Interaction of ligands with cytochrome *P*-450

On the 442 nm spectral species generated during the oxidative metabolism of pyridine

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(Received 23 November 1981/Accepted 29 January 1982)

When added to aerobic rabbit liver microsomal fractions fortified with an NADPH-generating system, pyridine initially produces a type II difference spectrum such as is observed with other aromatic amines. There is a time-dependent conversion of this perturbation into a new spectral species characterized by an absorbance maximum at 442 nm and a minor peak at 389 nm. Experiments with inhibitors of the cytochrome *P*-450-dependent electron-transport chain suggest that these species originate from binding to the haemoprotein of metabolic intermediate(s) derived from the amine substrate. Analysis of the incubation mixtures by t.l.c., high-pressure liquid chromatography, u.v.- and mass-spectrometry reveals the presence of a single metabolite arising from cytochrome *P*-450-catalysed oxidation of the heteroaromatic tertiary amine, which was identified as pyridine *N*-oxide, obviously accounting for adduct formation. This view is supported by comparative studies on the spectral changes generated by exogenous amine oxide with NADPH-reduced cytochrome *P*-450. Moreover, dithiothreitol, a potent *N*-oxidase inhibitor, strongly suppresses development of the 442 nm and 389 nm complexes. The ability of forming low-spin adducts with ferrous cytochrome *P*-450 absorbing around 440 nm appears to be an inherent property of different types of *N*-oxides. Considering the dipole character of the N^+-O^- function, a co-ordinate iron–oxygen bond is proposed to be formed in these complexes.

Certain types of nitrogenous compounds, such as alkylamines (Franklin, 1974a; Werringloer & Estabrook, 1975; Buening & Franklin, 1976; Franklin, 1976; Kawalek *et al.*, 1976), arylamines (Franklin, 1976; Hlavica & Aichinger, 1978), or hydrazines (Braun *et al.*, 1980; Hines & Prough, 1980) have been reported to undergo cytochrome *P*-450-dependent metabolic conversion associated with the formation of product–ferrohaemochromes absorbing in the range 438–455 nm. *N*-Oxidation seems necessary for the formation of such complexes (Franklin, 1974b; Jonsson & Lindeke, 1976; Hlavica & Aichinger, 1978; Lindeke *et al.*, 1979; Braun *et al.*, 1980). In the case of primary alkyl- and aryl-amines the spectral intermediates are assumed to result from interaction with ferrous cytochrome *P*-450 of nitroxide (Ebel *et al.*, 1975; Jonsson & Lindeke, 1976) or nitroso species (Jonsson & Lindeke, 1976; Mansuy *et al.*, 1977, 1978a) derived from the corresponding hydroxylamines by en-

zymic activation. However, the very slow rate in the presence of NADPH of complex-formation with certain nitrosoalkanes as compared with that with the corresponding alkylhydroxylamines (Jonsson & Lindeke, 1976) suggests caution regarding conclusions on the nature of the proximate ligands of cytochrome *P*-450. Further, nitrones, which can be regarded as counterparts to the nitroso compounds, appear to be incapable of forming product adducts without the prerequisite of metabolism (Lindeke *et al.*, 1979).

Mansuy *et al.* (1978b) believe that primary alkylhydroxylamines might be liganded to the central iron atom of ferric and ferrous cytochrome *P*-450 via their oxygen atoms, whereas the free pair of electrons of the nitrogen atom might contribute to binding in the case of the nitroso analogues.

Reports on the spectral manifestation of *N*-oxidation of tertiary-amine compounds by the cytochrome *P*-450 system are scarce. This lack of data might result from the general assumption that *N*-oxidation of tertiary amines is restricted to catalysis by a flavin-containing mono-oxygenase

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

(EC 1.14.13.8). However, evidence has accumulated indicating involvement of cytochrome *P*-450 in amine oxide formation from certain types of aliphatic (Hallström *et al.*, 1981b), aromatic (Hlavica & Kehl, 1977; Hlavica & Hülsmann, 1979), heteroaromatic (Schwartz *et al.*, 1978; Gorrod & Damani, 1979; De Graeve *et al.*, 1979) and alicyclic (Mattocks & White, 1971; Hallström *et al.*, 1981a) tertiary-amine substrates.

