Photooxidation of Tryptophan in the Presence of Riboflavin

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Solutions of tryptophan (free and bound in various peptides, with a Trp concentration of 1.0×10^{-3} M) containing 2.1×10^{-5} M riboflavin were exposed to fluorescent light, and the oxidation products, kynurenine (kyn) and *N*-formylkynurenine (NFK), were measured by HPLC. In the range pH 4–7.5, kyn and NFK accumulated most rapidly at pH 7.5. With dipeptides (Trp-Gly; Gly-Trp), the rate of Trp oxidation was greatest when Trp was bound on the carboxyl side. Binding of Trp within a tripeptide (Gly-Trp-Gly) resulted in the smallest reaction rate, while Trp bound on the amino side (Gly-Trp) exhibited a reaction rate intermediate between those of Gly-Typ-Gly and Trp-Gly. Of the three antioxidants tested, EDTA was the least effective in retarding oxidation of Trp, and sodium azide and L-ascorbic acid were equally effective.

Oxidative reactions have long been of interest in foods. Products of lipid and protein oxidation can affect the sensory qualities, shelf stability, nutritional value, and safety of foods. Oxidative reactions are usually avoided, if possible, but instances do occur where they are encouraged, e.g. protein oxidation during bread dough preparation and bleaching of milk for some kinds of cheeses.

Several amino acids are subject to oxidation (cystine, histidine, methionine, tyrosine, tryptophan), and conditions need not be severe (Cheftel et al., 1985; Feeney, 1980; Yong et al., 1980). These amino acids can be oxidized by peroxidizing lipids, by irradiation (ionizing or light) in the presence of oxygen, or by standard oxidizing agents such as peroxides (Yong and Karel, 1978a, 1978b, 1979; Yong et al., 1980; Finot, 1982).

Tryptophan (Trp) was chosen for this study since it is an essential amino acid and yields oxidation products of interest. Trp residues are easily oxidized by hydrogen peroxide or atmospheric oxygen, by photooxidation, or by irradiation in the presence of oxygen (Finot, 1982; Takahashi, 1984; Tassin and Borkman, 1980; Yong et al., 1980). Some of the oxidation products reported are kynurenine (kyn), *N*-formylkynurenine (NFK), 3-hydroxyanthranilic acid, and dioxidindole-3-alanine (Benassi et al., 1967; Gurnani et al., 1966; Walrant et al., 1975). kyn is carcinogenic when injected into animal bladders (Bryan et al., 1964; Matsushima et al., 1982), and Trp degradation products, as a group, inhibit growth of cultured mouse embryonic fibroblasts (Zigman et al., 1978) and exhibit mutagenic activities (Matsumoto et al., 1976). A tripeptide containing oxidized Trp has been shown to be less well absorbed than the unoxidized counterpart (Finot, 1982). Oxidative rupture of the 2,3-indole bond in Trp of proteins could lead to proteins containing NFK or kyn in place ot Trp. Such changes would be expected to lead to marked changes in the physical, chemical, and biological properties of these proteins.

The very reactive nature of the indole nucleus of Trp has led to extensive work on its photochemistry. Major differences in the studies have included state of the Trp (free vs. protein bound) and source of the light. Results of experiments involving flash photolysis (Bent and Hayon, 1975; Grossweiner and Usui, 1971; Lion et al., 1982; Pailthorpe and Nicholls, 1971; Pailthorpe et al., 1973; Santus and Grossweiner, 1972; Subramanyan and Tollin, 1972; Templer and Thistlethwaite, 1976), X-rays (Jayson et al., 1954), UV light (Asquith and Rivett, 1971; Fujimori, 1980; Pirie, 1971; Singh et al., 1984; Slawinski et al., 1980; Sun and Zigman, 1979; Tassin and Borkman 1980), and dye-sensitized illumination (Benassi et al., 1967; Brake and Wold, 1960; Gomyo and Fujimaki, 1970; Gurnani et al., 1966; Jori and Galiazzo, 1971; Nakagawa et al., 1981; Savage, 1971; Tanielian et al., 1984) have appeared in the literature. Care must be taken when comparing results from experiments using different sources of light (Fontana and Toniolo, 1976).

