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Oxidation of Tryptophan in the Presence of Oxidizing Methyl Linoleate

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Tryptophan (Trp) oxidation to kynurenine (kyn) and *N*-formylkynurenine (NFK) was studied in the presence of oxidizing methyl linoleate. The rate of Trp oxidation was found to depend on pH, ionic strength, and the presence or absence of metals and metal chelators. The presence of copper (5 ppm) increased the rate of oxidation, and EDTA decreased it. When Trp was present in a tripeptide (Gly-L-Trp-Gly) the rate of Trp oxidation was greater than that found in like samples where Trp was free instead of bound. Trp in control samples (no methyl linoleate) also oxidized relatively rapidly, but the rates were always less than those observed in samples containing Trp and methyl linoleate. Approximately 1–2% of the Trp in either free or bound form in a tripeptide (no added catalyst or inhibitor) was converted to kyn and NFK in just 72 h at room temperature.

Oxidation in foods has been a problem for centuries. It leads to changes in nutritional and functional properties and may even result in development of toxic substances (Fontana and Toniolo, 1976; Yong and Karel, 1979). Although lipids are considered the most oxidation-prone constituents of foods, some amino acids in free or combined forms are also susceptible to oxidation. The amino acids cystine, methionine, histidine, tyrosine, and tryptophan (Trp) are all susceptible to oxidation, and the conditions need not be severe (Yong et al., 1980). Protein-bound forms of these amino acids can also be oxidized under conditions that sometimes prevail in stored food. With regard to protein-bound Trp, storage for 4 weeks at 37 °C in the presence of oxidizing methyl linoleate resulted in a 25–30% increase in the chemically determined loss of Trp as compared to that encountered in control samples (Nielsen et al., 1985a,b). In another study, the kynurenine (a major degradation product of oxidized Trp) content of dolphin flesh increased from 0.9 to 3.5 μg/g during exposure of the fish to sunlight for 8 h (Takahashi, 1984). Thus, Trp can be oxidized in either free or bound form, and study of Trp in less complex forms has the advantage of facilitating separation and quantification of major degradation products.

Oxidation of proteins and amino acid residues occurs by a free-radical mechanism. Free radicals can be initiated by ionizing or ultraviolet radiation, by visible light, or by free-radical transfer from other molecules (Fontana and Toniolo, 1976; Schaich, 1980). Peroxidizing methyl linoleate is very efficient at transferring its free radicals to other substances including proteins and amino acids (Gunstone and Norris, 1983). Similar behavior would be expected from linoleate in foods, when it is bound in triacylglycerols and phospholipids.

Free tryptophan (Trp) in food and simple systems oxidizes readily in the presence of light or peroxidizing lipids, participates in the Maillard reaction, and is destroyed under the usual conditions for acid hydrolysis (Yong and Karel, 1979). The degradation products of Trp oxidation are similar regardless of whether the degradation is caused by peroxidizing lipids, ionizing radiation, or photooxidation (Yong and Karrel, 1979). Protein-bound Trp is less susceptible to oxidation than protein-bound lysine or methionine (Nielsen et al., 1985a).

Loss of Trp through oxidation is important because it is an essential amino acid; the resulting products can contribute to off-flavors in irradiated foods, can lead to the yellowing of wool in sunlight, can contribute to the development of yellow and brown cataracts in the lens of the human eye, and can reduce the activities of some enzymes; and at least two of the products (kynurenine and *N*-formylkynurenine) are suspected promoters of urinary bladder carcinogenesis in mice (Bryan, 1971; Friedman and Finley, 1971; Matsushima et al., 1982; Nielsen and Hurrell, 1984; Wolf, 1984; Yong and Karel, 1979).

Oxidation of proteins and amino acids is affected by many environmental factors such as pH, temperature, water activity, and the presence of catalysts or inhibitors (Erickson, 1982; Mitchell and Henick, 1962). Therefore the extent of oxidation and the rate at which it occurs is not easily predicted.

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The purpose of this research is to determine the rates of linoleate-catalyzed oxidation of free and peptide-bound Trp to kynurenine (kyn) and *N*-formylkynurenine (NFK) as influenced by pH, ionic strength, buffers, antioxidants, and metal catalysts. A companion study deals with riboflavin-sensitized photooxidation of free and peptide-bound Trp (Kanner and Fennema, following paper in this issue). Both of these studies involve quantitative measurements of kyn and NFK, and this has not been done in most other studies of this kind.

