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A COMPARISON OF ARYL HYDROCARBON HYDROXYLASE INDUCTION PHENOTYPES AMONG AND WITHIN CELL LINES.

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A COMPARISON OF ARYL HYDROCARBON HYDROXYLASE INDUCTION PHENOTYPES AMONG AND WITHIN CELL LINES

by

© Cynthia Jane Forster-Gibson

(née Forster)

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A COMPARISON OF ARYL HYDROCARBON HYDROXYLASE INDUCTION PHENOTYPES AMONG AND WITHIN CELL LINES

by

Cynthia Jane Forster-Gibson

ABSTRACT

Aryl hydrocarbon hydroxylase (AHH) activity in two cell lines - Hepa-1c1 and H4IIE-C3 was induced to similar levels by benzo(a)-anthracence. Absolute levels varied between experiments. Relative levels, determined within experiments, were stable between experiments. Nuclear translocation of Ah receptor occurred in these lines and the noninducible HTC-SR cell line. No receptor was detectable in the noninducible Vero cell line. The Ah receptor appeared to be necessary, but not sufficient for AHH induction.

Two subclones assessed for their level of inducible AHH activity, had significantly lower levels, two had approximately the same level, and three had significantly higher levels ($p < 0.005$) than their progenitor - Hepa-1c1. The relative level was 0.14 ± 0.09 for Hepa-1c1 subclone 1 (Hs-1), and 1.37 ± 0.48 for subclone 9 (Hs-9). These levels were stable: over 2.4 years (Hs-1), or 1 year (Hs-9); throughout the year; through routine cell storage at -80°C; and over approximately

190 (Hepa-1c1), 160 (Hs-1), and 150 (Hs-9) generations. The relative levels were similar when activity was induced with either 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), or benzo(a)anthracene (BA). The high percentage of variant subclones and the stability of the relative levels of AHH induction of Hs-1 and Hs-9 suggests that an epigenetic mechanism contributes to control of AHH induction. No inducible activity was seen in two Vero subclones.

There was no apparent correlation between relative level of induced AHH activity and: 1. quantity of total Ah receptor (cytosol plus nuclear) per mg protein; 2. receptor affinity for [^{3}H] TCDD (Kd values were 7.5 nM (Hepa-1c1), 7.7 nM (Hs-1) and 7.9 nM (Hs-9); 3. subcellular distribution of [^{3}H] TCDD; 4. specificity of binding and saturable nature of binding; or 5. lack of induction of receptor by BA. There was no significant difference in the quantity of nuclear receptor (per mg protein) between Hepa-1c1 and Hs-1, but significantly more nuclear receptor in Hs-9 compared to Hepa-1c1. Co-ordinate measurement of nuclear receptor and absolute induced AHH activity indicated a correlation of 0.62 between these parameters. The data suggest that the extent of the correlation between the quantity of Ah receptor translocating to the nucleus and level of induced AHH activity is mediated by other factors.

To my parents

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I wish to express my sincere appreciation to my supervisor, Dr. Michael J. Dufresne, for giving me the opportunity to develop and broaden my scientific skills, and for his enthusiastic support and direction. He has given generously in both a scientific and a personal capacity.

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LIST OF ABBREVIATIONS

Ah ... Aromatic hydrocarbon

AHH ... Aryl Hydrocarbon Hydroxylase

BA ... Benzo(a)anthracene

BNF ... Beta-naphthoflavone (5,6-benzoflavone)

BP ... Benzo(a)pyrene

DMSO ... Dimethylsulfoxide

Hs-n ... Hepa-1c1 subclone n

MC ... 3-methylcholanthrene

MEM ... Alpha Minimal Essential Medium - not enriched

PAH ... Polycyclic Aromatic Hydrocarbon

PB ... Phenobarbital

TCDD ... 2,3,7,8-tetrachlorodibenzo-p-dioxin

Vs-n ... Vero subclone n

INTRODUCTION

The metabolism of thousands of foreign compounds - including chemical carcinogens, drugs and environmental pollutants - and some endogenous substrates - such as steroids and fatty acids - is accomplished by a group of membrane-bound monooxygenases referred to as cytochromes P-450 (Tukey, et al., 1982). Cytochrome P_1-450 is one of a number of forms of cytochrome P-450 induced by polycyclic aromatic hydrocarbons (PAHs) (reviewed by Nebert, et al., 1982). The PAHs include such compounds as 3-methylcholanthrene (MC), benzo(a)anthracene (BA), benzo(a)pyrene (BP), beta-naphthoflavone (BNF), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), all of which are capable of inducing aryl hydrocarbon hydroxylase (AHH) activity. The assay of induced AHH activity is considered to be representative of the appearance of induced cytochrome P_1-450.

The study of the metabolism of PAHs and the nature of the induction process has been the focus of widespread attention due to the ubiquity of PAHs and to the toxic, mutagenic and/or carcinogenic capacity of various members of this class of compounds. The metabolism of PAHs is of interest due to the findings that, for example, the formation of the ultimate carcinogenic form of a PAH such as BP, MC, or BA is dependent on its metabolism via cytochrome P_1-450 (reviewed by Conney, 1982). The process of AHH induction is therefore also of interest since induction alters the balance of various forms of cytochromes P-450 present in the cell and, as discussed below, can thereby alter the

balance of carcinogenic to non-carcinogenic metabolites.

Metabolism of Polycyclic Aromatic Hydrocarbons

The metabolism of PAHs involves, to differing extents, both P-450 and P_1-450 cytochromes, as well as epoxide hydrolase and soluble transferases. Oxygen is inserted into PAHs by one of the monooxygenases to produce epoxides or arene oxides. These products are potent electrophiles capable of covalently binding to nucleic acids and proteins (Nebert & Atlas, 1978), or they may undergo further metabolism. Epoxide hydrolase can convert epoxides to dihydrodiols, or epoxides can convert non-enzymically to phenols. Dihydrodiols and phenols can then be conjugated with glutathione by soluble transferases to produce water-soluble, excretable products, or further metabolized by monooxygenases to produce diol-epoxides. Depending on the region of the molecule so metabolized, the diol-epoxide formed may represent the biologically active form of the parent compound (Zedeck, 1980).

The overall metabolic process is represented by the specific example of the metabolism of BP, illustrated in Fig. 1. Epoxides are formed at the "K-region" of the BP molecule (indicated in Fig. 1) primarily by cytochrome P-450. These 4,5-oxides react with glutathione more quickly than do non-K-region epoxides. Therefore, the K-region epoxide can be excreted more rapidly than non-K-region epoxides, which are converted to dihydrodiols before conjugation with glutathione or further epoxidation. Benzo(a)pyrene is primarily converted to the non-K-region 7,8-oxide by cytochrome P_1-450. As the ratio of P_1-450 to P-450

Fig. 1. Metabolism of Benzo(a)pyrene. Chemical structures of some of the known metabolites of benzo(a)pyrene (top, center with carbon atoms numbered from 1 to 12). The K region arene oxide (top, left) is formed predominantly by a form(s) of cytochrome P-450 other than P_1-450 and is subsequently converted to the diol by epoxide hydrolase. The 7,8-oxide is formed predominantly by P_1-450. Following diol formation via. epoxide hydrolase, the 7,8-diol-9,10-epoxide can presumbaly be formed by any species of P-450. Not shown is the in vivo formation of the phenols of BP at the 1-, 3-, 6-, 7-, 9- and perhaps other positions. The possible secondary oxygenations of diols in other positions are also not illustrated. (Modified from Thorgeirsson and Nebert, 1977.)

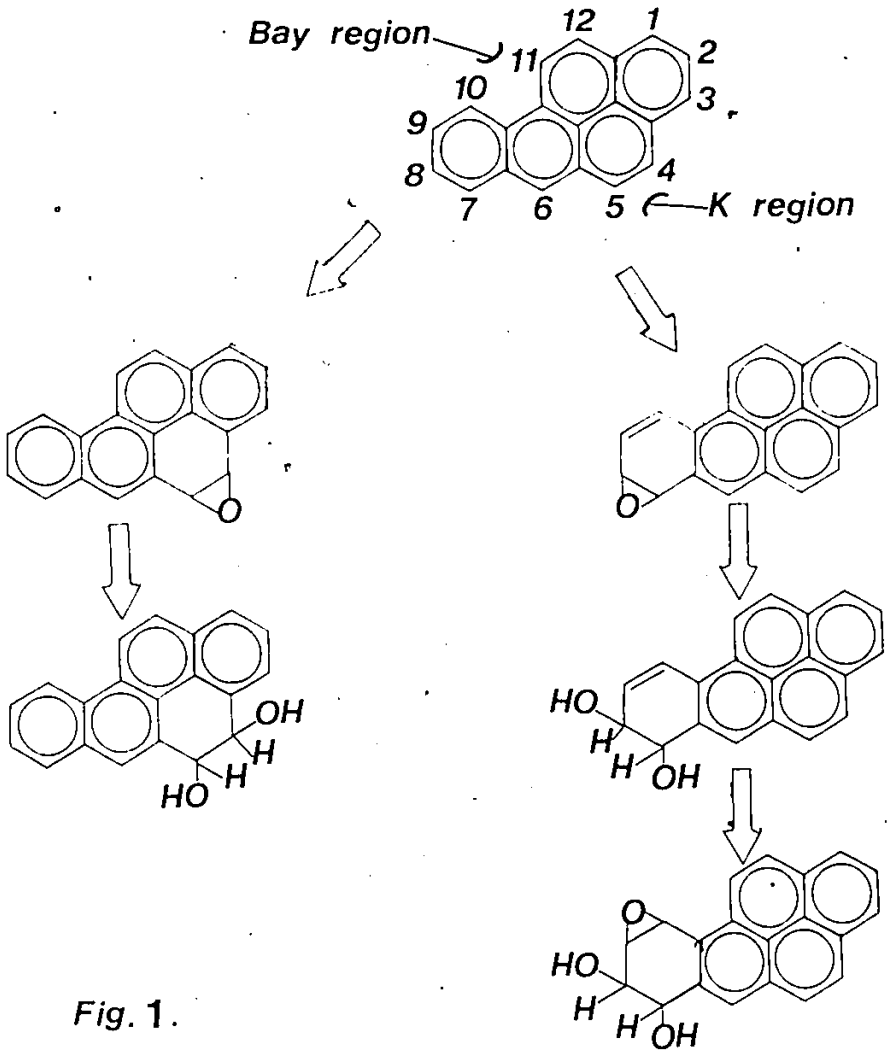
Bay region
12
1
11
2
10
3
9
4
8
7
6
5
K region
O
OH
H
HO
H
HO
H
H
OH
O
HO
H
H
OH

Fig. 1.

increases, so does the ratio of 7,8-oxide to 4,5-oxide, and, thereafter, the ratio of the metabolites of 7,8-oxide to the (eg. excretable) metabolites of the 4,5-oxide (Thorgeirsson and Nebert, 1977).

It has been postulated that the 7,8-diol-9,10-epoxide metabolite of the 7,8-oxide is the ultimate carcinogenic form of BP (Weinstein, et al., 1976; and reviewed by Conney, 1982), capable of binding to DNA in vivo . Therefore, an increase in the ratio of cytochrome P_1-450 to cytochrome P-450 may result in an increased ratio of carcinogenic to non-carcinogenic metabolites.

The ultimate carcinogenic form of many other potentially carcinogenic PAHs appears - like BP - to be a diol-expoxide with a highly reactive epoxide forming part of the bay region on a saturated angular benzo ring of a PAH (Conney, 1982). (Fig. 1 indicates the position of the bay region.) This may be the consequence of conversion of dihydrodiols to diol-epoxides in the same manner as occurs with BP (Conney, 1982).

As previously indicated, the toxic, mutagenic, carcinogenic, or harmless effect of exposure to a PAH depends to some extent on the balance of enzymes involved in the compound's metabolism. The enzyme balance in the whole animal, tissue, or cell is, in turn, altered by prior exposure to compounds that have the capacity to induce the formation of new cytochrome P-450 or P_1-450 molecules in inducible cells. Forms of the P-450 class of cytochromes, other than P_1-450, can be induced by a group of compounds that includes phenobarbital and pregnenolone-16α-carbonitrile. The P_1-450 cytochrome is induced by

PAHs including MC, TCDD, BNF and BA.

In control animals (i.e. those that have not been previously exposed to PAHs), metabolism of the PAH occurs by one or more forms of cytochrome P-450. After exposure to PAHs, those animals genetically capable of an induction response show induced AHH activity that reflects newly synthesized cytochrome P_1-450. Thus the ratio of cytochrome P_1-450 to P-450 is higher in induced than in uninduced animals. The metabolic paths consequently followed in the cells of induced versus uninduced animals varies as described above.

Polycyclic Aromatic Hydrocarbons in the Environment

Man is constantly being exposed to various levels of PAHs. For example, hydrocarbons such as the carcinogen BP are found in cigarette smoke, broiled fish and charcoal-broiled beef (reviewed by Nagao and Sugimura, 1978; and Conney, 1982), and in polluted air (Freeman, et al., 1971). The latter is primarily a consequence of forest fires and combustion of fossil fuels for heating and energy (Zedeck, 1980).

The halogenated PAH, TCDD, has been detected in the food chain (Baughman and Meselson, 1973). It has also recently come to wide public attention that TCDD has been detected as a contaminant of soil and water in certain areas of the world. Well-publicized examples include Seveso, Italy - where contamination was the consequence of a factory explosion (IARC Monograph, 1977), and Times Beach, Missouri - where contamination was the result of spraying contaminated waste oil

on roads and other public areas as a dust control (Sun, 1983). TCDD is a contaminant formed in the production of a number of chemicals, including the herbicide 2,4,5-T (IARC Monograph, 1977). It has been found to be mutagenic, teratogenic (IARC Monograph, 1977; and Hassoun and Dencker, 1982), and toxic (Knutson and Poland, 1982) in various test systems, at very low doses. TCDD is also a suspected carcinogen, although the effect on man is poorly defined (IARC Monograph, 1977).

Man's exposure to PAHs - some of which are metabolized primarily by induced AHH to carcinogenic forms - makes elucidation of the sequence of events involved in the induction of AHH particularly interesting. The possible association detected between high levels of induced activity and development of lung cancer in man (Kellermann, et al., 1973; Gahmberg, et al., 1979; McLemore, et al., 1979 and 1981; and Kouri, et al., 1982), serves to emphasize the importance of understanding induction as it relates to disease in man.

Model for Aryl Hydrocarbon Hydroxylase Induction

In 1977, Guenthner and Nebert proposed the model illustrated in Fig. 2. This model, and substantiating data obtained to date, are described below.

Upon entering a cell, a PAH is either non-specifically bound, or binds specifically with a receptor in the cytoplasm (Fig. 2), referred to as the Ah receptor. The PAH-receptor complex can then move into the nucleus. This movement has been shown to occur in mice (Okey, et al., 1979).

Fig. 2. Proposed Model for AHH Induction. The figure represents a cell in which polycyclic aromatic inducers evoke a pleiotypic response controlled by the Ah locus. The PAHs - MC, BA, BNF, or BP interact with a cytosolic receptor. The inducer-receptor complex, translocated into the nucleus, becomes the "effector", which turns on the integrator gene set. As a result, AHH activity increases and, it has been proposed, the cytosolic receptor is also induced. Reactive metabolites may bind covalently in the same cell, or in other cells, or other tissues to critical subcellular targets associated with tumours, mutation and toxicity. Reactive and nonreactive metabolites, and conjugated products are excreted from the cell. (Modified from Guenthner and Nebert, 1977.)

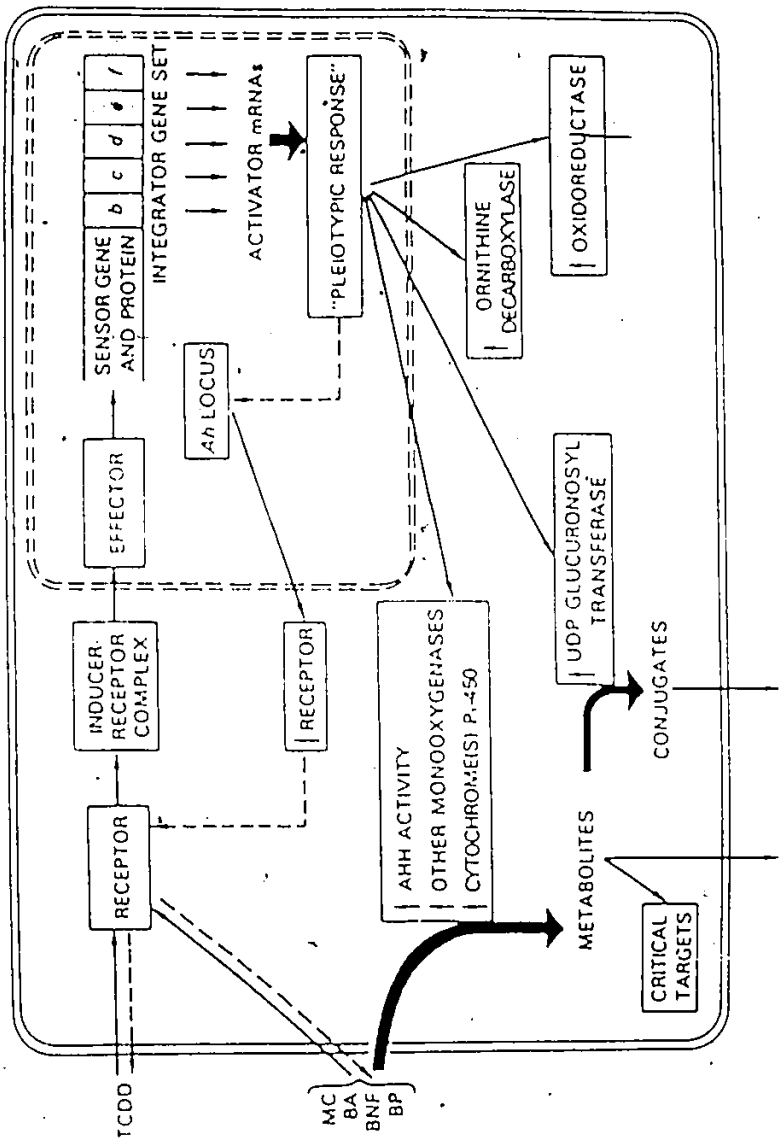
TCDD
MC
BA
BNF
BP
RECEPTOR
INDUCER-RECEPTOR COMPLEX
EFFECTOR
SENSOR GENE AND PROTEIN
INTEGRATOR GENE SET
b
c
d
e
f
Ah LOCUS
RECEPTOR
ACTIVATOR mRNAs
"PLEIOTYPIC RESPONSE"
AHH ACTIVITY
OTHER MONOOXYGENASES
CYTOCHROME(S) P-450
ORNITHINE DECARBOXYLASE
OXIDOREDUCTASE
UDP GLUCURONOSYL TRANSFERASE
CONJUGATES
METABOLITES
CRITICAL TARGETS

Fig. 2.

Transcription and translation are stimulated by interaction between the complex components and AHH-related structural genes (Nebert, et al., 1975). An increase in transcription was shown to occur by Tukey et al. (1981), who detected an increase in the amount of a 23S cytochrome P_1-450 mRNA in responsive mice treated with the cytochrome P_1-450 inducers, MC, or TCDD. The authors suggest that this is the result of increased transcription, rather than altered mRNA processing rates, or increased stability, and have recently shown (Tukey, et al., 1982) that a correlation exists between the amount of receptor measurable in the nuclei of liver cells from mice exposed to TCDD and the amount of cytochrome P_1-450 mRNA that was subsequently transcribed in these cells. An increase in translation may also occur, as suggested by the accompaniment of de novo protein synthesis with increases in, and/or appearance of new forms of, cytochrome P_1-450 (Haugen and Coon, 1976; and Althaus, et al., 1979).

The overall result of these events is an increase in AHH activity in the endoplasmic reticulum (Nebert and Atlas, 1978), and possibly nuclear membranes of the cell (Bresnick, 1978). As previously mentioned, this form of induction alters the balance of enzymes capable of metabolizing PAHs, tending to increase the ratio of carcinogenic to excretable metabolites (for PAHs that are potentially carcinogenic).

Genetic Variation

The degree of induced AHH activity has been found to vary with a wide array of factors. These factors include, among other things, the tissue being assessed and the age, hormonal status and genetic make-up of the test animal (reviewed by Thorgeirsson and Nebert, 1977). The genetic factors have been primarily investigated in inbred strains of mice.