The reaction mechanisms involved in photooxidation of Trp are complex and depend on numerous factors (e.g., pH, oxygen, concentration of Trp, wavelength of light, concentration of sensitizer). Despite these complexities, generalizations have been made and reaction schemes proposed (Asquith and Rivett, 1971; Fontana and Toniolo, 1976; Foote and Dobrowlski, 1984; Pailthorpe and Nicholls, 1971; Singh et al., 1984; Slawinski et al., 1980; Sun and Zigman, 1979).

In this study Trp, either free or bound in a peptide, is photooxidized in the presence of riboflavin, and the rate of accumulation of kyn and NFK is measured. The following factors are examined as they relate to the rate of this reaction: (1) pH and type of buffer; (2) temperature; (3) binding of Trp within a di- or tripeptide; (4) the presence of antioxidants. A study of this kind has not been reported in the literature. In a companion study (Krogull and Fennema, previous paper in this issue), Trp oxidation in the presence of oxidizing methyl linoleate was investigated.

MATERIALS AND METHODS

Sample Preparation and Handling. Test samples were designed to contain Trp and riboflavin at concentrations similar to those existing in milk. The approximate concentration of Trp in whole milk is 2.3×10^{-3} M (Brunner, 1976; Swaisgood, 1985). The concentration used in these model systems was always 1.0×10^{-3} M. This lower concentration was used to ensure that Trp-containing peptides could be solubilized.

The approximate concentration of riboflavin in whole milk is 4.0×10^{-6} M (Korycka-Dahl and Richardson, 1978; Swaisgood, 1985). The concentration used in these model systems was 2.1×10^{-5} M, in approximate accord with other experiments using riboflavin or proflavine as a photosensitizer (Benassi et al., 1967; Silva and Barrera

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Table I. Composition of Samples

type of sample	characteristics: 25 mL, 0.01 M phosphate buffer (pH 7.5)[a]	1.0×10^{-3} M Trp[b]	2.1×10^{-5} M riboflavin	light exposure[c]
experimental	×	×	×	×
control T	×	×		×
control TR	×	×	×	

[a] ACS grade; J.T. Baker Chemical Co., Phillipsburg, NJ. [b] Sigma grade; Sigma Chemical Co., St. Louis, MO. [c] Fluorescent light, 470 ft-c.

1985; Sluyterman, 1962; Vehara et al., 1971).

A pH of 7.5 was used throughout this study (except when pH was studied as a variable). This pH was selected since it provides conditions most amenable to dye-sensitized photooxidation (Iborra et al., 1977; Nakagawa et al., 1981; Papeschi et al., 1982; Sluyterman, 1962; Weil, 1965; Weil et al., 1951).

Test samples (Table I) were prepared by pipetting 25-mL portions of the appropriate stock solutions into 50-mL beakers containing 1-in. stir bars. These transfers were done in a dark room. Beakers containing the TR control samples were covered with aluminum foil thereafter. Beakers containing the other samples were covered with Saran wrap secured with rubber bands.

The beakers were transferred to a refrigerator (3 °C), and the light-exposed samples were placed such that their liquid surfaces were approximately 30 cm from a fluorescent light source (two 40-W, cool-white, Westinghouse), yielding an average intensity of 470 ft-c as measured by a Gossen Panlux electronic footcandle meter (Berkey Marketing Co., Inc., Burbank, CA).

The samples were stirred at a moderate rate magnetically. By placing the beakers directly on the magnetic devices and carefully regulating the stirring rate, a sample temperature of 20 ± 2 °C was maintained.

Photooxidation was allowed to proceed under these conditions for at least 72 h. At 24-h intervals pH and temperature were measured, and 1-mL samples were withdrawn for analysis by high-performance liquid chromatography (HPLC). The withdrawn samples were stored in aluminum foil covered beakers at 3 °C until analyzed.

Analysis of Tryptophan Degradation Products by HPLC. The HPLC unit used in these experiments was a Model 332 gradient liquid chromatograph (Altex Scientific, Inc., Berkeley, CA) equipped with either a fixed-wavelength director (Altex Science, Model 153) or an Isco V[4] variable-wavelength detector (Isco, Inc., Lincoln, NE).

A Hibar EC, LiChrosorb RP-18, 10 μm, 250 mm × 4.0 mm, analytical column (EM Reagents, Associate of E. Merck, Darmstadt, Germany) was used to separate reaction products.

