BIOORGANIC CHEMISTRY 16, 189–205 (1988)

Possible Occurrence of a β-Carboline Pathway in the Oxidative Catabolism of 5-Hydroxytryptamine: Chemical Approach and Structure Determination of a Yellow Substance and Related β-Carboline Derivatives[1]

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Received April 15, 1987

The reaction of 5-hydroxytryptamine with severalfold excess silver oxide in a methanol medium at 65 to 70°C for several hours gave 6-hydroxy-3,4-dihydro-β-carboline (**I**) as a main product. The yellow substance obtained in an aqueous system reported previously was identical to the latter compound with respect to NMR, field desorption (FD) mass spectroscopy, and other optical measurements. On the other hand, the same system in an ethanol medium gave 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (**II**) as a main product. It became clear from the following results that silver oxide reacted with methanol to produce formaldehyde, and thereafter oxidized the adduct of 5-hydroxytryptamine with formaldehyde to produce the yellow substance. The borohydride-reduced yellow substance was determined to be 6-hydroxy-1,2,3,4-tetrahydro-β-carboline. Anaerobic incubation of 5-hydroxytryptamine with formaldehyde in ethanol did not give rise to the yellow substance. Upon the addition of silver oxide or the addition of iron-EDTA under air to this system, the yellow substance was found. The C-1 unit of formaldehyde was utilized, being incorporated into the yellow substance. Although iron-EDTA catalyzed the oxidative reaction, EDTA itself could also act as a C-1 unit donor to a minor extent. The most powerful donor of the C-1 unit of the yellow substance in our experiments was glyoxylic acid. On aerobic oxidation of 5-hydroxytryptamine with glyoxylic acid, and more so together with iron-EDTA, the yellow substance was produced at a higher rate than with formaldehyde and iron-EDTA. Anaerobic incubation of 5-hydroxytryptamine with glyoxylic acid at 60°C at alkaline pH gave another yellow compound which was identified as 2-carboxymethyl-3,4-dihydro-β-carboline (**III**), on the basis of NMR and FD mass spectrometry. The borohydride-reduced compound was found to be 6-hydroxy-2-carboxymethyl-1,2,3,4-tetrahydro-β-carboline. The reduced compound was hardly autoxidizable. A common precursor for both the yellow substance and the anaerobically formed yellow compound was isolated and purified from the anaerobic incubation mixture of 5-hydroxytryptamine and glyoxylate at alkaline pH. The structure was found to be 6-hydroxy-1-carboxyl-1,2,3,4-tetrahydro-β-carboline (**IV**).

[1] A preliminary report was presented at the IVth FAOB Congress of Biochemistry, Singapore. This work was supported by Grant-in-Aid for Special Project Research (Metabolic Research of Blood Vessels) from the Ministry of Education, Science and Culture, Japan.

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0045-2068/88 $3.00

INTRODUCTION

Epinephrine and 5-hydroxytryptamine are both vasoconstrictive hormones, as well as neurotransmitters in brain tissues, and are derived from aromatic amino acids. The *O*-transmethylation of the hydroxyl group of the phenol of epinephrine as well as that of the indole of 5-hydroxytryptamine (*2, 3*) has been well established.

Indoleamine dioxygenase was reported to degrade 5-hydroxytryptamine in addition to tryptamine. However, the molecular activity for the former substrate was one two-hundredth ($\frac{1}{200}$) that for the latter (*4*). Monoamine oxidase was reported to act on 5-hydroxytryptamine, leading to the formation of indoleacetic acid derivatives (*5*).

Long ago an aminochrome of epinephrine, adrenochrome, was reported as an intermediate in the oxidative catabolism of epinephrine. In contrast, the corresponding oxidative pathway for 5-hydroxytryptamine catabolism has not been well investigated. We have searched for a possible analogous pathway for oxidative 5-hydroxytryptamine catabolism.

Adrenochrome was reported to have been prepared from epinephrine in the aqueous media such as a ferritin or iron–EDTA system (*6*) and another system involving xanthine oxidase, xanthine, and EDTA (*7*). In a previous paper (*1*) we reported the appearance of a strongly fluorescent yellow substance, on the use of xanthine oxidase, xanthine, and iron-EDTA in glycine buffer. It was reported (*8, 9*) that adrenochrome could be easily prepared in a single step by the chemical method in which silver oxide in methanol acts as an effective oxidant; therefore, we commenced the oxidation of 5-hydroxytryptamine by using silver oxide in order to obtain the yellow substance simply and to explore the mechanism of oxidative catabolism of 5-hydroxytryptamine.

In this paper, we report the formation of the yellow substance and related compounds from 5-hydroxytryptamine, by means of chemical preparative procedures, and describe the natures of these compounds, their basic properties and methods for obtaining them in simple ways.

MATERIALS AND METHODS

5-Hydroxytryptamine hydrochloride and the monohydrated sodium salt of glyoxylic acid were obtained from Sigma Chemicals. Silver oxide, from Wako Pure Chemicals, Osaka, was used in aqueous and in nonaqueous media. The reagents used for HPLC were of HPLC grade and others were of analytical grade. Formaldehyde was prepared by dissolving paraformaldehyde (Merck, Darmstadt, West Germany) in water and heating on a hot plate. Nash's reaction was performed to determine formaldehyde concentrations (*10*).

Spectrophotometry was carried out with a Hitachi 220 spectrometer using a cell of 1 cm in length and a digital output attachment.

Thin-layer chromatography was performed on glass plates precoated with nonfluorescent silica gel 60 (Merck), using various developing solvent systems, but

most frequently, an aqueous 5% NaCl solution and *n*-butanol–acetic acid–water (60 : 15 : 25). The fluorescent spots were detected under a uv irradiator (Atto Co., Tokyo) at wave lengths of 366 and 254 nm.

