Kinetics of the Formation and Decomposition of 1,1′-Ethylidenebis[L-tryptophan], an Eosinophilia Myalgia Syndrome-Associated Compound

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1,1′-Ethylidenebis[L-tryptophan] (EBT), an impurity found in L-tryptophan associated with eosinophilia myalgia syndrome (EMS), has been suggested as one of the causative agents for EMS. High-performance liquid chromatography was used to study the kinetics of EBT formation and decomposition in buffer systems. Initial EBT decomposition was first-order with respect to EBT and strongly pH-dependent. The pH profile suggested that decomposition involved the addition of one proton. The value of the critical pH, above which the rate of EBT decomposition remained constant, was determined to be 4.8. The rate of EBT decomposition increased with increasing temperature, and the activation energy was calculated to be 12.5 kcal/mol. The rate of EBT formation decreased with increasing pH and decreasing temperature. The presence of excess acetaldehyde increased EBT formation to a lesser extent than did the presence of excess L-tryptophan. The results of this study are consistent with the hypothesis that EBT was formed during the absorption stage of the cation exchange operation in the commercial process that resulted in the formation of EMS-associated L-tryptophan.

Keywords: *1,1′-Ethylidenebis[L-tryptophan]; peak E; L-tryptophan; eosinophilia myalgia syndrome; kinetics*

INTRODUCTION

The 1989 outbreak of eosinophilia myalgia syndrome (EMS) has been associated with the ingestion of L-tryptophan produced by a single manufacturer, Showa Denko, K.K., Tokyo, Japan (Belongia et al., 1990; Slutsker et al., 1990). Epidemiologic studies suggested that EMS may be associated with one or more impurities in the case-associated L-tryptophan (Slutsker et al., 1990). High-performance liquid chromatographic (HPLC) analyses of the case-associated L-tryptophan revealed more than 60 impurity peaks (Hill et al., 1993). Among these impurities, 1,1′-ethylidenebis[L-tryptophan] (EBT), also known as "peak E" or "peak 97" (Belongia et al., 1990), has been implicated by epidemiological data. The structure of EBT has been determined to be a dimeric form of L-tryptophan with an ethylidene bridge (Mayeno et al., 1990; Smith et al., 1991).

The physiological effects of EBT have been the focus of a number of investigations. Results of animal studies showed that EBT caused certain pathologic changes that were noted in human EMS. Love et al. (1993) reported that Lewis rats treated by gavage with either EBT or case-associated L-tryptophan developed significant myofascial thickening, compared with rats that received control L-tryptophan. Silver et al. (1994) showed that female C57BL/6 mice administered EBT intraperitoneally developed inflammation and fibrosis accompanied by an increase in the number of mast cells. In addition, Yamaoka et al. (1994) showed that EBT induces functional activation of human eosinophils and interleukin 5 production from T lymphocytes.

Ito et al. (1992) and Driskell et al. (1992) reported the decomposition of EBT in solutions simulating human gastric fluids and determined the structures of the decomposition products. EBT decomposed quickly in the simulated gastric fluids with a half-life of about 15 min. The final breakdown products were L-tryptophan and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA). MTCA has been reported to be a precursor of mutagenic *N*-nitroso compounds when tested in Ames assays (Wakabayashi et al., 1983). The metabolism of MTCA in rats and the urinary excretion of MTCA in humans have been studied (Adachi et al., 1991, 1993b; Ogawa et al., 1993).

Adachi et al. (1993a) investigated the metabolism and distribution of EBT in rats. They found that, unlike the fast breakdown observed in the simulated gastric fluids, about 26% of EBT remained in the large intestine 5 h after peroral administration without decomposition by gastric fluid in the stomach of rats. The EBT breakdown product MTCA was observed in the urine and in the large intestine.

A great deal of interest has focused on determining how EBT was formed during the commercial production of L-tryptophan. The EMS-associated L-tryptophan was produced from a fermentation process utilizing a recombinant strain of *Bacillus amyloliquefaciens*. The final L-tryptophan product was extracted from the broth through a series of purification steps, including ion-exchange chromatography, crystallization, reverse osmosis, and activated-carbon treatment. Belongia et al. (1990) reported that the production of the EMS-associated L-tryptophan was linked to changes in the manufacturing processes (the use of a new strain of *B. amyloliquefaciens* and a reduction in the amount of powdered activated carbon used). However, they were unable to determine how the EBT impurity was formed. Sakimoto and Torigoe (1992) measured EBT in a laboratory-scale process simulating Showa Denko's commercial production conditions. Their results showed that EBT was not present in the postfermentation broth and that it first appeared in the eluate from a cation-exchange column. However, it is not clear how processing conditions influenced the formation of EBT; this

S0021-8561(95)00421-3

requires an understanding of the chemistry of EBT formation and decomposition.

EBT is easily formed from the reaction of L-tryptophan and acetaldehyde in acidic conditions (Smith et al., 1991). Although the reaction of L-tryptophan and acetaldehyde has been studied for more than 40 years (Brehm and Lindwall, 1950), detailed studies of the kinetics of EBT formation and factors affecting the rate of its formation and decomposition have never been reported.

