VARIATION IN ARYL HYDROCARBON (BENZO[*a*]PYRENE) HYDROXYLASE ACTIVITY IN HETEROPLOID AND PREDOMINANTLY DIPLOID RAT LIVER CELLS IN CULTURE

JAMES P. WHITLOCK, JR., HARRY V. GELBOIN, and HAYDEN G. COON

From the Chemistry Branch and Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014. Dr. Whitlock's present address is the Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Maryland 20014.

ABSTRACT

Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase is present and inducible in Buffalo rat liver cells in culture. There is substantial variation in both basal and inducible hydroxylase activities among heteroploid subclones isolated from a heteroploid parent population, and among diploid subclones isolated from a diploid parent population. This variation is not related to differences in the growth characteristics of the subclones, or to differences in their chromosome number. The results indicate that substantial heterogeneity in both basal and induced hydroxylase activity develops during the growth of both heteroploid and diploid cell strains in culture. These findings indicate that diploid cell populations are not necessarily homogeneous with respect to aryl hydrocarbon hydroxylase activity. This observation may complicate the interpretation of experiments involving somatic cell hybridization or polycyclic hydrocarbon-induced transformation and/or cytotoxicity. This heterogeneity in hydroxylase activity develops rather rapidly (2–3 mo of culture), in the absence of any apparent mutational stress.

Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase is a substrate-inducible, microsomal enzyme complex which is found in most mammalian tissues (5, 9, 19, 24) and which has an important role in both the detoxification of carcinogenic polycyclic hydrocarbons and their activation to more reactive and carcinogenic forms (8, 10–12, 23). The hydroxylase system has properties typical of the drug-metabolizing, microsomal mixed-function oxygenases, which metabolize many xenobiotics, such as carcinogens, drugs, and pesticides (3, 4, 14). This enzyme complex has been extensively studied in cell culture, where hydroxylase activity is measured as the average activity of a cell population. We have examined the heterogeneity in hydroxylase activity of a strain of Buffalo rat liver cells which we have previously studied (25, 26). Hydroxylase activity in these cells has remained stable for several years. Certain substrains of the original cell strain BRL-3C4 (7) have become heteroploid, while others have remained predominantly diploid. For this study, we have isolated and propagated subclones from the heteroploid and diploid parental populations. These subclones all exhibit substantial differences in both basal and induced hydroxylase activity, which, we assume,

reflects variation that exists among the cells of the parental populations. We find that substantial heterogeneity in hydroxylase activity develops within both heteroploid and diploid cell populations.

EXPERIMENTAL PROCEDURE

Materials

Materials were obtained as follows: cell culture medium, serum, and antibiotics from Grand Island Biological Co., Grand Island, N. Y.; cell culture plasticware from Falcon Plastics Div. of BioQuest, Oxnard, Calif.; NADPH from Calbiochem, San Diego, Calif.; ribonuclease A and chromatographically purified collagenase (CLSPA) from Worthington Biochemical Corp., Freehold, N. J.; trypsin (1-300) from Nutritional Biochemicals, Cleveland, Ohio; dimethylsulfoxide from J. T. Baker Chemical Co., Phillipsburg, N. J.; benz[*a*]anthracene from K and K I aboratories, Plainview, N. Y.; benzo[*a*]pyrene from Eastman Kodak Co., Rochester, N. Y. Both polycyclic hydrocarbons were recrystallized from 95% ethanol and checked for purity by two-dimensional thin-layer chromatography using benzene-methanol (19:1) as solvents in the first direction and benzene-hexane (15:1) in the second direction.

Cell Culture

The liver of a 6-wk old female rat of the inbred Buffalo strain was minced, washed in saline, and dissociated by repeated treatments with trypsin, collagenase, and chicken serum (6, 7). The suspensions were washed, diluted in growth medium, and plated at densities (10^5–10^6 trypan blue–excluding cells per 100-mm petri dish) which permitted the growth of isolated colonies. Individual colonies, with homogeneous epithelial morphology, were trypsinized; suspensions of single cells were replated at a density of 200 cells per 100-mm petri dish, yielding clones. Plating efficiency was 10–20%. The parental cell strain (BRL 3C4) was originally diploid, and spontaneously became heteroploid during continuous culture. BRL 3C4 was selected for study because its aryl hydrocarbon hydroxylase activity was highly inducible by polycyclic hydrocarbons.

