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Tryptophan Degradation During Heat Treatments: Part 1—The Degradation of Free Tryptophan

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ABSTRACT

No tryptophan loss was observed when a 10 mM tryptophan solution was heated at 90 to 140°C for 1·5 to 24 h in the absence of oxygen. When the thermal treatment was carried out in air or in oxygen, tryptophan degradation followed first order kinetics. The energy of activation of the oxidative degradation was found to approximate 80 000 J mole^{-1}. The rate of degradation increased with pH in the range 5–8. Up to five degradation products were detected by gel chromatography and were found to be UV-absorbing but ninhydrin-negative. There was a good correlation between direct spectrofluorimetric and HPLC–UV determinations of tryptophan.

INTRODUCTION

Tryptophan is an essential amino acid for man and for many other animals. Some of its derivatives, like serotonin, have neurophysiological or pharmacological effects. It is therefore of interest to investigate whether or not free tryptophan and protein-bound tryptophan are degraded through food processing, and especially during thermal treatments.

Few data are so far available concerning the effects of thermal treatments on free tryptophan. Sugimura *et al.* (1977) have shown that, above 200 °C, various β-carbolines are formed, and that some of these

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have a marked mutagenicity (Ishii *et al.*, 1980; Moule, 1980; Nagao *et al.*, 1980; Matsukura *et al.*, 1981*a*, *b*). Haidar *et al.* (1981) have identified the gases formed by tryptophan pyrolysis at 850 °C.

The effects of moderate thermal treatments have been studied by Stewart & Nicholls (1974): at 100 °C, in the presence of air, the maximum rate of degradation of tryptophan was observed at pH 5·3; the degradation products were not identified.

Some authors (Brautigam & Severin, 1974; Dworschak & Orsi, 1977; Orsi & Dworschak, 1978; Lee *et al.*, 1979) have suggested that, when heated in the presence of reducing carbohydrates, tryptophan could undergo Maillard reactions, either through its α amino group or through the nitrogen atom of the indole ring. This latter reaction, if it occurred, for example in sterilized or dehydrated milk or in milk–cereal mixes, could have detrimental nutritional effects.

The present study investigates the degradation of tryptophan during thermal treatments in a 90–140 °C temperature range. The effects of oxygen, of time and of pH were also investigated. Tryptophan was determined mainly by HPLC and UV absorbance.

MATERIALS AND METHODS

Reagents

Tryptophan and tryptamine were from Merck, kynurenin and *N*-formyl kynurenin from Calbiochem. Nitrogen ('U', less than 5 ppm O_2) and oxygen ('R') came from l'Air Liquide.

Thermal treatments

Influence of temperature and oxygen

Two or four millilitres of a solution of tryptophan (10 mM) in distilled water are pipetted into 18 × 180 mm pyrex tubes, which are then heat-sealed at atmospheric pressure under air, nitrogen or oxygen. In the last two cases, before heat sealing, the tryptophan solution is first frozen in liquid nitrogen and evacuated under a vacuum of $0{\cdot}4 \times 10^{-3}$ bars while slowly reheated, in order to remove dissolved gases.

Thermal treatments are carried out in an air-oven at 90, 100, 110, 121, 125 or 140 °C (±1·5 °C) for 1·5, 3, 6, 9 or 24 h.

Influence of pH

Four millilitres of a solution of tryptophan (10 mM) in 0·1 M sodium phosphate buffers pH 5, 6, 7 or 8, are pipetted into pyrex tubes, which are then heat-sealed at atmospheric pressure under air. Thermal treatments are carried out at 121 ± 1·5 °C for 6·5, 10·5 or 24 h.

After the thermal treatment, the tubes are opened by heat shock, their contents filtered on Millipore filters HA 0·45 μm, and the filtrate analyzed.

High performance liquid chromatography

Ten-microlitre samples are put on the RP 18 (7 μm) column (room temperature) of a Spectra Physics SP 8000 analyzer. Elution is carried out at 1 ml min^{-1} with a mixture of methanol and 0·01 M sodium acetate buffer, pH 4 (15% and 85% v/v, respectively). Absorbance of the eluate is recorded at 278 nm (350 nm in the case of kynurenine).