This study examined the effects of pH, temperature, and reactant concentration on the kinetics of EBT decomposition and formation; the results helped to clarify the role of processing conditions on the formation of EBT in Showa Denko's production of L-tryptophan and also helped to interpret EBT metabolism data obtained from animal studies.

MATERIALS AND METHODS

Materials. EBT standard was purchased from Calbiochem (La Jolla, CA). L-Tryptophan and acetaldehyde were purchased from Sigma Chemical Co. (St. Louis, MO). The following buffers were used at different pH values: 0.01 M sodium phosphate, dibasic (pH 5−7); 0.01 M potassium acetate (pH 3−4); and 0.01 M sodium phosphate, monobasic (pH 1−2).

HPLC Analysis. Two columns, a Waters Nova-Pak C_{18} column (3.9 × 150 mm) and a Shodex Cosmosil C_{18} column (4.6 × 250 mm), were used with the Waters 600E multisolvent delivery system and the Waters 990 photodiode array detector. The Nova-Pak column gave better separation of the EBT decomposition products, peaks Y and X; the Cosmosil column gave better resolution of the EBT formation byproducts. The following chromatographic conditions for the Nova-Pak column were used: flow rate, 1 mL/min; temperature, ambient; buffer A, water; buffer B, acetonitrile; buffer C, 1% trifluoroacetic acid in water; gradient program, 90% A/10% C from 0 to 2 min, from 90% A/10% C at 2 min to 26% A/64% B/10% C at 37 min, hold at 26% A/64% B/10% C from 37 to 40 min.

The chromatographic conditions used by Sakimoto and Torigoe (1992) were used with minor modifications for the Cosmosil column: flow rate, 1 mL/min; temperature, ambient; buffer A, 0.01 M phosphoric acid; buffer B, acetonitrile/water, 80:20; gradient program, from 95% A/5% B at 0 min to 90% A/10% B at 13 min, from 90% A/10% B at 13 min to 75% A/25% B at 60 min, hold at 75% A/25% B from 60 to 63 min, from 75% A/25% B at 63 min to 100% B at 64 min, hold at 100% B from 64 to 73 min.

Time Course Studies. Decomposition of EBT was initiated by dissolving 1 mg of EBT in 1 mL of buffer at the desired pH. At each time interval, an aliquot of the solution was withdrawn and analyzed by HPLC.

The formation of EBT was followed by adding acetaldehyde to L-tryptophan solutions at defined temperature and pH. Aliquots of the reaction mixture were withdrawn at fixed time intervals and analyzed by HPLC.

For fast decomposition and formation reactions, the aliquots withdrawn from the reaction mixtures were mixed with 1 N NaOH to a final pH of 10−11 to stop the reaction and were analyzed by HPLC at a later time. There was no measurable EBT decomposition or formation under alkaline conditions.

RESULTS AND DISCUSSION

A. Decomposition of EBT. Figure 1 shows a typical chromatogram of EBT decomposition products obtained with the Nova-Pak column. Five peaks were formed as a result of EBT decomposition. This chromatogram is similar to those obtained by Ito et al. (1992) and Driskell et al. (1992). According to Ito et al. (1992), the first four peaks are L-tryptophan, peak Y, peak X, and peak X′, respectively. Using a slightly modified

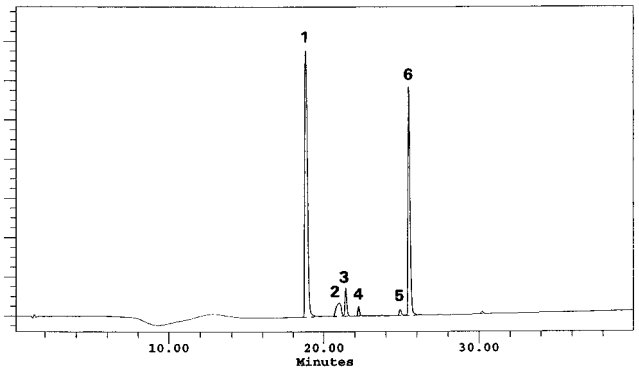

Figure 1. Typical HPLC chromatogram of EBT decomposition products, obtained at 220 nm with the Nova-Pak column. Peaks: 1, L-tryptophan; 2, peak Y or HET; 3, peak X or (−)-(1*S*,3*S*)-MTCA; 4, peak X′ or (−)-(1*R*,3*S*)-MTCA; 5, peak Z; 6, EBT.

