Genetic Differences in the Extent of Aryl Hydrocarbon Hydroxylase Induction in Mouse Fetal Cell Cultures

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SUMMARY

Benz[a]anthracene induces aryl hydrocarbon hydroxylase activity in secondary fetal cell cultures derived from various inbred mouse strains. The maximally inducible level of hydroxylase activity from the C57BL/6N strain is 4 to 6 times higher than that from the DBA/2N strain in cell cultures derived from the entire mouse fetus. There are no differences between C57BL/6N and DBA/2N cell cultures in the rate of uptake of benz[a]anthracene by the cells, in the binding of polycyclic hydrocarbon to cellular material, or in the rate of degradation of the induced hydroxylase activity. A 24-hour treatment of the cell cultures with benz[a]anthracene produces increases in the microsomal content of total protoheme and CO-binding cytochrome (about 88% and 120%, respectively) in C57BL/6N cells compared to about 31% and 33%, respectively, in DBA/2N cells.

In cells exposed to benz[a]anthracene plus cycloheximide and then grown in fresh growth medium, the rise in hydroxylase activity in C57BL/6N cells is at least 4 times greater than that from DBA/2N cells. In cells exposed to benz[a]anthracene and then treated with actinomycin D, the stimulatory phenomenon by the antibiotic is at least 4 times greater in C57BL/6N cells than that in DBA/2N cells.

The decreases in (a) formation of CO-binding hemoprotein, (b) spectral shift in the absorbance peak of the reduced cytochrome-CO complex, and (c) expression of induction-specific RNA may be related to the decreased extent of aryl hydrocarbon hydroxylase induction in DBA/2N cells.

The induction of aryl hydrocarbon hydroxylase activity in liver microsomes (2) converts a variety of polycyclic hydrocarbons to phenolic derivatives and is not specific for benz[a]pyrene. However, the substrate specificity of either the constitutive or the induced hydroxylase system from the various mammalian tissues has not been determined. For example, endogenous substrates such as steroids may be hydroxylated by this same enzyme system.

1 This enzyme system is also called benzpyrene hydroxylase and aryl hydroxylase. The nomenclature aryl hydrocarbon hydroxylase is preferred, since the mixed function oxygenase obtained from hamster fetal cell cultures has been examined in detail during the last 2 years (1-7). A certain sequence of events occurs during the stimulation of hydroxylase activity in cells grown in culture by polycyclic hydrocarbons such as benz[a]anthracene dissolved in the growth medium. The entrance of BA into the cell is rapid and independent of temperature; more than 50% of the eventual, net accumulation of intracellular polycyclic hydrocarbon is bound to cellular material in the first 2 min of exposure of the cells to BA (6).

About one-half of the total intracellular polycyclic hydrocarbon is associated with the nuclear fraction, but BA has a strong affinity for all subcellular fractions including cytoplasmic macromolecules (6). The process of microsomal oxygenase induction may be divided into at least two phases (4). In the initial phase, an induction-specific RNA is formed during which polypeptide synthesis is not required. Subsequently, translation involving this induction-specific RNA occurs where RNA synthesis is unnecessary. The first stage can be as short as 20 to 30 min. Thus, if transcription is prevented after the initial 30-min period, enzyme induction proceeds normally for the next 4 hours; however, inhibition of RNA synthesis before 20 min of exposure of the cells to BA produces no significant increase in hydroxylase activity.

An induction-specific RNA which is made during this initial phase presumably accumulates, especially if its translation is prevented (4). There is a close association between a spectrally distinct, microsomal cytochrome and hydroxylase activity. Both the appearance of this CO-binding cytochrome (5) and induction of the enzyme (7) are dependent upon protein synthesis rather than the presence of intracellular polycyclic hydrocarbon per se. This finding may represent the extranuclear manifestation, either directly or indirectly, of an induction-specific protein during the second phase of microsomal oxidase induction. Also, BA is metabolized immediately upon its entry into the cell by the constitutive hydroxylase, and subsequently by the induced oxygenase as well.

This metabolism of BA results in derivatives which are both covalently bound to cellular proteins, including cytochrome (6).

The abbreviation used is: BA, benz[a]anthracene (1,2-benzanthracene), as recommended by the American Chemical Society (8).
Material and excreted by the cell into the growth medium (6).
It is thus possible that a metabolite of BA, rather than the parent compound itself, is the active inducer.

There are genetic differences in the regulation of both constitutive and inducible levels of microsomal enzyme activity. Probably for this reason various mouse strains exhibit differences in sensitivity to drugs (9, 10). The basal levels and the variability in the induction of several hepatic drug-metabolizing enzymes by phenobarbital are widely different among six strains of rabbits (11). Also, in man, as well as in laboratory animals, there are many hereditary differences in the responses to drugs (12). A study of identical and fraternal twins (13) indicated that there is a genetic basis in man for differences in the rate of phenylobutazone metabolism. We recently reported (2) that both the constitutive hydroxylase activity and the extent of oxygenase induction by polycyclic hydrocarbons are very different in several tissues of six strains of mice. In the sequence of events occurring during aryl hydrocarbon hydroxylase induction, which step (or steps) are regulated by genetic differences? There are many possibilities. Thus, perhaps decreases in the extent of microsomal hydroxylase induction may be ascribed to (a) inability of the inducer to penetrate the cell, (b) fewer receptor sites for binding the inducer, (c) repression of synthesis of induction-specific RNA, (d) impaired transport of this RNA from the nucleus to the cytoplasm, (e) decreased rate of translation involving this RNA into induction-specific protein, (f) increased lability of either this RNA or protein, (g) impaired synthesis or assembly of other membrane components essential for hydroxylase activity (e.g. phospholipid or heme moieties), or perhaps (h) a diminished metabolic conversion of BA to active inducer. In this report the variation in hydroxylase induction is compared in fetal cell cultures derived from different mouse strains. By comparing such a genetic difference between cells in culture derived from the same species, we attempt to elucidate the mechanisms by which gene action regulates mammalian microsomal enzyme induction.

