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Fate of Inducer during Induction of Aryl Hydrocarbon Hydroxylase Activity in Mammalian Cell Culture

I. Intracellular Entry, Binding, Distribution, and Metabolism

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SUMMARY

The fate of the inducer benz[*a*]anthracene has been examined during the induction of aryl hydrocarbon hydroxylase, a microsomal enzyme system inducible by polycyclic hydrocarbons in hamster fetal cells grown in culture. Diffusion of the inducer into the cell is a passive phenomenon; a net accumulation of intracellular polycyclic hydrocarbon is reached after about 30 min of exposure of the cells to benz[*a*]anthracene. Less than 1% of the total intracellular polycyclic hydrocarbon content is bound covalently to cellular macromolecules. This binding process is enzymatic and can be inhibited by 2-diethylaminoethyl-2,2-diphenylvalerate HCl (SKF 525-A).

More than one-half of the covalently bound polycyclic hydrocarbon is located in the nuclear and microsomal fractions. About one-half of the physically bound polycyclic hydrocarbon is associated with the nuclear fraction, but this chemical has a strong affinity for all subcellular organelles and for cytoplasmic macromolecules. In cells derived from various hamster fetal tissues, there are differences in the maximal saturating levels of intracellular polycyclic hydrocarbon and in the rates of hydroxylase induction. Polar metabolites of benz[*a*]anthracene appear in the medium during the process of hydroxylase induction, and this accumulation is prevented by cycloheximide.

INTRODUCTION

Understanding the cellular mechanisms involved in microsomal enzyme induction may provide useful information for studying problems in pharmacology, endocrinology, and carcinogenesis. Aryl hydrocarbon hydroxylase[1] is a particulate enzyme system important in the metabolism of drugs, steroids, carcinogens, and insecticides (1–3). This mixed-function (4) oxidase is inducible in various mammalian cell cultures (5–11) by benz[*a*]anthracene and other polycyclic hydrocarbons dissolved in the growth medium.

[1] This enzyme system is also called *benzpyrene hydroxylase* and *aryl hydroxylase*. The nomenclature *aryl hydrocarbon hydroxylase is preferred*, since the enzyme obtained from rat liver microsomes, or from hamster fetal cells grown in culture, converts a variety of polycyclic hydrocarbons to phenolic derivatives and is not specific for benzo[*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.

Properties of the induced enzyme system from hamster fetal cell cultures have been reported (6). The effects of various inducers on hydroxylase activity and cell growth, the kinetics of induction and of enzyme activity decay, and the ability of the enzyme system to be induced in cells of different ages and origins have been described (7). Recently we have reported (10) that stimulation of hydroxylase activity in cell culture requires RNA synthesis initially and protein synthesis continuously, and that transcription and translation can be carried out independently of each other. During the process of aryl hydrocarbon hydroxylase induction, the appearance of a new, spectrally distinguishable, CO-binding cytochrome is dependent upon protein synthesis, rather than the presence of intracellular polycyclic hydrocarbon per se (8, 11).

In this cell-culture experimental system, one of the remaining areas in studying the mechanism of microsomal hydroxylase induction is the examination of the fate of the polycyclic hydrocarbon inducer. In this paper we describe the rate of entrance of inducer into the cell and the intracellular binding, localization, and excretion of the polycyclic hydrocarbon. In the accompanying paper (12) we report the possible relationship between intracellular polycyclic hydrocarbon content and the kinetics of hydroxylase induction and decay.

MATERIALS AND METHODS

The polycyclic hydrocarbons, BA[2] and benzo[*a*]pyrene, were purchased from Sigma and were purified by recrystallization from benzene. Recrystallized 3-hydroxybenzo[*a*]-pyrene was a generous gift of Dr. H. V. Gelboin, National Cancer Institute. SKF 525-A was generously supplied by Dr. J. H. Weisburger, National Cancer Institute. Cell culture supplies were obtained and prepared as described previously (6, 7). The standard complete medium consisted of 10% calf serum in Eagle's No. 2 minimal essential medium, pH 7.1, which contained 100 units of penicillin, 100 μg of streptomycin, and 10 units of Mycostatin per milliliter, 2 mM glutamine, and the amino acids arginine, aspartic acid, asparagine, glutamic acid, glycine, proline, and serine. The BA, initially dissolved in dimethyl sulfoxide, was dissolved in the growth medium, and the concentration of the polycyclic hydrocarbon in the filtered medium was determined spectrophotofluorometrically; control medium without BA was treated identically (6). Dimethyl sulfoxide at a final concentration of 0.25% in the growth medium had no measurable effect on cell growth or metabolism. "NCS" solubilizing reagent, uniformly labeled ^{14}C-protein hydrolysate (52 mCi/milliatom of carbon), generally labeled ^{3}H-BA (750 mCi/mmole), and ^{14}C-9-BA (56 mCi/mmole) were purchased from Nuclear-Chicago. Cycloheximide was obtained from the Cancer Chemotherapy National Service Center, National Institutes of Health. NADPH and NADH were purchased from Calbiochem. The National Institutes of Health Animal Supply provided pregnant hamsters estimated at 10–14 days of gestation.

