Spectrophotometric Evidence for a Hemoprotein in Fish Muscle Microsomes: Possible Involvement in Trimethylamine N-Oxide (TMAO) Demethylase Activity

Kirk L. Parkin[*][1] and Herbert O. Hultin

When fish muscle microsomes were reduced with sodium dithionite, characteristic absorption peaks appeared at 444 nm and very faintly at 560 nm. These peaks could also be generated by the presence of some of the components that are required for catalyzing microsomal enzyme-mediated trimethylamine N-oxide (TMAO) demethylation. Under certain conditions, the absorbance peaks underwent reoxidation in the presence of these cofactor components and TMAO. The reducible moiety was identified as protoheme by virtue of its absorption maxima at 557, 523, and 416 nm following solubilization in acid–acetone and reaction with pyridine, and it is proposed that it be named H-444 due to its unique Soret band. Reaction of the microsomes with CO after reduction with dithionite produced a Soret band shift from 444 to 428 nm. The hemin content of the fish muscle microsomes was 0.76 pmol mg^{-1} protein, some 3 orders of magnitude less than that observed in hepatic microsomes. This microsomal hemoprotein may possibly be involved in TMAO demethylation.

Biological demethylation reactions serve two major purposes in nature. In animals, enzymic demethylation systems in hepatic tissues are responsible for detoxifying xenobiotic compounds (Chambers and Yarbrough, 1976; Coon and Persson, 1980). The hepatic microsomal system is characterized by a complex organization of electron-transfer components, including cytochromes and flavoproteins (Estabrook, 1978). Cytochrome P-450 is a primary catalytic unit in these microsomal demethylation reactions (Coon and Persson, 1980; Schwen and Mannering, 1982). In methylotrophic microorganisms, demethylation reactions allow for assimilation of methylated amine carbon sources as C_1 fragments (Large, 1971; Colby and Zatman, 1973). Depending on the carbon source, C_1 assimilation requires a single- or multiple-enzyme system, including trimethylamine N-oxide (TMAO) demethylase (Colby and Zatman, 1975). The enzyme purified from *Bacillus* PM6 requires the presence of several cofactors but not other proteins for activity and is apparently not membrane associated (Myers, 1971; Myers and Zatman, 1971).

Reports of an enzyme catalyzing the demethylation of TMAO in fish have been concerned with activity in liver or kidney tissue (Harada, 1975; Gill and Paulson, 1982) or in a crude extract from muscle (Phillippy, 1983). The practical implications of this demethylation reaction is that the so-formed formaldehyde may react with muscle proteins and contribute to the freeze-induced textural toughening often observed in muscle of gadoid fishes (Castell et al., 1973; Gill et al., 1979). We have recently reported the presence of TMAO demethylase activity in a crude microsomal fraction of red hake (*Urophycis chuss*) muscle (Parkin and Hultin, 1982) and have characterized some of the basic requirements for, and the kinetic parameters of, TMAO demethylation by this enzyme system (Parkin and Hultin, 1986a). The active system is membrane associated, is stimulated by FMN, NADH, ascorbate, cysteine, or the nonphysiological redox components methylene blue and phenazine methosulfate, functions

through a cycling mechanism when iron is utilized as a cofactor, and is inhibited by azide and cyanide. This suggests that an electron-transfer mechansim is involved with a heme moiety essential to activity. In this report, we provide evidence for a membrane-associated hemoprotein in fish muscle microsomes that may have a role in TMAO demethylation.

MATERIALS AND METHODS

Fish. Fresh red hake were procured from day boats in Gloucester, MA. The fish were immediately transported to the laboratory on ice, filleted, and stored at –80 °C until use.

Chemical Reagents. Trimethylamine N-oxide (TMAO) and riboflavin were obtained from Eastman. Phenylmethylsulfonyl fluoride (PMSF), flavin mononucleotide (FMN), bovine serum albumin (BSA) fraction V, and type IX glucose oxidase (GOx) were purchased from Sigma. Grade I reduced nicotinamide adenine dinucleotide (NADH) was a product of Boehringer-Mannheim. Carbon monoxide (98–99% pure) and nitrogen were produced by Matheson. All other reagents were reagent grade or the best grade available commercially.

Microsomes. Crude muscle microsomes were prepared from fish as previously described (Parkin and Hultin, 1986a). Basically, this fraction was prepared from muscle that was minced and then homogenized in buffer (containing mercaptoethanol, EDTA, and PMSF) by a Brinkmann Polytron homogenizer. The fraction sedimenting between 21460- and $142100g_{max}$ initially and following a wash step (in 0.6 M KCl) was taken as the crude microsomal fraction.

Protein. Protein in the microsomal preparations was determined by a modified Lowry procedure (Markwell et al., 1978) using BSA as a standard.

Flavin Analysis. Flavin constituents in the microsomal fraction were analyzed by the riboflavin fluorescence method (Yagi, 1962; Cerletti and Giordano, 1971; Koziol, 1971). Microsomes were diluted to 10 mg of protein in 3 mL and were mixed with 3 mL of 20% TCA. After homogenization by 10 strokes in a Potter–Elvejhem tissue homogenizer, the mixture was cleared by centrifugation for 20 min at $2000g_{max}$. An aliquot was brought to pH 6.6 with 4 M K_2HPO_4 while another aliquot was incubated overnight at 40 °C to hydrolyze FAD to FMN prior to neutralization. All fluorescence measurements were per-

Fish Muscle Microsomal TMAO Demethylation

J. Agric. Food Chem., Vol. 35, No. 1, 1987   **35**
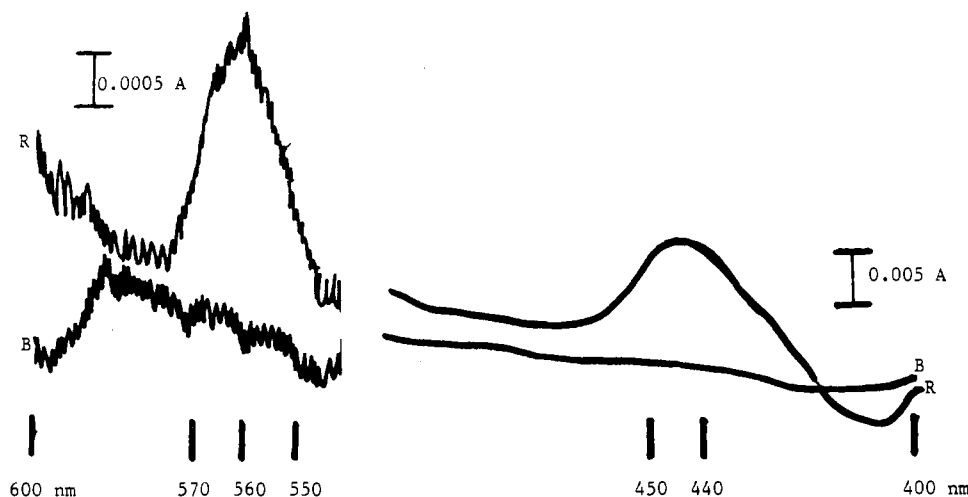
Figure 1. Dithionite reduction of microsomes. B = base line of equal light absorbance; R = scan following addition of crystalline sodium dithionite. Microsomal protein was 0.46 mg mL^{-1} in sample and reference.

formed with a Turner Model 111 fluorometer with wavelengths of excitation and emission of 445 and 560 nm, respectively.

