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Fluorometric assay of hepatic microsomal monooxygenases by use of 7-methoxyquinoline*

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Cytochrome P-450 monooxygenases constitute one of the most potent systems to deal with the metabolism of foreign substances. The hepatic microsomal monooxygenase system usually metabolizes nonpolar xenobiotics to more polar compounds. The monooxygenase system operating in hepatic microsomes is comprised of NADPH–cytochrome P-450 reductase and cytochrome P-450, flavo- and hemoproteins respectively. The central catalytic unit, cytochrome P-450, is subject to induction by a variety of chemicals which stimulate the biosynthesis of different forms of the cytochrome. Extensive reviews on the subject of chemical induction of cytochromes P-450 are available [1–3].

Many assays have been developed to determine the activities of the constitutive phenobarbitol (PB) and 3-methylcholanthrene (MC)-induced forms of the cytochrome P-450 monooxygenases. Only a few of these are direct assays, i.e. assays where enzymatic activity can be determined immediately. Fluorometric assays have been commonly utilized for the direct measurement of monooxygenases because of their sensitivity and the ability to select fluorophores that fluoresce in a wavelength range that obviates artifacts resulting from endogenous materials or other reaction components. Direct fluorescent assays include the O-dealkylation of coumarins (umbelliferones) [4–6], the O-dealkylation of phenoxazones (resorufins) [7–9] and the O-dealkylation of fluorescein [10].

While all of the above-mentioned substrates measure monooxygenase activity, two have been demonstrated to be specifically metabolized by the PB-induced (7-pentoxyresorufin) [9, 11] and MC-induced (7-ethoxyresorufin) [6, 8, 11, 12] monooxygenases. The reason for the high degree of metabolic specificity with the resorufin substrates is not immediately apparent. However, it is true that of the available fluorescent substrates the resorufins are the only ones that contain both oxygen and nitrogen atoms in the fluorophore ring system; the others contain oxygen atoms only. It would, therefore, be of interest to know if the presence of a nitrogen in the fluorophore ring system allows the specific metabolism of the substrate by certain cytochrome P-450 monooxygenases.

We have been investigating a series of 7-alkoxyquinolines to determine the effect of the nitrogen atom in the aromatic ring system on metabolic specificity. This report establishes 7-methoxyquinoline as a substrate for the assay of hepatic microsomal cytochrome P-450 monooxygenases. Synthesis, fluorescence characteristics, and comparison of metabolic activities with hepatic microsomal preparations from rats previously treated with MC and PB are presented.

Materials and Methods

Chemicals. NADPH, phenobarbital and Tris were from the Sigma Chemical Co. (St. Louis, MO). 3-Methylcholanthrene was purchased from Fluka AG (Buchs, Switzerland). 7-Quinolinol and iodomethane were obtained from Kodak Laboratory Chemicals (Rochester, NY).

Animals and sample preparation. Animals were obtained from a reproducing colony of Wistar rats at the Institute for Pharmacology and Toxicology, Marburg, FRG. Male rats of approximately 200 g were used in the experiments. Animals were induced with PB (0.1% in drinking water for 6 days) and MC (two i.p. injections of 30 mg/kg body weight, in peanut oil). Controls consisted of animals injected twice with 0.5 to 1.0 ml of peanut oil only. The animals were killed by cervical dislocation 2 days after the last 3-MC treatment. Hepatic tissues (two livers for each treatment group of rats) were homogenized in 4 vol. of 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl and 1 mM EDTA. The homogenates were centrifuged at 1000 *g* for 10 min and then the supernatant fractions were removed and centrifuged at 10,000 *g* for 10 min. The microsomal pellets were obtained by centrifuging the 10,000 *g* supernatant fractions at 100,000 *g* for 60 min, resuspending the resulting pellets in 20 mM Tris–HCl (pH 7.6, 150 mM KCl, 3 mM $MgCl_2$), and recentrifuging at 100,000 *g* for another 60 min. The microsomal pellets were finally suspended (1 ml resuspension buffer per g fresh liver weight) in 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl, 3 mM $MgCl_2$ and 15% glycerine.

Cytochrome P-450 concentrations were determined by

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the method of Omura and Sato [13] and protein concentrations were measured according to Lowry *et al.* [14].

