Red Pigment Produced by the Oxidation of L-Scorbamic Acid

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A crystalline red pigment was obtained by the oxidation of L-scorbamic acid which was thought to be an intermediate amino-reductone in the Strecker degradation of an α-amino acid with dehydro-L-ascorbic acid. The pigment which was soluble in water, MeOH, and EtOH, and insoluble in most other organic solvents was an ammonium salt and quite stable in a dry state, but it was easily decolorized in an acid solution to give L-scorbamic acid and dehydro-L-ascorbic acid as hydrolysis products. Its molecular formula was $C_{12}H_{16}N_2O_{10}$, and spectroscopic data (ir, nmr, uv, etc.), together with the results of quantitative analysis of hydrolysis products, proved it to be 2,2′-nitrilodi-2(2′)-deoxy-L-ascorbic acid monoammonium salt. It is a new pigment with a murexide-type chromophore.

An intense red coloration is observed when a mixed solution of dehydro-L-ascorbic acid (DHA) and an α-amino acid is heated. The phenomenon was first reported by Koppanyi *et al.* (1945), who heated L-ascorbic acid (ASA) with α-amino acid in the presence of H_2O_2. Since then, though considerable works (Nakabayashi and Shibata, 1967; Pecherer, 1951; Ranganna and Setty, 1968; Wurtz and North, 1963) have been done on this red pigment, its exact structure and formation mechanism are still unknown.

It was pointed out previously that L-scorbamic acid (SCA) might be a precursor of the red pigment (Kurata and Sakurai, 1966; Kurata *et al.*, 1971). SCA was first prepared by Micheel and Mittag (1937) as amorphous powder which produced a red pigment on oxidation, and two possible structures, namely a chain structure and a pyrazine ring structure, were proposed for the pigment. γ-Methyl-α-amino tetronic acid was also known to produce a red pigment, and a dihydropyrazine ring structure was proposed in this case (Lecocq, 1951). In this paper, isolation and characterization of the red pigment produced by the oxidation of SCA were described.

EXPERIMENTAL AND RESULTS SECTION

Prior to elementary analyses, all compounds were dried *in vacuo* over phosphorus pentoxide, unless otherwise stated. Melting points were uncorrected. Uv and visible spectra were recorded with a Hitachi recording spectrophotometer, Model EPS-3T. 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 or JNM-MH-60 spectrometer. Mass spectra were recorded on a Hitachi RMU-6L spectrometer with an ionizing voltage of *ca.* 70 eV.

Reagents and Chromatography. Anhydrous DHA was prepared as described previously (Kurata *et al.*, 1967). ASA and all other reagents were guaranteed grade (Kanto Chemical Co., Inc.) and used without further purification.

Both silica gel G (E. Merck) and cellulose (Serva) were used as adsorbents for thin-layer chromatography (tlc). Solvent systems containing mixtures of toluene–ethyl acetate (5:1–1:1) were used for silica gel layers, and two solvent systems, ethyl acetate–pyridine–water (10:4:3) and *n*-BuOH–EtOH–water (4:1:4 or 4:1:5), were used for cellulose layers. In the case of cellulose layers, colorless spots were visualized by spraying with a silver nitrate–ammonium hydroxide solution, followed by charring at 100° or by standing at room temperature for more than several hours.

