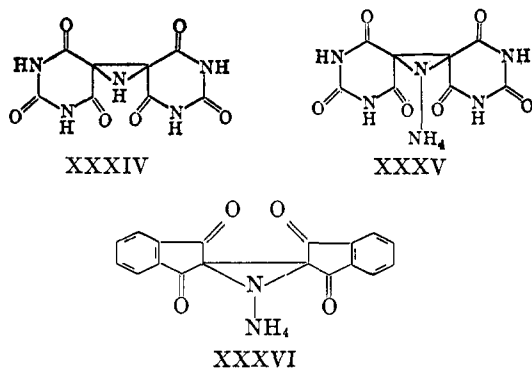


The theory would explain the apparent variety of colors in the ninhydrin reaction as being due to the variable absorption spectra of the free radicals (XXXIII), depending on the particular amino acid residue present. Moreover, the fading of the color could well be due to the decomposition of the free radicals. Retinger's theory is open to many objections, the most important being that salts of hydrindantin show no absorption in the visible range of the spectrum (44, 45), and that the visible and ultraviolet absorption spectra of Ruhemann's Purple and hydrindantin differ greatly in alkaline solution.

### 4. The theory of Woker and Antener

These authors were strongly influenced by the alloxan-ninhydrin analogy and represented the reac-

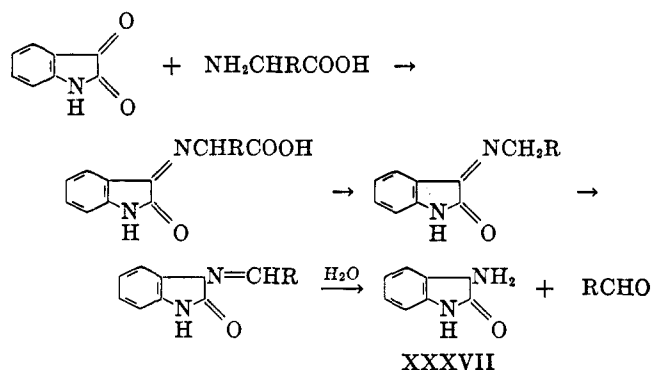
tions of these two compounds with amino acids by parallel schemes (96). They ignored the generally accepted formulas for murexide and purpuric acid and without experimental evidence proposed the less likely ethylenimine structures. Their suggested structures for purpuric acid (XXXIV), murexide (XXXV), and Ruhemann's Purple (XXXVI) are given below:



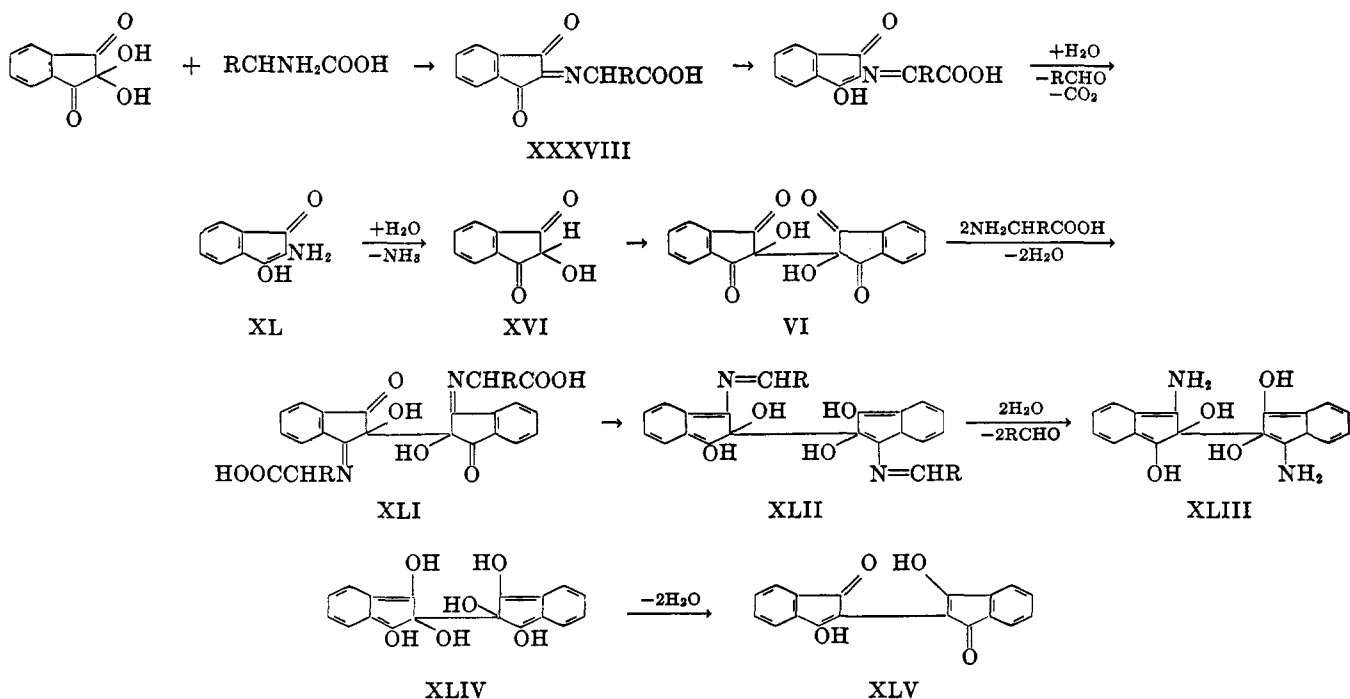
#### 5. The theory of Moubasher and Schönberg