The mobile phase consisted of 0.01 M sodium acetate (ACS) adjusted to pH 4 with acetic acid and either acetonitrile or methanol (both HPLC grade; EM Science, Cherry Hill, NJ). When methanol was used, the mixture consisted of 90% 0.01 M sodium acetate and 10% methanol. When acetonitrile was used, it comprised 5% of the mobile phase. Whether operating isocratically or by gradient, all components of the mobile phase were filtered and degassed prior to passage through the instrument at a rate of 1.2 mL/min. kyn and NFK were monitored at a wavelength of 254 nm.

kyn and NFK standards were injected each day that unknown samples were analyzed. It was also established at the beginning of these studies that the recorder response was linear with respect to concentrations of kyn and NFK over the range of concentrations obtained during photooxidation. In addition, it was established that the lower limit of sensitivity was approximately 10^{-7} g/mL buffer for both components (4.8×10^{-7} M NFK).

Retention times of kyn and NFK were obtained for every injection and compared to those of standards as a means of identification. Additionally, internal standards of kyn and NFK were regularly added to unknown samples to aid in the identification of these compounds.

Study of pH. Two buffer systems were used to establish pH 4, 5, 6, and 7.5. At pH 5 and 6, both buffers were tested to determine whether a buffer dependence existed. Potassium phosphate buffers, 0.01 M, were used at pH 5, 6, and 7.5 and sodium acetate buffers, 0.01 M, were used at pH 4, 5, and 6.

pH was monitored with a pH meter throughout the reaction period. With the exception of the potassium phosphate buffer at pH 5, pH fluctuated less than ±0.1 pH unit over the period of the experiment. With the pH 5 potassium phosphate buffer, the pH dropped by 0.2 pH unit during the 72-h reaction period.

Study of Temperature. Temperatures of 8 ± 2 and 20 ± 2 °C were investigated.

Study of Peptide-Bound Tryptophan. The three peptides used were glyclyl-L-tryptophan (Gly-L-Trp; monohydrate), L-tryptophylglycine (L-Trp-Gly), and glycyl-L-tryptophylglycine (Gly-Trp-Gly) (purity unknown, all from Research Plus, Inc., Bayonne and Denville, NJ). All were used without further purification. These peptides were chosen because glycine lacks an obstructive side chain that could otherwise affect the photoreaction.

The amount of peptide used in each experiment was such that the concentration of Trp was 1.0×10^{-3} M. Gly-L-Trp (0.007 g), L-Trp-Gly (0.0065 g), or Gly-Trp-Gly (0.008 g) each was combined with 25 mL of 0.01 M potassium phosphate in 50-mL beakers. Experiments were carried out at both 20 ± 2 and 8 ± 2 °C. Samples of 1 mL each were withdrawn every 24 h for hydrolysis and HPLC analysis.

Due to the hydrolysis procedure, there was always a 24-h lag period between hydrolysis and HPLC analysis. Hydrolysis with Pronase (Calbiochem, La Jolla, CA) was done essentially by the method of DeVries et al. (1980). A control containing Pronase alone was also tested to determine whether the enzyme contributed significant amounts of substances that interfered with the analysis of kyn and NFK. A standard containing both kyn and NFK was also exposed to the same hydrolysis treatment as a means of quantifying the results. In addition, free Trp (1.0×10^{-3} M) was photooxidized in the same way discussed earlier and exposed to the above-mentioned hydrolysis conditions. Control samples similar to those described earlier were included for the photooxidation studies of both the peptides and free Trp.

The retention times and parameters for peptide peaks were measured and compared to peaks from peptide standards to determine the precent hydrolysis achieved.

Study of Antioxidants. The types of samples listed in Table II were prepared in 50-mL beakers. They were prepared in the same manner as mentioned earlier, except that one of three antioxidants was added to the appropriate beakers as outlined above. The three samples without antioxidant were simply repeats of samples described earlier. Temperature was maintained at 8 ± 2 °C throughout the antioxidant study.

