Biochemical Pharmacology, Vol 23, pp 1319–1329 Pergamon Press, 1974. Printed in Great Britain.

INFLUENCE OF PYRIDINE AND SOME PYRIDINE DERIVATIVES ON SPECTRAL PROPERTIES OF REDUCED MICROSOMES AND ON MICROSOMAL DRUG METABOLIZING ACTIVITY*†

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(*Received* 12 *July* 1973; *accepted* 4 *October* 1973)

Abstract—Addition of pyridine, 3-(3-pyridyl)-propanol, reduced metyrapone and some other pyridine derivatives to dithionite reduced rat liver microsomes results in the formation of a six banded spectrum with absorption maxima at 425, 446, 527, 539, 555 and 568 nm. With metyrapone as the ligand, a 425 nm band can only be observed during development of the spectrum. The ratio of the two Soret bands depends on the pH. The 446 nm band is maximal at pH 7·8 in phenobarbital stimulated microsomes and decreases on both lowering and increasing the pH. For metyrapone the apparent spectral dissociation constant K_s for the absorbance change at 446 nm was 16 μM in microsomes from untreated animals and was decreased to 2·4 μM after phenobarbital pretreatment. In 3-methylcholantrene stimulated microsomes the K_s value for metyrapone was increased about 25 fold and amounted to 390 μM. The K_s value for reduced metyrapone in unstimulated microsomes was similar to that for metyrapone. Less pronounced pretreatment effects were, however, observed with this ligand leading to decreased K_s values in both PB and MC stimulated microsomes. Pyridine and 3-(3-pyridyl)-propanol were only bound to reduced cytochrome P-450 in millimolar concentrations. All tested ligands were able to inhibit microsomal drug metabolism. Metyrapone was the most potent inhibitor of drug demethylation exerting 50 per cent inhibition of *p*-nitroanisole demethylation at 4 μM and of aminopyrine demethylation at 86 μM. Aniline hydroxylation was inhibited by 50 per cent by millimolar concentrations of all inhibitors at aniline concentrations of 4 mM.

THE CARBON monoxide binding pigment of liver microsomes, cytochrome P-450, has reported to show optical properties which are unusual among hemoproteins. Reduced cytochrome P-450 forms a number of complexes with Soret bands at extremely long wavelengths at about 450 nm. The carbon monoxide complex has an absorption maximum at 450 nm which was known even before the enzymic function of the cytochrome had been recognized and which lead to the name P-450.[1]

A number of ligands produces a double banded Soret region by binding to reduced microsomes. Ethyl isocyanide (EtNC)[2] and other isonitriles[3] cause Soret bands at 430 nm and at 455 nm which have been ascribed to two interconvertible forms of cytochrome P-450 existing in a pH dependent equilibrium.[4] The ratio of the absorbance depends on the pretreatment of the animals with either phenobarbital (PB)

* The experiments were carried out with the financial support of the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg. Some of the equipment was provided by the Stiftung Volkswagenwerk.

† Part of this material was presented at the Joint Meeting of the German and the Scandinavian Pharmacological Societies, Copenhagen, 1971 (H. G. JONEN, R. KAHL and G. F. KAHL, *Acta pharmac. tox.* **29**, Suppl. 4, 24, 1971) and at the 5th Int. Congress on Pharmacology, San Francisco, 1972 (G. F. KAHL, H. G. JONEN and R. KAHL, *Abstracts of Volunteer Papers*, p. 118).

or 3-methylcholanthrene (MC).[5] Similar spectral changes have been observed after addition of some primary amines[6] and of high concentrations of pyridine and aniline[7] to reduced microsomes. Addition of piperonyl butoxide also leads to the appearance of a spectrum of the EtNC type in reduced microsomes,[8] and it has been shown that this spectrum is due to the formation of a metabolite of the insecticide.[9]