These workers realized that the ninhydrin reaction was a special case of the much more general Strecker reaction. The Strecker degradation (72, 77) refers to all degradations of  $\alpha$ -amino acids by carbonyl compounds and yields aldehydes or ketones containing one less carbon atom. They found (70) that ketones capable of effecting this reaction always contained the grouping  $-\text{CO}[-\text{C}=\text{C}-]_n\text{CO}-$ , where  $n$  was an integer or zero. Examples for which  $n = 0$  are alloxan, isatin, and perinaphthindan-1,2,3-trione hydrate. These three compounds give color reactions with amino acids. Of the

theories proposed to explain the Strecker degradation (2, 14, 38, 91), that of Schönberg, Moubasher, and Mustafa (73) is the most satisfactory. With isatin as an example, the reaction is supposed to proceed as follows:



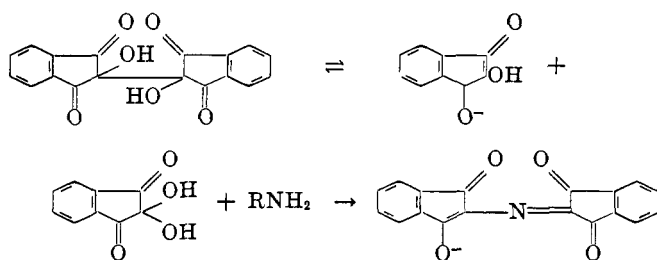
Compound XXXVII is of great significance; the analogous amine in the ninhydrin series provides a plausible intermediate for the formation of diketohydrindylidene-diketohydrindamine (DYDA) from ninhydrin. Any mechanism for the Strecker degradation must explain the formation of the Schiff base, the elimination of carbon dioxide, and the hydrolysis of the rearranged compound. It is doubtful whether one particular step determines the rate of reaction for all degradations. Baddar (3, 4) interpreted the Strecker degradation electronically, but he was severely criticized (49) on the grounds that he had provided insufficient experimental evidence. As an example of the Strecker degradation and the formation of Ruhemann's Purple, the mechanism of Moubasher and Ibrahim (50) is presented below. These workers consider that each of the compounds XXXVIII, XXXIX, XL, and XVI may be color con-



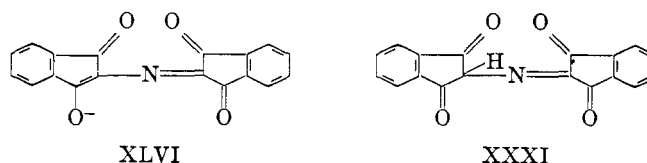
tributors. The varied colors observed in paper chromatography with different amino acids and ninhydrin could then be due to quantities of these compounds present along with the anion of DYDA (11). In support of this involved mechanism, only two new experimental observations are offered. 2,2-Bis(1-hydroxy-3-ketoindene) (XLV), a dark brown material, was obtained from the reaction of ninhydrin or hydrindantin with alanine. As far as the formation of hydrindantin (VI) their scheme follows the normal route observed for Strecker degradations, but beyond this point the mechanism is conjectural. No spectral data are offered, and in fact there is little resemblance between the absorption curves of 2,2-bis(1-hydroxy-3-ketoindene) (XLV) and those of aqueous solutions of ninhydrin and amino acids after reaction.