Experimental Procedure

Materials

The polycyclic hydrocarbons BA and benzo[a]pyrene, obtained from Sigma, were purified by recrystallization twice from benzene. Cell culture supplies were purchased and prepared as previously described (1), except for the use of Viokase (5) instead of trypsin for the dispersal of cells from minced fetal tissues. Viokase was purchased from Grand Island Biological Company, Grand Island, New York. The standard complete medium consisted of 10% calf serum in Eagle's number 2 minimal essential medium, pH 7.1, which contained 100 units of penicillin, 100 µg of streptomycin, and 10 units of Mycostatin per ml, 2 mM glutamine, and the amino acids arginine, aspartic acid, asparagine, glutamic acid, glycine, proline, and serine. The BA, dissolved in dimethylsulfoxide, was added to the growth medium, and the concentration of the polycyclic hydrocarbon was determined spectrophotofluorometrically. Control medium was treated identically (1). Dimethylsulfoxide at a final concentration of 0.25% in the growth medium had no detectable effect on cell growth, or RNA and protein synthesis. Nuclear-Chicago solubilizer (a solubilizing quaternary ammonium base), generally labeled [3H]-uridine (4.25 Ci per mmole), uniformly labeled [14C]-protein hydrolysate (52 mCi per milligram of carbon), and generally labeled [3H]-BA (750 mCi per mmole) were purchased from Nuclear-Chicago. Actinomycin D and cycloheximide were generously donated by the Cancer Chemotherapy National Service Center, National Institutes of Health. Inhalation grade carbon monoxide gas was purchased from The Matheson Company, Inc., East Rutherford, New Jersey. NADPH, NADH, and dithiothreitol (Cleveland's Reagent) were obtained from Sigma, St. Louis, Missouri. National Institutes of Health Animal Supply provided us with various strains of pregnant mice, estimated at 12 to 16 days of gestation.

Methods

Preparation of Cell Samples—Secondary cell cultures derived from the entire mouse fetus were used in all experiments. The individual cells derived from the entire fetus were prepared and passaged as had been previously described for hamster fetuses (1, 3). We attempted to match fetal size and gestational age for each experiment, because both the basal enzyme activity and the extent of hydroxylase induction by polycyclic hydrocarbons increase with increasing gestational age of the fetuses from which the cells are derived (3). The cultures were grown in 1 atmosphere of humidified air with 5% CO2 in a water-jacketed incubator from National Appliance Company, Portland, Oregon. The experiments were carried out during the logarithmic phase of growth, which is about 36 to 72 hours after the plating of the cells at a density of approximately 0.5 x 10⁶ per ml. The measurements of the incorporation of [3H]-uridine and [14C]-protein hydrolysate into perchloric acid- and trichloroacetic acid-precipitable material were used as parameters of cellular RNA and protein synthesis, respectively, as previously described (3, 4).

Enzyme Assay—Both the hydroxylase activity and protein concentration were determined in duplicate for the homogenate from cells scraped from one cell culture dish 100 mm in diameter, as previously described (1, 6). For determining enzyme activity, the reaction mixture in a total volume of 1.00 ml included 50 µmoles of Tris-chloride buffer, pH 7.5, 0.36 µmole of NADPH, 0.39 µmole of NADH, 3 µmoles of MgCl₂, 0.10 ml of cell homogenate (containing 0.3 to 1.0 mg of protein), and 80 µmoles of the substrate benzo[a]pyrene added in 40 µl of methanol just prior to incubation. Following the 30-min incubation at 37°, the alkali-extractable metabolites were examined with an Amico-Bowman model 4-8202 SPF recording spectrophotofluorometer (American Instrument Company, Baxter Laboratories, Silver Spring, Maryland); fluorescence corresponding to 3-hydroxybenzo[a]pyrene has an activation peak at about 396 nm and an emission maximum at about 522 nm (1). The fluorescence of a blank sample, to which benzo[a]pyrene had been added after the 30-min incubation and addition of acetone, was subtracted from the fluorescence of each experimental sample. One unit of aryl hydrocarbon hydroxylase activity has been defined (5-7) as that amount of enzyme catalyzing the formation per min at 37°, of
hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[a]pyrene. Protein concentrations were determined by a slight modification of the method as described by Lowry et al. (14), with crystalline bovine serum albumin as the standard.