Synthesis of 7-methoxyquinoline. Sodium metal (6.9 mmol) was added to a round-bottom flask containing 100 ml of absolute ethanol. The mixture was stirred at room temperature until the sodium metal was dissolved and then 7-quinolinol (6.9 mmol) was added. The flask was then fitted with a condenser, a nitrogen bubbler, and a heating mantle, and the mixture was heated to *ca.* 65° for 30 min under a nitrogen atmosphere. After cooling the mixture to room temperature, 2.6 ml of iodomethane (42 mmol) was added to the flask. The mixture was again heated to *ca.* 65° and was stirred overnight under nitrogen. The reaction mixture was cooled to room temperature and the ethanol removed via a rotary evaporator connected to a water aspirator. The resulting residue was dissolved in 400–500 ml of $CHCl_3$ and was subsequently washed with 0.1 M sodium bicarbonate until the aqueous layer contained no more blue fluorescence material. The organic layer was removed and dried by filtering through anhydrous sodium sulfate. The $CHCl_3$ was then removed by rotary evaporation. The crude product was dissolved in a minimum of $CHCl_3$ and streaked onto 1 mm thick preparative silica gel TLC plates (20 × 20 cm, Merck, Darmstadt, FRG). The TLC plates were developed in chambers with wicks containing $CHCl_3$:acetone (80/20). After the plates were developed they were viewed under UV light, and the violet fluorescing band ($R_f = 0.32$) was marked. The violet fluorescent band which contained the 7-methoxyquinoline was scraped from the plate and the product eluted with $CHCl_3$. After removing the $CHCl_3$ by evaporation, a golden brown oil, which was the product, remained in the flask. The product was subjected to TLC (analytical plates, 5 × 20 cm Merck) to determine purity. If needed, a second preparative TLC run was made. The oil was stored under an argon or nitrogen atmosphere until used. Typical yield was 20%. Structure was confirmed by mass spectral analysis using a Hewlett–Packard (Sunnyvale, CA) MSD 5970B mass spectrometer interfaced with a Hewlett–Packard GLC5890 gas chromatograph equipped with a 10 m OV-1 capillary column (Altech, Deerfield, IL). Conditions were as follows: flow rate, 1 ml He/min; 80°–300° linear temperature program over 50 min, 2 PSI head pressure; split was 40:1. Samples were injected in $CHCl_3$ and a single peak eluted after the solvent at 2.64 min. The fragmentation pattern was as follows: M^+ at 159 (100%); M^+-CH_2O at 129 (48%); M^+-C_2H_3O at 116 (71%); M^+-C_4H_6O at 89 (31%).

O-Demethylation of 7-methoxyquinoline assays. Either a Perkin–Elmer 204-A (Norwalk, CT) or a SLM-Aminco SPF 500C (Urbana, IL) spectrofluorometer was used in recording fluorescent spectra and the enzymatic production of 7-hydroxyquinoline (7-quinolinol). For enzyme assays, the excitation and emission wavelengths were 410 and 510 nm respectively. O-Demethylation of 7-methoxyquinoline was measured directly from reactions contained in the fluorometer cuvette. A typical reaction contained 2 ml buffer (20 mM Tris–HCl, pH 7.6, 150 mM KCl, 3 mM $MgCl_2$), 200–300 μg of microsomal protein, and 5–200 μM methoxyquinoline [added from a 20 mM stock solution made up in dimethyl sulfoxide (DMSO)]. The reactions were conducted at 25° and were initiated by addition of 500 nmol NADPH. The quantity of 7-quinolinol generated was determined by adding 5 μl of a 1 mM standard solution of 7-quinolinol in DMSO directly to the reaction mixture so that the reactions could be calibrated.

7-Methoxyquinoline interactions with cytochrome P-450. Difference spectra generated by the binding of 7-methoxyquinoline to oxidized microsomal cytochrome P-450 were recorded from 350 to 470 nm using an Aminco DW-2 UV-Vis spectrophotometer according to Schenkman *et al.* [15]. A tandem cuvette arrangement was utilized to obviate artifacts due to absorption of light by 7-methoxyquinoline [16].