Preparation of cell samples. All experiments described in this paper were carried out on secondary cell cultures (i.e., first subcultures). The individual cells, derived from hamster fetuses estimated at 10–14 days of gestational age, were prepared and passaged as previously described (6, 7). The fetal cells were grown in 1 atm of humidified air with 5% CO_2. The addition of inducer and/or inhibitors to the secondary cultures was always performed between 36 and 72 hr after the plating of the cells at a density of approximately 0.5×10^6 cells/ml; during this time the cells are in logarithmic growth. The measurement of the incorporation of ^{14}C-protein hydrolysate into cellular trichloracetic acid-precipitable material was used as a parameter of protein synthesis, as previously described (7). At the concentrations and lengths of exposure time employed, cycloheximide caused no irreversible effects. During any experiment in which one medium was replaced by another, the pretreatment medium was removed from the dish, the surface of the cell layer was washed three times

[2] The abbreviations used are: BA, benz[*a*]-anthracene (1,2-benzanthracene), as recommended by the American Chemical Society (13); SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate HCl.

with 4 ml of Dulbecco's isotonic phosphate buffer, and fresh medium containing the indicated additions was added. The cell homogenate was prepared in 0.25 M sucrose, and subcellular fractions were obtained by successive centrifugations of the homogenate at 400 × *g* for 10 min, 7000 × *g* for 15 min, and 78,000 × *g* for 90 min. We have not carried out biochemical measurements to determine the purity of the nuclear, mitochondrial, and microsomal fractions. However, more than 80% of both aryl hydrocarbon hydroxylase activity (6) and microsomal cytochrome b_5 and P-450 (11) are localized in the 78,000 × *g* supernatant fraction. Solid ammonium sulfate was used for the precipitation (14) of proteins from the 78,000 × *g* supernatant fraction at pH 7.0.

Enzyme assay. Both aryl hydrocarbon hydroxylase activity and protein concentration were determined in duplicate for the homogenate from cells scraped from one small cell-culture dish, as previously described (6). In the assay for 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 $MgCl_2$, 0.10 ml of cell homogenate (containing 200–800 μg of protein), and 80 nmoles 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 products were examined spectrophotofluorometrically (6), with activation at about 396 nm and fluorescence at about 522 nm. 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 (11) as that amount of enzyme catalyzing the formation, per minute 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 of Lowry *et al.* (15), with crystalline bovine serum albumin as a standard.

Measurement of radioactive polycyclic hydrocarbon in biological samples. In all experiments described in this paper, generally labeled ^{3}H-BA was used at a final specific activity of 50–75 μCi/μmole, while the ^{14}C-9-BA was used at a specific activity of 15–30 μCi/μmole in the growth medium. Thus, growth medium containing 1 pmole of ^{3}H-BA produced approximately 60–100 cpm by liquid scintillation technique, and medium containing 1 pmole of ^{14}C-9-BA produced between 30 and 40 cpm. In experiments lasting more than 12 hr, the possibility of a significant amount of tritium exchange with various cellular constituents was ruled out by the finding of identical results when either the ^{3}H- or the ^{14}C-labeled inducer was employed.

The rate of entrance and the total content of intracellular polycyclic hydrocarbon were measured in the following way. The cell surface in the 100-mm tissue culture dishes was washed three 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 BA. The cells were then harvested by scraping, and the packed cells were resuspended and washed once in 5 ml of cold Dulbecco's isotonic phosphate buffer. As many as nine repeated washings of the cell surface or of the packed cells produced breakage of a greater number of cells, as could be seen microscopically, but without a significant change in the association of polycyclic hydrocarbon with protein. Dialysis of a 3.5-ml suspension of intact cells or a 3.5-ml cellular homogenate (each containing approximately 14 mg of cellular protein) against 2 liters of isotonic phosphate buffer over a 24-hr period resulted in no change in the amount of polycyclic hydrocarbon associated with cellular proteins. The washed, packed cells were then homogenized in 0.25 M sucrose–0.5 M Tris-chloride buffer, pH 7.5, and the cellular homogenate (0.10-ml aliquots) was digested in 1.0 ml of Nuclear-Chicago solubilizing ("NCS") reagent. The radioactivity of duplicate or triplicate samples was determined in a Packard liquid scintillation counter. No attempt was made to determine whether the radioactivity was associated with the parent compound BA or with a metabolite of BA. By "intracellular polycyclic hydrocarbon," no distinction is

being made between inducer bound on the cell surface and that which entered the cell. Also, we are not concerned in this present paper with the relative affinity of the polycyclic hydrocarbon for the various cellular components, except for the measurement of covalent binding. Protein determinations and cell counts on each sample were carried out concomitantly.

The amount of polycyclic hydrocarbon covalently bound to cellular protein and nucleic acid was determined in the following manner. The cellular macromolecules were precipitated by the addition of 10 ml of cold 10% trichloracetic acid. The trichloracetic acid-insoluble material was then washed twice with cold 4% trichloracetic acid, once with cold ethanol, once with an ethanol-ether-chloroform (2:2:1) mixture, and three times with acetone. The residual radioactivity from trichloracetic acid-soluble material from cells harvested at zero time was subtracted from the values obtained after various periods of exposure to the radioactive BA. Any radioactive material which still remains associated with cellular protein and nucleic acid following this type of purification (16) is presumed to be covalently bound. This assumption is further supported by the data in this paper, which show the covalent binding to be the result of an enzymatic process that can be prevented by an enzyme inhibitor. Weighed amounts of the dried, acetone-insoluble residue were solubilized in 1.0 ml of "NCS," and the radioactivity of each sample was measured by liquid scintillation technique. Duplicate determinations of cell count, radioactivity in the trichloracetic acid-*soluble* fraction, and radioactivity in the trichloracetic acid-*insoluble* fraction were measured on identical dishes at each point in time.