Preparation of DHA Mono(phenylhydrazone), 2-(Phenylhydrazono)-DHA. An aqueous solution of DHA (Kurata and Sakurai, 1967) prepared by the oxidation of ASA (118 g) with *p*-quinone (72 g) was treated with phenylhydrazine hydrochloride (96 g) at *ca.* 60°, and, after cooling, precipitated orange-yellow crystals were collected by filtration, washed with water, dried *in vacuo* over silica gel, and crude crystals (*ca.* 100 g) were recrystallized from ethyl acetate: mp 165–166° (lit. 167–170°) (El Khadem and El Ashry, 1970; Pohloudek-Fabini and Fürtig, 1959; von Euler and Hasselquist, 1952), yield *ca.* 38 g. *Anal.* Calcd for $C_{12}H_{12}N_2O_5$: C, 54.54; H, 4.58; N, 10.60. Found: C, 54.59; H, 4.57; N, 10.50. Ir ν_{max}^{Nujol} 3400 (OH), 1759 (lactone C=O), 1669 cm^{-1} (5-membered ring C=O); nmr (solvent, pyridine-d_5; internal reference, Me_4Si) δ 4.26 (2 H, d, J = 7 Hz), 4.83 (1 H, t with a minor splitting of *ca.* 2 Hz, J = 7 Hz), 5.52 (1 H, d, J = 2 Hz); mass spectrum m/e (%) 264 (M^+, 62), 204 (88), 93 (71), 92 (100), 91 (64), 77 (74), 65 (65); uv λ_{max}^{EtOH} 233 nm (log ε 3.95), 246 (3.88), 255 (3.83), 390 (4.29). These ε values were calculated with the absorption spectrum obtained at the concentration *ca.* 0.8×10^{-4} *M*. In a highly diluted solution, the longest wavelength band appeared at 360 nm, with the disappearance of the absorption band at 390 nm. At intermediate concentration, the spectrum showed a maximum absorption at 376–378 nm, with a shoulder at 390 nm. The same phenomenon was also observed with the uv spectrum of 3-oxo-2-(phenylhydrazono)-γ-butyrolactone measured at lower concentration.

Preparation of 3-Oxo-2-(phenylhydrazono)-γ-butyrolactone. Tetronic acid was prepared by sodium amalgam reduction of α-bromotetronic acid obtained by the cyclization of α,γ-dibromoacetoacetic ester (Kumler, 1938; Wolff and Schwabe, 1896) and was coupled with benzenediazonium chloride in alkaline solution to give the titled compound. Recrystallization from ethyl acetate yielded yellow plates: mp 210–211° dec, (lit. 210°) (Wolff and Lüttringhaus, 1900). *Anal.* Calcd for $C_{10}H_8N_2O_3$: C, 58.82; H, 3.95; N, 13.72. Found: C, 58.60; H, 3.89; N, 13.44. Ir ν_{max}^{Nujol} 1762 (lactone C=O), 1672 cm^{-1} (5-membered ring C=O); uv λ_{max}^{EtOH} 230 nm (log ε 3.95), 246 (3.85), 255 (3.80), 389 (4.28). The longest wavelength absorption appeared at 356 nm in a extremely diluted solution.

Preparation of L-Scorbamic Acid, 2-Amino-2-deoxy-L-ascorbic Acid. Twenty grams of 2-(phenylhydrazono)-DHA was dissolved in 70% aqueous EtOH (*ca.* 600 ml) and was subjected to hydrogenolysis using palladium black (*ca.* 600 mg) as catalyst. A rapid hydrogen absorption was ceased within 3 hr, but the hydrogenolysis was continued for more than several hours. After the reaction was completed, the catalyst was removed by filtration and washed with 50–150 ml of freshly distilled or deoxygenated water. The filtrate was combined with washings and shaken once with a small volume of ethyl acetate and then

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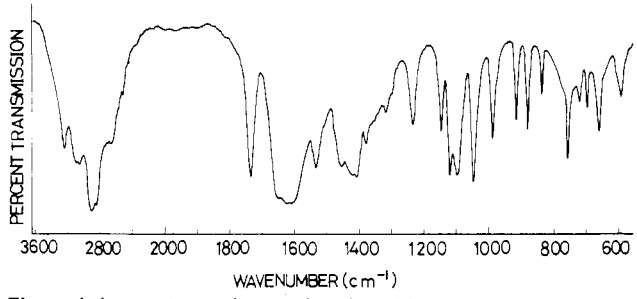


Figure 1. Ir spectrum of L-scorbamic acid.

