Nonenzymatic Discoloration in Dried Cabbage. II. Red Condensation Product of Dehydroascorbic Acid and Glycine Ethyl Ester

The cherry red color formed initially in the browning of freeze-dried cabbage has been studied using a model system consisting of dehydroascorbic acid (DHA) and glycine ethyl ester which produces a chromogen of similar color characteristics. Elemental analysis, gel elution, and spectral studies indicated that the reaction takes place in equimolar proportions to give a dimeric, unsaturated condensation product, the probable structure of which is discussed. The chromogen is labile, hygroscopic, unstable in aqueous or acidic medium, and is light and heat sensitive.

In fruits, wherein the pH is generally below 4.0, browning is mainly due to the decomposition of ascorbic acid (AA) to furfural (Huelin, 1953; Huelin *et al.*, 1971). Since the pH of the vegetables is generally above 4.2, this pathway of browning is inoperative. In the previous paper (Ranganna and Setty, 1968), it was shown that the development of a red-brown color in freeze-dried and in an alcoholic extract of cabbage was a nonenzymatic reaction involving Strecker degradation of amino acids by the oxidation products of AA, *viz.* DHA or 2,3-diketogulonic acid (DKG). As efforts to isolate the light cherry red coloring matter formed initially in the freeze-dried cabbage were not successful, a model system producing the same color characteristics as the chromogen was investigated.

EXPERIMENTAL SECTION

Glycine Ethyl Ester-DHA Reaction Product (Red Chromogen). Repeated attempts to prepare DHA by the procedures of Kenyon and Munro (1948) and Pecherer (1951) using iodine in absolute methanol gave poor yields and impure products. The use of absolute ethanol overcame this difficulty. Thorough shaking of DHA with alcohol two or three times until the filtrate was colorless followed by washing with cold water and finally with alcohol produced the pure compound, mp 225–227° after drying.

Glycine ethyl ester hydrochloride (Marvel, 1961) was converted to the free base in alcoholic sodium hydroxide and distilled under vacuum at 40°. The distillate containing 20.69 g of glycine ethyl ester was placed in an amber colored bottle and cooled in an ice bath, and 17.4 g of DHA was added. The mixture was shaken and heated on a water bath at 40° protected from moisture by a guard tube. Heating was continued until most of the DHA had dissolved (15–20 min) and then filtered. The resultant solution was deep cherry red in color. The solution was cooled in an ice bath, shaken with activated charcoal (Merck) for 30 min, and filtered using kieselguhr. The filtrate was concentrated under vacuum at 40° and the red chromogen precipitated using peroxide-free ethyl ether. The resultant precipitate which was sticky and gelatinous was dried in a vacuum desiccator over calcium chloride. The yield was 25.1 g.

The red chromogen did not crystallize by classical procedures or even when 5,6-*O*-isopropylidene DHA was used in the synthesis, instead of DHA. Hence, purification was effected by dissolving the dried product in absolute ethanol, precipitating with ethyl ether, centrifuging, and finally drying the residue in a vacuum desiccator. After three or four such precipitations, the resulting precipitate from ethanol–ether solution was not sticky but granular. Finally, the dry powder was dissolved in absolute alcohol and concentrated under vacuum, when precipitation of the chromogen occurred as concentration progressed. The precipitate was dried in a vacuum desiccator over phosphorus pentoxide. The recovery was very low (15%).

The following observations regarding the synthesis and purification of the red chromogen would be relevant. In the absence of a solvent, reaction between DHA and glycine ethyl ester was exothermic and the color turned brown. Similar color changes were observed in the presence of alcohol when the reaction temperature was 60–70°. Hence, mild heating conditions had to be used. Microcrystalline DHA is very sparingly soluble in absolute alcohol. The unreacted DHA could be easily filtered off. Traces of DHA in solution were not precipitated by ether. An excess of glycine ethyl ester, if present in the reaction product, would either be adsorbed on activated carbon or remain soluble in ether.

