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Tryptophan Side Chain Oxidase from *Pseudomonas*

OXIDATION OF SKATOLE TO INDOLE-3-CARBOXALDEHYDE VIA INDOLE-3-METHANOL*

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Tryptophan side chain oxidase from *Pseudomonas* was shown to catalyze two consecutive oxidations in which skatole was first oxidized to indole-3-methanol, and then to indole-3-carboxaldehyde; indole-3-methanol was the reaction product of skatole and also a substrate for the enzyme. A half-equivalent of oxygen was consumed for each reaction. The reaction also proceeded under anaerobic conditions with ferricyanide as an electron acceptor. Two equivalents of ferricyanide were reduced for each reaction.

Both reactions were assayed spectrophotometrically at a constant oxygen concentration (air-saturated buffer) at 25°C. Hyperbolic substrate saturation curves were obtained with both skatole and indole-3-methanol. The K_m values for skatole and indole-3-methanol were 15.4 and 43.5 μM, respectively, at pH 6.0. The ratio of the V_{max} for skatole to indole-3-methanol was 0.88. Each substrate inhibited the oxidation of the other. The apparent K_i values for skatole and indole-3-methanol were 16.7 and 44.1 μM, respectively. These results indicated that both reactions were catalyzed by a single enzyme. The reactions at various concentrations of oxygen were performed at a fixed concentration of the substrate, 0.2 mM skatole or 0.5 mM indole-3-methanol; the apparent K_m values for oxygen were 50 and 55 μM, respectively.

The substrate specificity of the enzyme was analyzed with special reference to 1- and 2-substituted skatole derivatives. The activity to 2,3-dimethylindole (0.5 mM) was one-half of that to skatole (0.5 mM). Neither 1-acetyl nor 1-methyl derivatives could serve as substrate or inhibitor for the enzyme.

A new hemoprotein that catalyzes the oxidation of various 3-substituted indoles was isolated from *Pseudomonas* (ATCC 29574) and crystallized (1, 2). The enzyme was termed "tryptophan side chain oxidase" (1). The enzyme catalyzed the reaction in which L-tryptophan was both decarboxylated and deaminated to yield 3-indolylglyoxal or 3-indolylglycolaldehyde (or both) as the major product (1). The enzymic formation of *N*-acetyl-α,β-didehydrotryptophanamide and dehydrotryptophan in peptides was demonstrated with *N*-acetyl-L-tryptophanamide and tryptophan-containing peptides as substrate, respectively (3, 4). On the other hand, Roberts and Rosenfeld reported that the same enzyme catalyzed the hydroxylation on the side chain of tryptamine and other indole derivatives, and proposed that the enzyme be termed "indolyl-3-alkane α-hydroxylase" (2).

In an attempt to clarify the mode of action of the enzyme, the reactions with skatole and indole-3-methanol as substrates were analyzed. These substrates are the simplest in their structures among the substrates (1), having a methyl or hydroxymethyl group as the side chain on C-3 of indole, and thus the complicated reactions involving the functional groups at the side chain of the substrate can be avoided. The substrate specificity of the enzyme is also presented with special reference to 1- and 2-substituted skatole derivatives.

EXPERIMENTAL PROCEDURES

Materials—Skatole (3-methylindole) and indole-3-carboxaldehyde were purchased from Nakarai Chemicals (Kyoto, Japan). The latter compound was recrystallized from ethyl alcohol/water (1:3). Indole-3-methanol from Aldrich Chemical Co. was recrystallized five times from chloroform. The melting point was 88°C (with decomposition) with no change on further recrystallization.

C_9H_9NO (147.17)

Calculated:	C 73.45, H 6.16, N 9.52
Found:	C 73.54, H 6.14, N 9.32

2-Methylindole from Nakarai was recrystallized from *n*-hexane. Indole was obtained from Wako Pure Chemicals (Osaka, Japan). 1-Acetyl-3-methyl-, 1-acetyl-2,3-dimethyl-, 1,3-dimethyl-, 1,2,3-trimethyl-, 2,3-dimethyl-, 2-cyclopropyl-3-methylindole, and 1,2,3,4-tetrahydrocarbazole were kindly provided by Drs. T. Matsuura and I. Saito of the Department of Synthetic Chemistry, Kyoto University Faculty of Engineering. 3-Methyldioxindole (2-oxo-3-methyl-3-hydroxyindoline) was a generous gift from The Institute of Food Chemistry (Osaka). Silicic acid (100 mesh powder) was from Mallinckrodt. Amberlite XAD-2 was from Rohm & Haas Co. All other chemicals were of reagent grade.

Tryptophan side chain oxidase was prepared from *Pseudomonas* (ATCC 29574) as described previously (1) except that an additional hydroxyapatite column chromatography was performed at the final step of purification.[1] Metapyrocatechase was kindly provided by Dr.

