Degradation of Transferrin in the Presence of Ascorbic Acid and Oxygen

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The liberation of ammonia in the reaction of transferrin and ascorbic acid in phosphate buffer at pH 7.4, 37°C was investigated.

It was found that transferrin is degraded in the reaction into smaller molecular weight polypeptides. The ammonia evolution was inhibited by catalase and by hydroxyl free radical trapping agents such as benzene, benzoate, and iodide. Dehydroascorbic acid and hydrogen peroxide in the presence of traces of ferric or cupric ions were also studied.

It is suggested that the ammonia evolution observed results from deamination of amino acids and polypeptide fragments as a result of the formation of reactive hydroxyl free radicals in the reaction mixture.

It has been reported that ascorbic acid under physiological conditions accelerates the degradation of transferrin and the pentapeptide GlyArgAsnArgGly. It was suggested that this degradation resulted from deamidation of glutaminyl and asparaginyl residues (Robinson et al., 1973). These observations are interesting since Robinson and coworkers have hypothesized that non-enzymatic sequence-controlled deamidation of amide residues in peptides and proteins is an important biological timing mechanism.

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that may control protein turnover and other biological processes (Robinson et al., 1970; Robinson, 1974; Robinson and Rudd, 1974). Furthermore, in vivo concentrations of ascorbic acid are subject to dietary control. We have carried out a series of experiments in order to learn more about the degradative effect of ascorbic acid on proteins, peptides, and amino acids. These experiments suggest that this degradation is not primarily deamidation, but is instead a breakage of the peptide chains and deamination of the resulting peptides.

Experimental Procedure

Human transferrin was obtained from Sigma Chemical Co. and Pentex Corporation. Catalase (45,000 units/ml), peroxidase, leucine aminopeptidase, GlyLeu, LeuAla, and pronase were purchased from Calbiochem, Inc. Cytochrome c Type III and superoxide dismutase were obtained from Sigma. Dehydroascorbic acid was obtained from Pfaltz & Bauer, Inc. Tertiary-butyloxy-carbonylhistidine was obtained from Fox Chemical Co., and sodium ascorbate was obtained from Merck and Co. or prepared by mixing equimolar quantities of ascorbic acid and sodium bicarbonate. Hydrogen peroxide was obtained as a 30 percent (v/v) "stabilized" solution from...
Ascorbic acid degradation of transferrin

J. T. Baker Chemical Co. Amino acids were the highest purity grade available from Schwarz Bioresearch, Inc. and Mann Research Laboratories.

Catalase was assayed spectrophotometrically with peroxidase and 2,2'-dimethoxybenzidine used to measure the hydrogen peroxide concentration. One unit of catalase was defined as the amount that catalyzed the decomposition of 1 u mole of H2O2 per minute at room temperature (23°). Phosphate buffer was prepared from 2.0 g of KH2PO4 and 8.8 g of Na2HP04 dissolved in one liter of distilled water which contained 16 mcg of both kana-mycin and neomycin sulfate or 1.0 ml of 88 percent solution of phenol as bacteria and mold retardants. Borate buffer was prepared by dissolving 0.1 mole of boric acid in distilled water, adding enough 10 M NaOH to bring the pH to 7.4, and adjusting the final volume to 1.0 liter.

Ammonia measurements were performed with an Orion Research ammonia-sensing electrode in combination with an Orion digital pH meter. Hydrogen peroxide was assayed according to the procedure of Spanyar et al. (1964) by using a titanic acid solution prepared by dissolving 2 g of titanium chloride in 400 ml of 25 percent (w/v) H2SO4 and stirring the cloudy solution with heating until completely clarified, or by using peroxidase and 2,2'-dimethoxybenzidine prepared by mixing 12 mg of peroxidase (59 units/mg) and 0.1 g of the chromagen in 100 ml of phosphate buffer (pH 7.4). Colorimetric determinations were performed with a Bausch and Lomb Spectronic 20 spectrophotometer with either a 0.2 ml flow-through cuvette or half-inch cuvettes; vapor-phase chromatography was performed with 1.9 m x 4 mm I.D. glass columns packed with either 15 percent FFAP on Chrom. W or 1.5 percent OV-101 on Chrom. G in a Varian Aerograph Model 2100 chromatograph. Amino acid analyses were performed with a Durrum D-500 Amino Acid Analyzer by injecting 40 ul of sample that had been previously adjusted to a pH of about 2.2 by the addition of 30 percent sulfosalicylic acid (10 ul per 100 ul of sample) and contained a known amount of a norleucine internal standard. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Weber and Osborn (1969). Enzymatic digestion of transferrin was carried out with leucine amino peptidase (Sigma) and pronase according to the procedure of Pisano et al. (1969). Hydrolysis of transferrin was carried out with 6 M HCl at 100° C in a sealed tube for 24 hours. The standard reaction mixtures for studying the degradative effect of 0.01 F ascorbate on transferrin contained 145 mg of transferrin per 100 ml of phosphate buffer (2.0 x 10-5 M assuming MW of 74,000) to which the required quantity of sodium ascorbate or other reactants was added. 25 ml screw-cap tubes were incubated in a 37° circulating water bath and 2.0 ml samples were removed at regular intervals to be analyzed immediately for ammonia or stored at -40° C for later analysis.