**Measurement of Polycyclic Hydrocarbon Associated with Cellular Material**—The level of intracellular BA (and metabolites) was determined as described previously (6, 7). Generally labeled \(^3\)H-BA was used at a final specific activity of 50 to 75 \(\mu\)Ci per \(\mu\)mole; thus, growth medium containing 1 pmole of \(^3\)H-BA produced approximately 0 to 100 cpm when measured by liquid scintillation technique. In the experimental cell culture system there is no significant amount of tritium exchange between \(^3\)H-polycyclic hydrocarbon and the various cellular constituents during the first 24 hours (6, 7).

The rate of entrance and the total content of intracellular polycyclic hydrocarbon were measured in the following way. The cell surface in each 100-mm tissue culture dish was washed 3 times with a total volume of 30 ml of cold, Dulbecco's isotonic phosphate buffer at the end of each designated time of exposure of the cells to \(^3\)H-BA. The cells were then harvested by scraping and packed by centrifugation. The packed cells were resuspended and washed once in 5 ml of cold, Dulbecco's isotonic phosphate buffer. There was a direct relationship between the number of times the cell surface or the packed cells had been washed and the amount of cells broken, as could be seen microscopically; however, as many as 9 repeated washings of either the cell surface or the packed cells did not significantly alter the association of polycyclic hydrocarbon with protein. Furthermore, the dialysis of a 3.5-ml suspension of intact cells or a 3.5-ml cellular homogenate (each containing about 14 mg of cellular protein) against 2 liters of isotonic phosphate buffer, pH 7.1, over a 24-hour period resulted in no change in the amount of polycyclic hydrocarbon associated with cellular material. The washed, packed cells were then homogenized in 0.25 M sucrose-0.05 M Tris-chloride buffer, pH 7.5, and the cellular homogenate (0.10-ml aliquots) was digested in 1.0 ml of the Nuclear-Chicago solubilizer. The radioactivity of duplicate or triplicate samples was measured in a Packard liquid scintillation counter. In this study, no attempt was made to determine whether the radioactivity was associated with the parent compound BA or a metabolite of BA. Also, we have not measured in this study the relative affinity of the polycyclic hydrocarbon binding to cellular material, except as described in the next paragraph. Protein determinations and cell counts on each sample were concomitantly carried out.

The amount of polycyclic hydrocarbon which is presumably bound covalently to cellular material was measured in the following way. The cellular macromolecules were precipitated by the addition of 10 ml of cold 10% trichloracetic acid to the monolayer of cells in the tissue culture dish. The trichloroacetic acid-insoluble material was then washed twice with cold 4% trichloroacetic acid, once with cold ethanol, once with an ethanol-ether-chloroform (2:2:1) mixture, and 3 times with acetone. Any radioactive material remaining associated with cellular protein and nucleic acid following this type of purification (15) is presumed to be covalently bound. The residual radioactivity from trichloroacetic acid-insoluble material from cells harvested at zero time was subtracted from the values obtained after various periods of exposure to \(^3\)H-BA. Weighed amounts of the dried, acetone-insoluble residue were solubilized in 1.0 ml of Nuclear-Chicago solubilizer, and the radioactivity of each sample was determined by liquid scintillation technique. Duplicate measurements of cell count and radioactivity in the trichloroacetic acid-insoluble fraction were carried out on identical dishes at each point in time.

In this paper for the sake of clarity, the total content of intracellular polycyclic hydrocarbon is expressed as 10\(^{-16}\) mole per cell and that presumed to be covalently bound as 10\(^{-18}\) mole per cell. No distinction is being made between polycyclic hydrocarbon bound on the cell surface and that which has entered the cell. Since we are using a heterogeneous cell population, we recognize that these values represent the average level of polycyclic hydrocarbon per cell. The maximal saturating level of intracellular polycyclic hydrocarbon differs by a factor of 2- to 3-fold, depending on cell type (6). In similar experiments performed at different times, the fact that there was good agreement in these values indicates that there is relatively little change from week to week in the heterogeneous cell population derived from the entire mouse fetus.

**Measurement of Alkali-soluble Metabolites of Benz[a]anthracene in Growth Medium**—The growth medium was precipitated with an equal volume of cold acetone; and the polycyclic hydrocarbon from the 2.0-ml aqueous-acetone mixture was extracted 4 times with 1.0-ml aliquots of hexane. The polar metabolites in the hexane-acetone phase were then extracted with 1 N NaOH, and radioactivity of 0.05-, 0.10-, and 0.20-ml amounts of the alkali fraction was determined in a Packard liquid scintillation counter. Not all of the derivatives of BA metabolized by aryl hydrocarbon hydroxylase are alkali extractable from a hexane-acetone mixture.

**Spectrophotometry**—The difference spectra of turbid microsomal fractions were measured as previously described (5) in 1-cm cuvettes at room temperature in an Amino-Chance dual wave length recording spectrophotometer, very generously made available by Mr. Robert Kate of the American Instrument Division of Baxter Laboratories, Silver Spring, Maryland. The 78,000 \(\times\) g pellet, or microsomal, fraction was prepared by successive centrifugations of the cell homogenate containing 0.25 M sucrose and 3 mM dithiothreitol, as described elsewhere (5). The fractions were suspended in 30% glycerol-0.25 M potassium phosphate buffer, pH 7.25; at protein concentrations between 3.0 and 10 mg per ml, the proteome and cytochrome determinations for the colorless microsomal suspensions from cell cultures were linear. A fixed, 0.5-mm slit opening was used, with a scanning speed of 0.5 mm per sec and the most rapid response time. Wave length measurements were standardized by the use of a holmium oxide crystal.