METHODS AND MATERIALS

Reagents. All chemicals used were of reagent grade unless otherwise specified.

Standard Procedure for Studies on Free Tryptophan. Samples were prepared by adding 0.2 g of Tween 80, 23 mL of buffer (either phosphate (0.01 M) at pH 4, 5, 6, or 7.5 or acetate (0.001, 0.01, 0.1 M) at pH 4, 5, 6, or 7.5), 17 mg of Trp (Sigma Chemical Co., St. Louis, MO; Sigma grade; 0.0033 M in final sample), and 2 mL of autoxidized methyl linoleate (prior to use stirred briskly for $2^1/_2$ h while exposed to air) to a 50-mL beaker. This resulted in a methyl linoleate to Trp ratio of 72.6:1. In the control samples 2 mL of distilled water was added in place of methyl linoleate.

Each sample was mixed for 1 min at maximum power with a Branson sonifer. The contents of each beaker were then transferred to 1000-mL flasks containing 3.8-cm stir bars, and each flask was stoppered with a cotton plug. Each flask was placed on a 2.5-cm piece of rigid foam insulation that in turn was situated on a magnetic stirrer. The samples were held at room temperature and exposed to continuous high-speed stirring throughout the experiment.

Subsamples were taken at 0, 24, 48, 72, and 96 h unless otherwise specified. Samples of 20 L were injected into an HPLC (Beckman 110A pumps, Beckman Model 153 detector, Beckman Model 420-30 controller, Waters Nova Pak C18 reversed-phase column, 5-μm particle size) and the eluents were monitored at a wavelength of 254 nm. Acetonitrile buffer (0.035 M at pH 4), flowing at a rate of 1.2 mL/min, was used as the mobile phase. The separated components were quantified as described later.

Addition of EDTA and Copper. The standard procedure for free Trp was followed using 0.01 M sodium acetate buffer at pH 4, 5, or 6. EDTA (20 ppm) and/or cupric sulfate (5 ppm) was added to the buffer. Samples were taken at 0, 24, 48, and 72 h.

Tripeptide Model System. The standard procedure for free Trp, using 0.01 M phosphate buffer at pH 7.5, was followed, except that 25 mg of tripeptide glycyl-L-tryptophylglycine (Gly-L-Trp-Gly) was added in place of Trp. The concentration of Trp in the final sample was 0.0031 M. Subsamples of 1 mL each were drawn at 0, 24, 48, 72, and 96 h and placed in 10-mL volumetric flasks. These samples were hydrolyzed as described below before being injected into the HPLC.

One milliliter of a Pronase solution Calbiochemical Co., La Jolla, CA; grade II; 8 mg/mL of sample) was added to each flask to achieve hydrolysis of the tripeptide. The flask was stoppered, covered with foil, and held in a water bath at 40 ± 1 °C for 24 h. This procedure resulted in 95–100% hydrolysis of the tripeptide. Samples were then injected into the HPLC. Samples containing Pronase but no tripeptide were also injected. Percent hydrolysis was determined by comparison with standard curves for the tripeptide and both dipeptides (Gly-L-Trp, L-Trp-Gly).

Calculations. Compounds responsible for the various peaks were identified by using internal standards [kyn from Sigma Chemical Co., St. Louis, MO; Sigma grade II. NFK from Calbiochemical Co., La Jolla, CA; 96% purity. L-Trp-Gly and Gly-L-Trp from Research Plus Laboratories, Bayonne, NJ; Absolute grade] and by retention times. Molarity of the sample components was determined from a standard curve (peak area vs. molarity of standards) for each compound.

For the tripeptide samples, the peak area of the Pronase blank was subtracted from that of the kyn peak since the Pronase and kyn peaks overlapped.

For each experiment a plot of log [(initial Trp) − (av kyn + NFK)]/(initial Trp) vs. time was prepared, based on three replicates at each time. The slope of each plot was determined by linear regression using a MINITAB computer program. The slopes of these plots were used to prepare plots of slope vs. pH for each system stuudied.