Early work with strains of mice responsive to inducers, and strains that were non-responsive (Nebert and Gielen, 1972; and Nebert, et al., 1975), suggested that at least two genes and six alleles were involved in the induction process. This is the simplest genetic model to explain the findings of three different patterns of inheritance of expression of AHH induction by MC. Depending on the strains of mice being crossed, autosomal dominant inheritance, additive inheritance, or lack of inheritance of AHH induction has been seen.

It has been suggested that, of the two genes thought to be required for AHH induction, one gene codes for a regulatory product and the other codes for a structural product(s). The regulatory product may be the receptor(s), referred to as the Ah receptor, that complexes with PAHs. As described previously in the model for induction, PAH binding to the Ah receptor is a required step in the process leading to increased cytochrome P_1-450 activity. For this reason, the Ah receptor has been described as a regulatory molecule. The structural product would be cytochrome P_1 - 450 - whose activity is induced.

In man, both monogenic (Kellermann, 1973a) and polygenic (Atlas, et al., 1976; and Okuda, et al., 1977) models have been proposed for the control of AHH induction. Presumably, as in mice, at least two loci must be involved - the regulatory receptor locus, and the structural cytochrome P_1-450 locus.

It should be noted that a number of different forms of cytochrome P-450 have been identified. Two or more of these isozymes appear to be under the control of the Ah locus, cytochrome P_1-450 being one of these forms (Nebert, et al., 1982). Each form of cytochrome may be coded for at a different genetic locus and the induction of each may be mediated by the same, or different receptors. The possibility for heterogeneity in genetic control of AHH induction increases with the identification of each regulatory or structural locus involved in the AHH system.

Research Description

The following work describes an analysis of the association between degree of induced AHH activity and Ah receptor characteristics in different cell lines - Hepa-1c1, H4IIE-C3, HTC-SR and Vero - and among clones of the same line - Hepa-1c1, or Vero. Hepa-1c1 and H4IIE-C3 are AHH inducible cell lines, while HTC-SR and Vero are noninducible (Guenthner and Nebert, 1977).

This analysis was undertaken in order to help substantiate the role of Ah receptor in AHH induction. Guenthner and Nebert had previously found essentially no difference in cytosolic [^{3}H] TCDD binding in the

above-mentioned cell lines, but did find an apparent increase in receptor binding during induction in the two inducible cell lines. However, the charcoal-binding assay used by Guenthner and Nebert to separate free from bound ligand (TCDD) was subsequently found to measure a large proportion of non-saturable binding sites. Okey, et al. (1979), showed that sucrose density gradiant analysis following dextran-charcoal binding of free ligand (TCDD) reliably separated a class of high affinity, low capacity sites from non-saturable binding. This modification was used in the work reported here in the hopes that it might serve to clarify and extend the results of Guenthner and Nebert.

In addition to an assessment of the role of Ah receptor in AHH induction, a clonal analysis was undertaken in order to investigate the control of the phenotypic expression of AHH induction. The existence of high levels of variation in sequentially subcloned hepatoma cells (BRL-3C4, Whitlock, et al., 1976); stable, poorly inducible variants of highly inducible, mutagenized hepatoma cells (Hepa 1c1c7, Hankinson, 1979); and unstable, poorly inducible variants of unmutagenized hepatoma cells (H4IIE-C3, Hankinson, 1980) indicated that both epigenetic and genetic mechanisms might exert control over the AHH induction process.

Practical Importance

Data suggesting a relationship between capacity for moderate to high levels of AHH induction and occurrance of lung cancer in man, high AHH levels and tumour incidence in mice, and, more specifically, the correlation between presence of an allele coding for Ah receptor and susceptibility to MC-induced lung adenomas in mice (Kouri, et al., 1980), has been used to suggest that assessment of the level of AHH induced activity could be used to reliably predict an individual's risk for developing lung cancer. Investigations in this regard have primarily assessed induction of AHH activity in cultured lymphocytes.

To date, however, the work has been fraught with methodological problems. These have resulted in the occurrence of high rates of variation between samples taken from the same individual at different times of the year; or monozygotic twins at the same, or different times of the year. Some of the causes of variation have been identified and include the necessity of using mitogens for the stimulation of lymphocyte proliferation and the use of different lots of serum to supply essential growth factors and/or nutrients (Kouri, et al., 1979). Recently (Kouri, et al., 1982), the successful use of rigorous standardization procedures to reduce variability has been documented. However, the nature of the standardization limits assessment to within assays. The degree of variability between assays remains high and may continue to limit to some extent the successful clinical application of this test system.

It was hoped that further information regarding the association between Ah receptor and level of induction of AHH activity drawn from the data collected in this report would help in determining the efficacy of establishing an individual's risk for lung cancer by evaluating his/her level of Ah receptor. Furthermore, an assessment of the degree of stability between experiments of specific induction phenotypes among inducible lines and inducible clones maintained in a controlled environment, could help to identify the importance of environment as a source of variation attendant to the AHH assay in lymphocytes.

EXPERIMENTAL PROCEDURES

Cell Line Maintenance - Hepa-1 was derived from a transplanted hepatoma BW7756 originally produced in the C57L/J mouse. Dr. G. Darlington generously gave the line to the Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health. The line was subsequently given to Dr. O. Hankinson who obtained, and kindly gave to us, the Hepa-1c1 clone (Hankinson, 1979). H4IIE-C3 and HTC-SR cell lines were generously provided by Dr. E. Brad Thompson, National Cancer Institute, Bethesda, MD. H4IIE-C3 is a rat cell culture line derived from Reuber hepatoma H-35 (Reuber, 1961). HTC-SR is a line derived from an ascites tumour, which in turn had been derived from the rat "minimal deviation" hepatoma 7288c (Thompson, et al., 1966). The Vero cell line, derived from African Green Monkey kidney, was purchased from the American Type Culture Collection Repository, Rockville, MD. All cell lines were maintained in alpha minimal essential medium (Stanners, et al., 1971), supplemented with 5% fetal calf serum and 50 μg of gentamicin per ml. Gentamicin is an antimycoplasmal agent. Cultures were kept at 37°C in an atmosphere of 10% CO_2 and 90% air.

Cells labelled with [^{3}H] TdR were assessed for presence of mycoplasma (Nardone, et al., 1965). Cells in logarithmic growth were washed with citrate saline and incubated with 1 μCi [^{3}H] TdR per ml (specific activity of [^{3}H] TdR was 47 Ci/mmol) for 24 hours. The cells were then washed and incubated in medium

containing 0.73 mg thymidine per ml for 10 minutes, 4 times. The cells were fixed, coated with a thin layer of emulsion - diluted 1g:1ml with water - in the dark (one safelight was used), and left at 4°C - wrapped well to prevent exposure to light - for approximately 7 days. Every few days plates were developed as follows. Plates were floated on developer (Microdol X) for 6 minutes, fixed in sodium thiosulfate for 7 minutes and rinsed in water. (Personal communication from Dr. Marlene Schwartz.)

Cells that are contaminated with mycoplasma show an inhibition in nuclear incorporation of [^{3}H] TdR and an appearance of isotope over the cytoplasm and along the cell margin. Our cells had heavily labelled nuclei. Mycoplasma were not detected using the above protocol.

Cell Culture Techniques i) Subculturing: Cells were kept routinely in culture by plating 2 - 25 cm^2 flasks per line with approximately 2, or 4 X 10^5 cells. When the cultures neared confluency their medium was decanted. The cell surface was washed with warm 0.015 M citrate saline, pH 7.8, and the cells treated with trypsin (0.05% for Hepa-1c1 and 0.25% in citrate saline for all other cell types) for five minutes at 37°C. Trypsinized cells were collected into approximately 8 ml of medium per flask and counted using a haemocytometer. Cells were pelleted by centrifugation at 1500 rpm for 6 minutes. The pellet was resuspended to give the appropriate cell concentration. Cells were plated into the required plasticware.

ii) Efficiency of Plating: Cells were plated in four-well Linbros at densities of 10^2 or 5 X 10^2 cells

per well. When most colonies contained 20 or more cells, the medium was decanted, cells were washed with citrate saline and stained with methylene blue. The methylene blue was made of 7×10^{-2} M methylene blue in 50% (v/v) methanol. The number of colonies (i.e. clones) per well could then be counted and efficiency of plating estimated as the number of colonies formed, divided by the number of cells plated, multiplied by 100. This basic procedure was followed in the analysis of cell growth in dialzyed fetal calf serum, or in the presence of benzo(a)pyrene.

iii) Growth Curves: The generation time of cells in culture was determined by plating cells at an initial density that would allow them to reach confluence (eg. 10^5 cells per 60 mm dish, or 10^6 cells per 100 mm dish). Thereafter, cells were trypsinized as described under "Subculturing" approximately every 8 to 12 hours, and the total number of cells calculated per plate, until the majority of the cells in the culture detached from the surface of the plate. The cell counts were plotted on a semi-logarithmic scale against the appropriate time point.

Cloning - When the cells were in logarithmic growth, their medium was decanted. The cell surface was washed with warm citrate saline and the cells treated with 0.05% trypsin for three to five minutes at 37°C. Trypsinized cells were collected into fresh medium and counted using a haemocytometer. Cells were pelleted by centrifugation at 1500 rpm for

six minutes. The pellet was resuspended and a dilution series was set up such that ten ml each of 100, 10 and 5 cells per ml of growth medium was obtained. Ninety six-well Linbro plates were then plated with 4 drops per well of the 5 or 10 cell per ml concentrations. Three wells of each Linbro were plated with 4 drops of the 100 cell per ml concentration as a growth control. All wells were screened within 24 hours to identify wells containing one cell. These wells were surveyed for colonial growth over a period of up to three weeks. Colonies were then successively subcultured into plasticware of increasing area until they could be maintained in 25 cm^2 flasks.

Chromosome Counts - Cell cultures in the log phase of growth were incubated at 37°C for one to three hours in the presence of 0.8 μg of colcemid per ml of fresh medium. The medium was decanted, cells washed with warm citrate saline, trypsinized, pelleted in fresh medium and resuspended in 0.025 to 0.075 M KCl (37°C). Cells were pelleted, fixed in cold methanol:acetic acid (3:1), and stained with fresh 4% Giemsa (v/v in distilled water). At least 20 metaphase spreads were counted per cell population.

Aryl Hydrocarbon Hydroxylase Assay - (Modified from Nebert and Gielen, 1972). Cells were plated at 10^6 cells per 100 mm culture dish. When the cells were in logarithmic growth, the medium was decanted from each dish, cells washed with citrate saline (0.015 M trisodium citrate, pH 7.8) at 37°C and medium containing: the appropriate concentration of

inducing agent in solvent (dimethylsulfoxide or acetone); solvent only; or no agent; was added. Eighteen to 24 hours later, cells were harvested by decanting medium, washing with cold PBS and scraping with a rubber policeman into cold PBS. PBS refers to phosphate buffered saline (Dulbecco and Vogt, 1954) containing 27 μM KCL, 1.5 mM KH_2PO_4, 0.15 M $Na_2HPO_4.7H_2O$ and 0.14 M NaCl. Cells were washed three times with PBS by centrifuging at 1000 X g for 10 minutes. The final pellet was resuspended in 250 to 500 μl (per plate) of glycerol phosphate buffer. Glycerol phosphate buffer contained 0.25 M K_2HPO_4 and KH_2PO_4 in 30% glycerol (v/v) (Gielen and Nebert, 1971). 100 μl of the cell suspension was added to 900 μl reaction mixture containing 0.1 M $MgCl_2$, 0.36 mM NADPH, 0.42 mM NADH, and 0.7 mg bovine serum albumin per ml in 0.2 M Tris buffer (pH 7.5). At 15 second intervals, 50 μl of 2 mM benzo(a)pyrene was added to each sample and each sample incubated for 20 minutes at 37°C in a shaking water bath. The reaction was stopped by the addition of 4.25 ml of cold hexane:acetone (3.25:1), followed by a 10 minute incubation at 37°C. One ml of the organic phase was extracted for 30 seconds with 3 ml of NaOH. Fluorescence was then determined using a Turner Model 430 spectrofluorometer at an excitation wavelength of 396 nm and emission wavelength of 522 nm. Typically, two values of aryl hydrocarbon hydroxylase activity were determined for each of three plates, for each variable tested. One unit of AHH activity is defined (Gielen and Nebert, 1971) as the amount of enzyme catalyzing per minute, at 37°C, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo(a)-pyrene recrystallized standard.

Routine standardization was accomplished using quinine sulfate in 0.1 N H_2SO_4 (Nebert, 1978). This was done due to difficulties in obtaining, using and storing recrystallized 3-hydroxybenzo(a)pyrene. There is a direct linear relationship between fluorescence of quinine sulfate in H_2SO_4 and 3-OH-BP (Fig. 3). Quinine sulfate fluoresces strongly in 0.1 N H_2SO_4 at the peak excitation and emission wavelengths of 3-hydroxybenzo(a)pyrene. The fluorescence of appropriate concentrations of quinine sulfate was determined directly. The fluorescence of appropriate concentrations of 3-hydroxybenzo(a)pyrene, dissolved in a minimum volume of benzene and brought up to volume with methanol, was determined after incubation for 20 minutes at 37°C of 3-hydroxybenzo(a)pyrene as a component of the AHH assay reaction mixture. 3-hydroxybenzo(a)pyrene took the place of BP in this reaction mixture, and boiled cell samples were used in place of active cell samples. The assay protocol routinely used after the 37°C incubation was followed and samples assayed for fluorescence.

Ah Receptor Assay: (See flow chart of major steps - Fig. 4)

i) Cells labelled in culture: Cells were plated at 10^6 cells per 100 mm culture dish. Seventy two hours later, cells were exposed to fresh medium containing 1 nM [^{3}H]TCDD (and/or other chemicals - as described in the "Results") in dimethylsulfoxide (1 μl per ml of medium). Cells were labelled for one hour at 37°C (or as described in the text). For these treatments, fetal calf serum was omitted from the medium.

Fig. 3. Comparison of 3-hydroxybenzo(a)pyrene and Quinine Sulfate Fluorescence. Fluorescence of quinine sulfate in 0.1 N H_2SO_4 and 3-hydroxybenzo(a)pyrene was determined as described in the text. 3-OH-BP represents 3-hydroxybenzo(a)pyrene.

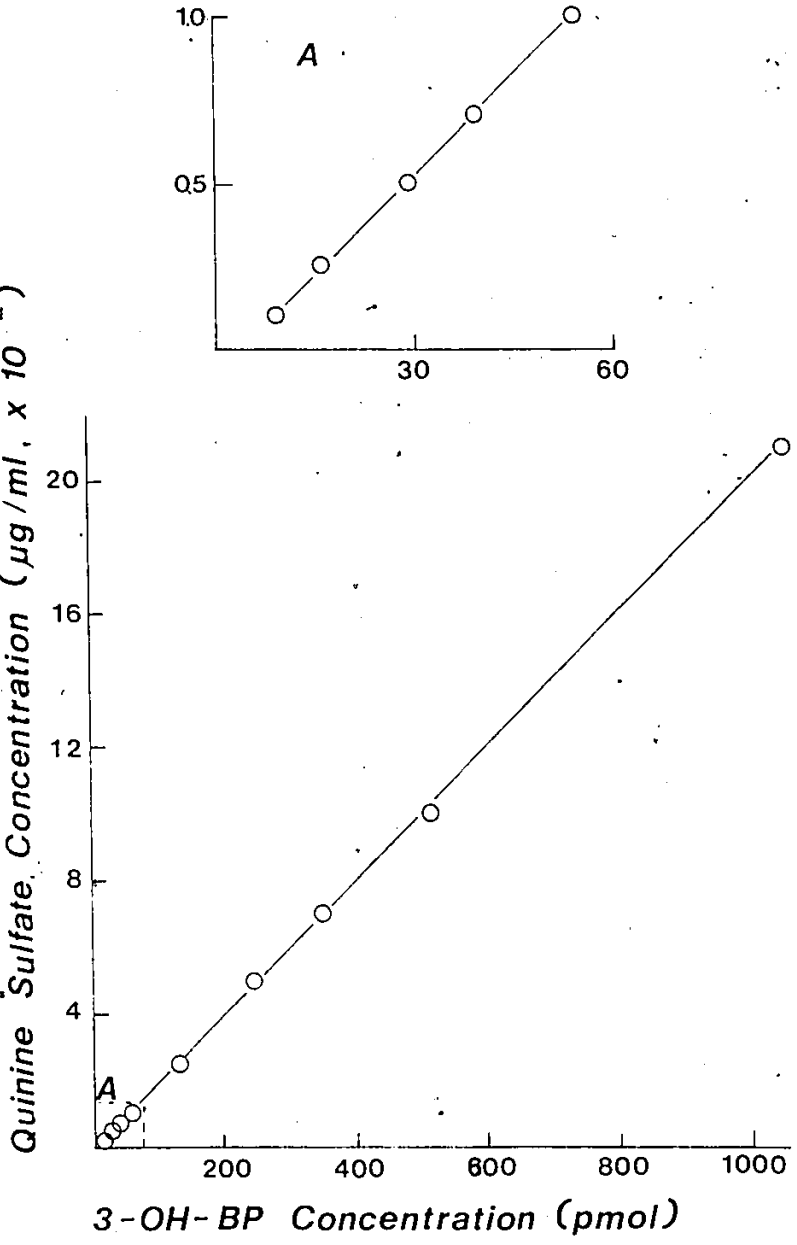
1.0
0.5
A
30
60
20
16
12
8
4
A
200
400
600
800
1000
Quinine Sulfate Concentration (μg/ml, x 10 2)
3-OH-BP Concentration (pmol)

Fig. 3.

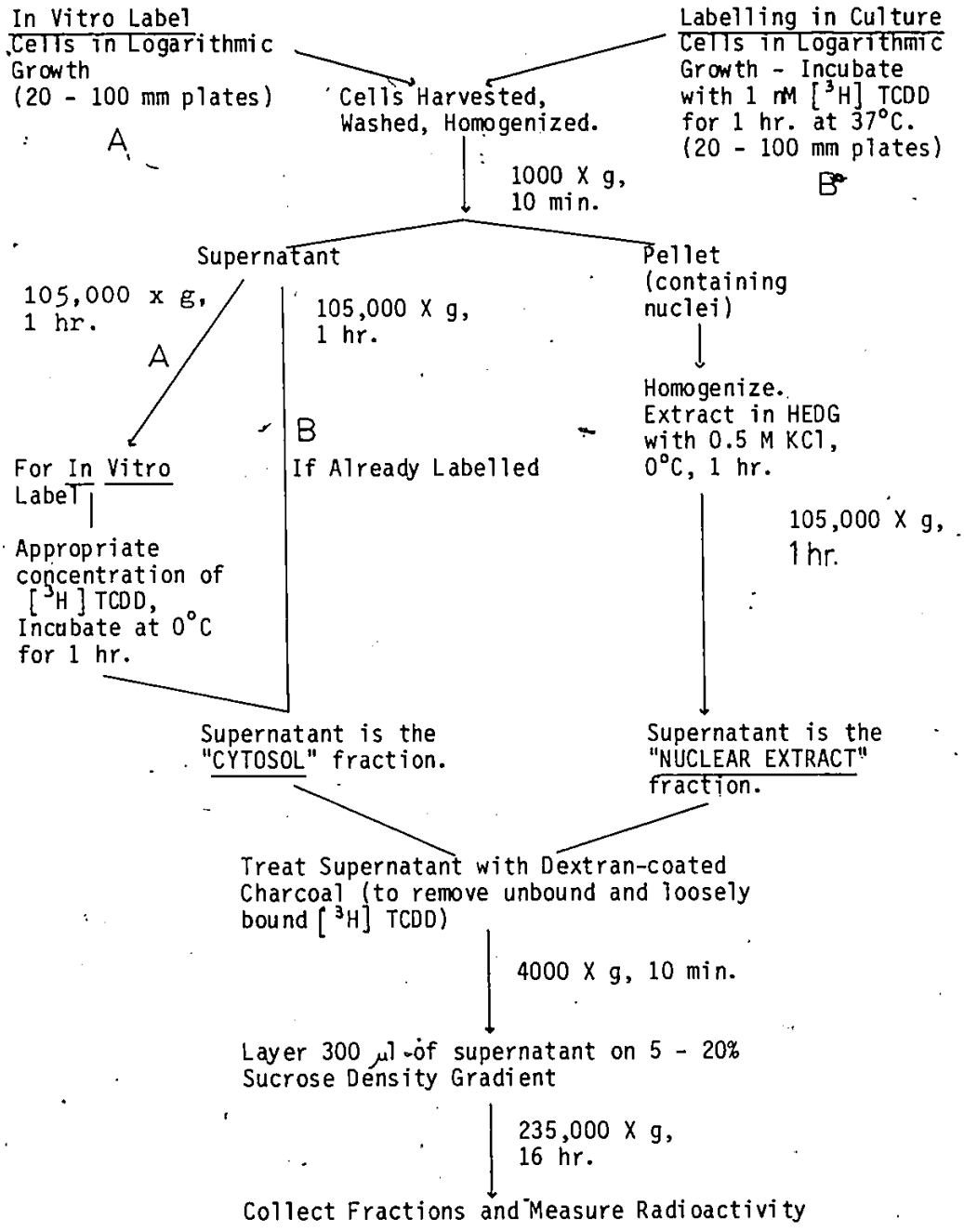

Fig. 4. Flow chart of Ah receptor assay - major protocol steps.

