Biochemical Pharmacology, Vol. 30, No. 7, pp. 757–762, 1981.
Printed in Great Britain.

0006-2952/81/070757-06 $02.00/0
Pergamon Press Ltd.

RANGE OF ENVIRONMENTALLY RESPONSIVE MONOOXYGENASE ACTIVITIES IN HUMAN PLACENTAL MICROSOMES DETERMINED BY DIRECT FLUORESCENCE TECHNIQUES

DAVID K. MANCHESTER*

Departments of Pediatrics and Pharmacology and Divisions of Perinatal Medicine and Clinical Pharmacology, School of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262, U.S.A.

(*Received* 7 *February* 1980; *accepted* 29 *August* 1980)

Abstract—Two direct fluorometric assays of *in vitro* xenobiotic oxidation have been adapted for use with placental microsomes. A total of 103 placentas were studied. NADPH-dependent disappearance of benzo[a]pyrene was found in the majority of placental preparations from pregnancies in which maternal smoking had occurred, but was observed only rarely in its absence. *O*-Deethylation of 7-ethoxyresorufin (ERR) was demonstrable in all cases. *O*-Deethylase activity correlated positively with benzo[*a*]pyrene disappearance ($r = 0.87 \pm 0.16$) when mothers smoked cigarettes and was significantly higher than activity in preparations from nonsmokers. A shift in apparent K_m toward ERR from 10^{-5} M to 10^{-7} M was observed as a consequence of maternal smoking. Addition of α-naphthoflavone markedly inhibited *O*-deethylation in induced placentas, whereas stimulation of *in vitro* metabolism occurred in noninduced placentas. These results establish operational definitions of induction in human placental monooxygenase systems and provide a basis for quantitative descriptions of fetal environmental responses.

In the years since Welch *et al.* [1] reported that human placental drug oxidations could be influenced by maternal cigarette smoking, several investigators have confirmed and expanded upon this observation. Nebert *et al.* [2] demonstrated a significant, though not perfect, correlation between *in vitro* placental hydroxylation of benzo[*a*]pyrene (BP) and the number of cigarettes smoked per day during pregnancy. Juchau *et al.* [3–5] reported that this maternal effect on fetal metabolism could occur early in gestation, that microsomal cytochrome P-450 systems located in syncytiocytotrophoblast are responsible, and that the P-450 hemoprotein found in placental microsomes demonstrates unusual spectral characteristics.

The term aryl hydrocarbon hydroxylase is often used to describe the monooxygenase system(s) that is induced by exposure to polycyclic aromatic hydrocarbons (PAH) such as those found in cigarette smoke. Pelkonen [6] has characterized the responses of two human fetal BP hydroxylation systems to *in vitro* inhibitors and concluded that the inducible system found in placenta closely resembles the PAH inducible systems described in laboratory animals. Other investigators [7] have expanded the spectrum of xenobiotics metabolized by this placental system.

Despite these efforts, the biologic significance and developmental role of inducible human placental aryl hydrocarbon metabolism remain unclear. Controlling factors, genetic and environmental, and the influence of the system, if any, on fetal outcome have yet to be identified. This report describes variations in environmentally responsive human placental xenobiotic metabolism assessed by two direct fluorometric assays. Experimental conditions that affect the reliability and reproducibility of these assays were identified. Activity of placental microsomes toward 7-ethoxyresorufin (ERR) was characterized in the presence and absence of maternal smoking and compared to BP metabolism. As a result, categories of "induced" and "noninduced" activity in human placentas were defined, and a range of environmentally responsive activity is described.

MATERIALS AND METHODS

Chemicals. 7-Ethoxyresorufin was synthesized from resorufin (Eastman Kodak Co., Rochester, NY) according to the method of Burke and Mayer [8] or purchased in crystalline form from Pierce Chemicals, Rockford, IL. Benzo[*a*]pyrene and α-naphthoflavone were purchased from the Aldrich Chemicals Co., Milwaukee, WI, and glucose-6-phosphate, NADP, glucose-6-phosphate dehydrogenase (Type V), aryl sulfatase (Type V), and β-glucuronidase (Type B-10) from the Sigma Chemical Co., St. Louis, MO.