Hildebrandt *et al.*[10] demonstrated that the pyridine derivative metyrapone (MP) also binds to reduced liver microsomes producing a single Soret band at 446 nm but double α and β bands. The same Soret band, but single bands in the visible region were obtained by addition of MP to a soluble cytochrome P-450 from Pseudomonas putida.[11] The MP induced spectrum in liver microsomes has been interpreted as indicating the presence of two forms of cytochrome P-450 within the microsomal membranes.[10]

The characteristics of MP binding to reduced microsomes have been related to its effects on drug metabolism.[10] This substance which has long been known to be an inhibitor of steroid biosynthesis,[12] also inhibits microsomal drug oxidation.[13,14] On the other hand, enhancement of type II substrate hydroxylation has been shown to occur in the presence of MP.[15] Similar effects have been described for EtNC which either inhibits or enhances aniline hydroxylation depending on the concentration employed.[16] An inhibitory action on both type I and type II metabolism has been observed for piperonyl butoxide.[17] Aniline has also been shown to inhibit microsomal drug metabolism.[18]

The present experiments were undertaken to verify if the characteristics of MP binding are also common to other pyridine derivatives and to study the influence of pH and pretreatment on the spectral effects which are produced by these substances. Further, the action of pyridine and its derivatives on drug metabolism was tested and correlated to their binding to reduced cytochrome P-450.

MATERIALS AND METHODS

Preparation of microsomes. Liver microsomes were prepared from male Sprague–Dawley rats of about 150 g body wt after pretreatment with 3 × 80 mg/kg sodium PB i.p. in 24 hr intervals or with 3 × 20 mg/kg MC dissolved in commercial salad oil i.p. in 12 hr intervals. Control animals received the same volume of saline i.p. The animals were sacrificed 24 hr after the last injection of PB or 36 hr after the last injection of MC and 12 hr after food had been withdrawn. Prior to homogenization in 3 vol of 0·25 M sucrose solution containing 0·02 M Tris–HCl buffer pH 7·4 and 0·005 M EDTA[19] the livers were perfused *in situ* with ice-cold saline to remove blood. Nuclear fragments and mitochondria were sedimented by centrifugation at 1600 *g* for 15 min and at 9000 *g* for 30 min. The microsomal pellet was obtained by centrifugation of the 9000 *g* supernatant at 105,000 *g* for 1 hr and was washed once. Protein concentration was determined according to Lowry *et al.*[20] For spectral recordings the pool was divided into portions of 5 mg protein and the portions were thawed immediately before preparing the sample to avoid variation of aging processes.

Spectrophotometric measurements. Spectrophotometric studies were performed in an Aminco Chance split beam spectrophotometer at 30 . For analysis of difference spectra 5 ml samples containing 1 mg protein/ml were reduced with 1 mg sodium

dithionite/ml and pH was then readjusted since considerable lowering of pH had been observed after dithionite addition. The sample was divided into two parts and a base line of equal light absorption was recorded. Addition of pyridine derivatives to the sample cuvette was performed in 2–100 μl of buffer. The same volume of buffer was added to the reference cuvette. Apparent spectral dissociation constants were obtained by double reciprocal plotting of the extinction difference at 446–490 nm against the ligand concentration.

The absence of hemoglobin and cytochrome P-420 from the microsomal preparations was verified by running difference spectra after bubbling of the sample cuvette with CO.

For recording pyridine hemochromogen spectra with hemin chloride this substance was dissolved in 1N NaOH and added to 0·067 M Soerensen phosphate buffer pH 7·4 to give a final concentration of 6×10^{-5} M. Difference spectra were recorded in dithionite reduced preparations.