Table I. Accumulation of kyn and NFK from Lipid-Catalyzed Oxidation of Free and Bound Trp[a] (24 h)

test condition		kyn + NFK as % of orig Trp (room temp, 24 h)	
buffer	pH	control[b]	sample[c]
0.01 M phosphate	4	0.015 ± 0.001	0.15 ± 0.001
0.01 M phosphate	5	0.06 ± 0.001	0.25 ± 0.001
0.01 M phosphate	6	0.06 ± 0.001	0.11 ± 0.02
0.01 M phosphate	7.5	0.14 ± 0.001	0.34 ± 0.01
0.001 M NaOAc	4	0.29 ± 0.001	0.59 ± 0.02
0.001 M NaOAc	5	0.15 ± 0.001	0.41 ± 0.02
0.001 M NaOAc	6	0.165 ± 0.014	0.35 ± 0.001
0.01 M NaOAc	4	0.21 ± 0.04	0.62 ± 0.02
0.01 M NaOAc	5	0.25 ± 0.001	0.66 ± 0.02
0.01 M NaOAc	6	0.23 ± 0.001	0.47 ± 0.01
0.01 M NaOAc	7.5	0.087 ± 0.02	0.18 ± 0.001
0.1 M NaOAc[d]	4	0.40 ± 0.001	0.82 ± 0.06
0.1 M NaOAc[d]	5	0.48 ± 0.01	0.71 ± 0.02
0.1 M NaOAc[d]	6	0.35 ± 0.04	0.55 ± 0.02
0.01 M NaOAc + Cu	4	0.96 ± 0.03	1.27 ± 0.06
0.01 M NaOAc + Cu	5	0.38 ± 0.02	1.49 ± 0.01
0.01 M NaOAc + Cu	6	0.22 ± 0.01	0.60 ± 0.01
0.01 M NaOAc + EDTA	4	0.09 ± 0.001	0.18 ± 0.01
0.01 M NaOAc + EDTA	5	0.03 ± 0.001	0.09 ± 0.003
0.01 M NaOAc + EDTA	6	0.03 ± 0.001	0.16 ± 0.01
0.01 M NaOAc + Cu + EDTA	4	0.19 ± 0.001	0.45 ± 0.001
0.01 M NaOAc + Cu + EDTA	5	0.09 ± 0.001	0.27 ± 0.001
0.01 M NaOAc + Cu + EDTA	6	0.0006 ± 0.001	0.19 ± 0.01
tripeptide[e]	7.5	0.001 ± 0.001	0.46 ± 0.02

[a] Means of triplicate determinations. Trp concentration 0.0033 M. [b] Controls contained phosphate or acetate buffer, water, Trp, and Tween 80. [c] Test samples contained phosphate or acetate buffer, methyl linoleate, Trp, and Tween 80. [d] Sample taken at 5 h. [e] Trp concentration in tripeptide 0.0031 M. NaOAc = sodium acetate buffer.

RESULTS

Subsamples of oxidized Trp were separated by HPLC, and a typical chromatogram is shown in Figure 1. Peak areas were compared to appropriate standard curves and the concentrations determined.

Data for 24- and 72-h time periods are shown in Tables I and II. It is somewhat surprising that this short storage period at room temperature resulted in relatively large amounts (about 1–3% based on original Trp) of kyn and NFK in samples to which catalysts were not intentionally added. The slope of the line of best fit for each plot (not shown) was determined (linearity was generally very good), and these slopes were plotted against pH as shown in Figures 2 and 3.

pH. As is evident from both figures, the rate of oxidation generally decreased as the pH was increased over the range 4–7.5. Exceptions were the samples that contained either sodium acetate and EDTA or phosphate buffer. In these samples, the rate decreased from pH 4 to 5 and then increased from pH 5 to 7.5.