Preparation of cytosol: After [^{3}H]TCDD labelling of cells, the medium was decanted and the cell surface rinsed with cold PBS. Cells were harvested by scraping with a rubber policeman into approximately 2 mls PBS per plate. Cells were washed three times with cold PBS by centrifuging at 1000 X g for 10 minutes. Cells from 20 plates were combined and the washed pellet resuspended in 2 ml HEDG buffer. HEDG buffer contained 25 mM Hepes (4-(2-hydroxymethyl)-1-piperazineethane-sulfonic acid), 1.5 mM EDTA (ethylenediaminetetraacetic acid), 1 mM dithiothreitol, and 10% glycerol (v/v), pH 7.6 (Okey, et al., 1979). Cells were homogenized with a Polytron PT-10. Cell counts were obtained using a haemocytometer, before and after homogenization. Disruption of cells was routinely greater than 95%. The homogenate was centrifuged at 1000 X g for 10 minutes. This 1000 X g pellet was used to obtain the nuclear extract. The supernatant, centrifuged at 105,000 X g for one hour, yielded the cytosolic fraction. The pellet constituted the "microsomal fraction".

Preparation of nuclear extract: The 1000 X g pellet described above (Preparation of cytosol) was resuspended and washed 3 times in HEDG buffer by centrifuging at 1000 x g for 10 minutes. The final pellet was resuspended in 1 ml HEDG buffer containing 0.5M KCl, pH 8.5, and homogenized. Nuclear receptor was extracted by incubating the extract for one hour on ice. The homogenate was then centrifuged at 105,000 X g for one hour.

Sucrose density gradient analysis: The cytosol and nuclear extracts obtained as described above were incubated for 15 minutes at

4°C with dextran-coated charcoal pellets. Dextran-charcoal was removed by centrifugation at 4000 X g for 10 minutes from a suspension of 0.05% dextran and 0.5% charcoal (Norit A) (w/v) in HEDG buffer. 300 µl of sample was layered onto a 5-20% sucrose gradient and centrifuged at 235,000 X g for 16 hours at 2°C in a Beckman SW60 Ti rotor. Fractions were collected (20 - 200 µl fractions) on an ISCO model 640 gradient fractionator. Radioactivity in each fraction was determined in a Beckman LS 7500 liquid scintillation counter and corrected for counting efficiency. All experiments were carried out two or more times to determine reproducibility.

Protein determination: Protein values for AHH and Ah receptor assays were determined by the method of Bradford (1976) using a BioRad protein assay kit. The BioRad assay measures protein concentration by the differential colour change that occurs on binding of protein to Coomassie Brilliant Blue G-250. When protein binding occurs, there is a shift in the maximum absorption for the dye from 465 nm to 595 nm. The dye reagent contains Coomassie Brilliant Blue G-250 in methanol and phosphoric acid. (Final concentrations - as per Bradford, 1976 - 0.01% (w/v) dye, 4.7% (w/v) ethanol and 8.5% (w/v) phosphoric acid.) The dye was filtered before use. 100 µl of protein sample was added to 5 ml of dye reagent, vortexed and absorbance at 595 nm determined. The standard used for determination of protein concentration was bovine gamma globulin (supplied by BioRad).

ii) Cells incubated with [^{3}H] TCDD in vitro for: "Modified Scatchard Analysis": Cytosol was prepared from cells in

the logarithmic phase of growth that had not previously been exposed to [^{3}H] TCDD. The same protocol was followed for preparation of cytosol as in "Cells Labelled In Culture". The appropriate concentration of [^{3}H] TCDD - as described in the "Results" - was added to cytosol samples and incubated at 4°C for one hour in the dark. Sucrose density gradient analysis of each sample (as described above) was then carried out.

Mathematical Analyses

All statistical tests used herein, including the "t" test, "F" test, least squares method for determining the equation of the regression line and calculation of correlation coefficients, are fully described in Mendenhall, et al., 1974.

Ah Receptor Calculations: i) Calculation of amount of [^{3}H] TCDD specifically bound (after labelling in culture or in vitro): The total disintegrations per minute (dpm) for the peak area of the gradient - representing specific [^{3}H] TCDD binding (i.e. eliminated by excess cold TCDD as described in "Results") were determined. From this, the level of radioactivity present in cells incubated with [^{3}H] TCDD and excess cold TCDD was subtracted. The value obtained is the number of dpm representing specifically bound TCDD. Disintegrations per minute were converted to fmol specifically bound [^{3}H] TCDD per mg protein as follows: Given the specific activity of the [^{3}H] TCDD

and correcting for decay, it was determined that

$$1 \text{ dpm} \quad 6.645 \times 10^{-18} \text{ mol } [^3H] \text{ TCDD}$$

Therefore, in a sample with 1000 dpm in the binding peak there would be 6.645×10^{-15} mol $[^3H]$ TCDD

Since the sample volume applied to the gradient was 300 µl, there would be $\frac{6.645 \times 10^{-15} \text{ mol}}{300 \text{ µl}}$

If the sample had 1 mg of protein per ml, the amount of specifically bound $[^3H]$ TCDD per mg protein would be

$$\frac{6.645 \times 10^{-15} \text{ mol}}{0.3}$$

or, 22.5 fmol

ii) Modified Scatchard Analysis

(Okey, et al., 1979): A Scatchard analysis compares the ratio of Bound to Free ligand (in this case, $[^3H]$ TCDD) to the concentration of Bound ligand. Bound ligand was calculated as the amount of $[^3H]$ TCDD specifically bound in fmol per mg protein, as described above. Free $[^3H]$ TCDD was calculated as the difference between total $[^3H]$ TCDD in the sample before charcoal treatment and specifically bound $[^3H]$ TCDD. From these data, a ratio of Bound/Free TCDD was determined for each value of specifically bound $[^3H]$ TCDD. The ratios are plotted against the amount of Bound $[^3H]$ TCDD.

The data were then assessed by a least squares linear regression analysis to obtain an equation for the line and a correlation co-efficient (r). The significance of the correlation so obtained can be calculated by a modified "t" test and assigned a probability value.

The high level of non-specific , non-saturable binding in this assay can cause some scattering of data points obtained for low levels of specific [^{3}H] TCDD binding. Data points giving a large degree of scatter were therefore not included in the calculations described herein.

Provided there was a good correlation between the Bound/Free and Bound values, the computed equation of the line could be used to determine affinity of binding (Kd) and number of receptor sites per sample (B_{max}) (Clark and Peck, 1977). The slope of the line is equal to $-\frac{1}{Kd}$. The x intercept gives the value for B_{max}.

The Kd value obtained herein was in fmol per mg protein and was, as is common practice, converted to a molar value. The number of receptor sites can be converted to number of receptors per cell, assuming one binding site per receptor, in the following manner:

If $n = 100$ fmol specifically bound [^{3}H] TCDD per mg protein

And the average amount of protein per cell for that sample was

$$0.3 \times 10^{-7}$$

The number of mol of specifically bound [^{3}H] TCDD per cell would be

$$30.0$$

(The average amount of protein per cell was determined on the basis of a total cell count just prior to homogenization and protein determination for the cytosol samples applied to the gradient.)

The mol of bound TCDD per cell was multipled by Avogadro's number (6.02252×10^{23}) to obtain a value of 1807 receptors per cell. The value was rounded to the nearest 100 since the sensitivity of the test was reduced by the method of determining the amount of protein per cell.

RESULTS

Analysis of Four Cell Lines

(1) Aryl Hydrocarbon Hydroxylase Induction Phenotypes

A modified version (personal communication to A.B. Okey from D.W. Nebert) of the AHH assay of Nebert and Gielen (1972) was used to assay activity in four cell lines. The lines were Hepa-1c1, derived from a mouse hepatoma; H4IIE-C3 and HTC-SR, both derived from rat hepatomas; and Vero, derived from an African green monkey kidney. Hepa-1c1 and H4IIE-C3 were confirmed in this laboratory to be inducible, Vero and HTC-SR were noninducible.

The inducible cell lines - Hepa-1c1 and H4IIE-C3 - were used to determine maximal assay conditions and thus appropriate modifications to the protocol in routine use in Dr. Okey's laboratory. In summary, modifications to the AHH assay initially used included: (1) the use of DMSO or acetone, rather than dioxane as solvent, in order to avoid induction of cells by exposure to solvent alone; (2) elimination of the step of homogenization of cells in favour of simple vortexing to increase the efficiency of the assay; (3) increasing the pH of the reaction mixture buffer (0.2 M Tris) from 7.25 to 7.5 - the apparent pH maximum for Hepa-1c1; and (4) decreasing the length of incubation of the reaction mixture (at 37°C) from 60 to 20 minutes.

All four cell lines were assayed for induction of AHH activity using the modifications discussed above. Table 1 gives specific activities for each cell line. Aryl hydrocarbon hydroxylase activity in H4IIE-C3 and Hepa-1c1 was inducible. Activity in HTC-SR and Vero was not inducible under the conditions used. Concentrations of TCDD and BA greater than those optimal for induction in Hepa-1c1 cells were used in order to determine whether Vero, or HTC-SR cells require greater inducer concentrations to increase AHH activity. The AHH activity in the Vero and HTC lines appeared, however, to be either uninducible, or much less inducible than Hepa-1c1 or H4IIE-C3.

(2) Comparison of the Nature of Inducibility in Hepa-1c1 and H4IIE-C3

Regardless of the assay conditions, Hepa-1c1 and H4IIE-C3 showed similar levels of BA-induced activity, when compared within experiments. The relative activity of H4IIE-C3 compared to Hepa-1c1 varied from 0.8 to 1.4 in different experiments (Table 2). As will be shown in the clonal analysis, this is a reasonably narrow range of variation.

When assayed in the same experiment, there was always an increase in activity when the concentration of BA was increased from 1.3 to 13 μM. Both lines were found to be inducible with 1.1μM MC.

Significant differences existed in the induction responses of Hepa-1c1 and H4IIE-C3 to exposure to varying concentrations of TCDD (Table 1). At 1, or 10 nM TCDD, the induced specificity activity of

Table 1. Aryl hydrocarbon hydroxylase activity in four cell lines.

Treatment[a]	Specific Activity ($\bar{X} \pm s$, N = 6)[b] H4IIE-C3	Hepa-1c1	HTC-SR	Vero
None	2.8 ±0.9	2.3 ± 0.3	0.2 ±0.3	0.9
0.1% DMSO	5.6 ± 0.2	2.6 ± 1.4	0.3 ±0.1	0.5 ± 0.2
1.3 μM BA	15.5 ± 0.4	16.8 ± 0.4	0.3 ±0.1	0.4 ± 0.2
13 μM BA	25.5 ± 2.3	22.3 ± 4.5	0.2	0.3 ± 0.1
1 nM TCDD	24.0 ± 0.7	54.0 ± 14.1	0.2	0.3 ± 0.3
10 nM TCDD	21.7 ± 2.7	39.0 ± 5.2	0.1	0.1 ± 0.1
100 nM TCDD	21.2 ± 1.9	18.2 ± 2.0	0.1	0.4

a: Cells in logarithmic growth were exposed to one of the agents listed as a component of their growth medium for 18 hours. At that time AHH activity was assayed as described in "Experimental Procedures".

b: Specific Activity: one unit equals that amount of enzyme catalyzing per minute, at 37°C, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo(a)pyrene recrystallized standard (Gielen and Nebert, 1971). N: represents the number of measurements obtained, per cell line, per treatment.

Table 2. Comparison of Hepa-1c1 and H4IIE-C3 aryl hydrocarbon hydroxylase induction responses.

Cell Line	N	Inducing Agent	Induced Specific Activity[c] ($\bar{X} \pm s$)	Relative Specific Activity[d]
Experiment 1[a]				
Hepa-1c1	6	1.3 μM BA	30.2 ± 3.5	1.0
H4IIE-C3	6		24.5 ± 7.0	0.8
Hepa-1c1	6	13 μM BA	37.3 ± 3.0	1.0
H4IIE-C3	6		49.4 ± 10.0	1.3
Experiment 2[a]				
Hepa-1c1	4	13 μM BA	13.3 ± 1.5	1.0
H4IIE-C3	4		17.5 ± 10.3	1.3
Experiment 3[b]				
Hepa-1c1	6	1.3 μM BA	14.6 ± 0.2	1.0
H4IIE-C3	6		12.7 ± 0.5	0.9
Hepa-1c1	6	13 μM BA	20.1 ± 4.3	1.0
H4IIE-C3	6		22.7 ± 1.4	1.1
Experiment 4[b]				
Hepa-1c1	6	1.1 μM MC	17.5 ± 4.7	
Hepa-1c1	6	11 μM MC	1.8 ± 1.5	
Experiment 5[b]				
H4IIE-C3	2	1.1 μM MC	10.0	
H4IIE-C3	2	11 μM MC	4.5	

a: Cells in logarithmic growth were exposed to one of the agents listed, as a component of their growth medium, for 18 hours. At that time, AHH activity was assayed as described in "Experimental Procedures" with the following exceptions: cells were homogenized, the pH of the reaction mixture was 7.25 and the 37°C incubation was for 60 minutes.

b: Cells in logarithmic growth were exposed to one of the agents listed, as a component of their growth medium, for 18 hours. At that time, AHH activity was assayed as described in "Experimental Procedures".

c: Induced Specific Activity was calculated by subtracting activity for solvent treated cells from that for cells treated with solvent and inducing agent.

d: Relative Specific Activity was determined for H4IIE-C3 compared to Hepa-1c1 within each experiment.

N: represents the number of measurements obtained.

H4IIE-C3 was significantly lower than that for Hepa-1c1 ($p < 0.05$ and $p < 0.025$, respectively). At 100 nM TCDD, however, there was no significant difference between the lines. Increasing the TCDD concentration above 1 nM resulted in a decrease in induced activity for Hepa-1c1. H4IIE-C3 cells, however, showed essentially no decrease from 1 to 10 to 100 nM in induced activity.

(3) Characteristics of Ah Receptor in Four Cell Lines

Data described in sections 3, 4 and 5 were obtained through collaborative work between Dr. M.J. Dufresne's laboratory (myself and J. Muncan) and Dr. A.B. Okey's laboratory (M.E. Mason and G.P. Bondy). As a graduate student in Dr. Dufresne's laboratory, my role included assisting in initiating the research, as well as aiding in, or carrying out experimental procedures. The data obtained through this collaboration have been published in the Journal of Biological Chemistry, 1980 (Okey et al., 1980). Data presented in Figs. 8 - 10 herein were collected subsequent to this publication, by myself, and confirm our previous findings.

The presence of Ah receptor in cell lines was detected by: (1) exposing cells in culture to medium containing [^{3}H] TCDD (for which the Ah receptor has a high affinity (Okey, et al., 1979), for one hour at 37 C; (2) treating the cytosol or nuclear extracts of these cells with dextran-coated charcoal (to eliminate unbound [^{3}H] TCDD); and (3) assessing binding by sucrose density gradient analysis. Centrifugation of samples on a sucrose density gradient separates specific

[^{3}H] TCDD binding from non-specifically bound [^{3}H] TCDD. The specifically bound [^{3}H] TCDD is represented by a binding peak that is eliminated in cells treated with both 1 nM [^{3}H] TCDD and an excess (100 to 1000nM) of unlabelled TCDD. According to these criteria, Ah receptor was detectable in the nucleus of Hepa-1c1, H4IIE-C3 and HTC cells, and the cytosol of Hepa-1c1 and HTC cells (Fig. 5, 6 and 7). Vero showed no TCDD receptor binding peak in the cytosol, or the nucleus, indicating a lack of detectable receptor (Fig. 8).

(4) Specificity of TCDD-Binding

The specific nature of the [^{3}H] TCDD binding thought to represent Ah receptor was assessed with further binding competition experiments. Cells were incubated for one hour at 37°C in the presence of 1 nM [^{3}H] TCDD and an excess (i.e. 100 or 1000 nM) concentration of competitor. The competitors used included the cytochrome P_1-450 inducers MC, BA, or BNF; or the cytochrome P-450 inducers PB, or pregnenolone-16α- carbonitrile. One would expect competition for binding between TCDD and any of the other chemicals that induce P_1-450, but not between TCDD and cytochrome P-450 inducers, if the specific [^{3}H] TCDD binding peak represents the Ah receptor. The results of such binding competition experiments are illustrated in Figs. 9 and 10 by: MC - a competitor representative of cytochrome P_1-450 inducers; and PB a competitor representative of cytochrome P-450 inducers.

The specific [^{3}H] TCDD binding peak in cytosol or nuclear

Fig. 5. Sucrose Density Gradient Detection of Specific [^{3}H] TCDD Binding in Cytosol and Nuclear Extracts of Hepa-1c1. Cells in logarithmic growth were incubated with 1 nM [^{3}H] TCDD, or 1 nM [^{3}H] TCDD plus 100 nM nonlabelled TCDD for one hour at 37°C. Cytosol and nuclear extracts were obtained, and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay". (Published data, Okey, et al., 1980.)

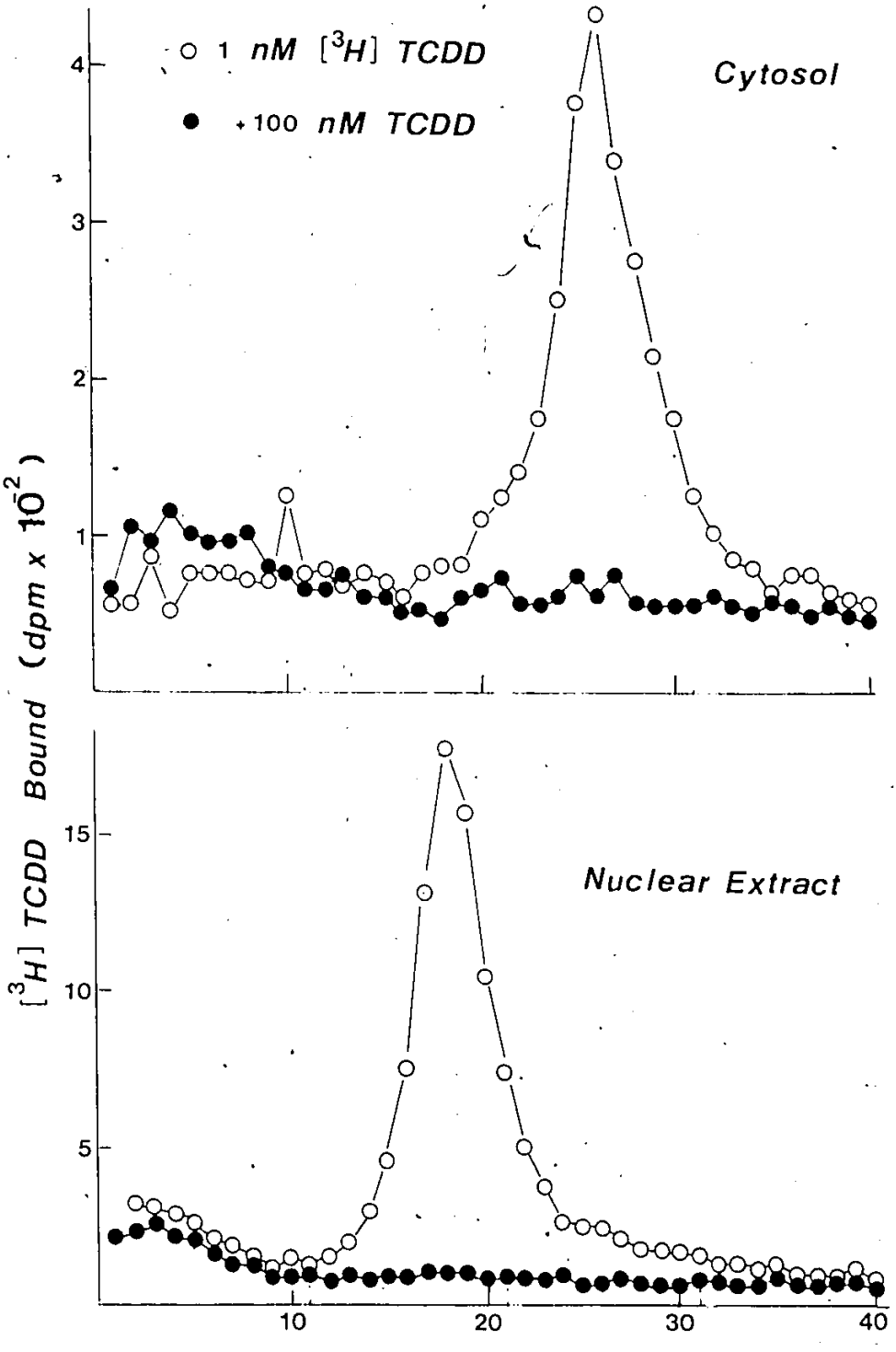
○ 1 nM [3H] TCDD
● +100 nM TCDD
Cytosol
Nuclear Extract
[3H] TCDD Bound (dpm x 10-2)
Fraction Number
1
2
3
4
5
10
15
20
30
40

Fig. 5.

