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THE RELATIONSHIP OF CYTOCHROME P-450 LEVELS AND ALKALOID SYNTHESIS IN *CLAVICEPS PURPUREA*

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Abstract—The presence of cytochrome P-450 and cytochrome b_5 in *Claviceps purpurea* is reported. The stimulatory effect of phenobarbitone caused an increase in cytochrome P-450 levels which were paralleled by increased levels of total alkaloid. Cyanide produced marked and sustained reductions in both cytochrome P-450 and total alkaloid. The results suggest a relationship exists between cytochrome P-450 and alkaloid synthesis.

INTRODUCTION

THE TIME and conditions favouring ergot alkaloid production have been studied by many workers using a variety of strains of *Claviceps purpurea*. Taber[1] and, more recently, Ramstad[2] have reviewed the biosynthesis of the ergot alkaloids. Zahid and Baxter[3] have reported on the stimulatory effect of phenobarbitone in increasing the incorporation of ^{14}C-tryptophan into total alkaloid. This effect is of considerable interest in light of the established effect of phenobarbitone on microsomal enzyme induction.[4] Comprehensive reviews of enzyme induction by Conney[4] and Mannering[5] have appeared recently. It has been reported[6,7] that many drugs such as barbiturates, insecticides and polycyclic hydrocarbons (e.g. 3,4-benzpyrene, 3-methylcholanthrene) increase the oxidative activity of microsomes and this is associated with an increase in the microsomal content of cytochrome P-450.

It is also significant that cyanide, which we have reported[3] to markedly decrease alkaloid synthesis, has been reported by Sato[8] to lower microsomal cytochrome P-450 levels. It was considered of interest, therefore, to establish the presence or absence of cytochrome P-450 in *C. purpurea* and, if present, to establish if a relationship exists between the levels of cytochrome P-450 and alkaloid in this organism.

RESULTS

A difference spectrum with a maximum at 450 nm (Fig. 1) typical of cytochrome P-450[9,10] was observed when the mycelial pellet preparation to which dithionite had been added was saturated with CO. The difference spectrum as illustrated in Fig. 2 and which is typical of

[1] W. A. TABER, *Lloydia* **30**, 39 (1967).
[2] E. RAMSTAD, *Lloydia* **31**, 327 (1968).
[3] N. ZAHID and R. M. BAXTER, *Biogenesis of Ergot Alkaloids; Regulatory Factors in Induction*, presented at A.Ph.A. Academy of Pharmaceutical Sciences, Miami, May 1968.
[4] A. M. CONNEY, *Pharmacol. Rev.* **19**, 317 (1967).
[5] G. M. MANNERING, *Pharmacological Testing Methods*, Marcel Dekker, New York (1968).
[6] H. REMMER and H. J. MERKER, *Ann. N.Y. Acad. Sci.* **123**, 79 (1965).
[7] S. ORRENIUS, *J. Cell Biol.* **26**, 725 (1965).
[8] R. SATO and T. OMURA, *J. Biol. Chem.* **237**, 2329 (1964).
[9] R. SATO and T. OMURA, *J. Biol. Chem.* **239**, 2370 (1964).
[10] D. GARFINKEL, *Arch. Biochem. Biophys.* **77**, 493 (1958).

that obtained with cytochrome b_5[9, 10] exhibited a small trough at 420 nm and a peak at 424 nm was obtained with a mycelial pellet preparation to which dithionite had been added. Although further proof of the presence of cytochrome P-450 and P-420 will be reported elsewhere, the characteristic difference spectra obtained allow for a tentative assumption of the presence of the cytochrome system in the mycelial pellet derived from the fungus *Claviceps*