Cells were grown in Ham's mixture F12 (15) containing 5% fetal calf serum (vol/vol), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in a 5% CO_2 humidified atmosphere in a National incubator (National Appliance Co., Portland, Ore.). Confluent monolayers were used for determination of hydroxylase activity. Fresh growth medium was added to the cultures 16 h before the start of each experiment. For long-term storage, cells were suspended in growth medium containing 10% dimethylsulfoxide (vol/vol), frozen slowly, and stored over liquid nitrogen. Cells were suspended at each serial passage in a solution of 12 U/ml collagenase, 0.1% trypsin, and 2% chicken serum.

For cloning, a confluent plate of cells was exposed for 45 min to the solution of collagenase, trypsin, and chicken serum, producing a suspension of single cells, with fewer than 1% doublets. After serial dilutions in growth medium, approximately 200 cells were placed in each of several 100-mm tissue culture plates. About 10–30% of the cells plated produced colonies. After 14–21 days of growth, well isolated colonies were removed by using cloning cylinders and propagated separately (21). The subcloned populations were assayed for hydroxylase activity 25–30 cell generations after the original clonal plating.

Chromosomes

Chromosome preparations were made by the technique of Rothfels and Siminovitch (22), except that suspensions of fixed cells were spread on dry slides at room temperature and greater than 40% relative humidity. About 20 well spread, unbroken metaphases were counted from each preparation.

Preparation of Medium Containing Benz[a]anthracene

Benz[*a*]anthracene was dissolved in dimethylsulfoxide (DMSO) and added to the medium; the final concentration of dimethylsulfoxide in the medium was 0.1%. This level of DMSO had no detectable effect on cell growth or hydroxylase activity.

Aryl Hydrocarbon Hydroxylase Assay

Confluent monolayers were washed twice with chilled Dulbecco's phosphate-buffered saline, collected by scraping into 5 ml of chilled phosphate-buffered saline and centrifuged at 1,000 *g* for 10 min at 0°C. The pellet was homogenized in 0.25 M sucrose-0.05 M Tris-HCl, pH 7.57, and an aliquot assayed for hydroxylase activity by the method of Nebert and Gelboin (18). Incubation mixtures contained in a vol of 1.0 ml: 50 μmol of Tris-HCl, pH 7.57, 3 μmol of $MgCl_2$, 0.5 mg of NADPH, 200 μl of cell homogenate, and 100 nmol of substrate, benzo[*a*]pyrene, added in 40 μl of methanol. Incubations were carried out for 30 min at 30°C and were performed under the illumination of a General Electric 25-W red bulb. The reaction was stopped by the addition of 1.0 ml of acetone, and the mixture was then shaken with 3.0 ml of hexane for 10 min. A 1.0-ml aliquot of the organic layer was extracted with 1.0 ml of 1 N NaOH, and the fluorescence of this extract was measured immediately at 396 nm excitation and 522 nm emission in an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Springs, Md.) and compared to the fluorescence of a standard 3-hydroxybenzo[*a*]pyrene solution. 1 U of hydroxylase activity catalyzes in 30 min the formation of phenolic products with the fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo[*a*]pyrene. The reaction was linear with respect to time of incubation and amount of cellular

protein. Protein was determined by the method of Lowry et al. (17), with ribonuclease A as standard.

RESULTS AND DISCUSSION

Previous results indicated that aryl hydrocarbon hydroxylase was inducible by polycyclic hydrocarbons in strain BRL 3C4 which spontaneously had become heteroploid (26). Benz[*a*]anthracene is relatively noncytotoxic and induces hydroxylase activity to high levels. Since the inducers are metabolized by the hydroxylase, their concentration in the medium is constantly decreasing during the induction period. Therefore, we used relatively short (4–8 h) induction times, when comparing the inducibility of different tissues; this procedure minimizes possible differences between tissues which metabolize the inducer at different rates. This procedure, however, does not distinguish between tissue variants having different kinetics of induction and those having different extents of induction.