RESULTS AND DISCUSSION

General Observations. In all studies, test samples showed a marked color change within 24 h. The characteristic yellow-green of riboflavin gradually gave way to

Table II. Composition of Samples Used for Testing Antioxidants

type of sample	characteristics				
	25 mL, 0.01 M phosphate buffer (pH 7.5)	1.0×10^{-3} M Trp	2.1×10^{-5} M riboflavin	antioxidant[a]	light exposure[b]
TR + antioxidant	×	×	×	×	×
TR	×	×	×		×
control TA	×	×		×	×
control T	×	×		×	×
control TRA	×	×	×	×	
control TR	×	×	×		

[a] Sodium azide (1.0×10^{-2} M), ethylenediaminetetraacetic acid (EDTA; 1×10^{-3} M), or L-ascorbic acid (5.7×10^{-3} M), all from Sigma Chemical Co., St. Louis, MO. [b] Fluorescent light, 470 ft-c.

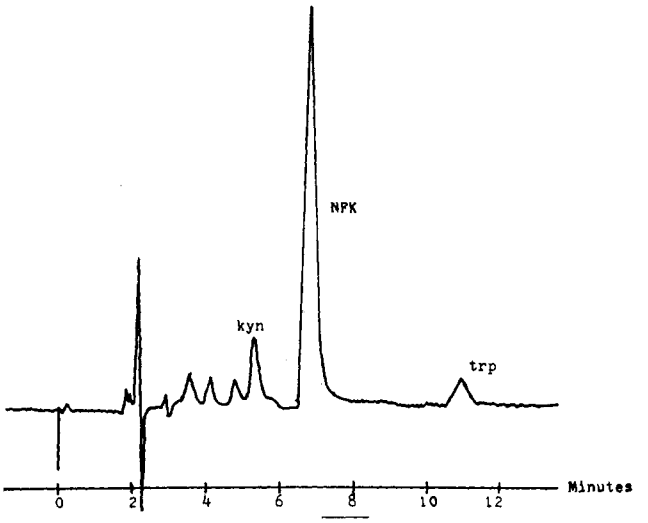

Figure 1. Separation of Trp and its photooxidation products by reversed-phase HPLC. Conditions: column, LiChrosorb RP-18, 250 mm × 4.0 i.d.; mobile phase, sodium acetate buffer (0.01 M, pH 4)–methanol (90:10); sample, 1.0×10^{-3} M Trp, 2.1×10^{-5} M riboflavin, 25 mL, 0.01 M phosphate buffer, 72-h reaction time; flow rate, 1.2 mL/min; detector, UV 254 nm, 0.02 Au.

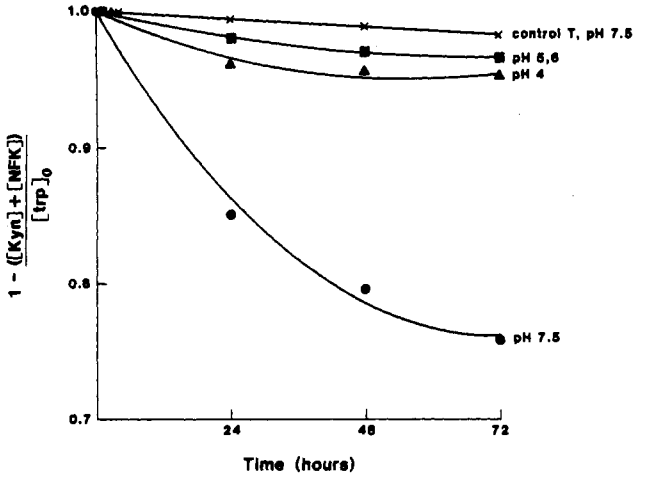

Figure 2. Influence of pH and buffer on the riboflavin-sensitized photooxidation of Trp to NFK and kyn. Ordinate values represent the fraction of Trp remaining (or converted to degradation products other than kyn or NFK). Conditions: temperature, 20 ± 2 °C; original Trp concentration, 1.0×10^{-3} M; original riboflavin concentration, 2.1×10^{-5} M; control, light exposed, no riboflavin present, pH 7.5 (control with greatest rate of reaction). Curves for test samples were drawn from mathematical formulas, whereas the curve for the control sample was fit visually. Experimental values are means of four replicates, and control values are means of two replicates.

a burntlike brown color. The light-exposed controls containing Trp (no riboflavin) turned from a clear solution to one that was slightly brown. The light-protected controls containing Trp and riboflavin (yellow-green tint) showed no color change during storage.