Gel chromatography

0·2 ml of the heat-treated tryptophan samples is analyzed on a 35 cm × 1 cm column of Biogel P_2 (Biorad). Elution is carried out with 0·01 N HCl at a flow rate of 0·6 ml min^{-1}. The per cent transmission at 280 nm is recorded with a Gilson analyzer.

Ion exchange chromatography

Tryptophan samples are also analyzed using a Technicon autoanalyzer, model NC 1, fitted with a 25 cm × 0·6 cm Chromobeads C_2 column (57 °C). The column is equilibrated with a sodium citrate buffer, (0·2 N in Na^+) pH 3·8. Elution is carried out with a pH 5 citrate buffer (0·8 N in Na^+) at a flow rate of 0·8 ml min^{-1}. The eluate is reacted with ninhydrin and the absorbance measured at 440 and 570 nm.

UV absorption spectra

These spectra are determined with 0·33 or 0·167 M heat-treated tryptophan solutions, using a DB-GT Beckman spectrophotometer.

Spectrofluorimetric measurements

The tryptophan content of the heat-treated solutions is also determined using a Jobin-Yvon JY 3 spectrofluorimeter with an excitation wavelength of 287 nm and an emission wavelength of 348 nm. The standard curve is established with 0·25 to 1 μM tryptophan solutions (the gain of the fluorimeter being set at 100).

RESULTS

Influence of oxygen, temperature and time

Typical HPL chromatographs from heat-treated solutions are shown in Fig. 1, A and B. Tryptophan is well separated from kynurenine.

HPLC determination indicates that tryptophan degradation occurs

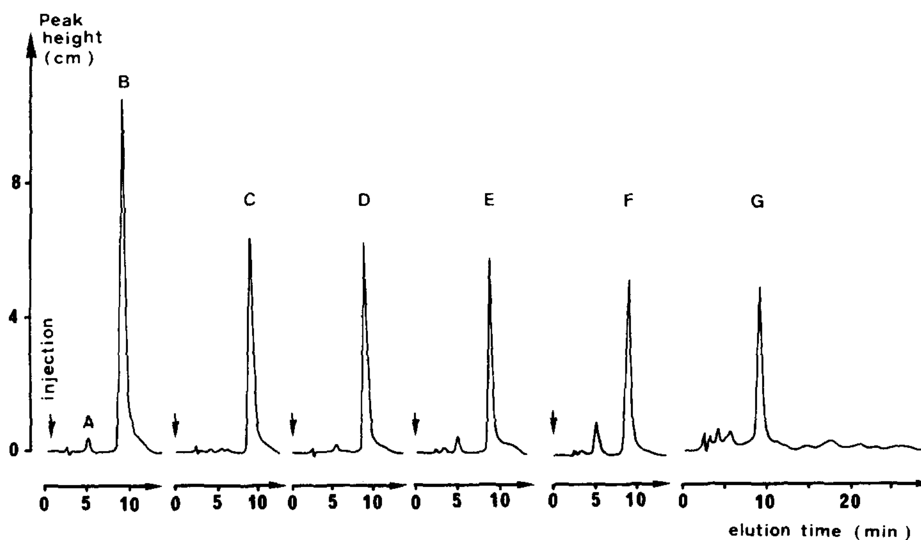


Fig. 1. HPL chromatographs of kynurenine, tryptophan and heat-treated tryptophan solutions (SP 8000: RP 18 column; 10 μl samples); elution with methanol–0·01 M sodium acetate buffer, pH 4, 15–85% v/v respectively; flow rate, 1 ml min^{-1}; absorbance at 278 nm). A: Kynurenine 5 mM; B: tryptophan 10 mM; C: tryptophan 10 mM, pH 5, heated for 24 h at 121 °C in air; D: tryptophan 10 mM, pH 6, heated for 24 h in air at 121 °C; E: tryptophan 10 mM, pH 7, heated for 24 h at 121 °C in air; F: tryptophan 10 mM, pH 8, heated for 24 h at 121 °C in air; G: tryptophan 10 mM, distilled water, heated for 24 h at 140 °C in oxygen. For this last sample the absorbance signal is amplified 8 times.