Furthermore, in this strain BRL 3C4 (h), the hydroxylase can also be induced without the addition of a polycyclic hydrocarbon, by causing a temporary (1–6 h) inhibition of protein synthesis. After release of the block in protein synthesis, hydroxylase activity increases to high levels. This increase is prolonged in the presence of actinomycin D, at a concentration which inhibits total RNA synthesis by greater than 90% (25). When both methods of hydroxylase induction are employed simultaneously (that is, temporary inhibition of protein synthesis plus exposure to benz[*a*]anthracene) the resulting increase in hydroxylase activity is greater than the sum of the increases which follow each treatment separately (25). This synergistic effect on hydroxylase activity suggests that these two modes of hydroxylase induction reflect two different mechanisms by which hydroxylase activity can be increased.

In the present study, we have used both of these methods of hydroxylase induction in each of the different subclones derived from the heteroploid parent line. Table I shows that in each subclone *(a)* hydroxylase activity increases after exposure to benz[*a*]anthracene; *(b)* hydroxylase activity increases after the release of a temporary inhibition of protein synthesis; *(c)* there is a synergistic effect on hydroxylase activity when these two methods are used simultaneously. Thus, each subclone responds to these different methods of hydroxylase induction. However, the subclones vary in the magnitude of the increases in hydroxylase activity produced by each method of induction. After an

TABLE I

*Aryl Hydrocarbon Hydroxylase Activity in Heteroploid Rat Liver Cell Subclones**

Subclone	Total chromosome no.‡	Treatment			
		None§	Benz[*a*]anthracene‖	Temporary inhibition of protein synthesis¶	Benz[*a*]anthracene + temporary inhibition of protein synthesis**
		U/mg protein			
Parents (BRL 3C4)	75 (70–84)	5	63	240	572
1	70 (57–75)	4	10	56	89
2	71 (67–77)	20	231	596	964
3	77 (70–82)	4	36	350	601
4	76 (66–79)	7	33	258	404
5	76 (73–80)	6	47	318	449
6	77 (73–81)	3	49	170	278
7	76 (71–85)	3	44	300	495
8	71 (65–78)	13	163	169	338

* Each value represents the average from duplicate plates of cells. Hydroxylase activity of identically treated plates of cells varied less than 15%.

‡ Values indicate means and ranges.

§ Hydroxylase activity at the beginning of the experiment.

‖ Cells were exposed for 8 h to medium containing benz[*a*]anthracene (1 μg/ml).

¶ Cells were exposed for 4 h to medium containing cycloheximide (10 μg/ml). They then received two 15 min washes with medium containing actinomycin D (1 μg/ml) and were exposed to actinomycin D for a total of 4 h.

** Same treatment as (¶), except medium also contained benz[*a*]anthracene (1 μg/ml).

8-h exposure to benz[*a*]anthracene, hydroxylase activity of subclone 1 is one-sixth that of the parent strain, while the activity of subclone 2 is almost four times that of the parent population. The hydroxylase activities in the other subclones range both above and below the activity in the parent strain. Similarly, there is a variation among the subclones in the magnitude of hydroxylase induction by temporary inhibition of protein synthesis; hydroxylase activity in subclone 1 is about one-fourth that of the parents, while activity in subclone 2 is more than double that of the parents. The other subclones from this heteroploid population have intermediate hydroxylase activities.

Induction by temporary inhibition of protein synthesis combined with exposure to benz[*a*]anthracene has the synergistic effect on hydroxylase induction in all subclones except number 8. Again, the extent of hydroxylase induction varies among the different subclones. The activity of subclone 1 is less than one-sixth that of the parents; that of subclone 2 is almost double that of the parents. Although the hydroxylase activity of a given strain may vary somewhat from experiment to experiment, the relative response of each strain is the same. These characteristic differences in response have remained reproducible during continuous culture for at least 5–6 mo. Thus, the results in Table I indicate that there is a qualitative similarity among the subclones in their responses to the different treatments known to induce hydroxylase activity in the parent strain. However, there is substantial variation among the subclones in the magnitude of these responses, suggesting that the parent cell population was heterogeneous with respect to its responses to such treatments.

