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MAGNETIC CIRCULAR DICHROISM STUDIES ON MICROSOMAL ARYL HYDROCARBON HYDROXYLASE: COMPARISON WITH CYTOCHROME b_5 AND CYTOCHROME P-450_{cam} †

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Running title: MCD of Cytochrome b_5, P-450_{cam} and Microsomes

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Abbreviations used are: MCD, magnetic circular dichroism; CD, circular dichroism; EPR, electron paramagnetic resonance; P-450 and $P\text{-}450_{cam}$ will be used to designate cytochromes P-450 from microsomes and *P. putida* respectively, while P-420 and $P\text{-}420_{cam}$ will refer to the apparently denatured forms of these enzymes having carbon monoxide absorption difference spectral maxima near 420 nm rather than 450 nm; DMBA, 7-12-dimethylbenz[a]anthracene; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

ABSTRACT

Magnetic circular dichroism (MCD) spectra are reported for the visible and near UV spectral regions of liver microsomes from dimethylbenzanthracene-treated rats. The sequential addition of NADH, dithionite, and carbon monoxide enables us to determine contributions to the MCD by cytochromes b_5 and P-450, which dominate the spectra. The MCD of the microsomal preparation is compared with that of purified oxidized and reduced cytochrome b_5 from pig liver and with the camphor-complexed and camphor-free oxidized, reduced, and reduced carbonmonoxy cytochrome $P\text{-}450_{cam}$ from Pseudomonas putida. The MCD spectra of the membrane bound cytochrome b_5 are similar to those of the purified protein, indicating that little or no alteration in the environment of the heme occurs during the isolation procedure. The soluble bacterial cytochrome $P\text{-}450_{cam}$ also appears to be a suitable model for microsomal P-450, although differences in the MCD intensity are observed for the two enzymes. It is concluded that MCD will be a useful technique for investigating redox and structural changes of hemoproteins in particulate systems.

Several polycyclic aromatic hydrocarbons, including common environmental carcinogens such as benzo(a)pyrene, are metabolized *in vivo* by an inducible enzyme system (Conney *et al.*, 1957) which causes hydroxylation to water-soluble products such as phenols and dihydrodiols (Sims, 1970). It is thought that reactive intermediates produced during this reaction are responsible for the carcinogenic effect shown by some aromatic hydrocarbons (Gelboin *et al.*, 1969). These intermediates may react with various cellular components to produce genetic or other transmissible damage (Kinoshita and Gelboin, 1972). The aryl hydrocarbon hydroxylase enzyme has been studied in several tissues such as skin, lung, and especially liver, as well as in some tissue culture systems (Nebert and Gelboin, 1969; Kinoshita and Gelboin, 1972). The enzyme is one of the microsomal mixed-function oxidases, a class of enzymes which plays an important role in the metabolism of several drugs and other exogenous compounds (Mason *et al.*, 1965), and also in the interconversion and degradation of endogenous metabolites such as steroids (Kuntzman *et al.*, 1964). The enzyme which hydroxylates benzo(a)-pyrene has been shown to be similar to, but distinct from, that hydroxylase principally induced by barbiturates (Gelboin *et al.*, 1972). These enzymes generally require both reduced pyridine nucleotide and molecular oxygen, as well as the hydroxylation substrate, and consist of a flavoprotein component which interacts with the pyridine nucleotide, and a heme protein of the P-450 type which interacts with the specific substrate (Lu *et al.*, 1972). The role of cytochrome b_5 as an intermediate electron carrier in the hydroxylase system has received considerable attention but has not yet been resolved (see Gillette *et al.*, 1972).

Chemical studies have identified epoxides (Grover et al., 1971), hydroperoxides (Shaw and Chen, 1972), and other reactive compounds as possible intermediates, but so far neither these nor spectroscopic methods have succeeded in delineating the main reaction pathway. We have recently begun an extensive study of the magnetic circular dichroism (MCD[1]) of a variety of hemoproteins and their derivatives and of some model heme compounds (Vickery, Nozawa and Sauer, in preparation). While the theory for the origin of the magneto-optical effects observed for heme complexes is not yet well developed, our results and those of some earlier workers suggest that the MCD spectra of hemoproteins can be used to determine the redox state, the spin state, and the identity of the axial ligands to the iron, complementing other experimental approaches. Many components of the electronic spectrum can be resolved, since the Faraday effect can have three possible origins, referred to as A, B, and C terms, for a given electronic transition: (A) a Zeeman splitting of a degenerate ground or excited state; (B) mixing of the ground or excited state with other excited states by the magnetic field; and (C) a temperature-dependent population difference in ground state levels whose degeneracy has been removed by the magnetic field; the Faraday A term is "S-shaped" and resembles the first derivative of the absorption spectrum; in contrast, the B and C terms are "bell-shaped" and approximate the shape of the absorption band (see Schatz and McCaffery, 1969, and Stephens, 1974, for recent reviews). While electron paramagnetic resonance (EPR) studies are also useful for investigating heme chemistry, they are restricted to Fe(III) complexes; also low temperatures are required due to the rapid spin-lattice relaxation of the iron in these compounds, whereas physiological temperatures and rapid kinetic

measurements can be obtained by the MCD technique. In addition, MCD experiments can be carried out with less than micromolar concentrations of hemoprotein. We have shown that difference MCD spectra can be used to follow oxidation-reduction changes in a complex mixture of cytochromes in chloroplast particles (Vickery et al., 1974) and wish to report here the application of MCD to the investigation of microsomal hemoproteins..

MATERIALS AND METHODS

Two hundred gram female Sprague-Dawley rats were given intravenously three doses of 4 mg of 7,12-dimethylbenzanthracene in cottonseed oil emulsion (gift of The Upjohn Co.), at 72-hour intervals. Forty-eight hours after the last injection, the rats were sacrificed by decapitation and the livers removed and stored at -70°C. Rats injected with a plain cottonseed oil emulsion were used as controls.

The livers were thawed, and washed in Tris-sucrose buffer (0.05 M Tris-HCl, 0.25 M sucrose, pH 7.5). They were then homogenized in 20 ml per liver of this buffer (1 minute at full speed in a Sorvall blender, cooling with ice). Cell nuclei and other large debris were removed by a low speed centrifugation, 600 x g for 10 minutes (Beckman J-21 centrifuge with 20 rotor at 3000 rpm). The mitochondrial fraction was removed by centrifugation at 7000 x g for 15 minutes. Finally the microsomal fraction was collected by centrifugation at 105,000 x g for 1 hour (Beckman L3-50 ultracentrifuge, 50 rotor). The microsomes were washed twice by resuspension in Tris-sucrose buffer and centrifugation at

105,000 x g. The microsomal preparation was resuspended in Tris-sucrose buffer and stored frozen in airtight vials under argon, at -70°C. The sample used for MCD studies had A_{360} = 1.6 using the conventional sample compartment in a Cary 14; with the Cary 1462 scattering attachment, the absorption at 360 nm decreased to 0.64 for the sample.

The aryl hydrocarbon hydroxylase assay used in all experiments was the standard fluorimetric estimation of benzo(a)pyrene hydroxylation products (Nebert and Gelboin, 1968), with the addition of NADH as well as NADPH. Each assay sample contained between 100 and 150 μg of protein. Protein concentrations were estimated by the method of Lowry (Lowry et al., 1951)., Microsomal P-450 concentrations were determined according to Omura and Sato (1964).