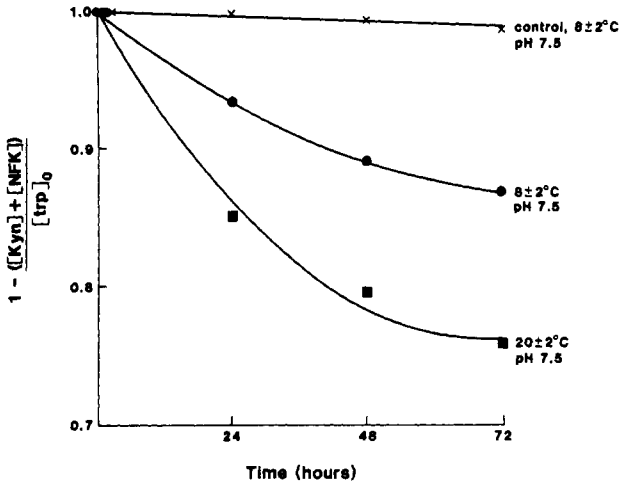

Figure 3. Influence of temperature on the riboflavin-sensitized photooxidation of Trp to NFK and kyn. Ordinate values represent the fraction of Trp remaining (or converted to degradation products other than kyn or NFK). Conditions: buffer, 0.01 M potassium phosphate, pH 7.5; original Trp concentration, 1.0×10^{-3} M; original riboflavin concentration, 2.1×10^{-5} M; control, light exposed, no riboflavin present, pH 7.5, 8 ± 2 °C (control with greatest rate of reaction). Curves for test samples were drawn from mathematical formulas, whereas the curve for the control sample was fit visually. Values are means of at least six replicates.

Changes in odor seemed to parallel changes in color. As the above-mentioned color changes took place, the smell became more and more burntlike. By the end of 72 h, the test samples had an extremely malodorous, bruntlike odor. The light-exposed controls containing Trp (no riboflavin) developed only a slight odor, while the odor of the light-protected controls did not change during 72 h of storage.

Typical High-Pressure Liquid Chromatograms. Regardless of whether methanol (10%) or acetonitrile (5%) was used in the mobile phase, each of the three components, kyn, NFK, and Trp, eluted within 12 min (Figure 1). The approximate retention times of the above components were 5.3, 6.8, and 11.0 min, respectively. Resolution was generally very good. By the end of 48 h several compounds with hydrophobicities greater than Trp appeared as peaks with retention times greater than 12 min. Riboflavin and its photoproducts usually required at least 15% acetonitrile (or 40% methanol) in the mobile phase to achieve elution within 25 min. If gradient HPLC was not used, riboflavin was allowed to accumulate on the column and washed off with 50% acetonitrile (or 70–80% methanol) every five to seven injections.

Results of Various Trials. Results of the various experiments are shown in Figures 2–5. The values that appear in these figures were calculated from

$$1 - [[NFK] + [kyn]]/[Trp]_0$$

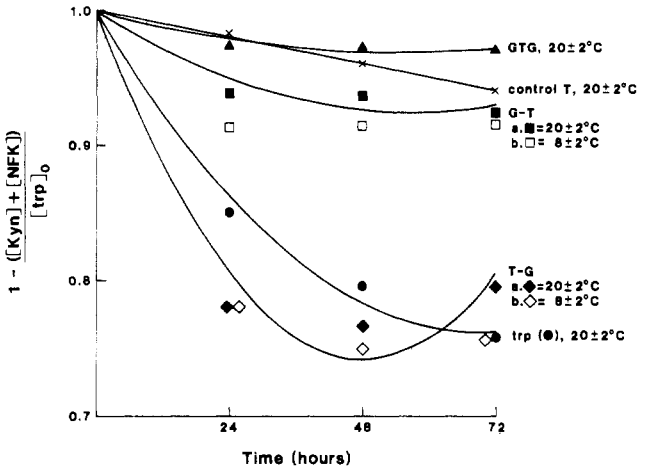

Figure 4. Rate of riboflavin-sensitized photooxidation of free and peptide-bound Trp to NFK and kyn. Ordinate values represent the fraction of Trp remaining (or converted to degradation products other than kyn or NFK). Conditions: buffer, 0.01 M potassium phosphate, pH 7.5; original Trp concentration, 1.0×10^{-3} M; original riboflavin concentration, 2.1×10^{-3} M; temperature, 20 ± 2, 8 ± 2 °C; control, T-G, light exposed, no riboflavin present, pH 7.5, 20 ± 2 °C (control with greatest rate of oxidation). Curves for test samples were drawn from mathematical formulas, whereas the curve for the control sample was fit visually. Values are means of a least three replicates.