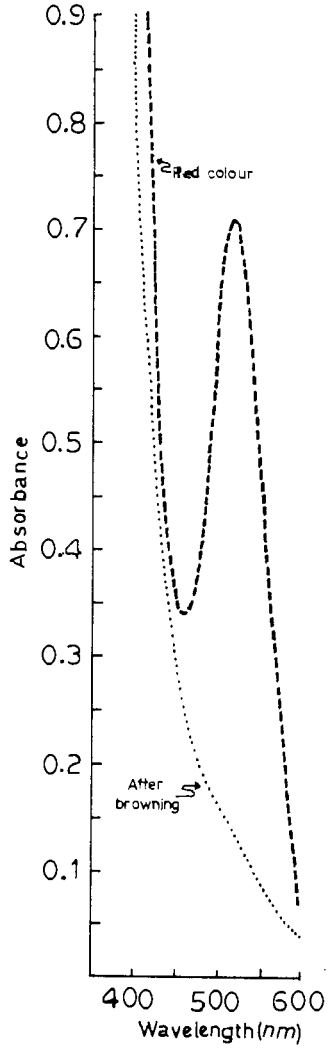


Figure 1. Absorption spectra of glycine ethyl ester–DHA reaction product.

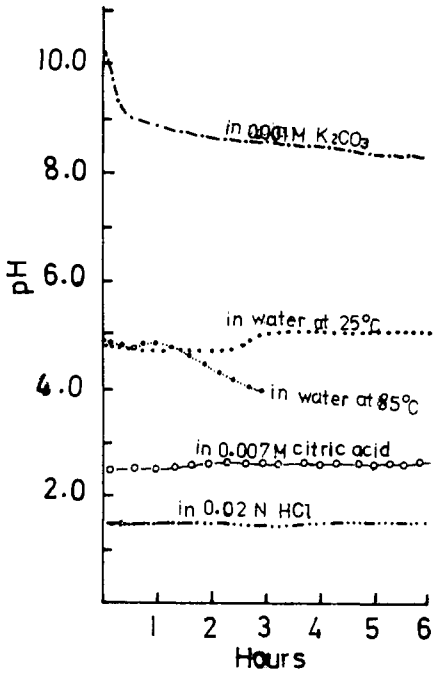


Figure 2. Changes in pH with added acid and alkali in aqueous medium.

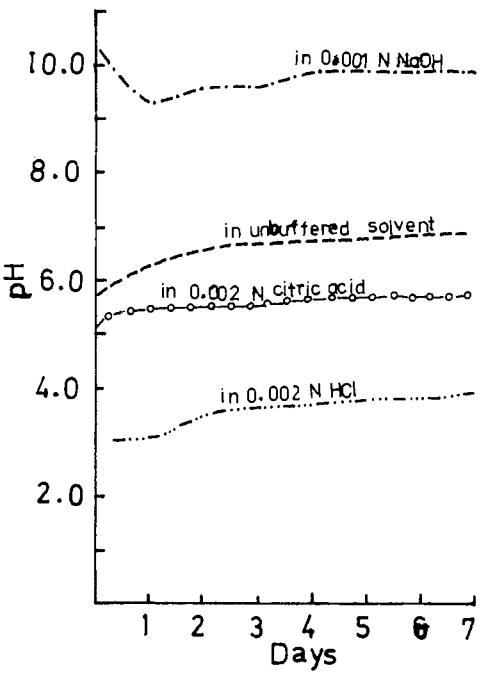


Figure 4. Changes in pH with added acid and alkali in alcoholic medium.

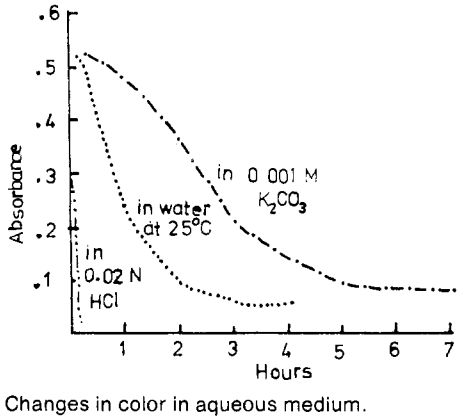


Figure 3. Changes in color in aqueous medium.

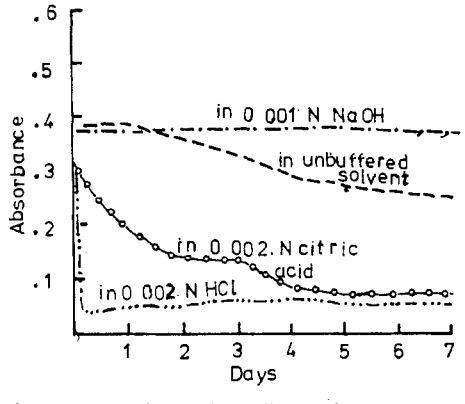


Figure 5. Changes in color in alcoholic medium.

The use of ethyl ether, though generally not favored for precipitation, was imperative as acetone, dioxane, ethyl acetate, petroleum ether, benzene, etc., were found unsuitable.

