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Oxygen Involvement in Betanine Degradation— Measurement of Active Oxygen Species and Oxidation Reduction Potentials

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ABSTRACT

The possible involvement of active oxygen species in the degradation of betanine was investigated using a variety of chemical and enzymatic tests. Neither singlet oxygen nor superoxide anion were implicated in the oxidative degradation of betanine. Betanine was destroyed in solutions containing photosensitized riboflavin. Oxidation–reduction (redox) potentials were measured in solutions containing betanine or its aglycone, betanidin. The lower potential of betanidin, relative to betanine, coincided with a greater susceptibility of the aglycone to destruction by molecular oxygen.

INTRODUCTION

The use of betalaine pigments as food colorants has been suggested but extensive use has been limited because of their instability (von Elbe, 1977). The presence of oxygen has been a major factor in accelerating betanine (major betalaine pigment in the red table beet) degradation (Attoe & von Elbe, 1984). The rapid destruction of betanine in the presence of reducing or oxidizing agents, including molecular oxygen, suggests that either active oxygen species or an oxidation–reduction mechanism is involved in the degradation. First-order reaction kinetics of betanine in the presence of a molar excess of oxygen have been described (Pasch & von Elbe, 1979;

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Attoe & von Elbe, 1982). In these studies, an induction period in the oxidation rate, typical of free radical chain autoxidations, was not observed. Also, the rate of pigment loss did not appear to be affected by the extent of oxidative degradation in the betanine samples. These observations indicate a direct electrochemical interaction between betacyanines and oxygen, rather than the slower peroxidative reaction found in lipid oxidation.

During the 1970s, research on the reactions of organic materials with atmospheric oxygen focused increasingly on the role of activated oxygen species. Improved methods of analysis, including electron spin resonance spectroscopy and free radical trapping procedures, have allowed detection of free radical species in many reaction systems. Comprehensive reviews have been published on the reactions of free radicals in biological materials (Pryor, 1976), and on active species in food constituents (Korychka-Dahl & Richardson, 1978*a*).

Oxidation–reduction (redox) potentials are a measure of the chemical energy available to transfer electrons from reactants with a lower potential to reactants with a higher potential. Clark (1960) reviewed the more significant findings for organic redox systems. Most redox potentials of organic compounds are associated with phenolic, amino and sulfhydral functional groups. Betacyanine molecules have both amino and phenolic functional groups, and therefore could be expected to possess redox potentials. The objectives of this study were: (1) to explore the possible involvement of active oxygen species in the oxidation reaction, and (2) determine whether betanine has a redox potential that can be measured and how this potential is affected by pigment concentration and solution pH value. The presence of a redox potential would suggest direct molecular oxygen interaction with the pigment.

MATERIALS AND METHODS

Sample preparation

Betanine was purified from beet juice as described previously (Attoe & von Elbe, 1981). Betanidin, the aglycone of betanine, was prepared from purified betanine by enzyme treatment with β-glucosidase (Worthington Biochemical Corp., Freehold, NJ). The reaction mixture, after holding at 25 °C for 4 h, was acidified with acetic acid to a pH value of 2·0. Betanidin

was separated by gel filtration (Bio-Gel P-2 Bio Rad Laboratories, Richmond, CA) using 1% acetic acid solution in water as the eluant. Pigment was dissolved in a 0·1 M phosphate/acetate buffer at the desired pH. Unless otherwise stated, pigment concentrations were between 0·02 and 0·03 mM. Betanine solutions were placed in 2 ml vials and the vials were sealed with rubber septa. When light exposure was needed, sealed 3·5 m quartz cuvettes were irradiated with fluorescent light at an intensity of 100 foot-candles. Duplicate samples were used for each condition unless a larger number of replicates is specified. Retained pigment was quantified using high performance liquid chromatography (HPLC) as described by Schwartz & von Elbe (1980).

Analysis for singlet oxygen

The amount of betanine remaining after light exposure for 148 h at 25 °C was quantified for solutions with and without singlet oxygen quenchers. The singlet oxygen quenchers used were: water dispersible β-carotene (Hoffman-LaRoche), sodium azide (Sigma Chemical), 1,4-diazabicyclo-[2,2,2]-octane (DABCO) (Aldrich Chemical Co) and histidine monohydrochloride (General Biochemicals, Inc.). A sufficient amount of each quencher was added to a buffered betanine solution to produce a 40 mM concentration.

