Quantification of cytochrome *P*-450-dependent cyclohexane hydroxylase activity in normal and neoplastic reproductive tissues

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(Received 13 August 1984/3 January 1985; accepted 8 January 1985)

It is well established that liver microsomal cytochrome *P*-450 participates in steroid metabolism and probably also in the metabolism of anti-oestrogens such as tamoxifen (Nolvadex). Thus it is possible that variations in cytochrome *P*-450 levels may influence the responsiveness of human breast and endometrial carcinomas to endocrine therapy. Therefore a simple sensitive spectrophotometric assay for determining levels of cytochrome *P*-450-dependent cyclohexane hydroxylation activity in breast and uterine microsomes (microsomal fractions) has been developed. Cyclohexane was chosen as a substrate because of the relatively high levels of cyclohexane hydroxylase activity in tumour microsomes and because cyclohexane serves as a substrate for several forms of cytochrome *P*-450. As previously described [Senler, Dean, Pierce & Wittliff (1985) *Anal. Biochem.* **144**, 152–158], a direct method utilizing isotope-dilution/gas chromatography–mass spectrometry was also developed in order to confirm the results of the spectrophotometric assay. The average activity (cyclohexane-dependent NADPH oxidation) for 139 human breast-tumour microsome preparations was 1.34 nmol/min per mg, which is in the range of that found in untreated mammalian liver (1–3 nmol/min per mg). Also, high enzyme activity was demonstrated in human ovary, normal uterus as well as uterine leiomyomas. Endocrine status appeared to influence enzyme levels, in that mammary tissue from virgin rats contained significantly ($P < 0.025$) higher amounts of activity than did tissues from either pregnant or lactating rats. Furthermore, carbon monoxide, as well as an antibody against rat liver cytochrome *P*-450, completely inhibited NADPH oxidation by breast-carcinoma microsomes. These results strengthen our hypothesis that tumours with high levels of cytochrome *P*-450 may have a reduced response to additive endocrine therapy.

Cytochrome *P*-450 is the name given to a family of haem-containing membrane-associated proteins that catalyse the mono-oxygenase (hydroxylation) reactions of a variety of xenobiotics, including drugs, pesticides, carcinogens and environmental pollutants, as well as endogenous substrates such as steroids (Gillette, 1966). The hydroxylation is carried out with molecular oxygen and reducing equivalents from NADPH, which are shuttled through a flavoprotein, cytochrome *P*-450 reductase (Lu & Coon, 1968). In addition, isoenzymes of cytochrome *P*-450 have variable and overlapping

substrate specificities. Cytochromes *P*-450 are found in the endoplasmic reticulum of many cell types, including brain (Sesame *et al.*, 1977), kidney (Armbrecht *et al.*, 1979), colon (Fang & Strobel, 1978), lung (Serabjit-Singh *et al.*, 1979), adrenal cortex (Estabrook *et al.*, 1963), skin (Poland *et al.*, 1974), testis (Betz *et al.*, 1976), and placenta (Ryan & Engel, 1957), although the highest concentrations are found in liver (Omura & Sato, 1964).

Not only does cytochrome *P*-450 exist in most tissues, but distribution studies have revealed that the enzyme can be detected in almost all forms of life. However, very little work has been performed concerning the existence and role of cytochrome *P*-450 in normal and neoplastic reproductive tissues (Hoffman *et al.*, 1979; Mason & Okey, 1981;

Abbreviations used: g.c.–m.s., gas chromatography–mass spectrometry; SDS, sodium dodecyl sulphate; AHH, aryl-hydrocarbon hydroxylation.

Osawa *et al.*, 1982). Furthermore, the probable involvement of cytochrome *P*-450 in carcinogenesis, either by converting procarcinogens into carcinogens, local metabolism of chemotherapeutic drugs or oestrogen synthesis by breast or uterine tumours, remains to be demonstrated in these tissues.