The total heme content was determined from the difference spectrum between the oxidized and \(N_a\)O\(_2\)-reduced forms of microsomal cytochromes (16); the method was modified slightly by including 0.1 mm ethylenediaminetetraacetate in the reaction mixture. The difference spectrum was obtained in 30% glycerol-0.25 M potassium phosphate buffer, pH 7.25; at protein concentrations between 3.0 and 10 mg per ml, the phenome and cytochrome determinations for the colorless microsomal suspensions from cell cultures were linear. A fixed, 0.5-mm slit opening was used, with a scanning speed of 0.5 mm per sec and the most rapid response time. Wave length measurements were standardized by the use of a holmium oxide crystal.

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Unpublished data.
Fig. 1. Kinetics of aryl hydrocarbon hydroxylase induction in secondary fetal cell cultures derived from four different mouse strains, C57BL/6N, NIH G.P., AKR/N, and DBA/2N. The size and gestational age of the fetuses used in making the primary cultures were identical. The inducer, 13 \( \mu \)M BA, was added to the cultures during logarithmic growth. In this figure and in all subsequent figures depicting enzyme activity, the ordinate is expressed as specific activity, units per mg of cellular protein. Each point represents duplicate determinations of both enzyme activity and protein content on a cellular homogenate.

Inducible Hydroxylase Activity in Fetal Cell Cultures from Difference Mouse Strains—We first examined the kinetics of aryl hydrocarbon hydroxylase induction in secondary cultures derived initially from fetuses of about eight different mouse strains. Genetic differences in the basal levels and in the hydroxylase activity inducible by polycyclic hydrocarbons among the various mouse strains in cell culture were similar to those found (2) in vivo. Fig. 1 shows representative examples of the kinetics of hydroxylase induction by BA from several of the genetically different mouse fetal cell cultures. In every case a maximal level of inducible hydroxylase activity was reached between 16 and 24 hours, while among the various strains we found large differences in this maximal specific activity. The rate of accumulated hydroxylase activity and maximal oxygenase levels inducible by BA in C57BL/6N cell cultures were approximately 4 times greater than those parameters in DBA/2N mouse fetal cells. The extent of hydroxylase induction in cell cultures derived from all other mouse strains examined was less than that found in C57BL/6N cells and greater than that seen in DBA/2N cells. Therefore, in the remainder of experiments summarized in this report, we used cell cultures derived from those two mouse strains, where the genetic difference in the variability of hydroxylase induction is maximal. The average generation time for the heterogeneous fetal cell cultures was estimated to be about 28 hours for both the C57BL/6N and the DBA/2N cells.6

Kinetics of Entry and Binding of Polycyclic Hydrocarbon in the Cell as Function of Initial Extracellular Concentration of Benz[a]anthracene—One possible reason for a genetic difference in the extent of hydroxylase induction between C57BL/6N and DBA/2N cells is a dissimilar rate in the intracellular uptake or binding of inducer. This possibility is examined in Fig. 2. Fig. 2A illustrates that in C57BL/6N and DBA/2N cells there were differences in the rate of net accumulation of aryl hydrocarbon hydroxylase activity inducible by either 13 \( \mu \)M or 1.3 \( \mu \)M BA. Starting with either of these two levels of BA in the growth medium, the rate of induced enzyme appearance in C57BL/6N cells was at least 4-fold greater than that in DBA/2N cells. A negligible rise in hydroxylase activity was seen in C57BL/6N cultures exposed to 0.13 \( \mu \)M BA for 6 hours.

Fig. 2B shows the rate of entry and binding of the total intracellular polycyclic hydrocarbon during the experiment depicted in Fig. 2A. With each of the three initial concentrations of BA in the medium, the maximal level of polycyclic hydrocarbon in the cell was attained between 30 and 120 min, and a significant decrease in this intracellular content was observed after this time. Also, at each of the three starting concentrations of BA in the medium, the intracellular level of polycyclic hydrocarbon in DBA/2N cells was always as high or higher than that in C57BL/6N cells at every point in time. Thus, the decrease in the extent of hydroxylase induction in DBA/2N cells does not appear to be due to a grossly diminished rate of uptake of BA into the cell. We have previously shown (7) that the appearance of the induced hydroxylase system is in some way responsible for the decrease in the content of polycyclic hydrocarbon in hamster fetal cells exposed to BA for more than 30 min. Therefore, the higher level of intracellular polycyclic hydrocarbon in DBA/2N cells seen in Fig. 2B may reflect the presence of less of this enzyme in DBA/2N cells than in C57BL/6N cells.