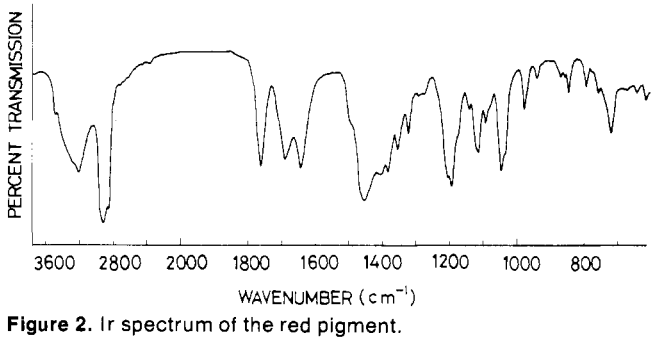


Figure 2. Ir spectrum of the red pigment.

twice with 150 ml of ethyl ether. The water layer was concentrated to a small volume under reduced pressure below 40°, and crystallization took place easily by seeding. Recrystallization from hot water yielded almost colorless needles: mp 180° dec; yield of crude crystals was about 60%. *Anal.* Calcd for $C_6H_9NO_5$: C, 41.14; H, 5.18; N, 8.00. Found: C, 41.33; H, 5.40; N, 8.24. Molecular weight was 175 (mass spectrum); pK = 6.8; $[\alpha]_D$ +135° (c 0.4, H_2O); ir ν_{max}^{Nujol} 3030 and 3130 (NH), 1738 (lactone C=O), 1610–1650 cm^{-1} (C=C, NH) (ir spectrum is given in Figure 1); uv $\lambda_{max}^{H_2O}$ 246.5 nm (log ϵ 4.19); nmr (100 MHz; solvent, D_2O; internal reference, DSS) δ 3.73 (2 H, d, J = 7 Hz), 4.03 (1 H, t with a minor splitting of *ca.* 2 Hz, J = 7 Hz), 4.63 (1 H, d, J = 2 Hz).

Condensation products with carbonyl compounds were prepared as follows. SCA was heated in boiling acetone for awhile and, after being cooled and evaporated, the condensation product was obtained as a colorless crystalline powder: mp 160–162°. *Anal.* Calcd for $C_9H_{13}NO_5$: C, 50.23; H, 6.09; N, 6.51. Found: C, 50.66; H, 6.15; N, 6.27. Ir ν_{max}^{Nujol} 3320 (OH), 1715, 1690 (weak), 1660 (weak), 1600 cm^{-1}. Similarly, on reaction with benzaldehyde, SCA readily gave a crystalline condensation product which was recrystallized from EtOH: mp 179–180°. *Anal.* Calcd for $C_{13}H_{13}NO_5$: C, 59.31; H, 4.98; N, 5.32. Found: C, 59.31; H, 5.04; N, 5.39. Ir ν_{max}^{Nujol} 3360 (OH), 1710, 1690, 1620 cm^{-1}.

Preparation and Characterization of the Red Pigment. SCA (*ca.* 2 g) in distilled water (50 ml) was shaken with *p*-quinone (*ca.* 1.3 g) in ethyl ether (200 ml) for about 15–20 min. The deep red water layer was washed several times with ethyl ether and filtered to remove quinhydron, concentrated to a small volume with a rotary evaporator at *ca.* 30–43° (bath temperature), and kept in a refrigerator overnight after seeding. The crude pigment (300–500 mg) obtained as a crystalline precipitate was washed with hot ethanol, and most of the residue was then dissolved in hot ethanol. The ethanol solution was filtered from an insoluble dark substance, concentrated to a small volume under reduced pressure, and kept in a refrigerator overnight after seeding. The pigment, which was obtained as colorless needles by recrystallization, turned to red when dried *in vacuo* over phosphorus pentoxide at room temperature. The colorless crystal obtained