The reaction product was chromatographed on tlc plates using alcohol, alcohol and ethyl acetate (1:1) or *n*-butyl alcohol, water, and ethanol (20:5:1.3) as solvents and then exposed to iodine vapor or sprayed with 5% sulfuric acid in ethanol and dried at 110°. After repeated precipitation of the chromogen, chromatographic examination revealed a single spot thus ensuring the purity of the compound.

2,4-Dinitrophenylhydrazine (2,4-DNPH) Derivatives. The 2,4-DNPH derivatives of DHA and the chromogen were prepared according to the method of Shriner *et al.* (1966) and recrystallized twice using 1:1 alcohol and acetone (Jackel *et al.*, 1951). The recrystallized sample was further purified by silica gel column chromatography using a mixture of toluene and ethyl acetate (1:1) as eluent (Kurata and Sakurai, 1967) and examined by tlc (Clegg and Morton, 1965).

Gel Elution. Fifty milligrams of the red chromogen dissolved in 0.5 ml of water was placed on a Sephadex G-10 column (30 cm × 1.5 cm) and eluted using 60% ethanol (fraction volume, 3 ml; elution rate, 1.75 ml/min). The color from each fraction was measured in a Spectronic 20 colorimeter at 510 nm.

Measurement of Spectra. Visible and uv spectra were taken in a Beckman DK-2 recording spectrophotometer. Nmr spectra were recorded in a Varian HA-100 analytical model spectrometer. The effect of light on the chromogen in solution was measured in a Beckman DK-2 recording spectrophotometer at 515 nm.

RESULTS AND DISCUSSION

Along with DHA, when glycine was used in place of glycine ethyl ester for synthesis, purification of the reaction product by chromatographic procedures (paper, cellulose, modified cellulose, silica gel, or ion exchange) was not successful due to rapid conversion of the red color to brown; irreversible adsorption of the coloring matter to the column when ion exchange resin, modified cellulose, or silica gel was used; and instability of the red color when water or aqueous buffer was used for elution. For similar reasons, efforts to isolate the chromogen from freeze-dried cabbage were also not successful.

General Properties of the Red Chromogen. The chromogen was fine granular, dull red in color and highly hygroscopic; mp 98–100° (melts and decomposes with foaming). It was soluble in water, ethanol, methanol, and ace-

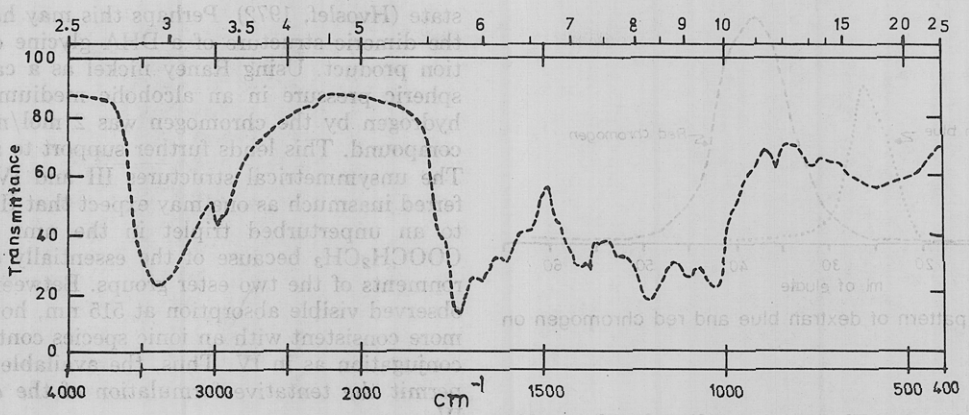


Figure 6. Ir spectrum of DHA–glycine ethyl ester reaction product (KBr pellet).