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[1] The specific activity of the enzyme was 2 to 3 times higher than that prepared previously (1); the visible and ultraviolet absorption

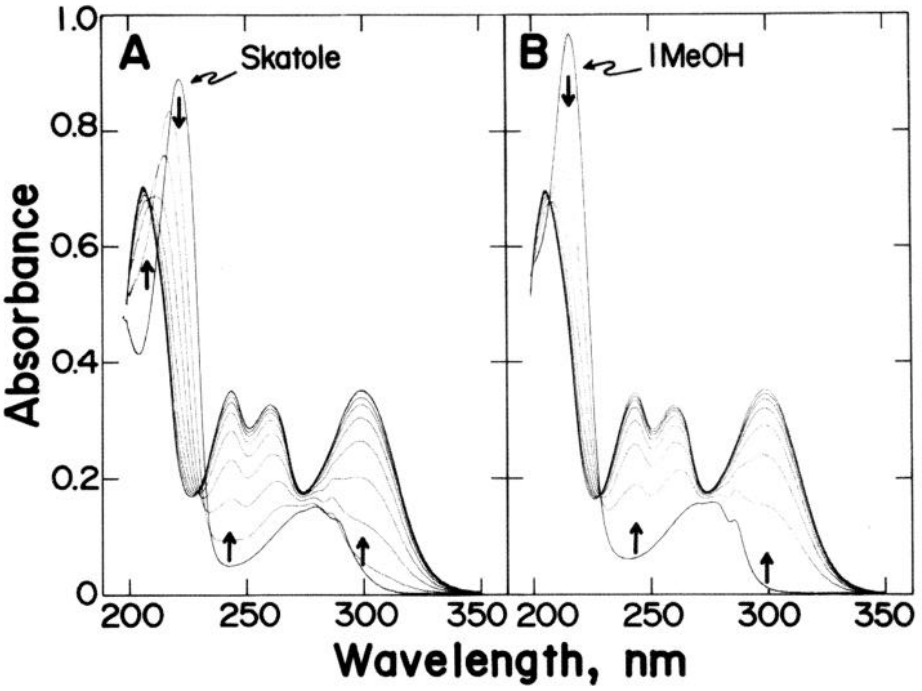

FIG. 1. Changes in ultraviolet absorption spectra during the enzymic oxidations of skatole (*A*) and indole-3-methanol (*B*). The sample cuvette contained either 28.7 μM skatole (*A*) or indole-3-methanol (*B*) and 8.6 μg of enzyme in 2.5 ml of 50 mM potassium phosphate buffer, pH 6.0. The reference cuvette contained all the components except the substrate. Each record was begun at 350 nm and completed in 1 min. The spectra of skatole (*A*) and indole-3-methanol (*IMeOH*) (*B*) were recorded prior to the addition of the enzyme. After the addition of the enzyme, successive records were begun at 3-min intervals up to 21 (*A*) and 18 min (*B*) at 25°C. The final records were begun at 45 (*A*) and 31 min (*B*), respectively. *Arrows* indicate the direction of the changes in absorbance.

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Methods—Indole-3-methanol was measured colorimetrically at 540 nm after reaction with Salkowski reagent (ferric chloride/perchloric acid) (5). To 0.2 ml of an ethyl alcohol solution containing 5 to 75 nmol of indole-3-methanol was added 0.8 ml of Salkowski reagent. Incubation was carried out at 25°C for 60 min. Indole-3-carboxaldehyde was determined according to the method of Stutz (6) with slight modifications. To 0.9 ml of an ethyl alcohol solution containing 5 to 100 nmol of indole-3-carboxaldehyde was added 0.1 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. After 20 min at 25°C, the absorbance at 550 nm was measured. Gas-liquid chromatography for the determination of skatole was performed with a glass U-shaped column (180 × 0.2 cm) packed with 5% SE-52 on 60/80 mesh Chromosorb W (AW-HMDS) in a Packard 7300 series gas chromatograph model 824, equipped with a hydrogen flame ionization detector. The inlet block temperature was set at 150°C, the column oven at 115°C, and the detector oven at 150°C. The flow rate of the carrier gas, nitrogen, was 30 ml/min. The retention time of skatole was 11 min.

Trimethylsilyl derivative of indole-3-methanol was prepared by incubating 0.15 mg of the compound with 0.2 ml of 25% *N,O*-bis(trimethylsilyl)acetamide in acetonitrile for 6 h at room temperature. The gas-liquid chromatography-mass spectrometry of the derivative was performed with a JEOLCO model JMS-D300 mass spectrometer interfaced with a JEOLCO JGC-20K gas chromatograph, equipped with a column (100 × 0.2 cm) packed with 2% OV-17 on 60/80 mesh Chromosorb W (AW-DMCS). The carrier gas was helium. The temperature of the column was programmed from 140°C to 200°C at a rate of 5°C/min. The ionization potential was 30 eV.

Silicic acid column chromatography was performed as described by Powell (7). Preparative silica gel thin layer plates of 0.3-mm layer thickness were prepared by applying an aqueous slurry of Silica Gel H (Merck) to glass plates (20 × 20 cm). Analytical thin layer chromatography was performed with precoated silica gel plates of 0.25-mm layer thickness (F_{254}, Merck). Solvent systems used were: A, chloroform/acetic acid (95:5); B, chloroform/methyl alcohol (93:7) saturated with 25% ammonia. Spots were detected under ultraviolet light at either 254 or 365 nm, or by spraying with Salkowski reagent or 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl.

Enzyme Assays—The reaction was performed at 25°C. The substrate was dissolved in ethyl alcohol as a stock solution; the final concentration of ethyl alcohol in the assay mixture was 1% unless otherwise noted.

spectra of these two preparations were indistinguishable from each other. The K_m values for skatole and indole-3-methanol, and the ratio of the V_{max} for skatole to indole-3-methanol were essentially the same as those obtained with the previous enzyme preparations.