Purified cytochrome b_5 prepared by trypsin treatment of pig liver microsomes was kindly supplied by Dr. R. Malkin of the University of California, Berkeley. Concentrations of cytochrome b_5 were determined according to the published extinction coefficients (Ozols and Strittmatter, 1964). The EPR spectrum of this preparation, measured in collaboration with Dr. A. J. Bearden, was essentially identical to that reported by other workers (Bois-Poltoratsky and Ehrenberg, 1967; Ikeda et al., 1974). The visible and near UV natural CD spectra of this sample were also in general agreement with the results for pig liver cytochrome b_5 obtained by Schnellbacher and Lumper, which were presented as reduced, or mean residue, ellipticity (Schnellbacher and Lumper, 1971). The Soret region CD spectra for the reduced forms of these preparations as well as calf liver ferrocytochrome b_5 (Morgan and Vickery, unpublished observation) resemble the first derivative of the Soret absorption band in contrast

to the predominantly negative CD reported for rabbit ferrocytochrome b_5 by Okada and Okunuki (1973). These results contradict the latter authors' proposal that b-type hemoproteins should exhibit only a negative Cotton effect associated with the Soret transition.

A purified preparation of the camphor complex of cytochrome $P\text{-}450_{cam}$ isolated from Pseudomonas putida was the generous gift of Dr. W. T. Morgan, Scripps Clinic and Research Foundation, La Jolla, California. The $A_{392\ nm}/A_{280\ nm}$ ratio for this sample was 1.34. Sufficient D-camphor was added to saturate all optical changes in studying the camphor complex. Camphor was removed by chromatography on Sephadex G-25. The sample was concentrated using ammonium sulfate and then dialyzed. Concentrations were measured using the published extinction values (Gunsalus et al., 1972). The natural CD results obtained with this preparation were similar to those reported previously (Peterson, 1971) except that the ellipticity values given by Peterson should be multiplied by 100 in order to conform to the conventional units of $[\theta]$ ($deg.\cdot cm^2\cdot decimole^{-1}$).

Fluorescence measurements were made with a Perkin-Elmer MPF-2A Fluorescence Spectrophotometer. Absorption spectra were obtained with a Cary 118 Recording Spectrophotometer. The instrument used for MCD measurements was designed in this laboratory and has been described elsewhere (Sutherland et al., 1974). A constant slit width of 0.5 mm for the Cary 14 monochromator was found to be sufficient to resolve all bands. A 1.0 cm pathlength cell was used for all samples, and peak absorbances were not allowed to exceed 1.6. An electromagnet operating at 14.3 kgauss was used in these experiments. A time constant of 0.3 sec and a scan rate of 30 nm per minute were routinely used. Natural CD was corrected

for by reversal of the direction of the magnetic field and subtracting the data for the two directions (Sutherland et al., 1974). Under these conditions the signal-to-noise ratio of the MCD spectra was significantly better than that of the CD spectra, and the latter are not shown. The CD signals observed are, however, large enough to make significant contributions to the optical activity even at considerably higher magnetic fields; for this reason, caution should be exercised in comparing the units and values used here and those used by other workers (vide supra). All figures show computer drawn curves (solid lines) or traces (dashed lines) of the experimental data. The results are expressed as $\Delta\varepsilon/H = \varepsilon_L - \varepsilon_R$ (= $[\theta]/3{,}305$) with the units of $(M \cdot cm \cdot Tesla)^{-1}$ or as $\Delta A/H = A_L - A_R$ $(cm \cdot Tesla)^{-1}$, where 1 Tesla = 10,000 gauss.

RESULTS AND DISCUSSION

Hemoproteins show intense and distinctive MCD spectra associated with the heme chromophore π-π^* and charge transfer transitions in the visible and near UV regions. Since the heme group has approximately D_{4h} symmetry, the porphyrin π states are nearly x-y degenerate and Faraday A terms are predicted for the near UV Soret band, the $Q_{0,1}$ or β bands, the $Q_{0,0}$ or α band and charge transfer bands involving the porphyrin. In addition, C terms will be found in paramagnetic derivatives (Briat et al., 1973, and Vickery, Nozawa and Sauer, in preparation).

Because of the mixing of the iron d orbitals with the porphyrin π system, the MCD spectra of heme compounds, like the absorption spectra, are very sensitive to the redox and spin states and the axial coordination of the iron. The MCD spectra are affected to a much lesser degree,

however, by differences in the protein crevice environment or solvent. This enables us to characterize heme complexes of unknown structure by comparison with known compounds. The Faraday parameters and MCD band shapes expected for each case will be discussed in interpreting the MCD spectra of purified cytochrome b_5 from pig liver and cytochrome P-450_{cam} from P. putida. These results will be used as a guideline for the assignment of the bands of the MCD spectra of the microsomes. The mammalian cytochromes b_5 examined thus far have been shown to be very similar in their spectral and chemical properties, so that the pig protein should serve as a suitable model for rat cytochrome b_5. The bacterial cytochrome P-450_{cam} catalyzes the hydroxylation of D-camphor in a reaction whose mechanism is believed to be analogous to the hydroxylation reactions carried out by microsomal cytochromes P-450, and the spectral properties of the soluble bacterial enzyme are similar to those of the particulate mammalian enzymes. Contributions from these two types of hemoproteins are expected to dominate the MCD spectra of microsomes, since the MCD of the other major components of microsomes are probably much weaker as indicated by studies on some flavin (Tollin, 1968, and Bayer et al., 1971) and non-heme iron proteins (see Marlborough and Hall, 1969; Sutherland et al., 1972; and Ulmer et al., 1973).

Cytochrome b_5--The MCD spectra of oxidized and reduced pig cytochrome b_5 are shown in Fig. 1. Essentially identical spectra were obtained with a trypsin-derived calf liver cytochrome b_5 (Vickery and Morgan, 1974). The results for ferricytochrome b_5 are in general agreement with those reported (Risler and Groudinsky, 1973), except that we observe somewhat weaker MCD intensities in the region of the Soret and

β bands and a more intense MCD associated with the α band. The "S-shaped" or derivative-like MCD curve in the Soret region of the oxidized cytochrome is typical of low spin ferrihemoproteins and can be used to estimate the amount of low spin components in situations where a spin state equilibrium exists (Vickery, Nozawa and Sauer, in preparation). As first reported for ferricytochrome b_2 (Briat et al., 1973), this band is not a Faraday A term but shows a temperature dependence indicative of two overlapping C terms of opposite sign and a crossover near the absorption maximum (Vickery and Morgan, 1974). The intensity of the Soret MCD of ferricytochrome b_5 is consistent with the iron existing completely in the low spin form. The visible MCD of ferricytochrome b_5 shows C terms associated with charge transfer bands in the 440-520 nm region and A and C terms for the Q bands (Vickery and Morgan, 1974). Because of the effects of axial ligation on the energies of these bands, an empirical analysis of the shape of the MCD curve in this region, ca., 440-570 nm, can be used to characterize the fifth and sixth ligands to the iron (Vickery, Nozawa and Sauer, in preparation). X-ray crystallographic studies on lipase-solubilized calf liver cytochrome b_5 show that the heme iron is axially coordinated to two imidazole side chains from histidine residues (Mathews et al., 1971), and the visible MCD of ferricytochrome b_5 is very similar to that of other bis-imidazole heme complexes (Vickery, Nozawa and Sauer, in preparation).