For HPLC, a Gilson Model 302 system or a Hitachi 635 was used. The former was used mainly for preparative procedures with a reverse phase of C-18 M & S pack (10 × 300 mm) (M & S Instruments, Osaka, Japan). Product detection and purity determination were carried out at a flow rate of 0.5 ml/min using a column of the same type with smaller dimensions (4.6 × 250 mm). The solvents used were mixtures of different amounts of methanol and 50 mM ammonium acetate. The absorbance was recorded at 250 nm unless otherwise specified. Purification was performed with linear methanol gradients from 10 to 30% for 40 min and then with 30 to 100% for 10 min in the case of 6-hydroxy-3,4-dihydro-β-carboline (**I**), and from 0 to 35% for 40 min and then 35 to 100% for 10 min in the case of 6-hydroxy-2-carboxymethyl-3,4-dihydro-β-carboline (**III**). For the later steps of purification, the concentration of ammonium acetate was reduced to 10 mM. The solution containing the purified product was lyophilized. To the resulting powder a few milliliters of distilled water was added, and lyophilization was repeated to free the product of ammonium acetate.

Proton NMR spectroscopy was carried out with a JEOL GX 270 spectrometer at 270 MHz at room temperature. Chemical shifts for protons are expressed in ppm downfield relative to those of trimethylsilane.

Mass spectrometry was performed with a JEOL D300 equipped with a JM-400 disc system. Usually a field desorption (FD) attachment was installed and a silica emitter was used for sample application. A positive potential of 3.5 to 5.0 kV was applied and the emitter current was increased at the rate of 4 mA per min up to 10 mA and then held for 5 min.

For mass number calibration, electron impact (EI) measurement of perfluorokerosene (PFK) was performed prior to each FD measurement. Acetylated 5-hydroxytryptamine was frequently used for examination of the FD mass spectrometry conditions. For FD mass spectrometry, acetylation of the hydroxyl group as well as the amino group was performed with a solution of a one-third volume of acetic anhydride in pyridine (*11*). Methylation was performed for carboxyl as well as hydroxyl groups using 2,2-dimethoxypropane (*12*). Trifluoroacetylation and methylation of the carboxylated β-carbolines in a single step were performed by using trifluoroacetic anhydride together with an equal amount of methanol instead of hexafluoroisopropanol (*13*).

RESULTS

Oxidation of 5-Hydroxytryptamine with Silver Oxide: Purification of the Products and Optical Properties of the Products

Fifty milligrams (0.28 mmol) of 5-hydroxytryptamine was dissolved in 40 ml of methanol or ethanol, and then 200 mg (0.86 mmol) of silver oxide was added. The reaction was carried out at 65 to 70°C for 4 h with stirring. Photochemical degrada-

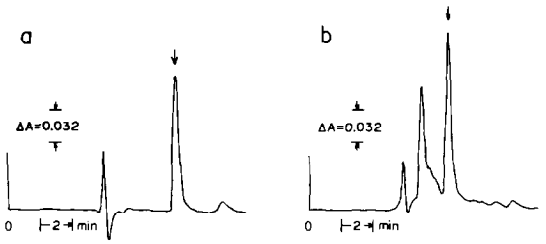

FIG. 1. (a) HPLC profile of the reaction products after the 4-h oxidation of 5-hydroxytryptamine in methanol with silver oxide. The solvent system comprised methanol and 50 mM ammonium acetate, pH 4.6, at the ratio of 1 : 2.5. The flow rate was 0.5 ml/min. Absorbance, 0.32 per full scale at 250 nm. Ten microliters of the sample was charged. The arrow denotes the peak of the yellow substance. (b) HPLC profile of the reaction products in the course of oxidation of 5-hydroxytryptamine in ethanol with silver oxide. The elution conditions were the same as those for a. The peak indicated by the arrow after 5-hydroxytryptamine denotes the retention time of the main reaction product, which was found to be an adduct of 5-hydroxytryptamine with acetaldehyde (see text).

tion was prevented by placing the reaction vessel in the dark. After the mixture had been cooled, 800 mg of Dowex, chloride form, 3 milliequivalents per gram, was added to precipitate silver ions according to Heacock and Scott (*8*).

The mixture in the methanol medium was passed through Whatman No. 2 filter paper. An aliquot of the filtrate was used for examination of the product. The reaction was monitored by TLC on a silica gel plate to check the yellow substance under uv irradiation: the R_f values for the yellow substance were 0.30 and 0.55 with the two developing solvent systems, 5% NaCl and *n*-butanol–acetic acid–water, respectively. For HPLC, an aliquot of the sample was dried under nitrogen, dissolved in 100 μl of the HPLC solvent, and then filtered to remove the insoluble; 10 μl was charged onto the HPLC column.

As shown in Fig. 1a, the yellow substance was found as a main product which exhibited strong fluorescence, and its R_f values on TLC coincided with those determined in a previous study (*1*). The first optical disturbance was derived from the solvent front and the peak of 5-hydroxytryptamine disappeared during the reaction.

For purification, the filtrate obtained from the main reaction mixture was dried in a rotatory evaporator, and then 6 to 7 ml of methanol–50 mM ammonium acetate (1 : 2) was added, followed by filtration through a HPLC filter. About 1 ml was charged onto the preparative column, and the main peak fractions corresponding to the yellow substance were collected.

The collected fractions with HPLC were evaporated to dryness to give 11 mg of powder. After lyophilization, the sample was dissolved in a smaller volume of the HPLC solvent and then subjected to HPLC for further purification. After freeze-drying, the product was washed with ethyl acetate on filter paper. Finally, a dark orange solid was obtained, which was stored at −20°C.