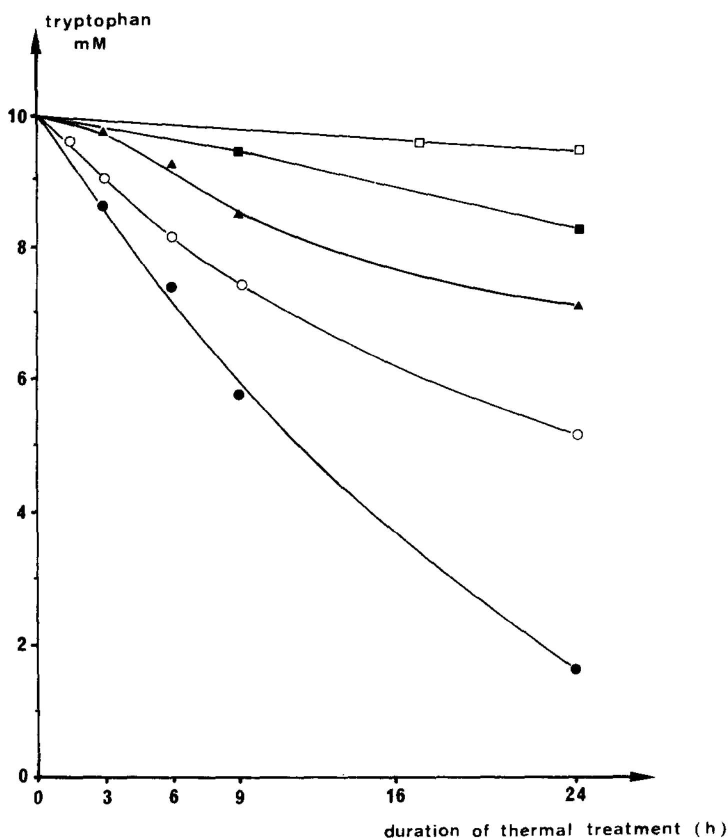


Fig. 2. Thermal degradation of tryptophan in the presence of air as a function of time and temperature. Determination by HPLC with UV detection. □—□ 90°C; ■—■ 100°C; ▲—▲ 110°C; ○—○ 125°C, ●—● 140°C.

TABLE 1

Residual Tryptophan Concentration (mM) after Heating a 10 mM Tryptophan Solution (in 0·1 M Sodium Phosphate Buffer, pH 5 to 8) at 121 °C in the Presence of Air. Influence of pH and Duration of Thermal Treatment. Determination by HPLC and UV Detection

Duration of thermal treatment	*Phosphate buffers*				*Distilled*
	pH 5	*pH 6*	*pH 7*	*pH 8*	*water*
0	10	10	10	10	10
6 h 30 min	8·07	8·26	8·15	8·20	8·30
10 h 30 min	7·39	7·49	7·3	7·35	7·58
24 h	6·25	6·07	5·69	5·17	5·88

only in the presence of oxygen. No tryptophan loss can be seen in tubes heat-sealed under nitrogen and heated for 24 h at 110, 125 or 140 °C. It therefore appears that the indole ring is heat stable in the absence of oxygen.

In the presence of air, tryptophan degradation increases with temperature and with time (Fig. 2 and Tables 1 and 2) and partly insoluble brown derivatives are formed. The reaction kinetics can be written schematically as follows:

$$\text{Tryptophan} + O_2 \rightarrow \text{oxidized derivative(s)} \rightarrow \text{insoluble brown polymers}$$

$$\frac{d[\text{oxidized derivative(s)}]}{dt} = k\,[\text{tryptophan}]^a \times [O_2]^b$$

Under nitrogen, $[O_2] = 0$ and the rate of reaction is equal to zero, as seen experimentally.

