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Ruhemann's Purple from Ninhydrin, Ascorbate, and Nitrite

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Ruhemann's purple is formed from nitrite, ascorbate, and ninhydrin with the formation of variable amounts of ammonia. The reaction begins with the nitrosation of ninhydrin to form nitrosoninhydrin, which will release nitric oxide. The nitrosoninhydrin is reduced by ascorbate to diketohydrindamine, which then couples with a second molecule of ninhydrin to form the pigment. The concentration of pigment is linear with nitrite over the range 1 to 10 mM, but ammonia production is not stoichiometric with initial nitrite.

INTRODUCTION

The formation of Ruhemann's purple (*1*) (1H-indene-1,3(2H)-dione, 2-[(3-hydroxyl-1-oxo-1H-inden-2-yl)imino, 7185-16-2) by the reaction of ninhydrin is the basis for the automated colorimetric determination of amino acids (*2*), and also is used for the determination of organic amines in general (*3*). While employing the reaction for the determination of amino groups in model meat-curing systems we discovered that the combination of ninhydrin, ascorbate, and nitrite resulted in the formation of a purple pigment. The reaction is of interest because it interferes in the determination of amines if nitrite is present, it can serve as a measure of nitrite in its own right, and it is a unique example of the complete reduction of an oxide of nitrogen by a biological reductant under relatively mild conditions. We therefore undertook a study of the reaction to determine the nature of the pigment and the mechanism of the reaction.

MATERIALS AND METHODS

The reaction was carried out in citrate buffer, pH 5.0, at 97.5°C for 20 min with 55 mM ninhydrin, 10 mM ascorbate, and 10 mM nitrite. For purposes of comparison, Ruhemann's purple was prepared by reacting 55 mM ninhydrin, 10 mM ascorbate, and 10 mM ammonia or histidine in pH 5.0 buffer. The nitrite solutions were tested for ammonia with Nessler's reagent. The ascorbate and ninhydrin were tested for ammonia by steam distillation from alkaline solutions. No ammonia was detected in any of the reagents, acids, or solvents, which eliminates ammonia contamination as a source of the observed pigment.

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To verify formation of a nitrosoninhydrin derivative we used the same hydrogen ion, ninhydrin, and nitrite concentrations, but left out the ascorbate, added 0.1 mM horse metmyoglobin, and ran the reaction at 20°C in nitrogen.

The pigments of the ninhydrin–ascorbate-nitrite and ninhydrin–ascorbate–ammonia reactions were purified by successive extractions into either chloroform or methylene chloride and back into citrate buffer, pH 5.0, until the absorption spectra of the two pigments remained constant through two successive extractions. The reacting mixtures were analyzed for ammonia by Kennedy's technique (*4*), which involved acidifying the pigment to release the ammonia, removal of the excess ninhydrin with hydrogen peroxide, alkalization, and distillation of the ammonia.

The stoichiometry of the reaction was investigated by varying the nitrite concentration from 0.5 to 20 mM and the ascorbate concentration from 2.5 to 30 mM with 55 mM ninhydrin. The pH was varied from 4.5 to 6.0 and the temperature from 60 to 90°C. The mechanism of the reaction was studied by prereacting the reagents two at a time for 20 min at 70°C, followed by the addition of the third reagent to start the formation of pigment.

RESULTS

After purification, the two pigments had identical visible spectra, with maxima at 570 and 40 nm, and minima at 455 and 355 nm. There were also three absorption maxima in the ultraviolet at 290, 252, and 220 nm, but these are benzene ring resonances, common to ninhydrin and all its derivatives, and therefore nonspecific. The two visible maxima are unique to Ruhemann's purple, and the spectra is always the same regardless of source or yield (*5–7*), unless derived from imines or sulfhydryl amines (*5*), uncleavable amines (*8, 9*), or amides (*10*). The spectra of these derivatives differ due to the organic amine, from which the pigment was derived, remaining attached to the nitrogen. The infrared spectra are, for the most part, nonspecific for ninhydrin and its derivatives, but do provide "fingerprint" identification. The ir spectra of the pigments derived from ammonia or nitrite are shown in Fig. 1, and indicate that the two pigments are the same.

As further evidence of the reduction of nitrite, we recovered ammonia from the reacting solutions employing Kennedy's procedure. The yields of ammonia were not correlated with the amount of pigment formed, however, and were highly variable, ranging from 30 to 60% of initial nitrite during the initial heating period. As discussed later, there are a number of competing reactions producing semistable intermediates taking place simultaneously in the nitrite–ascorbate–ninhydrin system which account for the poor yields and stoichiometry of the ammonia production.