The initial rate of the oxidation of skatole to indole-3-methanol was determined by measuring the increase in absorbance at 208 nm, an isosbestic point of indole-3-methanol and indole-3-carboxaldehyde (Fig. 1). A cuvette of a 0.2-cm light path contained the indicated concentration of skatole and 0.4 μg of enzyme in 0.5 ml of 50 mM potassium phosphate buffer, pH 6.0. The initial rate of the oxidation of indole-3-methanol to indole-3-carboxaldehyde was determined by measuring the increase in absorbance at either 300 or 320 nm. A cuvette of a 1-cm light path contained the indicated concentration of indole-3-methanol and 0.4 μg of enzyme in 1 ml of 50 mM potassium phosphate buffer, pH 6.0. The extinction coefficients of skatole and indole-3-methanol at 208 nm at pH 6.0 were taken as 16.34 and 23.91 mM^{-1} cm^{-1}, respectively; those of indole-3-methanol and indole-3-carboxaldehyde at 300 nm were 0.29 and 12.45 mM^{-1} cm^{-1}, and those at 320 nm were 0 and 4.12 mM^{-1} cm^{-1}, respectively; $\Delta\epsilon$ (indole-3-methanol/skatole) at 208 nm was 7.57 mM^{-1} cm^{-1}; $\Delta\epsilon$ (indole-3-carboxaldehyde/indole-3-methanol) at 300 and 320 nm were 12.16 and 4.12 mM^{-1} cm^{-1}, respectively.

The reactions at various concentrations of oxygen were performed as described above except that the buffer solution had been bubbled for 15 min with 0.96, 2.5, 5, 10, and 20% oxygen in a nitrogen carrier; after the addition of the substrate, the reaction mixture was bubbled for another 5 min. The reaction was started by the addition of the enzyme; the enzyme solution had been equilibrated with nitrogen gas.

Buffers (50 mM in concentration) used were: glycine/HCl, pH 2.5 to 3.5; sodium citrate, pH 2.5 to 5.3; sodium acetate, pH 3.5 to 5.5; sodium succinate, pH 4.0 to 6.0; potassium phosphate, pH 6.0 to 8.0; Tris-HCl, pH 7.0 to 8.5; sodium borate, pH 8.0 to 10.2.

Instruments—Ultraviolet absorption spectra were recorded on a Shimadzu recording spectrophotometer model UV-300 in a quartz cuvette of a 1-cm light path unless otherwise noted. Infrared absorption spectra were recorded on a JASCO recording spectrophotometer model IRA-1. The electron impact mass spectra were recorded on a Hitachi mass spectrometer model M52 by direct probe inlet technique. Melting points were determined with a Buechi melting point apparatus and were corrected.

RESULTS

Reactions with Skatole and Indole-3-methanol as Substrate—Fig. 1 shows a series of ultraviolet absorption spectra recorded at intervals during the enzymic oxidations of skatole (*A*) and indole-3-methanol (*B*). In both cases, the spectra at the completion of the reaction were the same as that of indole-3-carboxaldehyde. The spectral readings indicated that the conversions were quantitative. In Fig. 1*B*, isosbestic points at 208 and 228 nm are obvious, and the initial change in absorb-

ance at any wavelength tested is linear with respect to time. These results indicated that the product was formed without detectable accumulation of an intermediate. In Fig. 1*A*, however, no isosbestic points are observed; there is a lag in the increase in absorbance at 300 nm, which is indicative of the formation of indole-3-carboxaldehyde. These results suggested that certain intermediate(s) was formed during the reaction.

Identification of Reaction Product of Skatole—The reaction mixture contained 0.3 mmol of skatole and 4.5 mg of enzyme in 3000 ml of 50 mM potassium phosphate buffer, pH 6.0, containing 0.05% ethyl alcohol. Reaction was carried out at 25°C in three 1000-ml portions. The reaction was followed by the increase in absorbance at 300 nm. When the reaction was complete, each reaction mixture was extracted twice with 200 ml of ethyl acetate. The combined extracts were washed with 100 ml of water and evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of ethyl alcohol and applied to a column (1 × 60 cm) of silicic acid. The column was washed with 0.2% *n*-butyl alcohol in *n*-hexane (v/v). The product was eluted with 3% *n*-butyl alcohol in *n*-hexane (v/v). Portions of the main fractions were subjected to preparative thin layer chromatography with Solvent A to remove contaminants. A zone which absorbed ultraviolet light with an R_F value of 0.20 was scraped off the plates and extracted with ethyl acetate. The combined extracts and the main fractions from the silicic acid column were evaporated to dryness under reduced pressure. After being dried *in vacuo* over P_2O_5 at room temperature, the residue (36.9 mg) was recrystallized four times from ethyl alcohol/water (1:3). The needles (13.2 mg) melted at 197°C with decomposition. The mixed melting point with authentic indole-3-carboxaldehyde showed no depression.

C_9H_7NO (145.16)

Calculated: C 74.47, H 4.86, N 9.65
Found: C 74.45, H 4.86, N 9.61

The mass spectrum of the product showed a molecular ion peak at *m/e* 145 (*M*, relative intensity, 85%), a base peak at *m/e* 144 (*M* − 1, 100%), and a peak at *m/e* 116 (*M* − CHO, 40%). The infrared and ultraviolet absorption spectra of the product were identical with those of authentic indole-3-carboxaldehyde, respectively (Figs. 2 and 3). On thin layer chromatography, the product gave a single spot with any solvent system used, having an R_F value identical with that of indole-3-carboxaldehyde (Table I). Both the sample and the authentic compound gave an orange color with 2,4-dinitrophenylhydrazine reagent. On the basis of these results, the reaction product of skatole was identified as indole-3-carboxaldehyde.