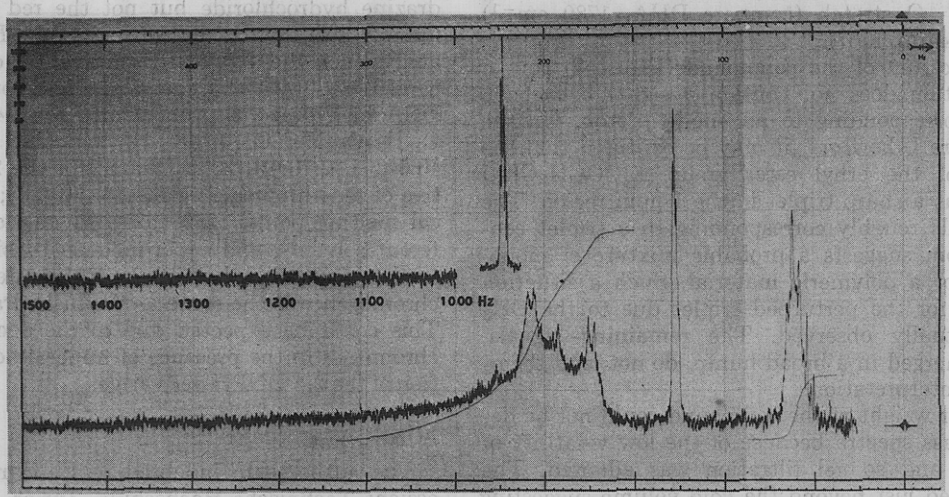


Figure 7. Nmr spectrum of DHA–glycine ethyl ester reaction product in Me_2SO-d_6.

tone but insoluble in nonpolar solvents. The solution in water was deep cherry red and tasted slightly bitter with a sour note. The absorption maximum was at 515 nm in the visible region (Figure 1) but there was no peak in the uv region. The color was photolabile. Absorbancy decreased by 0.1 unit at 515 nm in 60 min, while no decrease was observed in the sample kept in the dark. On browning, no peak was observed in the visible region (Figure 1). The absorption maximum in the visible region and the change observed on browning were similar to the color extracted from freeze-dried cabbage and from AA- or DHA–amino acid model systems (Ranganna and Setty, 1968).

Effect of Added Acid or Alkali on pH and Color. *(A) Aqueous Medium.* The pH of a 0.01 *M* solution (calculated on the basis of the molecular formula discussed later) was 4.8. On heating, the pH decreased rapidly (Figure 2) and the color was discharged (Figure 3). These changes were largely due to the breakdown of the chromogen into DHA and glycine ethyl ester as evidenced by a positive ninhydrin test and formation of a 2,4-DNPH derivative of DHA. Similar changes have been observed with *N*-D-glucosylglycine ethyl ester (Wolfrom *et al.*, 1949). On adding mineral acid, the color was discharged instantaneously (Figure 3), while with alkali the decrease was more gradual.

(B) Alcoholic Medium. The pH of a 0.02 *M* solution in alcohol was initially 5.8 which gradually increased during storage. Changes in pH in the presence of very dilute solutions of mineral (0.002 *N* HCl) or organic (citric, 0.002 *N*) acids were similar to those in the aqueous medium (Figure 4). In the alkaline medium (0.001 *N* NaOH), the pH gradually changed toward the acidic side. As compared to the aqueous medium, the color was more stable in the alcoholic medium, and preferably in neutral or alkaline pH (6.8 and above) than in the acidic pH (5.1 and below) (Figure 5). However, in the presence of a very low concentration of mineral or organic acid, the color faded very rapidly even in the alcoholic medium. The chromogen from the freeze-dried cabbage behaved similarly.

It has been shown above that the chromogen is unstable in the aqueous medium. In the dehydration of cabbage, AA–amino acid interaction occurred in the last stages of drying when the moisture content was relatively low (Ranganna and Setty, 1968). The alcoholic medium used in model systems simulated the low moisture conditions prevailing in the dried cabbage. A dry mixture of DHA, glycine, and Celite (1:3:6) left in a closed test tube for 10 days had developed light red color. This confirms our earlier observation that the AA–amino acid reaction resulting in the formation of red-brown color occurs in relatively low moisture conditions as in the case of the dehydrated vegetables.

Structure of the Red Chromogen. If the red chromogen, a condensation product of the reactants, is formed as a first step in the Strecker degradation (Schönberg and Moubacher, 1952), then structure I is probable. A structure wherein one of the ketonic carbonyls is enolized or hydrated in I cannot be ruled out. However, the elemental analysis does not support these. The ir spectrum (Figure 6) suggests that the product is either polymeric or a mixture of isomers. The broad band at 3200–3500 cm^{-1} may be assigned to the stretching frequency of the hydrogen bonded hydroxyl groups. The strong band at 1745 cm^{-1} may be ascribed to the C=O stretching frequency of an

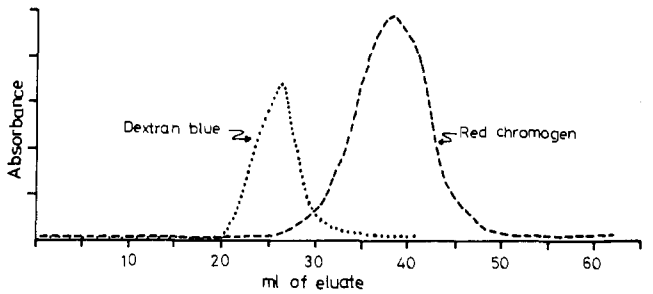
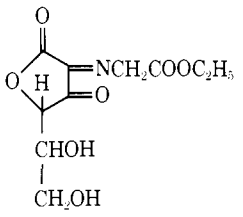


Figure 8. Elution pattern of dextran blue and red chromogen on Sephadex G-10.