# RESULTS

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We reported recently (6) that the hydroxylase system is responsible for polycyclic hydrocarbon which is presumably bound covalently to cellular material, since this binding in hamster fetal cells is blocked by a specific microsomal oxygenase inhibitor, 2-diethylaminoethyl-2,2-diphenylvalerate HCl, and does not occur in cell cultures which contain no constitutive or inducible hydroxylase activity. Fig. 2C depicts the rate at which polycyclic hydrocarbon is presumed to be bound covalently to cellular macromolecules during the experiment illustrated in Fig. 2, A and B. The covalently bound metabolites of BA represented 0.5 to 1.0% of the total concentration of polycyclic hydrocarbon in cells exposed to 13 μM BA, and ranged between 5 and 10% of the total in cells exposed to 0.13 μM BA. When the cells were treated with 13 μM BA and contained between 6 × 10⁻¹⁴ and 12 × 10⁻¹⁶ mole of total polycyclic hydrocarbon per cell, about 5 × 10⁻¹⁸ mole of polycyclic hydrocarbon per cell was bound covalently after about 30 min in C57BL/6N cells and after about 4 hours in DBA/2N cells. However, at levels of total polycyclic hydrocarbon between 0.05 × 10⁻¹⁴ and 2 × 10⁻¹⁴ mole per cell, the rate at which polycyclic hydrocarbon was

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Fig. 2. A, kinetics of aryl hydrocarbon hydroxylase induction. B, kinetics of uptake of the total content of intracellular polycyclic hydrocarbon, and C, kinetics of the amount of polycyclic hydrocarbon bound to trichloracetic acid-precipitable material, in cells exposed to three different, initial concentrations of RA dissolved in the growth medium. The secondary cell cultures were initially derived from entire fetuses from either the C57BL/6N (Curve C) or the DBA/2N (Curve D) mouse strain.
EXPOSURE TO BA (hours)

NANOMETERS

Fig. 5. CO-difference spectra of microsomal fractions obtained from fetal cells derived from either the C57BL/6N (C) or the DBA/2N (D) mouse strain during the induction of aryl hydrocarbon hydroxylase activity by 13 μM BA. The absorbance spectra are placed arbitrarily on this graph, and only differences within each single spectrum are relevant to changes in absorbance. Protein concentrations were 5.1, 7.2, and 7.0 mg per ml for the fractions from C57BL/6N (C) cells exposed to BA for 0, 12, and 24 hours, respectively, and 6.1, 6.0, and 7.2 mg per ml for the microsomes from DBA/2N (D) cells treated with BA for 0, 12, and 24 hours, respectively. Spectral maxima at 418.7 and 453.2 nm from a holmium oxide crystal are shown.

presumably bound covalently to cellular material was not different between C57BL/6N and DBA/2N cells. Perhaps the more rapid appearance of covalently bound metabolites of BA in C57BL/6N cells compared to that in DBA/2N cells exposed to 13 μM BA may be the result of a combination of factors, higher levels of intracellular BA in the presence of greater amounts of the constitutive hydroxylase. While enzyme induction does not occur in the presence of 0.13 μM BA initially in the medium (Fig. 2A), covalently bound polycyclic hydrocarbon was found in these cells (Fig. 2C). Thus, these data indicate that the basal levels of the microsomal oxygenase, even in DBA/2N cells, are sufficient to metabolize BA to detectable amounts of derivatives presumably bound covalently to cellular macromolecules.

We thus conclude from Fig. 2 that the uptake of BA and the degree of binding of polycyclic hydrocarbon are not impeded in DBA/2N cells. We also found no significant differences between the C57BL/6N and DBA/2N cells in the distribution of total polycyclic hydrocarbon, or that presumably bound covalently, in the nuclear, mitochondrial, microsomal, and cytoplasmic fractions. Therefore, within the limitations of our measurements, the decreased extent of the hydroxylase induction in DBA/2N cells is not due to differences in the uptake or binding of polycyclic hydrocarbon.

It was thought that perhaps the rate of hydroxylase induction in DBA/2N cells could be increased by treatment of the cells with excessive amounts of BA. However, we found that 50 μM BA, a concentration of polycyclic hydrocarbon that is close to saturation of the growth medium, depressed the rate of net accumulation of the induced enzyme in C57BL/6N cells by about 40% and did not appreciably alter the hydroxylase induction in DBA/2N cells.

Half-life of Induced Hydroxylase Activity—Fig. 3 illustrates that the half-life for the maximally induced oxygenase from either C57BL/6N or DBA/2N cells was about 2.4 hours. The rate of degradation of the induced enzyme was first order during the initial 6 hours of growth in fresh control medium. Whether the induced hydroxylase system was allowed to decay in cells grown in control medium alone or in cells where protein synthesis had been blocked by cycloheximide, similar half-lives were obtained. Thus, the decreased extent of hydroxylase induction in DBA/2N cells is not the result of a more rapid destruction of induced enzyme activity in these cells.

Metabolites of Benz[a]anthracene Excreted by Cells during Hydroxylase Induction—Fig. 4 shows that the rate of excretion of polar derivatives of BA in the growth medium by C57BL/6N cells is at least 4 times greater than that by DBA/2N cells. Thus, the rate at which metabolites of BA appear in the medium parallels the rate of net accumulated activity of the induced microsomal hydroxylase system (6). Cycloheximide, a protein synthesis inhibitor which prevents aryl hydrocarbon hydroxylase induction by BA (3-5, 7), and 2-diethylaminoethyl-2,2-diphenylvalerate HCl, a specific microsomal oxygenase inhibitor, both block the appearance of polar polycyclic hydrocarbons excreted by hamster fetal cells (6). Also, cells without constitutive or inducible hydroxylase activity excrete no polar metabolites of BA (6). Actinomycin D, which also inhibits hydroxylase induc-
fetal cell cultures with BA produces a spectrally distinct cytotoxic effect of 7,12-dimethylbenz[a]anthracene by embryonic rodent cells. The fetal cells were derived from either the C57BL/6N (C) or the DBA/2N (D) mouse strain.