Results

Irving and Robinson (unpublished) found that oxygen is required for the degradation of transferrin by L-ascorbic acid in phosphate buffer, pH 7.4 at 37°, and that dehydroascorbic acid also causes the degradation of transferrin, as measured by ammonia liberation. These observations suggest that a product resulting from the reaction of ascorbic acid or dehydroascorbic acid with oxygen, rather than ascorbic acid itself, is responsible for the reaction. The auto-oxidation of ascorbic acid is a complex process which is accelerated by traces of metal ions and which produces hydrogen peroxide in addition to many breakdown products of ascorbate (Baker et al., 1963; Aberg and Johansson, 1963; Taqui Khan and Martell, 1967).

In order to check the importance of metal ions in the degradation of transferrin, a solution in phosphate buffer was prepared which was 20 uM in transferrin and 0.01 in ascorbate and this solution was incubated at 37° in the presence of either EDTA, 20 u M FeS04,
or 13 μM CuSO₄. After 48 hours both the control which contained only 0.01 F ascorbate and the iron-treated samples had ammonia concentrations corresponding to 5 moles of ammonia per mole of transferrin. The EDTA-treated sample had slightly less ammonia, 4.6 moles/mole, and the copper-treated sample had 7.0 moles/mole. A control containing transferrin and 20 μM FeSO₄ but no ascorbate had 0.8 moles of ammonia per mole of transferrin, probably as a result of deamination. These results, which are shown in Figure 1, indicate that metal ions are probably not necessary for the reaction, but that copper ion catalyzes the reaction.

When the initial concentration of ascorbate was decreased to 0.001 F or increased to 0.1 F the initial rate of ammonia liberation remained about the same. Hence, the rate of ammonia liberation is zero or fractional order in ascorbate. This may result from a free radical mechanism for the reaction.

A source of free radicals could be hydrogen peroxide which is formed during the auto-oxidation of ascorbate by molecular oxygen. The hydrogen peroxide concentration in the phosphate buffer, at 37° in the presence of 0.01 F ascorbate, was measured spectrophotometrically using titanic acid for the reagent (Spanyan et al., 1964). The hydrogen peroxide concentration was initially about 50 μM, but varied over the course of several days as illustrated in Figure 2. Addition of cupric sulfate (0.3 mM) caused the peroxide concentration to double. Bubbling pure oxygen into the solution increased the peroxide concentration. A decrease in the ascorbate concentration to 0.001 F had little effect on the initial peroxide concentration. Addition of catalase decreased the peroxide concentration. Dehydroascorbic acid also produced hydrogen peroxide in the course of its auto-oxidation in the phosphate buffer, possibly as a result of its conversion to 2, 3-diketogulonic acid and subsequent reduction of molecular oxygen by the enediol form of this molecule (Kenyon and Munro, 1948). Other tests for the presence of
hydrogen peroxide in the auto-oxidizing ascorbate solutions such as oxidation of 2, 2'-dimethoxybenzidine in the presence of peroxidase (Bjorksten, 1966) and decrease in the 410 nm Soret band of catalase were also positive for hydrogen peroxide (Zito and Kertesz, 1961).