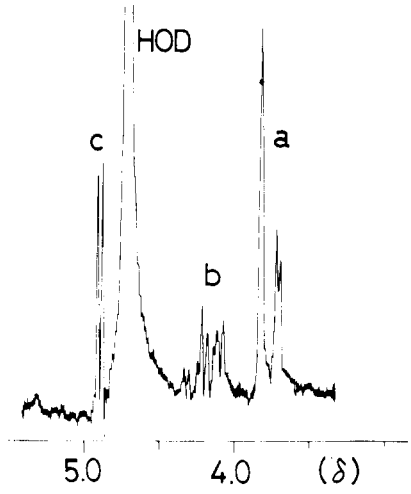


Figure 3. Nmr spectrum (60 MHz) of the red pigment. Relative intensities of bands a:b:c = 2:1:1.

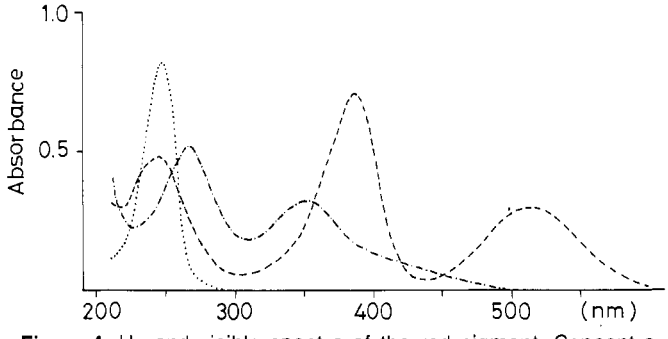


Figure 4. Uv and visible spectra of the red pigment. Concentration, *ca.* 5 × 10^{-5} *M*. - - - - - -, pH 6 (H_2O); . . . . . , pH 2 (0.01 *N*, HCl); ---------, pH 12 (0.01 *N*, KOH).

before drying gave the same red color when dissolved in a solvent such as water or methanol, and showed the same behavior on a thin-layer chromatogram as those of red crystals obtained after drying. The pigment showed no distinct melting point, and its crystal form was kept, even at a high temperature (over 200°), though discoloration and darkening took place at *ca.* 150–180°; yield *ca.* 100–300 mg. *Anal.* Calcd for $C_{12}H_{16}N_2O_{10}$: C, 41.38; H, 4.63; N, 8.04. Found: C, 41.49; H, 4.58; N, 7.91. Ir spectrum in Nujol mull is shown in Figure 2. Nmr (100 MHz; solvent, D_2O; internal reference, DSS) δ 3.76 (4 H, d, J = 6.5 Hz), 4.19 (2 H, t with a minor splitting of *ca.* 1.7 Hz, J = 6.5 Hz), 4.7 (HOD), 4.88 (2 H, d, J = 1.7 Hz). Nmr spectrum (60 MHz; solvent, D_2O; internal reference, DSS), uv, and visible spectra are given in Figures 3 and 4, respectively.

Esr spectrum of the pigment was measured in a solid state at room temperature with a Japan Electron Optics JES-ME-3X spectrometer, and the approximate radical concentration was estimated to be below 10^{18-19} spin/mole.

Molecular weight was determined by an osmometric method using a Hitachi Perkin-Elmer molecular weight apparatus Model 115, with the main oven temperature and bridge current at 60° and 15 μA, respectively. D-Glucose solutions of various concentrations were used for calibration, and deionized water was used as the solvent. Measurements were carried out with the pigment solutions of three different concentrations, and the average of three measured values, 175, was adopted as the molecular weight of the pigment without dissociation or association. Its exact molecular weight was estimated from its empirical formula.