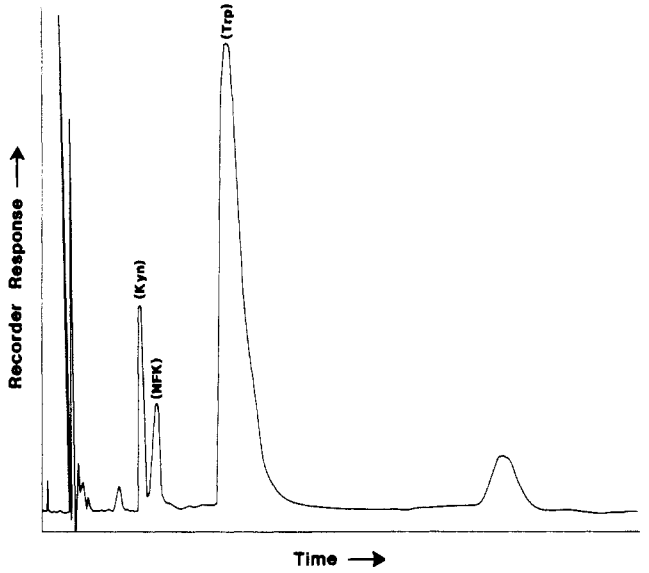

Figure 1. Typical chromatogram for sample of oxidized tryptophan. Conditions: column, C_{18} reversed phase; flow rate, 1.2 mL/min; mobile phase; acetonitrile; sample in sodium acetate buffer, pH 5.0; retention times, 3.2 min for kyn, 3.8 min for NFK, and 6.0 min for Trp.

Table II. Accumulation of kyn and NFK from Lipid-Catalyzed Oxidation of Free and Bound Trp[a] (72 h)

test condition		kyn + NFK as % of orig Trp (room temp, 72 h)	
buffer	pH	control[b]	sample[c]
0.01 M phosphate	4	0.35 ± 0.04	0.59 ± 0.1
0.01 M phosphate	5	0.36 ± 0.02	0.61 ± 0.01
0.01 M phosphate	6	0.26 ± 0.02	0.49 ± 0.04
0.01 M phosphate	7.5	0.37 ± 0.01	0.94 ± 0.03
0.001 M NaOAc	4	0.76 ± 0.04	1.97 ± 0.09
0.001 M NaOAc	5	0.73 ± 0.0001	1.79 ± 0.09
0.001 M NaOAc	6	0.62 ± 0.03	0.93 ± 0.03
0.01 M NaOAc	4	1.39 ± 0.04	2.57 ± 0.06
0.01 M NaOAc	5	1.09 ± 0.02	1.90 ± 0.02
0.01 M NaOAc	6	0.91 ± 0.01	1.32 ± 0.01
0.01 M NaOAc	7.5	0.37 ± 0.02	0.67 ± 0.02
0.1 M NaOAc[d]	4	1.47 ± 0.03	3.05 ± 0.02
0.1 M NaOAc[d]	5	1.64 ± 0.01	2.65 ± 0.001
0.1 M NaOAc[d]	6	1.06 ± 0.03	1.37 ± 0.02
0.01 M NaOAc + Cu	4	2.67 ± 0.03	6.09 ± 0.09
0.01 M NaOAc + Cu	5	1.33 ± 0.02	2.60 ± 0.07
0.01 M NaOAc + Cu	6	0.97 ± 0.02	2.10 ± 0.04
0.01 M NaOAc + EDTA	4	0.39 ± 0.03	0.53 ± 0.03
0.01 M NaOAc + EDTA	5	0.24 ± 0.03	0.59 ± 0.04
0.01 M NaOAc + EDTA	6	0.37 ± 0.001	0.77 ± 0.02
0.01 M NaOAc + Cu + EDTA	4	0.64 ± 0.001	1.02 ± 0.001
0.01 M NaOAc + Cu + EDTA	5	0.35 ± 0.001	0.64 ± 0.001
0.01 M NaOAc + Cu + EDTA	6	0.32 ± 0.001	0.42 ± 0.001
tripeptide[e]	7.5	0.56 ± 0.02	1.06 ± 0.02

[a] Means of triplicate determinations. Trp concentration 0.0033 M. [b] Controls contained phosphate or acetate buffer, water, Trp, and Tween 80. [c] Test samples contained phosphate or acetate buffer, methyl linoleate, Trp, and Tween 80. [d] Sample taken at 26 h. [e] Trp concentration in tripeptide 0.0031 M. NaOAc = sodium acetate buffer.

It is also evident that accumulation of kyn and NFK occurred more rapidly in the acetate buffer than in the phosphate buffer (Figure 2). The slower rate in the phosphate buffer is very likely attributable to its metal chelating ability (Labuza, 1971), and this point will be discussed more fully in a later section.

