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Cytochrome P-450 and Alkaloid Synthesis in *Claviceps purpurea*

S. H. AMBIKE and R. M. BAXTER

Abstract □ Tryptophan, 4-dimethylallyltryptophan, and various analogs of tryptophan were investigated for their effect on binding to cytochrome P-450 and on cytochrome P-450 and total alkaloid levels in *Claviceps purpurea*. These compounds were shown not to affect cytochrome P-450 levels, in contrast to phenobarbital which increased the levels, but rather exhibited stereospecific binding to cytochrome P-450. Those compounds (L-tryptophan and L-4-dimethylallyltryptophan), which showed the highest binding affinity and are known precursors, caused the greatest increase in total alkaloid levels. The significance of these findings is discussed.

Keyphrases □ Cytochrome P-450, alkaloid synthesis—*Claviceps purpurea* □ ^{14}C-L-Tryptophan, tryptophan analogs—cytochrome P-450 □ *Claviceps purpurea* growth—tryptophan analogs □ Colorimetric analysis—spectrophotometer

The occurrence of cytochrome P-450 in a clavine-producing strain of *Claviceps purpurea* has been previously reported (1). Phenobarbital-treated *C. purpurea* exhibited a parallel increase in the cytochrome P-450 and in total alkaloid. Cyanide produced a gradual but marked decrease in both cytochrome P-450 and alkaloid levels. The interconversion of cytochrome P-450 and P-420 has been utilized to provide additional evidence for its presence in *C. purpurea* (2).

Cytochrome P-450 is now recognized as an "oxygen-activating enzyme," participating in the hydroxylation of a wide variety of compounds (3, 4). Substrate interaction with cytochrome P-450 gives rise to two types of difference spectra which have been utilized to measure the nature and degree of interaction of cytochrome P-450 with various compounds. Type I spectral changes are characterized by a difference spectrum, with a trough at 420 mμ and a peak at 385 mμ, and are characteristic of that produced by the interaction of cytochrome P-450 with hexobarbital, aminopyrine, phenobarbital, and chlorpromazine. Type II spectral changes exhibit a difference spectrum, having a peak at 430 mμ and a trough at 390 mμ; the spectrum is produced by the interaction of aniline, nicotine, nicotinamide, *etc.*, with cytochrome P-450. The magnitude of the spectral changes is substrate concentration-dependent (5). It has been suggested (6) that the substrate forms complexes with the oxidized form of cytochrome P-450 and that the rate-limiting step of the reaction is the reduction of the substrate–cytochrome P-450 complex. However, it has also been suggested that the complex is more readily reduced by NADPH cytochrome P-450 reductase than is cytochrome P-450 in the absence of the substrate (7).

The present study is concerned with the possible role of cytochrome P-450 in alkaloid synthesis and extends the previously reported data. The binding of tryptophan, tryptophan analogs, isopentenyl pyrophosphate, and 4-dimethylallyltryptophan to cytochrome P-450 was studied using the difference absorbance. The effect of these compounds on the levels of cytochrome P-450 and total alkaloid and the correlation of these values with effects on binding are also reported.

EXPERIMENTAL

Binding of ^{14}C-L-Tryptophan to Cytochrome P-450—The method utilized was similar to that of Orrenius and Ernster (8) who studied

Table I—Binding Affinity of Cytochrome P-450 of *C. purpurea* and Alkaloid Levels

Compound[a]	Absorbance Difference, $A_{385-420}$	Amount of Cytochrome P-450 in μmoles, $A_{450-490}$	Total Alkaloid in mg./ Flask
Control	0.010	6.10	2.10
L-Tryptophan	0.038	6.51	4.08
D-Tryptophan	0.009	5.86	2.05
DL-Tryptophan	0.018	5.95	2.21
4-Methyl-DL-tryptophan	0.010	6.04	2.14
5-Methyl-DL-tryptophan	0.020	6.05	3.20
6-Methyl-DL-tryptophan	0.010	5.98	2.18
5-Fluoro-DL-tryptophan	0.012	6.20	2.04
4-Fluoro-DL-tryptophan	0.011	5.95	2.15
4-Dimethylallyl-L-tryptophan	0.042	6.09	4.45
Isopentenyl pyrophosphate	0.015	5.95	3.15

[a] Concentration = 2 m*M*.