Enzyme assays. Incubation mixtures for aminopyrine demethylation and aniline hydroxylation contained 0·9 μmole NADP, 15 μmoles glucose-6-phosphate, 1·4 units glucose-6-phosphate dehydrogenase, 2·5 mg microsomal protein and 0·067 M Soerensen phosphate buffer in a total volume of 2·5 ml. The reactions were started by the addition of substrate (1 mM aminopyrine and 4 mM aniline) and stopped by protein precipitation after 10 min (aminopyrine demethylation) or 20 min (aniline hydroxylation). The incubations were carried out at 30° and pH 7·4 (aminopyrine demethylation) or 7·8 (aniline hydroxylation). Aminopyrine metabolism was measured by formaldehyde production according to the method of Nash[21] as used by Cochin and Axelrod.[22] *p*-Aminophenol formation from aniline was measured by the method of Brodie and Axelrod[23] but ether extraction was omitted from the procedure as described by Imai *et al.*[24]

p-Nitroanisole demethylation was monitored continuously as described by Netter and Seidel[25] except that measurement was performed at 405 nm instead of 420 nm. The incubation was carried out at 30° and pH 7·8 in a total volume of 2·5 ml containing 1 μmole NADP, 5·75 μmoles glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 25 μmoles nicotinamide to assure constant demethylation rate in the first minutes, 2·5 mg of microsomal protein and 0·067 M Soerensen phosphate buffer. After a 5 min equilibration phase the reaction was started by the addition of NADP and followed for 90 sec before the inhibitor was added. Substrate concentration was 0·2 mM.

The lipophilic character of pyridine and its derivatives was tested by determining the partition coefficients between 1-octanol and water.[26] 10 ml of 0·02 M Soerensen phosphate buffer pH 7·4 saturated with 1-octanol and containing various concentrations of the substances to be tested were mixed with 1 ml of 1-octanol saturated with buffer. The mixture was shaken for 1 hr and then centrifuged. The concentration of the tested substance in the buffer layer was determined from the u.v. spectrum.

Cofactors were products of Boehringer, Mannheim. Pyridine, 3-(3-pyridyl)-propanol, and the pyridyl ethylenes were purchased from E. Merck, Darmstadt. MP (2-methyl-1,2-bis(3-pyridyl)-1-propanone, SU 4885) and reduced MP (2-methyl-1,2-bis(3-pyridyl)-1-propanol, SU 5236) were obtained from CIBA.*

* We thank Dr. J. J. Chart of CIBA Pharmaceutical Co., Summit, N.J., U.S.A., for kindly donating these substances.

RESULTS

Properties of the absorption spectrum. A six banded difference spectrum was recorded in dithionite reduced hepatic microsomes from untreated, PB pretreated, and MC pretreated animals after addition of pyridine and a number of pyridine derivatives including 3-(3-pyridyl)-propanol, 1-(2-pyridyl)-2-(4-pyridyl)-ethylene, 1-(2-pyridyl)-2-(3-pyridyl)-ethylene, reduced MP and nicotinamide. The position of the double peaks in the Soret region and in the visible region is shown in Fig.1. The nicotinamide spectrum was only observed with very high ligand concentrations exceeding 10^{-2} M. No hemochromogen spectrum was observed after addition of pyridine nucleotides to the reduced microsomal suspension.

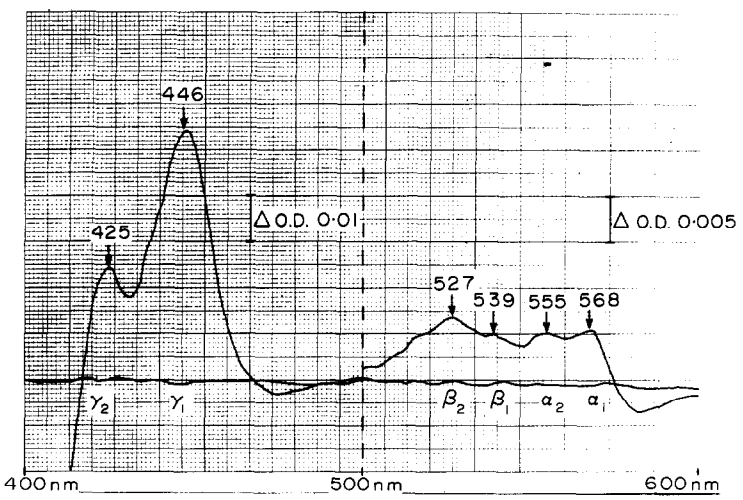

FIG. 1. Difference spectrum of reduced microsomes in the presence of 10^{-2} M 3-(3-pyridyl)-propanol. PB stimulated rat liver microsomes (1 mg protein/ml) were reduced with 1 mg sodium dithionite/ml and pH was readjusted to 7·4.

