ON THE MECHANISM OF THE REACTION OF NINHYDRIN WITH \( \alpha \)-AMINO ACIDS

II. A SPECTROPHOTOMETRIC STUDY OF HYDRINDANTIN REACTIONS*

BY DOUGLAS A. MACFADYEN AND NATHALIE FOWLER

(From the Rush Department of Biochemistry, The Presbyterian Hospital of the City of Chicago, Affiliated with the University of Illinois, Chicago)

(Received for publication, January 21, 1950)

Our purpose is to show by means of a quantitative method for determination of hydrindantin derivatives that previous concepts of the mechanism of the reaction of ninhydrin with amino acids are inadequate or erroneous, and to show facts supporting a new concept.

The main facts are as follows: (a) Hydrindantin forms when amino acids react with ninhydrin (Ruhemann (1); Abderhalden (2)); (b) it reacts with ammonium salts to give Ruhemann's purple ((1); Harding and Warnford (3); Harding and MacLean (4)); (c) ammonia is formed by ninhydrin deamination of amino acids ((1); MacFadyen (5)); (d) hydrindantin in dilute alkaline solution gives a red color, in concentrated alkaline solution a blue or purple color ((1); Retinger (6)). All these facts have been confirmed by us. The reaction of proline or hydroxyproline with ninhydrin is a special case, giving no purple color (Grassmann and von Arnim (7)), and no ammonia (5).

There are three main concepts according to Ruhemann (1), Retinger (6), and Harding et al. (3, 4). In the first the amino acid is oxidatively deaminized by ninhydrin, which is reduced to diketohydrindol; diketohydrindol and ninhydrin condense to form hydrindantin, which then combines with ammonia to give Ruhemann's purple. In the second, 2 moles of amino acid combine with hydrindantin and the compound splits into two identical nitrogen-free, purple-colored free radicals, analogously to the presumed compound formed from inorganic cations and hydrindantin in strongly alkaline solution. In the third, amino acids are distinguished from amines and ammonia because the former react faster chromogenically with ninhydrin. Amino acids decompose independently of ninhydrin into a glyoxal and ammonia; the glyoxal reduces ninhydrin to diketohydrindol and is oxidized to the corresponding \( \alpha \)-keto acid; ammonia combines with diketohydrindol to form diketohydrindamine, and diketohydrindamine condenses with ninhydrin to form Ruhemann's purple. The sequence of events is the same in the case of amines and ammonia.

* Supported by the Otho S. A. Sprague Memorial Institute.
except that a glyoxal cannot come from their decomposition but only from
the change of ninhydrin into o-carboxyphenylglyoxal.

Ruhemann's concept fails to account for the more rapid chromogenic
reaction of amino acids with ninhydrin (3, 4), which we have confirmed,
and also with hydrindantin, a new fact. Retinger's concept is not in
accord with the marked difference in adsorption spectra between Ruhe-
mann's purple (MacFadyen (8)) and hydrindantin in strongly alkaline
solution, as shown herein. The concept of Harding et al. is inconsistent
with the following facts: (a) a negligible amount of ammonia is evolved
from amino acids in the absence of ninhydrin under the conditions in
which ninhydrin causes evolution of CO₂, purple color, and NH₃ (5); (b)
the source of CO₂ cannot be the α-keto acid corresponding to the amino
acid, because evolution from keto acids is much slower than from amino
acids, according to Van Slyke, Dillon, MacFadyen, and Hamilton (9);
amino acids react faster than NH₃ with hydrindantin, as shown herein.
With respect to ammonium salts the concept is adequate: hydrindantin
formation from ninhydrin alone in aqueous solution at a pH as low as 5,
and hydrindantin cleavage into diketohydrindol and ninhydrin at a pH
above 4, can be shown by our method.

Our spectrophotometric method is quantitative in contrast to previous
ones (1, 3, 4), because exclusion of oxygen during the reactions prevents
fading of the red and blue colors of hydrindantin. Control of the oxygen
content and of the pH provides for a clear distinction between hydrindan-
tin reactions and those of ninhydrin. These controls are necessary, for,
on the one hand, oxidation of hydrindantin yields 2 moles of ninhydrin,
and, on the other hand, ninhydrin at certain pH values can form hydrin-
dantin by way of o-carboxyphenylglyoxal.