Analysis for superoxide anion

Betanine stability was measured at 36 °C and pH 7·0 in the presence and absence of 50 units/ml of superoxide dismutase (SOD) (Sigma Chemical Co.). Quadruplicate samples were analyzed after 22 h of degradation.

A quantitative assay for superoxide anion was made, based on the reduction of nitroblue tetrazolium chloride (NBT) (Sigma Chemical Co.) to diformazan. The diformazan was then dissolved in *N*,*N*-dimethyl formamide (DMF) (Eastman Organic Chemical) for spectrophotometric analysis at 560 nm. Betanine samples (284 μM) were adjusted to pH 7·0 and were illuminated for 22 h in the presence of NBT prior to spectrophotometric analysis. The nitroblue tetrazolium assay procedure has been described by Korycka-Dahl & Richardson (1978*b*).

The presence of superoxide anion O_2^- was further tested by a procedure based on the observation by Misra & Fridovich (1972*a*,*b*) that superoxide anion will cause the oxidation of epinephrine to adrenochrome under

alkaline conditions. Betanine and epinephrine were at concentrations of 0·31 mM and 0·64 mM, respectively, in a borax/sodium hydroxide buffer at pH 9·0 and 25 °C. After 20 h, adrenochrome formation was measured by light absorbance at 480 nm. Samples containing betanine or epinephrine (Aldrich Chemical Co.) alone were analyzed for comparison.

Analysis for peroxides

The effects of hydrogen peroxide, catalase, horseradish peroxidase and ferrous sulfate, a nonenzymatic peroxide decomposer, on betanine stability in solution were determined at 40 °C. Concentrations of 1 % and 10 ppm were used for H_2O_2 and Fe^{2+}, respectively. Approximately 30 000 units of catalase activity or 500 units of peroxidase activity were used for each milliliter of betanine solution.

A peroxide value determination was attempted on degraded betanine in solution. Betanine (0·48 mM) was degraded for 18 h in the presence of oxygen at 60 °C and pH 7·0. Ten milliliters of acetic acid were added to 16 ml of the pigment solution along with 1 ml of a gelatinized aqueous starch dispersion. This solution was thoroughly flushed with nitrogen in a sealed flask. A saturated potassium iodide solution (0·1 ml) was then injected into the flask. Free iodine, if present, was titrated with 0·0172 N sodium thiosulphate. The quantity of sodium thiosulphate was measured by using a calibrated microlitre syringe. Known amounts of H_2O_2 were added to degraded betanine samples to serve as positive controls.

A further analysis was made for H_2O_2 by using HPLC. The HPLC conditions were similar to those used for betanine analysis, except that light absorbance was measured at 254 nm instead of 546 nm. Betanine (0·33 mM) was allowed to partially degrade at room temperature for 5 days in the presence of oxygen. A 25 μl sample of the pigment solution was then chromatographed, causing H_2O_2, if present, to elute prior to betanine and its major degradation products. This procedure would allow the detection of H_2O_2 in highly colored solutions, so that complete thermal degradation of betanine is not needed. It was thought that H_2O_2 would be more stable, and therefore more likely to accumulate, at lower temperatures. As low as 2 ppm H_2O_2 could be detected when added to pigment solutions.

Analysis for hydroxyl radical

The amount of betanine remaining in solution was measured after degradation in the presence and absence of compounds known to readily

react with the hydroxyl radical (HO·). Levels of 1 % mannitol, ethanol or isopropanol were added to betanine solutions at pH 5·0 and solutions were held for 23 h at 40 °C. As positive controls, the HO· scavenger activity of these compounds was monitored by measuring betanine loss in solutions to which 10 ppm H_2O_2 and 10 ppm Fe^{2+} had been added to generate HO·. In these solutions, pigment retention was determined 3 min after the addition of H_2O_2 and Fe^{2+}. Five replicate samples were used for each condition.

Photosensitized destruction of betanine by riboflavin

A 0·1 mM concentration of riboflavin was dispersed in betanine solutions at pH 5·0; control solutions contained betanine alone. Either 0·1 mM water-dispersible β-carotene, 0·01 % butylated hydroxyanisole (BHA, National Biochemical Corp.) or 100 units/ml of superoxide dismutase was added to betanine solutions containing riboflavin to try to determine the mechanism of the photosensitized destruction of betanine. BHA was first dissolved in ethanol to aid in aqueous dispersion. Solutions were held in the dark for 1 h at 25 °C to determine any immediate effects that the additive might have on betanine stability. Samples were then placed in 1 cm quartz cells and exposed to a 100 foot-candle intensity of fluorescent light for a 4 h period. Some cells were flushed with nitrogen after sealing to determine whether oxygen was involved in the reaction.