Paper electrophoresis was carried out in AcOH–pyridine–H_2O (0.4:10:90) buffer solution (pH, 6.5) at 200 V

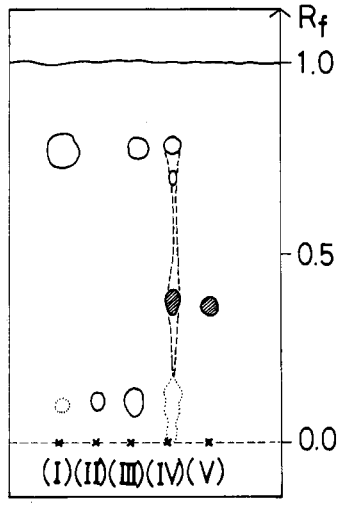


Figure 5. Chromatograms of cellulose thin layers developed with ethyl acetate–pyridine–water (10:4:3). ◍, red spots without visualization; O====O, visualized with $AgNO_3$–NH_4OH; (I), DHA. (II), SCA. (III), Red pigment treated with HCl. (IV), SCA treated with *p*-quinone. (V), recrystallized red pigment.

(5 mA) for 70 min using Toyo Roshi No. 51 filter paper and a Natsume electrophoresis apparatus. The pigment migrated toward the anode about 10 mm.

Detection of ammonium ion in the pigment was carried out by the usual qualitative analyses (Treadwell, 1949) using Nessler's reagent, cobalt sodium nitrite solution, or chloroplatinic acid solution. Aqueous solution of the pigment was positive to these three qualitative tests for ammonium ion. A Hitachi amino acid analyzer KLA-3A was used to determine the quantity of ammonia. The result revealed that 1 mol of the pigment contained 0.95 mol of ammonia.

Detection and determination of hydrolysis products of the pigment were performed as follows. Hydrolysis of the pigment took place readily when it was dissolved in a dilute hydrochloric acid (*ca.* 0.01 *N*) or its aqueous solution was treated with the acid. The color of the acid solution faded very rapidly, and the resulting colorless solution was subjected to the qualitative and quantitative analyses of hydrolysis products.

Detection of the hydrolysis products was made by tlc using cellulose layers. After visualization, formation of SCA and DHA was confirmed by comparison of the R_f values with those of the authentic samples (Figure 5). Moreover, to the acid solution of the pigment was added phenylhydrazine hydrochloride and the resulting product, 2-(phenylhydrazono)-DHA, was isolated and identified by tlc on silica gel and mass spectrometry.

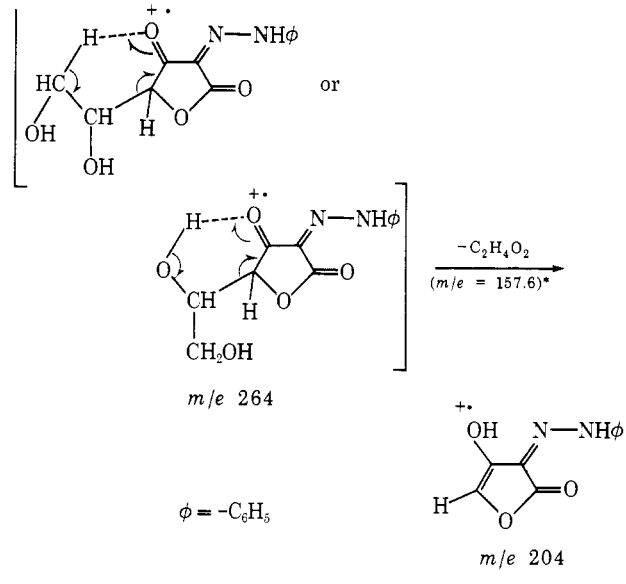
The uv spectrum of the acid-treated solution of the pigment showed only the absorption band due to SCA (Figure 4), and its concentration was determined from the $OD_{246.5\ nm}$ value. The quantity of SCA was also estimated by titration with KIO_3 solution, as in the determination of ASA (Ballentine, 1941). The results indicated that 1 mol of pigment gave 0.9–1.1 mol (uv spectrometric analysis) or 1.0–1.1 mol (titration) of SCA. The acid solution of the pigment was also submitted to usual quantitative determination of DHA using a 2,4-dinitrophenylhydrazine reagent (Mills *et al.*, 1949; Roe and Kuether, 1943; Roe *et al.*, 1948). Under the reaction conditions (37°, 3 hr) employed to produce DHA- or 2,3-diketo-L-gulonic acid (DKG) bis-2,4-dinitrophenylhydrazone (DNPH) from DHA, no DHA- or DKG-bis-2,4-DNPH was produced from SCA. It was revealed that 1 mol of the pigment yielded 1.1 mol of DHA, approximately half of which was estimated to be DKG by H_2S reduction.