In the presence of air or of oxygen, it can be assumed that the concentration of dissolved oxygen remains constant during the reaction. (Tryptophan loss is the same whether 2 or 4 ml of the tryptophan solution are placed in the 180 mm × 18 mm pyrex tube.) One can then write the first order kinetic equation:

$$\frac{d[\text{tryptophan}]}{dt} = k_{\text{apparent}} \times [\text{tryptophan}]$$

and

$$\ln[\text{tryptophan}] = -k_{\text{apparent}} \times t + \ln[\text{tryptophan}_0]$$

When the thermal treatment is carried out in the presence of air, the following regression lines and correlation coefficients can be calculated from the experimental results (k_{apparent} given in h^{-1}):

At 90 °C: $\ln[\text{trp}] = -2{\cdot}5 \times 10^{-3} \times t + 2{\cdot}30$ $r = 0{\cdot}999$

At 100 °C: $\ln[\text{trp}] = -8{\cdot}0 \times 10^{-3} \times t + 2{\cdot}31$ $r = 0{\cdot}992$

At 110 °C: $\ln[\text{trp}] = -15 \times 10^{-3} \times t + 2{\cdot}30$ $r = 0{\cdot}992$

At 125 °C: $\ln[\text{trp}] = -27{\cdot}9 \times 10^{-3} \times t + 2{\cdot}28$ $r = 0{\cdot}996$

At 140 °C: $\ln[\text{trp}] = -78 \times 10^{-3} \times t + 2{\cdot}28$ $r = 0{\cdot}995$

The energy of activation of the reaction can be calculated with the Arrhenius equation:

$$\ln k = -\frac{\text{Ea}}{\text{RT}} + \text{constant}$$

TABLE 2

Tryptophan Degradation after Heating a 10 mM Tryptophan Solution. Influence of pH, Temperature, Duration of Thermal Treatment and Atmosphere above Solution. Results are Given as Tryptophan Loss (% of Initial Concentration). A: Determination by HPLC and UV Detection; B: Spectrofluorimetric Determination

	110°C				*121°C Air*								*125°C*				*140°C*			
	Air		*O_2*		*pH 5*		*pH 6*		*pH 7*		*pH 8*		*Air*		*O_2*		*Air*		*O_2*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
3 h	0·9	0	7·1	6									9·7	9	16·6	22	13·5	22	38·7	35
6 h	6·9	6·5	17·2	15·5									18·3	18	33·4	30·5	26	35	60·4	40
9 h	15·6	13	19·2	24									25·8	30·5	47·2	37	42·5	46	68	56·5
24 h	29	30·5	49·5	52	37·5	35	39·3	35	43·1	39	48·3	43·5	48·9	52	79·4	74	84	78·5	94	80

from the experimental results, at various temperatures, the following equation is obtained:

$$\ln k = -9703 \times \frac{1}{T} + 20{\cdot}9 \qquad r = 0{\cdot}986$$

Therefore, the energy of activation of tryptophan thermal degradation in solution and in the presence of air is equal to 80 570 J mole^{-1}.

In the presence of oxygen, tryptophan degradation is greater than in the presence of air, for given thermal treatments (Fig. 3 and Table 2). From the experimental data and the following equation:

$$\ln [\text{tryptophan}] = -k'_{\text{apparent}} \times t + \ln [\text{tryptophan}_0]$$

the following regression lines and correlation coefficients can be calculated:

At 90 °C: $\ln [\text{trp}] = -5{\cdot}1 \times 10^{-3} \times t + 2{\cdot}3 \qquad r = 0{\cdot}996$

At 100 °C: $\ln [\text{trp}] = -11{\cdot}3 \times 10^{-3} \times t + 2{\cdot}3 \qquad r = 0{\cdot}999$

At 110 °C: $\ln [\text{trp}] = -27{\cdot}5 \times 10^{-3} \times t + 2{\cdot}29 \qquad r = 0{\cdot}995$

At 125 °C: $\ln [\text{trp}] = -66{\cdot}2 \times 10^{-3} \times t + 2{\cdot}29 \qquad r = 0{\cdot}999$

At 140 °C: $\ln [\text{trp}] = -114 \times 10^{-3} \times t + 2{\cdot}18 \qquad r = 0{\cdot}997$

The regression equation from the Arrhenius plot is as follows:

$$\ln k = -9503 \times \frac{1}{T} + 21 \qquad r = 0{\cdot}991$$

and the activation energy for tryptophan degradation in the presence of oxygen is equal to 78 920 J mole^{-1}.

Influence of pH

The thermal degradation of tryptophan was determined at 121 °C, in the presence of air, as a function of time and of pH. The sodium phosphate buffer was selected for its pH range (5 to 8) and because pH variations with temperature are minimal.