Our interest lies in the possibility that cytochrome *P*-450 may play a role in determining the responsiveness of human breast tumours as well as uterine tumours to additive endocrine therapy. It has been demonstrated that the presence of the oestrogen receptor in breast tumours is a relatively good predictive index of response to endocrine therapy (Maass *et al.*, 1980; Jensen *et al.*, 1971). However, in a significant fraction of patients whose tumours exhibit the oestrogen receptor, no response to endocrine therapy is observed (Maass *et al.*, 1980; Jensen *et al.*, 1971; Wittliff *et al.*, 1978). Wittliff and co-workers suggested previously that this lack of response may be due to 'defective' oestrogen receptors in certain tumours (Wittliff *et al.*, 1978).

In the present study, cyclohexane was used as a substrate to assay for cytochrome *P*-450-dependent hydroxylation activity in normal and neoplastic reproductive tissues. The alicyclic hydrocarbon cyclohexane was chosen as substrate in these studies because of its high rate of catalysis, its high efficiency of hydroxylation, and the fact that cyclohexane acts as a general substrate for most forms of cytochrome *P*-450 in rabbit liver microsomes (Nordblom & Coon, 1977; Lu *et al.*, 1970). Cytochrome *P*-450 activity (cyclohexane hydroxylase activity) is readily monitored spectrophotometrically by measuring NADPH oxidation at 340 nm. In order to validate the NADPH assay as well as to confirm that NADPH oxidation is coupled to cyclohexane hydroxylation, a g.c.-m.s. procedure was developed for detecting low levels of the hydroxylated product, cyclohexanol, in rabbit liver microsomes as well as in human breast and uterine microsomes (Senler *et al.*, 1985).

In the present study we assayed the microsomes from 139 biopsies of human breast carcinoma, normal and neoplastic uteri, normal ovary, as well as normal rat mammary gland during various stages of development, for cytochrome *P*-450-dependent hydroxylation activity. Furthermore, we compared this parameter with levels of the oestrogen and progesterone receptor in the same human breast tumour.

Materials and methods

Collection and handling of breast-carcinoma specimens

A successful method for obtaining human

breast-cancer specimens is in operation in co-operation with the University-affiliated and surrounding hospitals. The pathologist selects samples from the excised tissue at the time of operation which are adequate for both enzyme and receptor studies without compromising the patient's diagnostic evaluation. The specimens are kept on ice at all times, and after approval by the pathologist, are taken to the laboratory. A 300–1000 mg (wet wt.) portion of each specimen is placed in small plastic vials for analyses, frozen in liquid N₂ and stored at –86°C until the assays are performed.

Steroid-binding proteins in the frozen tissue are extracted in buffer with the Polytron using homogenizer by procedures that were reported previously (Wittliff *et al.*, 1978, 1980). Before determination of specific steroid-binding capacity, a representative sample of the breast-tumour biopsy is fixed in neutral formalin, stained with haematoxylin and eosin, and examined microscopically. Confirmation of tumour pathology and quantitative determination of cellular composition may be determined.

Preparation of tissue cytosols

Tissue cytosols were prepared at 0–3°C. Cold buffer consisting of 10 mM-Tris/HCl, pH 7.6, containing 1.5 mM-EDTA, 10 mM-monothioglycerol and 10% (v/v) glycerol was added to the minced tumour at a volume determined by the tissue wet weight (tissue/buffer, 1:3, w/v). Tissues were homogenized with several intermittent 5–10 s pulses of the Polytron (Brinkman Instruments, Lucerne, Switzerland) homogenizer. The homogenate was centrifuged at 105 000g for 30 min in fixed-angle rotors in a preparative ultracentrifuge. The supernatant was drawn carefully from beneath the lipid layer by using a Pasteur pipette and kept on ice until used. The pellet was frozen and later was processed further for microsome preparations.

Multipoint titration analyses for oestrogen receptor

Multipoint titration analysis using dextran-coated charcoal (Wittliff *et al.*, 1980) was used to determine the quantity of oestrogen receptors in each breast tumour. Each of these assays was quality controlled by simultaneous analysis of oestrogen receptors in a tissue reference powder (Wittliff *et al.*, 1980).

