Red Pigment Produced by the Reaction of Dehydro-L-ascorbic Acid with α -Amino Acid

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Received December 28, 1972

At the initial stage of the browning reaction of dehydro-L-ascorbic acid (DHA) with α -amino acid, a kind of red pigment was produced. The pigment was isolated as very hygroscopic red powder from non-aqueous reaction system, and its characterization was made. It was revealed that it had the same structure with that of the red pigment produced by the oxidation of L-scorbamic acid, an intermediate amino-reductone expected to be produced by Strecker degradation. Formation mechanism of the pigment which was considered to be an intermediate of browning reaction of DHA with α -amino acid was also discussed.

Browning reaction of dehydro-L-ascorbic acid (DHA) with amino acid is known to be involved in discolorations of some foods, especially in orange,¹⁾ lemon juice,²⁾ and dehydrated foods.^{3,4)} Although, several studies have been made on this browning system including model systems,^{5~7)} detailed mechanism of the reaction is still unknown.

When a mixed solution of DHA and an α -amino acid is heated, the development of red color is observed. The phenomenon was first reported by Koppányi *et al.*,⁸⁾ and since then, considerable works have been done on this red pigment.^{3,4,9~13)} However, there remain still some unsettled problems including its structure and formation mechanism, as the pigment has never been isolated in pure state owing to its highly labile character. In this study, isolation and characterization of the pigment were attempted, and re-examination of reaction conditions for the coloration was also made to resolve the apparent discrepancies observed in the early investigations.

EXPERIMENTAL

Spectroscopic methods. IR spectra were determined with a Japan Spectroscopic Model IR-S infrared spectrophotometer, and NMR spectra were measured with a Japan Electron Optics JNM-4H-100 spectrometer. UV and visible spectra were recorded with a

Hitachi recording spectrophotometer, Model EPS-3T. A Hitachi-Model 101 spectrophotometer was also used for the measurement of browning.

Chromatography. Cellulose (Serva) or microcrystalline cellulose (Avicell) was used as adsorbent in thin-layer chromatography (TLC) which was performed with two solvent systems, *i.e.* *n*-BuOH-EtOH-water (4: 1: 4 or 4: 1: 5) and EtOAc-pyridine-water (10: 4: 3). The following spray reagents¹⁴⁾ were used for the visualization of colorless spots: (1) Ninhydrin solution for amino acids, amines; (2) silver nitrate-ammonium hydroxide solution for reducing substances; (3) 2,4-dinitrophenylhydrazine-HCl solution for carbonyl compounds. In paper chromatography (PPC), Tōyō Rōshi No. 51 filter paper was used, and essentially the same solvent systems and visualization procedures as those employed in TLC were also used.

Reagents. Unless otherwise stated, guaranteed grade reagents (Kanto Chemical Co., Inc.) were used in this experiment.

Anhydrous DHA was prepared as described before.¹⁵⁾

5-Phenyl-3,4-diketo- γ -butyrolactone was prepared according to the method described by Dahn *et al.*¹⁶⁾

Triose reductone was prepared from D-glucose treated with NaOH,¹⁷⁾ and aqueous solution of its oxidized form was prepared by oxidation with iodine.

DHA (enol) solution (0.2 M) and 2,3-diketo-L-gulonic acid (DKG) solution (0.2 M) were prepared by treating aqueous solution of anhydrous DHA with one or two equivalents of KOH solution (1 N), respectively. Their pH values were adjusted to 5.6 before use.