Chemical reduction of cytochrome b_5 results in the loss of the strong C terms in the Soret region and the appearance of a sharp, intense A term corresponding to the α band, typical of low spin ferrohemoproteins. Overlapping A terms largely cancel one another in the region of the vibrational β bands. Values of the MCD intensity at selected extrema and zero crossings for oxidized and reduced cytochrome b_5 are given in Table I.

Cytochrome P-450$_{cam}$--MCD spectra of P-450$_{cam}$ were recorded first for the oxidized and reduced form of the camphor complex maintained during the isolation procedure and then for the oxidized, reduced and reduced-carbon monoxide complex of the camphor-free enzyme. The results obtained with the oxidized enzyme are shown in Fig. 2A. In the absence of substrate the enzyme exists almost completely in the low spin form, as evidenced by EPR (see Gunsalus, 1968; Tsai et al., 1970; and Peterson, 1971) and Mössbauer spectroscopy (Sharrock et al., 1973), and has the characteristic derivative C term MCD for the Soret band. Estimation of the percent low spin is difficult due to the number of factors affecting the intensity of the net C terms for this band (Vickery, Nozawa and Sauer, 1974). The visible MCD of the uncomplexed P-450$_{cam}$ is similar in its general shape to other low spin hemoproteins. Distinct differences in the band positions and intensities of the extrema can be seen, however, when the MCD of P-450$_{cam}$ is compared with the MCD of hemoprotein complexes where methionine-histidine (see Sutherland and Klein, 1972; Risler and Groudinsky, 1973; and Vickery et al., 1974b), histidine-histidine (Figure 1 and Risler and Groudinsky, 1973; Vickery and Morgan, 1974; and Vickery et al., 1974) or lysine-histidine (Vickery, Nozawa and Sauer, in preparation) coordination to the heme iron has been proposed. Mercaptide coordination from a cysteine residue has been suggested for cytochromes P-450 (Mason et al., 1965; Jefcoate and Gaylor, 1969; and Tsai et al., 1970) and may account for the observed differences. Additionally, the presence of a high spin component in the sample complicates this interpretation (vide infra).

When camphor is complexed with oxidized P-450$_{cam}$ the heme is converted to a predominantly high spin form (Gunsalus, 1968; Tsai et al.,

1970; Peterson, 1971; Keller et al., 1972; and Sharrock et al., 1973). This transition is clearly evidenced by the loss of the derivative-like C terms and the appearance of new A and C terms in the Soret region MCD (Fig. 2A). Changes are also observed in the visible region MCD. The visible MCD spectrum is very similar to that of ferrimyoglobin (Bolard and Garnier, 1972, and Vickery, Nozawa and Sauer, in preparation), and this may indicate a similar coordination of the iron by the protein. The shape of the visible absorption spectrum (Gunsalus et al., 1972) also resembles that of ferrimyoglobin. A change in the axial ligand(s) from mercaptide to a weaker field ligand, such as the imidazole of the proximal histidine in myoglobin, has been suggested as a mechanism of spin state changes upon substrate binding in cytochromes P-450 (Tsai et al., 1970). It is significant, however, that the Soret transition occurs at higher energy in camphor-complexed $P\text{-}450_{cam}$ than in ferrimyoglobin, and the Soret MCD differs in shape and intensity from that of ferrimyoglobin (Vickery, Nozawa and Sauer, in preparation).

The MCD associated with the charge transfer band near 648 nm in the camphor complex is considerably weakened but still present in the absence of camphor. In the absorption spectrum the peak at 648 nm with $\epsilon_{648}^{mM} = 5$ decreases to a shoulder with $\epsilon_{648}^{mM} = 2$ when camphor is removed. This band may reflect the presence of some high spin component in the camphor-free enzyme at room temperature, or possible failure to remove the camphor completely. However, other camphor-free preparations exhibit this absorption band (Peterson, 1971); and low field EPR spectra (Tsai et al., 1970), as well as quantitation of the low spin EPR signal (Peterson, 1971), have shown the presence of high spin components at low temperatures.

Reduction of P-450_{cam} produces further changes in the MCD spectra (Fig. 2B) which do not admit a simple interpretation. The Soret region spectra taken in the presence and absence of camphor are quite similar, indicating that, unlike the case for the oxidized protein, camphor binding causes no major changes in the properties of the heme group. In addition, we observed no differences in the CD spectra upon addition of camphor, by contrast with the slight differences reported by Peterson (1971). For the camphor-free enzyme the latter author found an absorption shoulder at 443 nm which was not seen in this work or that of others (Gunsalus et al., 1972). We determined the ratio A_{409}/A_{450} to be 2.6 both in the presence and absence of camphor. The MCD band shape does not resemble that reported for any other ferrohemoproteins. This might reflect an unusual coordination of the iron, such as by cysteine, in the reduced protein.

Inspection of the visible region curves reveals pronounced differences in the shape and intensity of the MCD of the reduced enzyme with and without camphor (Fig. 2B). The origin of the changes is not clear, but they may arise from the presence of some denatured form of the enzyme in the absence of camphor. It is noteworthy that the visible absorption spectrum of the camphor-free P-450_{cam} used in these experiments possessed a shoulder near 558 nm not observed in the camphor-complexed enzyme or by other workers (Gunsalus et al., 1972). Peterson (1971) observed a shoulder at longer wavelength which he attributed to a form of cytochrome P-420_{cam}, or denatured P-450_{cam}, and Yu and Gunsalus (1974) have prepared a form of P-420_{cam} possessing an α band at 556 nm by acetone treatment. Since typical low spin ferrohemoproteins exhibit an intense A term corresponding to a sharp α absorption band (see, for example, Fig. 1), the sharp component in the visible MCD of reduced camphor-free P-450_{cam} may represent a contaminant

with an α band close to 558 nm. Evidence for the presence of some form of cytochrome P-420$_{cam}$ in this preparation was obtained when CO was added to the enzyme from which camphor had been removed (see below and Fig. 2C). In addition, absorption spectra of the CO complex showed a small peak at 422 nm on the side of the main 446 nm band, which was reflected by a weak "S"-shaped curve in CD spectrum similar to that reported by Peterson (1971). The lack of a characteristic sharp α absorption band and intense Faraday A term in the reduced enzyme (Fig. 2B) may be a reflection of a high spin, out of plane iron in the heme group. This has also been suggested on the basis of room temperature nuclear magnetic resonance (Keller et al., 1972) and low temperature Mössbauer (Sharrock et al., 1973) studies on reduced P-450$_{cam}$.