Hemin Analysis. Hemin analysis of the microsomes was accomplished by the pyridine hemochrome method (Drabkin, 1942) on the residue following TCA extraction for flavin analysis. Hemin content was calculated on the basis of a molar extinction coefficient of 133×10^6 cm^2 mol^{-1} at 420 nm. The nature of the hemoprotein components of the microsomes was characterized by the procedure of Sakaguchi and Kawai (1978). Forty-four milligrams of microsomal protein were brought to 2 mL with water and homogenized in 10 mL of 4% HCl in acetone by five strokes in a Potter–Elvehjem homogenizer. Following centrifugation for 20 min at $2000g_{max}$, the cleared supernatant fraction was evaporated to <1 mL under nitrogen. The volume was brought to 1.5 mL with water, and 1.5 mL of pyridine and 0.3 mL of 1 M KOH were added. Following homogenization by five strokes in a Potter–Elvehjem homogenizer, samples were recentrifuged for 10 min. For the cleared supernatant fraction, a base line of equal light absorbance was obtained for this fraction in the reference and sample cuvettes between 600 and 400 nm. Immediately after reduction of the sample contents by dithionite, the spectrum between 600 and 400 nm was recorded again. The insoluble (residue) fraction resulting from acid–acetone extraction was suspended in 3 mL of 50% pyridine and 0.3 mL of 1 M KOH. Subsequent treatment was identical with the steps employed for the acid–acetone-soluble fraction.

Spectrophotometry. Spectrophotometric analysis was conducted on a Perkin-Elmer Model 556 spectrophotometer coupled with a Hewlett-Packard Model 7045A X-Y recorder. Microsomal protein was diluted to 0.50–1.38 mg mL^{-1} in 0.12 M NaCl, 25 mM histidine, pH 7.0, and equal portions were added to sample and reference cuvettes. Following the establishment of a base line of equal light absorbance, the effect on membrane spectral characteristics by the addition of various components was examined. With the observation that the rate of change in membrane absorbance spectra induced by the various components was on the order of minutes, repetitive scans were performed for each reaction. Scans were routinely performed from 500 to 400 nm and required about 50 s to complete. The spectrophotometer was calibrated with a solution of FMN in 0.1 M phosphate, pH 7.0, yielding an absorbance maximum of 445 nm (Dawson et al., 1969; Koziol, 1971). Unless otherwise noted, all samples contained microsomal

protein in 0.12 M NaCl and 25 mM histidine, pH 7.0. When included, glucose was added at 13 mM and GOx at 9 U mL^{-1} in all cases.

RESULTS

Spectrophotometric Characterization of Muscle Microsomes. The absolute absorption spectrum of red hake microsomes exhibited no obvious peaks or troughs over the range of 400–700 nm. When the crude microsomal suspension was reduced with crystalline sodium dithionite, a component in the membrane displayed a major absorbance peak at 444 nm and a minor peak at 560 nm, indicative of a heme functional group (Figure 1). Since the peak at 560 nm could only be detected at maximal sensitivity with the accompanying instrument noise, observations for the effects of various components and/or conditions on the oxidation–reduction of this component were routinely monitored at 444 nm.

Components involved in, or have an influence on, TMAO demethylation by the muscle microsomes were evaluated for their ability to promote changes in the spectral properties of the membrane suspensions. Some of these components absorb in the range of 400–500 nm: NADH (<430 nm); FMN (A_{max} 445–450 nm); FAD (A_{max} 450 nm); glucose oxidase (400–430 nm); FeCl$_3$ (at concentrations >50 μM). In the case of FMN, FAD and glucose oxidase, these components, when used, were added to both the sample and reference cuvettes unless otherwise noted. Interference of microsomal spectral characteristics by the presence of NADH was inconsequential since the wavelengths at which NADH absorbs did not obscure the observations concerning changes brought about (at 444 nm) in the microsomal suspensions. Concentrations of FeCl$_3$ in the range of 50–200 μM caused a smoothly rising base line of light absorbance as scans were performed from 500 to 400 nm. However, this did not obscure observations concerning spectral changes in the microsomes, and when base-line compensation was required, FeCl$_3$ was added to both sample and reference cuvettes. The presence of azide (anaerobically) or KCN induced peak formation at 432 nm when GOx was present; when these components were included with the sample microsomes, this interference was compensated for by the addition of like components to the reference cuvette.

Several conditions promoted reduction of the microsomal component absorbing at 444 nm (Table I). Some of these conditions allowed TMAO demethylase activity (in the presence of TMAO) while others did not [cf. Parkin
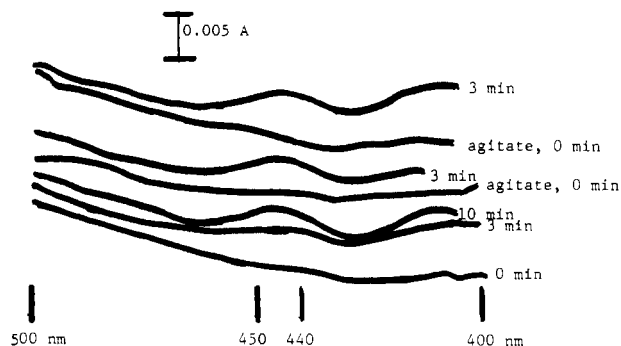
Figure 2. Anaerobic peak formation by 10 mM ascorbate and effect of agitation on peak stability. Sample and reference contained GOx and 0.62 mg of microsomal protein mL⁻¹. Sample also contained glucose. Agitation was performed by stirring contents with glass rod.

and Hultin (1982, 1986a)]. For example, NADH anaerobically, combinations of $FeCl_2$ or $FeCl_3$ and ascorbate and/or cysteine, and FMN and NADH under anaerobic conditions induced microsomal peak formation. These combinations are also capable of stimulating microsomal demethylase activity in the presence of TMAO. On the other hand, 10 mM ascorbate or 10 mM cysteine anaerobically, $FeCl_2$ and NADH, or 10 mM each of ascorbate and cysteine was capable of inducing peak formation yet do not support TMAO demethylation. Conversely, under anaerobic conditions, FMN did not induce microsomal peak formation, yet this has been shown to stimulate TMAO demethylation by the microsomes.