Fig. 1 shows the kinetics of hydroxylase induction after exposure to benz[*a*]anthracene, in the highly inducible subclones 2 and 8, the weakly inducible subclone 1, and subclone 6, whose response is similar to that of the parental population. These results further illustrate the differences between the subclonal populations in their responses to inducing conditions, again suggesting that the parental population was heterogeneous with respect to hydroxylase induction by benz[*a*]anthracene.

Fig. 2 indicates that both basal and benz[*a*]anthracene-induced hydroxylase activities remain quite constant in the parent strain and subclones 1 and 6 throughout a 2-wk period of growth. Hydroxylase activity in subclone 2, on the other hand, is apparently regulated differently

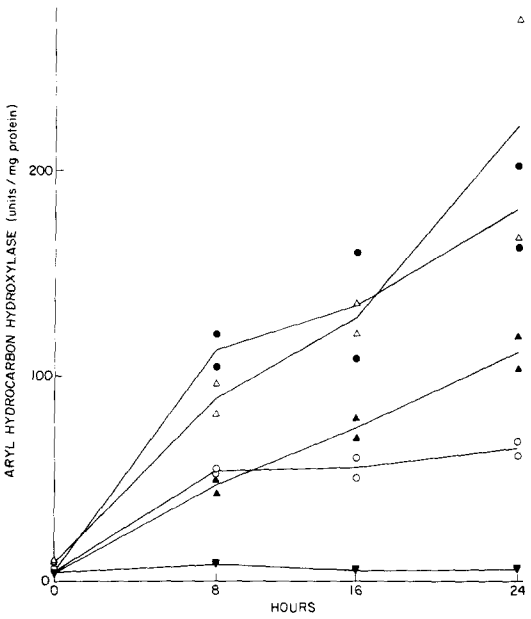

FIGURE 1 Kinetics of aryl hydrocarbon hydroxylase induction in heteroploid parental cells and subclones. Cells were exposed to medium containing benz[*a*]anthracene (1 μg/ml) for the times indicated. Each point represents the average of duplicate determinations of aryl hydrocarbon hydroxylase and protein concentration on a single plate of cells. ○, parents; ▼, subclone 1; △, subclone 2; ▲, subclone 6; ●, subclone 8.

during different phases of growth in culture. Each cell strain grew at similar rates and all became confluent at about 7 days after plating. In addition, the protein content of each plate of cells increased with time and was similar for each strain. Since each experiment (except for those described in Figs. 2 and 5) was done when the cells reached confluence, the observed differences in hydroxylase induction by benz[*a*]anthracene are not primarily related to different growth characteristics of the various subclones.

We have also examined the properties of the benz[*a*]anthracene-induced hydroxylase in the parent strain and subclones 1 and 2. In each of these strains, hydroxylase activity is NADPH-dependent and CO-sensitive, properties typical of the drug-metabolizing mixed-function oxygenases (6, 14). In addition, hydroxylase activity is inhibited by 7,8-benzoflavone, a potent inhibitor of this enzyme system (8, 13, 16, 28). Enzyme activity is destroyed by trypsin digestion, indicating the protein nature of the catalysis. Furthermore, the hydroxylase activities of the parent strain and sub-