Subjects. The study protocol was reviewed and approved by the University of Colorado Health Sciences Center Committee on Human Experimentation. All placentas were obtained from the delivery rooms at University Hospital, Denver, CO. The population giving birth at this hospital includes women followed in prenatal clinics at the Health Sciences Center as well as cases referred for a variety of perinatal complications.

* Address reprint requests to: D. K. Manchester, M.D., Department of Pediatrics, Container C-228, University of Colorado Health Sciences Center, 4200 East Ninth Ave., Denver, CO 80262, U.S.A.

Smoking histories were obtained from medical records and from postpartum interviews. Smoking, drug, and medical histories are routinely taken by nurses during the first prenatal clinic visit. Resident physicians, in addition, repeat these questions when each woman is admitted to the labor area. In most cases cigarette smoking was assessed by two independent observers. Whenever the history was unclear, postpartum interviews were conducted by the author. Women were considered smokers if they had smoked cigarettes within 2 weeks of delivery. Placentas from three women who discontinued smoking during their pregnancies were considered separately.

Tissue preparation. Placentas were examined immediately upon delivery, and 50–100 g of normal-appearing villous tissue was cut from the maternal surface. Several areas were sampled from each placenta and immersed in ice-cold 0.01 M sodium phosphate buffer, pH 7.0, containing 1.15% KCl. Adherent decidua and chorionic plate were not included.

After considering previously reported procedures for preparation of placental microsomes [9, 10], we found the following approach both convenient and reproducible. We recognize that some microsomal activity may be lost during this process. The buffer described above was used throughout, and all procedures were carried out on ice or in a cold room (4°). Fifty per cent homogenates of fresh, minced and washed villous tissue were made at high speed for 30 sec in a Waring blender. Homogenates were centrifuged at 4000 *g* for 10 min, and the resulting supernatant fraction was spun at 9000 *g* for 20 min. The 9000 *g* supernatant fractions were either stored at −70° or immediately used to prepare microsomes by ultracentrifugation (105,000 *g* × 60 min). Microsomes for assay were resuspended at a ratio of approximately 10 g of placental tissue to 1 ml of homogenizing buffer, the protein content was determined by the method of Lowry *et al.* [11].

Assays. All assays were performed in duplicate. Cytochrome P-450 carbon monoxide binding activity was assayed by difference spectroscopy (Varian, Cary 118 dual beam spectrophotometer) with the sample cuvette reduced (dithionite) and CO bubbled through both sample and reference cuvettes [12]. An extinction coefficient of 91 mM^{-1} cm^{-1} was assumed [13].

Benzo[*a*]pyrene metabolism was estimated by the direct fluorometric assay of Yang and Kicha [14]. This assay, which follows the disappearance of fluorescence produced by an interaction between BP and microsomes, was carried out at a constant protein concentration of 0.25 mg/ml. Heat-denatured microsomal protein was added when necessary. The reaction was followed at 37° in a water-jacketed cuvette in a Perkin–Elmer 44F spectrophotofluorometer. NADPH was provided by an enzymatic generating system. Final concentrations in the 2 ml reaction mixture are given in the tables. Benzo[*a*]pyrene, freshly dissolved in acetone, was added in 10 μl aliquots to yield final concentrations of 0.01 to 0.1 μM. Care was taken not to expose the BP to fluorescent light.

Fluorescence spectra, obtained with BP and the oxidation product 3-hydroxybenzo[*a*]pyrene, in this system were essentially identical to those reported by Yang and Kicha [14] using rat liver microsomes. Excitation and emission wavelengths were therefore set at 389 and 410 nm respectively. A detection limit of approximately 0.3 pmole BP metabolized · (mg protein)$^{-1}$ · min^{-1} was observed. This background loss of fluorescence occurred with heat-denatured placental microsomes in the presence and absence of the NADPH-generating system and was subtracted from the rate observed with untreated microsomes. Activity in each placenta assayed was linearly related to time and active microsomal protein concentration. Since optimal protein and substrate concentrations varied among placentas, standard curves were determined for the final set of conditions at the time of assay.