Peak formation that occurred under anaerobic conditions decayed upon mechanical agitation (stirring contents of cuvettes with a glass rod), presumably due to the incorporation of oxygen (Figure 2). Upon subsequent incubation, the peak would re-form to its original height.

In the absence of NADH and/or anaerobic conditions, reduction of the microsomal component by ascorbate and/or cysteine (in most cases) required iron (Table I). The only exception was that 10 mM each of ascorbate and cysteine prompted slight peak formation. Either $FeCl_2$ or $FeCl_3$ was effective at 2 μM in the presence of 2 mM each of ascorbate and cysteine; under these conditions, $FeCl_2$ achieved a faster reduction of the microsomal component than did $FeCl_3$. The presence or absence of TMAO had little, if any, influence on the reduction of the microsomal component under the conditions examined. A more definitive account of the influence of TMAO is presented in the following section.

Adding dithionite after incubation of the microsomes with FMN and NADH anaerobically or with iron, cysteine,
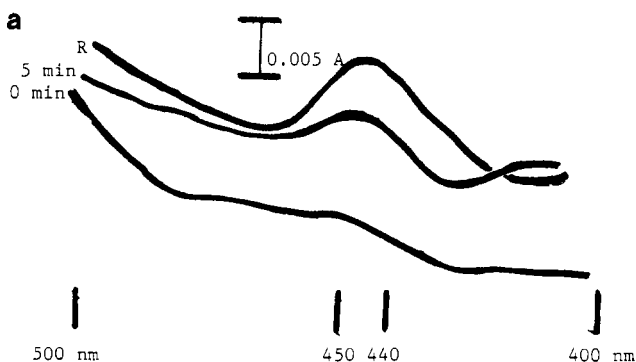
Table I. Effect of Various Combinations of Components on Microsomal Peak Formation at 444 nm[a]

| components	min req for peak formn	rel peak intens
anaerobiosis	10–15	slight
anaerobiosis, 400 μM NADH	1–3	moderate
anaerobiosis, 2–200 μM NAD(P)H, 0.67–67 μM FMN or FAD	1–3	strong
anaerobiosis, 10 μM $FeCl_2$	0–10	moderate
anaerobiosis, 10 mM ascorbate or cysteine	0–5	moderate
anaerobiosis, 100–200 μM $FeCl_3$	no peak within 10 min	
200 μM NADH[b]	no peak within 10 min	
100 μM NADH,[b] 1 μM FMN	no peak within 5 min	
anaerobiosis, 1 μM FMN	no peak within 15 min	
10 μM $FeCl_2$, 200 μM NADH	5–10	slight
10 μM $FeCl_2$ ($FeCl_3$), 2 mM ascorbate	5–10	moderate
10 μM $FeCl_2$ ($FeCl_3$), 2 mM cysteine ± 20 mM TMAO	1–5	moderate
2 μM $FeCl_2$, 2 mM ascorbate, 2 mM cysteine	5–10	strong
2 μM $FeCl_3$, 2 mM ascorbate, 2 mM cysteine	15–20	strong
10 mM ascorbate, 10 mM cysteine	10–15	slight
10 mM ascorbate or 10 mM cysteine	no peak within 10 min	
2 mM ascorbate, 2 mM cysteine ± 40 mM TMAO	no peak within 10 min	

[a] All incubations were conducted in 0.12 M NaCl, 25 mM histidine, pH 7.0, at ambient temperature (20–25 °C) and 0.46–0.73 mg of protein mL⁻¹ in sample and reference curvettes. Anaerobic conditions were obtained with 13 mM glucose and 9 U GO × mL⁻¹. NADH, NADPH, FAD, FMN, and GOx, when used, were added to both sample and reference curvettes. In the case of anaerobic conditions, glucose was added to sample curvette only. [b] NADH added to sample curvette only. Peak intensity was defined in relation to the peak induced in the microsomes by the addition of dithionite as follows: slight = 33%, moderate = 33–67%, and strong = 67–100% of the peak generated by dithionite, respectively.

and ascorbate enhanced peak formation (Figure 3a,b). In addition, after preincubation with iron, ascorbate, and cysteine, the addition of sodium dithionite produced a derby-shaped peak (Figure 3b).

Effect of TMAO on the Spectra of the Cofactor-Reduced Microsomal Hemoprotein. The spectral scans shown in Figures 4 and 5 describe the effect of TMAO on oxidation–reduction of the microsomal component. Under anaerobic conditions, FMN (0.67 μM) and NADH (2 μM) caused reduction of the microsomal component (Figure 4a). Up to 20 min was required for maximal reduction, and the reduced component was stable for at least an additional 20 min in the absence of TMAO. When TMAO was present, the time course of microsomal reduction (Figure 4b) was similar to that in the absence of TMAO. However,
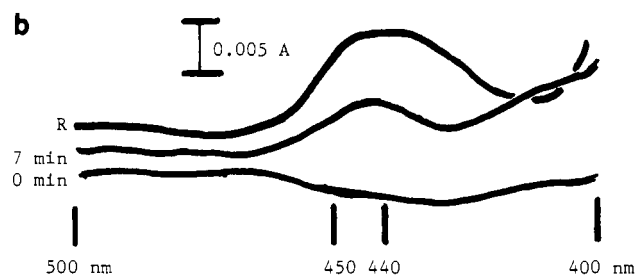
Figure 3. Effect of dithionite on the spectra of microsomal membranes following preincubation of microsomes with FMN and NADH [(a) sample and reference contained 200 μM NADH, 1 μM FMN, and GOx; sample also contained glucose] and ascorbate, cysteine, and iron [(b) sample and reference contained 2 mM ascorbate and 2 mM cysteine; sample also contained 2.5 μM $FeCl_2$]. R = addition of crystalline sodium dithionite. Sample and reference cuvettes contained 0.46 mg of microsomal protein mL⁻¹.