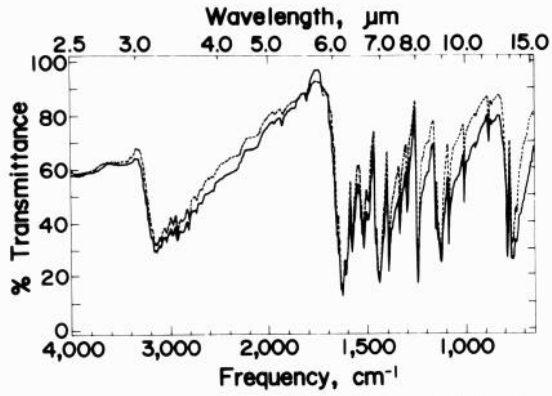


FIG. 2. Infrared absorption spectra of authentic indole-3-carboxaldehyde (——) and the reaction product of skatole (- - -) in KBr pellets.

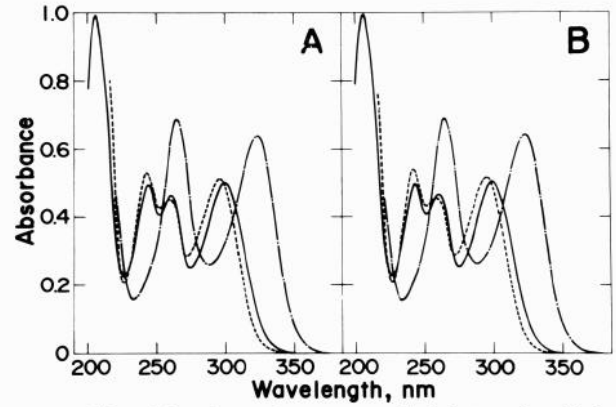


FIG. 3. Ultraviolet absorption spectra of indole-3-carboxaldehyde (*A*) and the reaction product of skatole (*B*) in 50 mM potassium phosphate buffer, pH 6.0, containing 0.4% ethyl alcohol (v/v) (——), 0.1 N NaOH containing 0.4% ethyl alcohol (-·-·-), and ethyl alcohol (- - -). The concentrations were 40 μM.

TABLE I

Thin layer chromatography of the reaction products, skatole, indole-3-methanol, and indole-3-carboxaldehyde

Twenty nanomoles of each compound were subjected to thin layer chromatography at room temperature with the following solvent systems: I, chloroform/acetic acid (95:5); II, chloroform/methyl alcohol/acetic acid (75:20:5); III, chloroform/methyl alcohol (93:7) saturated with 25% ammonia; IV, benzene/acetone (90:10); V, isopropyl alcohol/methyl acetate/25% ammonia (80:10:10). The length of run was 10 cm.

Compound	R_f values				
	I	II	III	IV	V
Product of skatole	0.12	0.75	0.33	0.13	0.83
Product of indole-3-methanol	0.12	0.75	0.33	0.13	0.83
Skatole	0.60	0.87	0.67	0.54	0.89
Indole-3-methanol	0.39[a]	0.71[a]	0.22	0.09	0.84
Indole-3-carboxaldehyde	0.12	0.75	0.33	0.13	0.83

[a] Decomposed.

Identification of Reaction Product of Indole-3-methanol—The reaction mixture contained 0.2 mmol of indole-3-methanol and 1.1 mg of enzyme in 400 ml of 50 mM potassium phosphate buffer, pH 6.0, containing 1% ethyl alcohol. The reaction was followed by the increase in absorbance at 300 nm at 25°C. When the reaction was complete, the product was extracted once with 200 ml and twice with 50 ml of ethyl acetate. After being washed with 50 ml of water, the combined extracts were evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of ethyl alcohol and subjected to preparative thin layer chromatography with Solvent A. The product (19.2 mg) was recrystallized three times from ethyl alcohol/water (1:3). The needles (10.3 mg) melted at 197°C with decomposition. The mixed melting point with authentic indole-3-carboxaldehyde showed no depression.

C_9H_7NO (145.16)

Calculated: C 74.47, H 4.86, N 9.65
Found: C 74.29, H 4.85, N 9.58

On thin layer chromatography, the sample gave a single spot with any solvent system used, having an R_F value identical with that of indole-3-carboxaldehyde (Table I). The infrared and ultraviolet absorption spectra were identical with those shown in Figs. 2 and 3, respectively. On the basis of these results, the reaction product of indole-3-methanol was identified as indole-3-carboxaldehyde.

Stoichiometry of Reaction—The reaction was performed

TABLE II

Stoichiometry of reaction

The reaction vessel contained either 250 nmol of skatole or 260 nmol of indole-3-methanol and 120 μg of enzyme in 2.5 ml of 50 mM potassium phosphate buffer, pH 6.0. Oxygen consumption was determined polarographically (8) at 25°C. When the oxygen uptake had ceased, the reaction mixture and the washings were extracted three times with 3 ml of ethyl acetate. After being isolated by thin layer chromatography with Solvent A, indole-3-carboxaldehyde (IAld) was determined as described under "Experimental Procedures."