Metyrapone fails to exert the γ_2 Soret peak at 425 nm in the fully developed spectrum at pH 7·8. An initial absorbance at 425 nm is, however, seen during the development of the spectrum. This initial absorbance gradually decreases while the absorbance at 446 nm concomitantly increases. The process is much faster in microsomes from untreated and PB pretreated animals than in those from MC pretreated animals where it can be followed conveniently over a period of about 5 min (Fig.2). A similar shift of the absorbance in the Soret region is observed with all tested ligands but does not lead to complete disappearance of the γ_2 band as in the case of MP.

The γ_1/γ_2 ratio depends on the pH of the medium and is maximal at pH 7·8 in PB stimulated microsomes. On lowering or increasing the pH the absorbance at 425 nm becomes more prominent (Fig.3). It was not possible under our conditions to restitute the γ_1/γ_2 ratio as measured at pH 7·8 after carefully increasing or decreasing the pH and then readjusting it to 7·8. The γ_1/γ_2 ratio was different for the different ligands. Only a small shoulder at 425 nm was observed in the spectrum induced by reduced MP at pH 7·8, while the γ_2 peak invariably exceeded the γ_1 peak in the case of 1-(2-pyridyl)-2-(3-pyridyl)-ethylene. On aging of the microsomal suspensions at

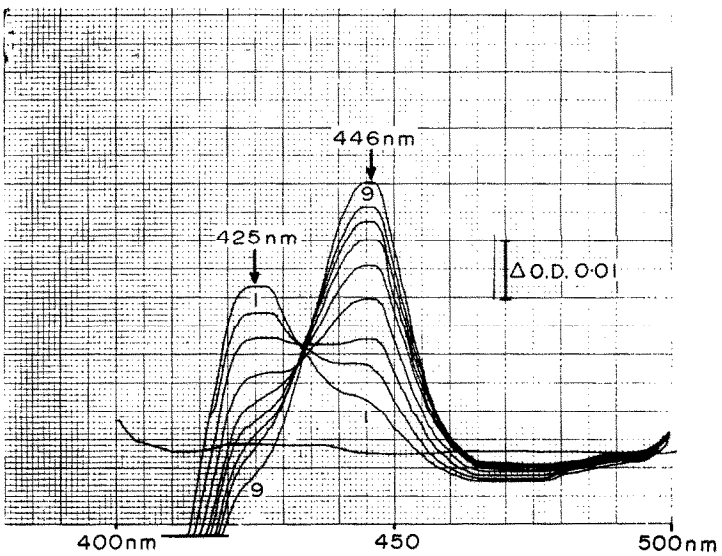

FIG. 2. Development of the MP induced difference spectrum in MC stimulated microsomes. 1 mg microsomal protein/ml was reduced with 1 mg sodium dithionite/ml and pH was readjusted to 7·8. Recordings were started 25 sec after addition of 2·5 mM MP. Spectra 1–8 were recorded in 25 sec intervals. Spectrum 9 was recorded 10 min after MP addition.