Results are given in Table 1; they show that tryptophan degradation after a 24 h thermal treatment increases from 38 to 48 % of the initial contents with increasing pH.

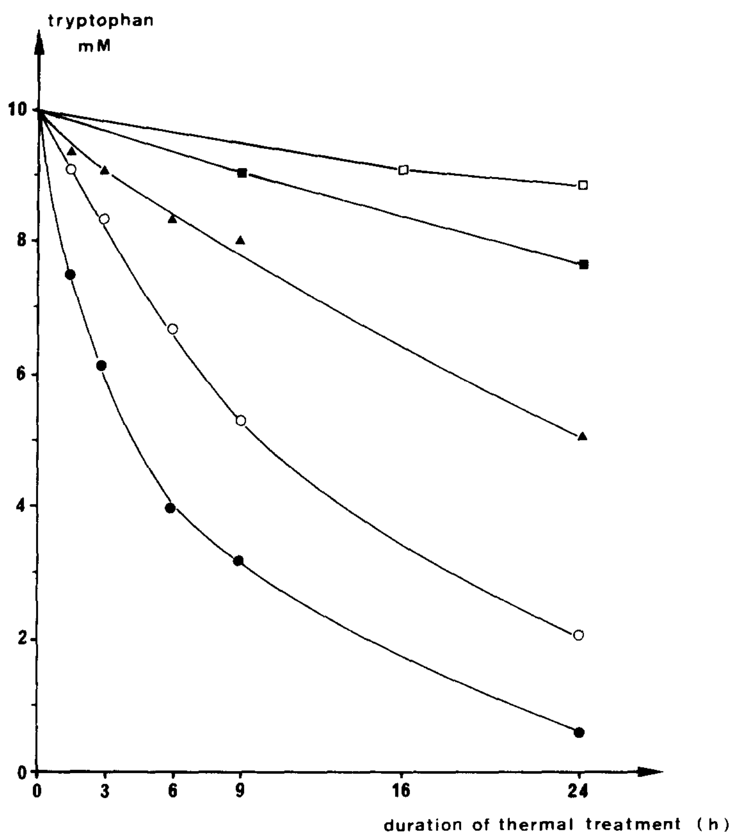


Fig. 3. Thermal degradation of tryptophan in the presence of oxygen as a function of time and temperature. Determination of HPLC with UV detection. □—□ 90 °C; ■—■ 100 °C; ▲—▲ 110 °C; ○—○ 125 °C; ●—● 140 °C.

Formation of tryptophan derivatives

It can be seen on the HPL chromatographs of Fig. 1 that new derivatives appear as tryptophan is degraded. Some of these derivatives are more polar than tryptophan, others have a lower polarity or a higher molecular weight (Fig. 1G). The concentration of one polar derivative appears to increase with the pH of the heated tryptophan solution (Fig. 1C, D, E and F). From measurements of the absorbance of the HPLC eluates at 350 nm (maximum absorbance wavelength of kynurenine), it can be concluded that no detectable amounts of kynurenine or *N*-formyl kynurenine are formed.

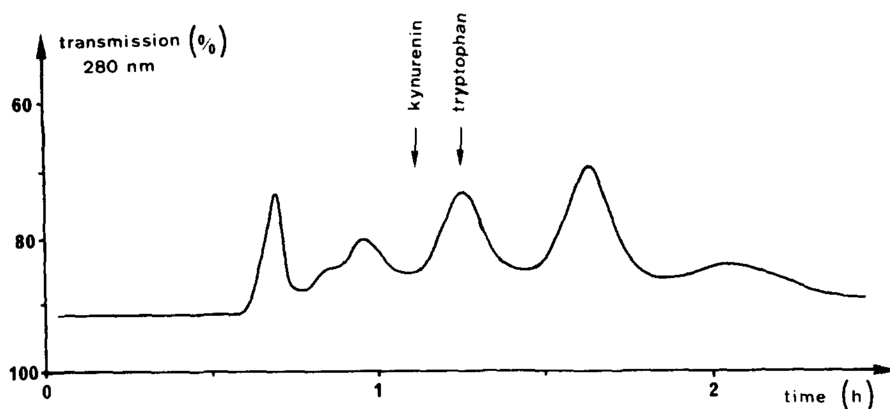


Fig. 4. Biogel P2 chromatograph of a 10 mM tryptophan solution heated at 140 °C for 24 h in the presence of oxygen. 0·2 ml sample; elution with 0·01 N HCl; flow rate: 0·6 ml min^{-1}; 35 cm × 1 cm column.