The probable structure of the red-colored derivative of hydrindantin is
indanone-enediol and not, as Ruhemann (10) believed, the monovalent
salt of intact hydrindantin. In the reaction of amino acids and hydrindan-
tin, 1 mole of indanone-enediol is used up for each mole of Ruhemann's
purple formed. The reaction with amines summarized in formulae I
could be either a simultaneous or a sequential condensation with inda-
none-enediol and ninhydrin. The alternatives will be considered in a sep-
arate paper on the order of reaction, which the present method has made
possible.

**Apparatus—**

1. Spectrophotometers. The Beckman model DU instrument, with
quartz prism, and the Coleman clinical instrument, model 6, were used.

2. Cuvettes and reaction vessels. For the Beckman instrument silica
for the measurement of ultraviolet absorption, Corex for visible light.
The length of the light path was 1 cm. For the Coleman instrument,
Hamilton vessels (11) were calibrated for length of the light path, which averaged 1.89 cm., and were used as reaction vessels as well.

\[
\begin{align*}
\text{C}_6\text{H}_4\text{C}\equiv\text{C}-\text{OH} & + \text{NH}_2\text{R} & + & \text{C}_6\text{H}_4\text{C}\equiv\text{C} - \text{OH} \\
\text{Indanone-enediol} & & \rightarrow & \text{Ninhydrin}
\end{align*}
\]

\[
\begin{align*}
\text{C}_6\text{H}_4\text{C}\equiv\text{C}-\text{N}=\text{C} & \\
\text{Ruhemann's purple}
\end{align*}
\]

Methods

Preparation of Hydrindantin—A solution of 0.5 gm. of ascorbic acid and 1 gm. of ninhydrin in 200 ml. of McIlvaine’s buffer (0.1 M) at pH 3 was heated to 90° and the crystals allowed to settle at room temperature. Recrystallization from hot acetone yielded 350 mg. of colorless anhydrous hydrindantin. The purity of the product was checked by elementary analysis and by its melting point (see Abderhalden (2)).

Preparation of Oxygen-Free Reaction Solutions—Since at room temperature water does not dissolve hydrindantin, it was dissolved in acetone in a concentration of 1 mg. per ml. 1 ml. was delivered to each Hamilton vessel and dried by passing a stream of nitrogen above, not in, the solution. 10 ml. of buffer solution, either alone as a control or containing another reagent under test, oxygen-free after a stream of nitrogen was passed through it for 5 minutes, were delivered to each vessel, which was lubricated and quickly closed so as to be air-tight. The gas was then removed by suction from a motor pump until the pressure was constant at about 2 mm. of Hg. Each vessel in the control and test group was again made air-tight and was immersed upright in a frame in a boiling, distilled water bath for a known time interval.

Spectrophotometric Readings—The optical density was recorded at wavelengths of 490 and 570 m\(\mu\) in the Coleman instrument. Sometimes the measurements were made as quickly as possible after removal from the boiling water bath, in order to record the optical density at a temperature
close to 100°. In such a case, it was found that three measurements could be taken comfortably in from 1 to 2 minutes. For the most part, they were taken at room temperature after cooling in a water bath, and again after passing air through the solutions for 3 minutes.

Absorption Spectra of Red and Blue Colors Derived from Hydrindantin Different from Ruhemann’s Purple (Fig. 1)—Solutions of hydrindantin either in Sörensen’s NaOH-borate buffer at pH 9.2 or in 0.4 N NaOH were prepared to be oxygen-free in cuvettes for the Beckman instrument.

The visible colors were constant for at least 48 hours, but ultraviolet absorption, without change in position of the maxima, slowly lessened until a constant value was reached at the end of 48 hours. The result is explained by hydrolysis of hydrindantin into diketohydrindol and ninhydrin, followed by irreversible transformation of ninhydrin into either o-carboxyphenylglyoxal or o-carboxymandelic acid, depending on the pH; either process can be detected by change in the ultraviolet absorption spectrum of ninhydrin (see MacFadyen (8)).