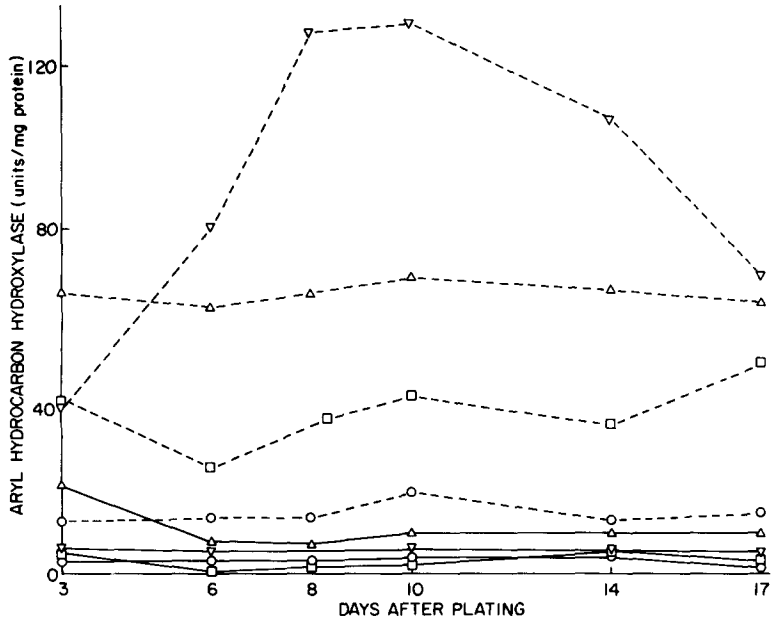

FIGURE 2 Aryl hydrocarbon hydroxylase activity in heteroploid parental cells and subclones after increasing times in culture. On day zero, cells were distributed at 2–5 × 10^5 cells/plate. At the indicated times thereafter, both basal hydroxylase activity (hydroxylase activity 16 h after a medium change) (solid lines) and induced hydroxylase activity (hydroxylase activity after 8 h exposure to benz[*a*]anthracene, 1 μg/ml) (dashed lines) were determined. At least two plates of cells were combined for each determination. Each point represents the average of duplicate determinations of both aryl hydrocarbon hydroxylase and protein concentration. △, parent; ○, subclone 1; ▽, subclone 2; □, subclone 6. Cells became confluent approximately 7 days after plating.

clones 1 and 2 have identical heat-inactivation curves (Fig. 3), suggesting similarity in their native conformation. Although these findings do not prove that each strain contains the identical enzyme complex, the similarity in properties suggests that they are the same.

We also tested the highly inducible subclone 2 for possible heterogeneity in hydroxylase activity by isolating sub-subclones (Table II). All six sub-subclones have hydroxylase activity, which is inducible both by exposure to benz[*a*]anthracene and by combined exposure to benz[*a*]anthracene and temporary inhibition of protein synthesis. Again, however, there is a variation in the magnitude of responses, with values ranging from one-third to twice that of subclone 2. The results also indicate that the overall hydroxylase activity of the subclone 2 population had decreased during the period required to isolate and propagate the sub-subclones. We do not know the mechanism of this decrease in activity or whether the average hydroxylase activity of the subclone 2 population will eventually stabilize at a still lower value, perhaps more like that of the parent population.

These results demonstrate heterogeneity in hydroxylase inducibility among the sub-subclonal populations and indicate that this heterogeneity appears during the time period required to isolate and propagate the sub-subclones (2–3 mo).

The parental cell population and the subclones in which these differences in hydroxylase inducibility were first observed were heteroploid (Table I). It was possible that the observed variation in hydroxylase activity was a property limited to heterploid cells and is due to differences in the chromosomal composition of the various cell strains. We, therefore, prepared subclones from a BRL 3C4 cell population known to be predominantly diploid, and examined these subclones for heterogeneity in hydroxylase activity.

Table III indicates that these predominantly diploid subclones also exhibit substantial variation in hydroxylase induction by benz[*a*]anthracene. The subclones also differ from each other in the