It will be shown in this report that the maximal saturating level of intracellular polycyclic hydrocarbon differs by a factor of 2–3-fold, depending upon cell type. However, throughout this paper, for the sake of clarity, the intracellular content of polycyclic hydrocarbon is expressed in terms of 10^{-16} *mole/cell.* Since we are using a heterogeneous cell population, we recognize that these values represent the *average* level of polycyclic hydrocarbon per cell. In similar experiments performed at different times, however, the fact that there was good agreement in these values indicates that there is relatively little change in the components of the heterogeneous fetal cell population from week to week.

Measurement of alkali-soluble metabolites of BA 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 four times with 1.0-ml aliquots of hexane. The polar metabolites in the hexane-acetone phase were then extracted with 1 N NaOH, and the radioactivity of 0.05-, 0.10-, and 0.20-ml amounts of the alkali fraction was determined in a Packard liquid scintillation counter. The possibility exists that not all of the derivatives of BA produced by aryl hydrocarbon hydroxylase are extractable by this procedure.

RESULTS

Temperature dependence of intracellular entry and binding of polycyclic hydrocarbon. Figure 1A shows the effect of temperature on the rate of accumulation of intracellular BA. The maximal level of polycyclic hydrocarbon in the cell was reached within 30 min at 37° and required more than 2 hr at 2°. More than 50% of the maximal saturating concentration was attained in the first 2 min at 37° and after about 10 min at 2°. Under the conditions of these experiments, there was about a 20-fold excess of extracellular BA at the time of equilibrium.

The change in the initial rate of influx of BA into the cell for a 10° increase in temperature, (i.e., Q_{10}) was about 1.1. Presumably, small, temperature-dependent changes in the cell membrane structure may occur, and such a phenomenon would produce Q_{10} values slightly greater than 1.0. However, these data indicate that the process of intracellular entry of BA is most likely nonenzymatic. In all experiments, a net accumulation of intracellular polycyclic hydrocarbon concentration was established during the first hour of exposure of the cells to BA—almost always at the 30-min point in time.

The maximal saturating level ranged between 4×10^{-16} and 12×10^{-16} mole of polycyclic hydrocarbon per cell in more than 20 experiments on secondary cultures of cells derived from the total hamster fetus.

Figure 1B illustrates the importance of temperature in affecting the rate of covalent binding of polycyclic hydrocarbon to cellular protein and/or nucleic acid. During the initial 5 min of exposure of cells to the inducer BA, a Q_{10} value of 3.3 was calculated for the rate of polycyclic hydrocarbon binding to trichloracetic acid-precipitable material. The amount of covalently bound polycyclic hydrocarbon per cell ranged between 0.5% and 1% of the total intracellular polycyclic hydrocarbon content.

Therefore, these data indicate that the entry of inducer into the cell is a passive process, while the covalent binding of polycyclic hydrocarbon to trichloracetic acid-insoluble cellular material probably requires an enzyme. Grover and Sims have shown (17) that, in the presence of an NADPH-requiring rat liver microsomal suspension *in vitro*, all eight polycyclic hydrocarbons tested were bound significantly to both protein and DNA. More recently, Gelboin has demonstrated that both benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene bind covalently to either DNA or RNA in a similar system *in vitro* containing NADPH and rat liver microsomes (18). The primary enzyme system which metabolizes polycyclic hydrocarbons is the microsomal aryl hydrocarbon hydroxylase (6). Several dozen oxidation derivatives of BA (19) and benzo[*a*]pyrene (20) have been identified. Thus, it is likely that the covalent binding of polycyclic hydrocarbon to trichloracetic acid-precipitable material, depicted in Fig. 1B, is caused by the aryl hydrocarbon hydroxylase in the fetal cells in culture. This hypothesis was tested by using a specific microsomal oxygenase inhibitor, SKF 525-A (21). This drug completely inhibits aryl hydrocarbon hydroxylase activity *in vitro*.[3]