The spectrum, Curve 1, Fig. 1, for the blue color is markedly different from the curve for Ruhemann’s purple (8) with respect to position and intensity of maxima. These differences invalidate Retinger’s concept (6).
Formation of Ruhemann’s Purple from α-Alanine and Hydrindantin Independent of \(\text{NH}_3\) Pathway (Table I)—At pH 5.3 at 100\(^\circ\), and anaerobically, the intensity of purple color in the reaction of hydrindantin, 0.3 mM, with the amino acid was 5 times that for ammonium salts in the same concentration, 0.56 mM. Therefore, the concepts of Ruhemann (1) and Harding et al. (3, 4), necessitating an \(\text{NH}_3\) pathway, are inadequate. The

### Table I

<table>
<thead>
<tr>
<th>Reaction time in boiling water bath (min.)</th>
<th>Observed optical densities, units (\times 10^3)</th>
<th>Hydrindantin disappearance</th>
<th>Formation of Ruhemann’s purple</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(P_{370})</td>
<td>(D^-O_{430})</td>
<td>(D^+O_{430})</td>
</tr>
<tr>
<td>10</td>
<td>360</td>
<td>283†</td>
<td>123</td>
</tr>
<tr>
<td>20</td>
<td>360</td>
<td>290</td>
<td>122</td>
</tr>
<tr>
<td>30</td>
<td>620</td>
<td>365</td>
<td>200</td>
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<td>50</td>
<td>880</td>
<td>441</td>
<td>278</td>
</tr>
<tr>
<td>60</td>
<td>1010</td>
<td>465</td>
<td>324</td>
</tr>
</tbody>
</table>

* For explanation of \(P_{370}, R_{430}\), computation of the factor 687, and for \(f_1\) and \(f_2\) see the text.
† For \((\text{NH}_4)_2\text{SO}_4\), the results, in chronological order, were 2.2, 5.0, 5.7, 9.5, 14.3, and 15.7.
‡ The third figure was estimated by interpolation.

Experimental details are discussed below in connection with the mechanism of the reaction.

Hydrindantin Red Color Disappears As Ruhemann’s Purple is Formed, Mole for Mole (Table I)—Difficulties of quantitative estimation of concentrations of Ruhemann’s purple and the red color from hydrindantin, together in reaction mixtures, were obviated in the following ways.

Ruhemann’s purple is decolorized at pH 5.3, even under anaerobic conditions, when its solutions are heated to boiling, but not significantly at room temperature. Therefore, estimates of the amount formed in a given
time interval, in contrast to the amount present, required measurements of the intensity of the purple color remaining in solution of known amounts of the sodium salt of diketohydrindamine diketohydrindylidene (8) under the conditions of the α-alanine reaction with hydrindantin including all reagents except α-alanine. Such tests provided us with factors, $f_1$, by which the optical density at $\lambda = 570 \text{ m\mu}$ observed at a given time interval of boiling was converted into the initial optical density before heating. Furthermore, while it is true that Ruhemann's purple obeys Beer's law (8), it was necessary to correct for spectrophotometric conditions of the Hamilton vessels and the Coleman instrument with respect to deviations from Beer’s law, but not in the case of the red color. For this purpose the sodium salt of diketohydrindamine-diketohydrindylidene was dissolved in buffer at pH 9, and the optical densities at varied concentration determined in the Hamilton vessels in the Coleman instrument. From these results factors, $f_2$, were computed which when multiplied by the optical density at $\lambda = 570 \text{ m\mu}$, in so far as it is referable only to Ruhemann’s purple, yielded the concentration of the sodium salt in micromoles per liter.

Application of the factors, depending on a clear distinction between the purple color due to the sodium salt and the red color due to hydrindantin, was made by taking advantage of two facts. Whereas Ruhemann’s purple is stable when oxygenated at pH 10 to 11 at room temperature, the red color is discharged in 3 minutes. Therefore, the optical density after oxygenation, measured at $\lambda = 570 \text{ m\mu}$, $D^{+o}_{570}$, when corrected in the usual manner for variation in length of the light path and translucency from vessel to vessel, is representative of Ruhemann’s purple and is denoted as $P_{570}$, whereas the optical density difference due to oxygenation, measured at $\lambda = 490 \text{ m\mu}$, $D^{+o}_{490} - D^{-o}_{490}$, when similarly corrected, is representative of the hydrindantin red color and is denoted by $R_{490}$.