### 6. The theory of MacFadyen

In 1944 MacFadyen stated that Ruhemann's Purple was due to the anion of DYDA, since the color appeared to depend on ionization (42, 44, 45). By studying the behavior of both ninhydrin and hydrindantin by spectroradiometric methods under rigorously controlled conditions of pH, etc., he obtained results which supported none of the previous theories of the ninhydrin reaction. He showed that the sodium salt of DYDA had the same spectrum as the ammonium salt (43) and that the sodium and potassium salts had the same set of absorption coefficients. Extraction of a dilute sulfuric acid solution (0.001 *N*) of the sodium salt with benzene gave DYDA·2H<sub>2</sub>O. When this benzene solution was extracted with dilute sodium hydroxide at pH 10, Ruhemann's Purple was formed in the aqueous phase. The ultraviolet and visible absorption spectra of solutions of the sodium salt of DYDA were identical with those of reaction mixtures of ninhydrin with primary amines, and the sodium salt was isolated from the reaction of ninhydrin with several amino acids (48). MacFadyen and Fowler (45) showed that ammonium sulfate or  $\alpha$ -alanine reacted with hydrindantin in acetate buffer at pH 7 and that the red color, due to hydrindantin, disappeared at a rate equal to the formation of Ruhemann's Purple. It appears that the nonenolic component of Ruhemann's Purple must be supplied by ninhydrin, which comes from the hydrolysis of hydrindantin, since only one molecule of the enolic component is used up for each molecule of Ruhemann's Purple formed.



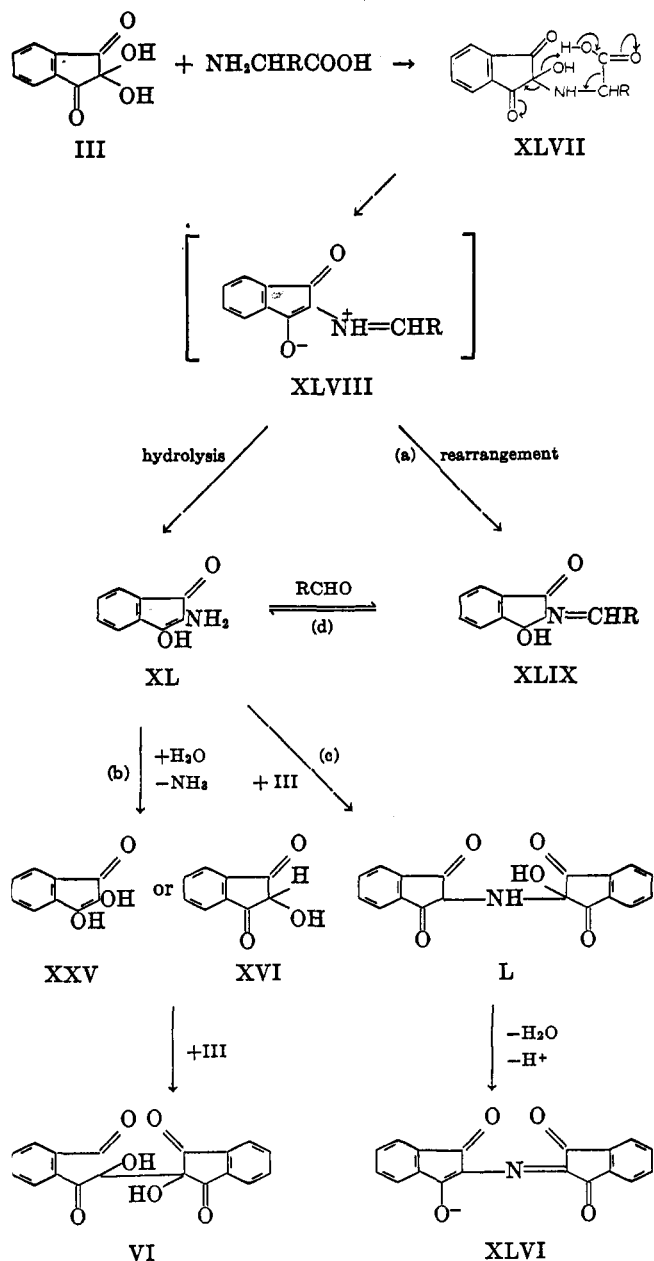
This scheme is very similar to that of Ruhemann and is open to many of the same objections. The authors give no details of the manner in which the condensation step is supposed to proceed, nor of the way in which the amino acid reduces ninhydrin to 2-hydroxy-1,3-indandione. Ruhemann's Purple is independent of the nature of the cation and must therefore be due to the anion of diketohydrindylidene-diketohydrindamine (XLVI). Their reaction scheme is supported by their



finding that the chromogenic reaction of  $\alpha$ -alanine with one molecule of hydrindantin is faster than with two molecules of ninhydrin, and that ammonia forms Ruhemann's Purple with ninhydrin in the presence of a reducing agent capable of producing some hydrindantin.