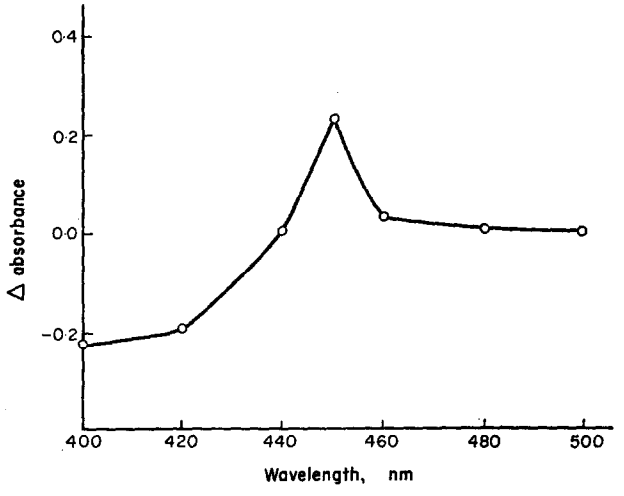

FIG. 1.

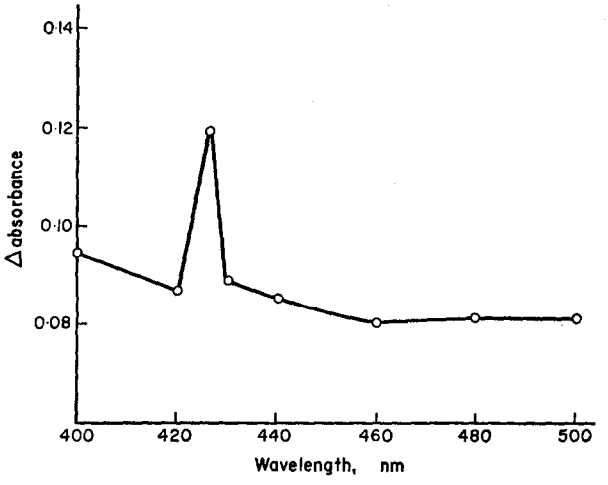

FIG. 2.

purpurea. The reactivity of reduced cytochrome P-450 and the lack of reactivity of cytochrome b_5 to CO allow for the estimation of cytochrome P-450 and b_5.[10]

The addition of phenobarbitone (1 mg/ml) increased whereas KCN (5 mcg/ml) markedly lowered the amount of cytochrome P-450 and b_5 in the mycelial pellet (Fig. 3). The addition of 3-methylcholanthrene resulted in a cytochrome P-450 exhibiting a characteristic absorbance maximum at 448 instead of 450 nm.

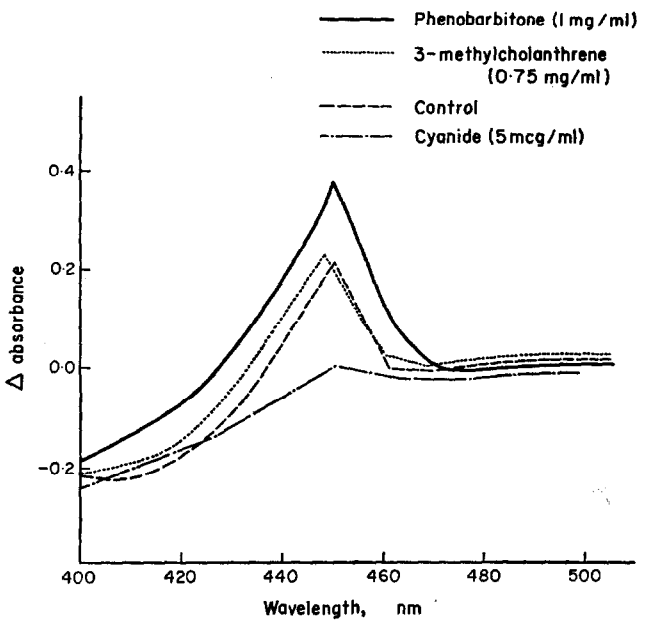

FIG. 3.