The experiments were carried out with 0.5 mg. of α-alanine (or 0.37 mg. of (NH$_4$)$_2$SO$_4$) and 1 mg. of hydrindantin in 10 ml. of 0.1 M acetate buffer at pH 5.3. The solutions, in duplicate, were heated to boiling, anaerobically, for a given time interval of 10, 20, 30, 40, 50, or 60 minutes. Then they were cooled in ice water for 4 minutes and brought to room temperature at 27°, about 25 minutes later. The optical densities in the absence of oxygen, $D^{-o}$, were recorded. The vessels were opened, the pH of the solutions changed to 10 to 11 by adding about 0.02 ml. of 40 per cent NaOH, and the red color was discharged by bubbling air through the solutions for 3 minutes. Then the optical densities, $D^{+o}$, were recorded. The observed data were converted to micromolar concentrations (X) of substance responsible for the red or purple colors as follows: In the case of the red color, $X = R_{490}/(\epsilon \times 10^{-6} \times l \times \alpha)$, where $\epsilon$ is the molar ab-
sorption coefficient at $\lambda = 490$ mm and at pH 9.2, assuming complete hydrolysis of hydrindantin into diketohydrindol, $l$ is the length of the light path in cm., and $\alpha$ is the ratio of optical density at pH 5.3 to that at pH 9.2. The numerical values were 1400, 1.89, and 0.55 respectively. The equation simplifies to $(X) = 687R_{90}$. In the case of the purple color, $(X) = P_{670} \times f_1 \times f_2$, previously described. For the time intervals 10, 20, 30, 40, 50, and 60 minutes the numerical values of $f_1$ were 1.31, 1.44, 1.58, 1.74, 1.91, and 2.10, respectively, and for $f_2$ were 24.8, 25.2, 25.8, 26.5, 27.4, and 27.5, respectively.

The disappearance of red color associated with formation of Ruhemann's purple was calculated from $R_{490}'$, obtained when the reaction mixture contained $\alpha$-alanine, and from $R_{490}''$, obtained when all reagents were present except $\alpha$-alanine. The disappearance, in terms of micromoles of hydrindantin per liter, $= 687(R'' - R')_{490}$.

In the case of $\alpha$-alanine, the results show that the disappearance of hydrindantin red color is proportional to the formation of Ruhemann's purple, within the limits of error of the method.

Red and Blue Colors from Hydrindantin Due to Diketohydrindol (Fig. 2)—Our claim that the red color evolved from hydrindantin, as well as the undisputed blue color (1), is due to diketohydrindol rests on the following facts. (a) The blue color is reversibly changed into the red by acid-base titration under anaerobic conditions, $pK' = 12.3$ at $25^\circ$. When the blue color is formed, the other component of hydrindantin, namely ninhydrin, is changed into $\alpha$-carboxymandelic acid (1) by irreversible internal oxida-
tion-reduction. This change is complete in a few minutes, but the color change is quantitatively reversible for days. Therefore, the change from blue to red does not necessitate the reformation of hydrindantin; the claim (10) that the red color is due to the monovalent anion of hydrindantin is invalid. (b) The same play of colors with change in pH was observed by Hassall (12) in connection with the hydrolysis of acetoxyindandione to diketohydrindol, which was identified by adding ninhydrin to acidified solutions from which hydrindantin was obtained. (c) In acid solutions of hydrindantin dimethyldihydroresorcinol accelerates the formation of the red color, which attains a constant intensity for a given concentration of hydrindantin. The explanation offered is hydrolysis of hydrindantin into the red color and ninhydrin, accelerated by combination with the resorcinol (see Fig. 2). The resorcinol combines with ninhydrin (8). The compound, inert to oxygenation, can be detected spectrophotometrically in the solutions after oxygenation. (d) Above pH 7, there is no difference in red color caused by dimethyldihydroresorcinol or by cooling the solutions from 100° to room temperature. If a red alkaline solution is acidified to pH 5.3, anaerobically, the color fades as hydrindantin is precipitated. The data could be interpreted, as Ruhemann concluded (10), to show that intact hydrindantin is responsible for the red color. However, at a pH, temperature, and time interval (pH 10, 25°, 24 hours) insuring complete irreversible transformation of ninhydrin to o-carboxyphenylglyoxal, acidification no longer causes reformation of hydrindantin, the fading of the red color being what would be expected from its titration curve, pK' = 5.2. In this case, addition of ninhydrin causes and is necessary for precipitation of hydrindantin. (e) By careful adjustment of the concentration of added hydrosulfite, the intensity of the red color from hydrindantin can be doubled.