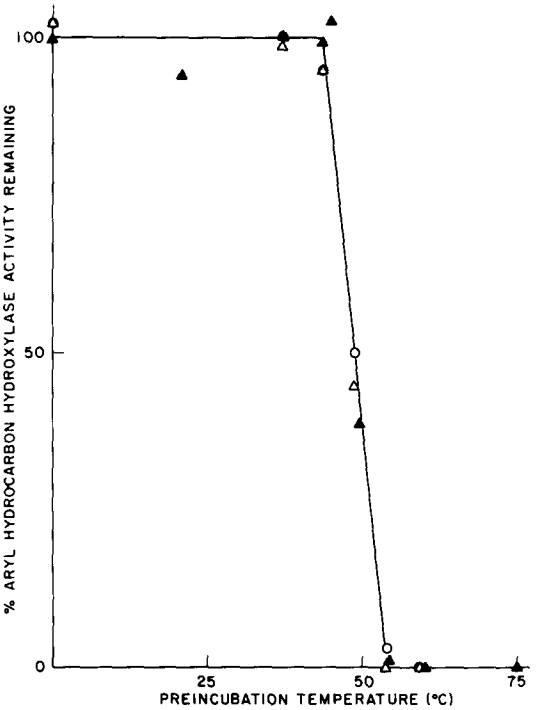

FIGURE 3 Heat stability of benz[*a*]anthracene-induced aryl hydrocarbon hydroxylase in heteroploid parental cells and subclones. Cells were exposed to benz[*a*]anthracene (1 μg/ml) for 8 h. Cell homogenates were preincubated for 5 min without NADPH or substrate. The standard incubation followed the preliminary incubation. ▲, parents; ○, subclone 1; △, subclone 2.

TABLE II

*Aryl Hydrocarbon Hydroxylase Activity in Rat Liver Cell Sub-subclones**

Sub-subclone	Treatment: None‡	Benz[*a*]anthracene§	Benz[*a*]anthracene + temporary inhibition of protein synthesis‖
		U/mg protein	
Subclone 2	7	93	546
A	2	103	362
B	7	86	455
C	8	177	573
D	2	28	260
E	5	57	380
F	4	38	378

* Each value represents the average of duplicate plates of cells. Hydroxylase activity of identically treated plates of cells varied less than 15%.

‡ Hydroxylase activity at the beginning of the experiment.

§ Cells were exposed for 4 h to medium containing benz[*a*]anthracene (1 μg/ml).

‖ Cells were exposed for 4 h to medium containing benz[*a*]anthracene (1 μg/ml) and cycloheximide (10 μg/ml). They then received two 15-min washes with medium containing benz[*a*]anthracene and actinomycin D (1 μg/ml) and were exposed to this second medium for a total of 4 h.

TABLE III

*Aryl Hydrocarbon Hydroxylase Activity in Predominantly Diploid Rat Liver Cell Subclones**

Subclone	Total chromosome no.‡	Treatment: Medium change§	Benz[*a*]anthracene‖
		U/mg protein	
Parents (BRL-3C4)	42 (40-42) 14/20	57	285
A	42 42 20/20	5	58
B	42 (41-43) 14/20	22	115
C	42 (40-43) 13/20	45	336
H	42 (41-43) 15/20	27	172
J	43 (42-44) 15/20	4	6
L	42 (40-43) 12/20	34	197
N	43 (41-43) 12/20	17	63
P	42 (41-43) 6/20	47	263

* Each value represents the average from duplicate plates of cells. Hydroxylase activity of identically treated plates of cells varied less than 15%.

‡ Values indicate means and ranges and the fraction with a chromosome number equal to the mean.

§ Cells were exposed for 8 h to fresh medium.

‖ Cells were exposed for 8 h to medium containing benz[*a*]anthracene (1 μg/ml).

kinetics of hydroxylase induction by benz[*a*]-anthracene (Fig. 4). Furthermore, these differences in hydroxylase activity are also present throughout the different phases of cell growth (Fig. 5). These subclonal differences have remained stable during several months of growth in culture. The results suggest that the cells which made up the diploid parental cell population were also heterogeneous with respect to hydroxylase induction by benz[*a*]anthracene. We also found that the predominantly diploid cell populations, in general, have higher basal and benz[*a*]anthracene-induced hydroxylase activities than the heteroploid populations; however, the hydroxylase in subclone J is apparently not inducible by benz[*a*]-anthracene.

Our results indicate that cell strains which differ substantially in aryl hydrocarbon hydroxylase activity can be isolated from both heteroploid and diploid BRL 3C4 populations. We infer, therefore, that the parental cell populations are composed of individual cell lineages which are heterogeneous with respect to hydroxylase induction. Furthermore, we cannot explain the heterogeneity simply in terms of chromosome dosage. The possibility remains that diploid clones and subclones are genetically different from one another, even though their chromosome numbers are identical. However, if we assume that diploid subclones have the same genetic constitution, then epigenetic events must regulate hydroxylase activity. We cannot account for this degree of heterogeneity as a result of independent mutations, unless the mutation rate is extremely high under these conditions of cell culture.