30° for periods of 5–60 min, the γ_1 band decreases while the α_2, β_2 and γ_2 bands increase (Fig.4). The time dependent changes were more prominent at 425 nm than at 446 nm in most microsomal preparations. In boiled microsomes only the α_2, β_2 and γ_2 bands were observed. All tested ligands lead to the formation of a type II difference spectrum in oxidized microsomes and were able to produce typical pyridine hemo-

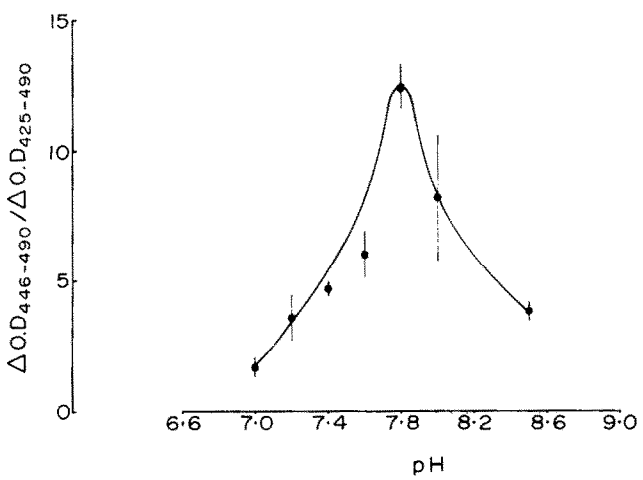

FIG. 3. Influence of pH on the γ_1/γ_2 ratio of 3-(3-pyridyl)-propanol induced difference spectra. PB stimulated rat liver microsomes (1 mg protein/ml) were reduced with 1 mg sodium dithionite/ml. The concentration of 3-(3-pyridyl)-3-propanol was 10^{-2} M. Soerensen phosphate buffer was used for estimations at pH 7·0 to 7·8. Glycine buffer was used for estimations at pH 8·0 and 8·6. Values are means ± S.E.M.

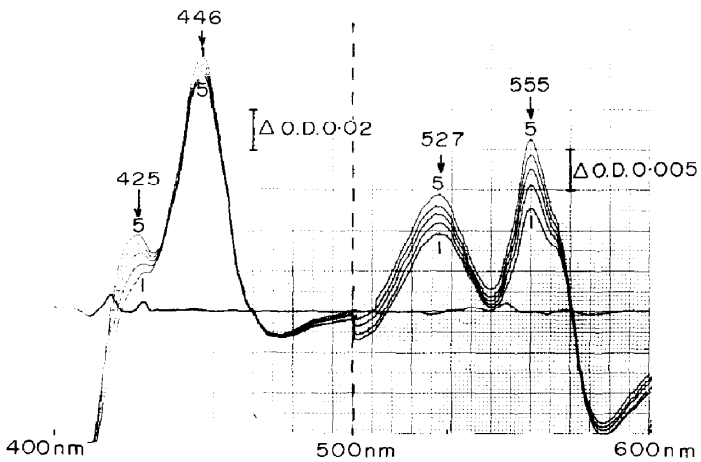

FIG. 4. Time dependent changes of the 3-(3-pyridyl)-propanol induced difference spectrum in reduced microsomes. PB stimulated rat liver microsomes (1 mg protein/ml) were reduced with 1 mg sodium dithionite/ml and pH was readjusted to 7·8. The recordings were performed in 2 min intervals. The 3-(3-pyridyl) propanol concentration was 5×10^{-2} M.

chromogen spectra with peaks at about 430, 527 and 557 nm with alkaline solutions of hemin chloride.

Pyridine and its derivatives are displaced from their binding to reduced cytochrome P-450 by CO as has been previously noted for MP by Hildebrandt *et al.*[10] Figure 5 shows that the Soret maximum at 446 nm gradually shifts to 450 nm when