In a previous paper (Hlavica & Aichinger, 1978) we reported on the formation, during cytochrome *P*-450-mediated metabolism of *NN*-dimethylaniline, of a product adduct absorbing at 448 nm that was attributed to binding to the pigment of the corresponding amine oxide. The present paper concerns the characterization of a spectral complex associated with mixed-function oxidation of pyridine.

Materials and methods

Chemicals

Chemicals used in the present study were obtained from the following sources: NADP⁺, NADH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from Boehringer (Mannheim, Germany); dithiothreitol, pyridine, pyridine *N*-oxide, 2-, 3- and 4-hydroxypyridine, 2- and 3-hydroxypyridine *N*-oxide, nicotinamide and nicotinamide *N*-oxide from EGA-Chemie (Steinheim, Germany); octylamine, butan-1-ol, ethanol, chloroform, ethyl acetate and diethylamine from Merck A.G. (Darmstadt, Germany). Metyrapone was kindly supplied by Ciba-Geigy (Wehr, Germany).

Preparation of microsomal fraction

Microsomal fractions from the livers of adult male and female Chinchilla rabbits, pretreated with phenobarbital (50 mg/kg for 7 consecutive days), were prepared by the method of Jagow *et al.* (1965). Haemoglobin was removed from the microsomal suspensions by two washings with 0.15 M-KCl. As a routine the microsomal pellets were suspended in 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, and stored at +4°C until use. The protein content of the individual preparations was determined by the method of Gornall *et al.* (1949), as modified by Szarkowska & Klingenberg (1963).

Preparation of antiserum to microsomal NADPH:cytochrome *c* (*P*-450) reductase

Detergent-solubilized rabbit liver microsomal NADPH:cytochrome *c* (*P*-450) reductase (EC 1.6.2.4) was purified to apparent homogeneity as described previously (Hlavica & Hülsmann, 1979). The enzyme was concentrated to a protein content of 10 mg/ml of 0.9% NaCl, emulsified with an equal volume of Freund's complete adjuvant (GIBCO, Grand Island, NY, U.S.A.), and injected sub-

cutaneously into different sites on the back of an adult male goat. At 3 weeks after the initial injection an intramuscular injection of 10 mg of the enzyme in 0.9% NaCl, mixed with an equal volume of complete adjuvant, was given. This was followed by two intravenous booster injections of 10 mg of reductase in 0.9% NaCl at 2-week intervals. Blood was taken 10 days after the last injection, and the potency of antiserum tested by immunodiffusion on 1% agarose in 0.9% NaCl.

Control serum was prepared from a non-immunized animal.

Measurement of pyridine-induced difference spectra

The basic incubation media contained: 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4; 1.2 mM-NADP⁺; 10 mM-glucose 6-phosphate; 3 mM-MgCl₂; 4 µg of glucose 6-phosphate dehydrogenase/ml of mixture, and 2 mg of microsomal protein/ml of assay medium. The mixture was equally divided between two cuvettes. After establishing the baseline of equal light absorbance, pyridine (final concn. 1 mM) was added to the contents of the sample cuvette and the difference spectrum recorded with a Cary 219 spectrophotometer at 25°C by repetitive scanning at definite time intervals; the optical path-length was 1 cm. Further additions to the basic incubation mixtures are specified in the appropriate legends to the Figures.

Analysis of metabolic products

Microsomal incubation mixtures (100 ml), containing 5 mM-pyridine and the NADPH-generating system described above, were incubated at 37°C for 60 min. The reaction was stopped by the addition of 10 ml of 3 M-HClO₄, and the precipitate removed by centrifugation at 10 000 *g* for 15 min. Subsequently the pH of the clear supernatant fraction was adjusted to 10 by the addition of 3 M-NaOH. This was followed by exhaustive extraction with excess of freshly distilled diethyl ether. After removal of the organic layer, the pH of the aqueous phase was adjusted to 2.4 by the addition of 3 M-HCl. The solution was extracted twice with an equal volume of butan-1-ol. The extracts were combined, dried with anhydrous K₂CO₃ and evaporated to dryness.

When catalytic amounts of reference compounds were added to the aqueous media and treated as described above, the recovery after extraction with butan-1-ol was as follows: 2-hydroxypyridine, 99%; 3-hydroxypyridine, 67%; 4-hydroxypyridine, 80%; pyridine *N*-oxide, 61%; 2-hydroxypyridine *N*-oxide, 78%; 3-hydroxypyridine *N*-oxide, 100%; nicotinamide, 26%; nicotinamide *N*-oxide, 100%.