Fish Muscle Microsomal TMAO Demethylation
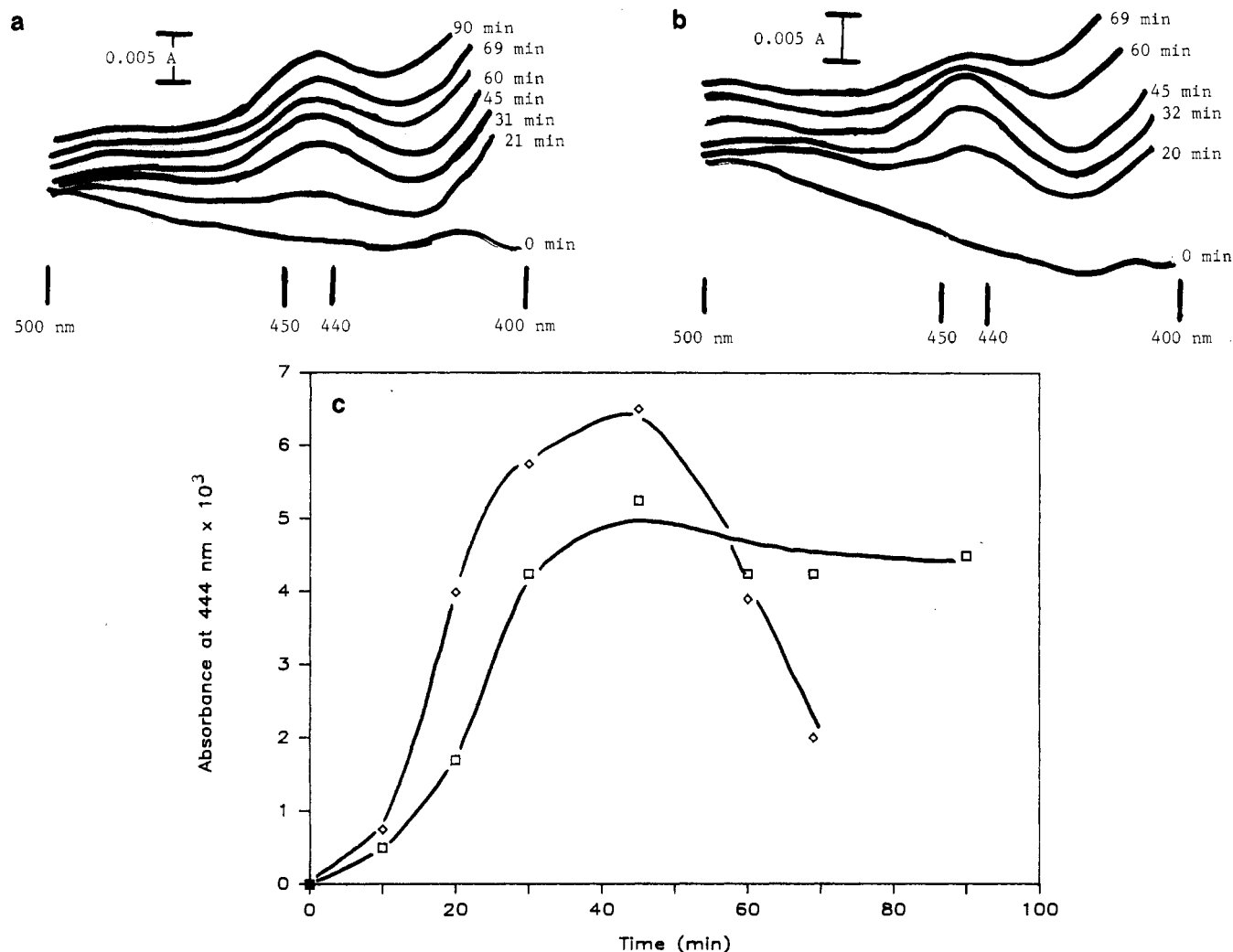
J. Agric. Food Chem., Vol. 35, No. 1, 1987   37
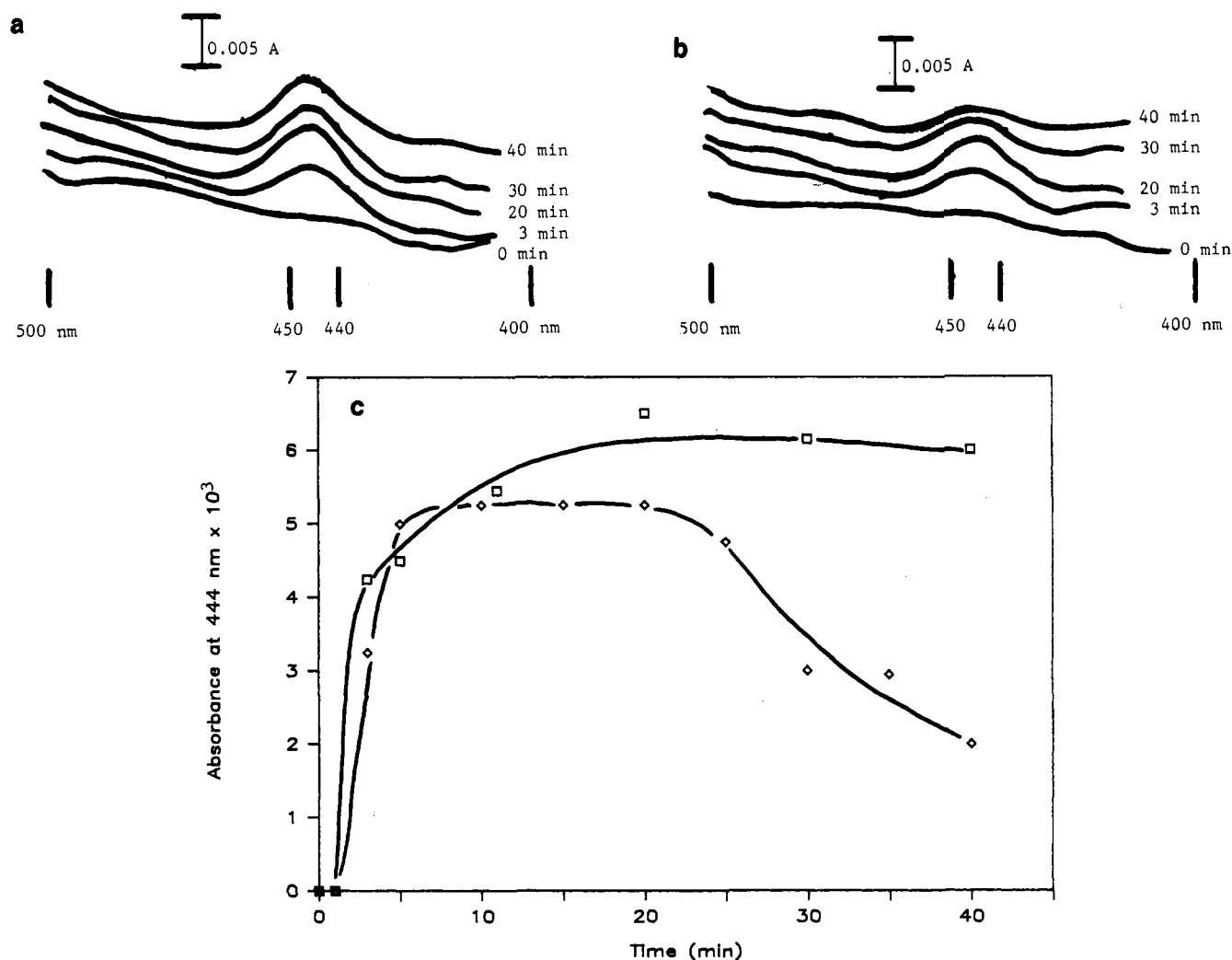
Figure 4. Changes in peak height of the microsomal component absorbing at 444 nm as affected by FMN, NADH, and TMAO. (a,b) Sample and reference contained 0.67 μM FMN, 2 μM NADH, GOx, and 0.73 mg of microsomal protein mL^{-1}; sample also contained glucose and, in (b), 40 mM TMAO. (c) Time course of changes in absorbance at 444 nm in the absence ($\square$) and presence ($\lozenge$) of TMAO.