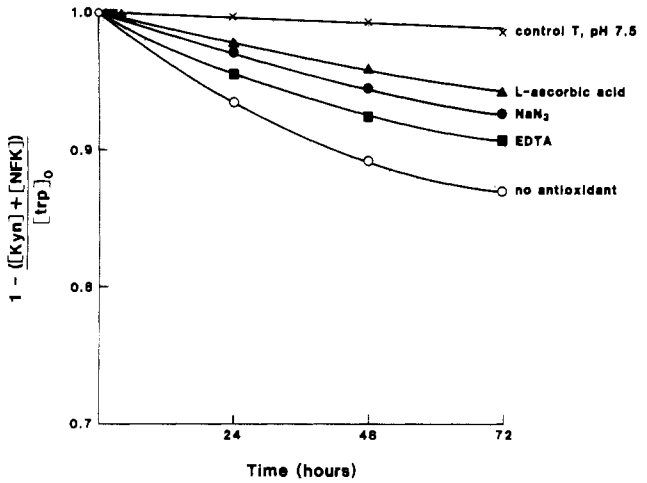

Figure 5. Influence of antioxidants on the rate of riboflavin-sensitized photooxidation of Trp to NFK and kyn. Ordinate values represent the fraction of Trp remaining (or converted to degradation products other than kyn or NFK). Conditions: buffer, 0.01 M potassium phosphate, pH 7.5; original Trp concentration, 1.0×10^{-3} M; original riboflavin concentration, 2.1×10^{-3} M; temperature, 8 ± 2 °C; antioxidant concentration, 0.01 M sodium azide, 0.001 M EDTA, 5.68×10^{-3} M L-ascorbic acid; control, light exposed, no riboflavin, 7.5 (control with greatest rate of reaction). Curves for test samples were drawn from mathematical formulas, whereas the curve for the control sample was fit visually. Values are means of at least three replicates.

where $[Trp]_0$ was the original concentration of Trp. In all of the test samples studied, the rate of photooxidation of Trp to NFK and kyn could be accurately represented by a quadratic equation of the type

$$y = \beta_1 t^2 + \beta_2 t + 1$$

Equations are presented for all studies with the exception of those involving controls, which did not obey the same type of equations. Curve similarity was determined by Hotelling's T^2 test (Pillai, 1983) using a 0.01 level of confidence.

The best fit quadratic equations (plotted in Figure 2) defining the influence of pH and buffer on the rate of riboflavin-sensitized photooxidation of Trp to NFK and kyn are as follows:

pH	buffer (0.01 M)	equation
7.5	KH_2PO_4	$y = 0.029t^2 - 0.17t + 1$
6.0	KH_2PO_4	$y = 0.0038t^2 - 0.023t + 1$
6.0	CH_3CO_2Na	$y = 0.0044t^2 - 0.025t + 1$
5.0	KH_2PO_4	$y = 0.0052t^2 - 0.025t + 1$
5.0	CH_3CO_2Na	$y = 0.0046t^2 - 0.026t + 1$
4.0	CH_3CO_2Na	$y = 0.0092t^2 - 0.043t + 1$

where y is represented by the fraction

$$1 - [[NFK] + [kyn]]/[Trp]_0$$

and t is in days.

As is evident from data in Figure 2, oxidation of Trp to NFK and kyn occurred most rapidly at pH 7.5, with the rate decreasing as the pH was decreased to 4–6. Furthermore, the photooxidation rate of Trp in the control sample (control T, pH 7.5) was significantly slower than that of any of the test samples.

All of the curves presented are statistically different; however, only one curve has been drawn to demonstrate the effects of pH 5 and 6 for both buffer systems since all combination of these four variables gave the same results.

Because none of the work cited in the literature involved measurement of the rate of photooxidation of trp to NFK and kyn, it is not possible to make a quantitative comparison of results reported here with any other results. However, a few qualitative comparisons can be made.

The rapid rate of Trp photooxidation at pH 7.5 is in accord with the results of several other investigators (Iborra et al., 1977; Nakagawa et al., 1981; Papeschi et al., 1982; Sluyterman, 1962; Weil, 1965; Weil et al., 1951). The reaction rate–pH relationship, yielding a minimum rate at pH 5–6 with greater rates at higher or lower pHs, is in approximate agreement with results of Sluyterman (1962).