In contrast to the reaction in the methanol medium, the reaction in the ethanol medium gave different products, as shown in Fig. 1b. In this case, the peak of 5-hydroxytryptamine remained, showing that the reaction took place slowly com-

pared to that in the methanol medium. In addition to the first optical disturbance, there were several minor peaks. Separation of the main product from 5-hydoxytryptamine and silver oxide in the ethanol medium was performed by preparative HPLC. Further purification procedures were similar to those for the yellow substance. A faint gray product was obtained after lyophilization.

The formation of formaldehyde with the methanol–silver oxide system was indicated by Nash's reaction. Under anaerobic conditions, 5-hydroxytryptamine reacted with formaldehyde to give a precursor of the yellow substance, but no formation of the yellow substance was observed. The addition of silver oxide caused decreases of both the precursor and 5-hydroxytryptamine and the appearance of a peak of the yellow substance on HPLC as shown in Fig. 2. The dashed arrows indicate the peak position of the authentic 5-hydroxytryptamine.

The absorption spectra of the yellow substance obtained were the same as those of the yellow substance presented in the previous paper (Fig. 3a). The molar extinction coefficient was determined with the purified compound to be 9.1 mM^{-1} cm^{-1} at pH 4.0 and 400 nm as shown in Table 1.

On acetylation of the yellow substance in a pyridine solution with acetic anhydride, a rapid absorbance change accompanied by the acetylation reaction was observed. When the yellow substance was dissolved in pyridine and a one-third volume of acetic anhydride was added, the color changed instantaneously from yellow to orange and then faded in about 10 min.

The absorption spectra of the purified main product from ethanol are shown in Fig. 3b. The calculated molar extinction coefficients are given in Table 1. The acetylation of this compound was carried out according to a procedure similar to

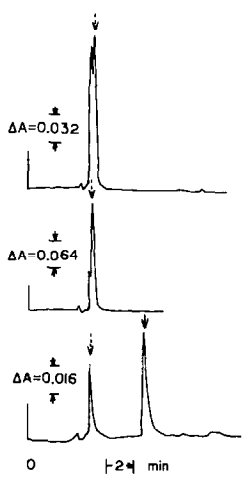

FIG. 2. HPLC profiles of the reaction of 5-hydroxytryptamine with formaldehyde in ethanol. The top trace shows the peaks of an intermediate and 5-hydroxytryptamine next to the solvent front in the course of the anaerobic reaction without silver oxide; the middle trace, sample of the top trace except that 5-hydroxytryptamine was added for confirmation of its peak assignment; the bottom trace, sample with silver oxide. The dashed arrow indicates the retention time of 5-hydroxytryptamine, while the solid arrow indicates that of the yellow substance. The reaction was performed at 55°C for 3 h. 5-Hydroxytryptamine, 8.8 mM; formaldehyde, 12 mM; silver oxide, when added, 15 mg.

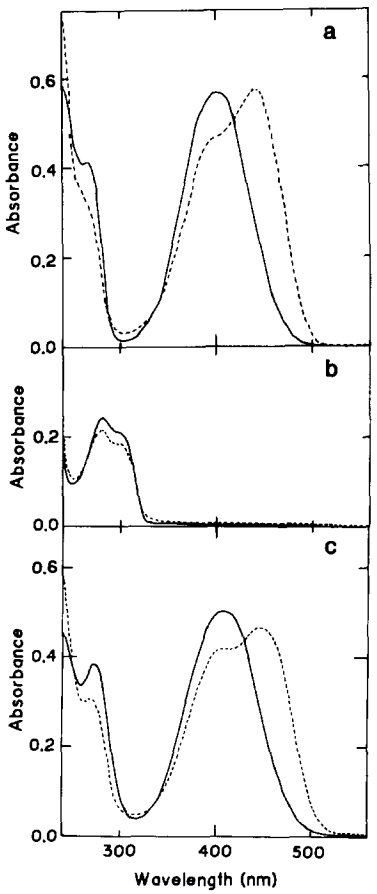

FIG. 3. Absorption spectra of the β-carboline derivatives derived from 5-hydroxytryptamine: (a) 6-hydroxy-3,4-dihydro-β-carboline (**I**); (b) 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (**II**); (c) 6-hydroxy-2-carboxymethyl-3,4-dihydro-β-carboline (**III**). The solvent systems used were 10 mM ammonium acetate, pH 4.0, for the solid curves and 10 mM ammonium acetate, pH 9.0, for the dashed curves.

TABLE 1

Molecular Absorbances of the Three β-Carboline Derivatives

	β-Carbolines					
	I		**II**		**III**	
pH	4.0	9.0	4.0	9.0	4.0	9.0
ε_1 (mM^{-1} cm^{-1})	9.1	9.5	—	—	11.4	10.5
Wavelength (nm)	400	440	—	—	410	445
ε_2 (mM^{-1} cm^{-1})	6.8	—	5.0	4.4	8.7	7.0
Wavelength (nm)	265	—	280	280	275	270

Note. **I**, **II**, and **III** represent 6-hydroxy-3,4-dihydro-β-carboline, 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline, and 6-hydroxy-2-carboxymethyl-3,4-dihydro-β-carboline, respectively.