O-Deethylation of ERR was monitored by following the production of resorufin in the presence of 5 mg of microsomal protein, according to the procedure of Burke and Mayer [8]. The 2 ml reaction mixture contained the NADPH-generating system described in Table 1. Optimal activity at 37° in 0.25 M sodium phosphate buffer occurred at pH 7.8. Substrate was added in ethanol, ethanol–propylene glycol (1:1, v/v), or in 10% albumin to yield final concentrations ranging from 0.001 to 50 μM. Fluorescence spectra of ERR and resorufin in this system were similar to those reported previously [8], with maximum emission for resorufin at 586 nm. Excitation at 510 nm rather than at shorter wavelengths minimized interference. Standard curves for resorufin were developed for each set of experimental conditions. Activities of placental microsomes toward ERR prepared in our laboratory and commercially prepared substrate were identical.

RESULTS

When villous tissue was placed in ice-cold buffer immediately after delivery, placental microsomal cytochrome P-450 concentrations ranged from 40 to 100 pmoles P-450/mg protein (N = 20). As shown in Fig. 1, loss of cytochrome P-450, and presumed conversion to P-420, occurred within a few minutes of delivery if the placenta was left at room temperature. Results with vaginally delivered placentas were comparable to those in placentas obtained at caesarian section. Since villous tissue is supported by maternal circulation [15], cellular function and microsomes appear to remain intact even though placentas which deliver vaginally are obtained several minutes after interruption of umbilical circulation.

Storage influenced both catalytic and cytochrome P-450 CO binding activities. Cytochrome P-450 CO binding activity was stable in microsomal suspensions stored overnight at −20° or −70°, but not thereafter. Benzo[*a*]pyrene disappearance and ERR *O*-deethylation were unchanged for up to 48 hr when 9000 *g* supernatant fractions were stored at −70°. With continued storage, activity toward BP decreased and was approximately halved by 5 days. Activity toward ERR, on the other hand, remained unchanged for as long as 2 months.

A total of 103 singleton placentas was studied.

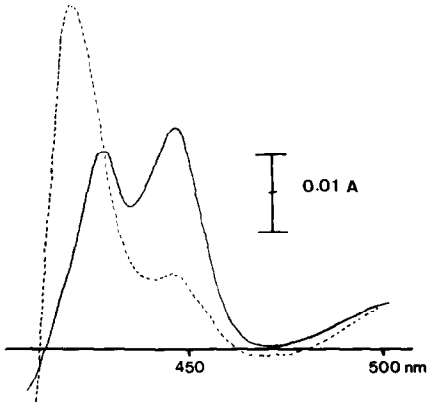

Fig. 1. Effect of tissue handling on CO difference spectra. The solid (——) line traces the spectrum observed when microsomes were prepared from placental tissue homogenized immediately after delivery. The same placenta was allowed to stand at room temperature for 15 min, and a second sample was taken and processed exactly as the first. The dotted (····) line traces the difference spectrum produced by the second sample. In each experiment, the sample and reference cuvettes contained 4.5 mg of microsomal protein in 0.01 M sodium phosphate, pH 7.0. Both cuvettes were bubbled with CO, and the sample cuvette was reduced with dithionite.

Adequate histories were available in 80 cases that delivered at term. All placentas were collected at the time of delivery and assayed within 48 hr. Thirty-six mothers gave a history of cigarette smoking, and two women who denied smoking cigarettes volunteered histories of marihuana smoking on a regular basis. Cigarette consumption was greater than one cigarette per day for all smokers. Complicated pregnancies, premature deliveries, and cases requiring chronic medication were considered separately.

Placental activities toward BP and ERR in placentas from smokers and nonsmokers are described in Tables 1 and 2. Microsomal activity toward BP varied between 0.3 and 100 pmoles BP metabolized · (mg protein)$^{-1}$ · min^{-1}. Benzo[*a*]pyrene disappearance was not detected in the majority of placentas from nonsmokers, whereas detection of activity was the rule when mothers smoked. Cigarette smoking also influenced activity toward ERR (Table 2). *O*-Deethylation was detectable in all placentas and a positive correlation between the two activities was found when mothers smoked (Fig. 2). Both placentas from the marihuana smokers demonstrated activity toward BP and had *O*-deethylase activity greater than 100 pmoles · (mg protein)$^{-1}$ · min^{-1}. Three women discontinued smoking during pregnancy, each at least 2 months prior to delivery. Benzo[*a*]pyrene disappearance was not detected in their placentas and *O*-deethylase activity was comparable to that of nonsmokers. Increased monnoxygenase activity toward either substrate did not necessarily correlate with increased cytochrome P-450 CO binding activity.