### 7. The present theory

From the results obtained in a study of the reaction of ninhydrin with imino acids (32), it is now suggested that the reactions of ninhydrin with amines, amino acids, and imino acids all proceed by the same mechanism. The interpretation is based on the mechanism of the Strecker degradation and explains the formation of Ruhemann's Purple and hydrindantin in the reactions with amino acids or amines (see page 46). It invokes a concerted electronic mechanism in the initial reaction of ninhydrin with the  $\alpha$ -amino acid and it will be observed that this avoids the enamine-vinylamine shift postulated by Moubasher and Ibrahim (50), which is improbable under these experimental conditions. The evidence for the formation of the first intermediate (XLVII) is strong; this reaction is the first step of the Strecker degradation, and in the reaction of ninhydrin with cyclic bases compounds analogous to the structure XLVII were isolated. The zwitter ion (XLVIII) is then produced by the electronic changes shown, which involve decarboxylation and dehydration. From this compound, the products of the reaction are formed by hydrolysis or rearrangement. The amine (XL) was isolated by Ruhemann, although he preferred to write it in the diketo form. He had shown that the compound was very reactive, and it is suggested that the three reactions in which it is depicted as participating proceed simultaneously. The aldehyde from the degradation recondenses with the amine to yield XLIX, and 2-hydroxy-1,3-indandione (XVI) or its tautomer (XXV), produced by further hydrolysis, combines with ninhydrin to furnish hydrindantin (VI). Finally, a further molecule of ninhydrin condenses with the amine to

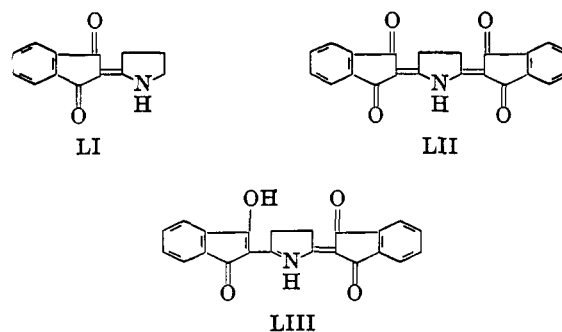


produce Ruhemann's Purple (XLVI). Somewhat related mechanisms have been discussed by Hammick (22), Hine (29), Sweeley and Horning (78), and Metzler, Ikawa, and Snell (46). At pH 1 to 2.5, the reaction proceeds chiefly by route (b), ammonia is evolved almost quantitatively, and no Ruhemann's Purple is formed (42). In solutions of pH 5, route (c) must predominate since, under these conditions, color formation is the basis of the analytical method of Moore and Stein (48). The reaction with imino acids (see below) follows the same path as far as the compound XLVIII. The reactions of ninhydrin are represented here as modifications of the Strecker degradation, which is interpreted as following route (b). This would be the path followed by the reaction of amino acids,  $\text{NH}_2\text{CHRCOOH}$ , with other carbonyl compounds such as the isatins, etc. (38,

39, 40, 41). The theory explains the appearance of aldehyde, carbon dioxide, ammonia, hydrindantin, and Ruhemann's Purple. It has the advantage that it suggests a common route for the reaction of ninhydrin with amines, imino acids, and amino acids, and it explains why these reactions proceed more readily with compounds which contain a carboxyl group adjacent to the nitrogen atom. The orange, brown, gray, and green colors (5, 11, 24) observed by various workers are almost certainly due to the presence of Schiff bases of the type of XLIX, formed via routes (a) or (d). Indeed, Ruhemann isolated several such condensation products including the compound XLIX (R = phenyl), which is orange in color.