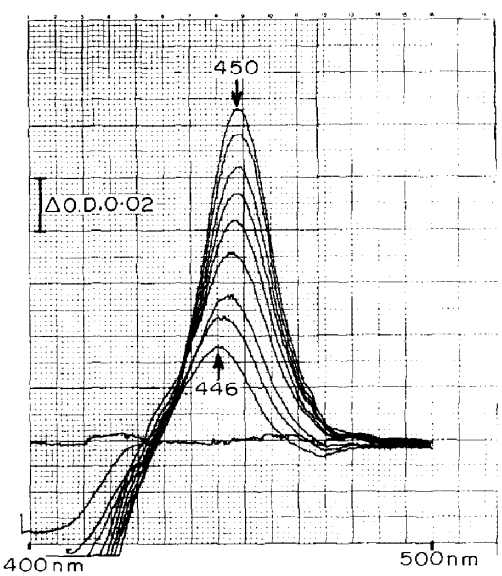

FIG. 5. Shift of the MP induced difference spectrum in reduced microsomes after addition of CO. PB stimulated rat liver microsomes (1 mg protein/ml) were reduced with 1 mg sodium dithionite/ml and pH was readjusted to 7·4. After addition of 5×10^{-5} M MP a spectrum with an absorption maximum at 446 nm was recorded. Adding of 0·5 μl portions of a CO saturated buffer gradually shifts the maximum towards 450 nm.

small portions of a CO saturated buffer are successively added to a microsonal suspension which contains MP. Displacement seems, however, not to be complete. When 2·5 mM MP is already present in the preparation before saturation with CO, the absorbance at 450 nm is only 71·4 ± 8·6 per cent (mean ± S.D.) of that measured in preparations from which MP is absent. On the other hand, when the CO peak is already developed no decrease of the 450 nm band can be achieved by the subsequent addition of MP or other pyridine derivatives.

Spectral dissociation constants. The γ_1 bands at 446 nm caused by pyridine and the other ligands tested were compared by means of apparent spectral dissociation constants K_s in microsomes from untreated, PB treated and MC treated animals (Table 1). Lineweaver–Burk plots did not result in linear functions when a ligand concentration range exceeding one order of magnitude was plotted. The K_s values given in Table 1 were obtained from plots with almost maximal ligand concentrations. For MP a K_s value of about 10^{-5} M was obtained in microsomes from untreated animals. In PB stimulated microsomes the affinity of reduced cytochrome P-450 for MP was considerably increased since K_s values in these preparations were found to be in the micromolar range. On the other hand, K_s values in MC stimulated microsomes were about 25 times higher than in unstimulated microsomes indicating that the high affinity of the cytochrome for MP is lost during MC pretreatment.

Less pronounced pretreatment effects were found for binding of reduced MP. The affinity for reduced MP in unstimulated microsomes was similar to that for MP but was increased by both PB and MC pretreatment in contrast to that for MP. It should be noted that the changes of K_s values for reduced MP were unimpressive as compared to those of K_s values for MP. Binding of pyridine and 3-(3-pyridyl)-propanol occurs only in millimolar concentrations of the ligands and is not changed by PB pretreatment. The same is true for 3-(3-pyridyl)-propanol after MC pretreatment, while an increase of the K_s value for pyridine was observed in MC stimulated microsomes. For all ligands the γ_1 Soret peak is slightly shifted to shorter wavelengths after pretreatment with MC being localized at 445 nm rather than at 446 nm under these conditions.

Titration of the absorbance at 425 nm was difficult because of its rapid increase with time which was obseved in some microsomal preparations. Thus, substrate dependency could not be determined exactly but it was obvious that K_s values would exceed those measured for the absorbance at 446 nm.

TABLE 1. APPARENT SPECTRAL DISSOCIATION CONSTANTS K_s OF PYRIDINE AND ITS DERIVATIVES IN REDUCED LIVER MICROSOMES*

	Control microsomes	PB stimulated microsomes	MC stimulated microsomes
Metyrapone	0·16 ± 0·02	0·024 ± 0·002‡	3·9 ± 0·8†
Reduced metyrapone	0·56 ± 0·08	0·28 ± 0·05†	0·14 ± 0·03†
3-(3-Pyridyl)-propanol	12·6 ± 2·4	12·8 ± 2·4	11·6 ± 1·7
Pyridine	13·7 ± 3·6	22·2 ± 4·9	45·5 ± 6·7‡

* Values × 10^{-4} M are means ± S.E.M.
† $P < 0·01$.
‡ $P < 0·001$.