The MCD of the reduced CO complex of P-450$_{cam}$ has distinct A terms at 448 nm and 422 nm associated with the Soret peaks of P-450$_{cam}$ and P-420, respectively. These bands are independent of temperature as expected for diamagnetic, low-spin ferrous heme and are similar to the intense Soret A terms observed in carbonmonoxy myoglobin (Vickery, Nozawa and Sauer, in preparation) and carbonmonoxy hemoglobin (Kobayashi et al., 1970). The δ, or N, absorption band occurs near 360 nm in carbonmonoxy P-450$_{cam}$ (Peterson, 1971) and probably represents a transition from a lower energy porphyrin π orbital (b_{2u}) to the same x-y degenerate $e_g^*(\pi)$ orbital excited state as the Soret transition. We therefore expect an A term for this band, but reduced in magnitude relative to the Soret MCD since the δ band is weaker and broader. The 345 nm peak and 377 nm trough may represent an A term associated with the δ band and superimposed upon a broader, negative MCD.

The visible region spectra are more difficult to interpret. The absorption spectrum of carbonmonoxy P-450$_{cam}$ does not exhibit the α and β bands seen in other CO-hemoproteins and the MCD lacks the distinct A term associated with the α band of carbonmonoxy myoglobin (Bolard and Garnier, 1972, and Vickery, Nozawa and Sauer, in preparation) and hemoglobin (Kobayashi et al., 1970). Since the intensity of the Faraday A term is proportional to the inverse square of the bandwidth, a broadened or weakened α band could account for the small amplitude of the A term observed near 566 nm. The features of the MCD spectra of P-450$_{cam}$ are summarized in Table I.

Microsomes--The MCD spectra in the near UV and visible spectral regions are shown for a microsomal preparation isolated from a DMBA-treated rat in Fig. 3, with characteristic wavelengths listed in Table II. The specific aryl hydrocarbon hydroxylase enzyme activity for this preparation was 1.4 nmoles/mg protein x 30 minutes, representing a 1.73-fold increase in specific activity over that of control rats. There was no increase in the amount of P-450 detectable by absorption difference spectroscopy. This is in agreement with the results of Nebert et al. (1972), who observed that on induction of aryl hydrocarbon hydroxylase with 3-methylcholanthrene there was no increase in overall P-450 concentration as determined by the method of Omura and Sato (1964). They did, however, detect an alteration in the type of P-450 as deduced from the spin state equilibrium (as measured by n-octylamine titration). The MCD spectra of the DMBA-induced microsomes were indistinguishable from those of the control microsomes, and only the former will be discussed.

In the absence of added reductants we assumed all components to be in a predominantly oxidized state since the terminal electron acceptor,

cytochrome P-450, is air-oxidizable. In order to separate the contributions of cytochrome b_5 and cytochrome P-450 to the overall MCD spectra, we used a stepwise reduction and complex formation procedure. NADH was introduced to reduce cytochrome b_5 followed by dithionite and carbon monoxide addition to reduce and complex cytochrome P-450.

The MCD spectrum of the oxidized microsomes has an S-shaped band in the Soret region (Fig. 3A and Table II). This band contains C term contributions primarily from low spin ferricytochrome b_5 (compare with Fig. 1 and Table I) and cytochrome P-450 (compare with Fig. 2A and Table I). The spectrum between 450 and 550 nm shows no distinct features; the trough at 580 nm probably corresponds to a low-spin or substrate-free form of cytochrome P-450 (compare Fig. 2A).

The addition of NADH reduces cytochrome b_5 as evidenced by the loss of intensity of the broad derivative-shaped band in the Soret region and the appearance of the narrow derivative-shaped A term in the visible region (Fig. 3B). The small, positive extremum near 426 nm probably corresponds to the peak seen in the MCD of purified ferrocytochrome b_5 (Fig. 1). The A term characteristic wavelengths of the α band MCD are also identical for the soluble and particulate reduced cytochrome (compare Table II with ferrocytochrome b_5, Table I). In addition, the fine structure in the β band MCD at 511, 520 and 527 nm are apparent in both (compare Fig. 1 and Table I with Fig. 3B, C and D). From the intensity of the α band A term and the loss of the Soret MCD upon NADH addition, the liver microsomal cytochrome b_5 concentration can be estimated to be 0.6 to 0.7 μM, or approximately 0.8 μmoles/mg protein. This is in good agreement with the value of 0.7 μM calculated from the microsomal difference absorption

spectrum (Fig. 4) with ε^{mM} for reduced minus oxidized cytochrome b_5 of 17 at 557 nm and 110 at 424 nm as determined for the purified cytochrome. The similarities in the MCD spectral properties may indicate both that the heme group in rat cytochrome b_5 is in an environment very similar to that of the pig (Fig. 1) and calf (Vickery and Morgan, 1974) proteins, and that removal of the cytochrome from the membrane does not result in changes in the heme chromophore.

The residual S-shaped C term MCD in the Soret region (Fig. 3B) has a peak and crossover near 410 and 418 nm, respectively, as observed for oxidized P-450_{cam}. Although contributions from ferrocytochrome b_5 overlap considerably, the concentration of microsomal P-450 can be estimated to be about 1 μM in this preparation assuming that the Soret MCD of the rat enzyme is similar to that of the bacterial enzyme. Comparison of the intensity of the 580 nm trough in the two systems, however, yields a microsomal P-450 concentration closer to 2 μM. The disagreement of the two methods of calculation may arise from differences between the rat enzyme and the bacterial model or, possibly, from the presence of P-420.

When dithionite is added, the S-shaped Soret MCD is transformed into a band shape resembling high-spin ferrous P-450_{cam} (Fig. 3C and Table I). The trough at 580 nm is also lost. A small increase in the amount of reduced cytochrome b_5 also seems to occur as judged from the intensity of the α band A term. Noteworthy is the lack of an intense positive MCD near 434 nm, where deoxy hemoglobin exhibits a strong peak (Djerassi et al., 1971; Kobayashi et al., 1973). Contamination of microsomal preparations with hemoglobin can be readily detected in this manner, as well as by observation of the α band A term of oxyhemoglobin centered at 575 nm.

The addition of carbon monoxide to the fully reduced sample causes further changes in the P-450 MCD but should not affect cytochrome b_5. The A terms with crossovers at 452 and 421 nm (Fig. 3D) probably correspond to P-450 and P-420, respectively. If microsomal carbonmonoxy P-450 and P-420 are assumed to have Soret A terms equal in intensity to that of $P\text{-}450_{cam}$, then the concentration of P-450 and P-420 each appears to be about 0.5 μM. Analysis of the absorption difference spectrum for the reduced carbonmonoxy minus reduced microsomes shown in Fig. 5, on the other hand, yields a P-450 concentration of 0.6 μM, with the amount of P-420 being considerably lower. Thus the shape and the intensity of the Soret MCD of microsomal carbonmonoxy P-450 is similar to that of carbonmonoxy $P\text{-}450_{cam}$. It also appears that the Soret MCD of microsomal carbonmonoxy P-420 must be more intense than that of carb onmonoxy P-450. However, measurement of the levels of P-420 by MCD are complicated both by overlap of the Soret spectrum with that of reduced cytochrome b_5 and by the lack of a suitable model system, and difference absorption spectroscopy tends to underestimate the amount of P-420 (Fujita et al., 1973). As was found for oxidized P-450, a higher estimate of the amount of the hemoprotein of approximately 2 μM is obtained by comparison of the 580 nm MCD trough in the microsome spectra with that of $P\text{-}450_{cam}$, indicating a difference between the MCD intensity of the microsomal and bacterial enzymes.