Table I

<table>
<thead>
<tr>
<th>Hours of exposure to BA</th>
<th>Total heme</th>
<th>Total CO-binding pigment</th>
<th>Hydroxylase specific activity</th>
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<td>units/mg cellular protein</td>
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Changes in Microsomal CO-binding Cytochrome During Hydroxylase Induction—Aryl hydrocarbon hydroxylase is one of the NADPH-linked, mixed function oxygenases which have as an active site for their oxidative function the CO binding pigment Pas, (16, 19, 20). The active form of this membrane-bound hemoprotein has a Soret maximum at about 450 nm upon combination of the reduced form with CO. Treatment of hamster fetal cell cultures with BA produces a spectrally distinct cytochrome, the reduced hemoprotein-CO complex of which absorbs maximally at about 446 nm (3, 5). The appearance of this cytochrome is associated with hydroxylase activity, because the blue spectral shift is dependent upon protein synthesis rather than the intracellular level of polycyclic hydrocarbon per se (5). This hypochromic spectral shift of the Soret maximum is detectable only after 8 or more hours of exposure of the cells to BA and maximal after about 20 hours (5). Whether these two spectrally distinguishable CO-binding pigments represent two hemoproteins or a single hemoprotein interconvertible between two forms is not known. The rate of formation of the new, spectrally distinct cytochrome and increases in the content of total microsomal heme and CO-binding pigments were therefore compared in C57BL/6N and DBA/2N cells during polycyclic hydrocarbon treatment.

Fig. 5 demonstrates the absorption spectra of the total microsomal CO-binding cytochromes from C57BL/6N and DBA/2N cells during 24 hours of exposure to BA. The hypochromic shift in the spectral peak was evident in C57BL/6N cells exposed to BA for 12 hours and maximal after 24 hours of treatment with the polycyclic hydrocarbon. However, the absorbance maximum from microsomes of the DBA/2N cells exposed to BA for 24 hours was between 448 and 449 nm. Treatment of either type of cells with BA for more than 20 hours produces no further blue spectral shift in these absorption maxima. The absorbance at about 420 nm is due to inactivation of the hemoprotein CO complex absorbing in the 446 to 450 nm region and reflects the unavoidable severity of conditions involved during the isolation of microsomes from these cells.

Table I summarizes the content of total heme and CO-binding cytochromes and the hydroxylase activity during the experiment depicted in Fig. 5. The concentration of the CO-binding cytochrome (or cytochromes) absorbing maximally in the 446 to 450 nm region was estimated by using an extinction coefficient of 91 mmole \( \times \) cm\(^{-1}\) for the difference in absorbance between the Soret maximum of the reduced hemoprotein-CO complex and the baseline at 490 nm, as previously reported (5). Exposure of C57BL/6N cells to BA for 24 hours produced about 88%, 120%, and 13-fold increases in total heme, CO-binding cytochromes, and aryl hydrocarbon hydroxylase activity, respectively. Similar treatment of DBA/2N cells caused about 31%, 33%, and 4-fold increases in total microsomal protoheme, CO-binding cytochromes, and hydroxylase activity, respectively. The smaller increases in total microsomal heme content relative to the increases in CO-binding pigment presumably reflect the smaller rise (5) in the amount of microsomal cytochrome \( b_6 \). It is of interest that, in the two types of cells under control conditions, the total content of both microsomal heme and CO-binding...
cytochrome is about the same, while treatment with BA subsequently produces a difference in response.

This finding is further supported by our studies in vivo. In liver from these two strains of mice, we found increases in the total microsomal protoporphyrin and CO-binding pigment following administration of polycyclic hydrocarbons that were similar to those in C57BL/6N and DBA/2N cells in culture. Also, while the absorption peak of the reduced cytochrome-CO complex from hepatic microsomes of the C57BL/6N mouse is shifted from about 450 nm to about 448 nm after polycyclic hydrocarbon treatment, we found no shift in this spectral peak from DBA/2N hepatic microsomes after similar treatment in vivo.

Therefore, while entry and binding of the polycyclic hydrocarbon is not different between the two types of cells, there is a difference in the rate of newly formed hemoprotein which is essential for aryl hydrocarbon hydroxylase activity. This genetic difference in the response to BA may be transcriptional or posttranscriptional in nature. We thus designed experiments in an attempt to distinguish between these two possibilities.