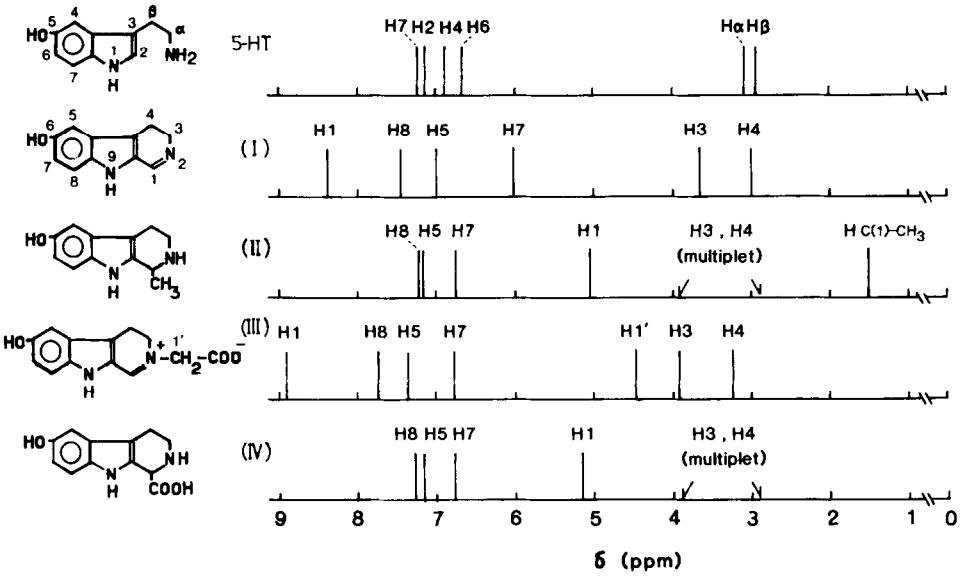

FIG. 4. Schematic representation of NMR patterns of the yellow substance and the related compounds: chemical shift values are listed in Table 2. NMR measurements were performed in a solvent system composed of dimethyl sulfoxide-d 6.0, 0.35 ml/D_2O, 0.1 ml. The NMR pattern of 5-hydroxytryptamine (5-HT) in the same solvent was presented at the top. The assignment of the chemical shifts of 5-hydroxytryptamine in D_2O or in $CDCl_3$ have been reported in the literature (*16, 17*).

that used for the yellow substance. No color change was observed during the acetylation reaction.

NMR and Mass Spectrometry of the Products Derived from 5-Hydroxytryptamine with Silver Oxide

The NMR spectrum of the yellow substance obtained according to the procedure mentioned above was the same as that reported in a previous paper (*1*).

A schematic representation of NMR data of the yellow substance together with the related compounds, which appear in the following sections, is shown in Fig. 4. Chemical shifts and the assignments are listed in Table 2. Assignments were made on the basis of the comparison of the chemical shifts of 5-hydroxytryptamine in $CDCl_3$ or D_2O (*16, 17*) and in our solvent system of dimethyl sulfoxide–D_2O with those of the observed values of the products.

FD mass spectrometry of the yellow substance demonstrated virtually a single mass species with peaks at 186 (m/z) and 187 (m/z + 1), which is identical to that of the yellow substance obtained enzymatically. The acetylated compound also showed a single species with peaks at 228 (m/z) and 229 (m/z + 1). Thus, from NMR and mass spectrometric data, the native and acetylated compounds were identified as 6-hydroxy-3,4-dihydro-β-carboline (cf. Fig. 10, **I**) and 6-acetoxy-3,4-dihydro-β-carboline, respectively. Additional support for this structure determination is the ninhydrin reaction which was negative for both compounds. In the case of acetylated 5-hydroxytryptamine, FD mass spectrometry showed a single

TABLE 2

NMR Data of Compounds **I**, **II**, **III**, and **IV**, Showing the Chemical Shifts as Expressed in ppm

Compound				
5-HT δ (ppm)	**I** δ (ppm)	**II** δ (ppm)	**III** δ (ppm)	**IV** δ (ppm)
	8.39 S		8.90 S	
7.24 D	7.46 D	7.21 D	7.74 D	7.26 D
7.15 S				
6.90 S	7.02 S	7.17 S	7.37 S	7.17 S
6.64 D	6.27 D	6.75 D	6.79 D	6.77 D
		5.05 Q		5.14 S
			4.47 S	
3.08 T	3.66 T	Multiplet 3.95–2.90	3.92 T	Multiplet 3.95–2.90
2.94 T	3.00 T		3.22 T	
		1.51 D		

Note. S, D, T, and Q denote singlet, doublet, triplet, and quartet patterns of spin–spin coupling, respectively.

species with peaks at 260 (*m/z*) and 261 (*m/z* + 1), indicating that two acetyl groups were introduced. Under the same conditions, only one acetyl group was introduced into the yellow substance.

The yellow substance was reduced by adding a few grains of sodium borohydride in a mixed solvent of methanol and 10 mM ammonium acetate (1 : 4), followed by purification by HPLC with the same solvent. The purified substance was found to be reversibly reoxidized, as shown in Fig. 5. The structure of this compound was assigned to be 6-hydroxy-1,2,3,4-tetrahydro-β-carboline on the basis of the results of FD mass spectrometry of its acetylated derivative: the values of *m/z* and *m/z* + 1 were 272 and 273, respectively.

On the other hand, the product purified from the reaction mixture of 5-hydroxytryptamine with silver oxide in ethanol showed values different from those of the yellow substance with respect to R_f on TLC (Table 3) as well as the retention time on HPLC. FD mass spectrometry showed that the molecular peaks of the product and the acetylated derivative were 202 (*m/z*) and 203 (*m/z* + 1), and 286 (*m/z*) and 287 (*m/z* + 1), respectively. The two acetyl groups were found to be incorporated into the reaction product in ethanol. NMR data are shown in Fig. 4 and in Table 2. The structure of the nonacetylated compound was assigned from these results to be 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (cf. Fig. 10, **II**). The rationale for this structure was given by the NMR experiments. As shown in Fig. 4, quartet methine proton linked to C-1 was seen at 5.05 ppm. 6-Hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline was found to be stable under air without autoxidation. The preparation of this compound by the Pictet–Spengler reaction has been already reported (*18*). It was separated from 5-hydroxytryptamine and 6-hydroxy-1,2,3,4-tetrahydro-β-carboline sensitively with HPLC (*19*).