Redox potential measurements

Redox potential measurements were made with a platinum indicating electrode and a calomel reference electrode. The electrodes were introduced through two of the openings of a 50 ml 3-neck flask, and the space around each electrode was sealed with Parafilm. The third opening held a glass thermometer and a pipette. The latter was used to introduce high purity nitrogen into the reaction solution. This center opening was loosely sealed to allow the escape of nitrogen gas, which was passed through the solutions at approximately 300 ml/min. A 40 ml volume of a 0·1 M phosphate/acetate buffer was placed inside the flask and maintained at 25 °C with an external heater. After oxygen was removed from the system by continuous nitrogen flushing, a known concentration of pigment was introduced for analysis. Ascorbic acid solutions were used for comparison.

Millivolt readings were indicated with a Model 601 digital ionanalyzer

(Orion Research, Cambridge, MA). The calomel reference electrode was calibrated against a standard hydrogen electrode. The value of +220 mV, determined for the calomel half-cell, was added to the observed potentials to obtain the potentials of the test solutions relative to the hydrogen half-cell at the pH value under study. A slope of −0·059 V per pH unit was assumed for both the hydrogen and calomel half-cells, so that the difference between these half-cells was considered independent of hydrogen ion concentration.

The response of the platinum electrode was sluggish in dilute solutions and required up to 2 h to establish a stable potential reading. A stable potential was arbitrarily defined as a change of less than 1 mV in a 5 min period. When duplicate samples were analyzed, the potentials differed by less than 5 mV.

RESULTS AND DISCUSSION

Analysis for singlet oxygen

Involvement of singlet oxygen in oxidation reactions can often be presumed when the reactions are inhibited in the presence of compounds that quench this species. Data showing the effect of these quenchers on the stability of betanine in solutions exposed to fluorescent light at 25 °C are presented in Table 1. After 148 h, betanine samples containing quenchers

TABLE 1
Effect of Singlet Oxygen Quenchers on Betanine Stability in Solution at 25 °C, pH 5·0, when Exposed to 100 Foot-candles of Fluorescent Light for 148 h[a]

Treatment	*% Betanine remaining*		
	Sample A	*Sample B*	*Sample C*
Betanine alone	38	39	39
Betanine + azide	26	28	27
Betanine + histidine	17	21	19
Betanine + β-carotene	36	42	39
Betanine + DABCO	0	0	0

[a] Betanine samples not exposed to light averaged 45% of pigment retained.

indicated no greater betanine stability compared with control samples. All quenchers either had no effect or had a negative impact on betanine stability. DABCO completely decolorized betanine solutions when used at a 1 % level. The observation that β-carotene had little effect on betanine stability during light exposure suggests that $^1O_2$ does not play a major part in betanine degradation under the conditions of this study. It is therefore unlikely that betanine is promoting its own destruction by the photosensitized generation of $^1O_2$. Other oxidative mechanisms must be responsible for the observed loss of betanine.

Analysis for superoxide anion

Superoxide dismutase is an enzyme that effectively inactivates superoxide anions and has been suggested to play an important role in protecting living cells from this radical. If O_2^- plays a significant part in betanine oxidation, the addition of SOD would be expected to stabilize the pigment. The effect of this enzyme on the rate of the oxidative degradation of betanine was studied at 36 °C and pH 7·0. Four betanine samples containing SOD averaged 36 % of pigment retained after 22 h, while four samples without SOD averaged 39 % retention. There was no statistically significant difference between the two treatments, indicating that O_2^- probably does not contribute to betanine degradation under these conditions.

To further test for the presence of O_2^-, the NBT reduction assay was used. In this assay, difficulty was encountered in that betanine appears to form a precipitate with NBT that is independent of the presence of O_2^-. This precipitate, when dissolved in DMF for spectrophotometric analysis, has a light absorption maximum in the vicinity of 520 nm. This wavelength of maximum light absorption is well below the 560 nm maximum produced when O_2^- reduces NBT to diformazan. Since this complex does not appear to require O_2^- for its formation, this assay cannot be used to quantify O_2^- in solutions containing betanine.