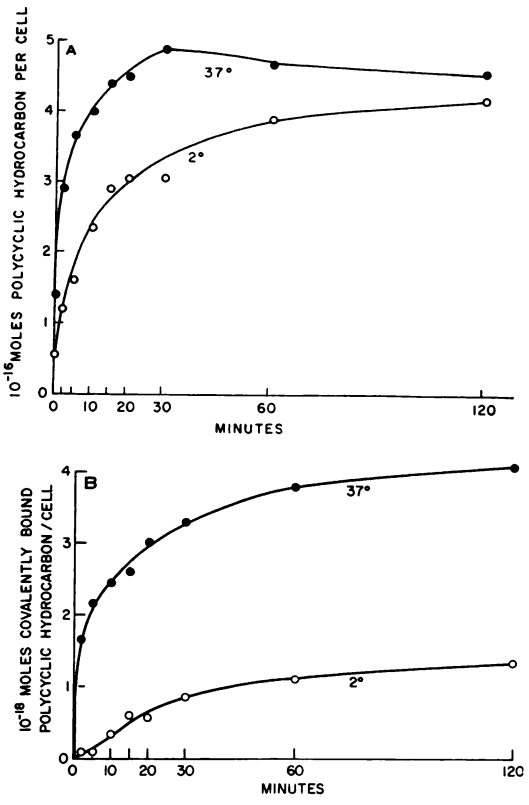

FIG. 1. *Effect of temperature on rate of entrance of inducer into the cell (A) and rate of polycyclic hydrocarbon binding to trichloracetic acid-precipitable cellular macromolecules (B)*

A concentration of 13 μM BA in the growth medium was used.

Figure 2 shows that the rate of covalent binding of polycyclic hydrocarbon to cellular macromolecules is inhibited more than 60% by 10^{-5} M SKF 525-A, and more than 80% by 10^{-7} M levels of this drug. Therefore, this finding strongly implicates the importance of the microsomal hydroxylase system in the mechanism of binding polycyclic hydrocarbon covalently to cellular nucleic acid and protein. A lower inhibition of microsomal enzymes by higher concentrations of SKF 525-A has been observed and discussed by others (21–23), but the reason for this effect is not known. Perhaps the binding of excess drug to nonspecific sites on the membranes may alter the apparent effectiveness of this inhibitor, which acts by binding to specific enzyme active sites. It is also possible that higher levels of SKF 525-A may stabilize hydroxylase activity by direct interaction with the microsomal membrane, or by an

[3] D. W. Nebert and L. L. Bausserman, unpublished data.

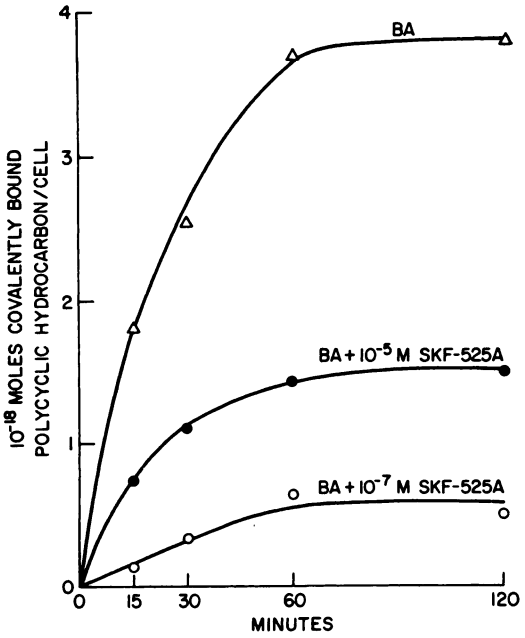

FIG. 2. *Effect of the microsomal enzyme inhibitor SKF 525-A on rate of polycyclic hydrocarbon binding to trichloracetic acid-precipitable cellular macromolecules*

The cells were exposed to the indicated concentrations of inhibitor dissolved in the growth medium, or control medium alone, for 30 min; then 13 μM BA with more SKF 525-A, or inducer alone, was added to the appropriate dishes of cells. At these levels of SKF 525-A for 2.5 hr, no toxic effects on the cells were observed.

indirect action such as slowing the normal rate of hydroxylase degradation.

Hepatoma tissure culture cells have no detectable constitutive, or inducible, aryl hydrocarbon hydroxylase activity (7). We found no significant level of covalently bound intracellular polycyclic hydrocarbon in these cells.[4]

Equilibrium between extracellular and intracellular polycyclic hydrocarbon. If polycyclic hydrocarbons have a strong affinity for cellular protein and nucleic acids, then, at lower levels of BA in the medium, there should be a more than proportionate accumulation of polycyclic hydrocarbons within the cell. The data summarized in Table 1 indicate that this view is correct. This situation does not persist for more than 30 min in cells treated with low concentrations of BA, however. The decline in intracellular polycyclic hydrocarbon content after 30 min is presumably due to the appearance of newly induced aryl hydrocarbon hydroxylase, and this phenomenon is examined in detail in the accompanying paper (12).

Subcellular distribution of intracellular polycyclic hydrocarbon. Table 2 lists the amount of polycyclic hydrocarbon bound to the various subcellular organelle fractions and to the soluble cell fraction precipitable by saturation with ammonium sulfate. These measurements reflect radioactivity associated with cellular material that is not removed by repeated washings with buffer. About 7 times more polycyclic hydrocarbon was associated with the particulate fractions than with the 78,000 × *g* supernatant fraction. The amount of radioactivity associated with protein in the 400 × *g* pellet fraction was 3 times greater than the specific radioactivity of the starting cellular homogenate. Thus, while the 400 × *g* pellet fraction accounted for less than 20% of the protein in the cell, more than 50% of the polycyclic hydrocarbon was localized in this fraction. More than 10% of the radioactivity of the starting homogenate resided in the post-microsomal supernatant fraction; this radio-

TABLE 1

Concentration of total intracellular polycyclic hydrocarbon as a function of BA concentration in growth medium

Growth medium containing the designated concentrations of ^{3}H-BA was added to dishes of hamster fetal cells. After 30 min at 37°, the cells were harvested and the concentration of intracellular polycyclic hydrocarbon was determined as described in MATERIALS AND METHODS.