Results and Discussion

Fluorescence properties. Uncorrected excitation and emission spectra of 7-methoxyquinoline and 7-quinolinol are shown in Fig. 1. 7-Methoxyquinoline had major excitation and emission maxima at 329 and 368 nm, respectively, in 50 mM potassium phosphate buffer at pH 7.4. The emission spectrum is broad, ranging from *ca.* 340 nm to *ca.* 540 nm with a shoulder at 420 nm. At the same conditions 7-quinolinol had excitation maxima at 330 and 398 nm. There is a single emission maximum located at 515 nm.

The fluorescence properties of the product, 7-quinolinol, were sufficiently different from those of the substrate and those of NADPH so that there is little or no interference from either these materials or from those aminoacids that may be present in the assay system. These properties make 7-methoxyquinoline a suitable substrate for the direct assay of hepatic microsomal monooxygenase systems.

Metabolism of 7-methoxyquinoline by rat hepatic microsomal monooxygenases. The reaction had different pH optima for different rat hepatic microsomal treatment groups; control microsomes had a pH optimum from 7.6 to 7.8, MC microsomes a pH optimum from 7.4 to 7.6, and PB microsomes a pH optimum from 7.9 to 8.1. The reaction was linear with regard to microsomal protein from 100 to 900 μg/ml reaction mixture (data not shown). Monovalent salt solutions such as KCl were slightly stimulatory (20–30%) up to 200 mM, after which they became increasingly inhibitory for the reaction. Addition of Mg^{2+} at 3 mM was slightly stimulatory, increasing the reaction rate by *ca.* 10–15% (data not shown). Therefore, for all of the following experiments, it was decided to use 20 mM Tris–HCl at pH 7.6 containing 150 mM KCl and 3 mM $MgCl_2$.

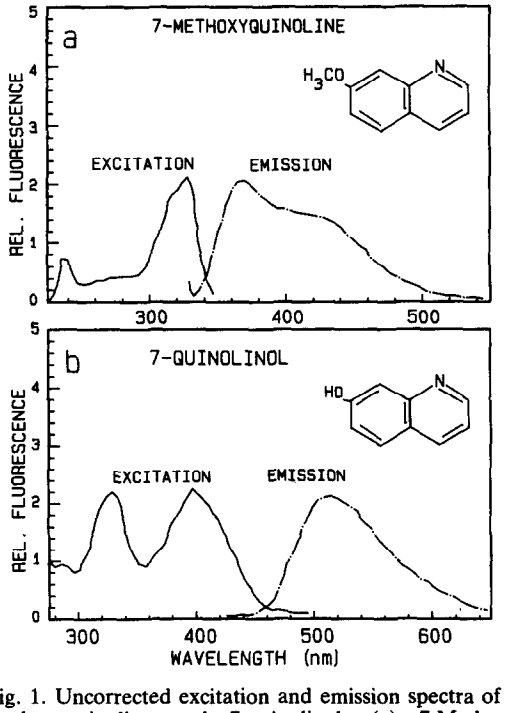

Fig. 1. Uncorrected excitation and emission spectra of 7-methoxyquinoline and 7-quinolinol. (a) 7-Methoxyquinoline (1 μM), emission spectrum recorded with the excitation set at 327 nm, excitation spectrum recorded with the emission set at 371 nm. (b) 7-Quinolinol (1 μM), emission spectrum recorded with the excitation set at 410 nm, excitation spectrum recorded with the emission set at 505 nm. Compounds were made up in 50 mM potassium phosphate buffer at pH 7.4.

Table 1 summarizes the effects of various inhibitors on the O-demethylation reaction with PB microsomes. It is clearly indicated that O_2 and NADPH were required for the reaction to occur, and that CO was inhibitory to the reaction. The addition of either metyrapone or hexobarbital inhibited the control, MC-induced, and PB-induced reactions (data not shown for control and MC).