The results shown in Figs. 3-5 were obtained at room temperature using a microscomal preparation which had been subjected to freezing and thawing prior to treatment. All spectra were stable during the course of the experiments and gave no evidence of time-dependent denaturations. Essentially identical MCD spectra were also observed when freshly prepared microsomes were utilized and all experiments were carried out at 4°. Under

these conditions as well, no effect of treatment of the rats with DMBA could be determined from the MCD spectra.

Conclusions--These studies illustrate the utility of MCD as a sensitive optical probe for the investigation of membrane-bound hemoproteins in particulate systems. The MCD spectra of oxidized and reduced cytochromes b_5, P-450 and P-420 can be clearly identified in the crude rat liver microsome spectra, and these cytochromes seem to account for all of the major spectral features observed. There did not appear to be any contributions to the MCD spectra from other microsomal components or from extraneous hemoproteins.

The effect of treatment with the carcinogen, DMBA, on these cytochromes was investigated by comparison of the MCD spectra of microsomes isolated from treated and control rats. Administration of polycyclic hydrocarbons is generally observed to cause the induction of a new type of P-450 hemoprotein referred to as P_1-450 by Sladek and Mannering (1966) or as P-448 by Alvares et al. (1967) because of its blue shifted CO-difference spectrum. No differences could be detected, however, between the MCD spectra of the induced and control preparations. This may simply reflect a failure of DMBA to induce sufficient quantities of new P-450 rather than an insensitivity of the MCD spectra to changes in the hemoprotein, since no changes were observed in absorption difference spectra either. The greater cytotoxicity of DMBA relative to other polycyclic hydrocarbons such as 3-methylcholanthrene (D. W. Nebert, personal communication) may have prevented the induction of sufficient P-448 type hemoprotein for a difference to be detected spectrophotometrically, although an increase of 70% in enzyme activity was observed. The use of higher magnetic fields to increase the MCD signal intensity as well as further signal averaging in

spectral regions where changes are anticipated should prove useful for detecting small changes in the spectra.

We have also compared the properties of the membrane-bound cytochromes with those of the related solubilized cytochromes in order to determine whether the isolated proteins retain the same heme structure as the bound forms. Cytochrome b_5 is an amphipathic protein consisting of a hydrophylic "head" group and a hydrophobic "tail" region; the heme-containing head portion is cleaved from the tail in the course of enzymatic extractions, but the intact molecule can be isolated with detergents (Ito and Sato, 1968; Spatz and Strittmatter, 1971). The protease and detergent solubilized cytochromes b_5 have essentially identical absorption spectra (Ho and Sato, 1968; Spatz and Strittmatter, 1971), redox potentials (Ito and Sato, 1968), and CD spectra in the Soret region (Okada and Okunuki, 1973), indicating that the tail peptide has little effect on the protein conformation surrounding the heme group. The nonpolar tail, however, is responsible for binding of cytochrome b_5 to the microsomal membrane (Strittmatter et al., 1973; Enomoto and Sato, 1973). This suggests that the polar, heme-containing portion may be oriented away from the membrane lipid matrix at the membrane surface (Strittmatter et al., 1972). If the head group of the cytochrome was embedded within the membrane of the endoplasmic reticulum, it might be expected that its removal and transfer to an aqueous environment would cause conformational changes in the protein which would lead to altered properties of the heme group. The MCD results obtained for the membrane-bound oxidized and reduced cytochrome b_5 are essentially identical to those of the solubilized protein with respect to both wavelength positions and ellipticities. This is consistent with the

proposed localization of the catalytic portion of cytochrome b_5 at the membrane surface, but could also simply reflect the stability of the hemoprotein. Comparison of the EPR signal near g=3 of the soluble pig (Bois-Poltoratsky and Ehrenberg, 1967; Ikeda et al., 1974) and calf cytochromes (Bearden and Vickery, unpublished) with the microsomal cytochromes b_5 of pig (Ehrenberg and Bois-Poltoratsky, 1968) and rat (Stern et al., 1973) also suggest that at least the low spin oxidized component present in the membrane is largely unaffected by extraction. Nebert and Kon (1973), however, have attributed to cytochrome b_5 an EPR signal observed in mouse liver microsomes shifted to significantly lower field from the cytochromes of other species. Studies on the effect of incorporation of a detergent solubilized cytochrome b_5 into artificial phosphatidyl choline vesicles did not reveal any changes in the properties of the hemoprotein, and also indicated that the heme portion of the molecule was exposed to an aqueous environment (Sullivan and Holloway, 1973).

The soluble bacterial cytochrome P-450_{cam} has been used as a model for mammalian cytochrome P-450: absorption spectra of P-450_{cam} are very similar to those of microscomal P-450 (Katagiri et al., 1968), and the EPR spectra of P-450_{cam} (Gunsalus, 1968; Tsai et al., 1970; Peterson, 1971) resemble both the low spin (Mason et al., 1965) and high spin (Jeffcoate and Gaylor, 1969) signals seen in microsomes. Attempts to analyze the contribution of P-450 to the MCD spectra of the microsomes, however, suggest that significant differences exist between the relative ellipticities in the Soret and visible regions of microsomal P-450 and P-450_{cam}, although the presence of P-420 and residual bound DMBA may complicate the interpretation of these results. The origin of the apparent increased MCD intensity

in the visible of microsomal P-450 compared to P-450_{cam} is not yet clear. We are currently investigating this difference as well as the effects of solubilization and substrate binding on the MCD of the microsomal aryl hydrocarbon hydroxylase.

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TABLE I: MCD Spectra of Pig Liver Cytochrome b_5 and *P. putida* $P\text{-}450_{cam}$

Sample	Soret Absorption λ_{max}(nm)	Soret MCD λ(nm)	Soret MCD $\Delta\varepsilon/H$ $(M\cdot cm\cdot T)^{-1}$	Visible Absorption λ_{max}(nm)	Visible MCD λ(nm)	Visible MCD $\Delta\varepsilon/H$ $(M\cdot cm\cdot T)^{-1}$
Ferri-cytochrome b_5	413	406	68	532	553	9
		413	0	(562)	562	0
		419	88		570	-14
Ferro-cytochrome b_5	423	415	-9	557	553	185
		426	20		557	0
		438	-6		561	-185
Ferri-$P\text{-}450_{cam}$	417	410	28	537	560	7
		418	0	570	568	0
		427	-26	645	579	-13
					645	0
					659	1
+ camphor	391	378	4	510	531	2
		397	-18	(540)	540	0
		416	5	648	555	-5
					645	0
					657	-3
Ferro-$P\text{-}450_{cam}$	409	422	-23	543	586	-11
+ camphor	410	423	-32	544	583	-5
Ferro-$P\text{-}450_{cam}$ + CO	447	442	63	551	530	6
		448	0		566	0
		455	-43		581	-12