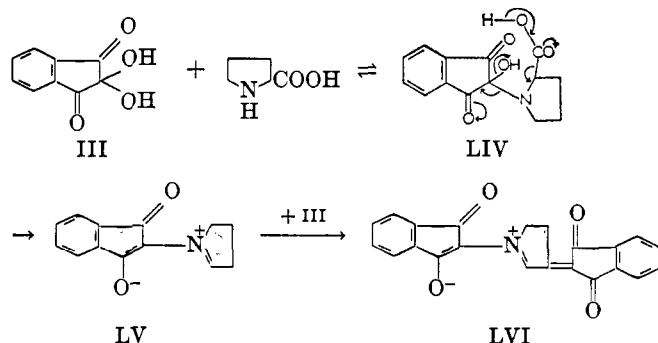
### C. REACTION WITH IMINO ACIDS

Whereas most of the common amino acids give purple colors, certain  $\alpha$ -imino acids such as proline and hydroxyproline give yellow colors (16, 17). This reaction is often used for identifications after chromatography on paper. After prolonged heating, the yellow proline spot may be transformed into purple-red. The chemistry of these colored compounds was first studied by Grassmann and Arnim (16, 17), who suggested that their formation might be used for the estimation of proline and hydroxyproline. Moore and Stein (48) realized that the cyclic  $\alpha$ -imino acids were anomalous in the method that they developed for the photometric determination of  $\alpha$ -amino acids with ninhydrin. Other authors (6, 76, 83, 90) evolved methods for estimating all naturally occurring cyclic  $\alpha$ -imino acids, on the basis of the absorption spectra of the colors they yield with ninhydrin. In the case of proline, for example, it was found that a yellow color ( $\lambda_{\text{max}}$ , 350  $\text{m}\mu$ ) was formed with ninhydrin in acetic acid at room temperature, whereas a reddish-purple color ( $\lambda_{\text{max}}$ , 515  $\text{m}\mu$ ) was formed at 100°C. If the latter reaction was carried out in neutral solution, the resulting compound showed maximum absorption at 550  $\text{m}\mu$ , and this was the compound which was investigated chemically by Grassmann and Arnim (16, 17). They suggested the structures LI and LII for the yellow and purple colors, respectively, and showed that the former was an intermediate in the formation of the latter.





These structures were accepted by later workers (20, 54, 56), and Troll and Lindsley (85) attempted to explain the pigment obtained in acetic acid as being due to an enol form (LIII) of LII. These structures have been revised recently (32), and it has been shown that the effect of ring size on the reaction of the cyclic  $\alpha$ -imino acids with ninhydrin causes the formation of a different type of stable condensation product from each of the four-, five-, and six-membered cyclic  $\alpha$ -imino acids. The reaction between ninhydrin and proline, which is outlined below, is envisaged as following the generalized mechanism described previously.



Compound LV is the yellow product by which proline is generally characterized on paper. Its color, spectra, and other properties closely resemble those of the enol-betaines described by Stafford (75). This mechanism, which involves the  $\alpha$ -carboxylic acid group of proline, explains why no similar intermediate is obtained in the reaction of ninhydrin and pyrrolidine. The purple condensation product (LVI) can be obtained from the intermediate LV or from the reaction of ninhydrin and pyrrolidine, and the suggested structure is substantiated by experimental evidence and by analogy with the proposed structure for isatin blue (31). Under normal conditions, however, the reaction of proline and ninhydrin only proceeds as far as the enol-betaine (LV).

Structures LI and LII can be criticized on several points. Condensations or rearrangements involving the  $\alpha$ -carbon atoms of cyclic  $\alpha$ -imino acids are of limited occurrence (30, 95), and the analogy drawn by Grassmann and Arnim (16) with migrations from nitrogen to carbon in the pyrrole series is clearly invalid. Furthermore, the observations that *N*-substituted imino acids do not give colored compounds and that characteristic pigments may be obtained from cyclic amines substituted at the  $\alpha$ -position make these structures no longer tenable. The more recent interpretation follows the suggested general mechanism for the ninhydrin reaction and explains why cyclic  $\alpha$ -imino acids react faster than the parent amines.

Pipecolic acid (LVII) and its derivatives are characterized by purple-red rather than yellow spots in paper chromatographic analysis by the ninhydrin method (18, 94).

The yellow intermediate corresponding to LV is less stable, although it can often be observed as a transient color, and the final condensation reaction, actuated by the *endo* double bond, furnishes the purple-red compound corresponding to LVI. This striking difference in the behavior of the five- and six-membered cyclic  $\alpha$ -imino acids is attributed to steric factors related to the ring size. The behavior of 2-azetidincarboxylic acid (LVIII) with ninhydrin differs from either of the



previous cases. This imino acid normally gives a brown color on paper, but if this condensation product (presumably the analog of LV) is treated with an excess of ninhydrin and heated, the color of the spot changes to blue. The main absorption of the new product corresponds to that obtained from the reaction of ninhydrin with a primary amino acid. 2-Azetidinecarboxylic acid is known (13) to undergo ring fission to homoserine under comparatively mild conditions, and it is possible therefore that the purple condensation product is the same as that derived from ninhydrin and homoserine.