Substrate added	IAld formed	Oxygen consumed
	nmol	
Skatole, 250 nmol	240	240
Indole-3-methanol, 260 nmol	260	115

TABLE III

Stoichiometry of reaction under anaerobic conditions

A Thunberg-type cuvette contained the indicated amount of either skatole or indole-3-methanol, 2 μmol of potassium ferricyanide, and 35 μg of enzyme in 1.5 ml of 50 mM potassium phosphate buffer, pH 6.0. The reaction mixture was made anaerobic by several cycles of degassing and flushing with argon. The content of residual oxygen was less than 0.5 nmol as estimated by the use of metapyrocatechase and catechol (9). The reaction was started by introducing the enzyme and ferricyanide from the side arm. The reduction of ferricyanide was followed at 25°C in terms of a decrease in absorbance at 420 nm, at which wavelength the extinction coefficient was taken as 1.02 mM^{-1} cm^{-1}. When the decrease in absorbance had ceased, the cuvette was immersed in a boiling water bath for 5 min. The reaction mixture and the washings were extracted twice with 2 ml of ethyl acetate. Indole-3-carboxaldehyde (IAld) in the extract was determined as described in the legend for Table II.

Substrate added	IAld formed	Ferricyanide reduced
	nmol	
Skatole, 190[a] nmol	170	700
Indole-3-methanol, 750 nmol	600	1130

[a] The amount of skatole was determined by the absorbance at 280 nm using the extinction coefficient of 5.20 mM^{-1} cm^{-1} after the reaction mixture was made anaerobic.

with either skatole or indole-3-methanol as the limiting substrate (Table II). Oxygen uptake ceased when 1 mol of oxygen had been consumed per mol of skatole initially present. One mole of indole-3-carboxaldehyde was formed per mol of skatole consumed. With indole-3-methanol as substrate, 1 mol of indole-3-carboxaldehyde was formed per mol of indole-3-methanol consumed with concomitant consumption of 0.5 mol of oxygen.

The reaction also proceeded under anaerobic conditions with ferricyanide as an electron acceptor. Table III shows that both skatole and indole-3-methanol were almost completely converted to indole-3-carboxaldehyde with concomitant reduction of 4 and 2 equivalents of ferricyanide, respectively.

Intermediate of Skatole Oxidation—To characterize the intermediate(s) of skatole oxidation, the products at various stages of the reaction were analyzed by thin layer chromatography. As shown in Fig. 4,[2] a spot (R_F = 0.18) that was different from either skatole or indole-3-carboxaldehyde appeared at the initial stage of the reaction. Its R_F value was the same as that of indole-3-methanol. Both the sample and the authentic compound gave a reddish brown color with Salkowski reagent. The compound found in this spot was extracted from silica gel with acetone. The ultraviolet absorption spectrum of the compound in ethyl alcohol was the same as that of indole-3-methanol. On thin layer chromatography, its R_F value was identical with that of indole-3-methanol with any solvent system cited in Table I. On incubation with the enzyme, the presumed intermediate was converted to indole-3-carboxaldehyde in the same way as shown in Fig. 1*B*. The sample and the authentic compound gave the same decomposition product, presumably 3,3′-diindolylmethane (11), when heated in H_2O at 100°C. A spot (R_F = 0.59) shown in Fig. 4 corresponded to this decomposition product. On gas-liquid chromatography-mass spectrometry, the trimethylsilyl derivative of the sample was indistinguishable from that of indole-3-methanol: the retention time, 6.0 min; mass spectrum: a molecular ion peak at *m/e* 291 (*M*, relative intensity, 90%), which corresponded to bis(trimethylsilyl)indole-3-methanol; *m/e* 290 (*M* − 1, 67%); *m/e* 202 (*M* − OSi $(CH_3)_3$, 100%). On the basis of these results, indole-3-methanol was identified as the intermediate of skatole oxidation.

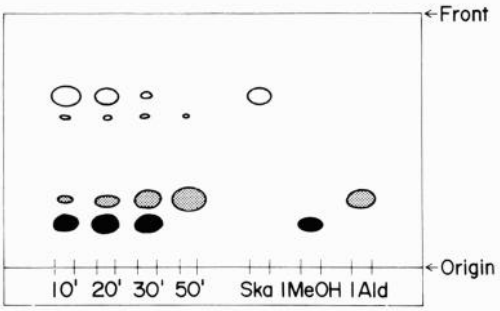


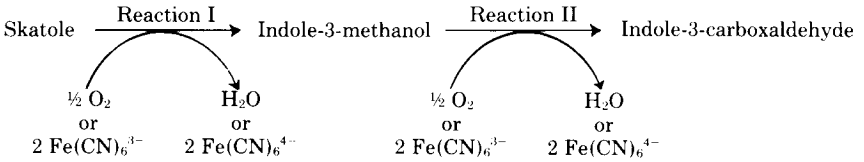
FIG. 4. Thin layer chromatography of the reaction products of skatole. Single side arm manometer vessels contained 1.5 μmol of skatole and 17 μg of enzyme in 3 ml of 50 mM potassium phosphate buffer, pH 6.0. The reaction was started by the addition of the enzyme from the side arm. The oxygen uptake ceased at 50 min of incubation as followed manometrically (10) at 25°C. At 10, 20, 30, and 50 min of incubation period, the reaction mixture and the washings were chilled in a bath of ethyl alcohol/water (1:1, v/v) containing dry ice. The products were extracted twice with 3 ml of ethyl acetate. The extracts were concentrated under a stream of nitrogen. One-tenth aliquot of each was subjected to thin layer chromatography with Solvent B. The authentic compounds were 50 nmol each of skatole (*Ska*), indole-3-methanol (*IMeOH*), and indole-3-carboxaldehyde (*IAld*). The spots were visualized by spraying with Salkowski reagent and 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl.