The residue of the butanol extracts was dissolved in a small volume of methanol, and the solution streaked on to t.l.c. plates (20 cm × 20 cm) pre-coated with silica-gel 60 F₂₅₄ (Merck). The chro-

matograms were developed twice with ethanol (system A); authentic compounds were co-chromatographed. Zones containing separated material were detected by inspection of the t.l.c. plates under u.v. light, scraped off and the substances eluted from the silica gel with methanol. The eluted material was restreaked on to t.l.c. plates, and these were run either in the solvent system ethyl acetate/ethanol/ammonia (50:40:1, by vol.; system B) or in the system chloroform/ethanol/diethylamine (80:30:1, by vol.; system C) (Damani *et al.*, 1978). Reference compounds were co-chromatographed. This procedure permitted the isolation of two compounds, hereafter called 'Product I' and 'Product II'. These were eluted from the silica gel with methanol, the u.v. absorption spectra of the centrifuged extracts being recorded between 230 and 340nm and compared with those of authentic material. The pooled samples from a series of analogous experiments were stored at -20°C until further analysis.

Analysis of metabolic products by h.p.l.c.

Microsomal lipids contaminating Products I and II were removed by reverse-phase h.p.l.c. on a μ -Bondapack C_{18} column (30cm $\times$ 0.4cm) attached to a Waters model 244 liquid chromatograph. The solvent system was methanol/water (1:4, by vol.); the flow rate was 2ml/min. Components were monitored by u.v. absorbance (254nm) and retention times compared with those of reference compounds. Fractions eluted from the column were collected, evaporated to dryness and stored at -20°C under N_2 .

Mass-spectral analysis of metabolic products.

Identification of Products I and II was achieved by electron-impact mass spectrometry using a CH 7-A Varian spectrometer operated at an electron energy of 70eV. The samples were introduced via the direct insertion probe. Spectra of authentic material were run for comparison.

Results and discussion

Spectral changes associated with the oxidative metabolism of pyridine

Addition of pyridine to an aerobic hepatic microsomal fraction from phenobarbital-treated rabbits fortified with an NADPH-generating system initially results in a type II spectral perturbation characterized by an absorbance maximum at 427nm and a trough around 398nm (Fig. 1); this difference spectrum is characteristic of the interaction of unchanged pyridine with cytochrome P-450 (Jefcoate *et al.*, 1969). Repetitive scanning of the sample reveals the gradual transformation with time of this spectral change to an optical intermediate exhibiting an additional absorbance band at

Fig. 1. Spectral changes associated with the mixed-function oxidation of pyridine in liver microsomal fractions from phenobarbital-treated rabbits

Microsomal suspensions were diluted with 0.15M $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4, to a final concentration of 2mg of protein/ml of mixture (8.7 μM -cytochrome P-450), supplemented with NADPH-generating system and equally divided between two cuvettes. After establishing the baseline of equal light absorbance, pyridine (final concn. 1mM) was added to the contents of the sample cell, and scans were taken 0.5 (—), 8 (—), 18 (· — ·) and 21 (····) min later.

442nm and a minor peak at 389nm (Fig. 1). The possibility that the 442nm absorption peak is due to a pyridine haemochrome of microsomal cytochrome b_5 (Raw *et al.*, 1958) can be safely ruled out; neither does it result from binding to cytochrome P-450 of endogenously generated CO, since the position of the Soret maximum is at variance with that of the carbonyl complex of phenobarbital-induced rabbit liver microsomal cytochrome P-450 (Lu & West, 1980).

Formation of the 442nm band is strongly dependent on the presence of NADPH in the assay

media, and is greatly suppressed when NADH or sodium dithionite substitute for the cofactor (Fig. 2a). Known inhibitors of the cytochrome *P*-450 system, such as CO, metyrapone and octylamine, as well as antiserum to NADPH:cytochrome *c* (*P*-450) reductase strongly impair development of the 442 nm spectral change (Figs. 2b and 2c). It is noteworthy that incorporation of non-immune serum into the incubation mixtures causes a lag phase in complex-formation; however, once triggered, formation of the 442 nm species appears to be enhanced by control serum (Fig. 2c). The reason for such phenomena remains obscure.

From the observations described above it is concluded that the 442 nm spectral species originates from binding to cytochrome *P*-450 of a metabolic intermediate arising from mixed-function oxidation of pyridine.