At ERR concentrations above 5 μM, a pattern of initial increase in fluorescence followed by a subsequent loss (Fig. 3) was frequently observed with microsomes from nonsmokers. This loss of fluorescence was only seen in preparations with low *O*-deethylase activity and nondetectable benzo[*a*]pyrene metabolism, but it was not a constant characteristic of this group. Burke and Orrenius [16] reported a similar pattern of loss of fluorescence when they measured ERR *O*-deethylase activity in cultured hepatocytes. In our experiments, loss of fluorescence occurred with both freshly prepared and older substrate. Fluorescence was unchanged for up to 20 min when the product, resorufin, was incubated with active microsomes under the assay conditions. Loss of fluorescence was not affected by

Table 1. Benzo[*a*]pyrene disappearance*

Maternal history	Number of placentas activity detectable	Number of placentas activity nondetectable
Smokers (N = 36)	30	6
Nonsmokers (N = 42)	17	25

* Assays were carried out in the presence of 1.25 mM $MgCl_2$, 2.5 mM glucose-6-phosphate, 0.25 mM NADP, and 1.25 units/ml glucose-6-phosphate dehydrogenase in 0.1 M sodium phosphate buffer, pH 7.4. Protein concentration was 0.25 mg/ml. BP (50 μM) was added to start the reaction. A statistically significant association was found between smoking history and detectable activity, $\chi^2 = 14.9$ ($P < 0.001$).

Table 2. 7-Ethoxyresorufin *O*-deethylation*

Maternal history	Activity [pmoles resorufin formed · (mg protein)$^{-1}$ · min^{-1}] Mean ± S.E.	Range
Smokers (N = 36)	188 ± 54†	3–1197
Nonsmokers (N = 42)	39 ± 6	2–227

* Assays were carried out in the presence of the NADPH-generating system described in Table 1 in 0.25 m sodium phosphate buffer, pH 7.8. Protein concentration was 2.5 mg/ml. ERR (2.5 μM) was added to start the reaction.

† Significantly different from nonsmokers ($P < 0.001$).

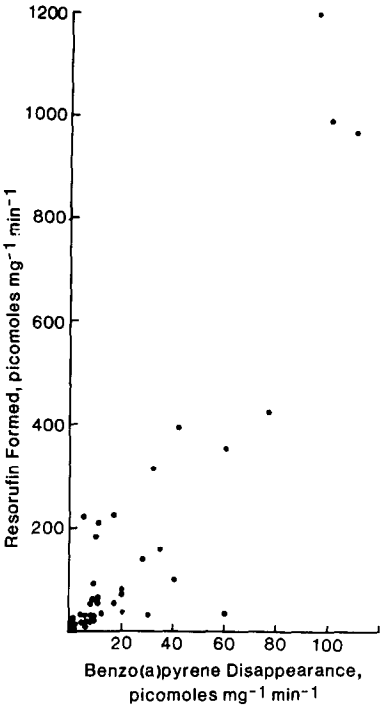

Fig. 2. Relationship between BP metabolism and ERR *O*-deethylase activity in placentas in which both activities could be observed. Material from smokers and nonsmokers is included. $r = 0.87 \pm 0.16$ ($P < 0.001$).

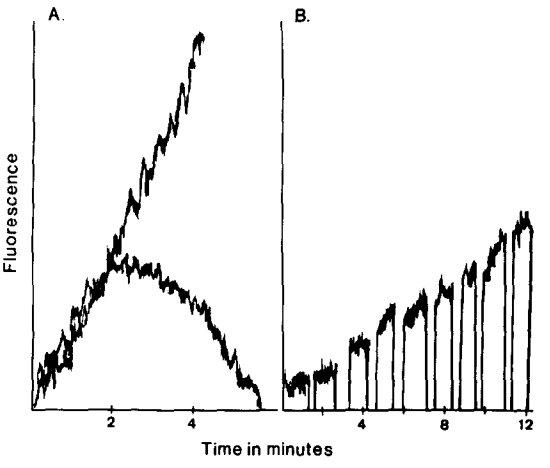

Fig. 3. Effects of albumin and stirring on loss of fluorescence during *O*-deethylation of ERR. Panel A: at 9.5 μM ERR, fluorescence at 586 nm was lost after 2–3 min of incubation. When the substrate was added in 10% albumin to the same microsomal preparation, the reaction remained linear. Panel B: in a second placental preparation, loss of fluorescence occurred despite addition of substrate in 10% albumin. In this case, the reaction became linear when the mixture was stirred intermittently. Each experiment was performed with a protein concentration of 2.5 mg/ml in the presence of 0.25 M sodium phosphate, pH 7.8, and the NADPH-generating system described in Table 1.