Preparation of tumour microsomes

Pellets from the cytosol preparation described above were thawed immediately before assay. The pellets were resuspended in a volume (equivalent to 4 times the original tissue weight) of 10 mM-

Tris/HCl buffer, pH 7.6, containing 2.5 mM-EDTA and 10% glycerol. At least 0.3 g of tissue was necessary to yield a sufficient amount of microsomes for each assay. The suspension was homogenized with a Polytron homogenizer at a setting of 7 for two 5 s bursts. The homogenized membranes were centrifuged at 10000g for 20 min and the supernatant was then centrifuged at 230000g for 30 min to isolate microsomal membranes. The pellets were resuspended (20 strokes in a 1 ml glass-pestle tissue grinder; Kontes Glass Co., Vineland, NJ, U.S.A.) in 0.6 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA and 20% (v/v) glycerol. The protein concentration of this final suspension was 1–5 mg/ml as determined (Lowry *et al.*, 1951) by using bovine serum albumin as a standard. Microsomes could be stored at -20°C for over 1 month without loss of cyclohexane hydroxylase activity. However, microsomes may be thawed one time only, after which considerable activity loss was observed. Liver microsomes from phenobarbital-treated rabbits were prepared as previously described (Wagner *et al.*, 1984).

The enzymic oxidation of succinate by indophenol (Green *et al.*, 1955) was used as a marker for determining mitochondrial contamination in human breast-tumour microsomal preparations. The average activity (reduction of indophenol) for five breast-tumour microsomal preparations was 3.0 nmol/min per mg, which is 28% of the average activity determined in five nuclear preparations (10.9 nmol/min per mg). Since the contamination varied from 0 to 55%, this relatively large average mitochondrial contamination observed in the microsomal preparations is probably a result of drawing variable amounts of the nuclear pellet into the supernatant used for microsome isolation. However, the mitochondrial contamination had no effect on cyclohexane-dependent NADPH oxidation, since all five nuclear preparations exhibited no cyclohexane hydroxylase activity, whereas the five microsomal preparations had an average activity of 818 pmol/min per mg.

Cyclohexane hydroxylation assay

Hydroxylase activity by breast-tumour microsomes was determined by measuring cyclohexane-dependent NADPH oxidation. The assay mixture was prepared by adding 100 μl of microsomes to 1.74 ml of solution containing (final concns.) 50 mM-Hepes buffer, pH 7.0, 15 mM-MgCl₂ and 0.17 mM-NADPH (Sigma, St. Louis, MO, U.S.A.). Then the mixture was divided into two 0.89 ml portions and 10 μl of 1 M-cyclohexane (Matheson, Coleman and Bell, Norwood, OH, U.S.A.) in methanol was added to one, while 10 μl of methanol was added to the other. The reaction was carried out at 30°C . At the end of 30 min, 100 μl of

10% (w/v) SDS (Sigma) were added to each portion and the difference in absorbance at 340 nm between the two samples was determined on a Beckman 35 or a Cary 219 spectrophotometer. Activity (NADPH oxidized) was calculated by using $6.22\text{ mM}^{-1}\cdot\text{cm}^{-1}$ as the molar absorption coefficient of NADPH. All non-specific NADPH oxidation activity that may occur in the absence of substrate is automatically corrected for, since the solutions differ only in that one contained cyclohexane. We have shown that the results of this assay are well correlated with cyclohexanol formation in microsomes from rabbit liver, human breast tumours and human uterus (Senler *et al.*, 1985).

Immunochemical studies

Cytochrome *P*-450 was purified from livers of phenobarbital-treated rats (Guengerich & Martin, 1980). Two bands were observed on SDS/polyacrylamide gels (Laemmli, 1970) with M_r values of 48000 and 53000 in this preparation [see Fig. 1 of the miniprint supplement to Gray (1982)]. The preparation contained no cytochrome *P*-450 reductase as determined by assay and by SDS/polyacrylamide-gel electrophoresis. A large amount of apo-(cytochrome *P*-450) was probably present, as shown by the relatively low specific content of 8.5 nmol/mg (pure whole protein has a specific content of 20 nmol/mg). Cytochrome *P*-450 (2.6 mg in 0.4 ml of 0.05 M-sodium phosphate buffer) was mixed with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI, U.S.A.) and injected subcutaneously at multiple sites on the back of a male New Zealand White rabbit. After 3 weeks the process was repeated, except with incomplete Freund's adjuvant, and again after another 3 week period. Antiserum was collected at the end of 7 weeks. Ouchterlony double-diffusion analysis (Dean & Coon, 1977) revealed two precipitin bands with the cytochrome *P*-450 preparation used for immunization and with solubilized microsomes from phenobarbital-induced rabbit livers. The antiserum precipitated approx. 10 nmol of purified rat liver cytochrome *P*-450/ml of serum. Inhibition of cyclohexane hydroxylation was observed by comparing cyclohexane-dependent NADPH oxidation by tumour microsomes in the presence of antiserum or control serum.