The best fit quadratic equations (plotted in Figure 3) defining the influence of temperature on the rate of Trp oxidation are as follows:

temp, °C	equation
8 ± 2	$y = 0.011t^2 - 0.076t + 1$
20 ± 2	$y = 0.029t^2 - 0.17t + 1$

As is evident from data in Figure 3, oxidation of Trp to NFK and kyn increased substantially as the temperature was increased from 8 to 20 °C. The rate of Trp oxidation in the control sample at 8 °C was appreciably slower than that of the test sample at 8 °C.

Experiments by Sluyterman (1962) and Weil (1965) demonstrated a similar temperature dependence. It should be pointed out, however, that the former investigator used proflavin as the sensitizer, while the latter used methylene blue. Also, neither investigator determined the rate at which Trp was photooxidized to NFK and kyn.

The best fit quadratic equations describing the influence that binding of the amino group, the carboxyl group, or both have on riboflavin-sensitized photooxidation of Trp to NFK and kyn are as follows (G = glycine, T = L-Trp):

peptide	temp, °C	equation
GTG	20 ± 2	$y = 0.0058t^2 - 0.027t + 1$
GTG	8 ± 2	$y = 0.0057t^2 - 0.025t + 1$
T-G	20 ± 2	$y = 0.064t^2 - 0.26t + 1$
T-G	8 ± 2	$y = 0.059t^2 - 0.26t + 1$
G-T	20 ± 2	$y = 0.013t^2 - 0.064t + 1$
G-T	8 ± 2	$y = 0.023t^2 - 0.096t + 1$
Trp	20 ± 2	$y = 0.029t^2 - 0.17t + 1$

Data for 8 and 20 °C are plotted in Figure 4, except for omission of the 8 °C data for sample GTG. Curves of best

fit are drawn only for the 20 °C data.

No significant difference exists between the rate of riboflavin-sensitized photooxidation of peptide-bound Trp (GTG) at 20 ± 2 and 8 ± 2 °C. A change in temperature from 8 to 20 °C had only a small influence on the rates at which G-T and T-G oxidized. At 20 ± 2 °C, the rate of oxidation of free Trp vs. Trp bound within the various peptides were all statistically different (0.01 level of confidence). The initial rate was greatest for Trp bound on the carboxyl side (T-G). Binding of both the α-amino and α-carboxyl groups of Trp resulted in the smallest reaction rate (GTG). It should be noted that the control that showed the greatest rate of reaction contained T-G (light exposed, no riboflavin, 20 ± 2 °C). This rate exceeded slightly that of Trp oxidation in the tripeptide.

The positive slope of the 20 °C curve for sample T-G after 48 h is believed to be real since this result was obtained in three separate experiments. Furthermore, this result is not regarded as surprising since this sample underwent the greatest conversion of Trp to kyn and NFK and would, therefore, be most likely to have significant amounts of kyn and NFK lost through subsequent conversion to other degradation products.

It should be noted that the extent of hydrolysis achieved during preparation for analysis was 85 ± 5% for the peptides GTG and G-T and 92 ± 5% for T-G. Therefore, the values reported for oxidation of Trp bound within these three peptides are underestimated as compared to the actual rates. A possible explanation for the great disparity in reaction rate between GTG and G-T on the one hand and T-G on the other is that more NFK and kyn remained peptide bound (were not hydrolyzed prior to analysis) in the former instance than in the latter. Indeed, several small peaks with retention times similar (but not equal) to the original peptides appeared on the chromatograms, but these peaks were not identified.

Several studies involving photooxidation of Trp and Trp-containing peptides have indicated that blocking of the α-amino group of Trp enhances the rate of Trp oxidation whereas blocking of the α-carboxyl group has no effect as compared to the rate of oxidation exhibited by free Trp (Benassi et al., 1967; Pirie and Dilley, 1974; Tassin and Borkman, 1980). These results are contrary to the results obtained here (Figure 4). The only explanation that can be offered is that important experimental conditions used by the other investigators were quite different from those used here. For example, Benassi et al. (1967) measured only kyn, used a pH of 4, used a different sensitizer, used an atmosphere of pure oxygen, and irradiated their samples much more intensely and for a substantially longer time than was done here. Pirie and Dilley (1974) did not use a sensitizer, and they irradiated their samples much more intensely than was done here. Tassin and Borkman (1980) did not use a sensitizer, they did use a very intense source of monochromatic radiation (290 nm), and they simply measured loss of substrate.