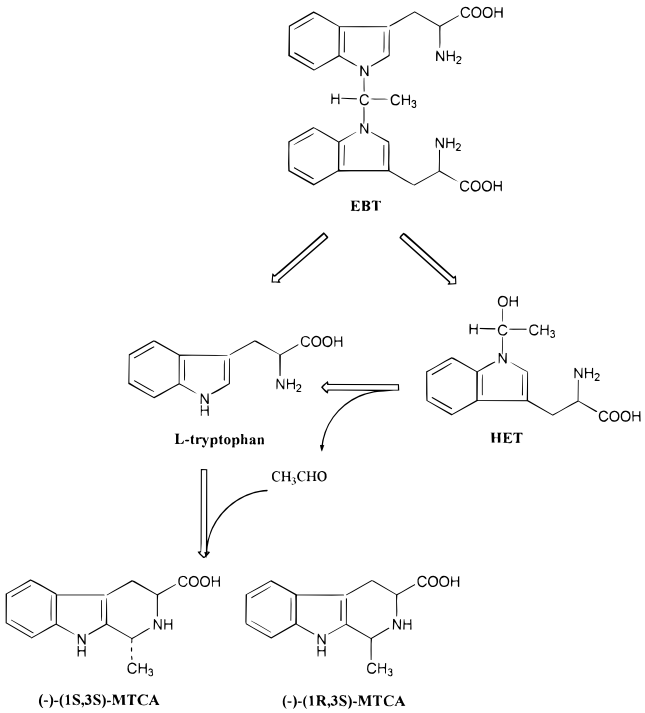

Figure 2. Likely EBT decomposition sequence.

elution condition, Ito et al. (1992) showed that peak Y was actually a combination of two isomer peaks, peaks Ya and Yb. When analyzed with the Cosmosil column, peaks Ya and Yb coeluted with peak X and appeared as two shoulders of peak X (chromatogram not shown). The structures of peaks Y, X, and X′ have been determined by Ito et al. (1992) and Driskell et al. (1992). Peak Y corresponds to 1-(hydroxyethylidene)-L-tryptophan (HET). Peaks X and X′ are structural isomers of MTCA. Peak X corresponds to (−)-(1*S*,3*S*)-MTCA, and peak X′ corresponds to (−)-(1*R*,3*S*)-MTCA. The structures of these compounds are shown in Figure 2.

The fifth peak, designated peak Z, appeared immediately before the EBT peak. Peak Z was present at a very low concentration during the entire time course of EBT decomposition. Its area was ≤1% of the initial EBT peak area. Peak Z was also observed by Driskell et al. (1992), who attributed it to an impurity. According to the time course study, the concentration of this compound gradually increased as EBT decomposition proceeded and gradually decreased when the concentration of L-tryptophan started to decrease. Therefore, peak Z probably is another decomposition product of

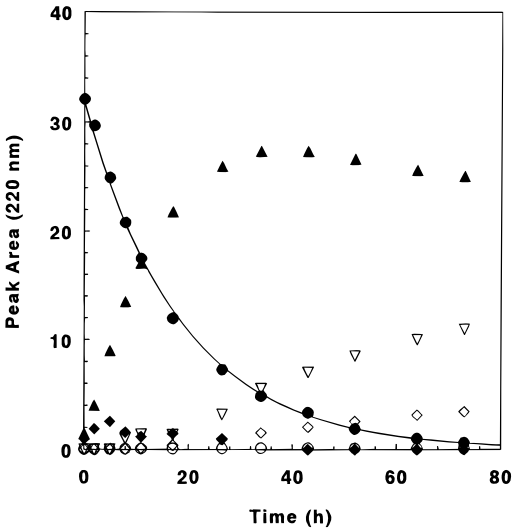

Figure 3. Time course of EBT decomposition at pH 2.0 and 25 °C: (●) EBT; (▲) L-tryptophan; (◆) HET; (▽) (−)-(1*S*,3*S*)-MTCA; (◇) (−)-(1*R*,3*S*)-MTCA; (○) peak Z.

EBT rather than an impurity. The structure of peak Z has not been determined; however, its spectrum showed two maxima, at 224 and 285 nm.

A typical time course of EBT decomposition is shown in Figure 3. On the basis of this plot, a likely sequence of EBT decomposition could be described as follows. EBT first decomposed to form L-tryptophan and HET. HET quickly decomposed to form L-tryptophan. During this time, both MTCA isomers formed gradually. When all of the EBT had disappeared, formation of the MTCA isomers continued and the concentration of L-tryptophan gradually decreased. These results suggest that the MTCA isomers were not formed directly from EBT but rather from EBT decomposition products.

Multiple interrelated reaction pathways from EBT to MTCA are possible. Driskell et al. (1992) proposed two mechanisms for formation of the MTCAs: (1) from HET by intramolecular rearrangement of the hemiaminal and subsequent cyclization; and/or (2) from reaction of the L-tryptophan produced by EBT decomposition and acetaldehyde produced by HET decomposition. [The presence of acetaldehyde in the reaction mixture was confirmed by nuclear magnetic resonance spectrometry (Driskell et al., 1992).] Results of the time course study favor the second mechanism because after HET had disappeared, the MTCA isomers continued to form and the concentration of L-tryptophan continued to decrease. A flow diagram of EBT decomposition is shown in Figure 2.

Adachi et al. (1993a) showed that the metabolism of EBT in rats also results in the formation of MTCA. The amount of MTCA measured in the large intestine of EBT-treated rats was significantly greater than that in the L-tryptophan-treated rats. This can be explained by the fact that MTCA is a major breakdown product of EBT but is formed from L-tryptophan only in the presence of acetaldehyde (see section B). The low acetaldehyde concentration present in rats will result in a lower concentration of MTCA in L-tryptophan-treated rats.