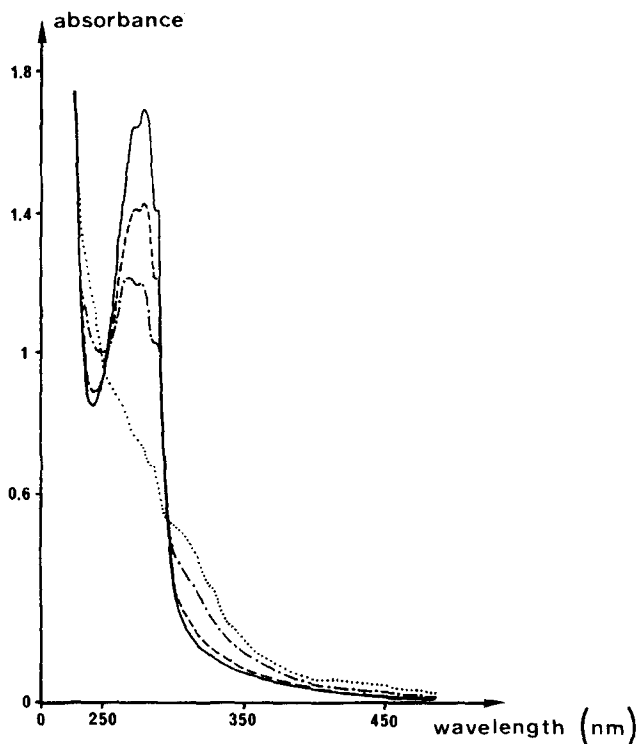


Fig. 5. UV absorption spectra of 10 mM tryptophan solutions heated at 140 °C in oxygen for various lengths of time. Measurement after 1/30 dilution with distilled water. —— 1 h 30 min; ---- 3 h; -·-·-·- 6 h; ······ 24 h.

Ion exchange chromatography of the 10 mM tryptophan sample heated at 140 °C for 24 h in the presence of oxygen shows both residual tryptophan and ammonia peaks, but no other ninhydrin-reactive components. It is likely that oxidative deamination of tryptophan occurs at the primary amino group of the molecule, leading to the formation of indole-3-pyruvic acid, itself unstable.

When the same heat-treated tryptophan sample is submitted to chromatography on Biogel P2, five UV-absorbing derivatives of tryptophan can be identified, three of them having higher molecular weights than tryptophan (Fig. 4). None of these derivatives corresponds to kynurenine.

The absorption spectra of 10 mM tryptophan solutions heat-treated under different conditions are shown in Figs 5 and 6. It can be observed that the 278 nm absorption maximum characteristic of tryptophan decreases as the duration of the thermal treatment increases (Fig. 5). Simultaneously, the 340–450 nm absorption increases, reflecting the formation of brown derivatives. These spectra show that it is not possible

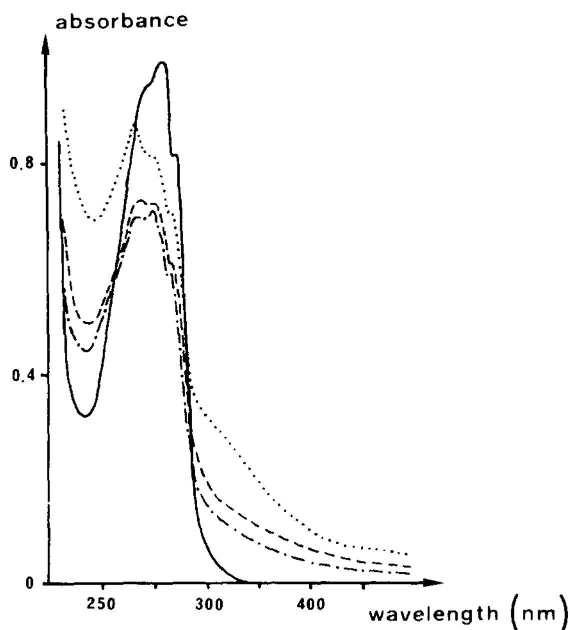


Fig. 6. UV absorption spectra of 10 mM tryptophan solutions of different pH heated for 24 h at 121 °C in the presence of air. Measurement after 1/60 dilution with distilled water. —— unheated tryptophan; ·–·– pH 5; – – – – pH 6; ------ pH 8.

to assess tryptophan degradation quantitatively by direct measurement of the 278 nm absorbance of the solutions. The absorption spectra of the heated tryptophan solutions of various pH are different (Fig. 6); this may indicate that the tryptophan derivatives formed are different.