Accumulation of Induction-specific RNA During Hydroxylase Induction—Fig. 6 illustrates microsomal oxidase activity in C57BL/6N and DBA/2N cells grown in fresh growth medium, following a 12-hour exposure of the cells to BA in the presence of the protein synthesis inhibitor cycloheximide. Allowing transcription in the absence of translation causes an induction-specific RNA to accumulate, and protein synthesis involving this RNA can then proceed when the cycloheximide and most of the polycyclic hydrocarbon are removed (4, 7). Replacement of the medium containing BA plus cycloheximide with fresh medium containing BA alone produced an immediate, linear increase in the hydroxylase activity in both types of cells, indicating the effective reversal of the block in translation. However, during the 10 hours of exposure to BA in the absence of cycloheximide, the rate of accumulation of microsomal oxygenase activity was more than 5-fold faster in C57BL/6N cells, compared to that in DBA/2N cells. Whether the BA plus cycloheximide had been replaced with fresh control medium or with BA-containing medium, a similar rise in net accumulated enzyme activity was seen in C57BL/6N cells for the first 2 to 3 hours. Under similar conditions with DBA/2N cells exposed to either control medium or BA alone, the small increase in net accumulated hydroxylase activity was about the same for at least 2 hours.

The inhibitor of DNA-dependent RNA synthesis, actinomycin D, prevents the induction of aryl hydrocarbon hydroxylase activity if the antibiotic is added during the first 20 min of exposure of hamster fetal cell cultures to BA (7). However, the later addition of actinomycin D not only is ineffective in blocking induction of the microsomal oxygenase, but the antibiotic is actually stimulatory (3, 4, 7). Also, when the hydroxylase activity is maximally induced by BA and then allowed to decay by growing the hamster cells in fresh growth medium, actinomycin D stimulates a return of induced enzyme activity for 7 to 10 hours (7). This stimulatory effect of actinomycin D has been recently reviewed (21) and appears most likely to be caused by the inhibition of synthesis of a labile translational repressor by relatively high concentrations of the antibiotic. Therefore, we have postulated (7) that this stimulatory phenomenon produced by actinomycin D is an effective method for estimating the relative amount of intracellular induction-specific RNA.

Fig. 7 shows the effect of actinomycin D on hydroxylase ac-
tivity in C57BL/6N and DBA/2N cells during induction of the enzyme by BA. The stimulation of microsomal oxygenase activity by high levels of the antibiotic in C57BL/6N cells exposed to BA for 12 hours was greater than that seen after 6 hours of treatment with BA. Actinomycin D caused a detectable rise in hydroxylase activity in DBA/2N cells after 12 hours of exposure to the polycyclic hydrocarbon, while no stimulatory effect was measurable in DBA/2N cells treated with BA for only 6 hours. In each case the stimulation of enzyme activity by actinomycin D was proportional to the level of induced oxidase activity at the time the antibiotic was added. Thus, the response to actinomycin D in C57BL/6N cells exposed to the inducer for 6 hours was greater than that found in DBA/2N cells treated with BA for 12 hours.

Hydroxylase Induction in Mixtures of Two Cell Types—Is there a factor, freely diffusible between cells in culture, that can influence the extent of microsomal oxygenase induction? Fig. 8 demonstrates that there is no such factor affecting hydroxylase induction in mixtures of varying proportions of C57BL/6N and DBA/2N cells. Cultures containing only C57BL/6N cells that were treated with BA for 12 hours had a specific hydroxylase activity of about 4.4 units, while a similar exposure of 100% DBA/2N cells to BA resulted in about 0.8 unit of enzyme activity. A dashed line is drawn between these two values. In mixtures of the two cell types, the extent of oxidase induction was additive and did not deviate widely from this dashed line at either end of the graph.

DISCUSSION

In this report a genetic difference, involving variations in microsomal enzyme induction, has been examined in secondary cell cultures derived from the same mammalian species, different strains of inbred mice. The use of such an experimental model may elucidate important concepts concerning the genetic regulation of enzyme induction in mammalian cells. A different type of experimental model that could be developed for achieving the same ends is cloning for mutants from an established cell line (22). However, genetic differences examined in secondary cell cultures instead of established cell lines are probably more closely related to these differences in vivo. For example, in this report we have found in secondary cultures that the magnitude of hydroxylase induction and the changes in the content of CO-binding cytochrome in response to polycyclic hydrocarbon treatment are similar to those parameters in vivo. Recently it was reported (23) that the concentration of cytochrome P450 in cells derived from an adrenal cell tumor decreases with subsequent passaging of the cells. There are decreases in the constitutive and inducible levels of aryl hydrocarbon hydroxylase (2) and in the content of microsomal CO-binding pigment associated with repeated subculturing of hamster fetal cells. Also, almost all established cell lines examined (2) have no basal or inducible levels of aryl hydrocarbon hydroxylase activity: two exceptions are the induction of the hydroxylase in m3T3 cultures (2) and measurable increases of this enzyme in HeLa cell cultures. The chances that a genetic difference in a clone of an established cell line reflects a situation identical with that in vivo, rather than an artifact of continuous subculturing, presumably will increase with the age of the cell line. For example, are such genotypes still eukaryotic and free from infection from bacterial L-forms (22)? Also, if spontaneous or viral transformation (24) has occurred, how is this phenomenon related to genetic regulation in vivo? Thus, pharmacogenetic studies with the inbred mouse in vivo and in early subcultures may provide a useful tool for examining the genetic control of microsomal enzyme induction.