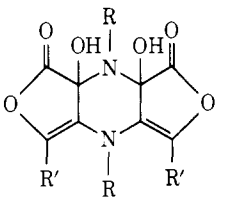
acyclic saturated ester. The corresponding band at 1230 cm^{-1} would suggest an acetate ester (C—O—C stretch; glycine moiety). The shoulder at 1780 cm^{-1} may be due to a lactone C=O stretch (compare DHA, 1780 cm^{-1}) (Kurata and Sakurai, 1967).

The nmr spectrum of the compound (Figure 7) run in Me_2SO-d_6 solution does not contain a signal between 6 and 15 ppm corresponding to an enolic proton. On the basis of structure I, however, it may be expected that the CH_3 protons of the ethyl ester group ($COOCH_2CH_3$) should appear as a sharp triplet in the 1-ppm region. The cluster of signals roughly corresponding to a triplet centered at 1.2 ppm suggests a probable mixture of monomeric isomers or a polymeric material. Such a situation would account for the perturbed triplet due to the CH_3 protons as actually observed. The remaining signals, somewhat submerged in a broad hump, do not lend themselves to ready interpretation.

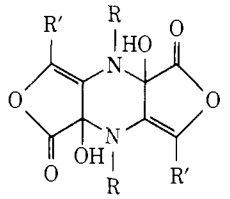
The molecular weight of the compound could not be determined by mass spectra because of the low volatility of the compound and so gel filtration was adopted. The chromogen eluted just beyond the void volume suggesting a molecular weight less than 700 (Figure 8). This would favor dimeric structures (II–IV) for the material rather



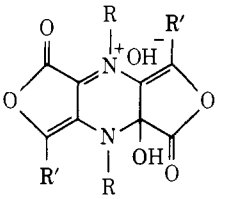
I



III



II



IV

Structure no.	*Anal.* calcd. % C	H	N
I, $C_{10}H_{13}NO_7$	46.33	5.02	5.41
II–IV, $(C_{10}H_{13}NO_7)_2 \cdot H_2O$	44.77	5.22	5.22
Found	44.06	5.24	5.34

than a polymeric structure. Based on crystallographic data, DHA is reported to be dimeric in the crystalline state (Hvoslef, 1972). Perhaps this may have a relation to the dimeric structure of a DHA-glycine ethyl ester reaction product. Using Raney nickel as a catalyst at atmospheric pressure in an alcoholic medium, the uptake of hydrogen by the chromogen was 2 mol/mol equiv of the compound. This lends further support to structures II–IV. The unsymmetrical structures III and IV are to be preferred inasmuch as one may expect that II should give rise to an unperturbed triplet in the nmr spectra for the $COOCH_2CH_3$ because of the essentially equivalent environments of the two ester groups. Between III and IV the observed visible absorption at 515 nm, however, would be more consistent with an ionic species containing extended conjugation as in IV. Thus, the available evidence would permit the tentative formulation of the compound as in IV.

Derivatives of DHA and Chromogen. DHA formed a derivative with semicarbazide hydrochloride or phenylhydrazine hydrochloride but not the red chromogen. DHA formed a bishydrazone with 2,4-DNPH. The elemental analysis of the 2,4-DNPH derivative of the chromogen agreed well with the calculated and found values of the DHA bishydrazone (*Anal.* Calcd for 2,4-DNPH of DHA, $C_{18}H_{14}O_{12}N_8$: C, 40.46; H, 2.64; N, 20.97. Found: C, 40.64; H, 2.76; N, 20.85. Found for the 2,4-DNPH derivative of red chromogen: C, 40.14; H, 2.68; N, 20.92). Identical melting points (289–292°), R_f on tlc plates using different solvents, and superimposable visible and ir spectra further confirmed that the 2,4-DNPH derivative of the red chromogen was the same as the bishydrazone of the DHA. This could be expected due to the decomposition of the chromogen in the presence of mineral acid in the preparation of the 2,4-DNPH derivative.

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