Nature of product(s) potentially involved in 442 nm cytochrome P-450 adduct formation

Large-scale incubation mixtures, containing hepatic microsomal fraction from phenobarbital-treated rabbits, NADPH-generating system and pyridine were extracted with butan-1-ol. As has been described in detail in the Materials and methods section, this procedure recovered catalytic amounts of all metabolites, that is, *C*- and *N*-oxygenated products (respectively) expected to arise from mixed-function oxidation of the heteroaromatic amine. Initial examination of the extracts by t.l.c. revealed the presence of two compounds (Products I

and II). These were subjected to further analysis by h.p.l.c., u.v.- and mass-spectroscopy. Identification of the material was achieved by comparison with data from authentic compounds. Thus Product I was unequivocally identified as pyridine *N*-oxide, accounting for approx. 5% of the parent amine, whereas Product II was recognized as representing nicotinamide, presumably generated through nucleosidase-catalysed hydrolytic breakdown of exogenous NADP⁺ (Tables 1 and 2). Despite the sufficient sensitivity of our analytical methods, we were unable to detect measurable amounts of *C*-oxygenated metabolic products in the assay media. This finding is not surprising in the light of a recent report by Damani *et al.* (1981), who succeeded in localizing NADPH-dependent pyridone formation in the hepatic 140 000 *g* supernatant fraction. Further, pyridine has not been found to undergo conversion in the rabbit to yield 3-hydroxypyridine (Smith, 1953). Pyridine *N*-oxide thus appears to represent the only metabolite accumulating in appreciable amounts during mixed-function oxidation of the amine substrate.

That nicotinamide produces the observed 442 nm adduct of ferrous cytochrome *P*-450 can be ruled out: (a) the amide is equally well generated in both the sample and the reference cell, the spectral contributions therefore being sufficiently balanced; (b) addition of nicotinamide to NADPH-reduced microsomal fractions results in a type II spectral change characterized by a Soret maximum at 427 nm (not shown). Alternatively, nicotinamide

Fig. 2. Kinetics of formation of the 442 nm product adduct of cytochrome *P*-450 under various conditions

For composition of the standard assay media, see the Materials and methods section. The specific experimental conditions were as follows: (a) control (O); omission of NADPH-generating system from the aerobic incubates (Δ); substitution of sodium dithionite (□; final concn. 1 mM) or NADH (●; final concn. 1 mM) for NADPH. (b) Gassing of the basic assay media with an atmosphere of CO/O₂ (4:1) for 5 min (O); addition of metyrapone (□) or octylamine (Δ) to the experimental cells, each to yield a final concentration of 1 mM. (c) Pretreatment of microsomal fractions with antiserum to NADPH:cytochrome *c* (*P*-450) reductase (10 mg of protein/ml of medium) for 15 h at 4°C (●); analogous treatment of microsomal suspensions with non-immune serum (○). Absorbance changes at 442 nm relative to 500 nm were monitored after the addition of pyridine (final concn. 1 mM) to the sample cuvette and plotted as a function of time. The experimental points represent the means of three determinations.

Table 1. *Characterization of Products I and II by means of t.l.c., h.p.l.c. and u.v. spectroscopy*

Compound	R_F	R_t (min)	λ_{max} . * (nm)
Product I	0.40†	2.3	262.1
Synthetic pyridine <i>N</i> -oxide	0.42†	2.2	262
Product II	0.64‡	2.8	256 sh§; 261.5; 268 sh
Synthetic nicotinamide	0.64‡	2.8	256 sh; 261.5; 268 sh

* In methanol.

† In chromatographic system C.

‡ In chromatographic system B.

§ sh = shoulder.

Table 2. *Major ion peaks in the direct-inlet mass spectra of Products I and II: comparison with authentic material*

The values in parentheses indicate relative intensity of the individual ion peaks.

Compound	m/e (Relative abundance, %)			
Product I	95 (100)	79 (12)	78 (9)	51 (17)
Synthetic pyridine <i>N</i> -oxide*	95 (100)	79 (11)	78 (8)	51 (17)
Product II	122 (100)	106 (60)	78 (83)	51 (48)
Synthetic nicotinamide	122 (100)	106 (64)	78 (93)	51 (51)

* See Cowan *et al.* (1978).

N-oxide, arising from microsomal *N*-oxidation of nicotinamide (Cowan *et al.*, 1978), could account for the 442nm spectral species. This possibility, however, could be excluded by failure to detect metabolic formation of the *N*-oxide. Furthermore, nicotinamide *N*-oxide, when incorporated into the assay media, elicits a weak type I spectral perturbation characterized by an absorbance band at 398 nm and a trough at 420 nm (not shown; Temple, 1971).

