

MECHANISM OF THE NINHYDRIN REACTION

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The detection and the quantitative estimation of α -amino acids (I) has long been accomplished by their reaction with ninhydrin (II) (2,2-dihydroxy-1,3-indandione). The reaction products include an aldehyde with one carbon atom less than the α -amino acid and carbon dioxide in stoichiometric amounts and varying amounts of ammonia, hydrindantin (IX) and a chromophoric compound, Ruhemann's Purple (diketohydrindylidenediketohydrindamine, VII). This pigment serves as the basis of detection and quantitative estimation of α -amino acids¹. Extensive use of the ninhydrin reaction is evidenced by its inclusion in both modern biochemistry and organic chemistry textbooks. However, the mechanism used to illustrate the reaction varies considerably among recent biochemistry and organic chemistry texts.

A survey of textbooks indicated two basically different mechanisms used to explain the formation of Ruhemann's Purple. The first (Scheme I), which follows in part the original hypothesis of Ruhemann², involves the oxidative deamination and decarboxylation of the amino acid and the subsequent condensation of the released free ammonia with one molecule each of ninhydrin (II) and hydroxydiketohydrindene (2-hydroxy-1,3-indandione, VIII). It is cited frequently in widely-used biochemistry textbooks, although it has been refuted by early^{3,4} and more recent studies^{5,6,7}. The current acceptable general mechanism for the ninhydrin reaction is illustrated in Scheme II, adapted from reference⁶. Here, ninhydrin (II) is shown to tautomerize to 1,2,3-indantrione (IIa) which forms a Schiff's base with the amino acid (I). The ketimine formed (reaction a) undergoes decarboxylation yielding the aldehyde and an intermediate amine (2-amino-1,3-indandione, VI), which was isolated by Ruhemann in his early studies². Condensation of this intermediate amine with another molecule of ninhydrin follows to form the expected chromophore, Ruhemann's Purple (VII). This mechanism accounts for the appearance of all the products obtained experimentally.

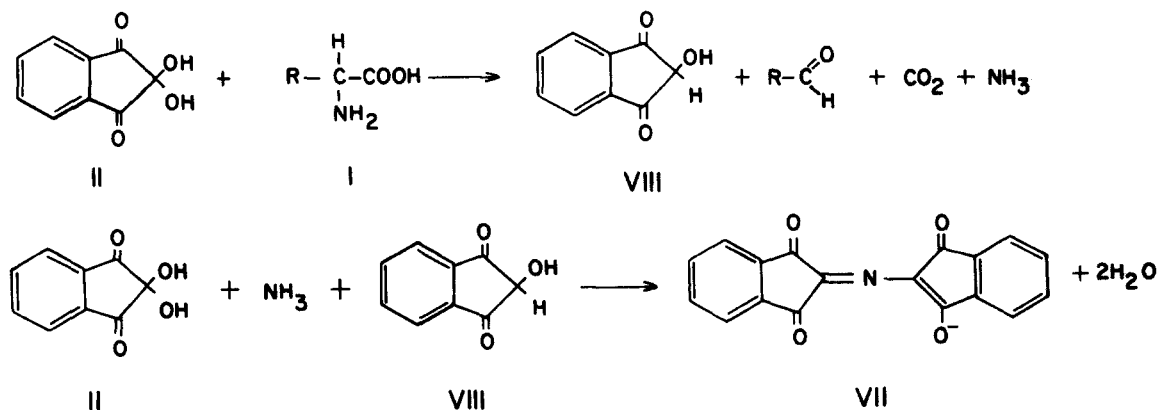
McCaldin³ in his 1960 review of the chemistry of ninhydrin suggested the involvement of the intermediate amine (VI). This possibility was supported later in the work of Friedman and Sigel⁴. McCaldin's mechanism differed from that depicted in Scheme II by involving initially the nucleophilic displacement of a hydroxy group of ninhydrin by an amino group. The initial step is more likely a Schiff's base-type condensation. Regardless of the path by which it is formed, the proposed mechanism involves an intermediate amine which is contrary to the sequence of reactions involving free ammonia as described in most biochemistry texts. The suggestion that free ammonia is not involved in the reaction of ninhydrin with α -amino acids is supported by experimental observations. It is known that ammonia reacts with ninhydrin, but at a much slower rate than amino acids⁸. Hence, the intermediacy of ammonia, as suggested