The nature of the reaction product was determined by pooling the reaction mixtures from control, PB and MC microsome experiments (*ca.* 150 ml), and extracting the pooled material with ethyl acetate (50 ml). The ethyl acetate extract was then dried by vacuum rotary evaporation. The residue was dissolved in 1 ml of ethyl acetate and a small amount (5 μl) applied to a 0.2 mm thick silica gel 60 thin-layer chromatograph plate (Merck). Authentic samples of 7-methoxyquinoline and 7-quinolinol were also applied to the plate. The plate was developed in $CHCl_3$/acetone (80/20) and migration of the materials was determined under UV light. Only two spots were observed for the reaction mixture extract. These spots coincided with the 7-methoxyquinoline ($R_f = 0.32$) and 7-quinolinol ($R_f = 0$) standards, indicating that 7-quinolinol was the reaction product.

Kinetic measurements. Apparent kinetic constants and V_{max} measurements were calculated for the O-demethylation of 7-methoxyquinoline for the various microsomal treatment groups. Values were obtained via Lineweaver–Burk plots using linear regression analyses to determine the intersects. The mean values ± SD are presented in Table 2. The K_m for the control group at 70.9 ± 4.8 μM was 3- and 5-fold higher, respectively, than for the PB and MC treatment groups, indicating that PB and MC type monooxygenases have a greater binding preference for 7-methoxyquinoline than do control microsomes. The V_{max} values are given on a per mg protein and per nmole cytochrome P-450 basis for comparison purposes. The V_{max} values for the MC treatment groups were the lowest of the treatment groups tested regardless of how the V_{max} value was calculated. The highest V_{max} values were with microsomes obtained from PB treatment regardless of how the values were calculated, i.e. per nmol cytochrome P-450 or per mg protein. If specific activities [160, 167, and 552 pmol 7-quinolinol · min^{-1} · (mg protein)$^{-1}$, respectively, for control, MC and PB microsomes] are compared for saturating substrate concentrations, the PB microsomal metabolism was 3- to 3.5-fold greater than those for the control and MC groups. The increase in specific activity for the PB group over the control group could be accounted for by the increase in cytochrome P-450 content of the PB microsomes (1.5 nmol cytochrome P-450/mg protein for PB vs 0.6 nmol/mg protein for the control). However, just an increase in microsomal cytochrome P-450 content will not always result in an increase in specific activity as the MC microsomes had about the same concentration of cytochrome P-450 as the PB group (1.6 nmol cytochrome P-450/mg protein for the MC group). These results indicate that there is a metabolic preference of PB hepatic microsomal monooxygenases over that of MC hepatic microsomal monooxygenases by a factor of about 3.5.

Spectral interaction of 7-methoxyquinoline with cytochrome P-450. It is well known that hemoproteins such as cytochrome P-450 exhibit spectrophotometric transitions that are associated with changes in the electronic configurations of the iron prosthetic group [17]. Binding of substrates or other ligands to the iron heme group will cause absorption changes in the Soret region of the UV-visible spectrum. Three different types of spectral changes are recognized for the binding of ligands to cytochromes P-450. These are classified as Type I (difference absorption peak at 385–390 nm and a minimum at *ca.* 420 nm), Type II (difference absorption minimum 390–405 nm and a maximum at 425–435 nm), and a Reverse Type I (also called modified Type II, difference absorption maximum at 420 nm and a minimum at 388–390 nm).

7-Methoxyquinoline interacted with control and MC microsomal cytochrome P-450 to produce Type I difference

Table 1. Effects of inhibitors on the O-demethylation of 7-methoxyquinoline by phenobarbitone-induced rat hepatic microsomal monooxygenases

Treatment	Assay conditions*	Activity†	% Inhibition
PB-induced	Enzyme only	0	
	Enzyme + NADPH	552.1	0
	Enzyme + NADPH + N_2	0	100
	Enzyme + NADPH + CO/O_2‡	135.4	76
	Enzyme + NADPH + 17.2 μM metyrapone	135.4	76
	Enzyme + NADPH + 250 μM hexobarbital	312.5	43

* Reaction mixture contained 0.3 mg microsomal protein/ml, 20 mM Tris, pH 7.6, 150 mM KCl, 3 mM $MgCl_2$, and 0.2 μmol 7-methoxyquinoline. Reactions were initiated with 0.1 μmol NADPH unless otherwise stated. Reaction volume was 2 ml.