TABLE 2. INHIBITION OF DRUG METABOLISM BY PYRIDINE AND ITS DERIVATIVES*

	50% inhibition of		
	p-Nitroanisole demethylation (μM)	Aminopyrine demethylation (mM)	Aniline hydroxylation (mM)
Metyrapone	4	0·086 ± 0·029	4·78 ± 1·28
Reduced metyrapone	12	0·255 ± 0·024	2·49 ± 0·50
3-(3-Pyridyl)-propanol	39	0·542 ± 0·062	3·45 ± 1·15
Pyridine	208	4·017 ± 1·460	4·46 ± 1·24

* PB stimulated microsomes. Substrate concentrations: 0·2 mM *p*-nitroanisole. 1 mM aminopyrine, 4 mM aniline. Values for *p*-nitroanisole demethylation are derived from regression lines with the data of all experiments performed. Values for aminopyrine demethylation and aniline hydroxylation are means ± S.E.M. obtained from regression lines of single experiments.

The control activities without inhibitors were 5·65 ± 0·25 nmoles *p*-nitrophenol/mg protein × min in *p*-nitroanisole demethylation, 9·35 ± 0·67 nmoles formaldehyde/mg protein × min in aminopyrine demethylation and 1·04 ± 0·07 nmoles *p*-aminophenol/mg protein × min in aniline hydroxylation.

Inhibition of drug metabolism and octanol/water distribution. MP and reduced MP are known to be inhibitors of microsomal drug metabolism.[13,14] In Table 2 their effect on *p*-nitroanisole demethylation, aminopyrine demethylation and *p*-hydroxylation of aniline is compared with that of 3-(3-pyridyl)-propanol and pyridine. MP was the most potent inhibitor and pyridine was the weakest inhibitor of both demethylation processes, but ten times higher concentrations of all inhibitors were required to achieve 50 per cent inhibition of aminopyrine demethylation as compared with *p*-nitroanisole demethylation.

Aniline hydroxylation was relatively insensitive to inhibition by pyridine and its derivatives. At an aniline concentration of 4 mM 50 per cent inhibition was only obtained with millimolar concentrations of all tested inhibitors.

The lipid solubility of pyridine and its derivatives was tested by determining their octanol/water distribution. The octanol/water partition coefficients are listed in Table 3.

TABLE 3. LIPID SOLUBILITY OF PYRIDINE AND ITS DERIVATIVES

	Octanol-water partition coefficient
Metyrapone	34·4 ± 1·1
Reduced metyrapone	22·1 ± 0·7
3-(3-Pyridyl)-propanol	4·1 ± 0·3
Pyridine	4·6 ± 0·3

Values are means ± S.E.M.

DISCUSSION

Binding of pyridine and some of its derivatives to reduced microsomes results in the appearance of a six banded spectrum with double α, β and γ bands. The α_1, β_1

and γ_1 bands which are located at rather long wavelengths as compared with other hemochromogen spectra have also been described to occur after addition of MP to purified bacterial cytochrome P-450.[10] From our results no conclusive evidence can be produced on the nature of the additional α_2, β_2 and γ_2 bands which seem to form a typical hemochromogen spectrum as it can be observed with a variety of hemoproteins.[27]

The possibility that these typical hemochromogen bands are due to pyridine binding to cytochrome b_5 has to be considered but some evidence against this assumption has accumulated from preliminary experiments with Nagarse digested microsomes (unpublished results). Other ligands of reduced cytochrome P-450 including EtNC[28] and primary aliphatic amines[6] are able to cause spectra with double Soret bands in microsomal preparations which are free of cytochrome b_5.