Stability of the Pigment in Aqueous Solution. Stability of the pigment was estimated by the measurement of decreasing OD values (513 nm) of the pigment solution held at 37° or heated at 98° for various periods of time. Results are shown in Figure 6, (A) and (B).

DISCUSSION

Preparation of SCA. As the yield of SCA by the synthetic route through 2-deoxy-L-ascorbic acid (Micheel and Mittag, 1937) was not sufficient for our needs, SCA preparation through DHA mono(phenylhydrazone) produced directly from DHA was attempted. It is well known that DHA treated with phenylhydrazine hydrochloride readily produces DHA mono(phenylhydrazone), together with DHA bis(phenylhydrazone) (von Euler and Hasselquist, 1952; Pohloudek-Fabini and Fürtig, 1959), but no conclusive evidence has been given for the assignment of the position of the hydrazono group in DHA mono(phenylhydrazone). Though the most probable site is expected to be the C-2 atom, located between two carbonyl groups and susceptible to nucleophiles, the C-3 position is not completely excluded. For instance, 1,2,3-cyclopentanetrione treated with phenylhydrazine gave 2-oxo-1,3-bis(phenylhydrazono)cyclopentane (Fatiadi and Isbell, 1967), suggesting the first nucleophilic attack on the carbon atoms of the outer-side carbonyl groups in the triketone system.

By comparison of the uv and ir spectra of DHA mono(phenylhydrazone) with those of 3-oxo-2-(phenylhydrazono)-γ-butyrolactone, it was concluded that the phenylhydrazono group in DHA mono(phenylhydrazone) was attached to the C-2 atom, and the strong McLafferty rearrangement peak (Budzikiewicz *et al.*, 1967) shown in its mass spectrum also supported this assignment.



It was reported that the uv spectrum of DHA mono(phenylhydrazone) showed the longest wavelength band at 376 nm (El Khadem and El Ashry, 1970) instead of at 390 nm, which was observed in this experiment. The discrepancy seemed to be mainly due to the formation of a dimer by an intermolecular hydrogen bond at a higher concentration, and the absorption band around 360 nm observed in a highly diluted solution was possibly due to the unassociated monomer.

SCA obtained as colorless needles was a weak acid with a slight acid taste, and was considerably stable in a dry state, even in the presence of air. In solution, however, it behaved as an amino-reductone, and easily underwent oxidation to yield a red coloration. As no reactions involved in the preparation seemed to give any significant changes in the steric environment around the C-5 atom, SCA was deduced to be a lactone of the L-series. Its specific dextro-

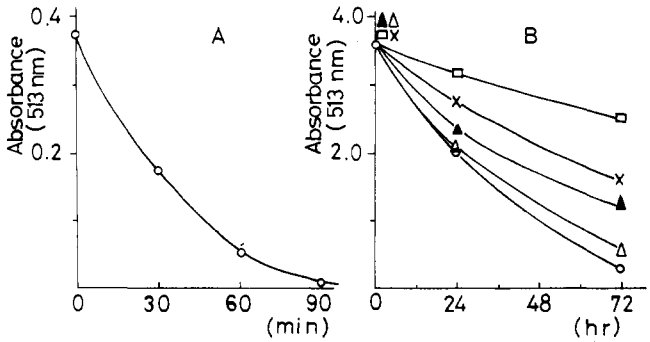


Figure 6. Stability of the red pigment in aqueous solution. (A), reaction temp, *ca.* 98°; concentration, *ca.* 6 × 10^{-5} *M*. (B), reaction temp, *ca.* 37°; concentration, *ca.* 5 × 10^{-4} *M*. —□—, 80% EtOH; —×—, 20% EtOH; —○—, aqueous soln; —▲—, in the presence of CH_3COONH_4 (0.01 *M*) added to the soln; —△—, in the presence of Ala (0.01 *M*) added to the soln.