Concentration of BA in medium	Total intracellular polycyclic hydrocarbon
μM	*(moles/cell) × 10¹⁶*
26	24
13	11
1.3	6.5
0.13	0.61
0.013	0.071
0.0013	0.016

[4] D. W. Nebert and E. B. Thompson, unpublished results.

TABLE 2

Fractionation of intracellular polycyclic hydrocarbon

Cells which had been treated with 4.3 μM ^{3}H-BA for 30 min at 37° were harvested and washed. The subcellular fractions and precipitates from ammonium sulfate saturation of the 78,000 × *g* supernatant fraction were prepared as described in MATERIALS AND METHODS.

Fraction	Total protein	Specific activity	Polycyclic hydrocarbon in each fraction	
	mg	*pmoles/mg protein*	*pmoles*	*% initial*
Homogenate	150	660	99,000	
400 × *g* pellet	27	1,900	51,000	52
7,000 × *g* pellet	24	560	13,000	13
78,000 × *g* pellet	23	540	12,000	12
78,000 × *g* supernatant	46	240	11,000	11
Total	120		87,000	88
$(NH_4)_2SO_4$ saturation of 78,000 × *g* supernatant				
0–20% pellet	5.2	250	1,300	12
20–50% pellet	18.5	220	4,100	37
50–100% pellet	6.4	410	2,600	24
100% saturated supernatant	12.0	14	170	1.5
Total	42		8,120	72

activity was distributed fairly equally among each of the three fractions precipitated by ammonium sulfate. Less than 0.2% of the total intracellular polycyclic hydrocarbon was not bound to either cellular organelles or soluble proteins.

These data demonstrate the ubiquitous and presumably nonspecific binding of polycyclic hydrocarbon to all fractions of the cell, as might be expected, because of the strong binding affinity of all polycyclic hydrocarbons. In each of these fractions, however, we have found[5] markedly different affinities of polycyclic hydrocarbons for the various cellular macromolecules which can be separated electrophoretically.

Table 3 summarizes the distribution of *covalently bound* polycyclic hydrocarbon among the same subcellular fractions examined in Table 2. The data of Figs. 1B and 2, and of refs. 17 and 18, indicate that the

[5] D. W. Nebert and L. L. Bausserman, manuscript in preparation.

TABLE 3

Fractionation of covalently bound intracellular polycyclic hydrocarbon

The subcellular fractions and the ammonium sulfate-precipitated fractions from the 78,000 × *g* supernatant solution were obtained from the experiment described in Table 2. The trichloracetic acid-insoluble material was washed as described in MATERIALS AND METHODS, and the radioactivity of each acetone-dried pellet was determined.

Fraction	Specific activity	Covalently bound polycyclic hydrocarbon in each fraction	
	pmoles/mg protein	*pmoles*	*% initial*
Homogenate	6.0	900	
400 × *g* pellet	14.0	380	42
7,000 × *g* pellet	5.8	140	15
78,000 × *g* pellet	9.8	230	25
78,000 × *g* supernatant	2.9	130	15
Total		880	98
$(NH_4)_2SO_4$ saturation of 78,000 × *g* supernatant			
0–20% pellet	5.9	31	24
20–50% pellet	4.9	91	70
50–100% pellet	0.51	4.1	3.1
100% saturated supernatant	0	0	0
Total		126	97

process of covalent binding probably requires the microsomal hydroxylase system. The specific radioactivity of the 400 × *g* pellet fraction was more than 2-fold greater than that of the starting homogenate; the only other fraction having a specific radioactivity higher than that of the starting material was the 78,000 × *g*, or microsomal, fraction. Among the soluble cytoplasmic macromolecules, the covalent polycyclic hydrocarbon was bound almost exclusively to material precipitable between 0% and 50% ammonium sulfate saturation.

If one considers the structural aspects of the cell, it would be reasonable to expect a relatively greater amount of chemically bound metabolite in the vicinity of the membrane-bound enzyme, which is responsible for formation of highly reactive intermediates. Our data are not entirely consistent with such a hypothesis. We have

shown that the covalently bound metabolites are present throughout the cell, but proportionately more exist not only in the microsomes but also in the 400 × *g* pellet, or nuclear, fraction. This finding is of interest because of a recent report (24) of microsomal enzyme activity in washed, detergent-treated nuclei of HeLa cells. The possibility also exists that the 400 × *g* pellet fraction in our study was contaminated with microsomes. About the same relative quantity of covalently bound polycyclic hydrocarbon was found in the supernatant fraction containing the cytoplasmic macromolecules precipitable by 0–50% ammonium sulfate saturation as in the 7000 × *g* pellet fraction.

Kinetics of hydroxylase induction and intracellular entry of polycyclic hydrocarbon in cells derived from various fetal tissues. We have previously reported (7, 9) differences in the ability to induce the hydroxylase system in fetal cell cultures derived from different species and from different tissues of the hamster fetus. The possibility that the entry rate and level of intracellular polycyclic hydrocarbon are related to the ability to induce the microsomal oxygenase activity was therefore investigated.