A third assay for O_2^- in oxidizing betanine solutions was based on the oxidation of epinephrine to adrenochrome by O_2^-. Samples containing both betanine and epinephrine should have a greater light absorption at 480 nm than the sum of the absorption by betanine and epinephrine alone, if betanine were producing O_2^-. Experimental samples showed no increase in light absorption at 480 nm, indicating that no substantial amounts of O_2^- were generated during the 20 h reaction period.

Formation of O_2^- has been demonstrated during the autoxidation of reduced flavins and quinones (Misra & Fridovich, 1972*b*), 6-hydroxydopamine (Heikkila & Cohen, 1973), thiols (Misra, 1974) and tetrahydropteridines (Nishikimi, 1975). Like betanine, all of these compounds are easily oxidized by oxygen in aqueous solutions. It is therefore reasonable to expect that O_2^- might form during betanine oxidation. In the present investigation, the presence of O_2^- could not be confirmed. Low yields of the radical and its known rapid dismutation at the lower pH values used in this study may have prevented its detection (McClune & Fee, 1976). Most studies that have detected O_2^- during oxidation of organic substrates have been conducted under alkaline conditions (pH > 9·0). These high pH values are impractical for studies of betanine oxidation because the pigment rapidly decomposes (Peterson & Joslyn, 1960).

Analysis for peroxides

Oxidation of organic materials often results in the formation of peroxides. Betanine could be involved in either a transfer of two electrons to O_2, resulting in H_2O_2, or the addition of O_2 to the betanine molecule, forming an organic peroxide.

Effects of H_2O_2, Fe^{2+}, catalase, and horseradish peroxidase on betanine stability at pH 5·0 and 40 °C are indicated in Table 2. The percentage of pigment remaining in solution was reduced from 63 to 31 by the presence of 1 % H_2O_2. However, the addition of only 10 ppm ferrous ion to solutions containing both betanine and H_2O_2 caused a complete decoloration of the pigment. Increased reactivity of H_2O_2 in the presence of Fe^{2+} can be explained by the production of highly reactive $HO^{\cdot}$ by the Fenton reaction. If peroxides were formed during betanine oxidation, this mechanism could explain the effect of certain metal ions in accelerating the loss of betanine. The destruction of these peroxides would be expected to influence the rate of betanine degradation.

Neither peroxidase nor catalase had a significant effect on betanine stability during the 14·5 h reaction period. Peroxidase enzymes have a hemeprosthetic group that catalyzes the destruction of H_2O_2 and organic peroxides. In these reactions, electron transfers may produce free radicals (Caughey, 1979). The radicals can then accelerate oxidation reactions. Catalase is an enzyme specific for H_2O_2 and causes a decomposition to O_2 and H_2O. Since these enzymes had little effect on betanine stability it

is likely that peroxides do not play a major role in the degradation mechanism. But their presence should not be discounted entirely. When catalase and Fe^{2+} were both added to betanine solutions, betanine was more stable than in the presence of Fe^{2+} alone. This may indicate that low levels of peroxide present in oxidizing betanine solutions can be decomposed to HO· unless they are first destroyed by catalase. Further evidence that trace levels of peroxide may be present was obtained by observing the effect of catalase on betanine stability over a longer

TABLE 2

Effects of Hydrogen Peroxide,[a] Ferrous Ion,[b] Catalase[c] and Peroxidase[d] on Betanine Stability during 14·5 h Storage at 40 °C, pH 5·0

Sample	*% Betanine remaining ± SD*[e]
Betanine	63 ± 1
Betanine + H_2O_2	31 ± 2
Betanine + Fe^{2+}	49 ± 0
Betanine + H_2O_2 + Fe^{2+}	0 ± 0
Betanine + peroxidase	65 ± 3
Betanine + catalase	64 ± 1
Betanine + Fe^{2+} + catalase	55 ± 2

[a] H_2O_2 concentration = 1 %.
[b] Fe^{2+} concentration = 10 ppm.
[c] Catalase concentration = 30 000 units/ml.
[d] Peroxidase concentration = 500 units/ml.
[e] SD based on triplicate samples.

degradation period. Using the same conditions (40 °C, pH 5·0), after 27 h the average pigment content remaining in the control samples was 42 %, while the pigment content in samples containing catalase averaged 47 %. This difference was significant at the 5 % level.