In order to evaluate the role of hydrogen peroxide in the degradation of transferrin by aerobic ascorbate solutions, a series of experiments were conducted in the presence of catalase. It was found that the addition of 9,000 units of catalase at 24-hour intervals was sufficient to completely inhibit the increased ammonia liberation in the presence of 0.01 F ascorbate. Smaller amounts of catalase, which was about 80 percent inhibited in the presence of 0.01 F ascorbate, caused partial inhibition of the reaction. Catalase also inhibited the dehydroascorbic acid degradation of transferrin. The results, illustrated in Figure 3, provide evidence that hydrogen peroxide may be involved in the degradation reaction.

**FIGURE 3**

**REACTION OF TRANSFERRIN AND ASCORBATE (0.01F) IN PHOSPHATE BUFFER, pH 7.4, 37°:**

- (a) 0.01F ASCORBATE + 20 μM TRANSFERRIN + 9000 units CATALASE AT TIME 0 AND 24 hours
- (b) 20 μM TRANSFERRIN + 9000 units CATALASE AT TIME 0 AND 24 hours
- (c) 20 μM TRANSFERRIN + 0.01F ASCORBATE

Hydroxyl free radicals could be present in the reaction mixture as a result of a reaction between hydrogen peroxide and metal ions (as in Fenton's reagent) or other mechanisms. In order to evaluate the role of hydroxyl radicals in the degradation of transferrin we studied the reaction in the presence of the hydroxyl radical trapping agents, benzene, benzoate, iodide, bromide, chloride, and ethanol. The results are illustrated in Figure 4. Ammonia evolution from the 20 μM transferrin under the usual conditions was inhibited by all of the reagents, especially by benzene, benzoate, and iodide. Vapor-phase chromatography (VPC) of the benzene-treated reaction mixture showed the presence of a compound which co-chromatographed with a sample of phenol and which was not present in the mixture prior to incubation. Similarly, salicylic acid in the mixture containing benzoate and ascorbate was identified by VPC. These results suggest that hydroxyl free radicals are involved in the ascorbate degradation of transferrin. They may arise secondarily to the production of hydrogen peroxide in the auto-oxidizing ascorbate solution.

**FIGURE 4**

**INHIBITION OF ASCORBATE-DEGRADATION OF TRANSFERRIN; 20 μM TRANSFERRIN, 0.01F ASCORBATE, PHOSPHATE BUFFER, pH 7.4, 37°**

- (a) ASCORBATE CONTROL
- (b) POTASSIUM CHLORIDE (62 mM)
- (c) ETHANOL (0.13M)
- (d) POTASSIUM IODIDE (10 mM)
- (e) BENZOIC ACID (5 mM)
- (f) POTASSIUM BROMIDE (25 mM)
- (g) BENZENE (SATURATED)
- (h) TRANSFERRIN CONTROL
Treatment of 20 uM transferrin (Sigma) in phosphate buffer with 0.01 F hydrogen peroxide at about 100-fold the concentration measured in the 0.01 F ascorbate did not increase ammonia evolution as compared to control. However, addition of a trace quantity (1.0 uM) of ferrous or ferric ions caused an increase in the ammonia concentration to 5 moles of ammonia per mole of transferrin after 24 hours. It is interesting that a sample of transferrin obtained from Pentex Corporation was degraded in the presence of 0.01 F hydrogen peroxide without addition of metal ions. The difference may be caused by differences in the amounts of iron contained in the two samples of transferrin. Atomic absorption spectroscopy showed that the Pentex sample contained 1/4 mole of iron per mole of protein while the Sigma sample contained 1/16 mole of iron per mole of transferrin. The results indicate that hydrogen peroxide is not the active agent in the reaction, but that free radicals resulting from the interaction of metal ions and hydrogen peroxide are probably involved.

Robinson et al. (1973) reported that the product resulting from the action of ascorbic acid and transferrin at 37° in pH 7.4 phosphate buffer increased in heterogeneity and negative charge with time and that less than 1 percent of the transferrin molecules remained in native form after three days. Similar results were obtained by us after 48 hours of incubation. We found 0 percent unaltered protein after this time.