Time Course of Reaction—Fig. 5*A* shows the time course of the reaction with skatole as substrate. There was a lag in the formation of indole-3-carboxaldehyde while skatole started to decrease linearly. Indole-3-methanol was at first formed linearly and then decreased. Throughout the course of the reaction, the amount of skatole consumed was almost equal to the sum of indole-3-methanol and indole-3-carboxaldehyde formed.[4] The oxygen uptake corresponded to the sum of indole-3-carboxaldehyde and one-half of indole-3-methanol formed.

These results together with those shown in Tables II and III indicate that the oxidation of skatole proceeds in two steps with indole-3-methanol as a dissociable intermediate. Skatole is first oxidized to indole-3-methanol (Reaction I), and the latter to indole-3-carboxaldehyde (Reaction II). A half-equivalent of oxygen or two equivalents of ferricyanide are consumed for each reaction:

[2] Besides the spots shown in Fig. 4, the chromatogram, however, included a spot at any incubation time analyzed with an R_F value of 0.57, which was detected by the yellow fluorescence under ultraviolet light (λ = 365 nm). The color hardly developed with either Salkowski reagent or 2,4-dinitrophenylhydrazine reagent. The nature of the compound found in this location was not further explored.

[4] While the conversions to indole-3-carboxaldehyde were almost quantitative at low concentrations of the substrates as shown in Table II, the yield was somewhat low (90%) mainly due to the formation of byproducts such as the yellow fluorescent compound described in Footnote 2 and 3,3′-diindolylmethane from indole-3-methanol.



SCHEME 1

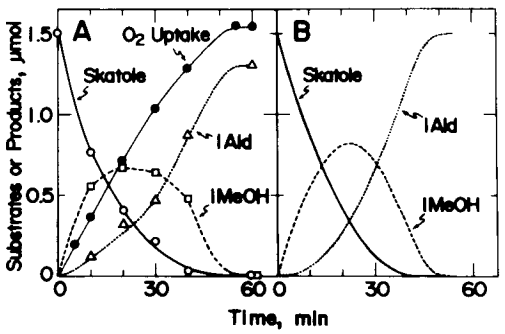


FIG. 5. *A*, time course of the reaction with skatole as substrate. The reaction mixture and the conditions for the reaction were the same as described in the legend for Fig. 4. The oxygen uptake was followed at 25°C. At intervals, the reaction was stopped by the addition of 15 μl of 0.2 M potassium cyanide.[3] The reaction mixture and the washings were applied to a column (0.5 × 10 cm) of Amberlite XAD-2, which had been equilibrated with 10% aqueous ethyl alcohol. Indole-3-methanol and indole-3-carboxaldehyde were eluted with 40% ethyl alcohol, and skatole with 95% ethyl alcohol. These compounds were determined as described under "Experimental Procedures." ○——○, skatole; □- - -□, indole-3-methanol (*IMeOH*); △·····△, indole-3-carboxaldehyde (*IAld*); ●——●, oxygen uptake. *B*, calculated time course of the reaction. The curves for skatole (——), indole-3-methanol (- - -), and indole-3-carboxaldehyde (····) were drawn according to the calculations from Equations 2 with V^{S}_{max} = 25.5 μM/min, s = 500 μM, and $m = p = 0$ at zero time, the kinetic parameters given in the text, and the volume of the reaction mixture of 3 ml.

Kinetic Analyses of Reactions I and II—The initial rates of Reactions I and II at various concentrations of each substrate were determined spectrophotometrically at a fixed oxygen concentration (air-saturated buffer) at 25°C (Fig. 6). A straight line was obtained on each double reciprocal plot. The K_m values for skatole and indole-3-methanol were 15.4 and 43.5 μM, respectively. The ratio of the V_{max} for skatole to indole-3-methanol was 0.88.

When a single enzyme acts on two different substrates present simultaneously in the reaction mixture, each will act as a competitive inhibitor of the other (12, 13). Fig. 7 shows the linear competitive inhibitions of Reactions I and II by indole-3-methanol and skatole, respectively. The apparent K_i values for skatole and indole-3-methanol were 16.7 and 44.1 μM, respectively. These values were comparable to their respective K_m values. Indole-3-carboxaldehyde (80 μM) did not inhibit either reaction (with 8 μM skatole or 20 μM indole-3-methanol as substrate) at pH 6.0.

These results conformed to the simple mechanisms:[5]

$$E + S \rightleftarrows (ES) \rightarrow E + M \qquad \text{(Reaction I)}$$

$$E + M \rightleftarrows (EM) \rightarrow E + P \qquad \text{(Reaction II)}$$

SCHEME 2

[3] To avoid the decomposition of indole-3-methanol by acid or heat, the reaction was stopped by the addition of potassium cyanide, an inhibitor of this enzyme. The recoveries of skatole, indole-3-methanol, and indole-3-carboxaldehyde were 80, 82, and 100%, respectively, through these manipulations.

[5] The concentration of oxygen was held constant, and the reaction was regarded as one substrate reaction.