Figure 3A depicts the rate of entry of BA into cells derived from various fetal hamster tissues. Each cell type in primary and secondary cell cultures was morphologically distinguishable from the others. The initial rate of inducer entering the cell was approximately the same for all types of cells; however, the level of saturation of intracellular polycyclic hydrocarbon was 2–3 times higher in cells derived from fetal liver than in cells from fetal limbs. Figure 3B shows that the rate of aryl hydrocarbon hydroxylase induction (i.e., enzyme accumulation) varied with the type of tissue from which the cells

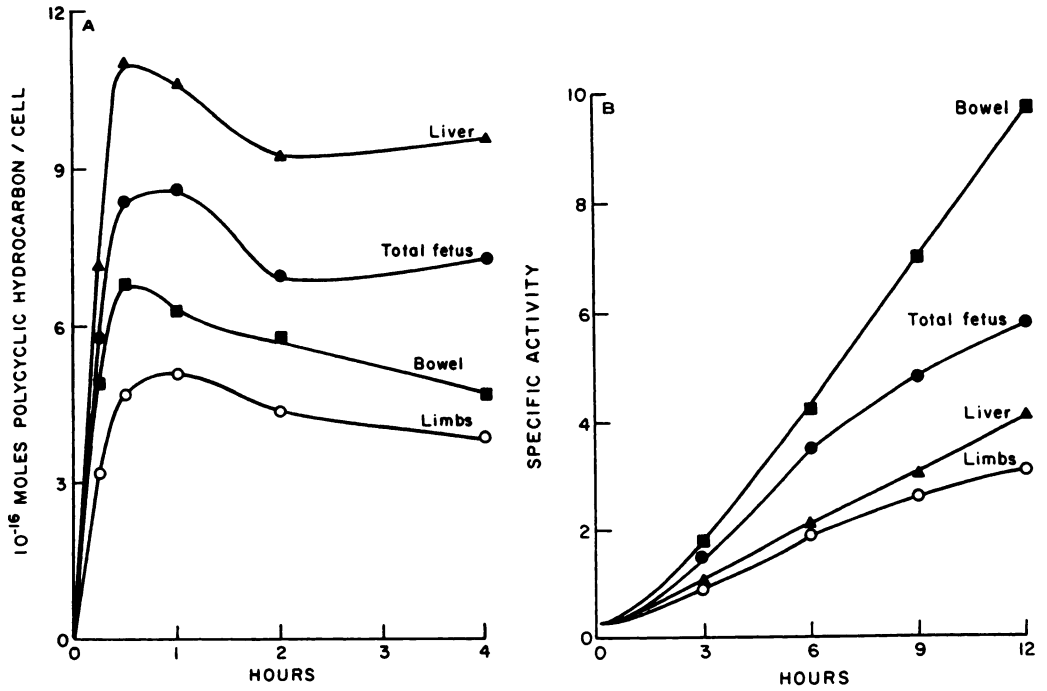

FIG. 3. *Kinetics of intracellular entry of polycyclic hydrocarbon (A) and aryl hydrocarbon hydroxylase activity (B) into cells derived from various hamster fetal tissues*

The specific activity of the enzyme is expressed in units per milligram of cellular protein. Each point represents duplicate determinations of both enzyme activity and protein content on a cellular homogenate. The secondary cell cultures derived from different fetal tissues were prepared as described previously (7).

had been derived. The enzyme system in cells derived from fetal hamster bowel was induced approximately 3 times faster than that from fetal limbs. The ability to induce hydroxylase activity and the entry rate of intracellular polycyclic hydrocarbon in fetal lung cells are not shown in Fig. 3, but were very similar to those values obtained in cells derived from fetal bowel. As expected, the kinetics of enzyme induction and the intracellular polycyclic hydrocarbon content for cells derived from the total fetus were between the maximal and minimal values found for cells derived from specific fetal tissues.

The reason for differences in the level of saturation of intracellular polycyclic hydrocarbon in the various cell types is not clear. Presumably, the equilibrium between the intracellular and extracellular polycyclic hydrocarbon concentration is dependent upon such factors as cell size and membrane content. Because a hepatocyte is larger and contains more lipophilic membranes than a connective tissue cell (25), it might be expected that cells derived from fetal liver incorporate more BA than cells derived from fetal limbs. Therefore, these data indicate that differences in the rate of hydroxylase induction and in the maximal level of intracellular polycyclic hydrocarbon do exist; however, there is no obvious relationship between the kinetics of enzyme induction and the level of intracellular polycyclic hydrocarbon in cells derived from different fetal tissues.

Polar polycyclic hydrocarbon metabolites excreted by cells during hydroxylase induction. The presence of water-soluble derivatives of intracellular polycyclic hydrocarbons (7, 26) and excretion of these products into the medium (27, 28) have been described in cell culture studies. In fact, in BA-treated fetal hamster cells there is always an alkali-extractable, fluorescent compound,[3] with an excitation peak at about 292 nm and emission peaks at about 419 nm and 610 nm, that is presumably due to a polar metabolite of BA.