Rat mammary-gland study

Sprague-Dawley (Holtzman, Madison, WI, U.S.A.) rats were used for all time-course experiments. Virgin animals were either 40 or 70 days of age. Mature females were examined for exfoliate cells obtainable from vaginal smears, and only females exhibiting at least two normal oestrus cycles were employed. Smears were also performed on breeding females. The presence of sperm in the

vaginal tract was taken as indicative of pregnancy, which was further confirmed when the animals were killed or, ultimately, by delivery of pups. The date that sperm was detected was designated day 1 of pregnancy. Pregnant animals at days 7, 14 and 20 were used. Lactating animals were 1, 10, 15 and 20 days *post partum*; all pups were weaned at day 21. Animals with mammary glands involuting were used 5 days after weaning. Except for virgin animals, all had previously delivered healthy litters. When the animals were killed, the six abdominal inguinal mammary glands were removed and placed on a glass slide over ice. No attempt was made to remove fat-pads or lymph nodes. Microsomes were prepared as described for breast-tumour pellets.

Human uterus and ovary study

Uteri and ovaries from non-pregnant premenopausal women (age range 20–48 years), removed for medically indicated reasons with informed consent, were obtained from the operating room and immediately placed in ice-cold physiological saline (0.9% NaCl). After examination by the pathologist, tissue sections were either used immediately or stored frozen in saline at -90°C . If a tumour were present, one sample of the leiomyoma and one sample of adjacent normal myometrium were taken from each uterine specimen. Since uteri (normal and leiomyoma) were received as whole-tissue samples, mincing was required before homogenizing, whereas ovary samples were in homogenate form. Microsomes were prepared as described for breast-tumour pellets.

Results and discussion

As mentioned above, the results of the g.c.-m.s. procedure (Senler *et al.*, 1985) have demonstrated that the spectrophotometric method (NADPH assay) provides a sensitive and accurate means of analysis of cytochrome *P*-450-dependent cyclohexane hydroxylation activity in normal and neoplastic human reproductive tissues. The correlation between cyclohexane-dependent NADPH oxidation and formation of cyclohexanol was 0.99 for rabbit liver, 0.90 for human breast tumours and 0.87 for human uterus (Senler *et al.*, 1985). Therefore the spectrophotometric method was utilized for all subsequent experiments because of the simplicity and speed of the measurement.

Characterization of the spectrophotometric cyclohexane-hydroxylation assay

Cytochrome *P*-450-dependent hydroxylation activity in normal and neoplastic reproductive tissues was measured by comparing NADPH consumption by breast and uterus microsomes in the

presence and absence of cyclohexane. Oxidation of NADPH was linear for 30 min, after which the activity decreased rapidly with three different breast-tumour preparations, as shown in Fig. 1. Since only a fraction of the total NADPH was consumed, this decrease in activity is probably due to denaturation of the enzyme system. Also, re-addition of fresh microsomes after 30 min stimulated additional NADPH oxidation. Maximal activity occurred at a temperature of 30°C (for a 30 min incubation), and the optimal pH for cyclohexane hydroxylation at 30°C was 7.0 (Fig. 2). This time, temperature and pH were used for all subsequent assays. By contrast, it was reported (Mason & Okey, 1981) that the rate of AHH catalysed by human breast-tumour microsomes was linear for incubation times up to 60 min. Furthermore, those authors also reported that AHH activity in tumour microsomes required a pH of 7.5 and a temperature of 37°C for optimal activity. These data would seem to suggest that the two assays measure levels of different *P*-450 isoenzymes.