Fig. 6. Sucrose Density Gradient Detection of Specific [^{3}H] TCDD Binding in Cytosol and Nuclear Extracts of H4IIE-C3. Cells in logarithmic growth were incubated with 1 nM [^{3}H] TCDD, or 1 nM [^{3}H] TCDD plus 100 nM nonlabelled TCDD for one hour at 37°C. Cytosol and nuclear extracts were obtained, and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay". (Published data, Okey, et al., 1980.)

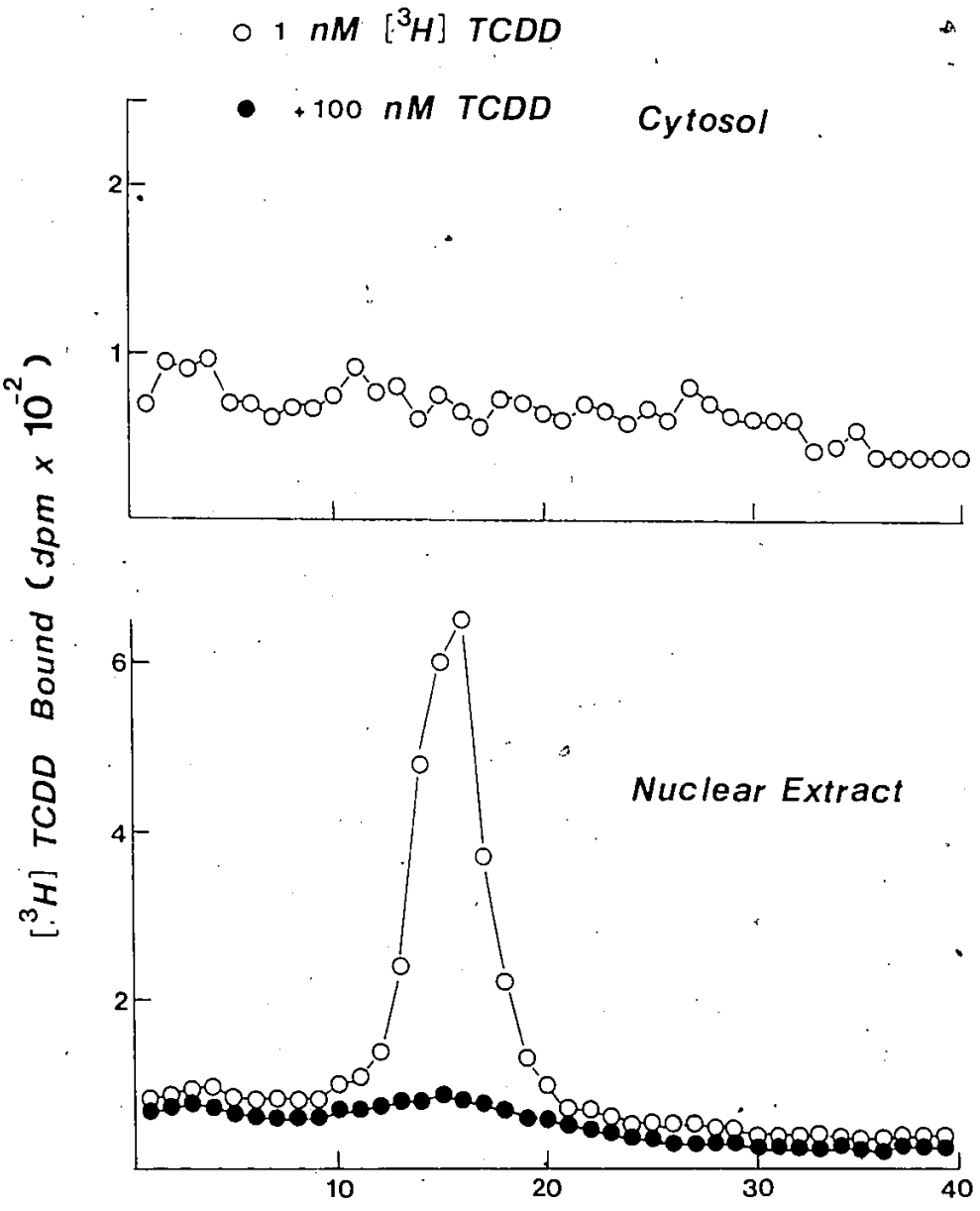
○ 1 nM [3H] TCDD
● +100 nM TCDD
Cytosol
Nuclear Extract
[3H] TCDD Bound (dpm x 10-2)
Fraction Number
2
1
6
4
2
10
20
30
40

Fig. 6.

Fig. 7. Sucrose Density Gradient Detection of Specific [^{3}H] TCDD Binding in Cytosol and Nuclear Extracts of HTC-SR. Cells in logarithmic growth were incubated with 1 nM [^{3}H] TCDD, or 1 nM [^{3}H] TCDD plus 100 nM nonlabelled TCDD for one hour at 37°C. Cytosol and nuclear extracts were obtained, and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay". (Published data, Okey, et al., 1980.)

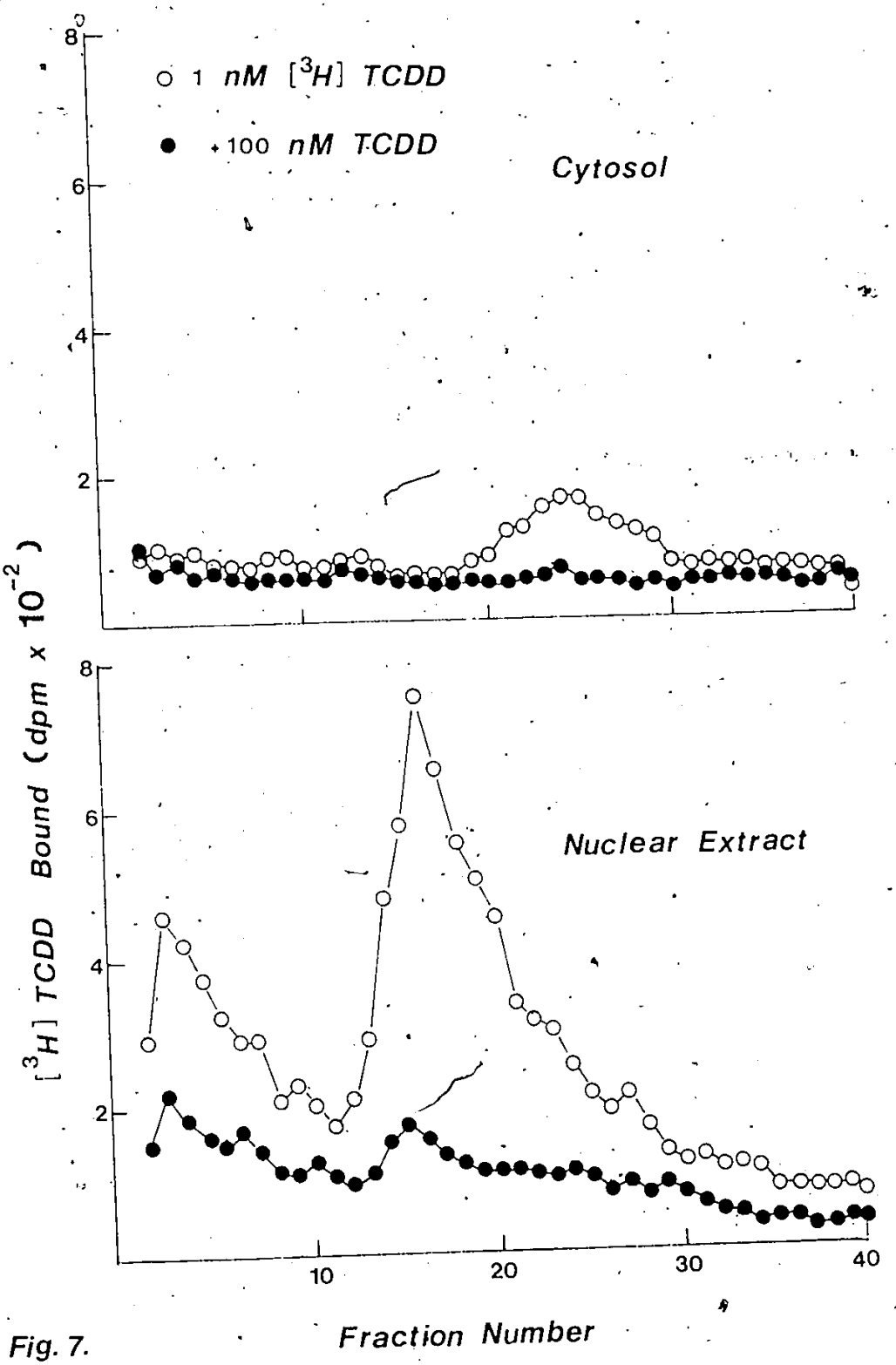
○ 1 nM [3H] TCDD
● +100 nM TCDD
Cytosol
Nuclear Extract
[3H] TCDD Bound (dpm x 10-2)
Fraction Number
8
6
4
2
10
20
30
40

Fig. 7.

Fig. 8. Sucrose Density Gradient Detection of Specific [^{3}H] TCDD Binding in Cytosol and Nuclear Extracts of Vero. Cells in logarithmic growth were incubated with 1 nM [^{3}H] TCDD for one hour at 37°C. Cytosol and nuclear extracts were obtained, and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay".

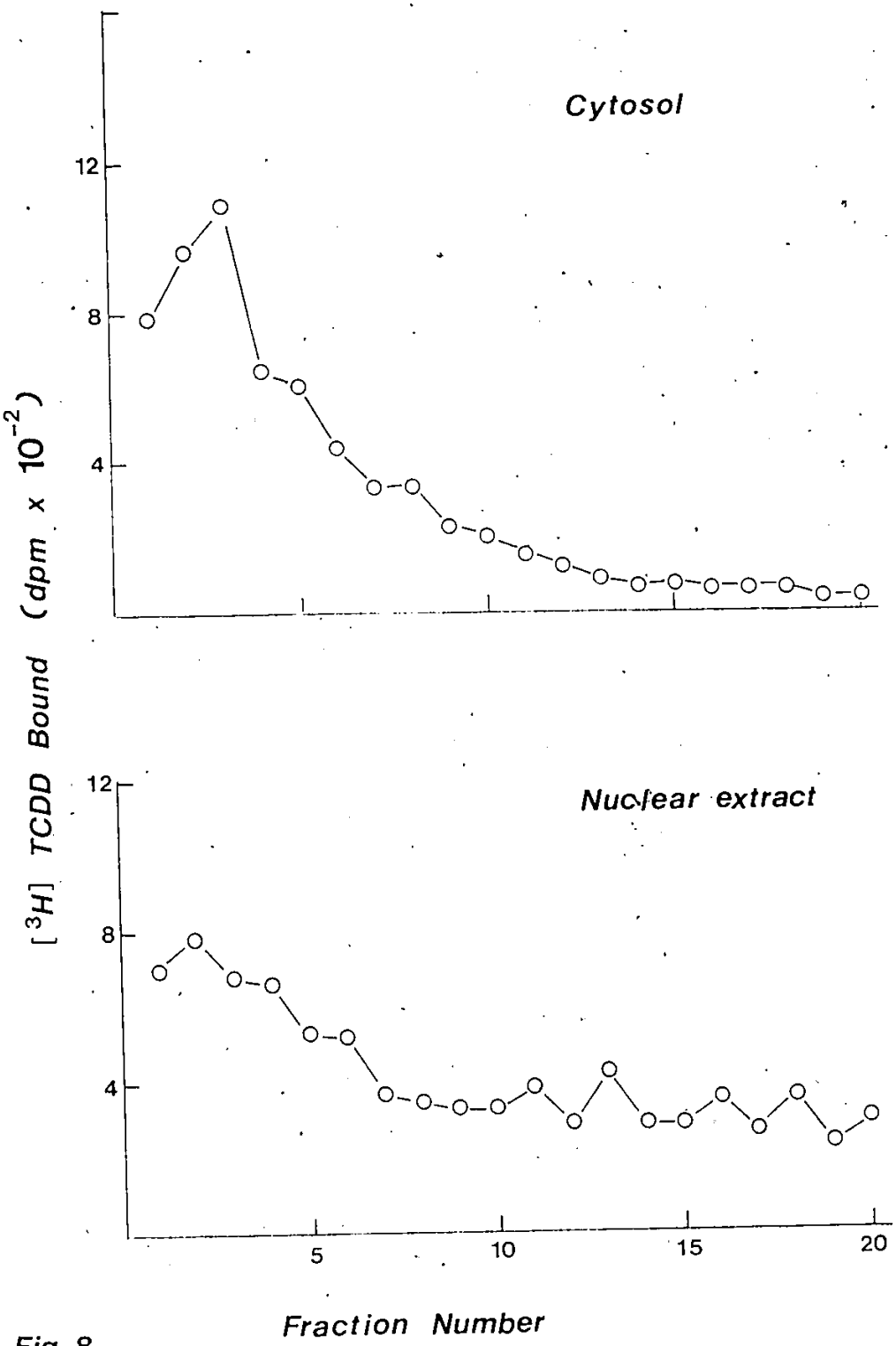
Cytosol
Nuclear extract
[3H] TCDD Bound (dpm x 10-2)
12
8
4
5
10
15
20
Fraction Number

Fig. 8.

Fig. 9. Competition for Specific [^{3}H] TCDD Binding - Using Excess Concentrations of Phenobarbital, or TCDD. Hepa-1c1 cells in logarithmic growth were incubated with 1 nM [^{3}H] TCDD, 1 nM [^{3}H] TCDD plus 1 μM nonlabelled TCDD, or 1 nM [^{3}H] TCDD plus 1 μM nonlabelled PB for one hour at 37°C. Cytosol and nuclear extracts were obtained, and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay". (Nuclear extract data, not shown.)

Fig 10. Competition for Specific [^{3}H] TCDD Binding - Using Excess Concentrations of Phenobarbital, or MC. Hepa-1c1 cells in logarithmic growth were incubated with 1 nM [^{3}H] TCDD, 1 nM [^{3}H] TCDD plus 100 nM PB, or 1 nM [^{3}H] TCDD plus 1 μM MC for one hour at 37°C. Cytosol and nuclear extracts were obtained, and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay". (Nuclear extract data, not shown.)

Fig. 9.

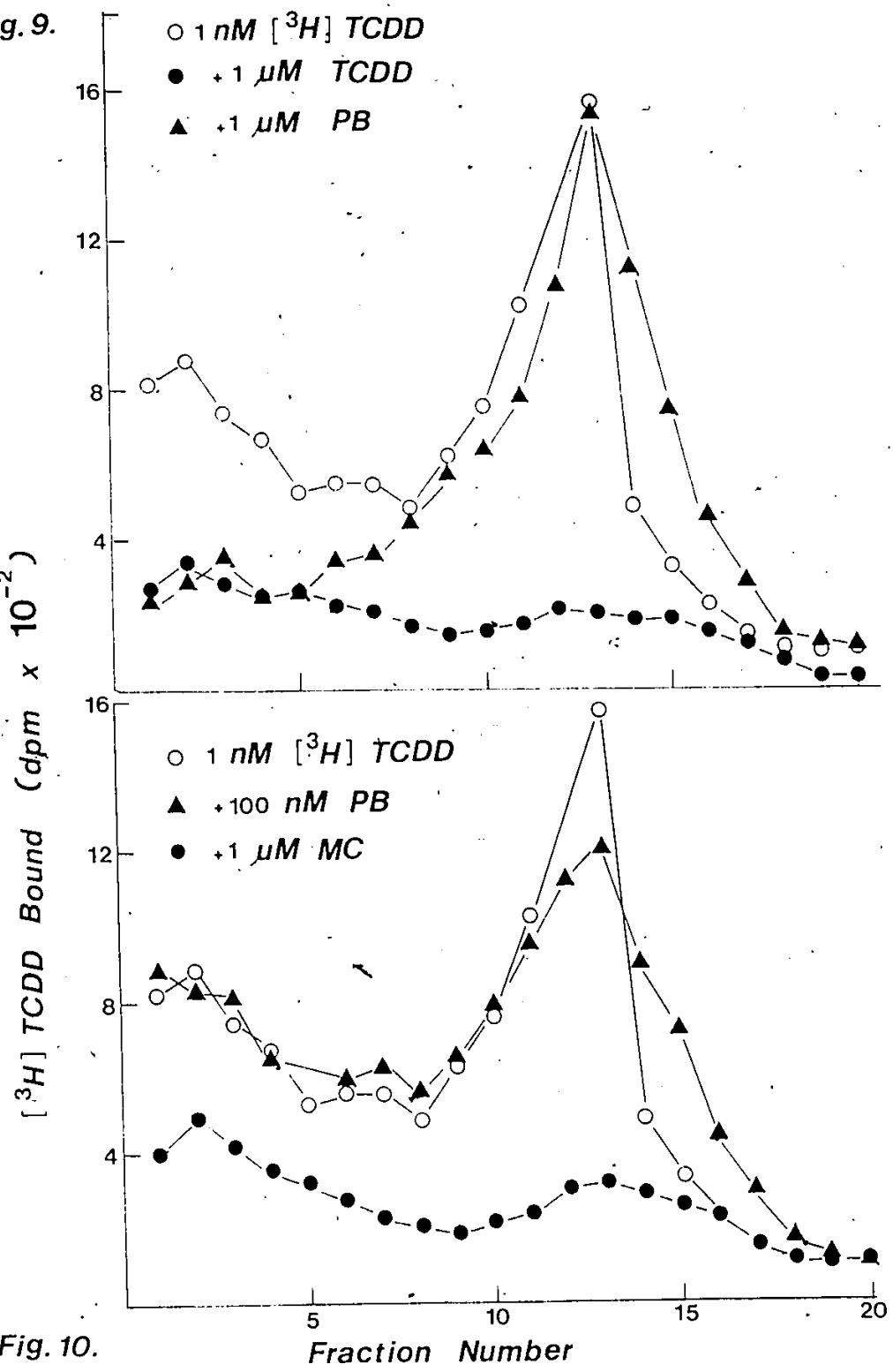
○ 1 nM [3H] TCDD
● +1 μM TCDD
▲ +1 μM PB
○ 1 nM [3H] TCDD
▲ +100 nM PB
● +1 μM MC
[3H] TCDD Bound (dpm x 10-2)
16
12
8
4
5
10
15
20
Fraction Number

Fig. 10.

extracts was eliminated in Hepa-1c1 cells incubated in the presence of excess MC, or BNF. The binding peak was greatly reduced in the presence of BA. (Differences in levels of competition seen with BA and MC are described in more detail for Hepa-1c1 and its subclones). Neither PB, nor pregnenolone-16α-carbonitrile, however, was capable of reducing the binding peak. These data illustrate the specific nature of the [^{3}H] TCDD binding.