TABLE 1  
Reactions of cyclic imino acids with ninhydrin\*

Ninhydrin and	Yellow Product	Purple Product
A. Proline.....	x	x
3-Carboxypyrrolidine.....	x	x
3-Methylproline.....	x	x
4-Methylproline.....	x	x
4-Hydroxymethylproline.....	x	x
1-Methyl-2-proline.....	—	—
1,1-Dimethyl-2-carboxypyrrolidine betaine.....	—	—
Kainic acid (47).....	x	—
B. 2-Carboxypiperidine (pipecolic acid).....	Transient	x
5-Hydroxy-2-carboxypiperidine.....	Transient	x
1,2,3,6-Tetrahydro-2-carboxypyridine (baikiain)...	Brown	Brown
C. 2-Azetidinecarboxylic acid.....	Brown	—

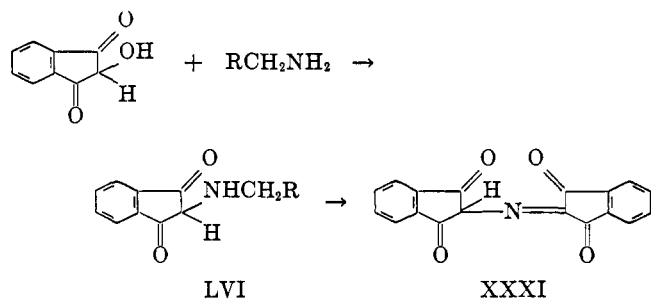
\* x represents a positive reaction; a dash represents a negative one.

The groups A, B, and C correspond to acids with five-, six-, and four-membered rings, respectively.

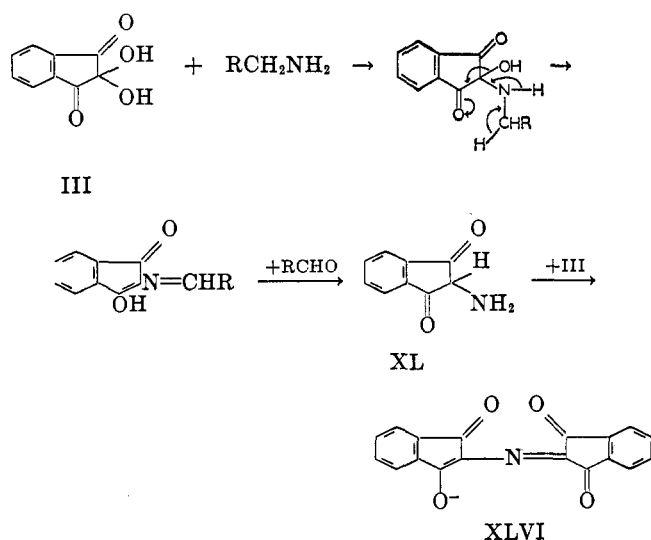
## D. REACTION WITH AMINES

### 1. Primary amines

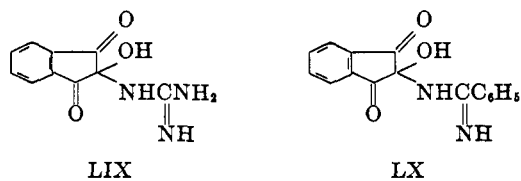
Neuberg (53) showed that many primary amines give Ruhemann's Purple with ninhydrin. Harding and MacLean extended their theory for amino acids and suggested that in alkaline solution ninhydrin could be converted to *o*-carboxyphenylglyoxal, which would reduce ninhydrin to 2-hydroxyindan-1,3-dione.



This would combine with the amine to form the amino compound LVI, which might condense further with ninhydrin to give diketohydrindylidene-diketohydrindamine (XXXI). The latter compound would give a blue color with excess of the amine by formation of a salt. Moubasher and Othman (51) suggested that the reaction proceeded by the Strecker degradation, but their interpretation of the formation of the purple color involved a double salt, which is unlikely. MacFadyen proposed that amines required the presence of both ninhydrin and reduced ninhydrin (43), and it was supposed that the reaction was either a simultaneous or a sequential condensation with ninhydrin and the enol form of reduced ninhydrin. The reaction of ninhydrin with primary amines can be considered as a special case of the general ninhydrin reaction outlined previously for the reaction of amino acids.



Evidence is provided by the isolation of several compounds corresponding to LVIII. Thus guanidine (44, 62) and benzamidine (86) form compounds LIX and LX, respectively, and urea, monomethylurea, and di-

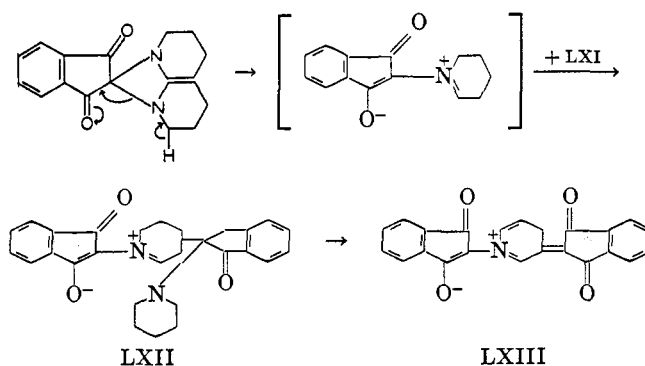


methylurea give analogous products (56). In attempts to clarify their behavior the color reactions of many amines have been summarized (5, 24). The diamines ornithine, cadaverine, and 1,6-diaminohexane are reported to yield Ruhemann's Purple in amounts corresponding to the reaction of only one amino group. Ethylenediamine does not react in the same manner however (98).