The rate vs. pH results reported here for samples containing phosphate buffer are qualitatively similar to those reported by Cuq and Cheftel (1983), Marcuse (1962), and Kanner and Fennema (following paper in this issue). In

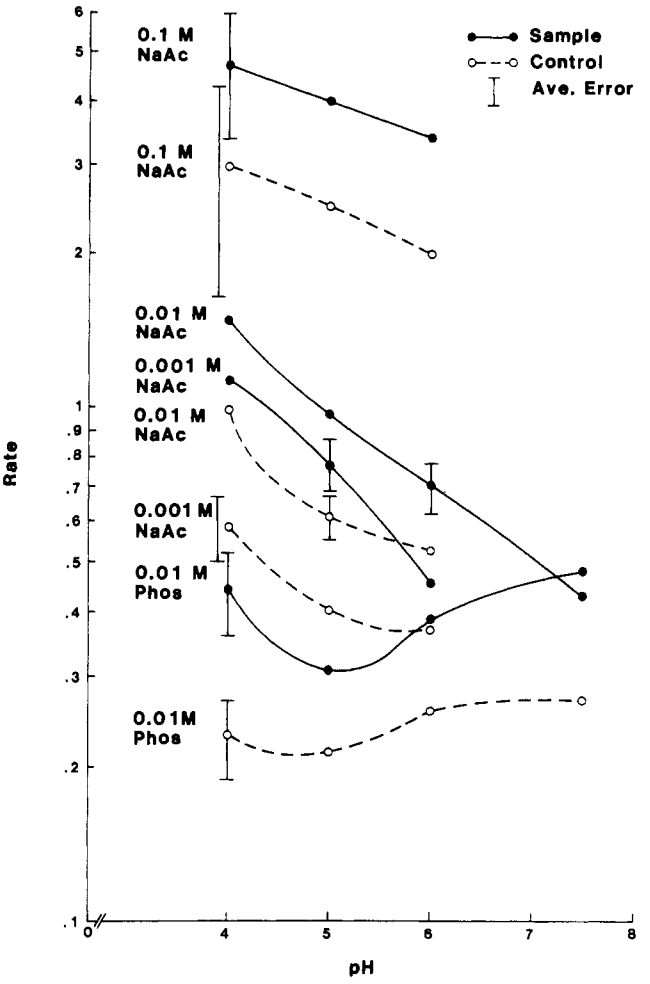

Figure 2. Rate of tryptophan oxidation (accumulation kyn and NFK) as a function of pH and ionic strength. Rates are slopes, (moles of kyn + NFK)/hour, from plots of log [(initial Trp) − (av kyn + NFK)]/(initial Trp) vs. time. The error was determined by calculating the mean differences in the slopes of triplicate samples. Control samples contained Trp, phosphate or sodium acetate buffer, and Tween 80. Treated samples contained Trp, phosphate or sodium acetate buffer, Tween 80, and methyl linoleate.

the study by Cuq and Cheftel (1983) the dependence of rate on pH was more pronounced than that observed here, but this is not unexpected since their experimental conditions and procedures were quite different from those used here (greater concentration of Trp, higher temperature, measured Trp remaining, lipids not present). In the study by Marcuse (1962) the dependence of rate on pH was quite close to that observed here, and this is no doubt attributable to the similarity in experimental conditions (Trp concentration, presence of lipids and an emulsifier), although he measured oxygen consumption rather than accumulation of oxidation products. In the study by Kanner and Fennema (following paper in this issue), the rate of accumulation of kyn and NFK was far greater at pH 7.5 than at any of the other pHs studied (4, 5, 6), rather than being a small difference as observed here. The discrepancy is probably attributable to experimental differences; i.e., the Kanner and Fennema study involved photooxidation of Trp in the presence of riboflavin.

Other studies involving Trp oxidation in an acetate buffer apparently have not been published.