L-Scorbamic acid (SCA) was prepared by hydrolysis of 2-(phenylhydrazono)-DHA obtained by the reaction of DHA with phenylhydrazine-HCl.¹⁸⁾

2,2'-Nitrilodi-2(2')-deoxy-L-ascorbic acid mono-ammonium salt (NDA) was prepared by the oxidation of SCA.¹⁸⁾ Anal. Calcd. for $C_{12}H_{16}N_2O_{10}$: C, 41.38; H, 4.63; N, 8.04. Found: C, 41.57; H, 4.74; N, 8.06%. IR $\nu_{\text{max}}^{\text{NaCl}}$: 3400~3200, 1760, 1690, 1640, 1410, 1350, 1210, 1190, 1120, 1050, 1030. NMR δ (100 MHz; solvent, D_2O ; internal reference, DSS): 3.76 (4H, doublet, $J \approx 6.5$ Hz), 4.19 (2H, triplet with a minor splitting of *ca.* 1.7 Hz, $J \approx 6.5$ Hz), 4.88 (2H, doublet, $J \approx 1.7$ Hz). The crystalline NDA was dissolved in MeOH-BuOH mixture, and solvents were removed under reduced pressure to dryness. The resulted amorphous NDA obtained as hygroscopic red powder was dried *in vacuo*, and its IR spectrum was taken in film.

Preparation of the red pigment

1) *Isolation from the reaction system of DHA-Leu.* A mixture of anhydrous DHA (1 mmole) and L-leucine (1 mmole) was heated in MeOH (*ca.* 50 ml) under reflux for several minutes, and the resulting red solution was rapidly cooled with dry ice-acetone to cease the reaction. The MeOH solution was concentrated under reduced pressure to a small volume, and subjected to the preparative TLC on microcrystalline cellulose layers. After development, the corresponding red zone was scraped off and extracted with MeOH. MeOH extract was concentrated, after the addition of *n*-BuOH, to a small volume under reduced pressure. The red precipitates obtained were washed with *n*-BuOH, then with Et_2O and dried *in vacuo* over phosphorus pentoxide for a night; yield was 1~2 mg.

2) *Isolation from the reaction system of DHA-formamide.* Anhydrous DHA (1.74 g) was heated in formamide (0.9~1.8 g) with constant stirring until deep red coloration took place, and just before the coloration reached to its maximum, the reaction mixture was rapidly cooled with dry ice-acetone to cease the reaction. The frozen reaction mixture was then melted and adsorbed on cellulose powder (*ca.* 5 g) which was packed in a small column. The cellulose column was washed with plenty of *n*-BuOH, then with dioxane to remove remaining formamide, and the pigment was eluted with MeOH. The MeOH solution obtained under reduced pressure, and the solvent was removed almost completely with the bath temperature below 30°C. The residue was washed in succession with dioxane, *n*-BuOH and Et_2O , and then dried *in vacuo*; yield was *ca.* 60~120 mg. The crude pigment thus obtained was dissolved in a small volume of MeOH, and the solution was filtered after standing overnight in a deep-freezer at -20°C. The filtrate which was strongly colored red was subjected to preparative TLC on cellulose layers. After development, the red zone was collected and extracted

several times with MeOH. The MeOH extract was concentrated after the addition of small volume of BuOH to facilitate the precipitation of the pigment. The chromatographically purified pigment obtained as hygroscopic red powder was washed with *n*-BuOH, then with Et_2O , and dried over phosphorus pentoxide *in vacuo*; yield was *ca.* 10~12 mg.

Browning of NDA and related compounds. To examine the roles of NDA in browning reaction, sample solutions of seven different model systems were prepared and heated in a boiling water bath. After heating for 2 or 5 hr, browning of each reaction mixture was estimated by values of absorbance at 438 nm.

RESULTS AND DISCUSSION

Conditions for coloration

The aqueous solution of DHA readily gave brown color when heated in the presence of α -amino acid, and the red coloration which took place at the first step of the reaction disappeared with the increment of browning, suggesting the red pigment to be an intermediate of the browning reaction (Fig. 1).

The coloration reaction of DHA with various nitrogen containing compounds including

FIG. 1. Discoloration of Aqueous DHA-Gly Solution (0.1 M).

Reaction condition: Heating in a boiling water bath for 2 (— · — · —), 5 (-----) or 10 (—) min.