a reoxidation of the reduced component began within 10 min after maximal reduction and continued for at least another 20 min (about 60% reoxidized at the end of the 40-min incubation). The time course of reduction and reoxidation under these conditions is graphically described in Figure 4c. The choices of concentrations of FMN and NADH were critical in allowing reoxidation of the microsomal component by TMAO to be observed. At 1 μM FMN and 4 μM NADH, similar observations were made in the presence and absence of TMAO, except that only 10 min was required for maximal reduction of the microsomal component. Reoxidation (in the presence of TMAO) did not begin until about 35 min hence. At 0.5 μM FMN and 1 μM NADH, only minimal reduction of the microsomal component was observed, preventing any conclusive evaluation of the effect of TMAO under these conditions. Under the conditions where the effect of TMAO could be evaluated (as in Figure 4), TMAO had to be incorporated into the membrane suspension at the beginning of the incubation (as opposed to its addition following reduction of the microsomal component). This was necessary since it was observed that simple agitation of the sample contents with a stirring rod following reduction lead to peak decay with no subsequent reformation (at these cofactor concentrations), even in the absence of TMAO.

When microsomes were incubated in the presence of 2 μM FeCl$_2$ and 1 mM cysteine (Figure 5a), maximal reduction of the microsomes was completed in about 30–45

min. The reduced component was stable for at least an additional 60 min. Under the same conditions in the presence of TMAO, maximal reduction again took place in 30–45 min (Figure 5b). However, the reduced component became substantially reoxidized after an additional 15–30 min. A direct comparison of the time course of reduction and reoxidation under these conditions is shown in Figure 5c. As was noted for the FMN–NADH system, the ability to observe the effect of TMAO on the microsomal component reduced by cysteine and FeCl$_2$ was critically dependent on the concentrations of these latter two components. At 0.5 mM cysteine and 2 μM FeCl$_2$, peak formation was minimal over the course of 45 min, preventing any conclusive evaluation of the effect of TMAO under these conditions. On the other hand, at 2 mM cysteine and 2.5 μM FeCl$_2$, maximal peak formation was observed within 20 min, but no peak decay was recorded for at least a 50-min period following, in either the presence or absence of 40 mM TMAO.

When microsomes were incubated anaerobically with 0.67 μM FMN and 2 μM NADH in the presence of 1 mM azide, no reduction of the microsomal component was apparent for at least 20 min (scan not shown). In the absence of azide, maximal reduction of the membrane component was achieved in 20 min (Figure 4a,b). Azide had no effect on the rate of microsomal reduction by 10 μM FeCl$_2$ and 2 mM cysteine in the presence or absence of oxygen (scans not shown).

Figure 5. Changes in peak height of the microsomal component absorbing at 444 nm as affected by $FeCl_2$, cysteine, and TMAO. (a,b) Sample contained 2 μM $FeCl_2$ and 1 mM cysteine in the absence (a) and presence (b) of 40 mM TMAO. (c) Sample and reference also contained 0.70 mg of microsomal protein mL^{-1}. (c) Time course of changes in absorbance at 444 nm in the absence ($\square$) and presence ($\lozenge$) of TMAO.
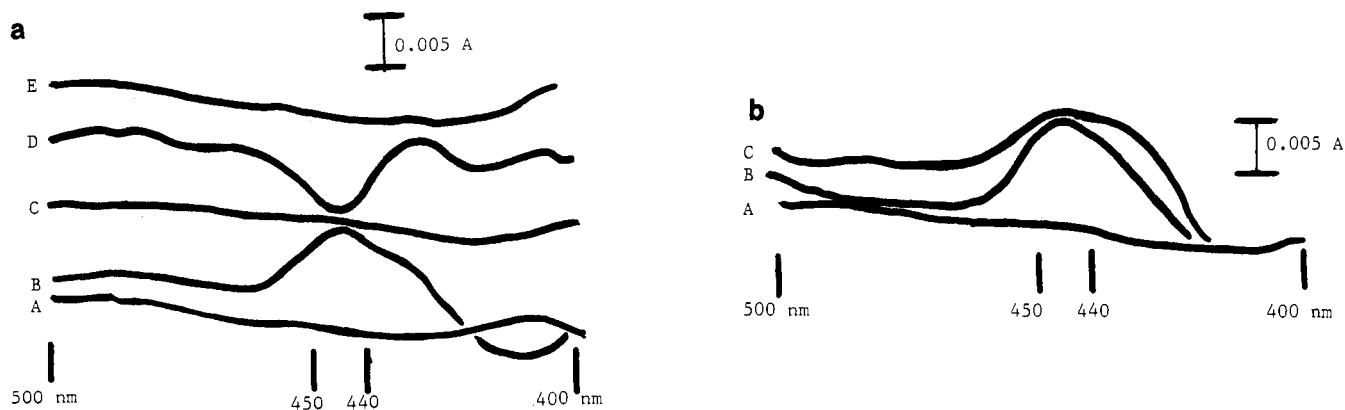
Figure 6. Effect of CO and KCN on microsomal membranes reduced with sodium dithionite. (a) Sample and reference contained 0.73 mg of microsomal protein mL^{-1} where A = base line, B = reduction of sample with dithionite, C = reduction of reference with dithionite, D = treatment of sample with CO, and E = treatment of reference with CO. (b) Sample and reference contained 0.46 mg of microsomal protein mL^{-1} where A = base line, B = reduction of sample with dithionite, and C = treatment of sample with 1 mM KCN.

Characterization of the Reducible Microsomal Component as Protoheme. The observation that the microsomal component exhibits absorption peaks at 560 and 444 nm upon reduction by dithionite would lead to its presumptive identification as a hemoprotein. An absorption peak corresponding to the β band was not detected.