With 0.01 F H_2O_2 and 10 uM FeCl_3 the transferrin was also extensively degraded. When electrophoresis was performed on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) (Weber and Osborn, 1969; Dunker and Reuckert, 1969) the ascorbate and hydrogen peroxide-treated samples of transferrin appeared to be heterogeneous with respect to molecular weight. The control sample that was incubated 24 hours showed a single band corresponding to that of pure transferrin with MW 74,000 (Roberts et al., 1966). The sample treated with 0.01 F ascorbate had a trace of material that corresponded to the native; protein, but the majority of material was smeared in the molecular weight range of 70,000 to 17,000. The peroxide-treated sample, like the ascorbic acid sample, gave no distinct bands of material but a faint smearing suggestive of random breakage of the polypeptide chain of transferrin. The results suggest that ascorbate (and peroxide plus metal ions) causes cleavage of the peptide chains in addition to loss of ammonia from the molecule.

In order to learn whether or not the increased ammonia evolution results from deamidation of glutaminyl and asparaginyl residues of transferrin, a sample of transferrin that had been incubated with 0.01 F ascorbic acid for 48 hours and that had liberated 8.3 moles of ammonia per mole of transferrin and a second sample that had been degraded in the presence of 0.01 F hydrogen peroxide and 10 mcg M ferric chloride (after 48 hours this sample had an ammonia concentration corresponding to 24.5 moles of ammonia per mole of transferrin) were subjected to enzymatic digestion and amino acid analysis. Hydrolysis with 6 M HCl demonstrated that the enzymatic digestion was approximately 75 percent complete in the controls and about 59 percent complete in the ascorbate and peroxide samples. The percent increase or decrease in amino acids in the experimental samples was calculated by normalization to recovery of the amino acids, Asp, Thr, Ser, Asn, Gin, Cly, Ala, Val, lie, Leu, Tyr, Phe. The results, listed in Table 1, indicate that asparagine and glutamine are not destroyed to a sufficient degree to account for the observed ammonia. This can be seen by looking at the acid to amide ratios, which would be expected to increase if the ammonia resulted from deamidation. These values are listed in Table 2. The acid to amide ratios tended to decrease compared to the control instead of increase. The observation that these ratios do not change in the direction expected leads us to conclude that most of the ammonia originating in
ASCORBIC ACID DEGRADATION OF TRANSFERRIN

TABLE 1

Concentrations (umoles/1) of amino acids resulting from enzymatic digestion of transferrin samples treated with 0.01 F ascorbate, 0.01 F hydrogen peroxide + 10 uM Fe + 3, and control; and percent increase or decrease of amino acids in experimental samples compared to control (normalized with Asp, Thr, Ser, Asn, Gin, Gly, Ala, Val, Isl, Leu, Tyr, and Phe).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control</th>
<th>Ascorbate</th>
<th>H2O2</th>
<th>% Change A.A.</th>
<th>% Change H2O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>22.9</td>
<td>16.8</td>
<td>17.3</td>
<td>-15.0</td>
<td>-15.7</td>
</tr>
<tr>
<td>Thr</td>
<td>56.6</td>
<td>52.6</td>
<td>51.8</td>
<td>7.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Ser</td>
<td>56.3</td>
<td>41.3</td>
<td>45.3</td>
<td>-15.0</td>
<td>-10.5</td>
</tr>
<tr>
<td>Asn</td>
<td>47.5</td>
<td>40.6</td>
<td>42.1</td>
<td>-1.0</td>
<td>-1.4</td>
</tr>
<tr>
<td>Glu</td>
<td>32.4</td>
<td>21.2</td>
<td>26.7</td>
<td>-24.0</td>
<td>-8.3</td>
</tr>
<tr>
<td>Gin</td>
<td>42.5</td>
<td>34.6</td>
<td>37.7</td>
<td>-5.5</td>
<td>-1.4</td>
</tr>
<tr>
<td>Gly</td>
<td>43.7</td>
<td>32.5</td>
<td>37.6</td>
<td>-13.9</td>
<td>-4.2</td>
</tr>
<tr>
<td>Ala</td>
<td>96.9</td>
<td>78.7</td>
<td>85.1</td>
<td>-5.8</td>
<td>-2.3</td>
</tr>
<tr>
<td>Val</td>
<td>76.9</td>
<td>70.8</td>
<td>72.9</td>
<td>6.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Cys</td>
<td>16.9</td>
<td>12.6</td>
<td>7.7</td>
<td>13.4</td>
<td>-49.1</td>
</tr>
<tr>
<td>Met</td>
<td>18.7</td>
<td>4.4</td>
<td>2.6</td>
<td>-72.7</td>
<td>-84.2</td>
</tr>
<tr>
<td>Isl</td>
<td>26.0</td>
<td>24.2</td>
<td>24.7</td>
<td>7.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Leu</td>
<td>95.5</td>
<td>92.2</td>
<td>93.4</td>
<td>12.0</td>
<td>8.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>64.9</td>
<td>57.3</td>
<td>57.1</td>
<td>2.3</td>
<td>-2.2</td>
</tr>
<tr>
<td>Phe</td>
<td>50.6</td>
<td>45.3</td>
<td>46.5</td>
<td>3.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Lys</td>
<td>61.8</td>
<td>44.9</td>
<td>48.3</td>
<td>-15.8</td>
<td>-12.9</td>
</tr>
<tr>
<td>His</td>
<td>30.4</td>
<td>12.1</td>
<td>4.9</td>
<td>-53.8</td>
<td>-81.9</td>
</tr>
<tr>
<td>Trp</td>
<td>5.7</td>
<td>4.2</td>
<td>0.6</td>
<td>-13.7</td>
<td>-87.7</td>
</tr>
<tr>
<td>Arg</td>
<td>27.6</td>
<td>20.9</td>
<td>22.3</td>
<td>-12.0</td>
<td>-10.1</td>
</tr>
</tbody>
</table>