Mechanism of the Reaction

When following the production of the pigment by simultaneous addition of nitrite, ninhydrin, and ascorbate, we observed a lag period which was assumed to

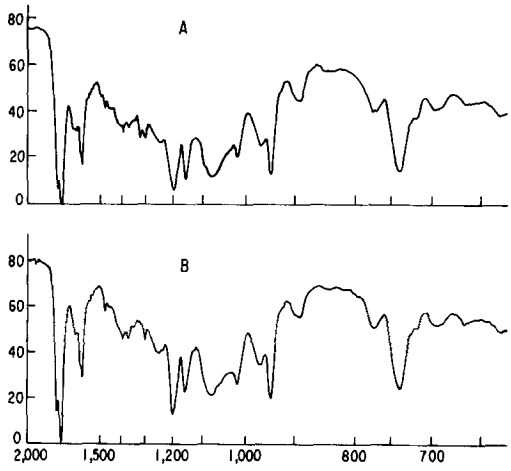

FIG. 1. Infrared spectra of the purple pigments produced from either (A) ninhydrin, ascorbate, and ammonia or (B) ninhydrin, ascorbate, and nitrite.

be due to the formation of a reaction intermediate. To determine the sequence of formation, we prereacted the reagents in pairs, then added the third reagent. The results are shown in Fig. 2. The prereaction of ascorbate and ninhydrin resulted in the formation of the red pigment, hydrindantin (*7*). When the curve for the reaction was corrected for the hydrindantin absorption, it showed a lag period (note insert) as did the reaction curve for the prereaction of ascorbate and nitrite. Only

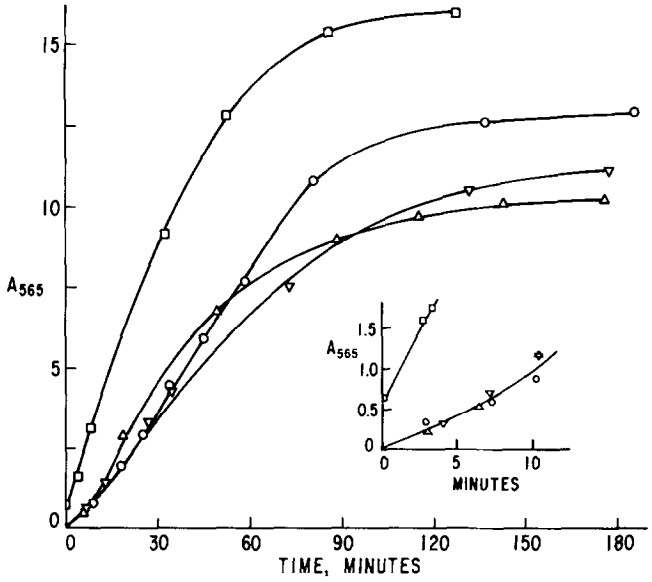

FIG. 2. The effect of prereacting reagent combinations on the formation of a purple pigment from ninhydrin, ascorbate, and nitrite. ○, all mixed simultaneously. □, ninhydrin/nitrite; △, ninhydrin/ascorbate; and ▽, ascorbate/nitrite prereacted 20 min at 70°C.

when nitrite and ninhydrin were prereacted did the formation of Ruhemann's purple start immediately. The prereaction of nitrite and ninhydrin also resulted in higher yields of pigment. The reaction sequence therefore begins with the nitrosation of ninhydrin to form nitrosoninhydrin (Fig. 3). Reasoning that nitrosoninhydrin would be unstable and dissociate to yield nitric oxide, in common with nitroso-reductants, we mixed nitrite, ninhydrin, and horse metmyoglobin in pH 5.5 buffer. We added the metmyoglobin as an indicator of nitric oxide formation since these two form the spectrally unique heme complex, nitrosylmyoglobin (NOMb) (*11*). The mixture resulted in complete formation of nitrosylmyoglobin at a rate of 0.015 mM NO/hr at 20°C, confirming the formation of nitrosoninhydrin.