Carbon monoxide and immunochemical-inhibition studies

Since it is well known that cytochrome *P*-450-dependent hydroxylation activity is inhibited by carbon monoxide, it was necessary to demonstrate

Fig. 1. Time course of cytochrome *P*-450-dependent hydroxylation of cyclohexane by breast-carcinoma microsomes

Cyclohexane-dependent NADPH oxidation was measured at the indicated times. Each point represents the mean value for two assays and different symbols indicate the results from three different tumours.

Fig. 2. pH optimum of cytochrome P-450-dependent hydroxylation of cyclohexane by breast-carcinoma microsomes

Cyclohexane-dependent NADPH oxidation was measured at the specified pH after a 30 min incubation at 30°C. Points represent the mean values of two determinations for three different tumour preparations. Hepes buffer was used at all pH values.

that NADPH oxidation is inhibited in the presence of carbon monoxide. Bubbling of carbon monoxide for 1 min before incubation for three breast tumours as well as control rabbit liver (phenobarbital-induced) inhibited cyclohexane-dependent NADPH oxidation completely. It is unlikely that this procedure lowered the oxygen concentration in the assay mixture sufficiently to inhibit hydroxylation. Therefore this inhibition is most likely due to interaction of carbon monoxide with cytochrome P-450. Further evidence for cytochrome P-450 involvement in breast tumour microsomes was demonstrated by immunochemical inhibition of cyclohexane-dependent NADPH oxidation, as shown in Fig. 3. A preparation of purified rat liver cytochrome P-450 containing two isoenzymes was used to elicit antibodies from a rabbit (as described in the Materials

Fig. 3. Influence of undiluted antiserum against rat liver cytochrome P-450 on cyclohexane hydroxylation by human breast-carcinoma microsomes

Increasing amounts of antiserum were added to the cyclohexane-hydroxylation assay with human breast-carcinoma microsomes, and cyclohexane-dependent NADPH oxidation was determined. Cyclohexane was the final addition to the assay mixture. No inhibition was observed when 100 μl of control serum was added to the assay instead of antiserum. The initial activity of the tumour microsomes was 500 pmol/min per mg.

and methods section). Interestingly, Guengerich *et al.* (1981) have reported that human liver cytochrome P-450 preparations cross-reacted with antibodies raised against phenobarbital-induced cytochrome P-450 from rat liver. As Fig. 3 shows, complete inhibition of cyclohexane-dependent NADPH oxidation resulted on the addition of cytochrome P-450 antibodies in three different tumour preparations (seven other preparations yielded similar results; results not shown). Furthermore, control serum had no effect on the activity. Also, cytochrome *c* reductase activity and NADPH oxidation independent of cyclohexane were not affected by the antiserum (results not shown). These results, coupled with the carbon-monoxide-inhibition studies, show that cyclohexane hydroxylation in breast tumours is carried out by cytochrome P-450. Although complete inhibition of cytochrome P-450-dependent activity

is not usually observed in liver microsomes with antibodies against cytochrome *P*-450 (Dean & Coon, 1977), the complete inhibition observed in breast-tumour microsomes may be the result of a different distribution of multiple forms of the cyclohexane in breast tissue.

Cyclohexane hydroxylase activity in normal and neoplastic reproductive tissues

In order to ascertain if there is any relationship between cyclohexane hydroxylase activity and endocrine status, rat mammary gland was examined at various stages of development (Fig. 4). Endocrine status appeared to influence enzyme levels in that mammary tissue from virgin rats contained significantly ($P < 0.025$) higher cyclohexane hydroxylase activities than did tissue from either pregnant or lactating rats [expressed either as specific activity (nmol/min per mg) or total activity/g wet wt.]. Thus it appears that cyclohexane hydroxylase levels were highest in virgin rats, progressively decreasing in pregnant and

Fig. 4. Cyclohexane-hydroxylation activity determined by NADPH-oxidation rates in rat mammary gland

Each microsomal preparation obtained from rats in one of ten developmental stages. All rats (Sprague-Dawley) were weaned at 21 days and killed at approximately the same age. Furthermore, all pregnancies were first pregnancies. Each bar represents the mean $\pm$ S.E.M. value for duplicate assays. Abbreviations used: d, day; PG, pregnant; LAC, lactating; Post-LAC, post lactation.