First-Order Kinetics. In Figure 3, the initial decrease in EBT concentration can be fitted with a curve (solid line) based on a first-order reaction, i.e., $d[EBT]/dt = -k[EBT]$. This means that the initial decomposition of EBT can be viewed as first-order with respect to EBT. The first-order rate constant k can be determined from the time course plot. The effects of pH and temperature on the value of k are discussed below.

Table 1. Effect of pH on EBT Decomposition As Shown by the First-Order Rate Constant Obtained at Various pH Values at 25 °C

pH	*n*	k (h^{-1}) ± SD
0.1	3	$(1.17 \pm 0.09) \times 10^{1}$
1.2	3	$(3.45 \pm 0.09) \times 10^{-1}$
2.0	2	$(4.59 \pm$ ND[a]$) \times 10^{-2}$
3.0	2	$(6.41 \pm$ ND$) \times 10^{-3}$
4.0	1	$(1.16 \pm$ ND$) \times 10^{-3}$
5.0	3	$(1.70 \pm 0.71) \times 10^{-4}$
6.0	2	$(0.81 \pm$ ND$) \times 10^{-4}$
7.0	3	$(1.68 \pm 0.14) \times 10^{-4}$

[a] ND, not determined.

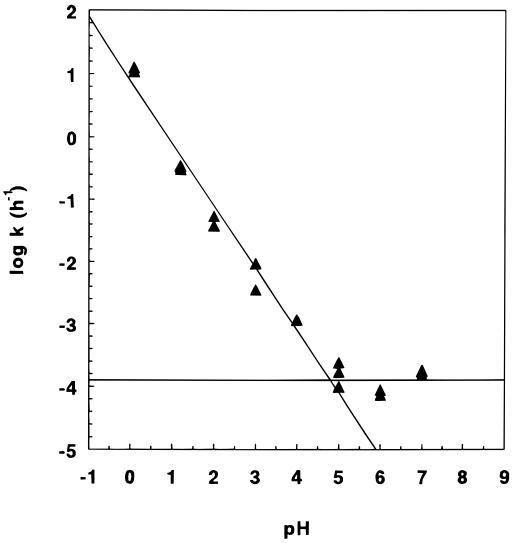

Figure 4. pH dependence of the first-order rate constant for EBT decomposition at 25 °C.

Effect of pH. The rates of EBT decomposition, HET formation and subsequent decomposition, and MTCA isomer formation all decreased as pH increased. Table 1 lists the values of k obtained at various pH values at 25 °C. As pH increased from 0.1 to 5.0, the value of k changed by a factor of 10^{-5}. However, at pH 5.0−7.0, the value of k remained approximately constant.

Figure 4 shows a plot of log k vs pH. The data obtained from pH 0.1 to 5.0 can be fitted with a straight line with a slope of −1, and the data from pH 5.0 to 7.0 can be fitted with a straight line with a slope of 0.

According to Dixon (1953), a slope of −1 in the pH profile indicates that the increase in the reaction rate is due to the addition of one proton to an ionizable group. In a similar sense, the pH profile for EBT shown in Figure 4 suggests that the presence of one proton facilitates EBT breakdown. As can be seen in Figure 2, protonation facilitates the elimination of the tryptophan molecule from EBT, thus accelerating decomposition. The value of the pH where the line with slope of −1 intersects the line with slope of 0 is the critical pH, above which the rate of EBT decomposition remained constant, namely, pH 4.8.

This pH dependence can be used to explain the different EBT decomposition rates observed in simulated gastric fluids (Ito et al., 1992) and rat stomach (Adachi et al., 1993a). The pH of the solution simulating human gastric fluids used by Ito et al. was 1.2. However, the pH in rat gastric fluid is about 5 (Calabrese, 1983). The rate of EBT decomposition at pH 1.2 was about 2000 times the rate at pH 5.0 (at 25 °C)

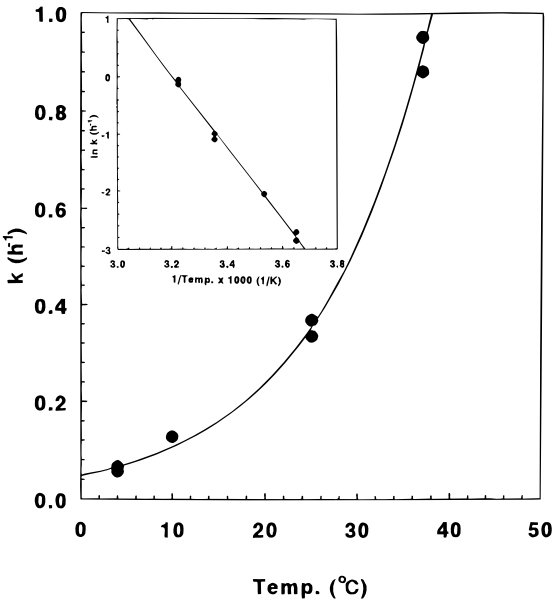

Figure 5. Temperature dependence of the first-order rate constant for EBT decomposition at pH 1.2. A plot of ln *k* vs 1/temp (in Kelvin) is shown in the inset.

(Figure 4). That is why only 15 min was needed for half of the EBT to decompose in the simulated gastric fluids, while 26% of the EBT remained in the rat large intestine 5 h after treatment. EBT should decompose much more rapidly in the human stomach than in the rat stomach. This difference should be considered when animal feeding data are used to explain EBT metabolism in humans.