(5) Translocation of the Ah Receptor

When Hepa-1c1 cells were incubated with [^{3}H] TCDD at 4°C for one hour, no receptor was detectable in the nuclear extract. With additional incubation at 37°C, however, receptor concentration in the nuclear extract gradually increased over a 55 minute period (Fig. 11). Concomitantly, the concentration of receptor in the cytosol decreased. These data indicate that a translocation of [^{3}H] TCDD bound receptor was occurring in a time and temperature-dependent manner.

Cytosol receptor was detectable in H4IIE-C3 cells only if the cells were incubated with [^{3}H] TCDD at 4°C, i.e. if translocation of receptor from the cytoplasm to the nucleus was blocked (data not shown). Conversely, nuclear receptor was detectable only after incubation at 37°C, i.e. after translocation from the cytoplasm has occurred.

(6) Choice of Parental Lines for Analysis of Clonal Variation

To assess the role of genetics in the control of phenotypic

Fig. 11. Time- and Temperature-Dependent Translocation of Specific [^{3}H] TCDD Binding from Cytosol to Nucleus in Hepa-1c1 Cells. Cells were incubated at 4°C for one hour in the presence of 1 nM [^{3}H] TCDD, then washed twice with cold citrate saline, cold fresh medium was added and cells were incubated for varying intervals at 37°C in the absence of [^{3}H] TCDD. Cytosol and nuclear extracts were prepared and specific binding was determined as described in "Experimental Procedures - Ah Receptor Assay".

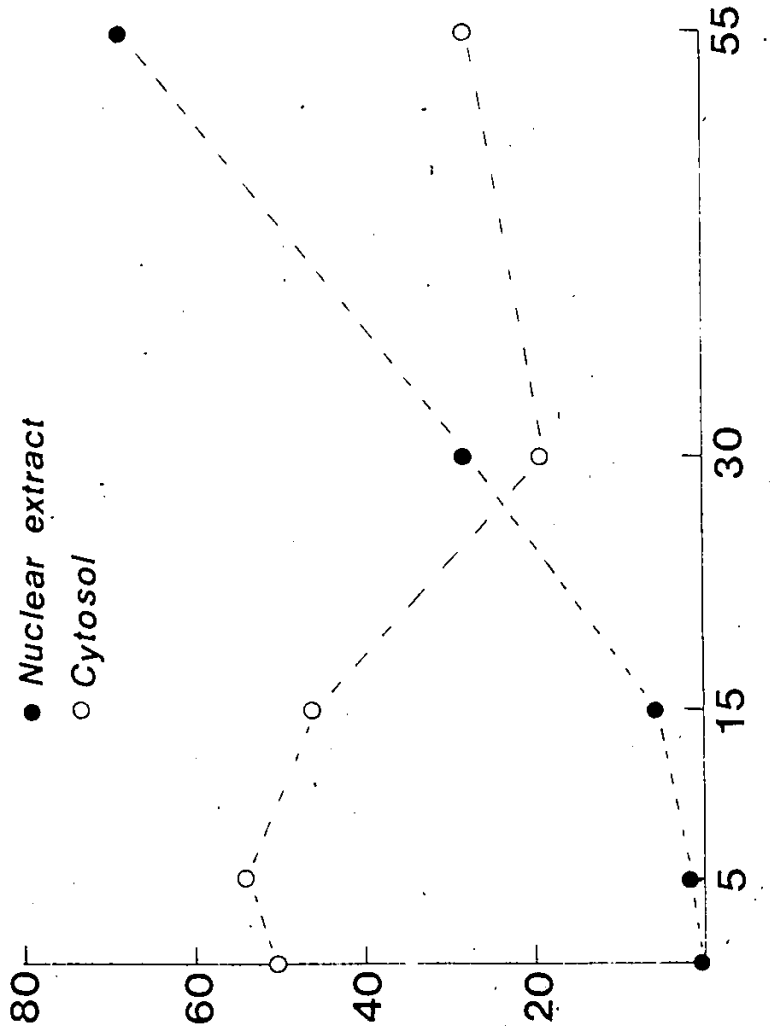
● Nuclear extract
○ Cytosol
80
60
40
20
5
15
30
55
TCDD-Specific Binding
(fmol per mg protein)
Time at 37°C (minutes)

Fig. 11.

expression of AHH induction, the analysis of variation in clones of an inducible and an uninducible cell line was undertaken. If genetic factors are a controlling element, one would expect to find similar levels of expression of induction in each clone. The level of induction characteristic of a particular clone would be expected to remain stable.

The information obtained on AHH induction and receptor characteristics was critical in deciding which of the four cell lines would be best suited to a clonal analysis of induction control. Lines with inducible or uninducible AHH activity and the appropriate Ah receptor characteristics expected on the basis of the proposed model of induction would be the most amenable.

Hepa-1c1 cells were inducible. Ah receptor (i.e. represented by specifically bound [^{3}H] TCDD) in Hepa-1c1 cells was detectable in the cytosol and capable of a time and temperature-dependent translocation to the nucleus. While H4IIE-C3 cells were inducible and showed receptor translocation to the nucleus, the requirement of labelling cells at 4°C to prevent translocation, as the only means whereby cytosol receptor could be detected, limited the nature of experiments that could be used to test the induction process in this line and allow comparison between lines.

The uninducible lines - Vero and HTC-SR - also showed interesting differences in receptor detection patterns. Presence of receptor in HTC cytosol and nucleus led to speculation that a block in the induction process occurs in these cells at some point after receptor-PAH cytosol to nucleus translocation has occurred. Vero

lacked detectable receptor in both cytosol and nucleus.

The lines chosen to be cloned were, therefore, Hepa-1c1 as the inducible line and Vero as the uninducible line most clearly representing the necessary elements of the AHH induction process as described in the model proposed by Guenthner and Nebert (1977).

Both lines were cloned by limiting dilution (as described in "Experimental Procedures"). Vero was cloned without difficulty, Hepa-1c1, however, proved difficult to clone. The possibility that clonal growth of isolated cells was inhibited by lack of essential growth factors, or presence of inhibitory factors in the particular lot of fetal calf serum used, was investigated.

Medium and serum, kindly supplied to us by Dr. O. Hankinson - who successfully cloned Hepa-1c1 - were compared with our medium and serum to assess their relative contributions to cloning efficiency. The medium from Hankinson was alpha minimal essential medium, already reconstituted, and the fetal calf serum, which he sent to us, had been heat inactivated.

Four well Linbros were plated to determine efficiency of plating. The latter was determined by plating cells at low density and counting the number of colonies formed (see "Experimental Procedures"). Efficiency of plating (EOP) equals the number of colonies formed, divided by the number of cells plated, and is expressed as a percentage. This can be construed as a measurement of cloning efficiency, with the understanding that it does not take into account the effect of the presence of neighbouring cells on clonal growth.

The data in Table 3 indicate that heat inactivated fetal calf serum (supplied by Hankinson) gave a higher EOP, in combination with either his or our medium (15-22 and 24-37%, respectively). than did our fetal calf serum in combination with either his or our medium (0-2 and 2-7%, respectively).

The relative importance of heat inactivation and source of fetal calf serum on cell growth is also indicated by the results in Table 3. The EOP in our heat inactivated or non-heat-inactivated fetal calf serum was the same. The lot and/or supplier of fetal calf serum, rather than heat inactivation per se, may therefore be of considerable importance in plating and cloning efficiency for this line.

Hepa-1c1 was successfully cloned using a new lot of fetal calf serum from a different supplier (Flow). It would appear that cell growth at low density was improved in the presence of fetal calf serum from a different source.

It should be emphasized that serum component - cell interaction(s), and not just serum constitution, are important in the support of cell growth. This was illustrated by the successful cloning of H4IIE-C3, and HTC-SR with serum that did not support clonal growth of Hepa-1c1.

Table 3: Comparison of cloning and plating efficiency of Hepa-1c1 cells, grown in various combinations of medium and serum.

Medium/Serum Combination[a]	Number of Cells Plated Per 60 mm Well	Number of Colonies Formed Per 60 mm Well	EOP (%)
A/1	10^2	15[a]	15
	5×10^2	109[a]	22
A/2	10^2	0[a]	0
	5×10^2	10[b]	2
B/1	10^2	36[b]	36
	5×10^2	141[b]	29
B/2	10^2	5[b]	5
	5×10^2	10[a]	2
B/3	10^2	2[a]	2
	5×10^2	2	1

a: Cells were plated at the indicated densities in one of a number of combinations of medium and serum. This was replenished every other day. When the majority of colonies contained at least 20 cells, the cells were fixed and stained in methylene blue for a colony count (see "Experimental Procedures").

A: Alpha minimal essential medium obtained from O. Hankinson.
B: Alpha minimal essential medium routinely used.

1: Heat inactivated fetal calf serum (10% of growth medium) from O. Hankinson.
2: Fetal calf serum routinely used (10% of growth medium).
3: Heat inactivated fetal calf serum. The fetal calf serum routinely used was heat inactivated at 55°C for one hour (10% of growth medium).

a: Average of two wells.
b: Average of four wells.

Analysis of Hepa-1c1 and Vero Subclones

The Hepa-1c1 and Vero populations being used in these investigations were themselves clones. Therefore, the clones derived from these populations were, technically, subclones. The subclones of Hepa-1c1 were designated Hs-n (n = 1 to 11). The subclones of Vero were designated Vs-1 and Vs-2.

Hepa-1c1 Subclones

(1) Assessment of Aryl Hydrocarbon Hydroxylase Assay

Hepa subclone 1 was assayed for AHH induction capacity. The lower level of induced AHH activity in Hs-1, compared to the Hepa-1c1 progenitor population, suggested that clonal variation in response to inducing agents existed. To assess the possibility that the reduced level of induction was due to differences in optimal assay conditions between Hepa-1c1 and Hs-1, conditions giving maximal AHH activity were determined for Hs-1. Both Hepa-1c1 and Hs-1 were: (1) optimally inducible at a reaction mixture buffer (o.2 M Tris) pH of 7.5; (2) showed linearity of AHH activity with protein concentration; and (3) showed similar optima for inducing agent concentrations. It was determined, that differences in induction capacity between Hepa-1c1 and Hs-1 were not due to differences in assay requirements. It was therefore assumed, on the basis of these findings and findings of similar maximal conditions for H4IIE-C3, that the assay conditions

would be appropriate for direct comparisons of AHH induction capacity between all Vero and Hepa subclones.

(2) Aryl Hydrocarbon Hydroxylase Induction

The level of induction of aryl hydrocarbon hydroxylase activity in Hepa-1c1 and seven subclones was assessed (Table 4). The subclones were 30 to 40 generations old (estimated from the single cell stage of the cloning procedure). A high percentage (71%) of the subclones showed variant levels of AHH induction compared to Hepa-1c1. Compared to Hepa-1c1, two subclones had significantly lower, two had similar and three had significantly higher levels ($p < 0.005$) of induced activity. Hs-1, showing the lowest level, and Hs-9, showing a level similar to the progenitor Hepa-1c1 population, were chosen for further assessment of their chromosome constitutions, the parameters of and restrictions on their growth, the nature and stability of their enzyme induction levels, and the quantity and nature of their Ah receptors.

(3) Generation Time and Chromosome Number

All three populations showed similar patterns of growth. Single or small groups of cells became attached to the surface of the plastic container and divided to produce colonies. Immediately after cells were plated, there was a lag phase before cell division commenced. After approximately 24 hours, the cell number began to increase, from

Table 4. Comparison of aryl hydrocarbon hydroxylase induction in Hepa-1c1 and subclonal populations. Cells were induced and assayed as described under "Experimental Procedures".

Population	Inducing Agent	Induced Specific Activity[a] ($\bar{X} \pm s$)	p[b]	Relative Specific Activity[c]
Experiment 1				
Hepa-1c1	6.5 μM BA	41.4 ± 4.9		
Subclone 1		12.6 ± 0.8	< 0.005	0.30
Subclone 9		49.9 ± 5.5	> 0.05	1.21
Experiment 2				
Hepa-1c1	1 nM TCDD	48.8 ± 2.0		
Subclone 5		33.8 ± 2.3	< 0.025	0.67
Experiment 3				
Hepa-1c1	6.5 μM BA	155.6 ± 35.5		
Subclone 2		361.3 ± 51.7	< 0.005	2.32
Subclone 7		955.7 ± 84.3	< 0.005	6.14
Subclone 8		170.3 ± 8.4	> 0.1	1.09
Subclone 11		254.5 ± 8.6	< 0.005	1.64

a: Calculated by subtracting units of specific activity for cells treated with solvent only, from units of specific activity in cells exposed to inducing agent.

b: Probability value, determined by t tests comparing $\bar{X} \pm s$ induced specific activity for each clone with that for Hepa-1c1 (within the same experiment).

c: Calculated by dividing the mean specific induced activity for each clone by that for Hepa-1c1 within the same experiment.

the number initially plated, and the culture entered a logarithmic growth phase (Fig. 12). Once the cells reached confluence, if the growth medium was not renewed, they began to die and lift off the surface. If the medium was renewed, the cells could be maintained as an actively growing population for at least two to four days. In the latter case, the cells readily grew on top of one another. They did not appear to be contact inhibited, under these conditions.

Generation times of cells in the logarithmic phase of growth, initially plated at 10^6 cells per 100 mm plate, were approximately 13 (Hepa-1c1), 14.5 (Hs-1) and 16.5 (Hs-9) hours.

An analysis of chromosome number (Fig. 13) indicated that Hepa-1c1, Hs-1 and Hs-9 cells had, respectively, modes of 58, 60 and 57 chromosomes per metaphase cell, with similar patterns of dispersion.

(4) Reduction of Serum Concentration

Variability has been described between serum lots and/or suppliers with respect to hormone (Milo, et al., 1976), protein, trace element, sugar (Honn, et al., 1975) and fatty acid (Boone, et al., 1974) content. These variations have been shown to affect cloning efficiency (Milo, et al., 1976; and this report) and AHH induction capacity (Kouri, et al., 1979). It was, therefore, decided to reduce serum concentration in the growth medium in order to reduce the rate of depletion of our stock of fetal calf serum and allow us to use one lot of fetal calf serum for the majority of the experiments to be undertaken.

Fig. 12. Growth Curves for Hepa-1c1, Hs-1 and Hs-9. Cells were plated at the density indicated at 0 hours in 100 mm plates. Cell counts were made, at the indicated intervals, of trypsinized cells, as described in "Experimental Procedures - Cell Culturing Techniques iii").

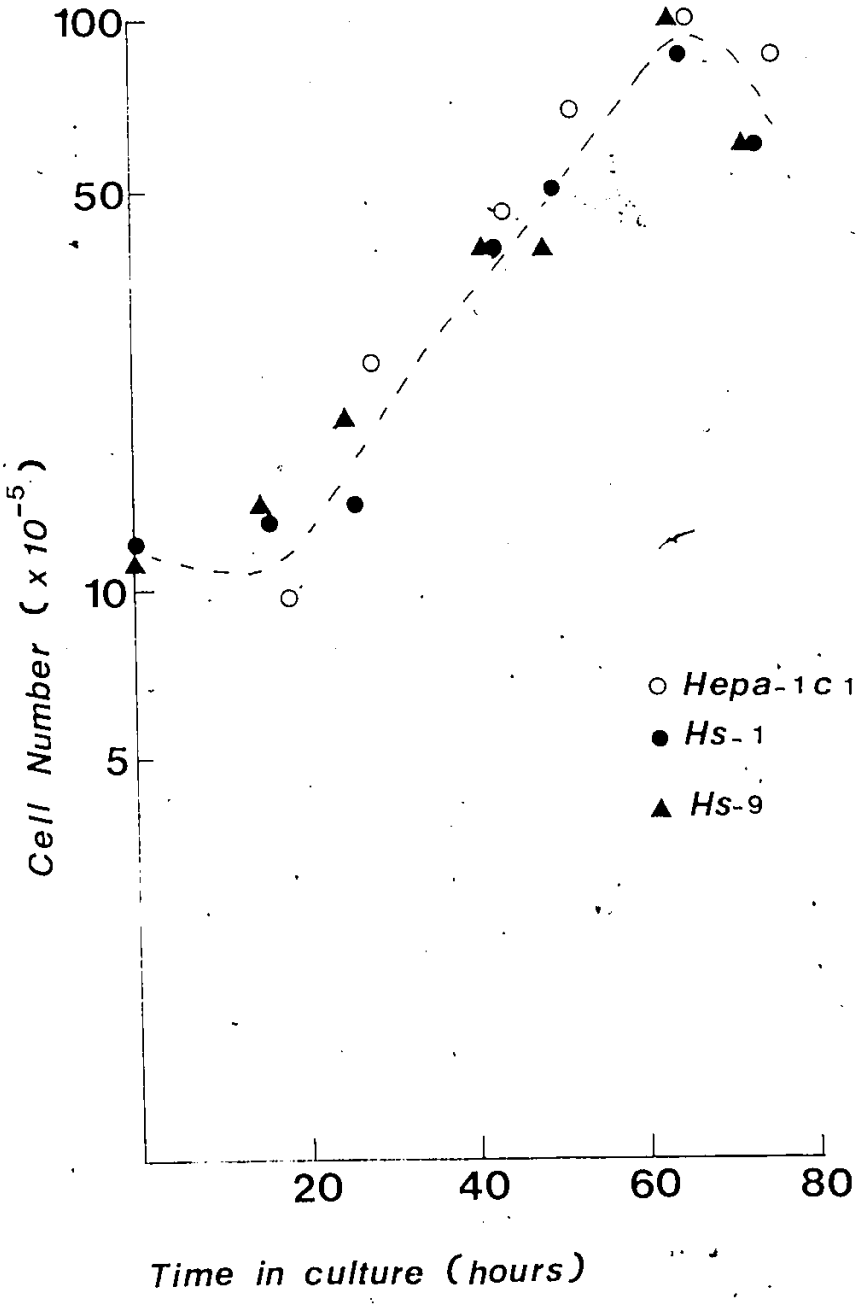
100
50
10
5
Cell Number (x 10⁻⁵)
20
40
60
80
Time in culture (hours)
Hepa-1c1
Hs-1
Hs-9

Fig. 12.

Fig. 13. Distributions of Numbers of Chromosomes Per Metaphase Cell for Hepa-1c1, Hs-1 and Hs-9. Cells in logarithmic growth were arrested in metaphase by the addition of colcemid to the growth medium. Chromosome spreads were obtained as described in "Experimental Procedures - Chromosome Counts", and the number of chromosomes determined for at least 50 metaphase cells.

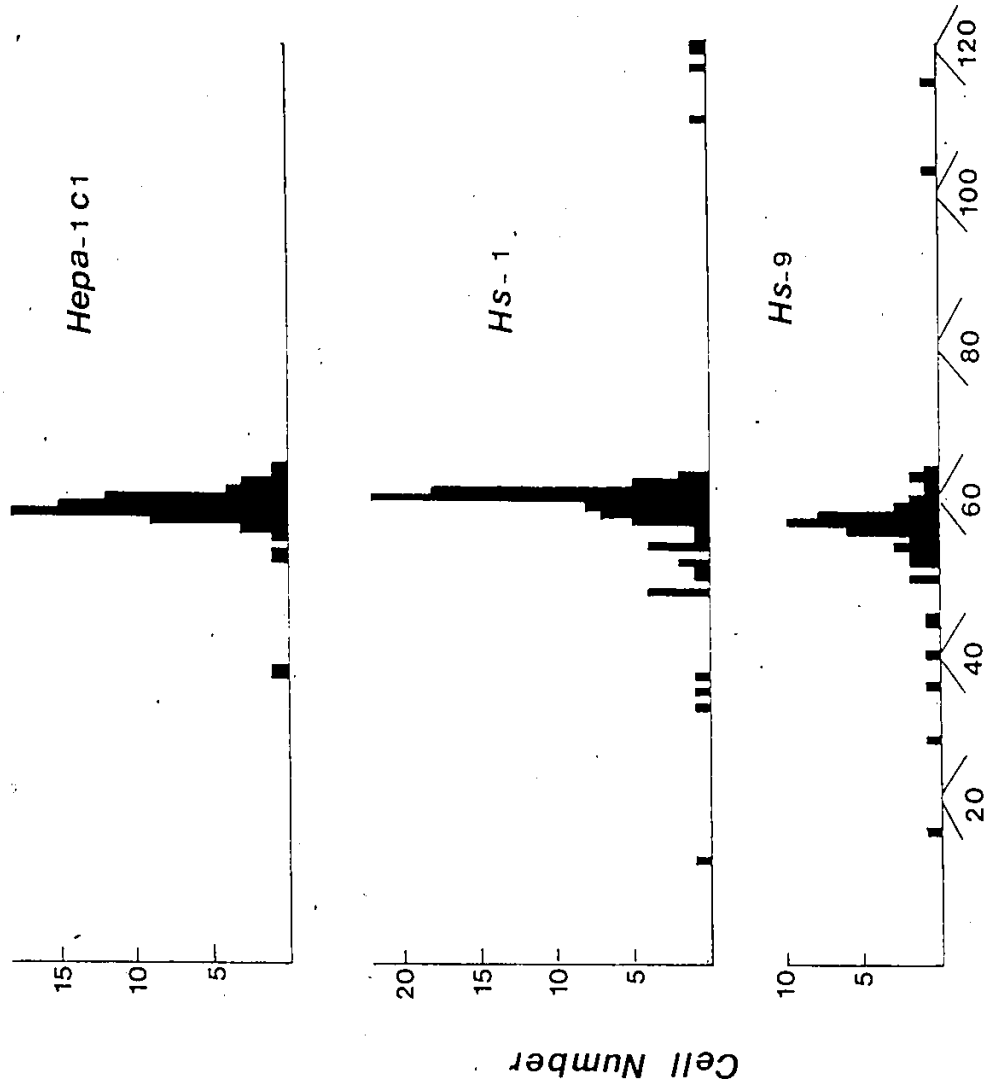
Hepa-1 c1
Hs-1
Hs-9
Cell Number
Chromosome Number per metaphase spread
15
10
5
20
15
10
5
10
5
20
40
60
80
100
120

Fig. 13.