Iodine determination of peroxides in a degraded betanine solution showed no detectable level of peroxide. Similarly, the HPLC method to detect H_2O_2 in solutions of partially degraded betanine indicated that no measurable amount of H_2O_2 was present. Either method would have been expected to show positive results if a greater than 10 % molar formation of peroxide from pigments had occurred. Peroxide yields may have been too low or peroxide decomposition too rapid for measurable quantities to accumulate in these reaction systems.

Analysis for hydroxyl radical

Effects of 1 % concentrations of mannitol, ethanol and isopropanol on betanine stability in solution are shown in Table 3. Betanine loss occurred by either spontaneous degradation at 40 °C for 23 h or by HO· produced by the presence of 10 ppm H_2O_2 and 10 ppm Fe^{2+} (Fenton's reagent). Fenton's reagent was used to generate HO· to demonstrate the effectiveness of HO· scavengers in stabilizing betanine when HO· was known to be present.

Mannitol, which has often been used as a scavenger for HO·, increased betanine stability both under spontaneous degradation conditions and in the presence of induced HO·. Ethanol and isopropanol, which have also been shown to have relatively high reactivities with HO· (Walling, 1975), stabilized betanine in the presence of induced HO· but had little or no effect during spontaneous degradation. The stabilizing effect of mannitol on betanine, during spontaneous degradation, provided presumptive evidence that HO could be involved in betanine degradation. However, the inactivity of the other two alcohols in this system, together with their demonstrated effectiveness in the presence of induced HO·, tend to discount the involvement of HO· in the spontaneous degradation of betanine. There is a need for further study to clarify the mechanism by which mannitol stabilizes betanine in solution.

TABLE 3

Effect of 1 % Concentrations of Hydroxyl Radical Scavengers on the Stability of Betanine in Solution when Betanine Degraded Spontaneously at 40 °C, pH 5·0, for 23 h in Oxygen-saturated Solutions, or in the Presence of HO· Induced by $H_2O_2 + Fe^{2+}$ (Fenton's reagent)

Sample	*% Betanine remaining ± SD*[a]	
	Spontaneous degradation	*Induced HO·*[b]
Betanine	37·3 ± 1·3	80 ± 1·1
Betanine + mannitol	42·4 ± 1·6	93·5 ± 1·6
Betanine + ethanol	36·7 ± 1·3	84·8 ± 1·0
Betanine + isopropanol	36·4 ± 1·4	86·5 ± 1·9

[a] Based on five replicate samples.

[b] Pigment quantified 3 min after the addition of 10 ppm H_2O_2 and Fe^{2+}.

Photosensitized destruction of betanine by riboflavin

Riboflavin is known to cause formation of active radicals when in solution and exposed to light. Rapid decolorization of betanine was found to occur when betanine solutions containing riboflavin were exposed to fluorescent light. The mechanisms that could be involved in this reaction include: a photosensitized production of singlet oxygen by riboflavin (Foote, 1976), a photosensitized generation of superoxide anion by riboflavin (Ballou *et al.*, 1969; Korycka-Dahl & Richardson, 1978*b*), or the reaction of photoreduced riboflavin with molecular oxygen to generate reactive oxygen adducts (Mager & Berends, 1966; Muller *et al.*, 1975). To see which mechanism was involved in betanine degradation, specific inhibitors were added to the solutions containing both riboflavin and betanine.

Involvement of singlet oxygen was tested by the addition of 0·1 mM water-dispersible β-carotene to the reaction solutions. β-carotene did not have a consistent stabilizing effect on betanine in the presence of photosensitized riboflavin. This indicates that the primary mode of betanine degradation in these systems probably does not involve singlet oxygen. Similarly, superoxide anion was discounted as the major participant in the photosensitized degradation of betanine because the addition of 100 units/ml of superoxide dismutase failed to have a notable impact on pigment stability.

As indicated by the data in Fig. 1, BHA effectively slowed the loss of betanine in solutions containing photosensitized riboflavin. Riboflavin did not cause any pigment degradation in solutions without exposure to light and no appreciable betanine loss occurred when betanine solutions without riboflavin were exposed to the 100 foot-candle intensity of fluorescent light during the 3 h reaction period at 25 °C. Since BHA acts to inhibit free radical oxidation reactions, the increased betanine stability in its presence suggests that riboflavin is generating free radicals by a Type I photosensitized reaction (Foote, 1976). By this mechanism, light exposure causes riboflavin to generate free radicals by a process that does not involve singlet oxygen.