† Activity is expressed as pmol 7-quinolinol produced per min per mg microsomal protein at 25°. Figures are the mean value of three determinations.

‡ $CO/O_2 = 80/20$.

Table 2. Apparent kinetic constants for 7-methoxyquinoline *O*-dealkylases in control, 3-methylcholanthrene, and phenobarbital microsomes

Treatment	K_m (μM)	V_{max} (nmol 7-quinolinol/min/mg protein)	V_{max} (nmol 7-quinolinol/min/nmol cytochrome P-450)
Control	70.9 ± 4.8	0.37 ± 0.02	0.39 ± 0.02
MC	14.2 ± 0.5	0.35 ± 0.01	0.28 ± 0.01
PB	22.4 ± 5.0	0.91 ± 0.13	0.42 ± 0.06

Values are the means ± SD of three to five determinations.

spectra. The difference absorption peaks and troughs were, respectively, 384–386 nm and 418–420 nm. The spectral interaction was weak with both MC and control microsomal preparations. Substrate binding with PB microsomal preparations produced difference spectra of the Reverse Type I variety with a trough at 390 nm and an absorption maximum at 416–417 nm; 7-quinolinol produced weak, Type I spectra with control, MC, and PB microsomes. Spectral binding constants (K_s) [15] could not be determined for 7-methoxyquinoline binding to control and MC microsomal cytochrome P-450; however, a K_s could be determined for the binding to PB microsomal cytochrome P-450. The apparent K_s was $22.6 \pm 9.4\ \mu M$ for 7-methoxyquinoline binding to PB microsomal cytochrome P-450. The K_s was in the same order of magnitude of the K_m for this substrate with PB microsomal monooxygenases.

It is interesting that 7-methoxyquinoline produces different types of binding spectra depending on the types of microsomes used. Gillette and Gram [18] observed that certain amine-containing compounds could cause both Type I and Type II spectral changes depending on the source of microsomes. In addition, Burke and Mayer [9] reported that phenoxazone produces Reverse Type I difference spectra, whereas the 7-alkoxyphenoxazone derivatives produce Type I difference spectra with murine hepatic microsomal cytochrome P-450 preparations. We cannot provide an explanation at this time for the two binding types observed for 7-methoxyquinoline with rat hepatic microsomal cytochrome P-450.

Suitability of 7-methoxyquinoline as a substrate for other microsomal monooxygenases. Preliminary tests were made to determine the suitability of 7-methoxyquinoline as a substrate for other monooxygenases. 7-Methoxyquinoline was metabolized in the same range as rat hepatic microsomes by rabbit and murine hepatic microsomes (i.e. 0.5 to 4 nmol 7-quinolinol formed per min per mg protein). Microsomes from a Rutgers diazinon-resistant housefly strain were also tested and gave a specific activity of 0.25 nmol 7-quinolinol formed per min per mg protein at saturating substrate concentrations.

It appears that 7-methoxyquinoline will be a useful tool to measure many different types of monooxygenases. Studies are currently underway to determine the usefulness of other 7-alkoxyquinolines for measuring cytochrome P-450 monooxygenases and whether or not the inclusion of a nitrogen atom in the fluorophore ring system confers specificity of metabolism by different microsomal preparations. These investigations indicate that certain 7-alkoxyquinolines are specifically metabolized by different cytochrome P-450 monooxygenases. One advantage the 7-alkoxyquinolines may have over the 7-alkoxyresorufins is that they may be more useful in *in situ* or single cell analyses. Experiments utilizing microspectrofluorometric equipment and tissue cultures inoculated with 7-alkoxyresorufins indicated that the product, resorufin, migrates out of the cell too rapidly to perform kinetic analyses (unreported data); 7-quinolinol may migrate from the cell more slowly.

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Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide

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The toxic effects of carbon monoxide (CO) are attributed to severe tissue hypoxia resulting from a decrease in intracellular oxygen tension [1]. Carbon monoxide interacts reversibly with hemoglobin to form carboxyhemoglobin which in turn decreases the oxygen carrying-capacity of the blood and shifts the oxyhemoglobin saturation curve to the left. As a result, the limited amount of oxygen transported by the blood is more tightly bound to hemoglobin and results in functional anemia. There are, however, several studies which suggest that CO exerts toxic effects independent of those associated with carboxyhemoglobin formation [2–5]. Since significant partial pressures of CO are found in several tissues upon CO inhalation [6–8], the interaction of CO with intracellular constituents may account for the occurrence of direct (i.e. carboxyhemoglobin independent) toxic effects.