α -amino acids (Gly, DL-Ala, L-Val, L-Leu, L-Ileu, L-Met, L-CySH, L-Ser, L-Thr, L-Phe, L-Try, L-Asp, L-Glu, L-Lys, L-His, L-Arg, etc.), formamide, ammonium salts (ammonium sulfate, ammonium chloride), amines (*n*-butylamine, *p*-toluidine, piperidine) was examined in the aqueous system, and it was revealed that almost all α -amino acids except L-proline gave red coloration, while formamide, ammonium salts and amines did not. Without solvent, however, formamide produced red coloration in the reaction with DHA. Since the red pigments produced from various α -amino acids and formamide gave the identical *Rf* value in PPC, for instance *Rf* = 0.12 with *n*-BuOH-EtOH-water (4:1:4) solvent system, and the same absorption maximum ($\lambda_{\text{max}}^{\text{H}_2\text{O}} = 513 \sim 4 \text{ nm}$) in their visible spectra, all these pigments were considered to be the same compound. The fact suggested that a common precursor leading to the formation of the red pigment might be produced in these reaction systems.

It is well-known that DHA is rather unstable in aqueous solution, and its lactone ring is easily hydrolyzed to give DKG.¹⁹⁾ As for the formation of red pigment from DKG,

there were some discrepancies in the previous studies.^{3,4,10,11)} Aqueous solutions of follow-

FIG. 2. Visible Spectra of ASA-Gly, DHA-Gly, DHA (enol)-Gly and DKG-Gly Solutions (0.1 M) Heated for Two min in a Boiling Water Bath.

(A).....: ASA-Gly solution
 - · - · - : DHA-Gly solution
 - - - - : DHA(enol)-Gly solution
 ———: DKG-Gly solution

FIG. 3. UV and Visible Spectra of Red Pigments.

- · - · - : Pigment obtained from DHA-Leu reaction system
: Pigment obtained from DHA-formamide reaction system
 - - - - : NDA
 Solvent: Water, Concentration: Arbitrary

ing reaction systems; L-ascorbic acid (ASA)-Gly, DHA-Gly, DHA (enol form)-Gly, DKG-Gly were prepared and heated at 95°C for two min. The initial pH of these four sample solutions was adjusted to *ca.* 5.6 just before the reaction. As shown in Fig. 2, the results indicated that the red coloration took place only in the case of DHA-Gly solution. Thus, it was evidenced that the coloration was due to the reaction of DHA (keto form) with α -amino acid and the presence of lactone ring was necessary for the formation of the pigment. Therefore, the optimum pH range for the coloration which was reported to be 5.8~6.0⁽¹⁾ seemed to reflect the counterbalance of two different effects: (1) At lower pH, the pigment was very unstable owing to the acid catalyzed hydrolysis;⁽¹¹⁾ (2) at neutral pH, the concentration of active species of DHA decreased due to enolization.

5-Phenyl-3,4-diketo- γ -butyrolactone which had the same type of lactone ring structure as that of DHA also gave a similar red color (513 nm, in dioxane) when reacted with an α -amino acid, while the oxidized form of triose reductone, a straight chain triketone, did not give a similar coloration. So, it may be concluded that a cyclic triketone system in which three carbonyl groups are forced to assume a nearly planar *s-cis* orientation is necessary for the coloration, and it is also deduced that contribution of the side chain

of lactone ring is not so significant.

Isolation and characterization of the pigment

Isolation of the pigment from the aqueous reaction mixture of DHA and an α -amino acid was extremely difficult owing to the highly labile character of the pigment in aqueous solution, and even a preparation obtained by repeated column and thin-layer chromatography still contained small amounts of impurities, and such a preparation did not give satisfactory results in elementary analyses. Wurtz and North⁽¹⁰⁾ reported the isolation of the pigment by adsorption on Norit followed by washing and elution, but their preparation also did not give the pure sample.