When the effect of riboflavin was observed using nitrogen-flushed solutions, it was found that photosensitized riboflavin had a greatly reduced influence on betanine stability. This evidence seems to contradict a Type I reaction because of a requirement for oxygen. The requirement for molecular oxygen can be explained by the formation of flavin–oxygen adducts by reduced flavins. Merkel & Nickerson (1954) have shown that

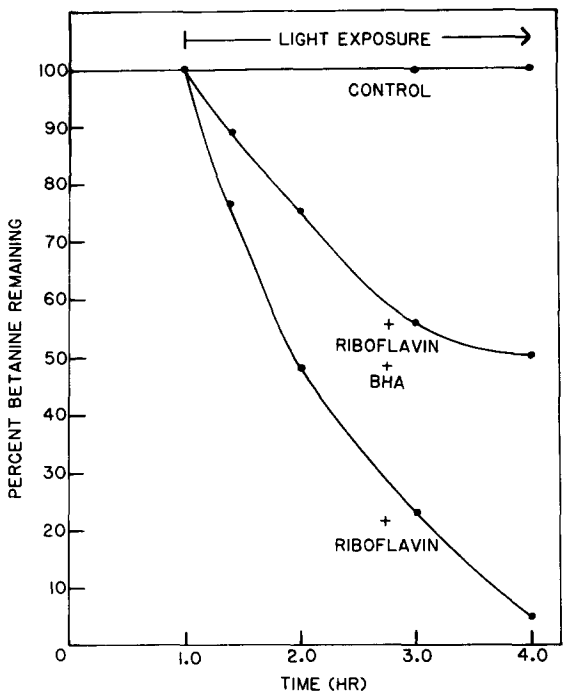

Fig. 1. Betanine degradation by photosensitized riboflavin at 25 °C, pH 5·0, in the presence and absence of 0·1 mM BHA.

light absorption causes a photoreduction of the riboflavin molecule. Reduced flavins spontaneously react with triplet oxygen to form reactive oxygen adducts (Mager & Berends, 1966; Muller *et al.*, 1975). The oxygen adducts can then give rise to free radicals by a variety of mechanisms. Since the reaction proceeds under oxygen, and at a lower rate when oxygen is limited, this suggests that it is photo-excited riboflavin which oxidizes the betanine, in a one or two electron step.

Electrochemical potential of betanine

The presence of a redox potential would suggest direct molecular oxygen interaction with the pigment.

Electrochemical potentials are expressed as standard reduction potentials ($E°$) when all reactants are at 1 M concentrations, at one atmosphere of pressure, and at 25 °C. According to the Nernst equation:

$$E = E° + \frac{RT}{nF} \ln \frac{\text{oxidant}}{\text{reductant}}$$

The observed potential is equal to the standard potential ($E = E°$) when the concentrations of oxidant and reductant are equal (i.e. ln (oxidant/reductant = ln 1 = 0). The resulting redox potential is known as the midpoint potential, because it is measured at the midpoint of an electrochemical reaction. Many redox reactions of organic compounds are not completely reversible, and therefore do not reach a definite equilibrium point. Betacyanine oxidation appears to be of this type. It was also noted that a low redox potential remained in betanine solutions after the color had degraded completely, indicating that the degradation products still had a reducing potential. Because of these factors, the midpoint concept could not be strictly applied to these pigments. Therefore, redox measurements were made with the pigment existing in its native reduced form, rather than a 50% oxidized and 50% reduced mixture. Several studies that were reviewed by Clark (1960) indicate that when redox substances are present entirely in their reduced form, their measured potentials are less well-poised and approximately 50 mV lower than the corresponding midpoint potentials.

Other difficulties encountered in measuring the potential of organic redox systems involve substrate concentration. The 1 M concentration of standard redox potentials may be impossible to attain because of low solubility or a lack of availability of the material. For these reasons, the redox potentials of organic compounds are often measured under nonstandard conditions. Complications can result from residual oxygen and a sluggish electrode response in solutions of dilute organic substrate. Such solutions are often poorly poised, and therefore small amounts of oxygen can have a major impact on the measured potential. This problem was described by Michaelis & Flexner (1928), while measuring the redox potentials of canned foods (Montville & Conway, 1982).