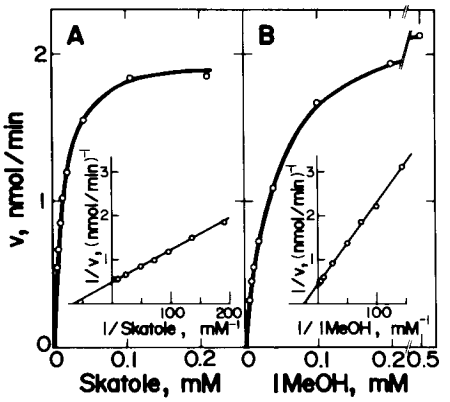


FIG. 6. *A*, initial rate of Reaction I as a function of skatole concentration. *Inset*, a double reciprocal plot of the results. *B*, initial rate of Reaction II as a function of indole-3-methanol (*IMeOH*) concentration. *Inset*, a double reciprocal plot of the results. The reaction was performed as described under "Experimental Procedures." Each point represents a mean of the duplicate determinations.

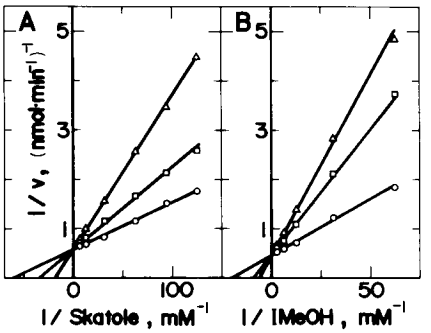


FIG. 7. Inhibitions of Reaction I by indole-3-methanol (*A*) and of Reaction II by skatole (*B*). The initial rate of the reaction was determined as described under "Experimental Procedures"; the oxidation of indole-3-methanol (*IMeOH*) was followed at 320 nm. For *A*, the concentrations of indole-3-methanol were: ○——○, 0 mM; □——□, 0.04 mM; △——△, 0.10 mM. For *B*, those of skatole were: ○——○, 0 mM; □——□, 0.02 mM; △——△, 0.04 mM. Each point represents a mean of the duplicate determinations.

In Scheme 2, E is a free enzyme, and S, M, and P are skatole, indole-3-methanol, and indole-3-carboxaldehyde, respectively. The steady state rate equations are given by:

$$v_I = \frac{V^{S}_{max} \cdot s}{K^{S}_{m}(1 + m/K^{M}_{m}) + s}$$

$$v_{II} = \frac{V^{M}_{max} \cdot m}{K^{M}_{m}(1 + s/K^{S}_{m}) + m}$$

EQUATIONS 1

where v_I is the rate of Reaction I and v_{II} that of Reaction II; s is the concentration of skatole and m that of indole-3-methanol; $K_m{}^{S}$ and $K_m{}^{M}$, and V^{S}_{max} and V^{M}_{max} are the Michaelis constants and the maximal velocities for skatole and indole-3-methanol, respectively.

The initial rates of the reaction at various oxygen concentrations were determined at a fixed concentration of skatole

(0.2 mM) or indole-3-methanol (0.5 mM). Hyperbolic saturation curves were obtained for the oxidations of both skatole and indole-3-methanol; the apparent K_m values for oxygen were 50 and 55 μM, respectively.

Calculated Time Course of Reaction—The integrated rate equations for Equations 1 are (14):

$$t = -\frac{1}{V^S_{max}}\left\{\frac{A_0}{1-\alpha}\frac{V^S_{max}}{V^M_{max}}s^{\alpha} + \left(1 - \frac{1}{1-\alpha}\frac{K^S_m}{K^M_m}\right)s + K^S_m \ln s\right\} + B_0$$

$$m = \frac{1}{1-\alpha}(A_0 s^{\alpha} - s)$$

$$p = C_0 - s - m$$

EQUATIONS 2

where $\alpha = V^M_{max} \cdot K_m{}^S / V^S_{max} \cdot K_m{}^M < 1$; p is the concentration of indole-3-carboxaldehyde; A_0, B_0, and C_0 are the integration constants.

The simulated time course of the reaction paralleled that in Fig. 5*A* (Fig. 5*B*).

Effect of pH—The initial rate of the oxygen uptake showed maximum at around pH 3.5 as assayed with 0.5 mM skatole as substrate. Below pH 5, the conversion of skatole to indole-3-carboxaldehyde was not quantitative due to the formation of byproducts including nonenzymic decomposition products of indole-3-methanol. The activities for both Reactions I and II decreased as pH increased above pH 5.5. The rates of the oxidation of skatole and indole-3-methanol at pH 9.0 were 21 and 18% of those at pH 6.0, respectively; both at pH 10.2 were less than 1%. The apparent K_m values for skatole and indole-3-methanol were 10.3 and 10.5 μM, respectively, at pH 9.0;[6] those for oxygen were 133 and 154 μM as determined with 0.2 mM skatole and 0.5 mM indole-3-methanol as substrate, respectively.