lactating rats, and again increasing during involution of the mammary gland. These results are interesting in light of the oestrogen-receptor content in the rat mammary gland during differentiation. It has been reported that oestrogen-receptor levels are higher in lactating females than in virgin or pregnant rats (Gardner & Wittliff, 1973), whereas other workers have reported variable concentrations of oestrogen receptors during pregnancy (Leung *et al.*, 1976; Forsythe & Hayden, 1977). Most recently, investigators in a comprehensive study of oestrogen- and progesterone-receptor levels during rat mammary-gland differentiation, demonstrated that oestrogen-receptor levels are indeed lowest in virgin rats (Wiehle & Wittliff, 1983). Also, they observed that oestrogen receptor levels rose during pregnancy and again after parturition, with the highest levels observed at late lactation, before returning to low levels in the involuting gland. Furthermore, these authors reported that progesterone-receptor levels were highly variable or undetectable in both pregnancy and lactation, with the highest levels observed for the 18-day involuting mammary gland. These data suggest that oestrogen, and perhaps progesterone, receptors are not involved in the regulation of cytochrome *P*-450 biosynthesis, since enzyme activities seem to oppose receptor levels in the rat mammary gland.

Cyclohexane hydroxylase activities determined at 30°C and pH 7 expressed as pmol of NADPH oxidized/min per mg of protein are shown for 139 breast tumours in Fig. 5. Activities ranged from less than 1 to more than 5000 pmol/min per mg of protein. The mean activity for the 139 breast tumours was 1340 ± 140 pmol/min per mg, with over 40% of the tumours assayed having activities in the range of those found in untreated rabbit liver microsomes. The levels of this enzyme activity determined in our laboratory ranged from 1000 to 3000 pmol/min per mg. Thus many tumours exhibited levels of cytochrome *P*-450 activity comparable with those in tissues (e.g. liver) actively engaged in drug metabolism.

As part of our study to examine the role of cytochrome *P*-450 in breast cancer, enzyme levels were related to established markers of endocrine response, i.e. the oestrogen- and progesterone-receptor levels. As demonstrated in Figs. 6 and 7, there was no apparent correlation between cyclohexane hydroxylase activity and oestrogen receptors ($r = 0.02$) and/or progesterone receptors ($r = 0.05$), as shown in Figs 6 and 7 respectively.

Cytochrome *P*-450 biosynthesis does not appear to be regulated by either oestrogen or progesterone receptors, since no correlation was found between receptor levels and cyclohexane hydroxylase activity. However, this is not to imply that there is no

Fig. 5. Distribution of cyclohexane-hydroxylation activity in breast-carcinoma microsomes. Activities are placed in one of nine categories for 139 breast carcinomas. Numbers above the bars refer to the percentage of total tumours in each activity category. Values are averages from duplicate or triplicate assays.

Fig. 7. Relationship between cyclohexane-hydroxylation activity and progesterone-receptor concentration from 127 breast-tumour specimens. Cyclohexane-dependent NADPH oxidation activities of less than 10 pmol/min per mg were scored as 10, as were progesterone-receptor levels below 10 fmol/mg of cytosol protein. Nine breast tumours had cyclohexane hydroxylation activity less than 10 pmol/min per mg and progesterone-receptor levels below 10 fmol/mg of cytosol protein. Least-squares analysis showed that the best fit line through the data had a correlation coefficient (r) of 0.05.

Fig. 6. Relationship between cyclohexane hydroxylation activity and oestrogen-receptor concentration from 125 breast-tumour specimens. Cyclohexane-dependent NADPH oxidation activities of less than 10 pmol/min per mg were scored as 10, as were oestrogen-receptor levels below 10 fmol/mg of cytosol protein. Least-squares analysis showed that the best-fit line through the data had a correlation coefficient (r) of 0.02.

relationship between these two activities in a given breast tumour from the standpoint of a patient's response to additive hormone therapy. For instance, a significant number of tumours had high levels of both receptors (oestrogen and progesterone) and enzyme activity.