A final problem that may be encountered when measuring the redox potential of organic substances is a nonlinear relationship between the pH value and the measured redox potential. This situation appears to be associated with ionizable groups that dissociate within the pH range being observed. Such a nonlinear effect of pH on observed potential was reported for ascorbic acid (Ball, 1937).

Effect of pigment concentration

Figure 2 shows the measured redox potentials of different betanine and betanidin concentrations in solution at pH 5·0. The redox potentials for ascorbic acid were included for comparison. Since it was impossible to use

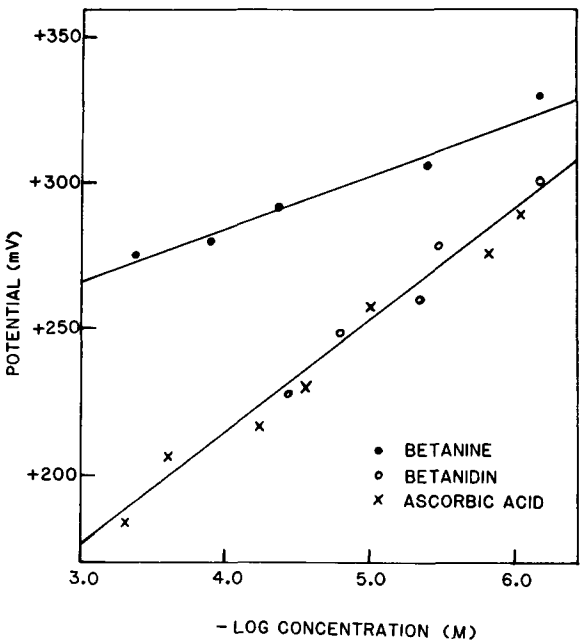

Fig. 2. Effect of concentration on the redox potentials of betanine, betanidin and ascorbic acid at 25 °C, pH 5·0. Potentials are relative to the hydrogen electrode at this pH value.

1 M concentrations of the pigments, a range of lower concentrations was used. It can be seen that the redox potentials vary with the logarithm of the concentration of the substance being measured. Higher concentrations resulted in lower redox potentials because redox compounds in their reduced forms act as reducing agents. Such a change in potential as a function of the logarithm of the concentration would be expected, based on the dependence of chemical potential on the activity of a substance.

$$\mu = \mu^\circ + RT \ln a$$

where: μ is the chemical potential of a component, μ° is the chemical potential of the component in its standard state, R is the ideal gas constant, T is the absolute temperature, a is activity.

In this equation, the chemical potential changes from its standard state value (1 M) according to the natural log of the activity of a substance. Molarity approximates activity in this experiment because of the dilute solutions used. Such a variation of the measured electrochemical

potential with the logarithm of the concentration of an organic compound has been reported in early redox potential studies (Dixon & Quastel, 1923; Michaelis & Flexner, 1928).

Effect of pigment structure

The regression line for the effect of betanidin concentration on the measured redox potential coincides with the data points obtained for reference ascorbic acid solutions. Apparently, the main electrochemical functional group of betanidin has a potential similar to the enediol group of ascorbic acid. Since the electrochemical potential of betanine differs from that of betanidin it can be assumed that the *o*-phenol of betanidin is responsible for this potential. Betanine has a glucose moiety linked at the C-5 position, resulting in a monophenol with a weaker reducing potential. At any given concentration, the redox potential of the aglycone is lower than that of the glycoside. This difference increases as the concentrations increase. By analogy, a comparison of the redox potentials of phenol and catechol (Frieser, 1930) showed that the *o*-phenol had a potential about 360 mV lower than the monophenol at an unspecified concentration.

The lower redox potential of betanidin in solution is reflected by its greater reactivity with oxygen, when compared to betanine in solution. Table 4 lists half-life values that were calculated for the degradation of betanine, betanidin, and their C-15 epimers, isobetanine and isobetanidin. Pigment solutions were either oxygen-saturated or nitrogen-flushed (Attoe & von Elbe, 1982). Little or no difference in stability was apparent between isomers. However, the half-life values of the glucosides were

TABLE 4
Half-life Values for Betacyanines at 40 °C, pH 5·0, in Oxygen-saturated and Nitrogen-flushed Solutions

Betacyanine	*Half-life value* ($h \pm SD$)[a]	
	O_2-saturated	*N_2-flushed*
Betanine	21 ± 2	82 ± 6
Isobetanine	19 ± 2	85 ± 8
Betanidin	$1{\cdot}2 \pm 0{\cdot}1$	26 ± 3
Isobetanidin	$1{\cdot}1 \pm 0{\cdot}1$	29 ± 4

[a] Based on triplicate samples.

approximately 17 times greater than those of the aglycones in the presence of oxygen. With the removal of oxygen, all of the betacyanine half-life values were increased several fold.