As the pigment was comparatively stable in non-aqueous solution, preparations by using non-aqueous solvents were attempted. The pigment produced from the reaction of DHA with L-Leu in MeOH was obtained as very hygroscopic deep red powder. Its UV and visible spectra, and IR spectrum which was forced to be taken in film (H₂O) owing to its highly hygroscopic character, were shown in Figs. 3 and 4, respectively.

Red pigment was also separated from DHA-formamide reaction mixture. Although its thin-layer chromatogram gave a single red spot, a small amount of impurities was also recognized when a large amount of the sample was charged. As this amorphous pigment

FIG. 4. IR Spectra of the Red Pigments (in films).

- : Pigment obtained from DHA-Leu reaction system
- - - : Pigment obtained from DHA-formamide reaction system
-: NDA

was easily degraded with discoloration when heated *in vacuo*, complete removal of water from the sample was unsuccessful, and two most reliable results of elementary analyses were as follows: *Anal.* (1) Calcd. for $C_{12}H_{18}N_2O_{11}(=C_{12}H_{16}N_2O_{10} \cdot H_2O)$: C, 39.34; H, 4.92; N, 7.65. Found: C, 39.41; H, 5.10; N, 7.15%. (2) Calcd. for $C_{12}H_{20}N_2O_{12}(=C_{12}H_{16}N_2O_{10} \cdot 2H_2O)$: C, 37.50; H, 5.21; N, 7.29. Found: C, 37.57; H, 5.43; N, 7.10%. NMR δ (100 MHz; solvent, D_2O ; internal reference, DSS): 3.76 (doublet, $J=6.5$ Hz), 4.19 (triplet with a minor splitting of *ca.* 1.7 Hz, $J=6.5$ Hz), 4.88 (doublet, $J=1.7$ Hz). The ratio of relative intensities of these three signals, from higher magnetic field to low-field, was approximately 2:1:1, and it was apparent that the pigment had the partial structure, $CH_2-CH-CH-$ in its molecule. As

shown in Fig. 3, the isolated pigment had characteristic three absorption bands ($\lambda_{max}^{H_2O} = 513 \sim 4, 386 \sim 7, 246 \sim 7$ nm) in UV and visible region, and these band positions were quite identical with those of the pigment from DHA-Leu reaction system. IR spectrum of the isolated pigment was also quite similar with that of the pigment from DHA-Leu system, as shown in Fig. 4, and suggestive of the presence of γ -lactone ring. Therefore, it was concluded that the pigments from both reaction systems, *i.e.* DHA-Leu and DHA-formamide, were the same compound.

Structure, formation mechanism and roles in browning

All these facts described above, together with the observations made by other researchers,^{4,9,20} suggested Strecker degradation of α -amino acid with DHA to be the key reaction of the coloration. The framework of Strecker degradation mechanism was extensively studied by Schönberg *et al.*^{20,21} If the pigment is produced in the course of Strecker degradation, it will not be unreasonable to infer that the amino-reductone produced by the amination of one of the carbonyl groups of DHA may

be a common precursor for the coloration and may play an important role in the formation of the pigment.^{11,12} Supposing the amination takes place at C-2 carbonyl, the corresponding amino-reductone will be 2-amino-2-deoxy-L-ascorbic acid²² (SCA). In the reaction with formamide, a carbonyl group of DHA might also undergo amination process like Leuckart reaction.²³

Recently it was revealed that the structure of the red pigment produced by the oxidation of SCA was 2,2'-nitrilodi-2(2')-deoxy-L-ascorbic acid monoammonium salt (NDA).¹⁸ Since NDA gave the same *Rf* values in PPC, for instance *Rf*=0.12 with *n*-BuOH-EtOH-water (4:1:4) solvent system, and showed the same behaviors on thin-layer chromatograms (Fig. 5) with those of the pigments isolated from DHA-Leu and DHA-formamide reaction systems, these pigments were considered to be identical. The results of elementary analyses of isolated pigment from DHA-formamide reaction system also seemed to support the structure. Further confirmation of their iden-

FIG. 5. Chromatograms of Red Pigments on Cellulose Layers.