The analysis of the cultures daily from day 10 to day 20 for cytochrome P-450 and b_5, total alkaloid and protein revealed that cytochrome and total alkaloid reached a maximum on day 16 in both the control series and the series to which phenobarbitone had been added. No significant increase in total protein was observed (Table 1, series I and II). It will be observed that a marked increase in cytochrome P-450 and total alkaloid occurred in the series II cultures to which phenobarbitone had been added and that this increase occurred on day 3 after the addition of phenobarbitone. The addition of cyanide resulted in a marked and sustained decrease in both cytochrome P-450 and alkaloid. No significant change occurred in protein concentration from day 10 to day 20. Thus, it is obvious that the increase in cytochrome P-450 resulting from the addition of phenobarbitone was paralleled by a corresponding increase in total alkaloid. Similarly, the decrease in cytochrome P-450 produced by cyanide was paralleled by a corresponding decrease in total alkaloid.

Barbiturates and polycyclic hydrocarbons stimulate drug metabolism by different mechanisms. The evidence to support this view has been reviewed and discussed by Conney.[4,11] The increase in cytochrome P-450 and b_5 induced by phenobarbitone and 3-methylcholanthrene can be inhibited by ethionine, a known inhibitor of protein synthesis. From Table 2 it will be observed that both phenobarbitone and 3-methylcholanthrene increased both cytochrome and total alkaloid. The stimulatory effect of 3-methylcholanthrene was, however, not as marked. Ethionine reversed the inductive effect of 3-methylcholanthrene on both cytochrome and total alkaloid.

The spectra of the cytochrome P-450 from the mycelial pellet preparation of *C. purpurea* which had been treated with 3-methylcholanthrene exhibited a characteristic absorbance maximum at 448 instead of at 450 nm (Fig. 3). When ethionine was added along with

[11] R. KUNTZMANN, W. LEVIN, M. JACOBSON and A. H. CONNEY, *Life Sci.* 7, 215 (1968).

3-methylcholanthrene, the peak shift from 450 to 448 nm induced by the latter was prevented, as has been reported.[11] L-Tryptophan, a known precursor of ergot alkaloids, although causing a small increase in total alkaloid, exerted no stimulatory effect on cytochrome P-450 or

TABLE 1. EFFECT OF PHENOBARBITONE, POTASSIUM CYANIDE ON THE CYTOCHROME, PROTEIN AND ALKALOID CONTENT IN *C. Purpurea*

Series	Days	Weight of the wet mycelial cells (g/flask)	Total protein* concentration (mg/100 g of cells)	Cytochromes (mμmoles) P-450	Cytochromes (mμmoles) b_5	Total alkaloids (mg/flask)
I—Control	10	1·59	70·2	4·55	4·07	1·79
	11	1·64	70·9	5·09	4·13	1·92
	12	1·65	71·2	6·08	4·52	1·98
	13	1·70	71·8	6·09	4·88	2·15
	14	1·80	72·4	6.46	5·54	2·35
	15	1·94	72·6	6·64	5·86	2·44
	16	1·96	72·6	6·70	6·02	2·41
	18	1·98	72·8	6·50	5·85	2·40
	20	2·01	70·4	6·09	5·70	2·38
II—Phenobarbitone (1 mg/ml)	10	1·56	73·4	5·64	3·09	1·92
	11	1·70	74·2	6·10	3·42	2·06
	12	1·81	79·2	6·46	3·67	2·34
	13	1·96	80·1	9·40	5·10	4·02
	14	1·96	80·1	10·20	5·20	4·04
	15	2·06	80·4	10·41	5·31	4·10
	16	2·08	80·6	10·51	5·70	4·12
	18	2·10	80·6	9·55	5·19	4·10
	20	2·17	80·8	9·28	5·16	4·04
III—Potassium cyanide (5 mcg/ml)	10	1·57	70·9	3·73	3·90	1·72
	11	1·58	70·4	3·61	3·40	1·50
	12	1·54	70·1	2·82	3·20	1·30
	13	1·50	69·2	2·73	3·09	1·25
	14	1·44	69·2	1·91	1·95	1·20
	15	1·40	68·8	1·73	1·63	1·10
	16	1·39	68·6	0·91	1·14	1·08
	18	1·35	68·4	0·90	1·13	1·04
	20	1·33	68·4	0·85	1·10	0·09

On day 10, after inoculation (see Methods), twenty-seven flasks were divided into three groups, each group consisting of nine flasks. One group served as a control to the second and third groups, phenobarbitone (1 mg/ml) and potassium cyanide (5 mcg/ml) were added respectively. Daily after the addition, one flask from each group was analyzed for cytochrome P-450, b_5, protein and total alkaloid. The results represent the average of duplicate series.