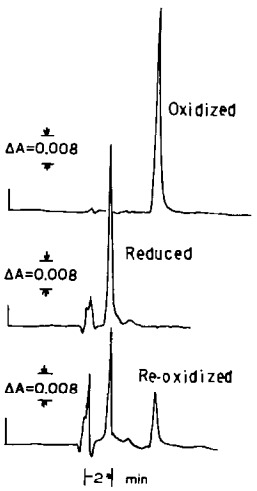

FIG. 5. Reversible reduction and reoxidation of the yellow substance. The top trace is the HPLC profile for the initial yellow substance. A few grains of sodium borohydride was added to the solution. The same amount of the test solution as for the initial mixture was subjected to HPLC. The middle trace is the profile for the borohydride-reduced yellow substance. After aeration of the reduced solution at 50°C for 20 min, the same amount of the sample was charged on the HPLC column. The bottom trace is for the yellow substance in the oxidized state. The solvent system was a mixture of 10 mM ammonium acetate, pH 4.6, and methanol, at the ratio of 2.5 to 1. Absorbance was monitored at 250 nm.

Formation of the Yellow Substance from 5-Hydroxytryptamine via the Adduct with Glyoxylic Acid

Glyoxylic acid was reported to be useful, by virtue of its effective fluorophore formation, for the histological detection of monoamines including tryptamine and 5-hydroxytryptamine (*15*).

TABLE 3

R_f Values of the β-Carboline Derivatives Derived from 5-HT

	β-Carbolines				
	I[a]		II	III	
	Oxidized	Reduced		Oxidized	Reduced
5% NaCl	0.30–0.33	0.53–0.56	0.58	0.36–0.39	0.63
n-Butanol–acetic acid–water	0.49–0.55	0.40–0.44	0.60	0.39	0.39

Note. The spots on silica gels were detected by irradiation of the monochromatic ultraviolet of 366 or 254 nm. **I**, **II**, and **III** represent 6-hydroxy-3,4-dihydro-β-carboline, 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline and 6-hydroxy-2-carboxymethyl-3,4-dihydro-β-carboline, respectively.

[a] Values are from Ref. (*1*).

When 5-hydroxytryptamine was incubated with glyoxylic acid at pH 8.5 and 50°C in 10 mM sodium pyrophosphate medium or 10 mM ammonium acetate medium, formation of 6-hydroxy-3,4-dihydro-β-carboline (I) was observed at a much higher rate than with formaldehyde. The time course of the formation of the yellow substance is shown in Fig. 6. The rates are higher with iron-EDTA than without. The reaction was followed by HPLC at 250 nm for concentration changes of 5-hydroxytryptamine and the product.

The HPLC pattern as well as the TLC and FD mass spectrometry patterns of the yellow substance produced in the reaction of 5-hydroxytryptamine with glyoxylic acid under air coincided completely with those obtained in the reaction with silver oxide in methanol. For preparation of the yellow substance, the reaction with glyoxylate was superior to that with silver oxide, and the yield was almost quantitative.

The peak of the yellow substance from 5-hydroxytryptamine incubated alone in an alkaline medium was undetectable on HPLC, even after 6 h at 50°C under aerobic conditions at pH 8.5. In the presence of 10 mM iron-EDTA, on the other hand, a small but constant increase in the peak height of the yellow substance was observed during the same reaction time. Although iron-EDTA was used as a catalyst for oxidation to produce the yellow substance, a minor portion of the yellow substance was derived from the reaction of 5-hydroxytryptamine and iron-EDTA, EDTA being a small C-1 unit donor.

The Anaerobic Formation of a Yellow Compound Other Than 6-Hydroxy-3,4-dihydro-β-carboline

A yellow compound different from the yellow substance mentioned above was obtained when 5-hydroxytryptamine was incubated anaerobically with glyoxylic

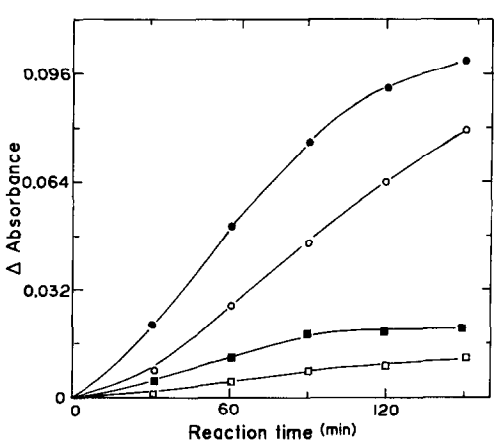

FIG. 6. The time course of formation of the yellow substance in the reaction mixture of 5-hydroxytryptamine with glyoxylate or formaldehyde in the presence or absence of iron-EDTA. ●, With glyoxylate with iron-EDTA; ○, with glyoxylate without iron-EDTA; ■, with formaldehyde with iron-EDTA; □, with formaldehyde without iron-EDTA. The concentrations were 5-hydroxytryptamine, 1 mM; glyoxylate, 1 mM; formaldehyde, 2 mM; iron-EDTA, 1.5 mM. The temperature was 50°C. Buffer, 0.04 M sodium pyrophosphate, pH 8.5. Absorbances were measured at 250 nm.