Effect of pH value

Figure 3 shows the effect of hydrogen ion concentration (pH) on the redox potential of betanine solutions. For comparison, literature values for the redox potentials of the standard hydrogen electrode and for the reduction of O_2 to H_2O are also shown. The values obtained for betanine were measured at a concentration of 0·012 mM. Using the concentration relationship indicated by Fig. 2, and extrapolating the redox potentials of betanine solutions to the theoretical standard concentration (1 M), would result in values approximately 100 mV lower than those reported in Fig. 3.

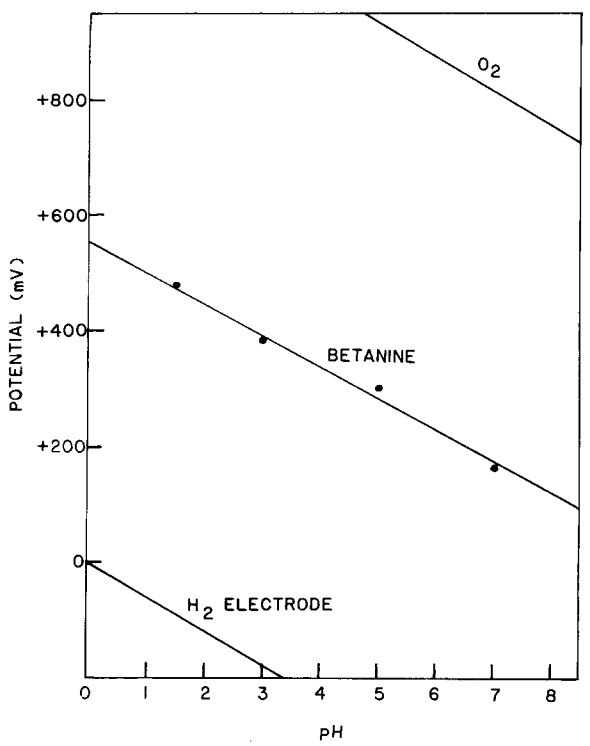

Fig. 3. Effect of pH on the redox potential of betanine.

From the equation

$$E = E^\circ + \frac{RT}{F} \ln [H^+] = E^\circ - 0{\cdot}059\,\text{pH}$$

where: E is the electrochemical potential, E° is the standard electrochemical potential, R is the ideal gas constant, T is the absolute temperature, F is the Faraday constant, $[H^+]$ is hydrogen ion concentration, it would be predicted that the redox potential of a compound would vary with the pH value and that the slope of the line formed by plotting pH versus E would be $-0{\cdot}059$ at 25 °C. The regression line shown in Fig. 3 was calculated to be $-0{\cdot}055$ for the effect of pH value on the measured redox potential of betanine. This value is in close agreement with the expected value and the difference is within experimental error.

CONCLUSION

Involvement of active oxygen species in the degradation of betanine could not be proven with great certainty. No active species accumulated to levels that could be clearly detected.

The small effects of catalase and mannitol in inhibiting the rate of betanine degradation provided tentative evidence that low levels of H_2O_2 and $HO^{\cdot}$ could be present. It is likely that none of the active species are required for betanine to oxidize in the presence of molecular oxygen, but the addition of these species to betanine solutions accelerated betanine degradation. This was especially true for hydroxyl radical, generated by Fenton's reagent, and for the photosensitized oxidation of betanine in the presence of riboflavin.

It can be concluded that the betacyanines have redox potentials that vary with the logarithm of pigment concentration and with the logarithm of hydrogen ion concentration, according to the principles of electrochemistry. Since a difference in electrochemical potential allows electrons to move, the existence of such potentials shows that betacyanines have a susceptibility to lose electrons to oxidizing agents such as molecular oxygen. Betanidin, compared with betanine, has a lower redox potential and a greater reactivity with molecular oxygen.

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