Effect of Temperature. The rate of EBT decomposition increased with increasing temperature. Figure 5 shows the variation of *k* with temperature at pH 1.2. The value of *k* increased from 0.067 h^{-1} at 4 °C (277 K) to 0.92 h^{-1} at 37 °C (310 K). The activation energy of EBT decomposition was calculated to be 12.5 kcal/mol, which is lower than the activation energy values for degradation of most food components (Lund, 1975). This suggests that EBT is less heat-stable than most food components.

B. Formation of EBT. The reaction of L-tryptophan with acetaldehyde yielded multiple byproducts including EBT. HPLC analyses showed that the amount of each peak formed varied with reaction conditions and duration. Under certain conditions, some peaks could not be seen. Figure 6 shows several representative chromatograms, obtained with the Cosmosil column, from the reaction of 0.03 M L-tryptophan with 0.015 M acetaldehyde at 25 °C at various pH values. More peaks were found at lower pH values. Peaks 3 and 4 were the predominant peaks at all pH values.

No attempt was made to determine the identities of these peaks. However, the retention times and the spectra of peaks 2a and 2b were the same as those of HET (i.e., peaks Ya and Yb). The retention times and the spectra of peaks 3 and 4 were the same as those of (−)-(1*S*,3*S*)-MTCA and (−)-(1*R*,3*S*)-MTCA, respectively.

The formation of EBT is a dynamic process. The amount of EBT present varies during the reaction. It is most convenient to use the maximum concentration formed to quantitate the reaction. The effects of pH, temperature, and reactant concentration on EBT formation are discussed below.

Effect of pH. The effect of pH on EBT formation was studied for two sets of reactant concentrations at 25 °C.

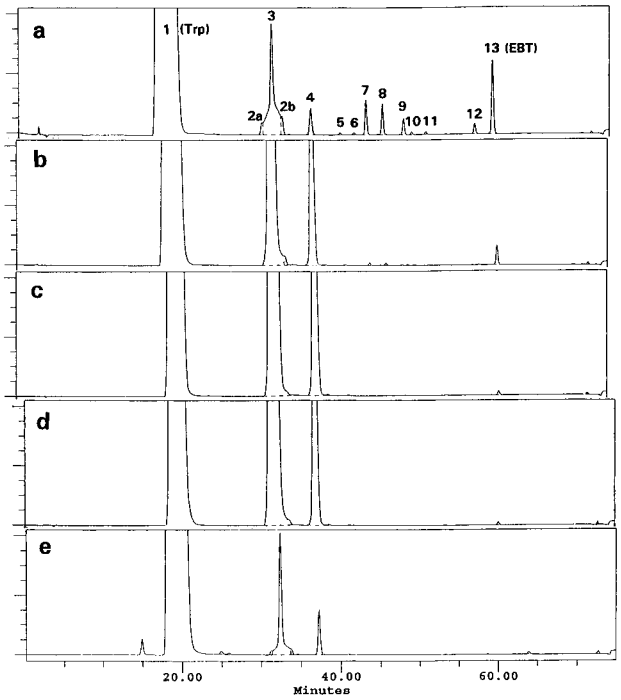

Figure 6. HPLC chromatograms at 220 nm, obtained with the Cosmosil column, from the reaction of 0.03 M L-tryptophan and 0.015 M acetaldehyde at 25 °C: (a) pH 0.1 at $t = 1.5$ h; (b) pH 1.2 at $t = 4.5$ h; (c) pH 2.0 at $t = 12$ h; (d) pH 4.0 at $t = 30$ h; (e) pH 7.0 at $t = 31.5$ h.

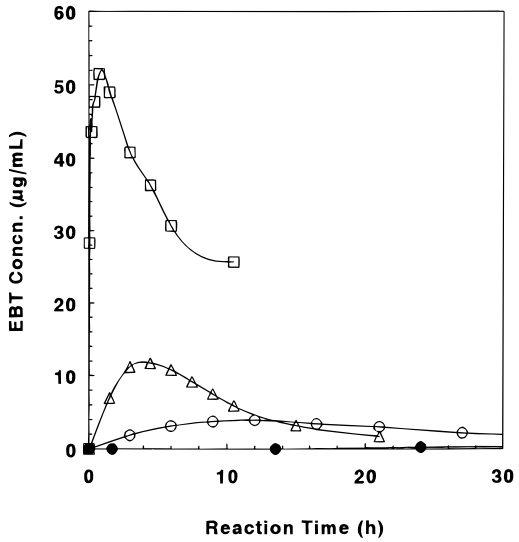

Figure 7. Time course of EBT formation at pH 0.1 (□), pH 1.2 (△), pH 2.0 (○), and pH 4.0 (●). Conditions: reaction of 0.03 M L-tryptophan with 0.015 M acetaldehyde at 25 °C.