the addition of inhibitors of sulfation (salicylamide, 0.6 mM) or glucuronidation (saccharo-1,4-lactone, 0.06 mM). Neither the linear reaction rate nor the loss of fluorescence, when observed, was influenced by inclusion of aryl sulfatase (10^3 units/ml) or β-glucuronidase (10^4 units/ml). Addition of albumin to the reaction mixture had no effect, but "solubilization" of the substrate in 10% albumin prior to incubation linearized many, but not all, reactions, probably by decreasing available substrate. In all circumstances, however, intermittent stirring of the reaction mixture restored linearity (Fig. 3B). These results are compatible with rapid, perhaps nonenzymatic, reduction or photodecomposition of the product. Intermittent stirring may reintroduce oxygen, or bring non-photodecomposed product into the light path.

A difference in *O*-deethylase reaction kinetics between two groups of placentas was suggested by a greater sensitivity of placentas from nonsmokers to variations in substrate concentration. Apparent Michaelis–Menten kinetic constants were determined in microsomal preparations from nine placentas: five with high rates of BP disappearance and *O*-deethylase activity (smokers), and four with nondetectable BP disappearance and low *O*-deethylase activities (nonsmokers). Activities were linear with time in all of these experiments, and substrate concentration was varied between 0.02 and 50 μM. Results were analyzed by the statistical procedure described by Wilkinson [17] and are recorded in Table 3. The weighted apparent mean K_m for placentas from smokers, 2×10^{-7} M, was significantly different ($P < 0.01$) from that for nonsmokers, 1.5×10^{-5} M, by *t*-test analysis [18]. The apparent maximum velocities overlapped.

According to these results, placentas were classified as having "high" or "low" apparent K_m values

Table 3. Kinetic studies of placental microsomal activity toward 7-ethoxyresorufin*

Group	Placenta	$K_m \pm$ S.E. ($M \times 10^{-6}$)	$V_{max} \pm$ S.E. [pmoles · (mg protein)$^{-1}$ · min^{-1}]
Low activity (nonsmokers)	A	13.7 ± 3.5	32 ± 11
	B	19.9 ± 5.0	170 ± 64
	C	10.9 ± 1.8	31 ± 3
	D	4.1 ± 1.8	13 ± 7
High activity (smokers)	E	0.2 ± 0.04	40 ± 3
	F	0.09 ± 0.02	83 ± 5
	G	0.08 ± 0.0007	110 ± 0.01
	H	0.10 ± 0.01	122 ± 7
	I	0.38 ± 0.01	12 ± 1

* K_m and V_{max} were estimated by the statistical method of Wilkinson [17].

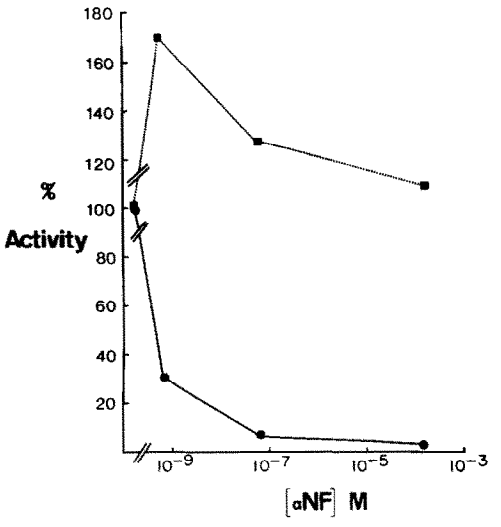

Fig. 4. Effect of α-naphthoflavone on ERR *O*-deethylase activity in microsomal preparations from two placentas. The circles indicate activity in a placenta from a woman who smoked approximately 20 cigarettes daily. The squares refer to a placenta from a nonsmoker who had little passive cigarette smoke exposure during her pregnancy. Initial activities were 104 pmoles resorufin formed · (mg protein)$^{-1}$ · min^{-1} (smoker) and 6.8 pmoles resorufin formed · (mg protein)$^{-1}$ · min^{-1} (nonsmoker). ERR concentration was 2.5 μM.