TABLE 2

Ratios of aspartic and glutamic acid concentrations to their amide derivatives resulting from enzymatic digestion of transferrin control and samples treated with 0.01 F ascorbate and 0.01 F hydrogen peroxide + 10 uM Fe + 3. Samples were incubated for 48 hours at 97°C in phosphate buffer, pH 7.4.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Control</th>
<th>Ascorbic acid</th>
<th>Hydrogen peroxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Asn</td>
<td>0.96</td>
<td>0.83</td>
<td>0.81</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>0.74</td>
<td>0.60</td>
<td>0.71</td>
</tr>
<tr>
<td>Asp + Glu/Asn + Gln</td>
<td>0.86</td>
<td>0.72</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Percent change in total acid/amide ratio:

\(-16\% \quad -11\%\)

the samples results from a reaction other than deamidation as suggested by Robinson et al. (1973).

Another possible source of ammonia is the nitrogen in the peptide bonds of the molecule. It is known that Fenton's reagent causes oxidative deamination of amino acids resulting in liberation of ammonia (Kalyankar et al., 1955; Johnson et al., 1951; Armstrong and Humphreys, 1967). Treatment of a 0.01 solution of alanine with 0.01 F ascorbic acid in phosphate buffer showed that ascorbate can effect the same or a similar reaction since ammonia was liberated in the solution over the course of several days. Catalase prevented the reaction, and the addition of 10 uM CuSO4
increased the ammonia liberation. 0.01 F H₂O₂ in the presence of 10 μM CuSO₄ resulted in a 20-fold increase in ammonia concentration compared to ascorbate after six days. The results are illustrated in Figure 5.

**FIGURE 5**

**REACTION OF ALANINE (0.01F) IN BORATE BUFFER, pH 7.4, (0.1M), 37° WITH:**
- △ 0.01F H₂O₂ + 10 μM CuSO₄
- ○ 0.01F H₂O₂ + 10 μM FeCl₃
- ▲ 0.01F ALANINE (CONTROL)
- ◇ 0.01F ASCORBATE + 0.63 mM EDTA
- ▼ 0.01F ASCORBATE + 10 μM CuSO₄
- ▽ 0.01F ASCORBATE + CATALASE

In order to investigate whether or not the N-terminal amino groups of peptides are deamidated in the presence of ascorbate, we treated 20 μM solutions of GlyLeu and LeuAla with 0.01 F ascorbate in phosphate buffer. The results of periodic ammonia determinations from the two samples are illustrated in Figure 7. The results show that ammonia is liberated from the two dipeptides at a rate which is greater than that observed for alanine. Amino acid

**FIGURE 7**

**LIBERATION OF AMMONIA FROM DIPEPTIDES**

GlyLeu AND LeuAla IN PHOSPHATE BUFFER (pH 7.4, CONTAINING 0.1% PHENOL), 37°
- ○ GlyLeu (2 mF) CONTROL
- □ LeuAla (2 mF) CONTROL
- △ GlyLeu (2 mF) + ASCORBATE (0.01F)
- ◇ LeuAla (2 mF) + ASCORBATE (0.01F)