* Protein was estimated by the method of Lowry.[12]

b_5 levels. However, when L-tryptophan and phenobarbitone or 3-methylcholanthrene were added in combination, total alkaloid was increased and a parallel increase in cytochrome P-450 and b_5 was observed.

[12] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. G. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

TABLE 2. EFFECT OF VARIOUS COMPOUNDS ON THE CYTOCHROME P-450, b_5, PROTEIN AND TOTAL ALKALOIDS

Compounds	Weight of the wet mycelial cells (g/flask)	Total protein* (mg/100 g of the cells)	Cytochrome (mμmoles) P-450	Cytochrome (mμmoles) b_5	Total alkaloids (mg/flask)
Control	2·04	81·2	6·67	5·07	2·51
Phenobarbitone (1 mg/ml)	2·15	83·4	11·80	5·91	4·48
3-Methylcholanthrene (3-MC) (0·75 mg/ml)	2·02	86·2	10·20	5·31	3·58
Ethionine (1 mg/ml)	1·97	61·2	5·82	4·98	2·40
Ethionine and 3-MC (1 mg/ml and 0·75 mg/ml)	1·99	72·1	7·02	5·10	3·10
L-Tryptophan (250 mg/ml)	2·02	84·8	6·41	5·02	3·10
Phenobarbitone and L-tryptophan (1 mg/ml and 250 mg/ml)	2·03	85·1	14·31	5·80	5·04
3-MC and L-tryptophan (0·75 mg/ml and 250 mg/ml)	1·98	82·1	11·20	5·28	3·40

The flasks were analyzed for cytochrome P-450, b_5, total protein and total alkaloids on day 6 after the addition of the additives. The results represent the average of duplicate series.

* Protein was estimated by the method of Lowry.[12]

DISCUSSION

Cytochrome P-450 is now recognized as a component of many mixed-function oxidase systems participating in the hydroxylation of a wide variety of compounds as, for example, steroids, many drugs, carcinogens and insecticides.[13] This component is presumed to be a hemoprotein containing protoheme as its prosthetic group in association with lipid and is found in the microsomal fraction of a number of tissues, most notably, liver, adrenal, and from certain bacterial systems.[13]

Despite several attempts by various workers, cytochrome P-450 has not been obtained in a highly purified form to date. Therefore, the absolute physical properties for cytochrome P-450 remain to be established, and the characterization of cytochrome P-450 and b_5 is dependent on their unique spectral properties. The characteristic spectrum for cytochrome P-450 as reported by Estabrook *et al.*[14] exhibits a trough at 440 nm and a single peak at 450 nm. Cytochrome b_5 exhibits a small trough at 420 nm and a single peak at 424 nm.

The characteristic absorption spectra obtained (Figs. 1 and 2) with the mycelial pellet preparation is indicative of the presence of cytochrome P-450 and b_5 in *Claviceps purpurea*. Thus, the CO-binding component in the mycelial pellet preparation of the control or phenobarbitone-treated cultures of *C. purpurea* exhibited the characteristic absorption peak at 450 nm and that in the mycelial pellet of the 3-methylcholanthrene-treated cultures exhibited an absorption peak at 448 nm (Fig. 3). This peak shift may be due to binding of 3-methylcholanthrene with cytochrome P-450 or may be due to the induction by 3-methylcholanthrene of a new CO-binding component with different spectral properties. Evidence for the formation of a new CO-binding pigment (cytochrome P-448) was obtained when *C. purpurea* was pre-treated with ethionine (Table 2). Thus, ethionine pre-treatment prevented the peak

[13] V. ULLRICH, B. COHEN, D. Y. COOPER and R. W. ESTABROOK, in *Structure and Functions of Cytochromes* (edited by R. OKUNUKI), p. 649, University of Tokyo Press, Tokyo (1968).