The nitrosation is followed by reduction of the nitrosoninhydrin intermediate to diketohydrindamine. This reaction could have been mediated either by ascorbate directly, or by hydrindantin which is also formed (Fig. 3) and is the reductant of choice in amino acid analyses. To test this possibility we added 27.5 mM hydrindantin to the nitrite–ninhydrin system, with and without ascorbate. Only in the presence of ascorbate was the pigment formed. Furthermore, in the prereaction experiments the longer ascorbate was allowed to prereact with ninhydrin to form hydrindantin, the lower the yields of pigment. Clearly, hydrindantin was not involved in the reaction. The reaction steps are therefore as shown in Fig. 3: (i) nitrosation of ninhydrin, (ii) reduction of nitrite to the equivalent of ammonia by the reduction of nitrosoninhydrin to diketohydrindamine by ascorbate, and (iii) coupling of the last with a second molecule of ninhydrin to form Ruhemann's purple. With the exception of the formation and reduction of the nitrosoninhydrin by ascorbate, this is essentially the same reaction sequence proposed for the formation of Ruhemann's purple from ninhydrin, ammonia or amines, and reductants (*7*).

Pigment and Reactant Concentrations

The linearity of the formation of pigment as a function of nitrite concentration was determined by varying the concentration from 0.5 to 20 mM at pH 5.0 in the presence of 55 mM ninhydrin and 10 mM ascorbate. The reaction was linear from 1

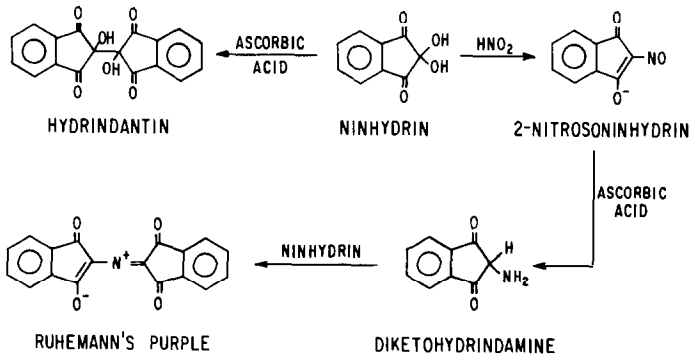

FIG. 3. Reaction sequence for the formation of Ruhemann's purple from ninhydrin, nitrite, and ascorbate.

to 10 mM. The regression equation is $A_{570} = 1.36\ [NO_2^-] + 1.04$, $r = 0.999$, $s_b = 0.06$, where the concentration of nitrite is millimolar, r is the correlation coefficient, and s_b is the standard deviation of the coefficient of the concentration term (*12*). At 20 mM nitrite pigment yields were about 40% low. The high intercept ($A_{570} = 1.04$) is due to the formation of hydrindantin in the reaction mixtures. The reaction at pH 5.0 was run at 60, 70, 80, and 90°C. The reaction at 60°C required over 2 hr and did not reach the pigment concentration attained by the higher temperatures. At 70, 80, and 90°C maximal color formation occurred at approximately 1 hr, 35 min, and 25 min, respectively. Color yields were about 1–3% lower at 70°C than at the higher temperatures, but the pigment once formed was stable for about 1/2 hr. At 80 or 90°C more pigment was formed, but the color faded rapidly. The reaction was also run at pH 4.0, 4.5, 5.0, 5.5, and 6.0. At pH 4.0 and 4.5, the 570-nm absorption maximum formed quickly but also faded quickly, with conversion being only about half that at pH 5.0. Above pH 5.0, the principal reaction was the formation of hydrindantin; a low absorption at 570 nm did develop after several hours but did not attain the pH 5.0 value. Maximal pigment formation occurred at 5 to 10 mM ascorbate. At higher ascorbate concentrations the principal pigment was hydrindantin, with but little Ruhemann's purple being formed.

DISCUSSION

The correspondence of the ultraviolet, visible, and infrared absorption spectra of the purple's pigment from nitrite with that from ammonia identifies the former as Ruhemann's purple. The linearity of pigment formation with limiting nitrite concentrations is proof that the pigment is derived from nitrite, and the formation of ammonia demonstrates that the nitrogen has been reduced from the +3 to the −3 oxidation state. The evidece, therefore, is clearly consistent with the formation of Ruhemann's purple from ninhydrin and nitrite with ascorbate as the reductant. With regard to this conclusion, two substantiating observations may be made. First, the only compound in the reaction mixture that could be steam-distilled to form the Nessler pigment was ammonia, and, second, the only possible source of the ammonia was the nitrite, since neither of the two reactants, ninhydrin and ascorbate, nor the buffer (citrate and sodium), contained nitrogen. It is from the foregoing observations and the known mechanisms of Ruhemann's purple formation that we proposed the reaction sequence shown in Fig. 3. The identification of the nitrosated intermediate as nitrosoninhydrin is based on the observation that ninhydrin will reduce nitrite to nitric oxide. All other nitrite reductants producing nitric oxide do so by forming a nitroso derivative, from which nitrite oxide is formed by a 1-electron transfer (*11, 13*). The intermediate is shown as the C-nitroso derivative, since in Ruhemann's purple the nitrogen is bonded with the 2-carbon of ninhydrin. With respect to formation of nitrosoninhydrin, Allen *et al.* (*14*) identified the product of a nitrosated β-diketone as the oximino derivative, which is tautomeric with the protonated form of nitrosoninhydrin, Fig. 4.