Imanaga (1955) reported that ascorbic acid reacts with histidine to produce ammonia as one of the products. In order to check the possibility that the ammonia originates from one of the nitrogens in the imidazole ring of histidine we treated histidine (0.001 F) in phosphate buffer, 37°, with 0.01 F ascorbate and a sample of t-BOC-histidine with ascorbate under identical conditions. The ammonia concentrations of the samples were assayed periodically and are shown in Figure 6. The results indicated that ammonia is liberated from histidine, but that blockage of the alpha-amino group prevents the ammonia liberation. This result along with small destruction of Lys, Trp, and Arg in the ascorbate-treated transferrin suggests that the bulk of the ammonia probably arises from nitrogens in the peptide bonds.
analyses of the products showed no evidence that the ascorbate treatment catalyzed hydrolysis of the peptide bonds, but showed that the peptides were partially converted to ninhydrin-negative derivatives.

Discussion

The results presented here are not consistent with the hypothesis that the increased evolution of ammonia amounting to several moles of ammonia per mole of transferrin in the presence of ascorbic acid and oxygen results from increased deamidation of amide residues in the protein. The ratio of amide to acid residues before and after treatment with ascorbic acid or hydrogen peroxide should increase if the ammonia results from deamidation, and it did not. Secondly, the heterogeneity of the treated transferrin observed upon electrophoresis appears to be as much a result of heterogeneity in molecular weight as of changes in the charge of the molecule. Lastly, treatment of model amides such as acetamide and N-acetyl-asparagine with ascorbic acid or hydrogen peroxide in the presence of metal ions under comparable conditions failed to show an increase in the rate of deamidation as compared to controls. It is unlikely that much of the ammonia results from the other nitrogen-containing amino acid residues, Lys, His, Trp, or Arg, since, our experiments excluded, histidine and treatment of a mixture of amino acids including lysine, tryptophan, and arginine with ascorbate showed only a small reduction in the concentrations of these amino acids and the production of no major unidentified ninhydrin-positive peaks.

We hypothesize that the ammonia evolution arises from deamination of peptide fragments that result from the treatment of transferrin with aerobic ascorbic acid. This mechanism could also explain the results obtained by Robinson, Irving, and McCrea with the labeled pentapeptide GlyArgAsnArgGly. They measured a more negatively charged form of the peptide by electrophoresis and estimated the half-life of deamidation by radioactive counting of the eluted band, but we have shown that ascorbate can effect deamination of N-terminal amino groups in peptides as well as amino acids, making it possible that they actually measured a deamination product rather than a deamidated product as they concluded. Both products would be expected to be more negatively charged than the native peptide and to have about the same electrophoretic mobility.

The evidence that the degradation of transferrin depends on hydroxyl radicals is: (a) that catalase inhibits the degradation but hydrogen peroxide alone is inactive, (b) that hydrogen peroxide + metal ions — a known hydroxyl radical generating system — causes degradation and ammonia evolution, (c) that hydroxyl radical trapping agents such as benzene and benzoate inhibit the degradation reaction, (d) that amino acids and peptides are deaminated in the presence of auto-oxidizing ascorbate, a reaction which is known to occur also in the presence of a hydroxyl radical source such as Fenton's reagent or ionizing radiation (Kalyankar et al., 1955; Johnson et al., 1951; Armstrong and Humphreys, 1967; Kakin, 1905; Neuberg, 1909; Stein and Weiss, 1949), and (e) that there is evidence that hydroxyl radicals generated by radiolysis cause cleavage of peptide chains and ammonia evolution from proteins (Garrison et al., 1962). Such peptide chain cleavage has also been reported for the degradation of catalase in the presence of ascorbic acid (Orr, 1967) and, subsequent to our work on transferrin, degradation of the basic protein of human myelin caused by aerobic ascorbic acid and involving breakage of the peptide chain was observed (Westall and Robinson, 1975). Several investigators have proposed that ascorbic acid under auto-oxidizing conditions forms hydroxyl free radicals which are known to cause deamination of alpha-amino acids. Yamazaki et al. (1960) have shown by electron spin resonance that free radicals are formed from ascorbic acid in the reaction with hydrogen peroxide with or without
peroxidase, and in the reaction with molecular oxygen in the presence of ascorbic acid oxidase (Yamazaki, 1962). Lagercrantz (1964) has similarly shown that a free radical is formed in auto-oxidizing solutions of ascorbate without added catalysts in the pH range of 6.6 to 9.6. This free radical was identified as monodehydroascorbic acid (MDA) by its hyperfine splitting pattern.