by Scheme I, is highly unlikely. In the pH range 1-2 and the presence of excess ninhydrin, ammonia is produced almost quantitatively but no color is formed, presumably due to the protonation of ammonia. However, aqueous solutions of amino acids do react in the pH range 5-7 to form Ruhemann's Purple¹. At this pH, insignificant hydrolysis of the intermediate amine occurs which leads to free ammonia. Hydrolysis of the amine under certain circumstances may account for the less than quantitative yield of color.

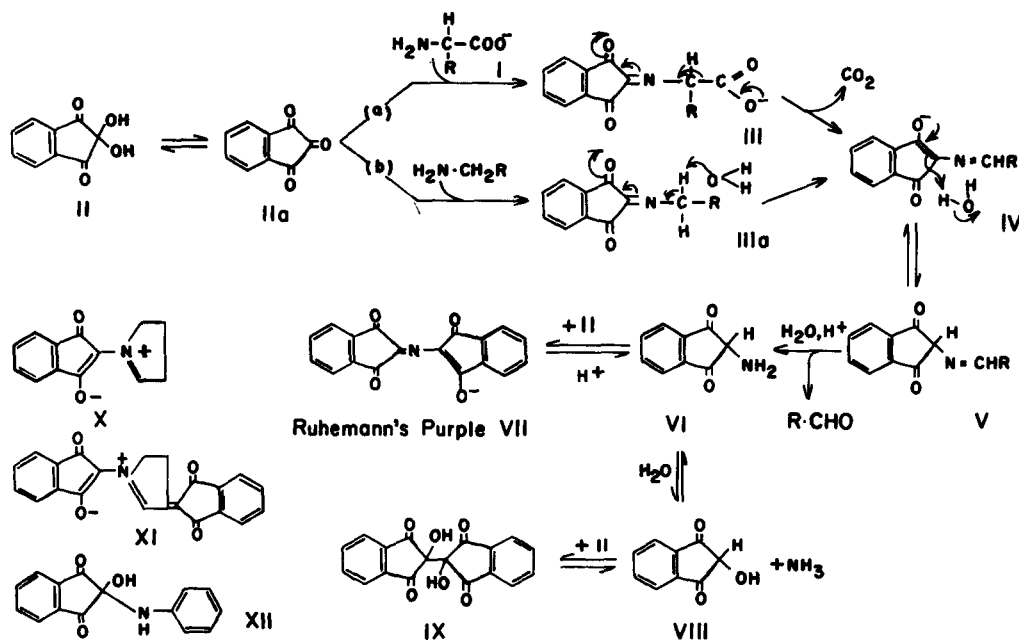
The kinetics of the ninhydrin reaction has been studied⁴. The reaction is first order in ninhydrin, first order in amino acid, and second order overall. Friedman and Sigel⁴ suggested that the rate-determining step is the initial nucleophilic-type displacement of a hydroxy group of ninhydrin and that the reactive species is the anionic form of the amino acid ($H_2NCHR\text{COO}^-$) rather than the zwitterion ($H_3H^+CHR\text{COO}^-$). A positively charged nitrogen at slightly acidic or neutral pH is not expected to behave as a nucleophile. Even though, at pH 6, the ratio of protonated to unprotonated amino groups is 4900 to 1 for alanine, nevertheless, the unprotonated amino group would seem to be the active species. Studies undertaken by Lamothe and McCormick⁷ have clarified certain aspects of the mechanism through their investigation of the effect of acidity on the ninhydrin reaction. With increasing pH, the concentration of the amino acid anion should increase progressively, thus resulting in an increase in the rate of reaction. Such is not the case. Since protonation of the carbonyl group allows for an increased reactivity toward nucleophilic agents⁹, a maximum rate should be observed when the product of the concentrations ($[R_2C=O^+H][H_2NCHR\text{COO}^-]$) is maximum. This was found to be the case by Lamothe and McCormick⁷. Thus, Scheme II incorporates the reactive species, 1,2,3-indantrione and the anion of the amino acid.