Before serum concentration in the growth medium could be reduced, it was necessary to determine whether such a reduction would alter AHH induction. Hepa subclone 1 cells, grown in the presence of 5 or 10% serum were assayed over a period of 5 days. The maximum level of activity reached in either 5 or 10% serum was similar (Fig. 14). Growth curves for cells grown in either 5 or 10% serum were also similar. Aryl hydrocarbon hydroxylase induction experiments were, therefore, carried out using cells grown in medium containing 5% fetal calf serum.

(5) Growth in the Presence of Benzo(a)pyrene

Growth in the presence of BP (i.e. BP resistance) has been used as a criterion to select for growth of mutagenized Hepa 1c1c7 (a subclone of Hepa-1c1) cells that have lost, or have a reduced capacity for AHH induction (Hankinson, 1979). Benzo(a)pyrene resistant cells do not metabolize BP to its toxic products because of their low control AHH activity and their lack of induction response to the PAH - BP.

Hepa-1c1, Hs-1 and Hs-9 populations were assessed for their resistance to BP (Table 5). As expected, Hepa-1c1 and Hs-9 cells showed essentially no growth in the presence of BP. Hs-1, had a low level of BP resistance, particularly when fetal calf serum, rather than dialyzed fetal calf serum was present.

Fig. 14. Comparison of Induced AHH Activity in Hs-1 Cells Grown in Medium Containing 5% or 10% Fetal Calf Serum. Cells were plated into medium containing 5%, or 10% fetal calf serum. Eighteen hours prior to each of the indicated intervals, the growth medium was replaced with medium containing the appropriate serum concentration and either 0.1% DMSO or 6.5 μM BA in DMSO. AHH activity was assayed (at the indicated intervals) as described in "Experimental Procedures - AHH Assay". Specific AHH Activity is defined as the amount of fluorescence equivalent to 1 pmol 3-hydroxybenzo(a)pyrene formed per mg minute per mg protein. The symbols represent: cells grown in 5% serum, treated with BA - open circles; cells grown in 10% serum, treated with BA - open triangles; cells grown in 5% serum, treated with DMSO - closed circles; cells grown in 10% serum, treated with DMSO - closed triangles.

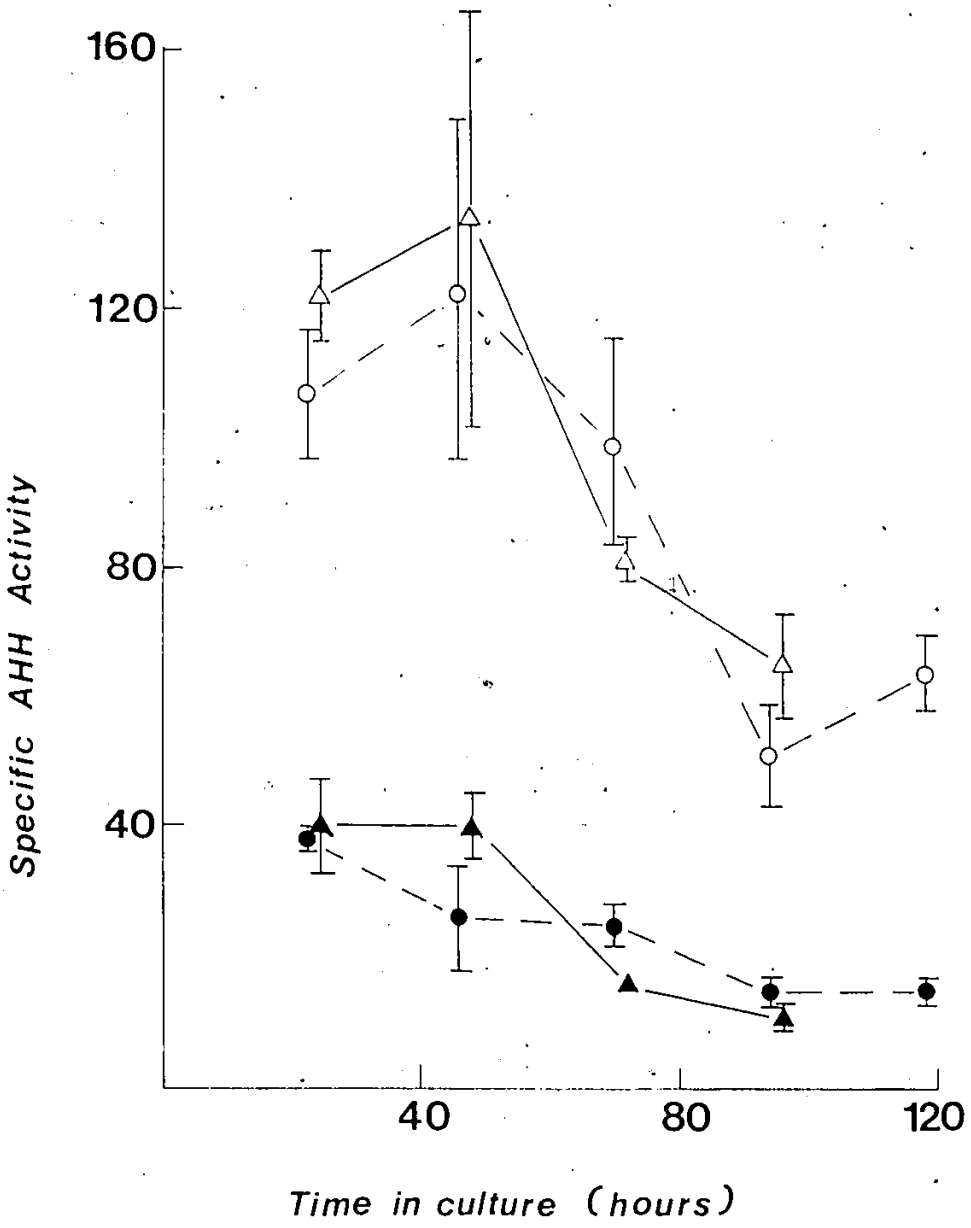
160
120
80
40
Specific AHH Activity
40
80
120
Time in culture (hours)

Fig. 14.

Table 5. Growth of Hepa-1c1, Hs-1 and Hs-9 in benzo(a)pyrene. Cells were plated at the indicated densities in one of the growth media listed on the table. The medium used was alpha minimal essental medium (MEM). Serum constituted 5% of the growth medium. The growth medium was replenished every other day. When the majority of colonies contained at least 20 cells, the cells were fixed and stained in methylene blue for a colony count (as described in "Experimental Procedures").

Table 5. Growth of Hepa-1c1, Hs-1 and Hs-9 in benzo(a)pyrene.

	Number of Cells Plated per 60 mm Well	Average Number of Colonies Formed Per Well	Efficiency of Plating (%)
Alpha MEM + Fetal Calf Serum			
Hepa-1c1	2×10^3	1023	51.2
Hs-1		883	44.2
Hs-9		517	25.9
Alpha MEM + Dialyzed Fetal Calf Serum			
Hepa-1c1	2×10^3	474	23.7
Hs-1		531	26.6
Hs-9		334	16.7
Alpha MEM + Fetal Calf Serum + 1 mM BP			
Hepa-1c1	2×10^3	1	< 0.1
	10^5	122	< 0.1
Hs-1	2×10^3	164	8.2
	10^5	>1800	1.8
Hs-9	2×10^3	1	< 0.1
	10^5	25	< 0.1
Alpha MEM + Dialyzed Fetal Calf Serum + 1 mM BP			
Hepa-1c1	2×10^3	1	< 0.1
	10^5	38	< 0.1
Hs-1	2×10^3	4	0.2
	10^5	418	0.4
Hs-9	2×10^3	1	< 0.1
	10^5	22	< 0.1

(6) Growth of Hs-1 in Dialyzed Fetal Calf Serum

The growth requirements of Hs-1 were assessed at generations 54, 60, 117 and 180, by comparing growth in the presence of dialyzed and non-dialyzed fetal calf serum. Generation number was calculated by multiplying the age (i.e. subculture number) of the line from the time of cloning, by the approximate number of divisions occurring between subculturing procedures. Between generations 60 and 117, the population developed a capacity for more vigorous growth, or initial efficiency of plating, in dialyzed fetal calf serum (Table 6). This change was apparently spontaneous, as the cells tested have been routinely subcultured in growth medium containing non-dialyzed fetal calf serum.

(7) Inhibition of Aryl Hydrocarbon Hydroxylase Activity by 7,8-Benzoflavone

Wiebel and Gelboin (1975) found that the synthetic flavonoid 7,8-benzoflavone (also referred to as alpha-naphthoflavone) inhibited the metabolism of BP when added to liver microsomes from MC-treated rats. However, when added to liver microsomes from untreated rats, the metabolism of BP was stimulated. Further work suggested that the 7,8-benzoflavone response depended on the presence of the P_1-450 cytochrome in the system. Nesnow (1979) demonstrated that 7,8-benzoflavone inhibits metabolism of BA by cytochrome P_1-450 and

Table 6. Variation in Hs-1 growth in medium containing dialyzed fetal calf serum.

Population	Generation Number[a]	Type of Fetal Calf Serum	Efficiency of Plating (%)	Relative Survival[b]
Hepa-1c1	200	Non-dialyzed	51.2	1.0
		Dialyzed	23.7	0.5
Hs-1	54	Non-dialyzed	33.3	1.0
		Dialyzed	0.8	0.1
	60	Non-dialyzed	30.2	1.0
		Dialzyed	1.6	0.1
	117	Non-dialyzed	31.8	1.0
		Dialyzed	18.3	0.6
	180	Non-dialyzed	44.2	1.0
		Dialyzed	26.6	0.6

a: All of the cells described above were assessed within the same experiment. The cells were plated at 2 X 10^3 per 60 mm dish in medium containing 5% of either non-dialyzed fetal calf serum, or dialzyed fetal calf serum (v/v). Colony counts were obtained as described in "Experimental Procedures" for determining the EOP (efficiency of plating) of the cells.

b: Relative Survival indicates the survival of each generation tested, for each cell line, in medium containing dialyzed fetal calf serum; compared to survival in medium containing non-dialyzed fetal calf serum.

stimulates cytochrome P-450 metabolism of BA. Thus the inhibitory or stimulatory effect of 7,8-benzoflavone on microsomal metabolism of BP appears to depend on the major form of cytochrome present. When 7,8-benzoflavone was added to cells, just prior to initiation of BP metabolism in the standard AHH assay, all three Hepa populations (Hepa-1c1, Hs-1 and Hs-9) showed inhibition of formation of hydroxylated BP metabolites (Fig. 15). At the highest concentration of 7,8-benzoflavone (6.5×10^{-5} M), the detectable AHH activity level dropped below that found for untreated cells. These data indicate that in all three populations, the increased level of metabolism of BP in BA-induced cells was the consequence of increased AHH-associated cytochrome P_1-450 involvement

(8) Stability of Variation in Level of Enzyme Induction

The level of induced AHH activity in each of the Hepa-1c1, Hs-1 and Hs-9 populations was assessed at varying intervals over approximately 190, 160 and 150 generations, respectively. As indicated in Table 7, assays were done at various times of the year over a 2.4 (Hepa-1c1 and Hs-1), or 1 (Hs-9) year period. Within these time intervals, cells were occasionally stored at -80°C (eg. between 10/82 and 1/83). It was found that actual levels of activity in untreated cells, cells treated with solvent alone, or cells treated with inducing agent plus solvent, varied widely between experiments. However, Hepa-1c1 and Hs-9 cells consistently had similar levels of induced specific activity, and Hs-1 had consistently less activity than Hepa-1c1 cells, within experiments.

Fig. 15. Effect of 7,8-benzoflavone on induced aryl hydrocarbon hydroxylase activity. Cells were exposed for 18 hours to 6.5 μM benzo(a)anthracene (BA) in 0.1% DMSO. At the time of assay, varying concentrations of 7,8-benzoflavone were added to the reaction mixture just prior to incubation at 37°C. Activity was determined as described under "Experimental Procedures".

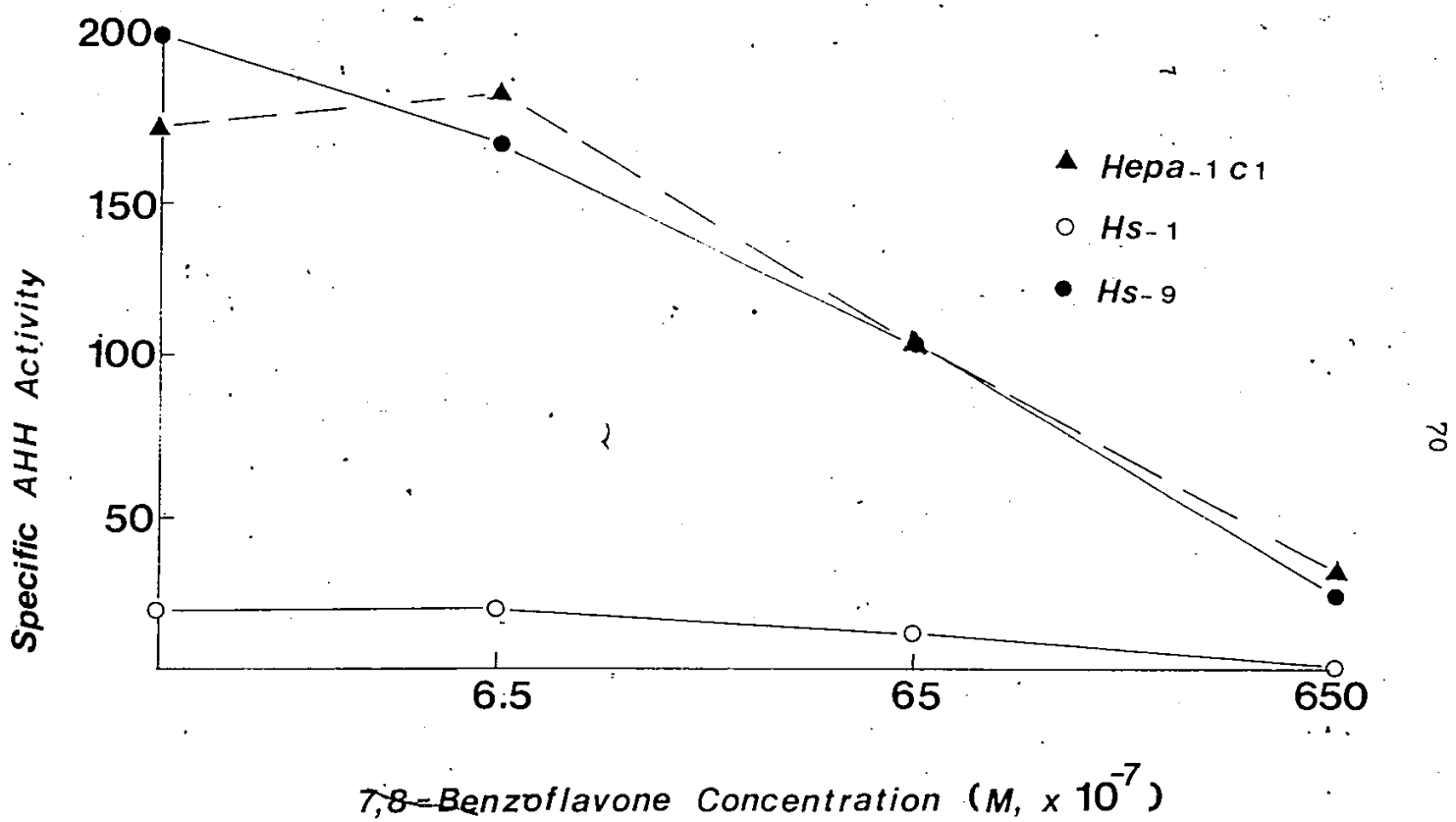
200
150
100
50
Specific AHH Activity
Hepa-1 c1
Hs-1
Hs-9
6.5
65
650
7,8-Benzoflavone Concentration (M, x 10^-7)

Fig. 15.

Table 7. Induction of aryl hydrocarbon hydroxylase activity in Hepa-1c1, Hs-1 and Hs-9.

Date (M/YR)	Population	Induced Specific Activity[a] ($\bar{X} \pm s$)	Relative Specific Activity[b]
		6.5 μM BA	
9/80	Hepa-1c1	197.2 ± 13.1	1.00
	Hs-1	51.0 ± 0.9	0.26
		1 nM TCDD	
1/82	Hepa-1c1	50.1 ± 2.0	1.00
	Hs-1	15.7 ± 0.5	0.28
	Hs-9	46.6 ± 1.8	0.93
		6.5 μM BA	
4/82	Hepa-1c1	15.1 ± 2.4	1.00
	Hs-1	2.4 ± 0.1	0.16
	Hs-9	17.0 ± 2.1	1.13
		6.5 μM BA	
9/82	Hepa-1c1	111.9 ± 9.2	1.00
	Hs-1	11.2 ± 0.7	0.08
	Hs-9	88.2 ± 19.0	0.78
1/83	Hepa-1c1	47.5 ± 5.2	1.00
	Hs-1	13.4 ± 1.2	0.30
	Hs-9	57.9 ± 7.8	1.21

a: Cells in logarithmic growth were incubated (at 37° C) in medium containing the indicated inducing agent, for 18 hours, and assessed for their AHH activity ("Experimental Procedures"). Induced Specific Activity was calculated by subtracting the activity for solvent treated cells from that for cells treated with solvent and inducing agent.

b: Relative Specific Activity was determined for Hs-1 or Hs-9 compared to Hepa-1c1 within each experiment.

When BA-induced specific activity levels in five experiments were compared, the mean relative specific activity for: Hs-1 compared to Hepa-1c1, was 0.14 (±0.09); and for Hs-9 compared to Hepa-1c1, was 1.37 (±0.48). Relative values were similar when TCDD was used as the inducing agent.

(9) Quantification of Ah Receptor

The differing response to inducing agents of Hs-1 compared to Hepa-1c1 and Hs-9 with respect to AHH activity, prompted an analysis of the quantity and cellular localization of Ah receptor in each population. Receptor was quantified in two ways: by measuring the fmol of [^{3}H] TCDD specifically bound per mg protein in cytosol or nuclear extracts from cells incubated with [^{3}H] TCDD in culture; and by determining the number of receptor molecules per cell using cytosol incubated with [^{3}H] TCDD *in vitro* (i.e. Scatchard analysis). The latter calculation involved determining the average amount of cytosolic protein per cell. The fmol of bound [^{3}H] TCDD per cell can then be determined and multiplied by Avogadro's number to determine the number of receptor molecules per cell (see "Experimental Procedures - Mathematical Analysis"). These calculations assume that there is one [^{3}H] TCDD binding site per receptor molecule.