the binding of ^{14}C-aniline to cytochrome P-450. The cytochrome P-450-containing mycelial pellet preparation (1) was incubated at 37° for 60 min. in 2.5 ml. of a reaction mixture containing 1 ml. of 0.05 *M* tromethamine (pH 7.5). One milliliter of 0.12 *M* KCl, 0.5 ml. of 1.67 *M* $Na_2S_2O_4$, and 1 ml. of 1 m*M* solution ^{14}C-L-tryptophan (sp. act. 9.0 mc./m*M*) were added. After incubation, the sample was diluted to 10 ml. with ice-cold 0.15 *M* KCl and centrifuged at 105,000×*g* for 60 min. The pellet was resuspended in 10 ml. of 0.15 *M* ice-cold KCl and recentrifuged. The washing was repeated three times or until the last washing was devoid of radioactivity. The washed pellet was suspended in 2 ml. of 0.15 *M* KCl and lyophilized. The residue was dissolved in 1 ml. of hyamine hydroxide and 10 ml. of scintillating solution (PPO, 4 g.; POPOP, 100 mg. dissolved in 1 l. of toluene). The radioactivity in counts per minute was determined according to the usual procedure, using a Packard liquid scintillation spectrometer. The results in counts per minute were utilized to determine the percentage of ^{14}C-L-tryptophan bound. The radioactivity in counts per minute was also determined for a 1 m*M* solution of ^{14}C-L-tryptophan and for the combined washings (three).

Effect of Carbon Monoxide on Binding of ^{14}C-L-Tryptophan to Cytochrome P-450—The washed pellet was suspended in 2.5 ml. of the reaction mixture. Carbon monoxide was bubbled into the mycelial pellet suspension for 30 sec. After centrifugation at 105,000 ×*g* for 60 min. and after three washings, the radioactivity was determined as described.

Determination of the Magnitude of Spectral Changes—The mycelial pellet, containing cytochrome P-450, prepared as described (2), was suspended in 2.5 ml. of the reaction mixture. One milliliter of a solution of L-tryptophan (range 0.5–6.0 m*M*/ml.) was added, followed by incubation at 37° for 60 min. The difference in absorbance was measured between the two wavelengths, 385 and 420 mμ. The difference absorbance for the binding of other compounds was determined in a similar manner. The results in Table I

Table II—Binding of ^{14}C-L-Tryptophan to Cytochrome P-450 of Control and Phenobarbital-Treated *C. purpurea*

Series	Source of Cytochrome P-450	Binding of ^{14}C-L-Tryptophan to Cytochrome P-450 in Mycelial Pellet,[a] %	^{14}C-L-Tryptophan in Supernatant,[a] %
1	Control	71.47	27.54
2	Phenobarbital-treated	85.14	14.43
3	Control plus carbon monoxide	11.13	88.41

[a] For details of preparation, see *Experimental.*

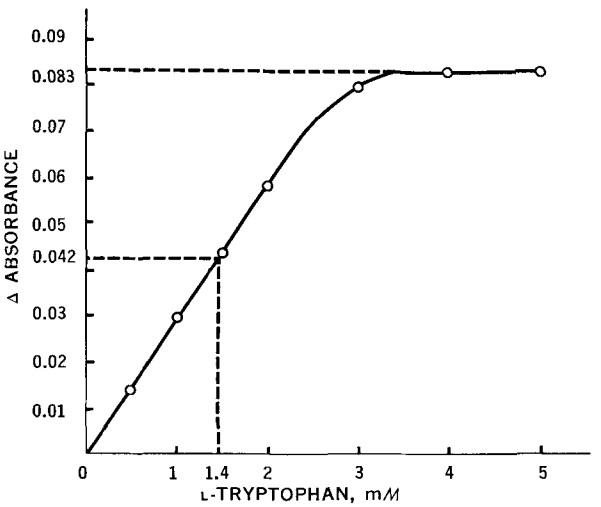

Figure 1—*Binding of* L-*tryptophan to cytochrome P-450 in the mycelial pellet of* C. purpurea.

were obtained using a 1-ml. solution of a concentration sufficient to give a final concentration of 2 m*M*.

Growth of *C. purpurea* in the Presence of Analogs—*C. purpurea* was grown as previously described (2). The analog of tryptophan and other additives were added to a final concentration of 2 m*M* on the 10th day. On the 16th day (6 days after additions), the medium was removed from the mycelial mat and used for estimating total alkaloids by the method of Taber and Vining (9). The preparation of the mycelial pellet was as previously described (2), and the estimation of cytochrome P-450 was by the method of Sato and Omura (10).