The presence of CO had an effect on the spectral characteristics of membranes reduced by dithionite (Figure 6a). Dithionite reduction of the sample cuvette contents resulted in the formation of a peak at 444 nm with a small shoulder at 430 nm. When the reference cuvette contents were reduced by dithionite, the original base line of equal light absorbance was reestablished. Purging CO into the

Fish Muscle Microsomal TMAO Demethylation

J. Agric. Food Chem., Vol. 35, No. 1, 1987   39
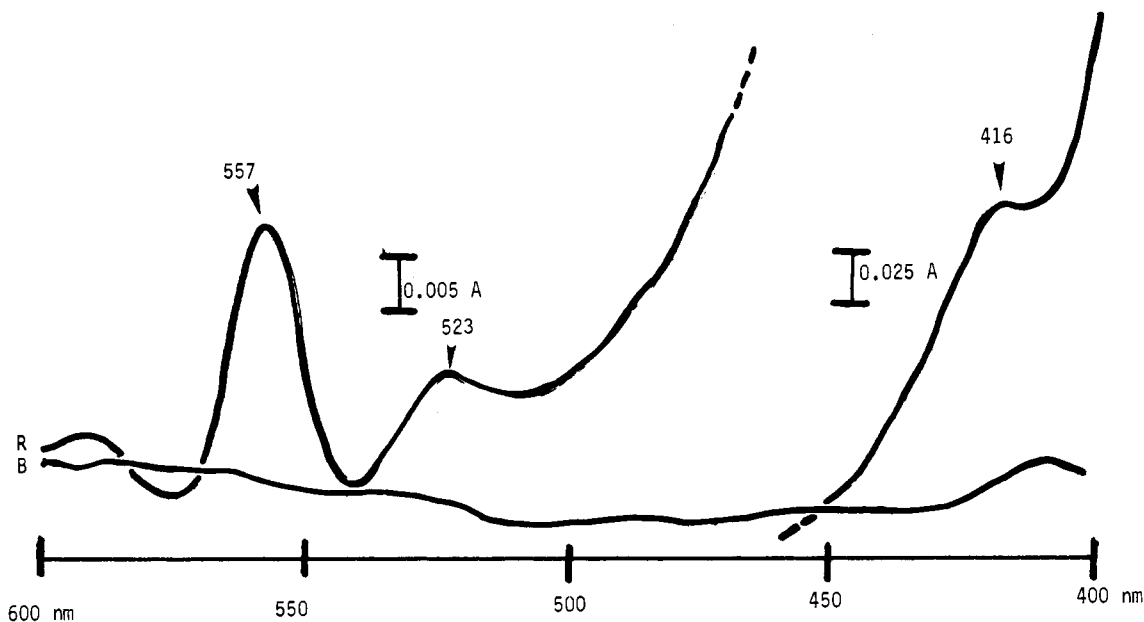
Figure 7. Reduced pyridine spectrum of the acid–acetone-soluble fraction of 44 mg of microsomal protein. B = base line of equal light absorbance, and R = spectrum following reduction of sample by crystalline dithionite.

reduced sample contents resulted in peak formation at 428 nm with no apparent shoulder and a trough at 444 nm. The base line became reestablished by the addition of CO to the reduced reference sample.

Cyanide (KCN) also induced spectral changes in the microsomes. After reduction of the sample by dithionite, the addition of KCN caused the formation of a derby-shaped peak (Figure 6b). Cyanide and CO had little effect on the spectral characteristics of microsomal suspensions reduced by (preincubated with) NADH and FMN, or ascorbate, iron, and cysteine (scans not shown). It was only in the presence of dithionite that these agents (KCN, CO) exerted their influence on the spectral parameters of the microsomes.

Heme analysis of the microsomes was accomplished by preparing acid–acetone-soluble and acid–acetone-insoluble fractions and subsequent formation of pyridine hemochrome derivatives. Most of the heme components were acid–acetone soluble, and this fraction appeared to be fairly pure in a moiety displaying absorbance maxima of 557, 523, and 416 nm (Figure 7). The presence of another heme component in this fraction is possible since a smaller absorbance maximum was observed at 590 nm. A minor membrane heme component was detected in the acid–acetone-insoluble fraction with absorbance maxima of 558, 553, and 419 nm (scan not shown).

Hemin and Flavin Contents of Hake Muscle Microsomes. The average content of hemin constituents was 0.76 pmol mg^{-1} protein (range of 0.54–0.98 for two membrane preparations assayed in duplicate). The flavin content was about 92 pmol mg^{-1} protein (range of 53–130 for four membrane preparations assayed in duplicate) and FAD was present in greater concentration than FMN (mean of 73% FAD, range of 54–92% of total flavin; mean of 27% FMN, range of 8–46% of total flavin).

DISCUSSION

The reducible muscle microsomal component exhibited an α band of 560 nm typical of protoheme (Smith, 1978; von Jagow and Sebald, 1980). Confirmation of this was provided by the reduced pyridine hemochrome spectrum of the acid–acetone-soluble preparation. An α band for the pyridine hemochrome derivative of 557 nm falls within the range (556–558 nm) for protohemin (I.U.B. Enzyme

Commission, 1979). The three absorbance maxima corresponded closely with that of protoheme from bacteria (Sperry and Wilkins, 1976) and hepatic H-450 (Kim and Deal, 1976). Procedures for identification and classification of hemoproteins are primarily based on the nature of the α peak, although it is acknowledged that difficulties may arise when identification is made in a complex environment such as a membrane system. Classification of the fish muscle microsomal hemoprotein on the basis of the α band would also obscure its distinguishing feature, namely its Soret band of 444 nm. To our knowledge, a Soret band of 444 nm has not previously been reported for any other hemoprotein (Smith, 1978; von Jagow and Sebald, 1980). This hemoprotein is not cytochrome P-450, since a spectral shift for the reduced heme upon combination with CO was to 428 and not 450 nm. Thus, we propose that this hemoprotein be classified as fish muscle microsomal H-444; a precedent for the use of this type of nomenclature has been established (Kim and Deal, 1976).

That the principal heme component identified by the pyridine hemochrome derivatives is identical with that absorbing at 444 nm for the dithionite-reduced microsomal suspensions is confirmed by several observations. The principal pyridine hemochrome was determined to be protoheme, and the absorbing component in the microsomal suspensions had an α band of 560 nm, also characteristic of the protoheme configuration (Smith, 1978; I.U.B. Enzyme Commission, 1979; von Jagow and Sebald, 1980). Both the pyridine hemochrome and dithionite-reduced absorbance spectra indicated only one major heme component in the microsomes. From Figure 6a and using the 121 mM^{-1} cm^{-1} extinction for the Soret band of reduced deoxymyoglobin (a protoheme constituent; Wood, 1984) for the sake of calculation, the dithionite-reduced component yielded a Soret band absorbance of 0.0065, equivalent to 0.74 pmol mg^{-1} microsomal protein. This agrees well with the 0.76 pmol mg^{-1} protein calculated from the pyridine hemochrome spectrum.