There was a considerable amount of variation in total specific receptor detectable (measured in fmol [^{3}H] TCDD bound per mg protein, Table 8, column A). When $\bar{X}$ ± s values were compared, there was no significant difference between Hepa-1c1 and Hs-1, or between Hepa-1c1

Table 8. Quantitative assessment of detectable specific [^{3}H] TCDD binding in Hepa-1c1, Hs-1 and Hs-9 cells. Cells in logarithmic growth were incubated for 1 hour at 37°C in 1 nM [^{3}H] TCDD. The quantity of specific binding was determined as described in "Experimental Procedures".

a: Total Specific Binding refers to the sum of cytosol and nuclear extract binding.

Table 8. Quantitative assessment of detectable specific [^{3}H] TCDD binding in Hepa-1c1, Hs-1 and Hs-9 cells.

Population	Total Specific Binding (fmol [^{3}H] TCDD bound per mg protein)[a] A	Specific Binding in Nuclear Extract (per cent of A) B
Hepa-1c1	86	31
	178	42
	110	51
	85	11
	186	61
	99	41
$\bar{X} \pm s$	124 ± 46	40 ± 18
Range	78 - 170	22 - 58
Hs-1	52	73
	103	54
	138	86
	141	79
	71	18
	114	48
$\bar{X} \pm s$	103 ± 36	60 ± 25
Range	67 - 139	35 - 85
Hs-9	92	63
	174	69
	94	69
	300	90
	300	75
	239	85
	112	58
$\bar{X} \pm s$	187 ± 93	73 ± 12
Range	94 - 280	61 - 84

and Hs-9, at $p = 0.05$, using the "t" test; or among all three populations using the "F" test. The proportion of total specific receptor represented by binding in nuclear extracts (column B) appears to increase from Hepa-1c1 to Hs-1 to Hs-9. The difference between Hepa-1c1 and Hs-1 was not significant, at $p = 0.05$. The difference between Hepa-1c1 and Hs-9 was significant ($p < 0.005$). The same results were obtained if absolute $X \pm s$ values were compared, ($p < 0.025$ for the comparison of Hepa-1c1 and Hs-9.)

A scatchard analysis of specific binding in vitro, in cytosol from cells not previously treated with TCDD, was carried out. The ratios of bound to free [^{3}H] TCDD are plotted against the concentrations of bound [^{3}H] TCDD for each population in Fig. 16. From a linear regression analysis ("Experimental Procedures - Mathematical Analyses"), values for receptor affinity for TCDD (Kd) and number of receptor sites (B_{max}) were estimated (Table 9). Receptor affinity (Kd) was approximately the same for all three populations. Hepa-1c1 and Hs-1 populations had similar estimated maximal numbers of receptors per mg protein. The value for Hs-9 may be an underestimate, since the degree of non-specific binding at TCDD concentrations greater than 4 nM increasingly obscured quantitation of specific binding in Hs-9. (Hs-1 and Hepa-1c1 binding peaks were well defined at 17.2 nM TCDD.) The estimated number of receptors per cell was similar in all three populations.

(10) Subcellular Distribution of [^{3}H] TCDD

In order to determine the availability of [^{3}H] TCDD for binding in the cytoplasm and nucleus, as well as the per cent of total [^{3}H] TCDD

Fig. 16. Scatchard Curves of Specific [^{3}H] TCDD Binding in Hepa-1c1, Hs-1 and Hs-9. Cytosol was obtained from cells not previously exposed to inducer or radioligand (see "Experimental Procedures - Ah Receptor Assay). Cytosol samples with 1 to 2 mg protein per ml were incubated with [^{3}H] TCDD at final concentrations of 0.1 to 17.2 nM for 1 hour at 0°C. The amount of specifically bound [^{3}H] TCDD was then determined. The Scatchard plots compare the ratio of specifically bound to unbound TCDD, to the concentration of specifically bound TCDD.

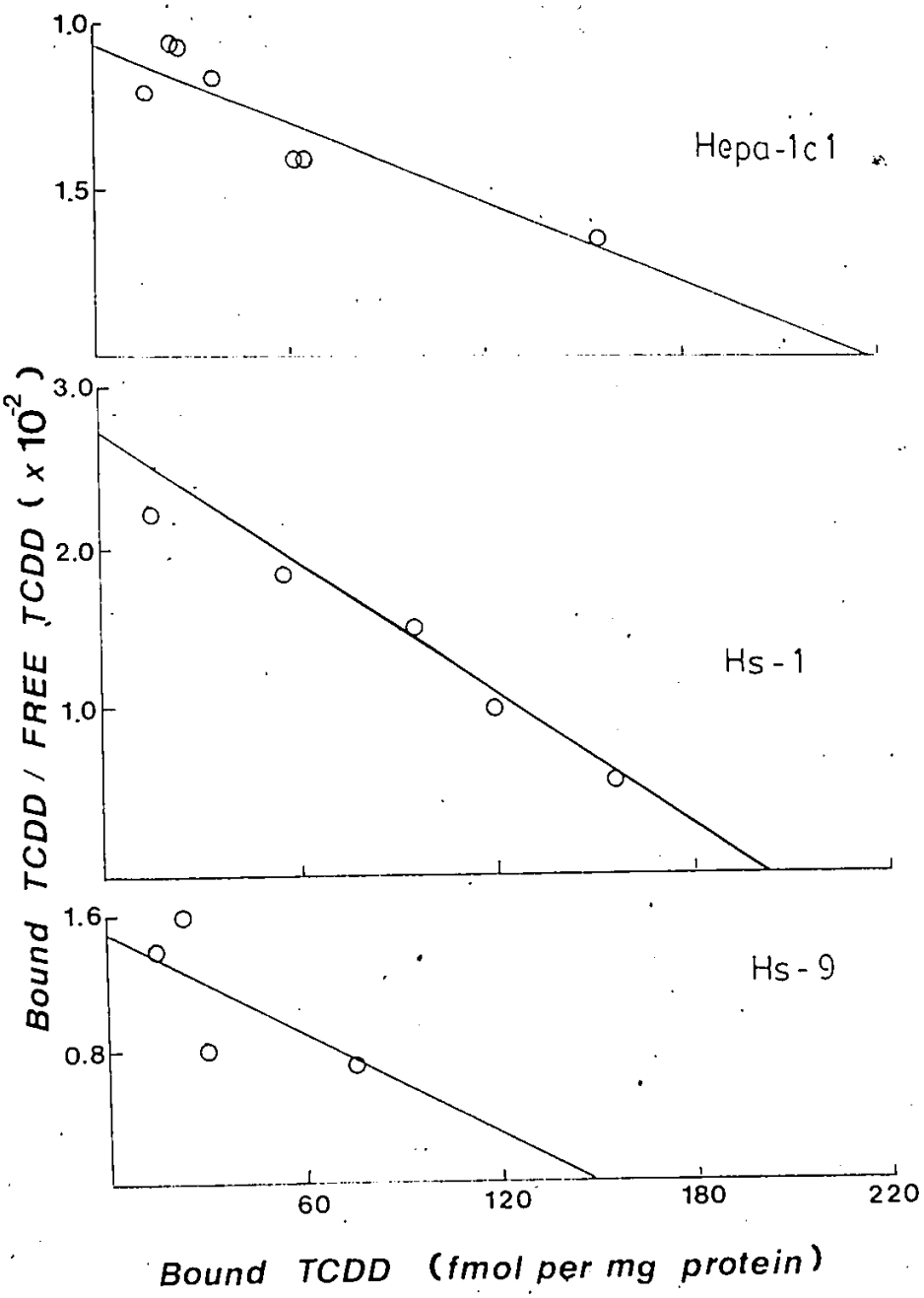
1.0
1.5
Hepa-1c1
3.0
2.0
1.0
Hs-1
1.6
0.8
Hs-9
Bound TCDD / FREE TCDD (x 10^-2)
60
120
180
220
Bound TCDD (fmol per mg protein)

Fig. **16.**

Table 9. Scatchard analysis of [^{3}H] TCDD binding in Hepa-1c1, Hs-1 and Hs-9.

Population	B_{max} (fmol [^{3}H] TCDD bound per mg cytosol protein)	Kd (nM)	Number of Receptors Per Cell
Hepa-1c1	239	7.5	3500
Hs-1	202	7.7	4000
Hs-9	150	7.9	3000

Cytosol was obtained from cells in logarithmic growth that had not previously been exposed to inducing agents or radioligand (see "Experimental Procedures"). Cytosol samples with 1 to 2 mg protein per ml were incubated with [^{3}H] TCDD at final concentrations of 0.1 to 17.2 nM for 1 hour at 4°C. The amount of specifically bound [^{3}H] TCDD was then determined as described in "Experimental Procedures". The values given in the above table were obtained from a linear regression analysis of the data illustrated in Fig. 16 (see "Experimental Procedures - Scatchard Analysis").

Table 10. Subcellular distribution of [^{3}H] TCDD in Hepa-1c1, Hs-1 and Hs-9 cells. Total radioactivity in duplicate samples of each subcellular fraction was determined by liquid scintillation counting of cells collected 1, 2 or 3 days after plating. Fractions were prepared from cells that had been incubated in culture in 1 nM [^{3}H] TCDD for 1 hour at 37°C, as described in "Experimental Procedures".

Table 10. Subcellular distribution of [^{3}H] TCDD in Hepa-1c1, Hs-1 and Hs-9 cells.

	Total [^{3}H] TCDD								
	Day 1			Day 2			Day 3		
	Hepa-1c1	Hs-1	Hs-9	Hepa-1c1	Hs-1	Hs-9	Hepa-1c1	Hs-1	Hs-9
	(fmols/ 10^6 Cells)								
Total Cell Homogenate	1214	1239	977	615	617	617	348	266	323
	% of Total Cell Homogenate								
Cytosol									
Total	7.2	7.0	8.3	5.2	4.9	4.4	4.5	4.3	4.5
After Charcoal Adsorption	2.3	1.6	2.8	1.6	0.9	0.9	1.8	0.8	1.4
Specifically Bound	0.16	0.06	0.08	0.39	0.15	0.11	0.53	0.08	0.2
Washed Nuclei	21.3	22.2	17.8	18.1	22.2	18.5	18.3	27.7	20.0
Nuclear Extract									
Total	1.0	1.2	1.3	1.2	1.1	1.3	1.4	1.8	2.0
After Charcoal Adsorption	0.1	0.1	0.2	0.1	0.1	0.1	0.3	0.4	0.6
Specifically Bound				0.02	0.01	0.03	0.08	0.09	0.09

within the cell that could be shown to be specifically bound, the subcellular distribution of labelled TCDD was determined. The level of radioactivity per cell was measured for the total cell homogenate, cytosol, washed nuclei and nuclear extracts; one, two and three days after cells were plated, as described in Table 10. Distributions of label were similar within and between populations whether calculated as the per cent of label present in total cell homogenate or the actual fmols of [^{3}H] TCDD present per 10^6 cells, for each fraction measured. The level of radioactivity associated with microsomal extracts (measured in three separate experiments) represented 21.3 ± 3.7% of total radioactivity in the cell homogenate. The amount of [^{3}H] TCDD present as specifically bound [^{3}H] TCDD always represented less than 1% of the total [^{3}H] TCDD present in the cell homogenate.

(11) Movement of Specifically Bound [^{3}H] TCDD Over Time

The movement of specifically bound [^{3}H] TCDD was followed by incubating cells with [^{3}H] TCDD for one hour at 4°C, then allowing movement from cytosol to nucleus to occur by washing the cells twice with cold citrate saline and incubating at 37°C for varying time periods in [^{3}H] TCDD-free medium (see "Experimental Procedures", Fig. 3). The translocation of TCDD-receptor complex from the cytosol to the nucleus in Hepa-1c1, Hs-1 and Hs-9 was shown to be both time and temperature-dependent by the following results: no detectable translocation occurred when the cells were held at 4°C; and specific binding in the cytosol and nuclear extracts showed a general pattern of

binding in the cytosol and nuclear extracts showed a general pattern of decrease and increase, respectively, over one hour at 37°C in all cell populations. Fig. 17 indicates the per cent label found in the nuclear extract at each time point, compared to total specific label detected at each time point. The increase in nuclear binding was similar in Hepa-1c1 and Hs-1. Hs-9, under these conditions, showed a greater degree of nuclear binding at each time point than either Hs-1 or Hepa-1c1.

(12) Co-Ordinate Measurement of Ah Receptor and Aryl Hydrocarbon Hydroxylase Induction

Hepa-1c1, Hs-1 and Hs-9 cells were each plated at the same concentration, at approximately the same generation number, on the same day for separate Ah receptor and AHH assays. The same day that 6.5 μM BA was added to culture plates, the Ah receptor content for each population was determined by routine incubation of cells in culture in [^{3}H] TCDD (i.e. 1 hour at 37°C). The experiment was repeated twice. Levels of BA-induced AHH specific activity and fmols [^{3}H] TCDD specifically bound per mg protein are compared in Fig. 18. An equation for a regression line was calculated by the least squares method and a correlation co-efficient, r, of 0.62 was obtained. The correlation was significant at $p < 0.05$. An r value of 0.62 indicates that, while receptor content and level of induced AHH activity are related - as the model would lead us to expect - there are other factors involved in

Fig. 17. Time- and temperature-dependent translocation of specifically bound [^{3}H] TCDD into the nucleus of intact Hepa-1c1, Hs-1 and Hs-9 cells. Each point is the average ($\pm$ s) of two experiments. Cells were incubated at 4 C for one hour in the presence of 1 nM [^{3}H] TCDD, then washed twice with cold citrate saline, cold fresh medium was added and cells were incubated for varying intervals at 37°C. Cytosol and nuclear extracts were prepared as described under "Experimental Procedures". Nuclear binding is represented here as the per cent of total (cytosol plus nuclear) specific binding at each 37°C time point.

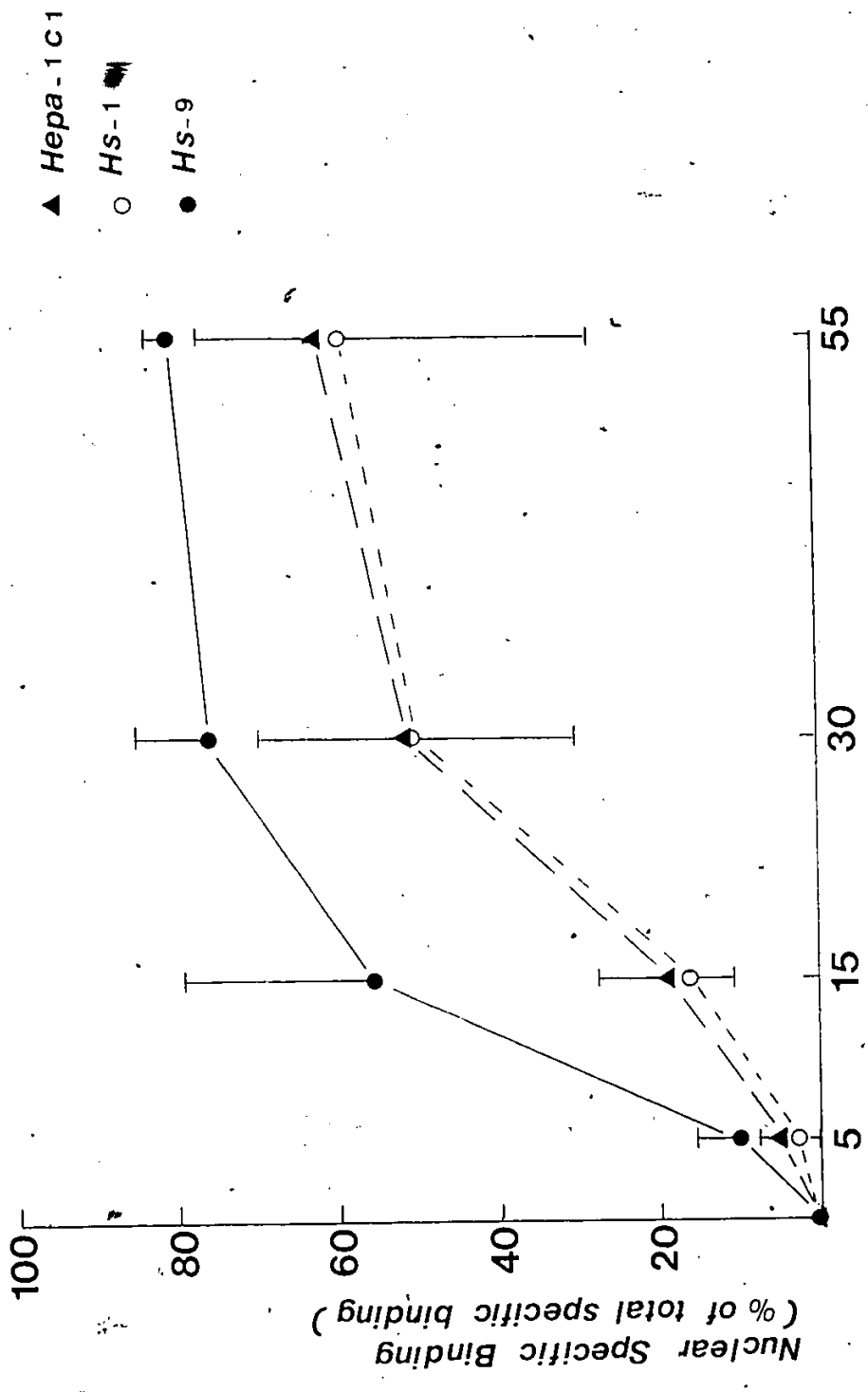
▲ Hepa-1 c1
○ Hs-1
● Hs-9
Nuclear Specific Binding
(% of total specific binding)
100
80
60
40
20
5
15
30
55
Time at 37°C (minutes)

Fig. 17.

Fig. 18. Comparison of Induced AHH Activity and Quantity of Specific [^{3}H] TCDD Binding in Nuclear Extracts of Hepa-1c1, Hs-1 and Hs-9. Cells in logarithmic growth, which had been plated on the same day, at the same density, were either: incubated for 1 hour at 37°C in the presence of 1 nM [^{3}H] TCDD and assayed for Ah receptor content; or washed and their medium replaced with medium containing 6.5 μM BA (or solvent alone) for AHH assay 18 hours later. (Assays were carried out as described in "Experimental Procedures".)

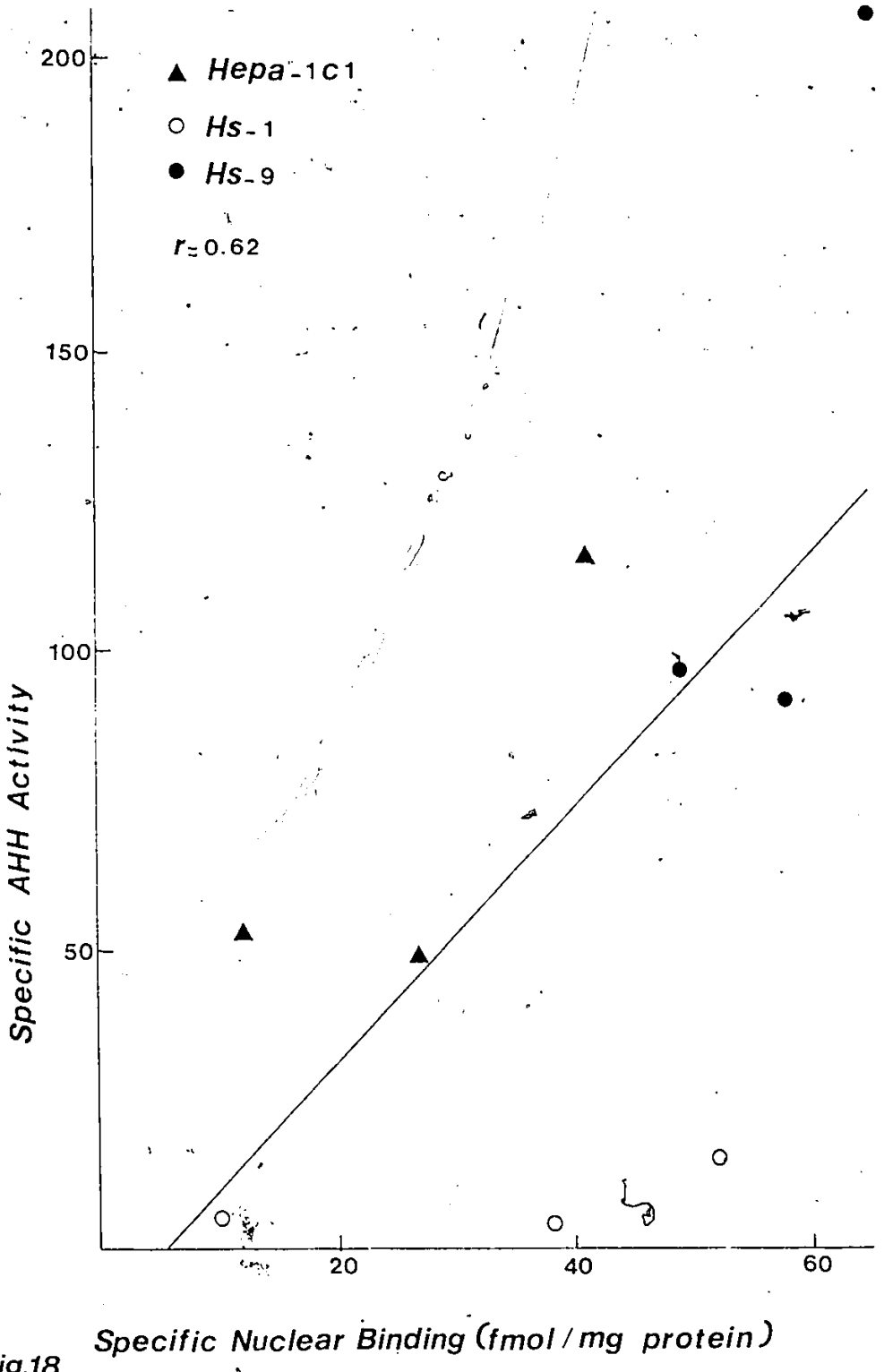
▲ Hepa-1c1
○ Hs-1
● Hs-9
r=0.62
200
150
100
50
20
40
60
Specific AHH Activity
Specific Nuclear Binding (fmol / mg protein)

Fig.18.

controlling the level of induced activity in these populations.