Duke and Killick [9] and Scharf *et al.* [10] have reported data which suggest that CO may inhibit the contractility of vascular smooth muscle. These reports are consistent with studies conducted in our laboratory which show that CO relaxes coronary and aortic vascular smooth muscle preparations [5, 11–13]. The ability of CO to decrease cellular calcium levels and relax vascular smooth muscle is not due to hypoxic or functional hypoxia nor mediated by adrenergic influences, adenosine or prostaglandins [5]. Since an elevation in cyclic nucleotide levels has been associated with a decrease in cellular calcium content and relaxation of vascular smooth muscle [14], the present study was conducted to determine if exposure of cultured aortic smooth muscle cells to CO is associated with alterations in cellular cyclic GMP levels.

Methods

Cell culture procedure. Male Sprague–Dawley rats (250–300 g) were obtained from Sasco, Inc. Animals were maintained in individual cages, and food and water were provided *ad lib*. Primary cultures of rat aortic smooth muscle cells were prepared as previously described [15]. Briefly, segments of thoracic aortae (18–24 mm) were excised from the animals and cleaned of clotted blood and connective tissue. The vessels were subjected to a series of enzymatic digestions to isolate medial smooth muscle cells. Cells were plated at a density of $1.8–2.0 \times 10^5$ cells in 35 mm petri dishes and grown in Medium 199 supplemented with fetal bovine serum, 10%; glutamine, 2 mM; penicillin, 10,000 units/ml; streptomycin, 10 mg/ml; and amphotericin, 50 μg/ml.

Treatments. Confluent cultures were exposed to a mixture containing air enriched with 5% CO_2:21% O_2:74% N_2 (control) or 5% CO_2:5% CO:21% O_2:69% N_2 (CO) for 30 or 60 min. Methylisobutylxanthine (50 mM) was added to the cultures to inhibit cyclic GMP phosphodiesterase [16]. At the end of the desired exposure periods, the culture medium was removed rapidly and 1 ml of ice-cold trichloroacetic acid (6%) was added. Samples were frozen and processed for cyclic GMP determination as described by Hirata *et al.* [17]. Cultures were subjected to three successive freeze–thaw periods. Cells were scraped off and centrifuged at 10,000 *g* for 15 min at 4°. The supernatant fraction was removed and combined with 10 ml of H_2O-saturated diethylether. The tubes were vortexed at room temperature for 60 sec and subjected to three successive ether extractions. Samples were incubated for 5 min at 80° to drive off all traces of ether. A 500-μl aliquot of the sample was lyophilized and resuspended in 500 μl of 0.05 M sodium acetate buffer (pH 6.2). Cellular cyclic GMP levels were assayed by radioimmunoassay (New England Nuclear).

Results and Discussion

Previous studies in our laboratory have shown that CO-induced relaxation of vascular smooth muscle is associated with a decrease in cellular calcium levels [13]. This vascular response is not secondary to hypoxia nor mediated by autonomic or humoral influences [5]. Furthermore, the ability of CO to relax vascular smooth muscle is not endothelium dependent since denuded aortic preparations respond to the same extent as intact vessels [12]. The results presented herein show that CO caused a time-dependent increase in the levels of cyclic GMP in cultured aortic smooth muscle cells (Fig. 1). Although no calcium measurements were conducted in the present study, the elevation of cGMP correlates temporally with a decrease in cellular calcium levels and a relaxation of aortic smooth muscle preparations under similar experimental conditions [12, 13]. Collectively, these results raise the possibility that carbon monoxide acts directly or through a second messenger system to alter vascular smooth muscle cell metabolism.

The concentration of CO used in this study was relatively high. However, our earlier studies demonstrating that CO relaxes vascular smooth muscle [5, 11–13] have all been conducted at 2.5 to 5% CO. Interestingly, these concentrations of CO are within the range used by both Ayres