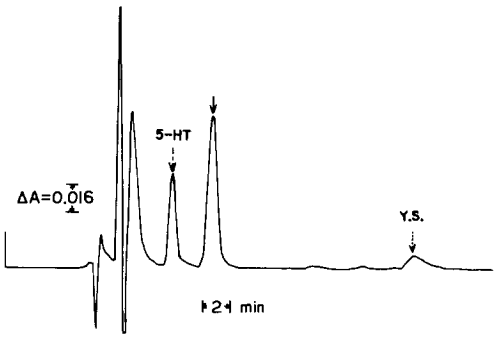

FIG. 7. Formation of another yellow compound on anaerobic incubation of 5-hydroxytryptamine with glyoxylate. The reaction was carried out in a Thunberg tube in ethanol at 50°C. Concentrations were 5-hydroxytryptamine, 10 mM; glyoxylate, 13 mM. After incubation for 210 min, 50 μl of the mixture was diluted six times with a mixture of methanol and 10 mM ammonium acetate at the ratio of 1 to 4. Fifteen microliters was charged on the HPLC column. The solid arrow indicates the peak of the product. The effluent was of the same methanol and ammonium acetate composition. The yellow substance (I) detected, which is denoted as Y.S., was formed due to insufficient anaerobicity or after exposure to air for further experiments.

acid. To investigate the formation of the compound, a Thunberg-type anaerobic tube was used in which 9 mg of 5-hydroxytryptamine in 0.02 M sodium pyrophosphate, pH 8.5, or ethanol was mixed with 6 mg of glyoxylic acid, sodium salt, in the same buffer or ethanol under anaerobic conditions. On incubation at 50°C for 3.5 h, a yellow color developed. The yellow compound also showed strong fluorescence but gave a peak on HPLC different from 6-hydroxy-3,4-dihydro-β-carboline (I), as shown in Fig. 7. The reaction was terminated by acidification with acetic acid and autoxidation was prevented. The compound corresponding to this peak was separated by reverse phase HPLC on a C-18 column. The solvent system was bubbled with nitrogen gas prior to HPLC procedure, and the fractions separated from those containing 5-hydroxytryptamine and other compounds were combined and lyophilized. About 2 mg of the purified compound was obtained, which was also dark orange in the dried solid state.

Optical, NMR, and FD Mass Spectrometry of the Yellow Compound Produced Anaerobically with Glyoxylic Acid and 5-Hydroxytryptamine

The anaerobically formed yellow product revealed properties different from those of the yellow substance mentioned above. Its R_f values are listed in Table 3. The absorption spectra are shown in Fig. 3c; the curves are similar to those for the yellow substance, as mentioned above, except the red shift of the peaks by 5 to 10 nm. The values of molecular absorption coefficients are given in Table 1. In the course of acetylation of this yellow compound, there was no appearance of an orange color, in contrast to the color change observed for the former yellow substance. It did not volatize in the FD ionization chamber unless it was methylated.

The results of FD mass spectrometry for methylated derivatives of the oxidized

and reduced compounds, which were prepared with dimethylpropane, showed the *m/z* values to be 259 and 261, respectively. The NMR data are shown in Fig. 4 and in Table 2. From these results, the anaerobic product was identified as 6-hydroxy-2-carboxymethyl-3,4-dihydro-β-carboline (cf. Fig. 10, **III**). After it had been purified by HPLC, it was found to be stable at 50°C against further autoxidation. When it was reduced with a few grains of sodium borohydride, neither reoxidation by air nor oxidative decarboxylation took place at 50°C in several hours.

Preparation of the Precursor of the Two Yellow Compounds and Determination of Its Structure

During the anaerobic formation of the yellow compound (**III**), a semicrystalline precipitate was seen at an earlier stage of the reaction. A slightly yellowish compound was isolated from the yellow mixture by filtration after acidification of the mixture to prevent autoxidation. Purification of the compound was achieved by recrystallization from acidic ethanol. This compound was converted under air to the yellow substance (**I**), mentioned in the preceding sections, quantitatively, even at room temperature. The result of autoxidation accompanied by decarboxylation is illustrated in Fig. 8. Autoxidation hardly occurred at acidic pH.

When incubation of the purified product with glyoxylic acid was carried out under anaerobic conditions, the reaction gave rise to the anaerobically formed yellow compound as seen in Fig. 9. The absorbance change at 450 nm was ascribed to the formation of the anaerobically formed yellow compound (**III**), because the retention time on HPLC revealed only appearance of the latter compound (**III**), not compound **I**. Even a short exposure of the reaction mixture to air caused appearance of compound **I**.

The structure of the compound was studied by FD mass spectrometry, after derivatization to the trifluoroacetylated form of the methyl ester. The molecular

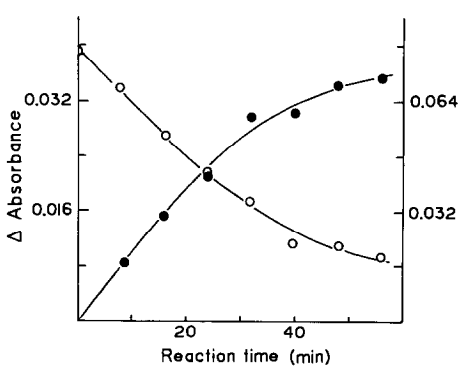

FIG. 8. Formation of the yellow substance (**I**) from the purified precursor (**IV**). The ordinate on the left-hand side denotes the decrease in the absorbance of the precursor, while that on the right-hand side denotes the increase in the absorbance of the yellow substance. The reaction was carried out at 40°C in 0.1 M ammonium acetate, pH 8.7. Absorbance was measured at 250 nm. Open circles denote the decrease in the concentration of the precursor (left ordinate), whereas filled circles denote the increase in that of the yellow substance (**I**) (right ordinate).