From nitrosoninhydrin to diketohydrindamine is a 5-electron reduction medi-

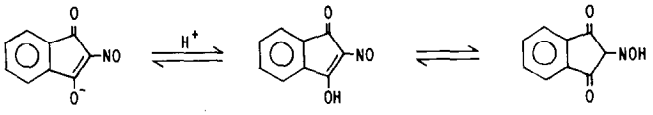

FIG. 4. Protonation and tautomerization of nitrosoninhydrin.

ated by ascorbic acid (since ninhydrin alone will not drive the reaction). We were not able to determine the molecularity or any characteristics of the reduction mechanism, since increasing ascorbate concentrations resulted in variable amounts of Ruhemann's purple being formed, with indeterminate kinetics. The coupling of the diketohydrindamine with a molecule of ninhydrin then forms Ruhemann's purple.

Limitations on Reduced Pigment Formation

The pigment is produced from nitrite, ascorbate, and ninhydrin in a highly complex system. In addition to ninhydrin, nitrite nitrosates ascorbate to form nitrosoascorbic acid, another source of nitric oxide (*11*). Through oxidation, nitric oxide may be recycled back to nitrite. Hardy *et al.* (*15*) proposed the direct regeneration of nitrite in systems containing ascorbate to account for the observation that there was no net loss of nitrite during the nitrite oxidation of ascorbate in air. This reconversion of nitric oxide to nitrite allows for eventual conversion of all of the initial nitrite to ammonia through Ruhemann's purple formation, but it means that, at intermediate periods in the reaction, some of the nitrite is in other forms not available for pigment and ammonia production. Ascorbate loss through hydrindantin formation also results in lowered pigment yields, particularly at higher ascorbate concentrations.

The diketohydrindamine intermediate is a very reactive nucleophilic species (*6, 7*) and, as soon as it is formed, would be expected to react with the nitrosating species to form the nitrosamine, *N*-nitrosodiketohydrindamine. The formation of the nitrosamine, in which form the nitrite is lost for pigment formation, would account for the lessened formation of Ruhemann's purple in strongly acid solutions. Its absence would account for the increased yield of pigment when ninhydrin and nitrite were preincubated. In this case, more of the nitrosating species would be in the nitrosoninhydrin form before the reduction to *N*-nitrosodiketohydrindamine, and therefore less nitrosating species to form the nitrosamine. Pigment fading was faster in acid solutions and in the presence of high concentrations of nitrite, both of which suggest that nitrosation is also involved in pigment destruction, probably at the nitrogen bridge. In view of the complexity of the system, it is less unusual that the reaction resulted in poor stoichiometry of ammonia production than it is that the production of pigment was linear with nitrite over any concentration range. Nevertheless, such linearity is not unusual. For example, even if the reaction conditions are reasonably well controlled, the ninhydrin reaction does not give the same pigment yields with different amino acids, yet may be used for quantitative determination of the individual acids. The Griess reaction

for nitrite is the standard determinative technique, almost never goes to completion (*15*), but with good technique can determine nitrite concentrations ±0.3%.

The formation of Ruhemann's purple may be an alternative to the Griess reaction for the determination of nitrite. The Griess reaction is carried out in mild acid (2.75–3.00 pH) conditions, which increases the reactivity of the nitrite, but cleaves labile nitrite-containing compounds (*17*). The reaction reported here goes at a more neutral pH (5.0), with less cleavage of other compounds, and may prove of utility in combination with the Griess reaction in determining different forms in which nitrite occurs in nature.

SUMMARY

Data are presented to show that nitrite, ascorbate, and ninhydrin react to form Ruhemann's purple and eventually ammonia. The reaction proceeds through nitrosation, reduction, and coupling reactions, in that order. The reaction may be used to determine nitrite quantitatively under nearly neutral conditions (pH 5.0). The reaction is a unique example of a complete reduction of nitrite to ammonia (a 6-electron step) by organic and biologic compounds.

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