TABLE II: MCD Spectral Characteristics of DMBA-induced Rat Liver Microsomes

Additions	Soret		Visible	
	λ(nm)	$\Delta A/H$ $(cm \cdot T)^{-1}$	λ(nm)	$\Delta A/H$ $(cm \cdot T)^{-1}$
None	407	82	560	13
	413	0	573	0
	421	-80	580	-35
NADH	410	28	553	117
	417	0	557	0
			561	-100
			580	-33
NADH + dithionite	422	-20	553	127
			557	0
			561	-113
NADH + dithionite + CO	414	30	553	123
	421	0	557	0
	425	-15	561	-108
	444	20	580	-22
	453	0		
	460	-10		

FIGURE LEGENDS

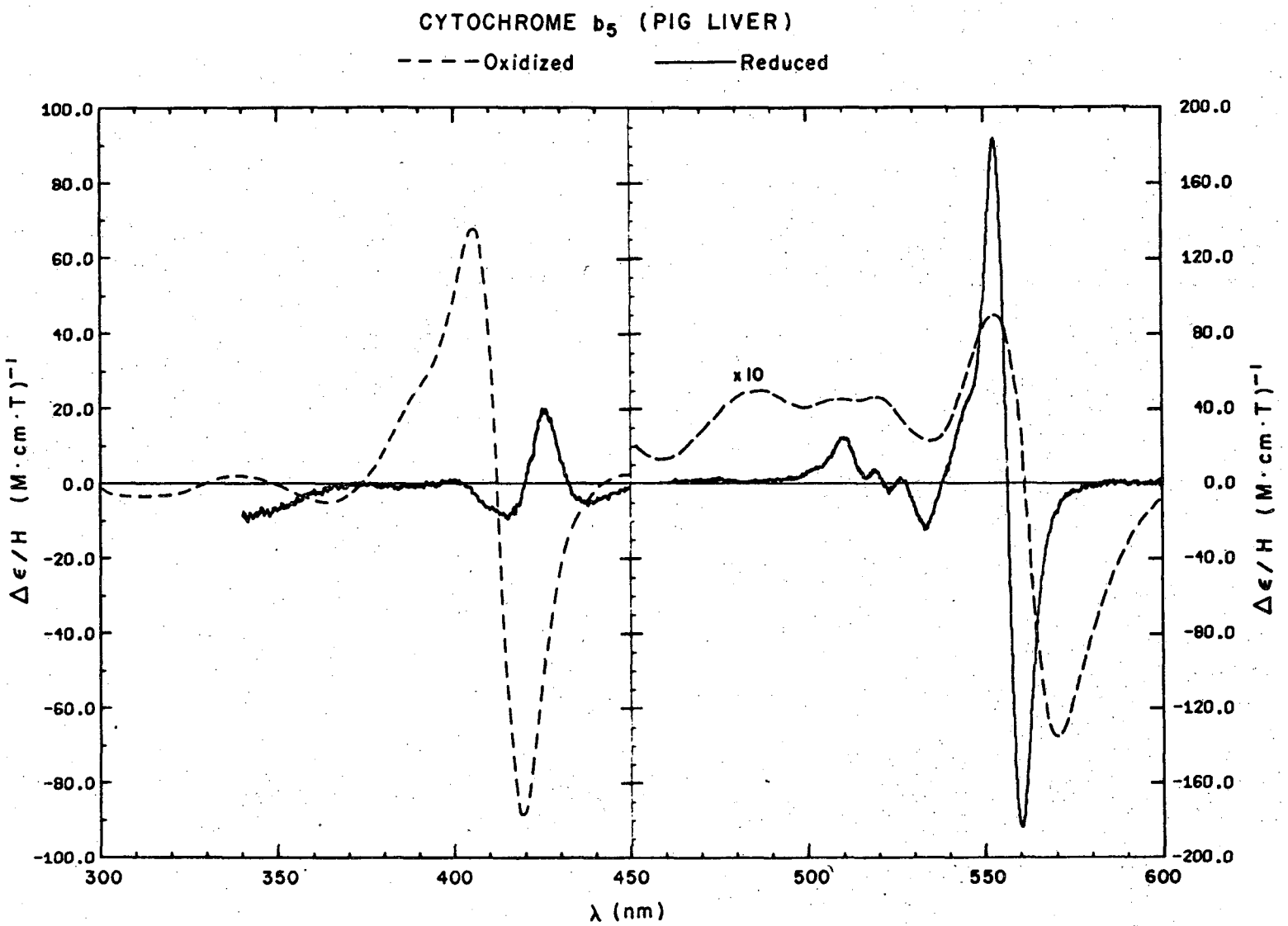
FIG. 1. MCD spectra of pig liver cytochrome b_5. Solvent: 0.1 M sodium phosphate, pH 7.5; (- - -) trace of computer-drawn curve for oxidized form; (——) computer plot of data for dithionite-reduced form; room temperature. Note that the spectrum of ferricytochrome b_5 in the 450-600 nm region has been multiplied by 10.

FIG. 2. MCD spectra of P. putida cytochrome P-450(cam). Solvent: 0.1 M sodium phosphate, pH 7.0; (- - -) in the presence of excess D-camphor; (——) camphor removed; room temperature.

FIG. 3. MCD spectra of DMBA-induced rat liver microsomes. Microsomes were suspended at a protein concentration of 0.8 mg/ml in 0.05 M Tris-HCl, 0.25 M sucrose, pH 7.5, and spectra recorded at ambient temperature (near 22°).

FIG. 4. Difference absorption spectra of NADH-treated versus untreated DMBA-induced rat liver microsomes. Sample and conditions as Fig. 3.

FIG. 5. Difference absorption spectra of dithionite-CO-treated versus dithionite-treated DMBA-induced rat liver microsomes. Sample and conditions as Fig. 3.



XBL735-4802 A

Fig. 1.