In addition to human breast tumours, human uterus (normal and neoplastic) and human ovary were chosen for determining levels of cyclohexane hydroxylase activity. All three human tissues examined (breast, uterus, ovary) had average activities in the range of those found in untreated mammalian liver (Table 1). As also shown in Table 1, leiomyomas (uterine fibroid tumour) contained significantly higher cyclohexane hydroxylase activity than did non-tumorous uterus ($P < 0.01$) when expressed in terms of specific activity, but not in terms of total activity ($P > 0.1$). This result suggests that other protein levels may be decreased in the leiomyomas. Moreover, it is of interest that leiomyomas are known to contain higher levels of oestrogen and progesterone receptors, which make leiomyomas more sensitive to endogenous and exogenous oestrogen and progesterone than normal

Table 1. *Summary of cyclohexane hydroxylase activity in various human and rat reproductive tissues*

Results are means $\pm$ S.E.M. for the numbers of animals given in parentheses. Results in group VI are specific-activity ranges determined in our laboratory for untreated and phenobarbital-induced rabbit liver microsomes.

Group	Tissue	Specific activity (nmol/min per mg of protein)
I	Human breast tumours	1.34 ± 0.14 (139)
II	Human uterus leiomyoma	2.87 ± 0.26 (17)
III	Non-tumorous human uterus	1.60 ± 0.11 (30)
IV	Human ovary	1.04 ± 0.23 (6)
V	Rat mammary gland	3.03 ± 0.64 (52)
	(A) Virgin	5.96 ± 1.27 (9)
	(B) Pregnant	1.05 ± 0.22 (17)
	(C) Lactating	0.24 ± 0.05 (21)
	(D) Post lactating	0.46 ± 0.16 (5)
VI	Rabbit liver	
	(A) Untreated	1–3
	(B) Phenobarbital-induced	7–20

myometria (Soules & McCarty, 1982; Wilson *et al.*, 1980).

It is now generally accepted that receptor determination is helpful in selecting patients for chemotherapy or hormone therapy (Maass *et al.*, 1980; Jensen *et al.*, 1971; Wittliff, 1984). However, in a significant fraction (approx. 40%) of patients whose tumours exhibit the oestrogen receptor, no response to endocrine therapy was observed (Maass *et al.*, 1980; Jensen *et al.*, 1971; Wittliff *et al.*, 1978). It has been suggested (Wittliff *et al.*, 1978) that this lack of response may be due to 'defective' oestrogen receptors in certain tumours. On the basis of the results of the present study, it is possible that women with breast or uterine tumours containing oestrogen receptors and high cytochrome *P*-450 activity may be less responsive to therapies utilizing tamoxifen or other hormonal agents by metabolizing them to inactive metabolites. Thus the oestrogen-receptor mechanism would be rendered ineffective. Future considerations need to include metabolism studies in tissue systems which display high enzyme activity (i.e. breast and uterus) utilizing radiolabelled oestrogens and anti-oestrogens, as well as testing our hypothesis by analysis of patient response to administrative endocrine therapy and correlating this with cytochrome *P*-450 activity.

We greatly appreciate the contribution of Dr. Ronald D. Wiehle of the Hormone Receptor Laboratory, who provided the tissues for the study with rodent mammary

tissues. Dr. Robert D. Gray, Department of Biochemistry, is acknowledged for his generosity in supplying the purified cytochrome *P*-450 from rat liver. We thank Mr. Steven L. Wagner for assisting in preparation of the control microsomes from rat liver. We also appreciate the efforts of Dr. George Barrows, Department of Pathology, and Dr. Glen Hofmann and Dr. Joseph Sanfilippo, Department of Obstetrics and Gynecology, in the collection of the human tissue specimens. T. I. S. is a recipient of a Homberger Graduate Fellowship. This study was supported in part by U.S. Public Health Services grants CA-19657 and CA-34211 from the National Cancer Institute.