rotation quite agreed with the so-called Hudson's lactone rule (Hudson, 1910). From the spectroscopic data, the amino-reductone was considered to assume the enaminol form which might be comparatively stabilized by conjugation and chelation. SCA readily reacted with carbonyl compounds such as acetone or benzaldehyde to give condensation products, suggesting the role of an enaminol as a potential nucleophile in a browning reaction involving Strecker degradation.

Preparation and Characterization of the Red Pigment. When the aqueous solution of SCA was treated with *p*-quinone, an intense red coloration took place almost instantly, and the red pigment produced was isolated and recrystallized from EtOH. The colorless crystals obtained by recrystallization turned red under dryness, and this phenomenon might be due to the loss of crystallization solvent which was possibly included in the colorless crystals and likely to hinder a suitable orientation of the chromophores required for coloration. Because the pigment migrated toward the anode in paper electrophoresis and its aqueous solution also showed positive to usual qualitative tests for ammonium ion, the pigment was thought to be an ammonium salt which easily dissociated into two ionic species in solution. Owing to its low volatility, measurements of the mass spectrum of the pigment were unsuccessful, and attempts to make volatile derivatives failed due to its labile character. So, determination of the molecular weight of the pigment was carried out by the osmometric method, and it was revealed that the molecular weight was 348, if a complete dissociation of the molecule took place. If no dissociation occurred, its molecular weight should be 174, and the pigment was regarded to be a radical from its empirical formula. The esr spectrum of the crystalline pigment, however, showed that the organic radical concentration in the pigment was too low to consider the pigment to be a radical.

Absorption bands in the ir spectrum (Figure 2) around 3200 and 1410 cm^{-1} suggested the presence of an ammonium ion in the molecule (Bellamy, 1958) and those at *ca.* 3400–3200 (br) and 1760 cm^{-1} suggested the presence of intermolecularly hydrogen-bonded polymeric hydroxyl groups and lactone rings, respectively. The identification of two bands appearing around 1690 and 1640 cm^{-1}, which were probably due to conjugated $\nu_{C=O}$, $\nu_{C=C}$, and/or $\nu_{C=N}$, was rather difficult, and conclusive assignments of these bands were not made in this work. As shown in Figure 3, its 60 MHz spectrum, which was very similar to that of ASA (Szymanski and Yelin, 1968), gave an AB_2 system $\Delta\nu_J = 3.8$ (Jackman and Sernhell, 1969) in a higher field, and each peak in the A portion was split further into doublets by first-order coupling with the adjacent proton which appeared on the low-field side of the HOD band.

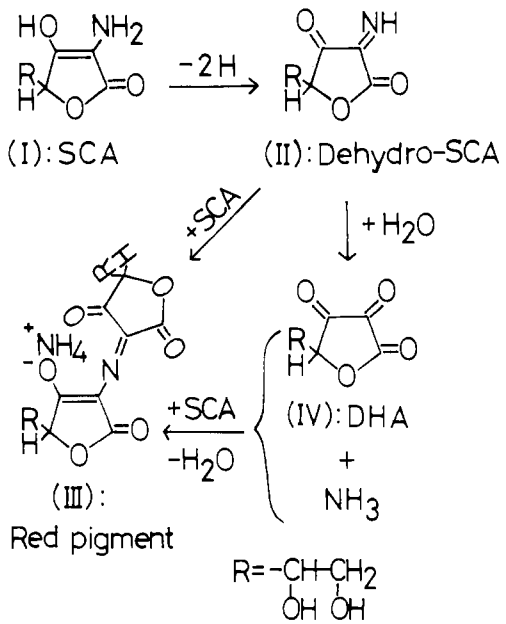


Figure 7. Possible formation mechanism of the red pigment.