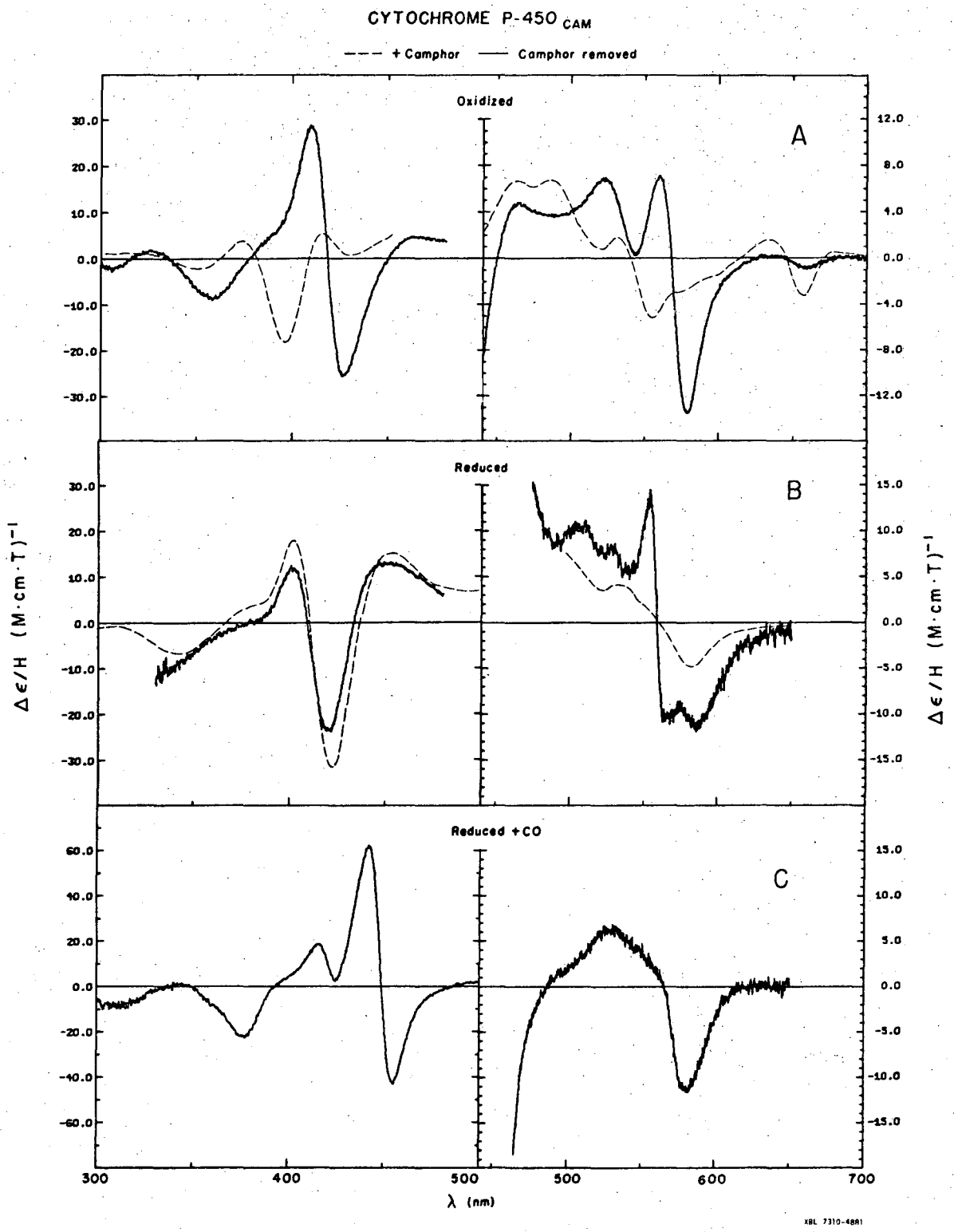

CYTOCHROME P-450 CAM
+ Camphor
Camphor removed
Oxidized
A
Reduced
B
Reduced +CO
C
Δε/H (M·cm·T)⁻¹
λ (nm)
XBL 7310-4881

Fig. 2.

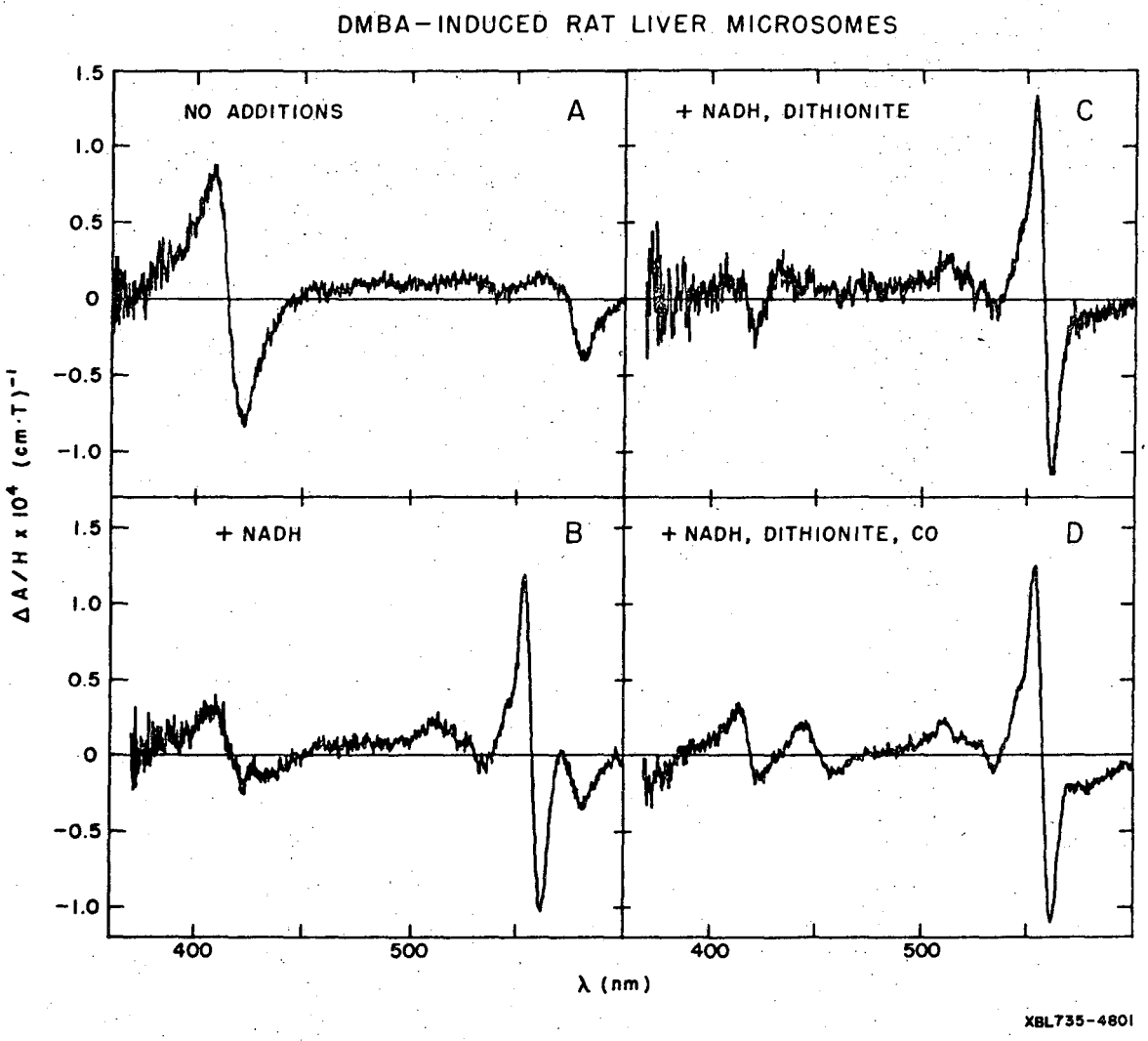
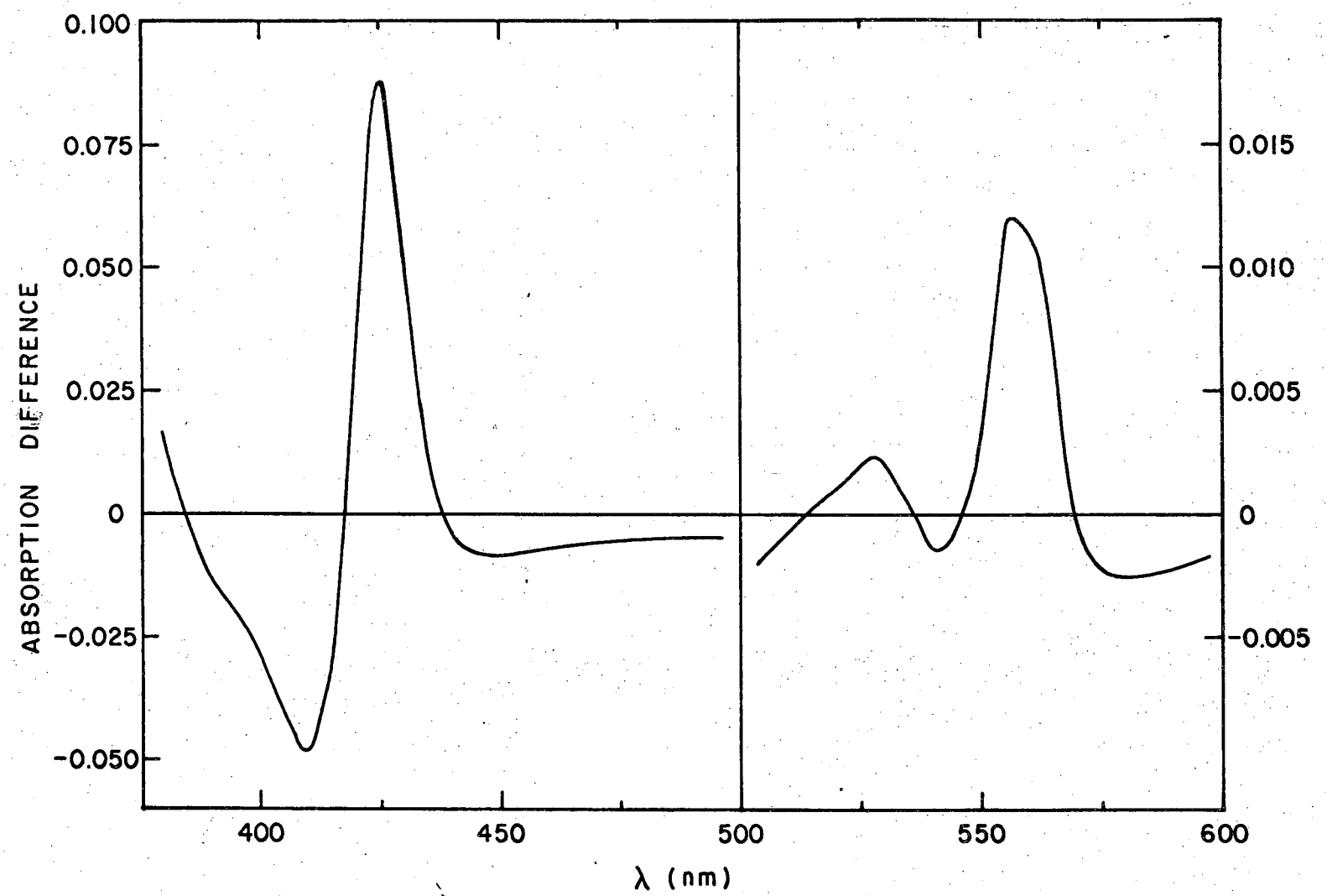