RESULTS

A comparison of the binding capacity of cytochrome P-450 in the mycelial pellet preparation from control and phenobarbital-treated *C. purpurea* revealed an increased binding capacity for L-^{14}C-tryptophan for the cytochrome P-450 in the mycelial pellet from phenobarbital-treated *C. purpurea*. Treatment of the mycelial pellet with carbon monoxide in the presence of dithionite resulted in a release of most of the bound L-^{14}C-tryptophan (Table II). Figure 1 illustrates the increase in binding with increasing tryptophan concentration expressed as an absorbance difference ($A_{385-420}$). Maximum binding was observed at a concentration of 5 m*M* of L-tryptophan, with one-half maximum being observed at 1.4 m*M* concentration of L-tryptophan.

Over a pH range from 4.0 to 9.5, binding was maximal at pH 5–5.5 and decreased by approximately 30% at pH 4.0 and 9.5. Table I summarizes the relative binding affinity of cytochrome P-450 as measured by absorbance difference for tryptophan, isopentenyl pyrophosphate, 4-dimethylallyltryptophan, and certain analogs of tryptophan. The effects of these compounds on cytochrome P-450 and total alkaloid levels in *C. purpurea* when added during the alkaloid-producing period are also indicated. It will be observed that the binding affinity is greatest for L-tryptophan and relatively low for D-tryptophan. The analogs of tryptophan used were the DL-isomers; therefore, the values recorded for these should be compared with those recorded for DL-tryptophan. None of the compounds in Table I produced any significant changes in the cytochrome P-450 levels from those of the control. However, L-tryptophan and 4-dimethylallyl-L-tryptophan, which are known precursors of ergot alkaloids, exhibit the highest binding affinities for cytochrome P-450 as well as causing the greatest increases in total alkaloid. The 5-methyl analog of DL-tryptophan exhibited a binding affinity similar to that of DL-tryptophan, but its addition during the alkaloid-producing period of growth of *C. purpurea* resulted in a greater increase in total alkaloid than that produced by DL-tryptophan.

The effects of various analogs of tryptophan when added during the alkaloid-producing period of growth of *C. purpurea* on cytochrome P-450 on total alkaloid levels and the affinity of the cyto-

Table III—Effect of Tryptophan Analogs[a] on Binding Affinity of Cytochrome P-450 for Tryptophan and Alkaloid Levels

Compound[b]	Absorbance Difference, $A_{385-420}$	Amount of Cytochrome P-450 in μmoles, $A_{450-490}$	Total Alkaloid in mg./ Flask
4-Methyl-DL-tryptophan	0.020	6.14	3.12
5-Methyl-DL-tryptophan	0.044	6.10	5.90
6-Methyl-DL-tryptophan	0.030	5.95	3.14
4-Fluoro-DL-tryptophan	0.024	6.10	3.10
5-Fluoro-DL-tryptophan	0.039	5.87	3.87
4-Dimethylallyl-L-tryptophan	0.048	6.14	6.10

[a] Analogs (2 m*M*) and L-tryptophan (2 m*M*) added on the 10th day to the culture of *C. purpurea*. Binding affinity of cytochrome P-450 of mycelial pellet, cytochrome P-450, and alkaloid levels determined on the 16th day (6 days after additions). [b] Concentration = 2 m*M*.

chrome P-450 for L-tryptophan are summarized in Table III. No significant differences were observed in the cytochrome P-450 levels. The cytochrome P-450 of *C. purpurea* grown in the presence of 4-methyl-DL-tryptophan and 4-fluoro-DL-tryptophan exhibited a significantly reduced affinity for L-tryptophan and also a lower total alkaloid. The known precursor 4-dimethylallyl-L-tryptophan, under the same conditions, increased the affinity of the cytochrome P-450 and a significant increase in total alkaloid resulted. The nonprecursor analog, 5-methyl-DL-tryptophan, produced an increase [tryptophan 0.038 (Table I) *versus* 0.044 (Table III)] in binding affinity over that exhibited by the cytochrome P-450 from *C. purpurea* which had not been exposed to any additive and produced an increase in total alkaloid nearly equivalent to that produced by 4-dimethylallyl-L-tryptophan.

The binding affinity of cytochrome P-450 of *C. purpurea* grown in the presence of L-tryptophan exhibited a markedly enhanced affinity for isopentenyl pyrophosphate over that exhibited by cytochrome P-450 from *C. purpurea* which had not been exposed to any additive [0.051 (Table IV) *versus* 0.015 (Table I)]. D-Tryptophan, 5-methyl-DL-tryptophan, and 5-fluoro-DL-tryptophan appeared not to exert a significant effect.

DISCUSSION

The binding of tryptophan to the cytochrome P-450 of the mycelial pellet of *C. purpurea* was both pH and temperature dependent. Maximum binding was observed at a pH of approximately 5.5. Orrenius and Ernster (8) reported that binding to cytochrome P-450 is pH dependent. At 37°, maximum binding was observed after 60 min. The extent of binding observed was 30% of maximum after 15 min., 50% after 30 min., and 80% after 45 min.