Although less likely, 442nm complex-formation might result from binding to the haemoprotein of dipyrindyl produced through condensation of pyridyl radicals generated during cytochrome P-450-dependent one-electron oxidation of pyridine. However, addition of dipyrindyl to fortified liver microsomal fractions results in a type I difference spectrum exhibiting an absorption peak at 407 nm and a trough at 422 nm (not shown).

As there is no reasonable alternative remaining, the 442nm metabolic product adduct obviously arises from binding to cytochrome P-450 of a *N*-oxidized intermediate of pyridine.

In order to confirm this concept, the influence on 442nm complex-formation of dithiothreitol, a known *N*-oxidase inhibitor (Beckett *et al.*, 1971; Jonsson & Lindeke, 1976), was studied (Fig. 3). The agent modifies the characteristics of the initial 427nm complex; the Soret band exhibits a slight blue shift; unless there is a difference in the

Fig. 3. *Influence of dithiothreitol on the development of the 442 nm spectral change*

Microsomal suspensions in 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, containing 2 mg of protein/ml of mixture (8.7 μM-cytochrome P-450) and NADPH-generating system were supplemented with dithiothreitol (final concn. 1 mM) and divided between two cuvettes. Spectra were recorded 0.5 (—), 5 (---) and 10 (- · - ·) min after incorporation of pyridine (final concn. 1 mM) into the contents of the sample cell.

Fig. 4. Difference spectra produced by pyridine *N*-oxide with rabbit liver microsomal fractions

Microsomal fractions were adjusted to a protein concentration of 2 mg/ml of mixture (8.7 μ M-cytochrome *P*-450) with 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, and divided between two cuvettes. Pyridine *N*-oxide (final concn. 10 mM) was added to the sample cuvette and the spectrum recorded (—). Alternatively the amine oxide was added to microsomal suspensions either treated with sodium dithionite (....) or supplemented with NADPH-generating system (---). All scans were taken immediately after substrate addition.

absorption coefficients, complex-formation is enhanced, but the complex formed appears to be less stable as compared with that produced in the absence of the sulphur compound (cf. Fig. 1).

As expected, dithiothreitol strongly impairs development of the 442 nm spectral intermediate, only a weak shoulder in the difference spectrum showing up about 8 min after the addition of pyridine to the contents of the sample cuvette. Similarly, the 389 nm absorbance band, regularly observed in the spectra (cf. Fig. 1), is abolished.

We further looked for the spectral changes generated by the addition of pyridine *N*-oxide to microsomal suspensions (Fig. 4), although there is some difference between this stable end product and its corresponding reactive intermediate. Accordingly, relatively high concentrations of the *N*-oxide were necessary to obtain measurable perturbations.

When added to microsomal fractions containing oxidized cytochrome *P*-450, the *N*-oxide produces a type II difference spectrum with an absorption peak at 427 nm and a trough at 394 nm, which corres-

Fig. 5. Comparison of the catalytic capacities towards pyridine and *NN*-dimethylaniline of highly purified flavin-containing amine oxidase

Flavoprotein amine oxidase (EC 1.14.13.8) was purified as described previously (Hlavica & Hülsmann, 1979). The reaction mixtures contained 1 mM-NADPH and 18 μ g of enzyme/ml of solution in 0.15 M-KH₂PO₄/Na₂HPO₄, pH 8.5. Pyridine (●) or *NN*-dimethylaniline (○) (each to give a final concn. of 3 mM) were added as indicated by 'S' and the arrow. *N*-Oxygenating activity was measured in terms of NADPH oxidation by monitoring the decrease in A_{366} .