Ring-substituted Indole Derivatives as Substrate—The activities of tryptophan side chain oxidase on 1- and 2-substituted indole derivatives were determined by measuring the oxygen uptake polarographically at pH 6. Either of the following indole derivatives was incubated with 38 μg of enzyme: 1-acetyl-3-methyl, 1-acetyl-2,3-dimethyl-, 1,3-dimethyl-, 1,2,3-trimethyl-, 2-cyclopropyl-3-methylindole, 3-methyldioxindole, and 1,2,3,4-tetrahydrocarbazole (each at 0.1 mM), and 2,3-dimethyl- and 2-methylindole (at 0.5 mM). The activity to 2,3-dimethylindole was one-half of that to skatole (0.5 mM). 1-Substituted indole derivatives did not serve as substrate. The activities to 2-cyclopropyl-3-methylindole and 1,2,3,4-tetrahydrocarbazole were negligible at pH 6.0. The enzyme was not active toward either 2-methylindole or 3-methyldioxindole. Neither these indole derivatives, except 2,3-dimethylindole, nor indole itelf served as inhibitor for the enzyme as assayed spectrophotometrically at pH 6.0 with either skatole or indole-3-methanol as substrate.

DISCUSSION

Our results presented herein indicate that a single enzyme, tryptophan side chain oxidase, catalyzes the oxidation of both skatole and indole-3-methanol. The oxidation of skatole involves the sequential formation of indole-3-methanol and indole-3-carboxaldehyde. The enzyme acts on other substrates in a similar manner: *N*-acetyl-β-hydroxytryptophanamide, a predominant reaction product of *N*-acetyl-L-tryptophanamide at low pH, is oxidized to *N*-acetyl-β-oxotryptophanamide (15); β-hydroxytryptamine, the reaction product of tryptamine (2), is further oxidized to β-oxotryptamine.[7] The kinetic analyses suggest that both Reactions I and II are catalyzed at a single active site. The enzyme acts on either of these substrates in a random manner (Scheme 2); Reactions I and II are independent of each other. A half-equivalent of oxygen is consumed for each reaction. Oxygen serves as an electron acceptor, and can be replaced by other electron acceptors such as ferricyanide. As shown under "Stoichiometry of Reaction," a half-equivalent of oxygen appears to be reduced to water by 2 equivalents of electrons from either of the substrates. These results are consistent with previous results (1, 16).

The minimal substrate requirements for the oxidation appear to be a side chain of one carbon atom, methyl or hydroxymethyl group, linked to C-3 of indole. 1-Substituted indoles do not appear to bind the enzyme, since these derivatives serve as neither substrate nor inhibitor for the enzyme. The enzyme is less specific with regard to C-2 since substitution of a methyl group at C-2 retains the activity. As indole does not serve as either substrate or inhibitor, a side chain at C-3 appears to be indispensable not only for the oxidation but also for the binding to the enzyme.

The precise mechanisms by which skatole is converted to indole-3-methanol are still unknown. Reaction I, however, can be assumed to involve dehydrogenation followed by hydration. Although the primary dehydrogenation product has not been detectable, one may consider 3-methylidene-3H-indole as a hypothetical intermediate. The participation of such a compound has been postulated in various substitution reactions and oxidation at the side chain of indole (17, 18). Hydration to this type of intermediate would yield indole-3-methanol. The involvement of indole NH proton in the dehydrogenation is at present obscure.

A similar mechanism may apply to the oxidation of other substrates having the side chain of more than two carbon atoms at C-3 of indole; the initial dehydrogenation would occur at 3-indolylmethyl group. Again 3-alkylidene-3H-indole may be considered as an intermediate. From such an intermediate, a vinylindole such as *N*-acetyl-α,β-didehydrotryptophanamide (3) would be readily formed with deprotonation at the side chain α-carbon and subsequent rearrangement of electrons. The intermediate otherwise would be hydrated to yield a hydroxy compound such as *N*-acetyl-β-hydroxytryptophanamide or β-hydroxytryptamine. Rosenfeld *et al.* (16) proposed a similar view with regard to the initial dehydrogenation step. Rosenfeld *et al.* (16) and Maelicke *et al.* (19) reported that the major reaction products of *N*-acetyl-L-tryptophanamide were 3-alkylidene indoline[8] and oxazoline type compounds. Our own findings, however, showed that the major product of the reaction was *N*-acetyl-β-hydroxytryptophanamide under similar conditions (15). The mechanisms whereby L-tryptophan yields 3-indolylglyoxal and/or 3-indolylglycolaldehyde, ammonia, and carbon dioxide (1) are under investigation.

Skatole is a well known metabolite of indole-3-acetic acid as seen in microorganisms in the large intestine (20, 21). At high doses, it causes a pulmonary edema and emphysema in cows and goats (21). There have been some reports on the enzymic oxidation of this compound (22, 23) as well as its *in vivo* metabolites (24, 25). The precise metabolic fate of this compound, however, has not been fully understood. Indole-3-methanol has been reported to be a metabolite of both indole-3-acetic acid and indole-3-carboxaldehyde in pea seedlings

[6] The absorption spectrum each of skatole, indole-3-methanol, and indole-3-carboxaldehyde was essentially the same between pH 6 and 9; the reaction was assayed spectrophotometrically as described under "Experimental Procedures."

[7] H. Ushiro, unpublished observations.

[8] The structure of the product proposed by Rosenfeld *et al.* (16) corresponds to 3-alkylidene-3*H*-indole (17) or 3-alkylidene indolenine rather than 3-alkylidene indoline.

(26), while the enzymes responsible for these reactions have not been clarified. Indole-3-carboxaldehyde is an oxidation product of indole-3-acetic acid in many plants (27), and is excreted into the urine of rats fed D-tryptophan (28). The formation of indole-3-carboxaldehyde from skatole either *in vitro* or *in vivo* has not been reported. The physiological significance of the reactions reported herein is at present unknown.

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