Carbon monoxide, which is known to form an enzymically inactive complex with reduced cytochrome P-450 (12), prevents binding of the compound which, once bound, is released in the presence of dithionite and carbon monoxide. Thus, an enzymically functional cytochrome P-450 is necessary for the binding to cytochrome P-450 and for active hydroxylation (8). From Table II, it will be observed that the extent of binding of ^{14}C-L-tryptophan was greatly diminished by carbon monoxide. The addition of dithionite had no effect on the extent of binding, but treatment with carbon monoxide resulted in the release of a large proportion of the already bound ^{14}C-tryptophan.

The binding of tryptophan to the cytochrome P-450 of the mycelial pellet of *C. purpurea* exhibits a high degree of stereospecificity. The data of Table I indicate that the cytochrome P-450 exhibits a relatively high binding affinity for L-tryptophan in contrast to D-tryptophan. The binding affinity exhibited for 4-dimethylallyl-L-tryptophan is at least of the same order as that exhibited for L-tryptophan or greater. These results are particularly significant with respect to the biosynthesis of the ergolene nucleus, since it has been established that ^{14}C-L-tryptophan is more efficiently incorporated into the ergolene nucleus than is ^{14}C-D-tryptophan. In addition, Agurell (13) has reported that 4-dimethylallyl-L-tryptophan is more efficiently utilized than 4-dimethylallyl-D-tryptophan or L-tryptophan. From Table I, it is also apparent that those compounds which exhibited the greatest degree of stereospecific binding also produced the greatest increase in total alkaloid.

Table IV—Effect of Tryptophan Analogs[a] on the Binding Affinity of Cytochrome P-450 for Isopentenyl Pyrophosphate and Alkaloid Levels

Compound[b]	Absorbance Difference, $A_{385-420}$	Amount of Cytochrome P-450 in μmoles, $A_{450-490}$	Total Alkaloid, mg.
L-Tryptophan	0.051	6.10	6.30
D-Tryptophan	0.020	5.85	3.20
DL-Tryptophan	0.030	5.90	4.10
5-Methyl-DL-tryptophan	0.020	5.80	3.38
5-Fluoro-DL-tryptophan	0.025	6.04	4.04

[a] Analogs (2 m*M*) and isopentenyl pyrophosphate (2 m*M*) added on the 10th day to culture of *C. purpurea*. Binding affinity of cytochrome P-450 of mycelial pellet, cytochrome P-450, and alkaloid levels determined on the 16th day (6 days after additions). [b] Concentration = 2 m*M*.

Although various routes have been proposed for the formation of ergot alkaloids, the route proposed by Arigoni (14) would appear of particular interest relative to the results reported here. The pathway proposed by Arigoni is one in which 4-dimethylallyltryptophan is hydroxylated to form an intermediate which is then converted to agroclavine. Thus the results presented in this and earlier reports suggest a role for cytochrome P-450 in the formation of the ergolene nucleus. The most obvious involvement of cytochrome P-450 on the basis of present evidence would be in a reaction in which 4-dimethylallyltryptophan is hydroxylated. However, the high binding affinity exhibited by cytochrome P-450 of the mycelial pellet for L-tryptophan (Table I) and the greatly enhanced affinity for isopentenyl pyrophosphate of the cytochrome P-450 of the mycelial pellet from *C. purpurea* grown in the presence of L-tryptophan (Table IV) suggest the possible involvement of cytochrome P-450 at an earlier stage.

The effect of growing *C. purpurea* in the presence of analogs of tryptophan on the binding affinity of the cytochrome P-450 of the mycelial pellet for L-tryptophan and their effect on cytochrome P-450 and total alkaloid levels are indicated in Table III. No significant changes in cytochrome P-450 levels were produced by any of the compounds. However, those compounds that increased the binding affinity for L-tryptophan (5-methyl-DL-tryptophan and 4-dimethylallyl-L-tryptophan) caused a significant increase in total alkaloids. Those analogs (4-methyl-DL-tryptophan and 4-fluoro-DL-tryptophan) that reduced the binding affinity tended to cause a reduction in total alkaloids. These results offer a possible explanation for earlier observations (15) that the 4-methyl and 4-fluoro analogs inhibited alkaloid synthesis relatively selectively and that such inhibition was only partially reversible by tryptophan. Protein synthesis was not significantly affected by these analogs; thus the effect of 5-methyl-DL-tryptophan in increasing total alkaloid (Table IV) and in increasing the incorporation of ^{14}C-L-tryptophan into agroclavine (15) may be explained in part by its effect on the binding affinity of cytochrome P-450 for L-tryptophan or perhaps 4-dimethylallyl-L-tryptophan.