The best fit quadratic equations defining the influence of various antioxidants on the rate of photooxidation of Trp are as follows:

antioxidant	equation
none	$y = 0.011t^2 - 0.076t + 1$
0.01 M sodium azide	$y = 0.0025t^2 - 0.032t + 1$
0.001 M EDTA	$y = 0.0067t^2 - 0.051t + 1$
5.68×10^{-3} M L-ascorbic acid	$y = 0.0013t^2 - 0.023t + 1$

These equations are shown graphically in Figure 5. As expected, the rate of reaction without antioxidant was greater than that with it, regardless of which antioxidant was used. None of the antioxidants was sufficiently inhibitory to reduce the rate of oxidation to that of the control sample. The curve for EDTA is statistically different (0.01 confidence level) from those for L-ascorbic acid and sodium azide. The curves for L-ascorbic acid and sodium azide are not statistically different. The curve for the control sample is statistically different from all other curves.

The reduction in reaction rate in the presence of EDTA suggests that the system contained some metal ions. However, a direct effect of EDTA on the photooxidation reaction cannot be ruled out, and this possibility was not investigated by altering EDTA concentration.

Sodium azide exerts its antioxidant effect by acting as a $^1O_2$ scavenger (Cadenas and Sies, 1984), and L-ascorbic acid exerts its effect by functioning as an electron donor, a metal chelator, and/or an oxygen scavenger (Nawar, 1985). Since sodium azide retarded but did not entirely inhibit the photooxidation reaction, it seems likely that the reaction proceeds to some extent, but not entirely, by a type II ($^1O_2$) mechanism. This is in accord with suggestions of other researchers (Korycka-Dahl and Richardson, 1978; Nilsson et al., 1972; Saito et al., 1977) that dye-sensitized photooxidation need not proceed by means of $^1O_2$ alone.

Since the antioxidant effect of L-ascorbic acid involves several mechanisms, it is not possible to determine the major mechanism by which it functioned. A possible reason for its failure to completely inhibit the reaction is that it probably degraded with time, thus losing its potency as an antioxidant.

ACKNOWLEDGMENT

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Registry No. EDTA, 60-00-4; NFK, 1022-31-7; kyn, 343-65-7; L-Trp, 73-22-3; Trp-Gly, 7360-09-0; Gly-Trp, 2390-74-1; Gly-Trp-Gly, 23067-32-5; L-ascorbic acid, 50-81-7; riboflavin, 83-88-5; NaN_3, 26628-22-8.

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Identification and Quantification of Pyridoxine–β-Glucoside as a Major Form of Vitamin B_6 in Plant-Derived Foods[1]

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A high-performance liquid chromatographic procedure was adapted to the fluorometric measurement of a glucoside conjugate of vitamin B_6 compounds in foods. 5′-*O*-β-D-Glucopyranosylpyridoxine was identified by HPLC and NMR methods as the major glycosylated form of the vitamin. This conjugate, which has been shown to exhibit incomplete metabolic utilization as vitamin B_6, was found to comprise 5–70% of the total vitamin B_6 in selected fruits and vegetables. Pyridoxine–β-glucoside was not detected in animal-derived foods including meats, human milk, and cow's milk.

The existence of conjugated or "bound" forms of vitamin B_6 in various foods of plant origin has been suggested by the results of several studies. Yasumoto et al. (1977) isolated and identified 5′-*O*-β-glucopyranosylpyridoxine from rice bran. These workers evaluated the biological activity of the synthetic pyridoxine–β-glucoside (PN–glucoside) and reported that the compound was well absorbed in vitro and was well utilized as vitamin B_6 in bioassays with deficient rats (Tsuji et al., 1977). Kabir et al. (1983a) devised an indirect microbiological assay procedure for the quantitation of β-glycosylated forms as well as total vitamin B_6 in foods. The results of this assay and studies of vitamin B_6 bioavailability in human subjects suggested that β-glycosylated forms of the vitamin were not biologically available (Kabir et al., 1983b). In contrast to the results of Tsuji et al. (1977), we have recently shown that PN–glucoside is well absorbed but undergoes little metabolic utilization in rats (Ink et al., 1986).

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