ponds to a change in the spin state of cytochrome *P*-450 from high to low (Mitani & Horie, 1969). Pyridine *N*-oxide, however, does not elicit a definite spectral change in microsomal preparations pretreated with sodium dithionite; this is not a consequence of conversion, in the assay mixtures, of the *N*-oxide into the parent amine, since pyridine *N*-oxide was found to be fairly stable in the presence of the reductant. It has to be kept in mind that dithionite also destroys the product adducts arising from metabolic transformation of aryl amines, but preserves those formed from alkyl amines (Franklin, 1976; Hlavica & Aichinger, 1978). Most interestingly, pyridine *N*-oxide gives rise to formation of a mixture of low- and high-spin haemochromes (Mitani & Horie, 1969), characterized by Soret bands at 408 and 442 nm, when incorporated into assay media containing an NADPH-generating system. These spectral changes could be argued to represent an 'oxidized-versus-reduced' difference spectrum of cytochrome *b*₅. This possibility, however, can be safely ruled out, since further stepwise addition of NADPH to the contents of the experi-

mental cell does not change the situation, and pyridine *N*-oxide does not impair reduction of cytochrome *b*₅. The discrepancy in complex-formation between dithionite- and NADPH-reduced microsomal fractions might be related to a selective conformational change of cytochrome *P*-450 induced by coupling of NADPH:cytochrome *P*-450 reductase to the haemoprotein (French *et al.*, 1980); such transition might facilitate access of the *N*-oxide to the hydrophobic core of the pigment.

Assuming the 442 nm-absorbing metabolic adduct to result from interaction with cytochrome *P*-450 of a *N*-oxidized intermediate of pyridine, comparison of the spectral changes generated by exogenous pyridine *N*-oxide in NADPH-reduced microsomal preparations (Fig. 4) with those elicited by endogenously formed *N*-oxy product (Fig. 1) would suggest that, in the latter process, formation of a low-spin haemochrome of cytochrome *P*-450 predominates. Nevertheless, the 389 nm absorption peak regularly apparent in the difference spectra shown in Fig. 1 points at a small portion of cytochrome *P*-450 existing in the complexed high-spin form; that pyridine *N*-oxide by itself accounts for this absorption can be ruled out (Table 1). Our data do not permit us to decide whether such a mixture of spin forms might be formed with a single haemoprotein species or multiple types of cytochrome *P*-450 co-existing in rabbit liver microsomal membranes (Lu & West, 1980). Clearly, the trough around 423 nm, seen in the difference spectrum resulting from interaction of pyridine *N*-oxide with NADPH-reduced cytochrome *P*-450 (Fig. 4), cannot be observed under metabolic conditions (Fig. 1), since, in the composite spectrum, the high background of absorbance originating from residual 427 nm species more than compensates for the relatively small decrease in absorbance caused by binding to the haemoprotein of catalytic amounts of *N*-oxy product.

In order to ensure that formation of the 442 nm product adduct is not the consequence of *N*-oxidation of pyridine by the microsomal flavo-protein amine oxidase (EC 1.14.13.8), with subsequent binding to cytochrome *P*-450 of the *N*-oxide formed, experiments with purified rabbit liver microsomal flavin-containing mono-oxygenase were conducted. As shown in Fig. 5, the enzyme readily *N*-oxygenates *NN*-dimethylaniline, a substrate most frequently used to assay the system (Ziegler, 1980), but is incapable of mediating *N*-oxidation of pyridine. This finding is in accord with data reported for the porcine oxidase (Ziegler, 1980).

The ability to form low-spin complexes with ferrous cytochrome *P*-450 absorbing in the vicinity of 440 nm appears to be an inherent property of different types of *N*-oxides: tiaramide *N*-oxide, an alicyclic compound, produces a 442 nm haemo-

chrome that is destroyed by the addition of CO (Sugiura *et al.*, 1974); *NN*-dimethylaniline *N*-oxide generates a difference spectrum with an absorbance maximum at 440–447 nm (Hlavica & Aichinger, 1978). This uniformity in spectral behaviour might be based on a common mode of binding to the haem site of all these *N*-oxides. Considering the relatively strong dipole character of the N⁺–O[–] function [pyridine *N*-oxide: μ (dipole moment) = 4.24 D (debyes) = $\sim 14 \times 10^{-28}$ cm·C; Ochiai, 1967], a coordinate Fe–O bond is proposed to be formed in these complexes. This view is endorsed by numerous studies on the donor properties of pyridine *N*-oxide (Hieber & Lipp, 1959; Carlin, 1961; Quagliano *et al.*, 1961) and some of its derivatives (Lever *et al.*, 1962; Sigel & Brintzinger, 1963) towards iron. It is noteworthy that the oxyferro intermediate of cytochrome *P*-450 also exhibits an absorption in the difference spectrum at 442 nm (Bonfils *et al.*, 1979).

We thank Miss Hildegard Wahler for expert technical assistance. The financial support of the Deutsche Forschungsgemeinschaft (grant no. H1 1/10) is gratefully acknowledged.

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