The binding affinity of cytochrome P-450 of the mycelial pellet of *C. purpurea* for isopentenyl pyrophosphate was altered when *C. purpurea* was grown in the presence of tryptophan and tryptophan analogs. The increase in affinity for isopentenyl pyrophosphate when L-tryptophan was added to the growth medium was the most significant finding observable from the data in Table IV. A stereospecific effect was observable in the alterations in binding affinities (compare L-tryptophan and D-tryptophan).

The results reported here and elsewhere (1) indicate that an increase or decrease in total alkaloid may be paralleled (or the result of): (*a*) an increase or decrease in cytochrome P-450 levels or (*b*) an increase or decrease in binding affinity of cytochrome P-450 for the alkaloid precursors (L-tryptophan and isopentenyl pyrophosphate or 4-dimethylallyl-L-tryptophan). Either or both of these may play a role in the initiation of alkaloid synthesis in *C. purpurea*, which is known to follow the active synthesis of primary metabolites such as protein. Thus, an increase in cytochrome P-450 levels in the presence of the precursors might result in the initiation of alkaloid synthesis. Equally as possible would be the initiation of alkaloid formation as a result of an increase in the binding affinity of existing cytochrome P-450 for alkaloid pre-

cursors. The results in Table IV might allow for the speculation that the increased availability of tryptophan resulting from reduced protein synthesis could sufficiently alter the binding of cytochrome P-450 for isopentenyl pyrophosphate that alkaloid synthesis might proceed, whereas at lower levels of available tryptophan such would not occur because of the low affinity of the cytochrome P-450 for isopentenyl pyrophosphate. Based on the observation that benzyl thiocyanate, which has been shown to enhance tetracycline formation (16), increased total alkaloid in *C. purpurea* to almost the same degree as phenobarbital (15), it is suggestive that cytochrome P-450 may be involved in the formation of certain other secondary cell metabolites in addition to that which has been discussed in this report.

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DRUG STANDARDS

Detection of Decomposition and Analytical Interferences in Pharmaceutical Preparations Containing Corticosteroids

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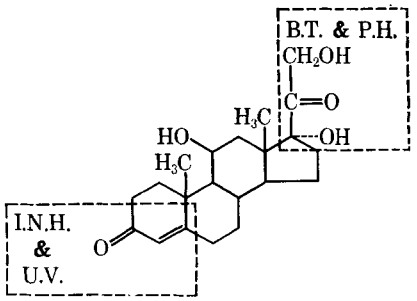
Abstract □ Since the blue tetrazolium and phenylhydrazine reagents for corticosteroids react with the intact side chain at C_{17}, and the isonicotinic acid hydrazide method and UV spectrophotometry depend upon conjugation in Ring A at the other end of the molecule, the analytical results by the four methods give information concerning decomposition caused by oxidation of the C_{17} side chain and by deconjugation in Ring A. Methods are proposed which allow the detection and determination of both acidic and neutral decomposition products. Measurement of the variation of absorbance with time can be used to detect unidentified interferences in the blue tetrazolium, phenylhydrazine, and isonicotinic acid procedures. The extent of interference of several substances which interfere in at least one of the color reactions is reported. Several examples of the use of the proposed methods to detect and determine decomposition and/or interference are given.

Keyphrases □ Corticosteroids, decomposition determination—methods compared □ Decomposition, corticosteroids—C_{17} side-chain oxidation determination □ Interference—corticosteroid analysis □ Blue tetrazolium, phenylhydrazine, isonicotinic acid hydrazide, UV spectrophotometry methods—analysis

The detection of decomposition and of analytical interferences in pharmaceutical corticosteroid preparations is important in the correct determination of the composition of such preparations. The usual methods of analysis for undecomposed corticoid hormones include the blue tetrazolium reaction (BT) (1–5); the phenylhydrazine–sulfuric acid–alcohol reaction (PH) (6, 7), which is also known as the Porter-Silber reaction; the isonicotinic acid hydrazide reaction (INH) (8), which is also known as the Umberger reaction; and UV spectrophotometry. As is shown in Structure I, the BT and PH reagents react with the C_{17} side chain, while the INH and UV methods depend upon the conjugation of the carbonyl group at C_3 with the double bond between C_4 and C_5 in Ring A of the steroid nucleus. Since



hydrocortisone—portions of molecule measured by the following methods: INH = isonicotinic acid hydrazide, BT = blue tetrazolium, PH = phenylhydrazine H_2SO_4, and UV = ultraviolet

I