First, 0.03 M L-tryptophan and 0.015 M acetaldehyde were allowed to react in 1 N HCl (pH −0.1) and at pH 0.1,1.2, 2.0, 4.0, and 7.0. (The maximum solubility of L-tryptophan at pH 7.0 is <0.04 M.) A typical plot of EBT formation at various pH values is shown in Figure 7. After L-tryptophan and acetaldehyde were mixed, EBT formed rapidly and then gradually decayed. pH affected both the rate and the amount of EBT formed. Generally, the lower the pH, the higher the amount of EBT and the more rapidly it was formed. Maximum EBT concentrations obtained at various pH values are listed in Table 2. The time at which the maximum concentration was obtained is also listed. The maximum concentration decreased from 51.64 μg/mL at pH 0.1 to 4.04 μg/mL at pH 2.0. The time at which the

Table 2. Effect of pH on EBT Formation As Shown by the Maximum EBT Concentration and the Time at which This Concentration Was Obtained during the Reaction of 0.03 M L-Tryptophan and 0.015 M Acetaldehyde at 25 °C

pH	*n*	max EBT concn (μg/mL) ± SD	time at max concn (h)
0.1	3	51.64 ± 0.31	0.8
1.2	2	11.71 ± ND[a]	4.5
2.0	2	4.04 ± ND	12.0
4.0	3	>2.54 ± ND	432.0
7.0	2	<0.05[b]	430.0

[a] ND, not determined. [b] The detection limit was 0.05 μg/mL.

Table 3. Maximum EBT Concentration Obtained during the Reaction of 0.24 M L-Tryptophan and 9.1 × 10^{-5} M Acetaldehyde at Various pH Values and Temperatures

temp (°C)	pH	*n*	max EBT concn (μg/mL) ± SD	time at max concn (h)
37	0.1	6	9.95 ± 1.66	0.2
	1.75	3	0.37 ± 0.11	1.5
25	0.1	4	17.60 ± 1.89	0.4
	1.0	6	3.14 ± 0.70	1.7
	1.75	5	0.27 ± 0.04	5.0
	2.0	4	0.17 ± 0.02	5.0
	3.0[a]	1	<0.05[b]	80.0
10	0.1	3	18.99 ± 2.84	1.2
	1.75	5	0.34 ± 0.08	15.0

[a] Under these conditions, the L-tryptophan was only partially soluble. [b] The detection limit was 0.05 μg/mL.

maximum concentration occurred increased from 0.8 h at pH 0.1 to 12.0 h at pH 2.0. At pH 4.0, the concentration of EBT was still increasing after 432.0 h. No measurable EBT was formed at pH 7.0 in 430.0 h.

According to Sakimoto and Torigoe (1992), the maximum concentrations of L-tryptophan and acetaldehyde found in a model commercial process were 0.24 M and 9.1 × 10^{-5} M, respectively. The effect of pH on EBT formation was also studied at these concentrations. At 25 °C, the maximum EBT concentration decreased from 17.60 μg/mL at pH 0.1 to 0.17 μg/mL at pH 2.0 (see Table 3).

Effect of Temperature. Table 3 shows the maximum EBT concentrations and the times at which these concentrations were obtained when 0.24 M L-tryptophan reacted with 9.1 × 10^{-5} M acetaldehyde at 10, 25, and 37 °C. When the reaction was performed at pH 0.1, the maximum EBT concentrations were 9.95 μg/mL at 37 °C, 17.60 μg/mL at 25 °C, and 18.99 μg/mL at 10 °C. The time at which the maximum concentration occurred increased from 0.2 h at 37 °C to 1.2 h at 10 °C. These results suggest that the rate of EBT formation increased with temperature. The maximum EBT concentration was lower at a higher temperature because EBT decomposition was faster at this temperature.

When the reaction was performed at pH 1.75, the maximum EBT concentrations obtained at all three temperatures were similar; however, it took longer to reach the maximum concentration at the lower temperatures.

Effect of Reactant Concentration. Table 4 shows the effect of reactant concentration on EBT formation. When 0.03 M L-tryptophan reacted with 0.015 M acetaldehyde at pH 1.2 and 25 °C, the maximum EBT concentration obtained was 11.71 μg/mL. When the concentration of L-tryptophan was doubled or increased 10-fold, the maximum EBT concentration increased 4- or 20-fold, respectively. However, a 2-fold (or 10-fold) increase in the concentration of acetaldehyde caused about 40% (or a 2.3-fold) increase in the maximum EBT concentration. This result indicates that EBT formation was increased less by the presence of excess acetaldehyde than by the presence of excess L-tryptophan.

Table 4. Effect of Reactant Concentration on EBT Formation (Reactions Performed at pH 1.2 and 25 °C)

molarity		*n*	max EBT concn (μg/mL) ± SD	time at max concn (h)
L-tryptophan	acetaldehyde			
0.03	0.015	2	11.71 ± ND[a]	4.5
0.06	0.015	2	38.24 ± ND	3.0
0.3	0.015	2	220.5 ± ND	1.6
0.03	0.03	3	17.16 ± 2.22	3.5
0.03	0.15	2	27.06 ± ND	2.0

[a] ND, not determined.

pH had a major effect on EBT formation. EBT formation is greatly enhanced under very acidic conditions. When 0.24 M L-tryptophan reacted with 9.1 × 10^{-5} M acetaldehyde, measurable EBT formed only at pH <2.0. According to Sakimoto and Torigoe (1992), in their process simulating commercial production conditions, the only unit operation at which the pH was <2.0 was the absorption stage of cation-exchange chromatography (performed at pH 1.75 and ambient temperature). Therefore, it is reasonable to conclude that EBT was formed during the cation-exchange operation as a result of the acidic environment.