It has been claimed that the two Soret bands due to isonitrile binding correspond to two interconvertible states of the reduced cytochrome the ratio of which depends on the experimental conditions employed.[4,5] The same may be true for pyridine binding but it should be noted that a number of differences exists between the pyridine spectrum and the isonitrile spectrum with respect to the γ_1/γ_2 ratio. With pyridine and its derivatives as the ligands this ratio is maximal at pH 7·8 in PB stimulated microsomes and decreases on both lowering or increasing the pH. The pH dependency is quite different for the EtNC spectrum in microsomal preparations[4] as well as in partially purified cytochrome P-450 preparations[28] where gradual decrease of the 455/430 nm ratio occurs with increasing pH.

It has been described that after irradiation at $-30°$ of carbon monoxide treated microsomes which contain EtNC the γ_1 band at 455 nm appears very rapidly and then gradually decreases while the γ_2 band at 430 nm concomitantly increases.[29] Development of the pyridine spectrum can conveniently be followed at $+30°$ if MC stimulated microsomes are used. The pyridine γ_2 band at 425 nm is most prominent immediately after addition of the ligand and then decreases while the γ_1 band at 446 nm increases. It is remarkable that the behaviour of the EtNC γ_1 band resembles that of the pyridine γ_2 band and vice versa during pH changing and during development of the spectrum. This is not true for the aging processes of the spectrum since during standing at room temperature the γ_1/γ_2 ratio decreases in both the pyridine spectrum in reduced microsomes and the EtNC spectrum in reduced preparations of partially purified microsomal cytochrome P-450.[28]

The unusual spectral properties of reduced cytochrome P-450 complexes have been explained in different ways. It has been discussed that they result from heme aggregation within the specifically oriented membrane subunits.[30] On the other hand, a specific coordination geometry of the heme in which the iron is located out of plane of the porphyrin skeleton has been taken into reason.[27] The double banded hemochromogen spectrum observed with pyridine as well as with other ligands may be due to the presence of both this specific state of the cytochrome and another state in which some disarrangement of the specific geometry has taken place. It may be interesting in this connection that the γ_2 band of the pyridine spectrum is especially prominent under experimental conditions like changing of pH and aging which one might assume to favour some disorganization of membrane structure.

Pretreatment of the animals with MC profoundly influences binding of MP to reduced cytochrome P-450. An effect of MC pretreatment has also been

described on the Soret region of the EtNC spectrum[5] where the γ_1 band is increased under these conditions. This is in contrast to the MC effect on the MP spectrum where the γ_1 band which appears after addition of small amounts of the ligand is considerably decreased as would be expected from the largely increased K_s value. There is some evidence that this effect of MC pretreatment is rather due to changes in the phospholipid environment of the heme moiety than to the synthesis of a MC specific cytochrome.*

Binding of pyridine and its derivatives to reduced cytochrome P-450 was related to an inhibitory action on drug metabolism. A comparison of the octanol/water partition coefficients with the K_s values in PB stimulated microsomes and with the concentrations required for 50 per cent inhibition of drug demethylation supports the impression that binding and inhibitory effect of pyridine and its derivatives in PB stimulated microsomes correlate to some extent with their lipid solubility.

The effect of MP on type II substrate metabolism has previously been reported to depend on the MP concentration employed, activation of these reactions occurring at intermediate MP concentrations.[15] In our experiments, activation was not observed at all tested MP concentrations at an aniline concentration of 4 mM. The action of both MP and reduced MP but not of pyridine and 3-(3-pyridyl)-propanol on aniline hydroxylation does, however, critically depend on the substrate concentration. At aniline concentrations as high as 32 mM an enhancing effect of MP and reduced MP on aniline hydroxylation has been observed.[31] No conclusions concerning a possible correlation of the activation effect with a specific state of cytochrome P-450 can be derived from our results.

Acknowledgements—The able technical assistance of Mrs. G. Schams and Miss C. Ross is gratefully acknowledged.

* R. Kahl, H. G. Jonen and G. F. Kahl, manuscript in preparation.

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