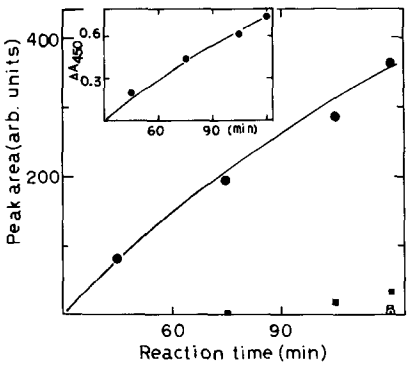

FIG. 9. The time course of the conversion of the precursor to the anaerobically formed yellow compound. The amount of 2 mg of purified compound was dissolved in 50 mM ammonium acetate, pH 6.55, and incubated in a Thunberg anaerobic tube with 1.5 mg of glyoxylic acid in a total volume of 3 ml. At the times indicated, the visible spectrometric measurements were performed. Aliquots for HPLC were taken out from the Thunberg tube and diluted with the solvent for HPLC. The reaction temperature was kept at 58°C. The integrated areas of the uv absorption at 250 nm of HPLC were plotted against time. The peak areas were plotted in arbitrary units. The increases of the absorbance at 450 nm at the same time intervals are shown in the inset in the upper left. The control, filled squares, without glyoxylic acid.

peaks at 438 (*m/z*) and 439 (*m/z* + 1) corresponded to the trifluoroacetylated derivative of the methyl ester of the precursor of the yellow substance. Peaks at 342 and 284 were observed in addition to the peak at 438. These peaks corresponded to the methylated monotrifluoroacetate of compound **IV** and the monotrifluoroacetate of the decarboxylated form of **IV** (see Fig. 10), respectively. The NMR data are presented in Fig. 4 and in Table 2. Based on these experimental results from FD mass spectrometry and NMR, the structure of the nonderivatized form was assigned to 6-hydroxy-1-carboxyl-1,2,3,4-tetrahydro-β-carboline (cf. Fig. 10, **IV**).

DISCUSSION

In the search for the oxidative metabolic pathway of 5-hydroxytryptamine, we used silver oxide to achieve direct oxidation of the indole ring expected from the analogy with adrenochrome formation. We failed, however, to observe iminoquinone formation, and therefore it is likely that the indole ring is resistant to direct oxidation by silver oxide under our experimental conditions. Instead, oxidation of the solvent, methanol or ethanol, by silver oxide took place first. The adduct of 5-hydroxytryptamine with formaldehyde or acetaldehyde was then formed, from which various products were derived: namely, 6-hydroxy-3,4-dihydro-β-carboline (**I**) or 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline (**II**), in the methanol or ethanol medium, respectively. The latter have been reported to appear in rat urine as enantiomers possibly due to an enzymatic reaction (*20*). The production of 1-methyl-1,2,3,4-tetrahydro-β-carboline through condensation and decarboxylation on the reaction of tryptamine with pyruvic acid was reported

(*21*). Oxidative decarboxylation of this sort of compound was reported in connection with alkaloid biosynthesis (*22*). However, the rate of reaction of 5-hydroxytryptamine with pyruvic acid, leading to the formation of the yellow substance via 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline, was rather slow under our experimental conditions when compared to the case with glyoxylate. 6-Hydroxy-1,2,3,4-tetrahydro-β-carboline was oxidized by silver oxide to the yellow product. Therefore, silver oxide is a useful oxidant for double bond formation at the C-1 position, but not at the C-3 position. This type of reaction with silver oxide in methanol medium gave the yellow substance (**I**) as the main product, as can be seen in Fig. 1a. In the aqueous system, however, there were several products that need to be structurally identified. Recently, effective oxidation by permanganate was reported for the syntheses of 3,4-dihydro-β-carboline derivatives other than 6-hydroxy-3,4-dihydro-β-carboline (*23*).

The properties of the yellow substance obtained in this study completely match those of the yellow substance reported in the preceding report (*1*) (Fig. 3a and Table 2 and 3).

In the case of the reaction with glyoxylic acid, the preceding event is also Schiff base formation followed by ring closure. When the autoxidation rates to the yellow substance are compared, it can be pointed out that the oxidative decarboxylation occurred at a higher rate than oxidation of 6-hydroxy-1,2,3,4-tetrahydro-β-carboline, as can be seen in Figs. 5, 6, and 8.

There have been several reports concerning β-carboline formation in relation to the histochemical technique of fixing indole ethylamines in tissues (*14, 15*). Glyoxylic acid was reported to be better for the staining of tryptamine than formaldehyde (*15*). The structures of the fluorophores were proposed on the basis of the results of EI–mass spectrometry. There still remains some ambiguity as to accurate structure of the yellow substance, especially regarding its occurrence in biological processes. We have carried out chemical experiments to determine the structure of the yellow product, which was obtained on the enzymatic and aqueous catabolism of 5-hydroxytryptamine (*1*). The yellow product obtained was identical to the main product of the reaction in the methanol system with 5-hydroxytryptamine and silver oxide, and it was found to be 6-hydroxy-3,4-dihydro-β-carboline (**I**), as judged by the results of both NMR and FD mass spectrometry. From the results obtained, a quinoneimine structure is unlikely for the yellow substance. Four aromatic-ring protons but not five protons besides C-3 and C-4 protons were observed, implying that the C-4a proton of the quinoneimine structure would be unreasonable.

Iron-EDTA in alkaline medium was found to be effective for 5-hydroxytryptamine catabolism with the xanthine oxidase system. In the chemical reaction without xanthine oxidase, for which the solution contained 5-hydroxytryptamine and iron-EDTA in pyrophosphate or ammonium acetate, the yellow substance was produced. In this case, the C-1 unit was presumably provided by EDTA itself through degradation of the ligand under aerobic conditions. However, the rate of production of the yellow substance from iron-EDTA and 5-hydroxytryptamine was very slow compared with the case with formaldehyde or glyoxylate.