References

- Armbrecht, A. J., Birnbaum, L. S., Zenser, T. V., Maltammal, M. B. & David, B. B. (1979) *Arch. Biochem. Biophys.* **197**, 277–284
- Betz, G., Tsai, P. & Weakley, R. (1976) *J. Biol. Chem.* **251**, 2839–2841
- Dean, W. L. & Coon, M. J. (1977) *J. Biol. Chem.* **252**, 3255–3261
- Estabrook, R. W., Cooper, D. Y. & Rosenthal, O. (1963) *Biochem. Z.* **338**, 741–755
- Fang, W. F. & Strobel, H. W. (1978) *Arch. Biochem. Biophys.* **186**, 128–138
- Forsythe, I. A. & Hayden, T. J. (1977) *Symp. Zool. Soc. London* **41**, 135–163
- Gardner, D. G. & Wittliff, J. L. (1973) *Biochemistry* **12**, 3090–3095
- Gillette, J. R. (1966) *Adv. Pharmacol.* **4**, 219–261
- Gray, R. D. (1982) *J. Biol. Chem.* **257**, 1086–1094
- Green, D. E., Mii, S. & Kohout, P. M. (1955) *J. Biol. Chem.* **217**, 551–567
- Guengerich, F. P. & Martin, M. V. (1980) *Arch. Biochem. Biophys.* **250**, 305–379
- Guengerich, F. P., Wang, P., Mason, P. S. & Mitchell, M. B. (1981) *Biochemistry* **20**, 2370–2378
- Hoffman, A. R., Paul, S. M. & Axelrod, J. (1979) *Cancer Res.* **39**, 4584–4587
- Jensen, E. V., Block, G. E., Smith, S., Kyser, K. & DeSombre, E. R. (1971) *Natl. Cancer Inst. Monogr.* **34**, 55–70
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leung, B. S., Jack, W. M. & Reiny, C. G. (1976) *J. Steroid Biochem.* **7**, 84–85
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lu, A. Y. H. & Coon, M. J. (1968) *J. Biol. Chem.* **243**, 1331–1332
- Lu, A. Y. H., Strobel, H. W. & Coon, M. J. (1970) *Mol. Pharmacol.* **6**, 213–220
- Nordblom, G. D. & Coon, M. J. (1977) *Arch. Biochem. Biophys.* **180**, 343–347
- Maass, H., Jonat, W., Stolzenbach, G. & Trams, G. (1980) *Cancer* **46**, 2835–2837
- Mason, M. E. & Okey, A. B. (1981) *Cancer Res.* **41**, 2778–2782
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2379–2385
- Osawa, Y., Tochigi, B., Higashiyama, T., Yarborough, C., Nakamura, T. & Yamamoto, T. (1982) *Cancer Res.* **2**, 3299–3306

- Poland, A. P., Glover, E., Robinson, J. R. & Nebert, D. W. (1974) *J. Biol. Chem.* **249**, 5599–5606
- Ryan, K. J. & Engel, L. L. (1957) *J. Biol. Chem.* **225**, 103–114
- Senler, T. I., Dean, W. L., Pierce, W. M. & Wittliff, J. L. (1985) *Anal. Biochem.* **144**, 152–158
- Serabjit-Singh, C. J., Wolf, C. R. & Philpot, R. M. (1979) *J. Biol. Chem.* **254**, 9901–9907
- Sesame, H. A., Ames, M. M. & Nelson, S. D. (1977) *Biochem. Biophys. Res. Commun.* **78**, 919–926
- Soules, M. R. & McCarty, K. S. (1982) *Am. J. Obstet. Gynecol.* **143**, 6–11
- Wagner, S. L., Dean, W. L. & Gray, R. D. (1984) *J. Biol. Chem.* **259**, 2390–2395
- Wiehle, R. D. & Wittliff, J. L. (1983) *Comp. Biochem. Physiol.* **76**, 409–417
- Wilson, E. V., Yang, F. & Rees, E. D. (1980) *Obstet. Gynecol.* **55**, 20–24
- Wittliff, J. L. (1984) *Cancer* **53**, 630–643
- Wittliff, J. L., Lewko, W. M., Park, D. C., Kute, T. E., Baker, D. T., Jr. & Kane, L. N. (1978) in *Hormones, Receptors and Breast Cancer* (McGuire, W. L., ed.), pp. 325–359, Raven Press, New York
- Wittliff, J. L., Wiehle, S. A., Sandoz, S. P., Fisher, B. & Durant, J. R. (1980) in *Cytotoxic Estrogen in Hormone Receptor Tumors* (Raus, J., Martens, H. & Leclercq, G., eds.), pp. 147–163, Academic Press, London