Figure 4 shows that the appearance of polar products of BA in the growth medium was almost completely prevented by cyclohexamide. The amount of polar metabolites in the medium was about 24 times greater in BA-treated cells than in cells exposed to inducer plus cycloheximide for 12 hr. We have previously noted (7, 10) that both actinomycin D and cycloheximide, added simultaneously with the inducer BA, inhibit the induction of aryl hydrocarbon hydroxylase in cell culture. Also, the microsomal oxygenase inhibitor SKF 525-A prevents the accumulation of polar derivatives of BA in the growth medium.[3] Furthermore, we have

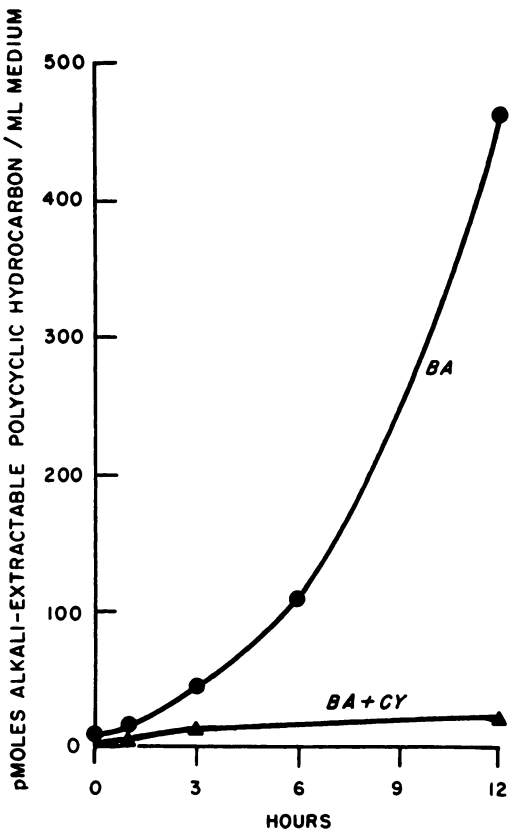

FIG. 4. *Rate of appearance of alkali-extractable polycyclic hydrocarbons in growth medium during stimulation of aryl hydrocarbon hydroxylase activity by 13 μM inducer (BA) in the presence or absence of 3.5 μM cycloheximide (CY)*

Each point represents the average of three determinations, the values not differing from each other by more than 10%. The presence of 3.5 μM cycloheximide in the growth medium prevented more than 90% of protein synthesis during the entire period during which the inhibitor was present.

found[4] that there is no appearance of water-soluble metabolites of BA in the medium of hepatoma tissue culture cells, which contain no constitutive or inducible aryl hydrocarbon hydroxylase activity. These data, therefore, illustrate that the induced microsomal oxidase is very active in metabolizing intracellular polycyclic hydrocarbon to alkali-extractable products. Diamond *et al.* (27) found that the amount of 7,12-dimethylbenz[*a*]anthracene metabolized and excreted by normal hamster fetal cells and by HeLa cells is much greater than the amount of covalently bound products that can be detected within the cells at any given time. Our data are in agreement with these observations. After 12 hr of exposure of the cells to BA, we estimate that about 7×10^{-16} mole of polar metabolites per cell accumulated in the growth medium while about 4×10^{-18} mole of covalently bound polycyclic hydrocarbon remained in the cell.

DISCUSSION

We have shown here that the inducer diffuses passively from the growth medium into the cell, where the BA has a strong affinity for all of the subcellular organelles and for cytoplasmic macromolecules: about one-half of the total intracellular polycyclic hydrocarbon is associated with the nuclear fraction. A net accumulation of intracellular polycyclic hydrocarbon is established within about 30 min of exposure of cells to BA. There is an enzymatic process, most probably both the control and induced aryl hydrocarbon hydroxylase systems in the microsomes, whereby small amounts of the polycyclic hydrocarbon derivatives (less than 1% of the total intracellular polycyclic hydrocarbon) are bound covalently to cellular material. More than one-half of these metabolites covalently bound to cellular material are found in the nuclear and microsomal fractions. Most of the metabolites are excreted by the cell.

A peak in the intracellular covalently bound products of BA is reached during the first hour: approximately 4×10^{-18} mole/cell, which is between 2×10^{6} and 3×10^{6} molecules/cell. At least two-thirds of this level is attained during the first 30 min. We have previously shown (7) that in the presence of BA there is a lag period of about 35 min before a significant increase in aryl hydrocarbon hydroxylase activity is detectable in hamster fetal cell cultures. Thus, the control enzyme system must be responsible for at least most of the intracellular covalently bound polycyclic hydrocarbon. The fact that a maximal level is reached in about 1 hr suggests that essentially all of the reactive sites on the cellular protein and nucleic acid have been saturated in this period of time. The data in this paper also suggest that metabolism of the inducer continues after the intracellular saturation of covalently bound derivatives, with the metabolites accumulating in the growth medium.