Ionic Strength. The rate of Trp oxidation increased as the ionic strength of the sodium acetate buffer was increased from 0.001 to 0.1 M (Figures 2 and 3). Accounts

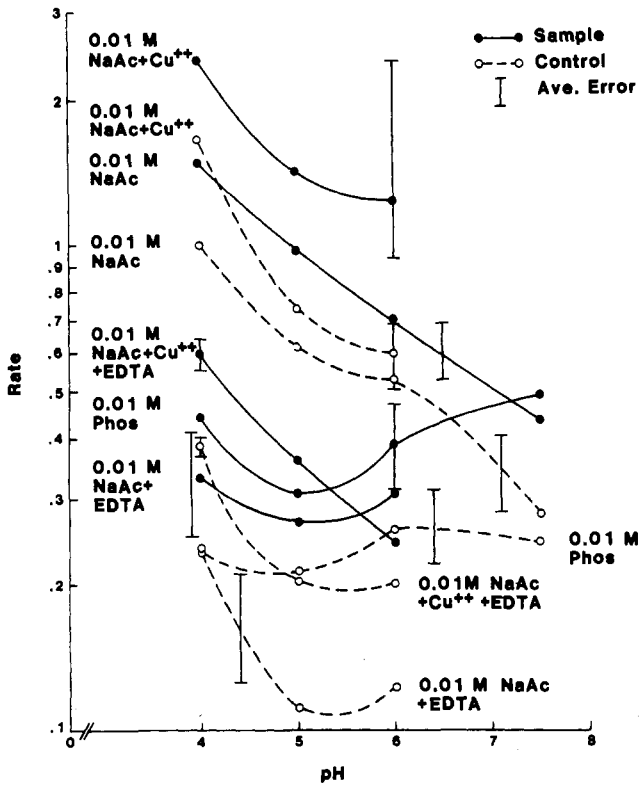

Figure 3. Rate of tryptophan oxidation (accumulation of kyn and NFK) as a function of pH, copper, and EDTA. Rates are slopes, (moles of kyn and NFK)/hour, from plot of log [(initial Trp) − (av kyn + NFK)]/(initial Trp) vs. time. The error was determined by calculating the mean differences in the slopes for each of the triplicate samples. EDTA was used at a concentration of 20 ppm and copper at a concentration of 5 ppm. Control samples contained Trp, phosphate or sodium acetate buffer, Tween 80, plus EDTA, Cu, or both EDTA and Cu. Treated samples contained Trp, phosphate or sodium acetate buffer, Tween 80, and methyl linoleate, plus EDTA, Cu, or both EDTA and Cu.

of other model systems employing different ionic strengths of sodium acetate buffer were not found in the literature.

The sodium acetate buffer apparently contained trace amounts of some metals judging from the reduction in rate when EDTA was added (Figure 3). Therefore, it is probable that the increased rate of oxidation resulting from increased buffer concentration was, in reality, the result of an increased concentration of trace metals. It is well-known that trace metals will decrease the induction period and increase the overall rate of oxidation of lipids and this, in turn, would likely increase the rate of Trp oxidation (Labuza, 1971; Tjhio and Karel, 1969).

Metal Catalysts. Added copper (5 ppm) increased the rate of Trp oxidation (Figure 3). Although comparable studies were not found, the result is in agreement with that of Farag et al. (1978). They studied a model system containing linoleic acid, Trp, and water and found, not surprisingly, that the rate of oxygen uptake (a direct measurement of Trp was not made) increased greatly when copper was added. Copper decreases the activation energy of the initiation step of lipid oxidation by directly transferring free radicals to Trp (Labuza, 1971; Schaich, 1980). The amount of copper used in the study reported here is not much greater than that often found naturally in foods, so the results have probable relevance to real situations.

Chelation. When EDTA (20 ppm) was added to Trp samples containing 0.01 M sodium acetate buffer, or when phosphate buffer was used in place of sodium acetate, the rate of oxidation, at almost all pHs studied, decreased

Table III. Comparison of Oxidation Rates of Tryptophan in Free and Bound Forms[a]

system	oxidn rate (20 °C, 96 h): kyn + NFK, mol/h control[b]	sample[c]
Gly-L-Trp-Gly, 0.01 M phosphate buffer, pH 7.5[d]	0.348 ± 0.04	0.508 ± 0.04
free Trp, 0.01 M NaOAc buffer, pH 7.5[e]	0.279 ± 0.08	0.442 ± 0.08
free Trp, 0.01 M phosphate buffer, pH 7.5	0.260 ± 0.08	0.480 ± 0.08

[a] Means of triplicate determinations. [b] Controls contained phosphate or acetate buffer, water, Trp and Tween 80. [c] Samples contained phosphate or acetate buffer, methyl linoleate, Trp, and Tween 80. [d] Trp concentration in tripeptide 0.0031 M. [e] Free Trp concentration 0.0033 M.

substantially compared to that in 0.01 M sodium acetate (Figure 3). Both EDTA and phosphate buffer are chelating agents that inactivate metal catalysts by blocking their reaction sites (Labuza, 1971).