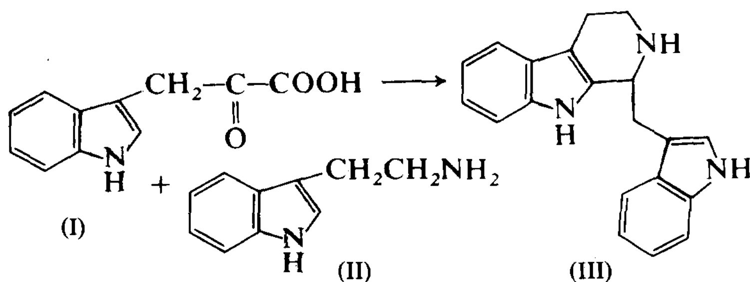
In some experiments, tryptophan was also determined by spectrofluorimetry of the heated solutions. The results are given in Table 2, and can be compared with those obtained after HPLC and measurement of the 278 nm absorbance. Discrepancies appear in some cases: values from spectrofluorimetric measurements higher than those from HPLC probably indicate the presence of fluorescent derivatives of tryptophan.

DISCUSSION

The present study indicates that free tryptophan is not degraded by heating at 140 °C for several hours unless oxygen is present. In the presence of air or oxygen, tryptophan degradation increases with the pH in a 5–8 range. These results are different from those of Stewart & Nicholls (1974) who studied the kinetics of tryptophan (0·2 mM) degradation at 100 °C in the presence of air, and found a maximum rate of degradation at pH 5·3; however, they used a spectrofluorimetric method which may not be specific for tryptophan.

The release of ammonia observed in the present experiments suggests that oxidative deamination occurs at the α amino group of tryptophan. It is likely that resulting carbonyl derivatives react with each other or with the α amino groups of remaining tryptophan to form condensation products of brown colour and low water solubility.

Some of the condensation products formed could be the indolyl methyl tetrahydro/1H/carbolines (III) shown by Gaignault *et al.* (1978) to result from the condensation of indolyl-3-pyruvic acid (I) and tryptamine (II) derivatives.



(I) and (II) could result from the oxidative deamination and decarboxylation of tryptophan, respectively.

Gaignault *et al.* (1978) have shown that the above-mentioned β carbolines have neurosedative and anticonvulsive effects. It would be of interest to determine if they form during the thermal treatments of tryptophan, and also to assess their possible mutagenicity.

Although it is likely that the indole ring of tryptophan is modified by severe thermal treatments in the presence of oxygen, no derivatives from such reactions could be found in the present study. Various authors (Previero & Coletti-Previero, 1967; Fontana & Toniolo, 1977) have shown that strong oxidizing agents split the C_2—C_3 bond of the indole ring, with the formation of *N*-formyl kynurenine and then of kynurenine. These two derivatives were not identified in the present experiments, even when tryptophan was heated at 140 °C in the presence of oxygen. These results are in agreement with those of Yamakawa *et al.* (1979) who did not observe any opening of the indole ring when indolyl-3-acetic acid was heated at 130 °C for 30 min in the presence of air.

From a practical standpoint, the loss in free tryptophan remains very moderate since it takes 12 h at 140 °C in air or 4 h at 140 °C in oxygen to reach 50 % tryptophan degradation. Industrial or home cooking in the presence of air or steam sterilization would therefore not cause any significant loss in free or in protein-bound tryptophan unless the food contains chemical species able to react with the indole ring or able to catalyze reactions of the indole ring with oxygen or other substances.

The thermal degradation of peptide or protein-bound tryptophan is studied in a subsequent paper.

ACKNOWLEDGEMENTS

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