We are concerned with the response of microsomal hydroxylase activity to a stimulus caused by the inducer, or pharmacological agent, benz[*a*]anthracene. The process of hydroxylase induction requires RNA synthesis initially and protein synthesis continuously (7, 10). Most likely, the inducer must bind to a receptor substance prior to eliciting the response of RNA synthesis, followed by protein synthesis and enzyme induction. Whether the inducer binds physically or chemically to the receptor sites remains to be elucidated. It would seem reasonable, however, that the interaction of inducer and receptor, as is the case with enzyme and substrate, is carried out by physical binding, which is readily reversible. In most studies of the intracellular distribution of polycyclic hydrocarbons (29–37), however, the emphasis has been on localization of only the covalently bound material.

The active site for microsomal hydroxylation of BA is the CO-binding hemoprotein cytochrome P-450 (1, 2, 8, 11, 38), one of several components of the hydroxylase system. After 30 min of exposure of the hamster fetal cells to 13 μM BA, there are approximately 12 pmoles of cytochrome P-450 (10) and about 2700 pmoles of physically bound polycyclic hydrocarbon[3] per milligram of microsomal protein. Therefore, of the total amount of polycyclic hydrocarbon physically bound to the microsomal membranes, we estimate that less than 0.5%

is specifically bound to the active sites of cytochrome P-450 at any given time.

Our study points out certain advantages of studying the mechanism of microsomal enzyme induction in cell culture, where the concentrations and exposure times of inducer and inhibitors can be rigidly controlled. With the knowledge of the rate of metabolism, the rate of entry and binding, and the subcellular distribution of the inducer in cell culture, we are thus able to examine these parameters during specific experiments involving the induction and decay of aryl hydrocarbon hydroxylase activity.

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REFERENCES

1. A. H. Conney, *Pharmacol. Rev.* **19**, 317 (1967).
2. J. R. Gillette, *Advan. Pharmacol.* **4**, 219 (1966).
3. H. V. Gelboin, *Advan. Cancer Res.* **10**, 1 (1967).
4. H. S. Mason, *Advan. Enzymol.* **19**, 79 (1957).
5. L. J. Alfred and H. V. Gelboin, *Science* **157**, 75 (1967).
6. D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.* **243**, 6242 (1968).
7. D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.* **243**, 6250 (1968).
8. D. W. Nebert, *Biochem. Biophys. Res. Commun.* **36**, 885 (1969).
9. D. W. Nebert and H. V. Gelboin, *Arch. Biochem. Biophys.* **134**, 76 (1969).
10. D. W. Nebert and H. V. Gelboin, *J. Biol. Chem.* **245**, 160 (1970).
11. D. W. Nebert, *J. Biol. Chem.* **245**, 519 (1970).
12. D. W. Nebert and L. L. Bausserman, *Mol. Pharmacol.* **6**, 304 (1970).
13. A. M. Patterson, L. T. Capell and D. F. Walker, "The Ring Index," Ed. 2. American Chemical Society, Washington, D. C., 1960.
14. A. A. Green and W. L. Hughes, *Methods Enzymol.* **1**, 67 (1955).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. P. Siekevitz, *J. Biol. Chem.* **195**, 549 (1952).
17. P. L Grover and P. Sims, *Biochem. J.* **110**, 159 (1968).
18. H. V. Gelboin, *Cancer Res.* **29**, 1272 (1969).
19. E. Boyland and P. Sims, *Biochem. J.* **91**, 493 (1964).
20. P. Sims, *Biochem. Pharmacol.* **16**, 613 (1967).
21. M. W. Anders and G. J Mannering, *Mol. Pharmacol.* **2**, 319 (1966).
22. J. R. Fouts and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **119**, 197 (1957).
23. J. R. Gillette, *Progr. Drug Res.* **6**, 13 (1963).
24. M. K. Bach and H. G. Johnson, *Biochemistry* **6**, 1916 (1967).
25. D. W. Fawcett, "An Atlas of Fine Structure: The Cell." Saunders, Philadelphia, 1966.
26. L. N. Andrianov, G. A. Belitsky, O. J. Ivanova, A. Y. Khesina, S. S. Khitrovo, L. M. Shabad, and J. M. Vasiliev, *Brit. J. Cancer* **21**, 566 (1967).
27. L. Diamond, C. Sardet and G. H. Rothblat, *Int. J. Cancer* **3**, 838 (1968).
28. L. Diamond and H. V. Gelboin, *Science* **166**, 1023 (1969).
29. G. Calcutt, *Brit. J. Cancer* **8**, 554 (1954).
30. G. Calcutt and S. Payne, *Brit. J. Cancer* **8**, 560 (1954).
31. G. Calcutt and S. Payne, *Brit. J. Cancer* **8**, 710 (1954).
32. P. M. Bhargava, H. I. Hadler and C. Heidelberger, *J. Amer. Chem. Soc.* **77**, 2877 (1955).
33. P. M. Bhargava and C. Heidelberger, *J. Amer. Chem. Soc.* **78**, 3671 (1956).
34. G. Calcutt, *Brit. J. Cancer* **12**, 149 (1958).
35. P. Brookes and P. D. Lawley, *Nature* **202**, 781 (1964).
36. L. M. Goshman and C. Heidelberger, *Cancer Res.* **27**, 1678 (1967).
37. E. Bresnick, R. A. Liebelt, J. G. Stevenson and J. C. Madix, *Cancer Res.* **27**, 462 (1967).
38. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2370, 2379 (1964).