The level of heme in the fish muscle microsomes averaged 0.76 pmol mg^{-1} protein. This is 3 orders of magnitude less than the 0.4–0.7 nmol of cytochrome mg^{-1} of protein existing in hepatic microsomes (Estabrook and Cohen, 1969; Ziegler et al., 1969) and the 0.29 nmol of cytochrome mg^{-1} of protein in sarcoplasmic reticulum from slow-twitch

40 *J. Agric. Food Chem.,* Vol. 35, No. 1, 1987

Parkin and Hultin

rabbit muscle (Salviati et al., 1981). However, the level of heme in fish muscle microsomes is similar to the 0.8 pmol of hemin mg^{-1} of protein reported for chicken breast muscle microsomes (Player and Hultin, 1977).

Research in this laboratory has demonstrated the presence of two discreet cofactor systems involved in TMAO demethylation in cytosolic (Phillippy, 1983) and microsomal (Parkin and Hultin, 1982, 1986a) fractions from red hake muscle. There is a question of whether these cofactor systems share a common catalytic unit or whether they are specific for different proteins or enzymes catalyzing a common reaction. The research described in this report may shed some light on this question in that evidence is provided that both sets of cofactors could reduce microsomal H-444. In vitro reduction of H-444 could be accomplished at low concentrations of cofactors (0.67 μM FMN, 2 μM NADH; 2 μM FeCl$_2$, 1 mM cysteine) compared to what is needed to demethylate TMAO (5 μM FMN, 400 μM NADH; 100 μM FeCl$_2$, 1 mM cysteine) at detectable rates (ca. at least 0.05 μmol of DMA formed mg^{-1} of protein per h). Therefore, in vitro, the reduction of microsmal H-444 appears to require lower concentrations of these components than does TMAO demethylation; maximal reduction of H-444 was observed at lower cofactor concentrations than was maximal TMAO demthylation. The cofactor concentrations required to reduce H-444 are in the range found in fresh red hake muscle in situ (Phillippy, 1983). Previous evidence for a common catalytic unit shared by these cofactor systems comes from observations that (1) similar degrees of purification of TMAO demethylase activity take place for each of these systems in the course of subcellular fractionation and partial purification by detergents, (2) the cofactor systems are competitive, and (3) similarities are shared between these cofactor systems with respect to inhibitor sensitivities (Parkin and Hultin, 1982, 1986a,b).

That the reduced microsomal component may be involved in TMAO demethylation was supported by the observation that, under certain conditions, the presence of TMAO was associated with a reoxidation of this component regardless of which cofactor system was responsible for originally reducing it. From our experiments it was not possible to discern whether TMAO was directly responsible for H-444 reoxidation or some other component was responsible for electron withdrawal from the reduced heme. It was noted that several conditions capable of reducing H-444 could not support TMAO demethylase activity. On the other hand, with the single exception of anaerobic incubation of FMN and membranes, all conditions examined that were capable of promoting demethylase activity were also capable of reducing H-444. Therefore, a good correlation was found between demethylase-supporting capability and H-444 reducing capacity for the cofactor mixtures evaluated. However, H-444 reduction and TMAO demethylation did not have identical requirements, and it cannot be concluded that H-444 plays a role in demethylation. If it does, then FMN likely functions at a site other than H-444. If TMAO demethylation takes place by an electron-transfer mechanism as previously suggested (Harada, 1975), then H-444, if it is involved in the microsomal system, may be only one of several proteins involved in the overall process. In addition, its role in the membrane may not be confined to this process alone. H-444 reoxidation by TMAO can only be observed under conditions where peak formation (reduction) is slow (10–30 min) and the supply of reducing equivalents to the microsomal component either exhaustible or rate limiting. If H-444 reduction is involved in TMAO demethylation,

it appears not likely to be the rate-limiting step. The time needed for reoxidation of an *Escherichia coli* cytochrome involved in TMAO reduction to trimethylamine (Sakaguchi and Kawai, 1978) was similar to that for reduced microsomal H-444. The *E. coli* TMAO-reducing system is membranous and composed of *b*- and *c*-type cytochromes, and an electron-transfer pathway is responsible for TMAO reduction (Bragg and Hackett, 1983).

Some other interesting correlations were made between the ability of cofactor and inhibitor combinations to reduce H-444 and promote microsomal TMAO demethylase activity based on the results from this and previous studies (Parkin and Hultin, 1982, 1986a,b): (1) NADH and FMN each stimulate activity only under anaerobic conditions and act synergistically. NADH alone can reduce the membrane cytochrome only under anaerobic conditions, the rate and extent of reduction is enhanced by the presence of FMN or FAD. H-444 reduction and microsomal demethylase activity by these components is prevented by O$_2$. (2) Azide prevents activity and H-444 reduction by FMN plus NADH under anaerobic conditions. Under anaerobic conditions, TMAO demethylation by ascorbate, cysteine, and iron is not completely inhibited by azide (40%), and the rate of H-444 reduction is unaffected. In the presence of oxygen, azide has no effect on demethylase activity and H-444 reduction by either cofactor system. Therefore, azide probably binds to H-444 only under anaerobic conditions. (3) Combinations of iron, cysteine, and/or ascorbate reduced H-444 and also supported demethylase activity in the presence of TMAO. Iron functions catalytically through a cycling mechanism, with ascorbate and cysteine probably functioning as external reductants; in the presence of low levels of these latter components, H-444 reduction in the presence of Fe^{2+} is faster than Fe^{3+}, indicating that Fe^{2+} is the active form.

We also have evidence that might lead one to suggest that H-444 is not involved in TMAO demethylation by the microsomes. Following partial purification of activity with SDS (and other detergents; Parkin and Hultin, 1986b), H-444 could not be detected in either the resultant particulate or supernatant fraction. It is likely that this component becomes denatured by detergent treatment. It was noted that when SDS or Triton X-100 was added to a suspension of dithionite-reduced membranes, the peak at 444 nm instantaneously disappeared (SDS) or was diminished to 10% of its original height (Triton X-100). This autoxidizable nature in the presence of O$_2$ and/or detergent has also been noted for other hemoproteins, including hepatic H-450 (Kim and Deal, 1976) and P-450 (Omura and Sato, 1962). It may be possible that H-444 functions in the partially purified fraction, even in the denatured state, as it has been shown that nonenzymic heme-catalyzed demethylations take place at a rate of 1–2% of those enzyme catalyzed (Machinist et al., 1966). If H-444 is involved in TMAO demethylation and it is not rate limiting to the overall reaction (as indicated by our data), its presence in the denatured form may still be able to drive the reaction. On the other hand, it is possible that H-444 is not associated with microsomal TMAO demethylase activity. As a result of purification by detergents (Parkin and Hultin, 1986b) it may become solubilized and removed from the resultant particulate and purified fraction.