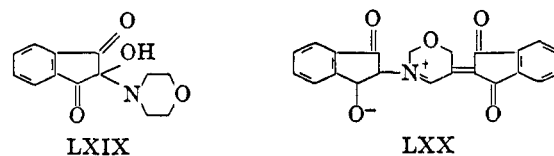
## 2. Secondary amines

The reactions of cyclic secondary amines were originally discussed by Grassman and Arnim (16, 17). They observed that pyrrolidine and piperidine formed purple-red pigments identical with those obtained from proline and pipecolic acid, and that colorless intermediate products could be isolated. These intermediates were readily converted into the purple-red compounds by the action of heat or in the presence of acetic anhydride, and it was suggested that the intermediate compounds were 2,2-diamino-1,3-indandiones (LXI). A similar interpretation to that suggested for the reaction of an  $\alpha$ -imino acid with ninhydrin was put forward.

The nature of these reactions was investigated more recently and a variation of the general mechanism was proposed for the conversion of the 2,2-diaminoindan-1,3-dione into the pigment. Piperidine is taken as an example. This conforms to the general ninhydrin reaction and implies that the ninhydrin units are not fused



to the  $\alpha$ -positions of the piperidine nucleus. A range of intermediate products may be obtained with different cyclic secondary amines. Morpholine, for example, with ninhydrin yields the compound LXIX (32), which can be converted to a purple-red pigment (LXX).



The spatial arrangement of atoms is thought to be rather congested in the latter compounds, since 2,6-dimethylpiperidine, hexamethylenimine, tetrahydro-

quinoline, and certain other cyclic secondary amines do not form compounds of the types described above.

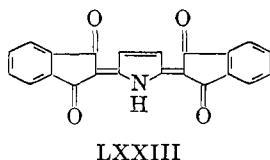
The secondary amino acid sarcosine is reported to be unreactive (73), but more recent work has shown this to be incorrect (97). Formaldehyde may be obtained from the reaction of ninhydrin and sarcosine, ephedrine, adrenaline, and other *N*-methyl amines. It is suggested that in each case the reaction proceeds through the formation of methylamine, which is then degraded in the normal manner. These reactions can be explained by the mechanism outlined earlier in this article, since other work (33) has shown that under different conditions *N*-methylbenzylamine yields benzaldehyde and not formaldehyde, as reported by Yamagishi and Yoshida.

### 3. Tertiary amines

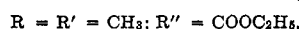
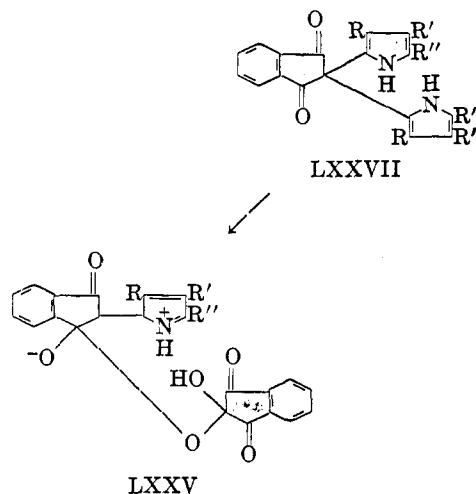
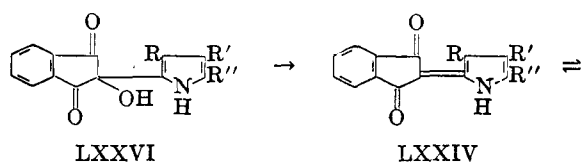
These do not react with ninhydrin.

#### E. REACTION WITH PYRROLES

Pyrroles react with ninhydrin to yield pigments which were assigned similar structures to those obtained from isatin (16, 17). It was suggested that compounds of structure LXXIII could be obtained from the reaction of ninhydrin (or isatin) with pyrrole, pyrroline, or pyrrolidine.