The reduced β-carboline, which was prepared from the yellow substance, 6-hy-

droxy-3,4-dihydro-β-carboline, by adding a few grains of sodium borohydride, was oxidized with iron-EDTA and oxygen to reproduce the yellow substance. That the rate was higher with iron-EDTA than that without substantiates that iron-EDTA catalyzes the formation of the yellow substance from the reduced carboline under air. Under anaerobic conditions, 6-hydroxy-3,4-dihydro-β-carboline was hardly produced, and if produced, it was only due to the insufficient anaerobicity.

When the rate of enzymatic formation of the yellow substance (cf. Fig. 10, **I**) is compared with that of nonenzymatic, i.e., chemical, formation, it was found that the rate of the enzymatic reaction was high, with a remarkable yield being obtained within 20 min at 30°C; the dehydrogenation of the reduced carboline was presumably caused by the participation of xanthine oxidase system used in those experiments.

The formation of the other yellow compound, 6-hydroxy-2-carboxymethyl-3,4-dihydro-β-carboline on reaction of 5-hydroxytryptamine with glyoxylic acid at alkaline pH under anaerobic conditions, is very interesting. In this case, iron-EDTA had no effect on the rate of formation of the compound. The mechanism of the formation of 2-carboxymethyl-3,4-dihydro-β-carboline (**III**) was proposed on the basis of the results of EI–mass spectrometry (*15*).

The precursor for both the yellow substance and the anaerobically formed yellow compound was first isolated, purified, and found to be 6-hydroxy-1-carboxyl-1,2,3,4-tetrahydro-β-carboline, because it was obtained in a high yield only under anaerobic conditions. The decarboxylation of 1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid was reported in brain homogenate, probably through the catalysis of pyridoxal-5′-phosphate (*24*).

As it is well known that glyoxylic acid is a key substance in biochemical reactions such as glycine metabolism, the glyoxylic acid cycle, and β-hydroxyaspartate formation, the adduct of glyoxylic acid with 5-hydroxytryptamine may play a meaningful role in the catabolism of the latter. In our previous study (*1*), glycine was converted to glyoxylic acid and served as the C-1 unit in the yellow substance.

Thiazoline-2-carboxylate, which was formed through the condensation of cysteamine with glyoxylic acid and subsequent oxidation by D-amino acid oxidase, was found to be a strong inhibitor for dopamine β-hydroxylase (*25*). Adducts of glyoxylic acid with various nucleophiles have been reported to be good substrates for the peroxisomal oxidases (*26*).

In the scheme (Fig. 10), we have summarized the oxidative catabolic pathway of 5-hydroxytryptamine and given the structural formulas of the compounds related to this pathway, which were confirmed in this study.

We are currently searching for the physiological occurrence of these derivatives of 5-hydroxytryptamine in the biochemical processes and investigating the actions of the products on 5-hydroxytryptamine uptake in synaptosomes. Several papers have appeared concerning the inhibitory actions of tetrahydro-β-carbolines on monoamine oxidase (*27, 28*). Two metabolic pathways for tetrahydro-β-carboline were investigated in rats (*29*). Very recently, enhancement of performance in learning and memory tasks due to a β-carboline, methyl-β-carboline 3-carboxylate, was reported (*30*).

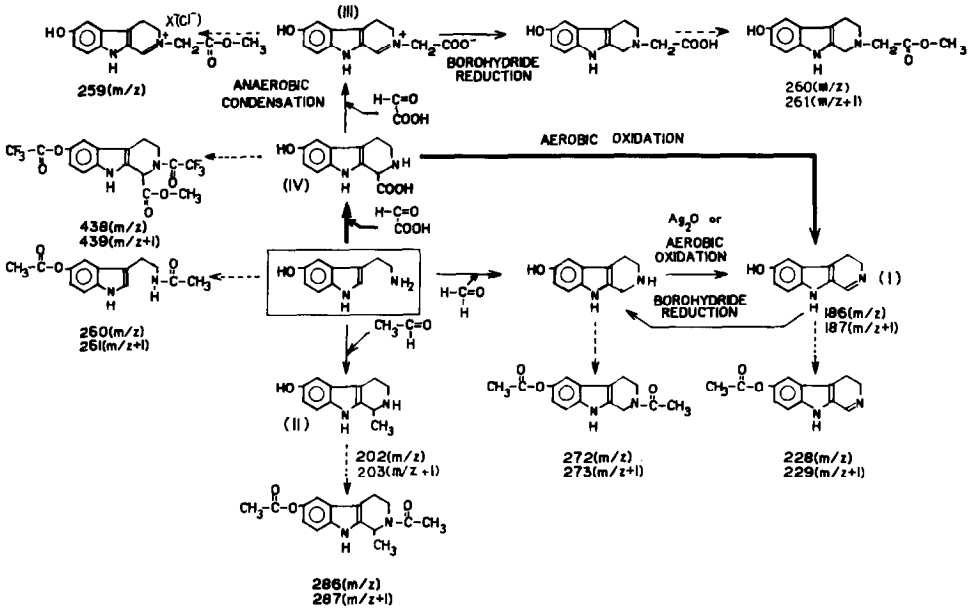

FIG. 10. Schematic presentation of the reaction processes for 5-hydroxytryptamine with formaldehyde, glyoxylic acid, and acetaldehyde. Roman numerals denote the key compounds. Dashed lines denote the derivatization for structure assignment by FD mass spectrometry. The thick lines denote the reaction showing the highest reaction rate. Peak heights of $m/z + 1$ are greater than or comparable to those of m/z described in the formulae. Even though data are based on low-resolution FD mass spectrometry, $m/z + 1$ peaks are due to protonated form.

ACKNOWLEDGMENT

We are grateful to Dr. Yoshihiro Miyake, Chairman of the Department of Biochemistry, National Cardiovascular Center Research Institute, for his interest and encouragement in this study.

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