by comparing activities at 2.5 and 1 μM ERR. All placentas tested, that had activity toward both substrates, had "low" apparent K_m values. The majority of placentas from nonsmokers had "high" apparent K_m values. Two placentas that did not have detectable activity toward BP were classified as having "low" apparent K_m values and showed no decrease in activity at substrate concentrations as low as 0.2 μM; in both cases *O*-deethylase activity was low (3.3 and 17.6 pmoles/mg protein per min), and the mothers had denied cigarette smoking.

Addition of α-naphthoflavone markedly inhibited *O*-deethylase activity in "low" apparent K_m placental preparations (Fig. 4). The responses in "high" apparent K_m preparations were variable, ranging from stimulation to as much as 30–40 per cent in the presence of 0.6 mM α-naphthoflavone.

Additional placentas were collected from complicated pregnancies and pre-term deliveries. Both "high" and "low" apparent K_m classes of activity were observed in placentas delivered as early as 24 weeks gestation; beyond 24 weeks, gestational age did not appear to be a major determinant of activity.

DISCUSSION

In this study a positive history of maternal cigarette smoking was associated with an increased likelihood of detection of BP metabolism and a nearly 4-fold increase in microsomal ERR *O*-deethylase activity. Increased BP metabolism in placentas from women who smoke has been reported previously by several laboratories [1–3, 6]. Enhanced *O*-deethylase activity in these same placentas has not been reported previously, but it is not surprising since this activity increases after treatments that induce benzo[*a*]pyrene hydroxylation in other microsomal systems.

Our laboratory is interested in environmental and genetic factors that influence placental metabolism. This investigation set out to describe inducible aryl hydrocarbon metabolism in a representative sample of human placentas. The sample was subdivided according to gestational age and history of maternal smoking. The majority of pregnancies delivered at term. Smoking histories may not have been entirely accurate, but ascertainment by interview was considered acceptable since our purpose was to identify and characterize a spectrum of placental activity in a large sample rather than to study environmental responses *per se*.

A shift in apparent K_m for ERR is consistent with the induction of a specific form(s) of cytochrome P-450. Evidence for multiple forms of human placental cytochrome P-450 has been presented by Bellino and Osawa [19]. A similar shift in apparent K_m for ERR in liver from rats treated with β-naphthoflavone is considered by Warner and Neims [20] to result from induction of a distinct form(s) of cytochrome P-450 whose kinetic behavior in a reconstituted system differs significantly from that of other forms of hepatic cytochrome P-450. On the other hand, Gurtoo *et al.* [21] have attributed an apparent decrease in K_m for BP in cultured lymphocytes to changes in relative protein concentration brought about by xenobiotic exposure. Kinetically significant changes in placental cytochrome P-450 content might be difficult to detect in our system since the vast majority of protein present is not cytochrome P-450.

Maximum velocities in the two groups of placentas compared in Table 3 overlap. The confidence with which V_{max} is estimated in the nonsmoking group, however, limits further interpretation. This extensive interindividual variability is typical of placental activities and may reflect the contributions of determinants of overall activity in addition to heterogeneous components of cytochrome P-450 systems.

Addition of α-naphthoflavone to reaction mixtures resulted in either stimulation or inhibition of *O*-deethylase activity. The overall response of intact rat liver microsomes is known to vary between these extremes as a function of xenobiotic induction [22]. We observed marked inhibition when mothers smoked, and less inhibition or stimulation when mothers gave a negative history. Variation in the effect of α-naphthoflavone on placental activity may reflect relative concentrations of heterogeneous oxidase forms [20].

Differences in stability at cold temperatures between BP metabolism and *O*-deethylase activity raise questions about additional heterogeneity within environmentally responsive placental monooxygenase systems. Guenther and Nebert [23] have presented evidence for two forms of PAH inducible cytochrome P-450 in liver. On the other hand, the assay of BP metabolism that we used measured a fluorescent complex involving both the substrate and intact microsomes, and we cannot rule out alteration of non-cytochrome P-450 related components of this fluorescent complex by storage.