Lamothe and McCormick⁵ have also studied the effect of hydrindantin (IX) concentration on the color yield. At low concentrations of ninhydrin, the hydrolysis rate of the intermediate amine (VI) becomes significant and the amino nitrogen is released as ammonia with the concomitant formation of hydroxydiketohydrindene (VIII). This is converted to hydrindantin (IX) on reaction with ninhydrin. The addition of hydrindantin to the ninhydrin reagent should give a nearly quantitative yield of Ruhemann's Purple at the optimum pH due to the suppression of the amine (VI) hydrolysis.

Steric factors associated with the amino group have been studied and the reactivities of glycine, α -alanine, and α -amino-isobutyric acid were compared⁴. In this series, the steric environment changes from zero to one to two methyl groups and the relative observed rates of reaction are 100, 24.2 and 0.38 respectively. The differences in reactivity can be attributed to steric factors almost completely, although polar effects may play a minor role. For instance leucine



Scheme I



SCHEME II

and α -methyl methionine, both of which have identical pK_2 values, exhibit a reactivity ratio of 37:1⁴.

A recent biochemistry text (1977) portrays a reaction between hydroxydiketohydrindene (VIII), labelled incorrectly as hydrindantin, ammonia and ninhydrin to give Ruhemann's Purple without indicating the intermediacy of the amine (VI). This contradicts recent proposals^{5, 6} that ammonia actually reacts with hydroxydiketohydrindene (VIII) to give the intermediate amine (VI), i.e. the reverse reaction of the amine hydrolysis.

The reaction of imino acids with ninhydrin proceeds initially in a similar manner to that of α -amino acids. Imino acids, e.g. proline and hydroxyproline, react with ninhydrin to give a yellow color. At higher temperatures ($\sim 100^\circ\text{C}$), the yellow compound (X) is transformed to the purple-red compound (XI)¹⁰.

Neuberg¹¹, a contemporary of Ruhemann, showed that primary amines give Ruhemann's Purple on reaction with ninhydrin. The general mechanism can be adapted to primary amines as shown in Scheme II. The scheme portrays a path (route b) where the common aldimine (IV) is formed, followed by hydrolysis to the intermediate amine (VI) and finally the formation of Ruhemann's Purple (VII) in the normal manner. Although Ruhemann's Purple is obtained with primary amines, it is interesting to note that other colored products have also been isolated. With aniline, Friedman¹² isolated a compound found to have a structure corresponding to (XII). A mechanism for the formation of this compound can be easily written.

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NOMENCLATURE OF MULTIPLE FORMS OF ENZYMES

The IUPAC-IUB Commission on Biochemical Nomenclature has now issued new recommendations for the naming of multiple forms of enzymes and their report has been published in the *Journal of Biological Chemistry*, 1977, **252**, 5939-5941. There is an important change from the earlier recommendation of the 1964 Committee that "multiple enzyme forms in a single species should be known as isoenzymes (or isozymes)". It is now recommended that the term

multiple forms of the enzyme should be used as a broad term covering all proteins catalyzing the same reaction and occurring naturally in a single species, while the term *isoenzyme* or *isozyme* should apply only to those multiple forms of enzymes arising from genetically determined differences in primary structure and *not* to those derived by modification of the same primary sequence. Thus malic dehydrogenases in the cytosol and in the mitochondria are isoenzymes but not the family of chymotrypsins derived from chymotrypsinogen or allosteric modifications of enzymes. There are recommendations for the naming of interconvertible enzymes using *o* for the original form and *m* for the modified ones so that, for example, the two well known forms of phosphorylase become *o*₂-phosphorylase and *m*₄-phosphorylase. There is a useful table indicating six groups of multiple forms of enzymes with different reasons for multiplicity. Those lecturing on isoenzymes should consult the report.