DMBA-INDUCED RAT LIVER MICROSOMES
NO ADDITIONS
A
+ NADH, DITHIONITE
C
+ NADH
B
+ NADH, DITHIONITE, CO
D
1.5
1.0
0.5
0
-0.5
-1.0
ΔA/H x 10⁴ (cm·T)⁻¹
400
500
λ (nm)
XBL735-4801

Fig. 3.



XBL7312-4979

Fig. 4.

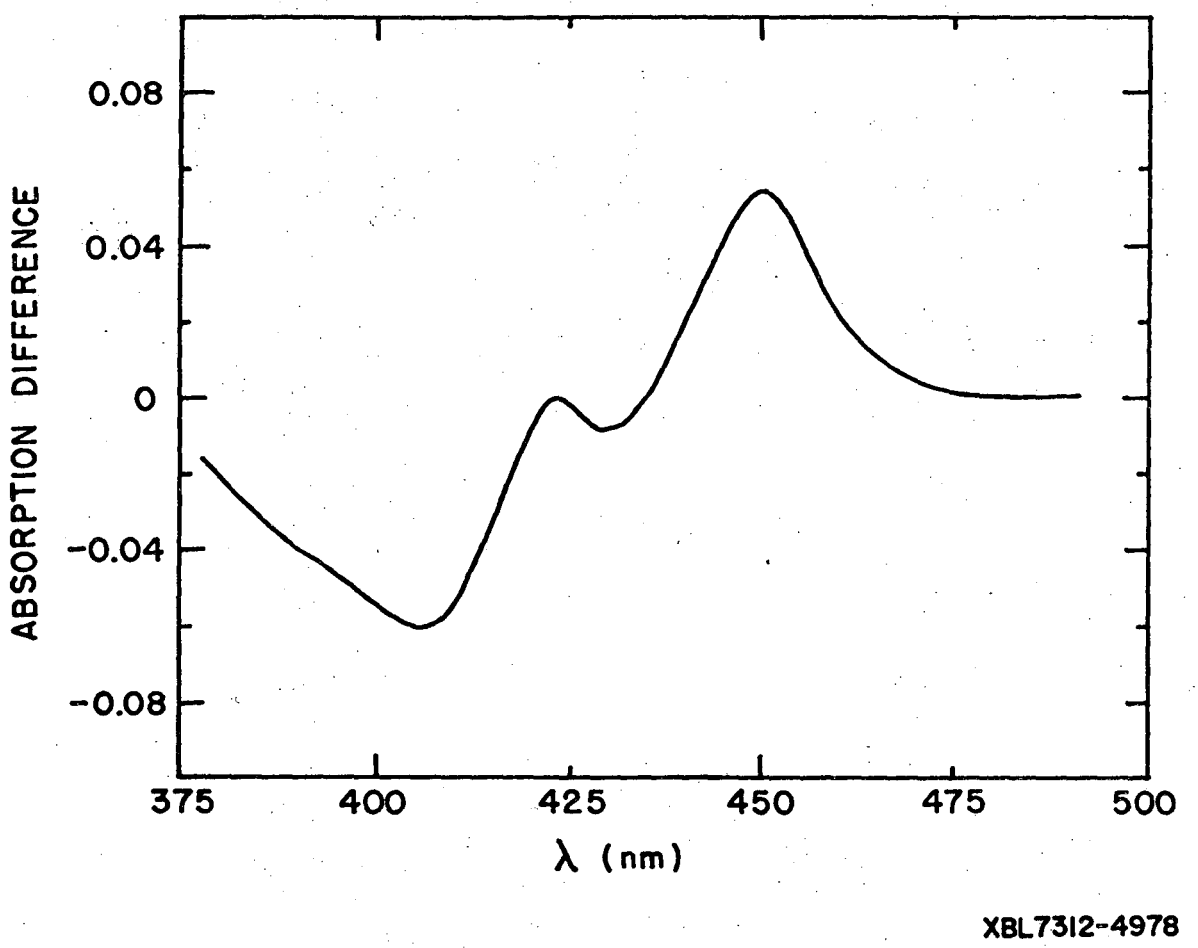

0.08
0.04
0
-0.04
-0.08
ABSORPTION DIFFERENCE
375
400
425
450
475
500
λ (nm)
XBL7312-4978

Fig. 5.

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