Having shown that the red and blue colors of hydrindantin solutions are derivatives of diketohydrindol readily convertible under anaerobic conditions one to the other and to diketohydrindol, simply by change in pH (pK' = 5.2 and 12.3), we conclude that the colors are due to the anions of diketohydrindol. Enolization of diketohydrindol would allow for two ionizable groups. Therefore, the red color is attributable to the monovalent anion, the blue color to the divalent anion. The ease of oxidation on exposure to air is consistent with the indene structure formable by enolization. The enediol formulae shown in II provide for resonance which would explain the difference in color and the chemical behavior of the substances.

On reduction with hydrosulfite the red and blue colors disappear but careful oxygenation restores their original intensity. On the other hand,
the effect of oxygenation is not reversible in the case of the blue color and
is only reversible in the case of the red color if ninhydrin is present in solu-
tion. Irreversibility is explained by oxidation of the red color to o-car-
boxyphenylglyoxal and of the blue color to o-carboxymandelic acid.

\[
\begin{align*}
\text{Pale yellow} & \quad \text{Red} & \quad \text{Blue} \\
\text{(II) Colored forms of indanone-enediol} & \\
\end{align*}
\]

**Mechanism of Reaction of \(\alpha\)-Alanine with Hydrindantin**—The disappearance of the red color in this reaction may now be reconsidered. It is
ascribed to conversion of indanone-enediol into Ruhemann's purple, the
anion of diketohydrindamine-diketohydrindylidene. The alternative, re-
duction of Ruhemann's purple \((C_{18}H_{30}O_N)^{-}\), which would become colorless
while the enediol was oxidized to colorless ninhydrin, is untenable, because
the chromogenic reaction of \(\alpha\)-alanine is faster with 1 mole of hydrindantin
\((C_{18}H_{19}O_6)\) than with 2 moles of ninhydrin \((C_9H_6O_4)\). The non-enolic
component of Ruhemann's purple must be supplied by ninhydrin, also
from hydrolysis of hydrindantin, for only 1 mole of indanone-enediol is
used up for each mole of Ruhemann's purple formed. The details of the
mechanism, summarized in formula I, will be discussed in a paper on the
order of the reactions of amines with hydrindantin and ninhydrin.
SUMMARY

1. The red and blue colors formed by dissolving hydrindantin in alkaline solution have been studied spectrophotometrically under controlled conditions of oxygen content, pH, and temperature. The results indicate that the red color represents the monovalent anion of indanone-enediol and the blue color represents the divalent anion.

2. At pH 5.3, the sodium salt of diketohydrindamine-diketohydrindyli-dene is formed in the reaction of α-alanine with hydrindantin but not by way of intermediate ammonia formation. Indanone-enediol is used up in the reaction mole for mole of Ruhemann's purple formed.

3. Of previous concepts of the mechanism of the reaction of amino acids and other amines with ninhydrin, only that proposed with respect to ammonium salts by Harding, Warneford, and MacLean (3, 4) is supported by the present results.

BIBLIOGRAPHY

ON THE MECHANISM OF THE REACTION OF NINHYDRIN WITH α-AMINO ACIDS: II. A SPECTROPHOTOMETRIC STUDY OF HYDRINDANTIN REACTIONS
Douglas A. MacFadyen and Nathalie Fowler


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