(I) NDA
 (II) Pigment from DHA-Leu system
 (III) Pigment from DHA-Formamide system
 Solvent systems; (A) EtOAc-pyridine-water (10:4:3)
 (B) BuOH-EtOH-water (4:1:5)

tity was obtained by comparing the spectral data. NMR spectrum of NDA was identical with that of the pigment from DHA-formamide system, UV and visible spectra of NDA were very similar to those of isolated pigments as shown in Fig. 3. As shown in Fig. 4, IR spectrum of the amorphous NDA was also quite identical with those of the pigments isolated from two reaction systems. All the facts mentioned above strongly indicated that these pigments were the same compound. The slight shift in band positions and differences in absorption intensities observed in UV spectra of these pigments suggested possible contamination of the isolated pigments from two reaction systems by impurities, such as SCA which had strong absorption maximum at 246.5 nm.

On reduction with H_2S or by catalytic hydrogenation, the aqueous solution of the pigment readily lost its color with the disappearance of absorption bands at around 513 and 386 nm, and the colorless solution partly restored its color through immediate oxidation by aeration. L-CySH, an amino acid capable of reducing DHA to ASA,²⁴ also caused decoloration which was probably due to the reduction by $-SH$ group, and this partly explained the anomalous behaviors of L-CySH in the formation of the pigment described in previous studies.^{3,10}

The possible formation mechanism of the pigment in the reaction of DHA with an α -amino acid may be as follows; at first, SCA will be produced in the course of Strecker

SCHEME 1. Possible Formation Mechanism of the Red Pigment.

degradation of an α -amino acid in the presence of DHA. SCA thus formed possibly undergoes oxidation¹⁸ to yield dehydro-L-scorbamic acid (dehydro-SCA), and the pigment will be probably produced by the reaction of dehydro-SCA with SCA as in usual exchange reaction, transalkylidenation, of imines with primary amines.^{25, 26} Since dehydro-SCA is considered to be partly hydrolyzed to give DHA and ammonia in aqueous system, a portion of the pigment may be also formed from equimolar amounts of ammonia and the condensation product of SCA with DHA. On reaction with SCA, DHA very readily produc-

TABLE I. BROWNING OF AQUEOUS SOLUTION OF NDA AND/OR RELATED COMPOUNDS

Materials (Concentration)	Reaction time	Browning (Absorbance at 438 nm) ^{b)}	
		2 hr	5 hr
NDA (0.005 M)		1.77	2.58
DHA (0.01 M)-NH ₃ (0.01 M)-SCA (0.01 M)		2.79	4.08
DHA ^{a)} (0.02 M)-Ala (0.02 M)		0.46	0.53
DHA ^{a)} (0.019 M)-Ala (0.019 M)-NDA (0.0005 M)		0.54	0.72
DHA (0.02 M)-NH ₃ (0.02 M)		0.67	0.74
DHA (0.019 M)-NH ₃ (0.019 M)-NDA (0.0005 M)		0.94	1.08
DHA ^{a)} (0.02 M)		0.37	0.41

^{a)} Initial pH (6.0~6.5) was adjusted with NaOH soln.

^{b)} Reaction temp.: ca. 98°C.

ed the pigment, but enol form of DHA or DKG did not.

From the results shown in Table I, under the same concentration of reactants, NDA was considered to produce more intense brown color than DHA-alanine system or DHA-NH₃-SCA system which was composed of identical components of the pigment itself. This may imply the accelerated degradation of DHA moiety of NDA. Addition of a small amount of NDA to DHA-alanine or DHA-NH₃ model system caused comparatively increased browning. From these results, the pigment NDA, was postulated to be an intermediate in browning reaction of DHA with an α -amino acid at relatively high concentration.

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