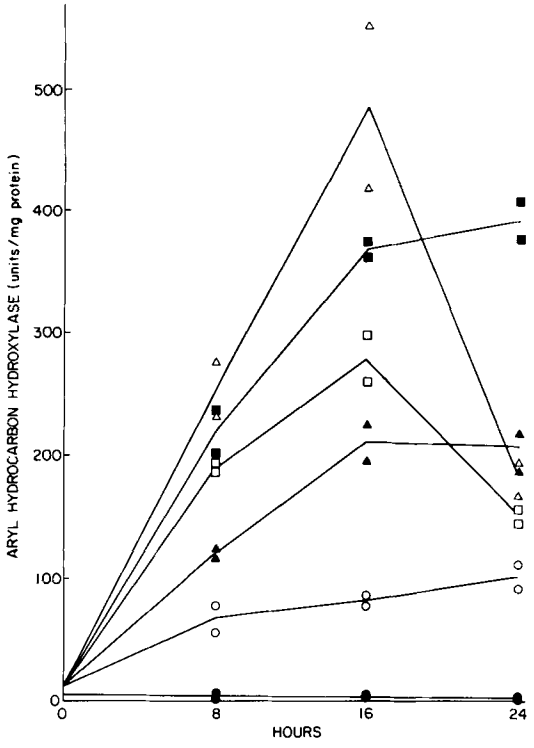

FIGURE 4 Kinetics of aryl hydrocarbon hydroxylase induction in near-diploid parental cells and subclones. Experimental conditions were identical to those described in Fig. 1. △, parents; ○, subclone A; ■, subclone C; ▲, subclone H; ●, subclone J; □, subclone L.

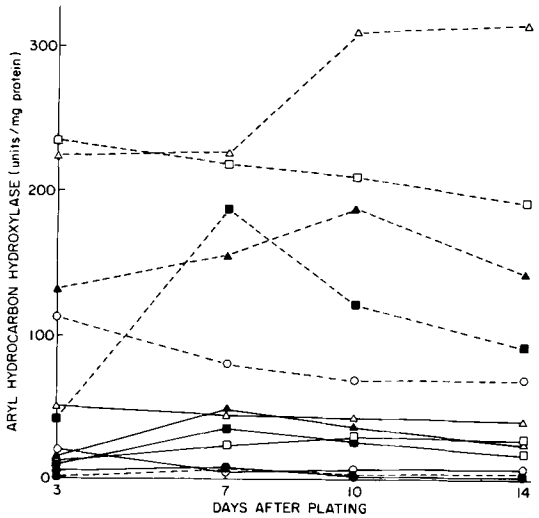

FIGURE 5 Aryl hydrocarbon hydroxylase activity in near-diploid parental cells and subclones after increasing times in culture. Experimental conditions were identical to those described in Fig. 2. △, parents; ○, subclone A; ■, subclone C; ▲, subclone H; ●, subclone J; □, subclone L.

It is likely that heterogeneity in hydroxylase activity has been underestimated in the interpretation of experiments involving polycyclic hydrocarbon-induced cytotoxicity and/or transformation, since the hydroxylase is involved in converting these compounds to detoxified products as well as to more reactive or carcinogenic forms (8, 10–12, 23). Potential heterogeneity in hydroxylase inducibility of parental cell populations must also be considered in the interpretation of experiments involving differences in hydroxylase activity between somatic cell hybrids, since some hybrids may be formed from cells whose hydroxylase activity differs substantially from that of their respective parental population's mean activity (2, 27).

Variability in tyrosine aminotransferase inducibility between subclones of hepatoma tissue culture cells (1) and in albumin production between subclones of Fu5 hepatoma cells (20) has also been reported. Heterogeneity within parental cell populations may prove to be more characteristic of mammalian cells in culture than has previously been assumed. Whether such heterogeneity reflects the situation in vivo remains to be determined.

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