We speculate that a possible mechanism for the formation of hydroxyl radicals in auto-oxidizing solutions of ascorbate might be the reaction of MDA and H$_2$O$_2$ as follows:

$$\text{MDA}^* + \text{H}_2\text{O}_2 \rightarrow \text{HO}^* + \text{H}_2\text{O} + \text{DHA}$$

It is well known that hydroxyl free radicals are formed in the reaction of hydrogen peroxide and metal ions such as iron (II). Since hydrogen peroxide is present at low concentration (40 — 120 uM) in the ascorbate solutions under the conditions studied it is possible that hydroxyl radicals form via the above reaction due to traces of metal ions present in the buffer solutions or in the transferrin itself. The reaction can be illustrated as follows:

$$\text{H}_2\text{A} + \text{O}_2 \rightarrow \text{DHA} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{C} > \text{2} + \text{M}^n \rightarrow \text{HO}^* + \text{HO}^- + \text{M}^n + 1$$

Another mechanism for the production of hydroxyl radicals might be the reaction of superoxide and hydrogen peroxide as illustrated (Yamazaki et al., 1960; Beauchamp and Fridovich, 1970).

$$\text{HA}^- + \text{O}_2 - \% \text{MDA}^* + \text{O}_2^- \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{HO}^* + \text{HO}^- + \text{M}^n + 1$$

However, this latter mechanism is probably not important in the ascorbic acid degradation of transferrin since addition of superoxide dismutase (112 units/ml of transferrin solution) did not inhibit the ammonia evolution in the presence of 0.01 F ascorbate. However, addition of catalase would be expected to inhibit the formation of hydroxyl radicals by any of the above mechanisms, since it would destroy the peroxide.

Catalase was also shown by Uden-friend et al. (1954) to inhibit the ascorbic acid-dependent hydroxylating system consisting of ascorbate, iron (II), EDTA, and oxygen or hydrogen peroxide. Bres-low and Lukens (1960) showed that ascorbic acid functions in this system as a source of hydrogen peroxide and as a reducing agent to convert iron (III) back to iron (II), which then reacts with peroxide to form the active hydroxyl radicals. Similarly, Matsumura and Pigman (1965) who studied the depoly-merization of hyaluronic acid in auto-oxidizing solutions of ascorbate, observed that the reaction is inhibited by catalase and accelerated by traces of copper and iron. They suggested that a radical mechanism is involved. Berneis (1963) has shown that ascorbic acid in the presence of oxygen causes degradation of DNA. That degradation is suppressed by the addition of catalase or peroxidase, and it has been postulated that the active agent is hydroxyl radical. It has also been shown that ascorbic acid in combination with hydrogen peroxide forms a powerful bacteriocidal mixture (Ericsson and Lundbeck, pp. 493-506, 507-527, 1955; Miller, 1969). The bacteria-killing power of this mixture has been attributed to hydroxyl radicals since: bacteria-killing is inhibited by free radical trapping agents (Miller, 1969). Furthermore, many of the other known or suggested biological reactions of ascorbic acid, many of which include hydroxylation or oxidation of heterocyclic compounds (Cuchhait et al., 1963; Lehninger, 1970; Rahandraha and Rat-sinamanga, 1955; Moriyama, 1957; Torii and Moriyama, 1955; Lerner, 1955; Sebrell and Harris, 1954; Brodie et al., 1954; Subramanian et al., 1973) could involve formation of hydroxyl radicals. It has been suggested that the degradative property of aerobic ascorbic acid solutions may be one of the principal biological functions of ascorbic acid (Robinson and Richheimer, 1975).

Further investigation should be done in order to determine the mechanism by which ascorbic acid degrades proteins, since ascorbic acid is often added to culture media and to enzyme preparations to test for activation, inhibition, or redox effects. It is possible that at least
some of the effects of ascorbate in such systems are the result of cleavage of peptide chains or modification of amino acid residues. It would be of special interest from the standpoint of the protein sequence chemist if ascorbate-cleavage of peptide bonds is found to be selective in nature.

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