EMS-implicated L-tryptophan samples contained about 20−130 ppm of EBT (Toyo'oka et al., 1991). Sakimoto and Torigoe (1992) reported that 55 ppm of EBT (micrograms per gram of L-tryptophan) was present in the eluate from the cation-exchange process. However, as shown in Table 3, no more than 0.27 μg of EBT/mL was formed when 0.24 M L-tryptophan reacted with 9.1 × 10^{-5} M acetaldehyde in solution at pH 1.75 and 25 °C, corresponding to 5.6 ppm based on the total L-tryptophan concentration. This is about $^1/_{10}$ the value obtained by Sakimoto and Torigoe in the cation-exchange operation.

It is possible that absorption of L-tryptophan on the ion-exchange column resulted in a higher local L-tryptophan concentration and therefore caused more EBT formation. Another possibility is that the cation exchanger effectively inactivated the α-amino groups of L-tryptophan and prevented them from acting as alternative nucleophiles for acetaldehyde, as opposed to the indole nitrogen, thereby allowing more EBT formation. A detailed study of the formation of EBT in a cation-exchange column is currently under way.

CONCLUSIONS

HPLC analyses of the decomposition of EBT revealed five peaks. Four of these have been identified as L-tryptophan, HET, and (−)-(1*S*,3*S*)- and (−)-(1*R*,3*S*)-MTCA. The fifth peak (peak Z) appeared at a very low concentration and has two absorption maxima, at 224 and 285 nm.

One likely decomposition sequence, suggested by the time course study, is that EBT first decomposed to L-tryptophan and HET. HET then decomposed to form L-tryptophan and acetaldehyde. The MTCA isomers were formed from the subsequent reaction of L-tryptophan with acetaldehyde.

The initial decomposition of EBT was a first-order reaction. A plot of the logarithm of the first-order rate constant against pH revealed two regions with different pH dependences. At pH <4.8, the value of log *k* decreased linearly with a slope of −1. At pH >4.8, the value of *k* did not change with pH. This pH profile

suggests that an addition of one proton to the EBT molecule was involved in the decomposition.

The pH of gastric fluid in humans is much lower than that in rats. EBT will decompose much more rapidly in humans than in rats. This difference should be considered when animal feeding data are used to explain EBT metabolism in humans.

EBT was formed from the reaction of L-tryptophan and acetaldehyde under acidic conditions. The rate of EBT formation decreased with increasing pH and decreasing temperature, and the presence of excess acetaldehyde increased EBT formation to a lesser extent than did the presence of excess L-tryptophan.

In the commercial process that resulted in the production of EMS-associated L-tryptophan, the only unit operation that was performed under acidic conditions was the cation-exchange chromatography. Therefore, it is likely that EBT was formed during the absorption stage of the cation-exchange operation. At the concentrations of L-tryptophan (0.24 M) and acetaldehyde (9.1×10^{-5} M) found in the commercial process, the maximum amount of EBT formed in solution was 0.27 μg/mL (5.6 ppm with respect to the initial L-tryptophan concentration) at pH 1.75 and 25 °C. This value is only about $^1/_{10}$ of that observed by Sakimoto and Torigoe (1992) in the cation-exchange process, indicating that the rate of EBT formation in a column matrix differs from that in solution.

ACKNOWLEDGMENT

I am grateful to Drs. Samuel Page, Mitchell Smith, Terryl Farrell, and Joseph Betz for providing technical information and to Dr. Steven Gendel for helpful comments and suggestions about the manuscript.

LITERATURE CITED

Adachi, J.; Yamamoto, K.; Ogawa, Y.; Ueno, Y.; Mizoi, Y.; Tatsuno, Y. Endogenous formation of 1-methyl-1,2,3,4,-tetrahydro-β-carboline-3-carboxylic acid in man as the possible causative substance of eosinophilia-myalgia syndrome associated with ingestion of L-tryptophan. *Arch. Toxicol.* **1991**, *65*, 505−509.

Adachi, J.; Naito, T.; Ueno, Y.; Ogawa, Y.; Ninomiya, I.; Tatsuno, Y. Metabolism and distribution in the rat of peak E substance, a constituent in L-tryptophan product implicated in eosinophilia-myalgia syndrome. *Arch. Toxicol.* **1993a**, *67*, 284−289.

Adachi, J.; Ueno, Y.; Ogawa, Y.; Hishida, S.; Yamamoto, K.; Ouchi, H.; Tatsuno, Y. Acetaldehyde-induced formation of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid in rats. *Biochem. Pharmcol.* **1993b**, *45*, 935−941.

Belongia, E. A.; Hedberg, C. W.; Gleich, G. J.; White, K. E.; Mayeno, A. N.; Loegering, D. A.; Dunnette, S. L.; Pirie, P. L.; MacDonald, K. L.; Osterholm, M. T. An investigation of the cause of the eosinophilia-myalgia syndrome associated with tryptophan use. *N. Engl. J. Med.* **1990**, *323*, 357−365.