Therefore, it was apparent that almost the same type of glycol moiety as that of ASA was present in its structure, and no enolization of the C-3 (or C-3′) carbonyl group involving a proton on the C-4 (or C-4′) occurred to any detectable extent. Other signals due to protons attached to double bonds or aromatic rings were not observed in its spectrum.

As its molecular formula was $C_{12}H_{16}N_2O_{10}$ and 1 mol of ammonium ion was contained in the molecule, the remaining part of the structure was thought to be an anion of the condensation product of SCA with DHA. In the formation of the condensation product, the nucleophilic attack of the amino group of SCA was expected to take place at the carbonyl C-2 atom of DHA, as is already shown in the formation of 2-(phenylhydrazono)-DHA. Therefore, though the details in the stereochemistry of the structure remained to be solved, 2,2′-nitrilodi-2(2′)-deoxy-L-ascorbic acid monoammonium salt (Figure 7, III), a structure with a murexide-type chromophore, was proposed for the pigment. The uv and visible spectrum of the pigment, given in Figure 4, showed three main regions of absorption, namely around 244–245 nm (log ε 4.02) with a shoulder at *ca.* 233–234, 386–387 (4.23), and 513–514 (3.86). An aqueous solution of murexide also showed three main absorption bands around 248 nm (log ε 4.22), 324 (3.80), and 522 (4.02), and the longest wavelength band position was fairly close to that of the pigment, considering the large difference in substituents around the chromophoric system, suggesting the presence of a similar chromophore in the pigment as is found in murexide.

The proposed structure was further substantiated by examining the hydrolysis products of the pigment. Formation of SCA and DHA was positively confirmed by their behavior in tlc (Figure 5), and their yields were also determined as described in the Experimental Section. Though the result of DHA determination showed an unexpectedly high DKG content, the original form of DKG in the molecule was thought to be DHA because the conversion of DHA to DKG was known to be irreversible under the experimental conditions employed (Borsook *et al.*, 1937; Herbert *et al.*, 1933; Penney and Zilva, 1943). From the results of these qualitative and quantitative analyses, the chain structure and the structures with pyrazine or dihydropyrazine ring were completely denied, and the structure of the pigment was concluded to be as proposed above.

Since the chromatographic and spectroscopic properties of the pigment were essentially identical with those of the red pigment produced by the reaction of DHA with α-amino acid, both pigments were considered to be the same compound (Kurata *et al.*, 1973).

Formation Mechanism and Stability of the Pigment. When SCA was oxidized with *p*-quinone, formation of DHA was observed by tlc, as shown in Figure 5, and this suggested the occurrence of hydrolysis of dehydro-SCA, the oxidized form of SCA, during the oxidation reaction in aqueous solution. Although the same pigment was produced when SCA was treated with lead dioxide in anhydrous *N,N*-dimethylformamide or MeOH, DHA was not detected by tlc in these cases. So it may be deduced that the hydrolysis of dehydro-SCA is not an essential reaction for the coloration. Therefore, as shown in Figure 7, the pigment seems to be partly produced by the reaction of dehydro-SCA with SCA, as in the case of reaction, *trans*-alkylidenation, of imines with primary amines (Dayagi and Degani, 1970; Smith, 1965), though its formation from equimolar amounts of ammonia and the condensation product of SCA with DHA may be also possible, especially in an aqueous system.

The pigment was not so stable in aqueous solution, and the color of a highly diluted solution disappeared almost completely within 90 min when heated at a high temperature (Figure 6A); however, it was much more stabilized at a lower temperature, in 80% ethanol solution, or in the presence of an ammonium salt (Figure 6B). The coloration of the pigment was most stable at pH 5–6. On the other hand, the crystalline or amorphous solid pigment was quite stable under dry state even in the atmosphere, and the sample which had been kept at room temperature over silica gel for more than 1 year showed virtually no change in its chromatographic and spectroscopic properties.

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