We noted other interesting spectral properties of fish muscle microsmal H-444. A derby-shaped peak appeared when H-444 was reduced in the presence of cyanide. A similar derby-shaped peak appeared when dithionite was added to the microsomes preincubated with iron, ascor-

Fish Muscle Microsomal TMAO Demethylation

J. Agric. Food Chem., Vol. 35, No. 1, 1987 **41**

bate, and cysteine. This effect of dithionite was not observed when its addition followed preincubation of the microsomes with FMN and NADH. These observations may indicate that although the two sets of cofactors may be capable of reducing H-444, their mechanisms may differ. The derby-shaped peak may imply the existence of two species of H-444 with the shape of the peak due to a contribution from each of the species. The species absorbing at the lower wavelength may be a liganded form of H-444; a spectral shift to lower wavelength was observed when the microsomes were incubated with KCN or CO in the presence of dithionite. Thus, there may be more than one functional species of H-444; if it participates in TMAO demethylation, this feature may be related to the difference between the cofactor systems with respect to their reaction requirements (e.g., dependency on oxygen tension). Multiple forms of cytochrome *b* are believed to account for its ability to accept electrons from a variety of sources (Wainio, 1970).

Registry No. TMAO, 1184-78-7; TMAO demethylase, 9076-66-8; hemin, 16009-13-5; protoheme, 14875-96-8.

LITERATURE CITED

Bragg, P. D.; Hackett, N. R. *Biochim. Biophys. Acta* **1983**, *725*, 168.
Castell, C. H.; Smith, B.; Dver, W. J. *J. Fish Res. Board Can.* **1973**, *30*, 1205.
Cerletti, P.; Giordano, M. G. *Methods Enzymol.* **1971**, *18B*, 285.
Chambers, J. E.; Yarbrough, J. D. *Comp. Biochem. Physiol., C: Comp. Pharmacol*, **1976**, *55C*, 77.
Colby, J.; Zatman, L. J. *Biochem. J.* **1973**, 132, 101.
Colby, J.; Zatman, L. J. *Biochem. J.* **1975**, 148, 513.
Coon, M. J.; Persson, A. V. In *Enzymatic Basis of Detoxification*; Jakoby, W. B., Ed.; Academc: New York, 1980; Vol. 1, p 117.
Dawson, R. M. C.; Elliot, D. C., Elliot, W. H., Jones, K. M., Eds. In *Data for Biochemical Research*, 2nd ed.; Oxford University: London, 1969; p 200.
Drabkin, D. L. *J. Biol. Chem.* **1942**, *146*, 605.
Estabrook, R. W. *Methods Enzymol.* **1978**, *52*, 43.
Estabrook, R. W.; Cohen, B. In *Microsomes and Drug Oxidations*; Gillette, J. R., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., Mannering, G. J., Eds.; Academic: New York, 1969; p 95.
Gill, T. A.; Paulson, A. T. *Comp. Biochem. Physiol., B: Comp. Biochem.* **1982**, *71B*, 49.
Gill, T. A.; Keith, R. A.; Smith-Lall, B. *J. Food Sci.* **1979**, *44*, 661.

Harada, K. *J. Shimonoseki Univ. Fish.* **1975**, *23*, 161.
International Union of Biochemistry, Enzyme Commission In *Enzyme Nomenclature 1978*; Academic: New York, 1979; p 594.
Kim, I. C.; Deal, W. C. *Biochemistry* **1976**, *15*, 4925.
Koziol, J. In *Methods Enzymol.* **1971**, *18B*, 253.
Large, P. J. *Xenobiotica* **1971**, *1*, 457.
Machinist, J. M.; Orme-Johnson, W. H.; Ziegler, D. M. *Biochemistry* **1966**, *5*, 2939.
Markwell, M. A. K.; Hass, S. M.; Beiber, L. L.; Tolbert, N. E. *Anal. Biochem.* **1978**, *87*, 206.
Myers, P. A. Ph.D. Dissertation, University of Reading, 1971.
Myers, P. A.; Zatman, L. J. *Biochem. J.* **1971**, *121*, 10P.
Omura, T.; Sato, R. *J. Biol. Chem.* **1961**, *237*, PC1375.
Parkin, K. L.; Hultin, H. O. *FEBS Lett.* **1982**, *139*, 61.
Parkin, K. L.; Hultin, H. O. *J. Biochem. (Tokyo)* **1986a**, *100*, 87.
Parkin, K. L.; Hultin, H. O. *J. Biochem. (Tokyo)* **1986b**, *100*, 97.
Phillippy, B. Q. Ph.D. Dissertion, University of Massachusetts, 1983.
Player, T. J.; Hultin, H. O. *J. Food Biochem.* **1977**, *1*, 153.
Sakaguchi, M.; Kawai, A. *Bull. Jpn. Soc. Sci. Fish.* **1978**, *44*, 999.
Salviati, G.; Salvatori, S.; Betto, R.; Margreth, A. *Biochem. J.* **1981**, *197*, 515.
Schwen, R. J.; Mannering, G. J. *Comp. Biochem. Physiol., B: Comp. Biochem.* **1982**, *71B*, 431.
Smith, L. *Methods Enzymol.* **1978**, *53*, 202.
Sperry, J. F.; Wilkins, T. D. *J. Bacteriol.* **1976**, *125*, 905.
von Jagow, G.; Sebald, W. *Ann. Rev. Biochem.* **1980**, *46*, 281.
Wainio, W. W. In *The Mammalian Mitochondrial Respiratory Chain*; Academic: New York, 1970, p 238.
Wood, P. M. *Biochim. Biophys. Acta* **1984**, *768*, 293.
Yagi, K. In *Methods of Biochemical Analysis*; Glick, D., Ed.; Interscience: New York, 1962; Vol. 10, p 319.
Ziegler, D. M.; Mitchell, C. H.; Jollow, D. In *Microsomes and Drug Oxidations*; Gillette, J. R., Cosmides, G. J., Estabrook, R. W., Fouts, J. R., Mannering, G. J., Eds.; Academic: New York, 1969; p 173.