[14] H. REMMER, H. GREIM, J. B. SCHENKMAN and R. W. ESTABROOK, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 2, p. 703, Academic Press, New York (1967).

shift to 448 nm in the CO-binding pigment induced by 3-methylcholanthrene. These observations are in agreement with those made by Kuntzman[11] in the case of the cytochrome P-450 from rat-liver microsomes.

The maximum increase in cytochrome P-450 and total alkaloid occurred on the sixth day after the addition of phenobarbitone and a close parallelism existed between the increase in cytochrome and total alkaloid, although protein remained relatively constant in the same time period (Table 1). The addition of L-tryptophan to the cultures produced a small increase in total alkaloid but no change in the cytochrome content. In combination with phenobarbitone, however, a definite increase in both total alkaloid and cytochrome P-450 occurred (Table 2). This confirms the earlier observation made by Zahid and Baxter[3] on the stimulatory effect of phenobarbitone on the incorporation of ^{14}C-L-tryptophan into total alkaloid. It was reported at that time that there was an increased incorporation of L-tryptophan into all the alkaloids of this strain of *C. purpurea*. The combination of 3-methylcholanthrene and tryptophan, although producing an increase in cytochrome P-450, did not produce as marked an increase in total alkaloid (Table 2).

EXPERIMENTAL

Organism and Growth Conditions

A clavine-producing strain of *Claviceps purpurea* (obtained from M. Abe) was grown on a modified Abe's medium at 27° for 10 days for the production of inoculum. The seed cultures were homogenized under sterile conditions in a Waring blendor and 1·0 ml inocula were transferred to 250-ml Erlenmeyer flasks containing 50 ml of the medium. The cultures were grown (stationary) in the dark at 25°. After 10 days a well-developed mat was formed.

Addition of Compounds

Compounds were added on day 10 when the alkaloids were detectable as noted from the rate curve.

Extraction and Determination of Alkaloids

The medium was separated from the mycelial mat and was used to estimate total alkaloid. The extraction procedure used was that of Taber and Vining[15] and the alkaloid content was determined using Van Urk's reagent using agroclavine as a standard.

Preparation of Mycelial Pellet

The mycelial mat, after separation from the medium, was washed several times with 1·15% ice-cold KCl solution. The mat was homogenized with 4 vol. of 1·15% (isotonic) KCl solution. The homogenate was centrifuged in a refrigerated centrifuge (4°) at 9000 *g* for 20 min. The supernatant was then centrifuged at 105,000 *g* for 60 min. The mycelial pellet thus obtained was suspended in 5 ml of ice-cold 1·15% KCl solution.

Estimation of Cytochrome P-450 and b_5

The content of cytochrome P-450 and b_5 present in the mycelial pellet was determined[9] by measuring the difference spectrum of the mycelial pellet preparation using a Beckman DU Spectrophotometer. To a 1·6 ml sample of the mycelial pellet in each of three cuvettes was added 1·2 ml of 0·1 M phosphate buffer (pH 7·4). One of these constituted the reference cuvette. To the contents of the second cuvette were added a few crystals of sodium dithionite (sample I) and to the contents of the third cuvette was added a few crystals of dithionite and sample was then saturated with CO by bubbling the gas through the sample for 30 sec (sample II). The content of P-450 was determined by the difference spectrum between *sample II* and *sample I* at 450 and 490 nm. The content of cytochrome b_5 was determined by the difference spectrum of sample I and reference cell at 424 and 408 nm. An extinction coefficient of 91 Mm^{-1} cm^{-1} for the difference in absorption between 450 and 490 nm was used for cytochrome P-450 and an extinction coefficient of 161 Mm^{-1} cm^{-1} for b_5 absorbance between 424 and 408 nm was used.[10]

[15] W. A. TABER and L. C. VINING, *Can. J. Microbiol.* **4**, 611 (1958).