We presume that genetic information in some way affects the extent of aryl hydrocarbon hydroxylase induction by BA so that in C57BL/6N cells the enzyme is inducible to levels 4 to 6 times higher than that in DBA/2N cells. How is this genetic regulation accomplished? We have found that the rate of uptake of BA by either type of cell is approximately the same. Gross measurements of polycyclic hydrocarbon binding to cellular material are about the same. However, these determinations represent 10^7 to 10^9 molecules per cell. Thus, if specific receptor sites for the polycyclic hydrocarbon inducer number only 10^6 or 10^7 per cell, we could not identify such a small number of specific binding sites within the cell where 100% or more of the polycyclic hydrocarbon is non-specifically bound. Therefore, we cannot rule out entirely the possibility that DBA/2N cells possess a smaller number of receptor sites for the inducer than do the C57BL/6N cells. Another possible situation is that the number of receptor sites for BA is the same in the two types of cells but that the dissociation constant is greater in DBA/2N cells; this hypothesis is unlikely since a 4-fold increase in the initial concentration of BA in the growth medium did not affect the rate of hydroxylase induction in these cells.

The rates of excretion of polar metabolites of BA in Fig. 4 are similar to the curves of hydroxylase induction depicted in Fig. 1 for these two types of cells in culture. If the decreased extent of hydroxylase induction in DBA/2N cells due to the decreased formation of a derivative of BA, where this derivative rather than the parent compound is the active inducer? If this were the case, then one would expect from Fig. 1 that, as more hydroxylase activity appears in the DBA/2N cells, the rate of net enzyme accumulation and the maximally inducible level of hydroxylase would eventually approach that in C57BL/6N cells. Fig. 4 shows that the amount of polycyclic hydrocarbon metabolites excreted by DBA/2N cells after 12 hours of treatment with BA is about the same quantity as that excreted by C57BL/6N cells after about 5 hours of exposure to BA. Yet, the curve of hydroxylase induction from DBA/2N cells subsequent to the first 12 hours of exposure to BA is not similar to that from C57BL/6N cells after the first 5 hours of treatment with BA. Thus, it would appear that the metabolism of BA to polar products is dependent upon the rate at which hydroxylase activity is induced, rather than the reverse possibility. However, there is a possible exception to this conclusion, if the metabolites produced by the hydroxylase system in C57BL/6N cells differ from those formed by the enzyme in DBA/2N cells. More than a dozen products of BA formed by the rat liver microsomal system have been identified (25, 26). Therefore, it is conceivable that a specific metabolite which is the active inducer may be formed 4 to 6 times faster by the oxidase system in C57BL/6N cells than by that in DBA/2N cells.

In Fig. 7 if the stimulation by actinomycin D were the same in C57BL/6N and DBA/2N cells after 6 or 12 hours of exposure to BA, one might conclude that relatively equal amounts of induction-specific RNA are present in the two types of cells. Such a conclusion would support an hypothesis that a post-transcriptional block in DBA/2N cells is responsible for the diminished ability to induce hydroxylase activity. However, from the results depicted in Figs. 6 and 7, we suggest that there is proportionately less induction-specific RNA in DBA/2N cells...
than in C57BL/6N cells. The other possible interpretation of these data is that we cannot measure the relative amount of induction-specific RNA in DBA/2N cells because translation of the RNA is rate-limiting in these cells. This possibility can only be examined adequately by the use of a protein-synthesizing system in vitro.

The maximally induced levels of hydroxylase activity are about 5 times higher in C57BL/6N cells than in DBA/2N cells. The increase in microsomal CO-binding pigment is almost 4 times greater in C57BL/6N cells than that in DBA/2N cells (120% versus 33%). Thus, the magnitude of increase in CO-binding pigment is strikingly similar to the magnitude of hydroxylase induction. Furthermore, it is of interest that the less than maximal hypochromic shift in the spectral peak of the reduced hemoprotein-CO complex from DBA/2N cells is about one-fourth of the 4-nm blue spectral shift in the microsomal spectrum from C57BL/6N cells. Therefore, we conclude that the extent of aryl hydrocarbon hydroxylase induction is probably related to the rate of formation of the new CO-binding cytochrome, the reduced hemoprotein-CO complex of which absorbs maximally at about 446 nm.

In comparing the various parameters of aryl hydrocarbon hydroxylase induction in C57BL/6N and DBA/2N cells, we have found several diminutions in the DBA/2N cells that may be related, such as (a) the decreased extent of hydroxylase induction, (b) the diminished formation of total microsomal protoporphyrin and CO-binding pigments, (c) the less than maximal blue spectral shift in the absorption maximum of the reduced hemoprotein-CO complex, and (d) indirect evidence for a decreased expression of induction-specific mRNA. Any diminution in induction-specific RNA may reflect a decrease in transcription or any posttranscriptional block. Possibilities of the latter include impaired transport of the RNA from the nucleus to the cytoplasm, decreased rate of translation involving this RNA into induction-specific protein, and increased rate of degradation of either the new RNA or the new protein. The induction-specific protein may be new enzyme protein or may be involved in protoporphyrin synthesis or in the formation of another membrane moiety necessary for the microsomal electron chain (19, 20) such as phospholipid. Conversely, this new protein may be a nonheme component of the hydroxylase system, either activating or stabilizing enzyme activity.

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Genetic Differences in the Extent of Aryl Hydrocarbon Hydroxylase Induction in Mouse Fetal Cell Cultures
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