Brehm, W. J.; Lindwall, H. Carboline syntheses. *J. Org. Chem.* **1950**, *15*, 685−694.

Calabrese, E. J. *Principles of Animal Extrapolation*; Wiley: New York, 1983; pp 46−47.

Dixon, M. The effect of pH on the affinities of enzymes for substrates and inhibitors. *Biochem. J.* **1953**, *155*, 161.

Driskell, W. L.; Ashley, D. L.; Grainger, J.; Sirimanne, S. R.; Mazzola, E. P.; Page, S. W.; Needham, L. L.; Hill, R. H. Identification of decomposition products of 1,1′-ethylidenebis-[L-tryptophan], a compound associated with eosinophilia-myalgia syndrome. *Bull. Environ. Contam. Toxicol.* **1992**, *48*, 679−687.

Hill, R. H.; Gaudill, S. P.; Philen, R. M. Contaminants in L-tryptophan associated with eosinophilia myalgia syndrome. *Arch. Environ. Contam. Toxicol.* **1993**, *25*, 134−142.

Ito, J.; Hosaki, Y.; Torigoe, Y.; Sakimoto, K. Identification of substances formed by decomposition of peak E substance in tryptophan. *Food Chem. Toxicol.* **1992**, *30*, 71−81.

Love, L. A.; Rader, J. I.; Crofford, L. J.; Raybourne, R. B.; Principato, M. A.; Page, S. W.; Trucksess, M. W.; Smith, M. J.; Dugan, E. M.; Turner, M. L.; Zelazonski, E.; Zelazonski, P.; Sternberg, E. M. Pathological and immunological effects of ingesting L-tryptophan and 1,1′-ethylidenebis[L-tryptophan] in Lewis rats. *J. Clin. Invest.* **1993**, *91*, 804−811.

Lund, D. B. Heat processing. In *Principles of Food Science. Part II. Physical Principles of Food Preservation*; Fennema, O. R., Ed.; Dekker: New York, NY, 1975; Chapter 3.

Mayeno, A. N.; Lin, F.; Foote, C. S.; Loegering, D. A.; Ames, M. M.; Hedberg, C. W.; Gleich, G. J. Characterization of "peak E," a novel amino acid associated with the eosinophilia-myalgia syndrome. *Science* **1990**, *250*, 1707−1708.

Ogawa, Y.; Adachi, J.; Tatsuno, Y. Accumulation of 1-methyltetrahydro-β-carboline-3-carboxylic acid in blood and organs of rat. A possible causative substance of eosinophilia-myalgia syndrome associated with ingestion of L-tryptophan. *Arch. Toxicol.* **1993**, *67*, 290−293.

Sakimoto, K.; Torigoe, Y. Study of mechanism of peak E substance formation in process for manufacture of L-tryptophan. Report submitted to U.S. Food and Drug Administration, April 1992.

Silver, R. M.; Ludwicka, A.; Hampton, M.; Ohba, T.; Bingel, S. A.; Smith, T.; Harley, R. A.; Maize, J.; Hehes, M. P. A Murine model of the eosinophilia myalgia syndrome induced by 1,1′-ethylidenebis[L-tryptophan]. *J. Clin. Invest.* **1994**, *93*, 1473−1480.

Slutsker, L.; Hoesly, F. C.; Miller, L.; Williams, L. P.; Watson, J. C.; Fleming, D. W. Eosinophilia-myalgia syndrome associated with exposure to tryptophan from a single manufacturer. *JAMA, J. Am. Med. Assoc.* **1990**, *264*, 213−217.

Smith, M. J.; Mazzola, E. P.; Farrell, T. J.; Sphon, J. A.; Page, S. W.; Ashley, D.; Sirimanne, S. R.; Hill, R. H.; Needham, L. L. 1,1′-Ethylidenebis-(L-tryptophan), structure determination of contaminant "97"-implicated in the eosinophilia-myalgia syndrome (EMS). *Tetrahedron Lett.* **1991**, *32*, 991−994.

Toyo'oka, T.; Yamazaki, T.; Tanimoto, T.; Sato, K.; Sato, M.; Toyoda, M.; Ishibashi, M.; Yoshihira, K.; Uchiyama, M. Characterization of contaminants in EMS-associated L-tryptophan samples by high-performance liquid chromatography. *Chem. Pharm. Bull.* **1991**, *39*, 820−822.

Wakabayashi, K.; Ochiai, M.; Saito, H.; Tsuda, M.; Suwa, Y.; Nagao, M.; Sugimura, T. Presence of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, a precursor of mutagenic nitroso compound, in soya sauce. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 2912−2916.

Yamaoka, K. A.; Miyasaka, N.; Inuo, G.; Saito, I.; Kolb, J-P.; Fujita, K.; Kashiwazaki, S. 1,1′-Ethylidenebis(tryptophan) (peak E) induces functional activation of human eosinophils and interleukin 5 production from T lymphocytes: association of eosinophilia-myalgia syndrome with a L-tryptophan contaminant. *J. Clin. Immunol.* **1994**, *14*, 50−60.

Received for review July 7, 1995. Accepted March 13, 1996.[⊗]

JF950421D

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1996.