It is clear from this study that human placental

microsomal activities toward ERR as well as toward BP vary considerably. In order to concentrate on genetic and environmental components of this variability, *in vitro* sources of error must be minimized. We have, therefore, imposed stringent protocols for collection and handling of tissue. Our use of the fluorescence assays described represents a compromise. Although both activities measured appear to reflect maternal environment, neither assay is ideal. Their advantage is that, under circumstances where activity can be expected to vary several-fold, they allow rapid assessment and adjustment of experimental conditions. Direct monitoring ensures that the activity ascribed to a given placenta can be measured under optimal conditions and that reproducibility of results can be quickly determined.

In addition to describing a range of activity in human placentas, this study provides a basis for categorical definitions of placental metabolism. Placentas can be operationally defined as "induced" if they show activity toward both substrates, have "low" apparent K_m values toward ERR, and are inhibited by α-naphthoflavone. The groups of induced and noninduced placentas so defined reflect the strong influence of maternal smoking, but they do not correspond in all cases with maternal smoking histories. Interesting exceptions suggest that maternal cigarette smoking may not be the only source of inducing agents and that certain fetuses fail to respond to cigarette smoke exposure. Surveys of placental activity by the above methods are underway in sample populations in which environmental exposure is more rigorously documented. Placental xenobiotic metabolism may be a practical tool with which to study human genetic and environmental interactions.

Acknowledgements—The author would like to acknowledge the excellent technical contributions of Elizabeth H. Jacoby to this study and the assistance of Joseph Gal, Ph.D., in the synthesis of ERR. This study was supported by Grant HD 12936-01 from the National Institutes of Health.

REFERENCES

1. R. M. Welch, Y. E. Harrison, A. H. Conney, P. J. Popper and M. Finster, *Science* **160**, 541 (1968).
2. D. W. Nebert, J. Winkler and H. V. Gelboin, *Cancer Res.* **29**, 1763 (1969).
3. M. R. Juchau, *Toxic. appl. Pharmac.* **18**, 665 (1971).
4. M. R. Juchau and E. A. Smuckler, *Toxic. appl. Pharmac.* **126**, 163 1(973).
5. E. Hodgson and M. R. Juchau, *J. Steroid Biochem.* **6**, 669 (1977).
6. O. Pelkonen, in *Biological Reactive Intermediates* (Eds. D. J. Jollow, J. J. Kocsis, R. Snyder and H. Vainio), p. 148. Plenum Press, New York (1977).
7. J. Kapitulnik, W. Levin, P. J. Poppers, J. E. Tomaszewski, D. M. Jerina and A. H. Conney, *Clin. Pharmac. Ther.* **20**, 557 (1976).
8. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **2**, 583 (1974).
9. P. R. Zachariah, Q. P. Lee, K. G. Symms and M. R. Juchau, *Biochem. Pharmac.* **25**, 793 (1976).
10. N. G. Smith and M. C. Brush, *Nature, Lond.* **252**, 302 (1974).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. B. Schoene, R. A. Fleischmann, H. Remmer and H. F. v. Oldershausen, *Eur. J. clin. Pharmac.* **4**, 65 (1972).
13. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
14. C. S. Yang and L. P. Kicha, *Analyt. Biochem.* **84**, 154 (1978).
15. S. Getzowa and H. Sadowsky, *J. Obstet. Gynaec. Br. Emp.* **57**, 388 (1950).
16. M. D. BUrke and S. Orrenius, *Biochem. Pharmac.* **27**, 1533 (1978).
17. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
18. N. M. Downie and R. W. Heath, *Basic Statistical Methods*, p. 123. Harper & Row, New York (1959).
19. F. L. Bellino and Y. Osawa, *J. Steroid Biochem.* **9**, 219 (1978).
20. M. Warner and A. H. Neims, *Drug Metab. Dispos.* **7**, 188 (1979).
21. H. L. Gurtoo, N. B. Baker, B. Paigen, M. B. Havens, J. Minowada and H. J. Freedman, *Cancer Res.* **39**, 4620 (1979).
22. F. J. Wiebel, J. C. Leutz, L. Diamond and H. V. Gelboin, *Archs Biochem. Biophys.* **144**, 78 (1971).
23. T. M. Guenther and D. W. Nebert, *Eur. J. Biochem.* **91**, 449 (1978).