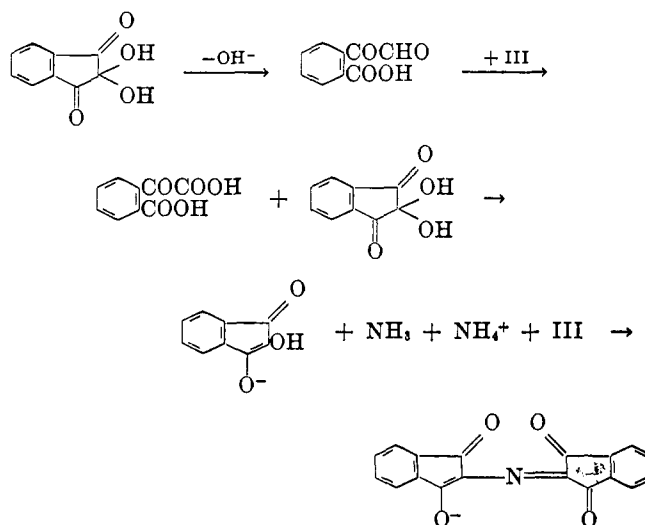


Recently Treibs, Herrmann, and Meissner (84) have reconsidered these ideas and have shown that two pigments are produced from the reaction of pyrroles with ninhydrin. The two compounds have similar ultraviolet spectra, but the analyses showed that two compounds were produced for which the structures LXXIV and LXXV were suggested. A sequence of reactions was described by these workers. Initially the yellow compound LXXVI is formed, from which "ninhydrin blue 1" can be obtained by treatment with hydrobromic acid. Further addition of the pyrrole yields the yellow diadduct LXXVII, and this produces "ninhydrin blue 2" (LXXV) on similar treatment with hydrobromic acid. The formation of an ether linkage in LXXV would be unique in the chemistry of ninhydrin and under acid conditions such a reaction is thought to be unlikely.



#### F. REACTION WITH AMMONIUM SALTS

All ammonium salts give a purple color with ninhydrin, provided the solution is sufficiently concentrated (25, 53). The color was shown to be the same as that obtained from amino acids, and in water containing 0.008 per cent of ammonia was claimed (15) to give a distinct positive reaction. Several of the earlier analytical procedures are useless, because all traces of ammonia and ammonium salts were not initially removed. Harding and Warenford (25) deduced the following mechanism for this reaction, and the work of MacFadyen and Fowler also supports it.



#### V. ANALYTICAL USES OF NINHYDRIN

The possible use of ninhydrin for the analysis of amino acids was suggested by Ruhemann (62), and since that time it has found wide application in identification especially after the introduction of paper chromatography. The qualitative methods have seen little refinement since 1910, but attempts were soon made to use the ninhydrin reaction as a quantitative method of analysis.

The most satisfactory quantitative method was evolved by Van Slyke, Dillon, MacFadyen, and Hamilton (87). This method measured the quantity of carbon dioxide evolved from the amino acids at pH 1 to 5 with great accuracy. Every known amino acid obtained from protein hydrolysis can be analyzed by this method. Since both the carboxyl and amino functions are involved in the reaction, the method has been widely used for estimating free amino acids present in protein digests.

Attempts were made to estimate amino acids in a quantitative manner by measuring other products of the ninhydrin reaction. MacFadyen (42) showed that at pH 1 to 2.5 no Ruhemann's Purple was formed and the amino group of the amino acid appeared as ammonia. However, the yields were never more than 90 per cent, and the method involves many precautions which make it rather unsatisfactory.

Determination of the aldehyde produced from the amino acid appeared a possible method (1). Virtanen and Laine (88) used this reaction to estimate certain specific amino acids which yielded aldehydes that could be determined easily. This is not the case with all aldehydes, however, and the  $\alpha$ -imino acids proline and hydroxyproline do not form aldehydes.

A colorimetric method for estimating amino acids depending on an estimation of Ruhemann's Purple was initially investigated by Harding and Warneford (25). These workers showed that a colorimetric method of estimation seemed limited by their inability to obtain a reproducible color standard. Their results agreed with those obtained by the Van Slyke method. Moore and Stein (48) set out to improve this method and were able by the addition of stannous chloride or hydrindantin to obtain a constant color yield for a given amino acid. Peptides, primary amines, and ammonia could also be estimated by this method. The procedure which was designed for use in conjunction with their chromatographic work relies on the development of Ruhemann's Purple. The reaction was carried out in a citrate buffer at pH 5 and 100°C. The absorption maximum of the purple color was estimated at 570 m $\mu$ . Later the method was modified to include estimation of proline and hydroxyproline. The accuracy of the method is approximately  $\pm 2$  per cent, which is comparable with the Van Slyke method.

Ninhydrin has found certain other uses in the detection of compounds in biological fluids, e.g., urinary indican (36, 82).

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