When both copper (5 ppm) and EDTA (20 ppm) were added to a 0.01 M sodium acetate buffer system, the rate of oxidation decreased by more than 50% compared to that observed when only copper (5 ppm) was added (Figure 3). A similar study was not found in the literature. However, Labuza et al. (1971) measured oxygen uptake in a freeze-dried model system containing methyl linoleate, protein, EDTA, and cobalt, and the results were in general agreement with those reported here.

Tripeptide Model System. The rate of oxidation of the tripeptide (Gly-L-Trp-Gly) sample in phosphate buffer at pH 7.5 was 0.508 mol/h for the test sample and 0.348 mol/h for the control (Table III). The control samples consisted of tripeptide in phosphate buffer and Tween 80 (no methyl linoleate). The rate at which the peptide-bound Trp underwent oxidation is greater than that found for free Trp in either the sodium acetate or phosphate buffer. Trp in the tripeptide may have exhibited a greater rate of oxidation than that of free Trp because free Trp has antioxidative properties (Marcuse, 1962).

Although reports of other studies dealing with oxidation of peptide-bound Trp in the presence of lipids were not found, several studies involving photooxidation of Trp have indicated, as was found here, that blocking of the α-amino group of Trp enhances the rate at which it undergoes oxidation (Benassi et al., 1967; Pirie and Dilley, 1974; Tassin and Borkman, 1980). However, Kanner and Fennema (following paper in this issue), in a study involving riboflavin-sensitized photooxidation of Trp in di- and tripeptides obtained results contrary to the above.

Controls. It can be seen that Trp oxidizes readily in control systems when methyl linoleate was not present (Figures 2 and 3). However, the oxidation rate of controls never equalled or surpassed that of the treated samples at any given reaction time. Since lipid was not added to the control systems, initiation of oxidation must have occurred through the presence of trace amounts of contaminating lipids (unlikely) or contaminating metals or through inadvertent exposure to light. Since the flasks that contained the control samples were covered with foil up to the point at which a subsample was removed for injection into the HPLC, photooxidation would also seem unlikely. Thus, the most likely explanation for the behavior of the control samples is the presence of trace amounts of contaminating metals in the Trp, water, buffer, or Tween 80 components. Metals will transfer free radicals to Trp (Fontana and Toniolo, 1976), thus accelerating the rate of oxidation as compared to that of control samples

in which trace metals are not present.

General Discussion. From the data in Figures 2 and 3, it is evident that Trp oxidizes readily and that the rate is dependent on pH, ionic strength, water activity, buffer concentration, and temperature.

It was noted by Karel (1980) that high or low water activities act to accelerate the rate of both lipid and protein oxidation. All model systems tested here had very high water activities, which is a point of difference as compared to most proteinaceous foods since the latter usually have intermediate water activities. It is likely, therefore, that the rates of oxidation found in these experiments are greater than those that would be found in typical proteinaceous foods, with the exception of very dry foods, which can oxidize very rapidly.

It should also be noted that the experiments reported here involved small, shallow samples that were stirred to encourage contact with air. These conditions would also tend to produce a greater rate of oxidation than that occurring in typical foods.

Although the samples in this study were held at room temperature, this is not necessarily an exaggerated test condition with respect to rates of oxidation since many commercial foods are exposed to temperatures more stimulatory to oxidation (freezing, high-temperature processing).

In conclusion, it can be stated that Trp is highly susceptible to oxidation and this is of concern since (1) Trp is an essential amino acid that is not abundant in foods and (2) the major oxidation products (kyn, NFK) are suspected carcinogens. In the results reported here approximately 1–2% of the Trp in either free form or bound in a tripeptide (no added catalyst or inhibitor) was converted to kyn and NFS in just 72 h at room temperature. Since commercial foods are stored for much longer periods than 72 h, it is possible that considerable amounts of kyn and NFK may accumulate, if care, in the form of proper packaging and storage conditions, is not exercised.

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Registry No. EDTA, 60-00-4; kyn, 343-65-7; NFK, 1022-31-7; L-Trp, 73-22-3; Gly-L-Trp-Gly, 23067-32-5.

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