(13) Specificity of [^{3}H] TCDD Binding

Specificity of binding was assessed by determining the effect of competitors on binding of [^{3}H] TCDD in cells simultaneously exposed to 1 nM [^{3}H] TCDD and excess concentrations of unlabelled TCDD, BA, or MC (cytochrome P_1-450 inducers), or phenobarbital (a cytochrome P-450 inducer). As indicated in Table 11, BA reduced binding to a similar extent in all three populations. The extent of reduction was consistently greater in the nuclear extract than in the cytosol, when competition in the cytosol was less than 100%.

Complete loss of cytosolic or nuclear binding occurred in the presence of excess TCDD, or MC (Table 12). On the other hand, binding was close to control levels in the presence of excess phenobarbital.

(14) Effect of Benzo(a)anthracene Pretreatment on Ah Receptor Quantity

In order to confirm whether or not PAHs could induce Ah receptor, receptor was quantified after cells were exposed in culture to a representative PAH. Hepa-1c1, Hs-1, or Hs-9 cells were exposed to

Table 11. Competition by benzo(a)anthracene for binding of [^{3}H] TCDD to receptor.

Population	Concentration of BA (nM)[a]	Degree of Competition (%)[b] Cytosol	Nuclear Extract	Total[c]
Hepa-1c1	100	39	84	58
	1000	66	100	69
Hs-1	100	38	79	60
	1000	100	100	100
Hs-9	100	27	91	76
	1000	39	98	92

a: Cells in logarithmic growth were exposed to fresh medium containing 1 nM [^{3}H] TCDD plus 100 or 1000 nM BA. The cells were then assayed for specific [^{3}H] TCDD binding (see "Experimental Procedures - Cells labelled in culture").

b: The degree of competition was calculated as the per cent of [^{3}H] TCDD specifically bound in cytosol or nuclear extracts in the absence of competitor, relative to that bound in the presence of competitor.

c: Total competition was determined by comparing the sum of cytosol and nuclear binding in the absence of BA, to that in the presence of BA.

Table 12. Effect of excess 3-methylcholanthrene or phenobarbital on binding of [^{3}H] TCDD to receptor.

Population	Competitor[a]	Competitor Concentration (nM)	Degree of Competition[b] (%)
Hepa-1c1	TCDD	1000	100
	MC	1000	95
	PB	100	11
		1000	15
Hs-1	TCDD	100	100
		1000	100
	MC	100	100
		1000	100
	PB	100	26
		1000	0
Hs-9	TCDD	1000	96
	MC	1000	91
	PB	100	12
		1000	26

a: Cells in logarithmic growth were exposed to fresh medium containing 1 nM [^{3}H] TCDD plus 100 or 1000 nM TCDD, MC or PB. The cells were assayed for specific [^{3}H] TCDD binding (see "Experimental Procedures - Cells labelled in culture").

b: The degree of competition was calculated as the per cent of [^{3}H] TCDD specifically bound in cytosol or nuclear extracts in the absence of competitor, relative to that bound in the presence of competitor.

6.5 μM BA in DMSO or DMSO alone for 18 hours. Cells were then washed and exposed to [^{3}H] TCDD for one hour. The quantity of specific cytosol and nuclear binding (fmol [^{3}H] TCDD bound per mg protein) was determined. Table 13 indicates the total amount of receptor detected in each population following treatment with 6.5 μM BA with or without a citrate saline wash of the cell surface, followed by incubation in BA-free medium for two hours. This procedure removes free BA from cells and allows metabolism of most of the remaining BA to polar products (Nebert and Bausserman, 1970). None of the cell populations show induction of receptor by BA pretreatment. Hs-1 cells, pretreated with BA, with or without a two hour chase, consistently had less receptor than untreated or DMSO pretreated controls. The BA-free incubation period appeared to be more important for removing residual BA in the poorly inducible Hs-1 cells than in Hepa-1c1 or Hs-9.

Vero Subclones

(1) Aryl Hydrocarbon Hydroxylase Induction

The level of induction of aryl hydrocarbon hydroxylase activity in Vero and two Vero subclones was assessed (Table 14). Vero progenitor and subclone populations showed no significant variation in AHH inducibility. None of the populations were inducible under the conditions used.

Table 13. Effect of benzo(a)anthracene pretreatment on specific [^{3}H] TCDD binding in Hepa-1c1, Hs-1 and Hs-9.

Pre-label Treatment[a]	Total Specific [^{3}H] TCDD Bound (fmol/mg protein)		
	Hepa-1c1	Hs-1	Hs-9
Experiment 1			
DMSO	203	86	179
BA in DMSO, 2 hr. BA-Free[b]	171	65	160
Experiment 2			
BA in DMSO	165	31	102
BA in DMSO, 2 hr. BA-Free[b]	143	73	101

a: Cells were incubated for 18 hours in medium containing 0.1% DMSO (dimethylsulfoxide), or 6.5 μM benzo(a)anthracene (in DMSO) prior to routine incubation in 1 nM [^{3}H] TCDD and assay for receptor content.

b: After 18 hours in medium containing BA in DMSO, cells were washed with warm citrate saline (37°C) and incubated in inducer-free medium for 2 hours prior to incubation in 1 nM [^{3}H] TCDD and assay for receptor content.

Table 14. Vero aryl hydrocarbon hydroxylase activity in progenitor and subclonal populations.

Treatment	Specific Activity ($\bar{X} \pm s$, N = 6)[a]					
	Progenitor		Subclone 1		Subclone 2	
0.1% DMSO	0.9	0.8	1.8	1.1	0.2	0.3
1.3 μM BA	0.7	0.3	1.0	1.1	0.3	0.5
6.5 μM BA	1.0	0.1	0.4	0.3	0.2	0.3
13 μM BA	0.3	0.2	0.2	0.3	0.6	0.6

a: One unit of specific activity equals that amount of enzyme catalyzing per minute at 37°C, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmol of 3-hydroxybenzo(a)-pyrene recrystallized standard (Gielen and Nebert, 1971).

N: represents the number of measurements obtained, per cell line, per treatment.

(2) Ah Receptor

Neither the Vero progenitor population, nor Vs-1 had any detectable receptor in cytosol or nuclear extract.

(3) Chromosome Number

Modal chromosome numbers were determined for Vero, Vs-1 and Vs-2. The chromosome numbers were as follows: Vero - 58, Vs-1 - 55, and Vs-2 - hypotetraploid. Data on distribution of chromosome numbers per metaphase spread are given in Fig. 19.

Fig. 19. Distributions of Numbers of Chromosomes Per Metaphase Cell in Vero, Vs-1 and Vs-2. Cells in logarithmic growth were arrested in metaphase by the addition of colcemid to the growth medium. Chromosome spreads were obtained as described in "Experimental Procedures - Chromosome Counts", and the number of chromosomes determined for at least 20 metaphase cells.

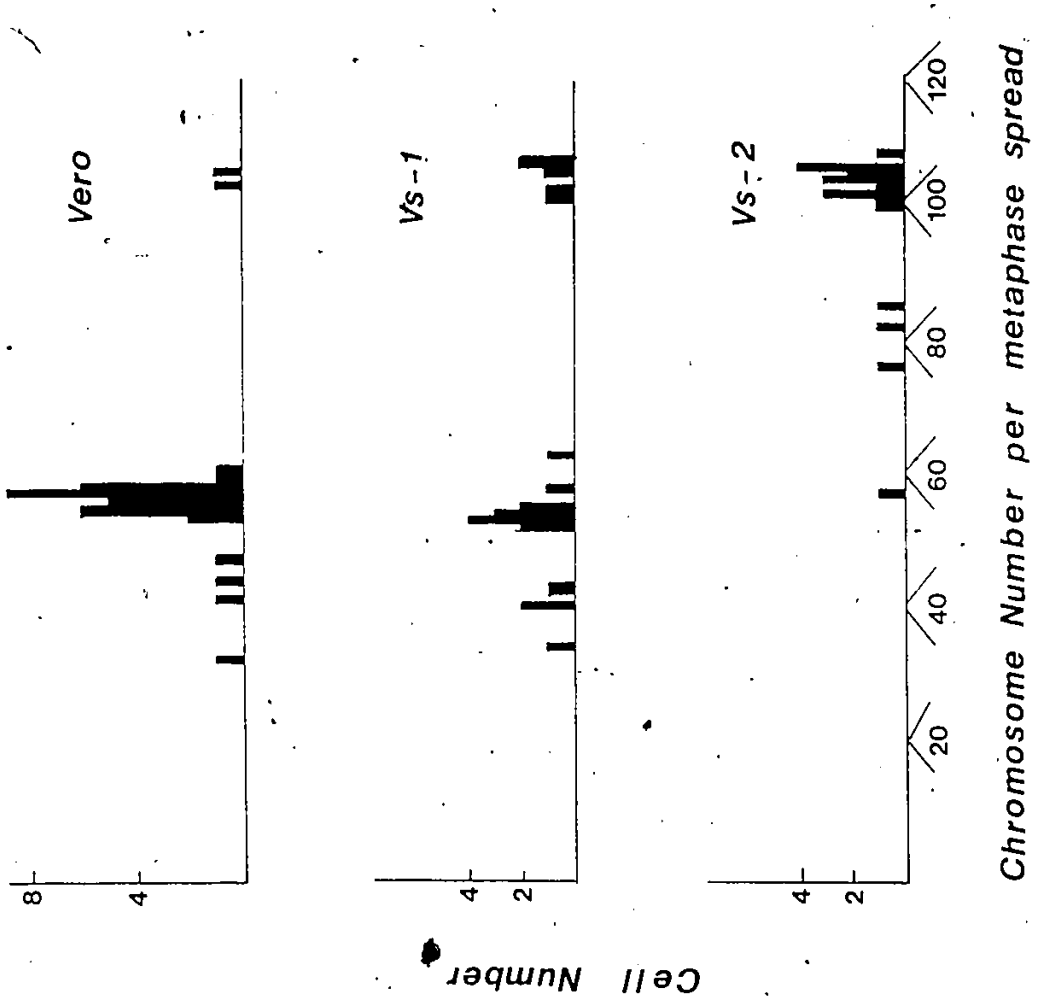
Vero
Vs-1
Vs-2
Cell Number
8
4
4
2
4
2
20
40
60
80
100
120
Chromosome Number per metaphase spread

Fig.19.

DISCUSSION

Analysis of Four Cell Lines

Assessment of the induced AHH activity in Hepa-1c1 and H4IIE-C3 cells revealed both similarities and differences between the lines with respect to their response to inducing agents. The inducible lines, when compared to each other within the same experiment, were found to have very similar levels of induced specific activity when induced with BA in either DMSO or acetone. Between experiments, the absolute levels of specific activity varied, but the relative values for H4IIE-C3 compared to Hepa-1c1 remained quite similar. These findings emphasize the usefulness of a comparison control in AHH induction assays for proper assessment of data from separate experiments.

Both lines had higher levels of induced activity when pretreated with 13 μM, rather than 1.3 μM BA. There was a significant difference between the TCDD-induced activities of these lines at 1 ($p < 0.05$), or 10 nM ($p < 0.025$) TCDD. As TCDD concentrations increased from 1 to 100 nM, induced AHH activity in Hepa-1c1 cells decreased, but activity in H4IIE-C3 cells remained approximately the same. This may have been due to differences in sensitivity to TCDD's toxic effects, rather than a specific facet of the induction capacities of these lines.

For both Hepa and H4IIE-C3, TCDD was a much more potent inducer of AHH activity than BA. Maximum induction was obtained at TCDD concentrations approximately 6.5×10^3 fold less for Hepa and at least 100 fold less for H4IIE-C3 than the maximally inducing BA

concentration. The available data for MC-induced AHH activity indicates a potency for induction approximately 10 fold higher than BA for both lines. These differences may be related to possible differences in Ah receptor affinity for each compound (Bigelow and Nebert, 1982).

Ah Receptor Characteristics

Hepa-1c1, H4IIE-C3, HTC-SR and Vero were assessed for the presence of Ah receptor by sucrose density gradient analysis - a method developed by Okey, et al. (1979) and previously used with tissues from whole animals. This method combines the treatment of labelled samples with dextran-coated charcoal to remove free [^{3}H] TCDD, with centrifugation of samples on sucrose density gradients to separate specifically bound [^{3}H] TCDD from the large amount of non-specifically bound [^{3}H] TCDD. The former is considered to represent Ah receptor.

The receptor characteristics differed for each cell line. The inducible lines, Hepa-1c1 and H4IIE-C3, had clearly demonstrable receptor in nuclear extracts. Hepa-1c1 also had cytosol receptor, as the proposed model of induction would lead one to expect.

The specificity of [^{3}H] TCDD binding was established by comparing binding in the presence or absence of excess concentrations of cytochrome P_1-450 inducers (TCDD, MC, BNF or BA) or cytochrome P-450 inducers (PB or pregnenolone-16α-carbonitrile). Binding was eliminated, or greatly reduced, in the presence of cytochrome P_1-450 inducers but essentially unchanged in the presence of cytochrome P-450 inducers.

These data establish the nature of the [^{3}H] TCDD binding as specific to cytochrome P_1-450 (and AHH) inducers.

The translocation of receptor from the cytosol to the nucleus of Hepa-1c1 cells was shown to be both time and temperature dependent. This information was helpful in investigating the existence of cytosol receptor in H4IIE-C3 cells.

H4IIE-C3 had almost no detectable receptor in the cytosol when assayed under routine conditions (i.e. after a one hour incubation in culture with 1 nM [^{3}H] TCDD at 37°C). When cells were incubated in culture with 1 nM [^{3}H] TCDD for one hour at 4°C, a small peak of cytosol receptor binding was obtained. Since this procedure was found to prevent [^{3}H] TCDD-bound receptor translocation into the nucleus, it seems reasonable to assume that cytosol receptor was not detected after the 37°C incubation because the majority of detectable receptor had moved into the nucleus by one hour.

The noninducible lines - Vero and HTC-SR - differed from each other and the inducible lines. Vero had no detectable specific [^{3}H] TCDD binding in cytosol or nuclear extracts. HTC-SR had both nuclear and cytosol receptor. Lack of induction in HTC-SR would appear, therefore to be dependent on a block in the induction process occurring after the translocation of the receptor-PAH complex into the nucleus. For example, a block could occur because the complex might not be capable of associating with chromatin in order to stimulate transcription, the mRNA for cytochrome P_1-450 might not be properly processed for transcription, or the induced cytochrome P_1-450 might not be functional due to altered structure.

The data reported suggest therefore that the Ah receptor is a necessary element of the induction process, but its presence and translocation to the nucleus are not sufficient for induction to occur. Furthermore, as described in the "Results" ("Hepa-1c1 Subclones - 15"), the Ah receptor in the inducible Hepa-1c1 line does not, itself, appear to be inducible. Thus it appears that an AHH inducible cell maintains an Ah receptor pool in order to be able to respond to the presence of PAHs in its cytoplasm by inducing cytochrome P_1-450 activity.

Analysis of Hepa-1c1 and Vero Subclones

Hepa-1c1 Subclones

Spontaneous Variation in Level of Inducible Aryl Hydrocarbon Hydroxylase Activity

A high degree of spontaneous variation in expression of induced AHH activity was found in seven Hepa-1c1 subclones. Five out of the seven subclones had significantly different induced activities compared to the progenitor Hepa-1c1 population. In order to further assess this variation, Hepa-1c1 and subclone populations Hs-1 and Hs-9 were extensively analyzed for their level of induced AHH activity and their Ah receptor characteristics. Hs-1 and Hs-9 were chosen from the seven subclone populations as representatives of variant and non-variant populations, respectively.

The absolute specific activity of induced AHH in all three populations varied widely from experiment to experiment. However, the relative levels of AHH induction in Hs-1 and Hs-9, when compared to Hepa-1c1 among experiments remained stable. This stability was seen at different times of the year, over a large number of generations and before and after routine storage of viable cells at -80°C. Relative levels of induction were similar with either BA or TCDD as the inducing agent.

Variation in absolute induced activity, but stability of relative levels of induced activity was also seen by Whitlock (1976) in BRL-3C4

rat hepatoma cells. The combination of these characteristics indicates that the cause of variation in absolute activity lies in a factor(s) common to all three populations with respect to their handling in culture, or aspects of the AHH assay itself. All populations to be assayed were plated on the same day, at the same density. Two days after plating, medium containing the inducing agent was added successively to plates for each population over a period of approximately 20 minutes per population, and cells were harvested within the same time periods and in the same order 18 hours later to minimize differences in time of exposure to the inducing agent. Prior and subsequent to AHH assays, the populations were subcultured on the same day, at the same density, in order to ensure that each population was approximately 3 generations older at each successive subculture. The medium the cells were grown in contained the same lot of fetal calf serum throughout the experiments reported here for the Hepa-1c1 progenitor and its subclones. It would therefore seem likely that the factor(s) responsible for variation in absolute induced AHH activity values is inherent in the AHH assay procedure.

The assay should be rigorously assessed with respect to the possible involvement of, for example, fluctuations in fluorometer sensitivity, stability of the BP substrate, or degree of incident daylight (the assay is carried out in the absence of fluorescent light) which might individually, or together, result in variation in detection of AHH activity. It might also be advantageous to express AHH activity as an AHH/cytochrome c activity ratio rather than as a value per mg protein (Kouri, et al., 1982). The cytochrome c activity is a good

estimate of cell number and viability and may provide a more accurate estimation of AHH activity .

In order to assess possible causes for variation in relative activities of subclones compared to Hepa-1c1 and the stable nature of those relative activities, the following factors were taken into consideration.

i) Selection

During the Hepa-1c1 cloning procedure, no intentional selection pressure was applied. The subclones were unselected in so far as they were not exposed to any condition (eg. presence of BP in the medium) that selects for a specific cellular characteristic (eg. lack of capacity to metabolize BP to toxic metabolites).

However, growth of isolated cells may itself be a form of selection for cells that lack a dependence on cell density for cell growth. Density dependence may be the result of lack of essential factors in the growth medium. If the latter can be supplied by secretion from the cells themselves, the density of plating could limit the effective concentration of the factor and thus limit cell growth. The finding that different lots of fetal calf serum supported plating efficiency to different extents for Hepa 1c1, suggests that the fetal calf serum constitution may be a limiting factor in growth of isolated cells. That is, the appropriate constitution could effectively eliminate

density dependence and allow growth of isolated cells.

It is reasonable to assume, therefore, that the cloning of Hepa-1c1 cells depended on the addition of an essential growth factor(s) to the medium by the lot of fetal calf serum used. This might, to some extent mitigate the unavoidable possibility that surviving clones were selected for density independent growth as a consequence of genetic change.

ii) Variation in Gene Expression

Variation in gene expression in cell lines has been previously cited and attempts made to determine frequencies for spontaneous or induced mutation. Povey, et al. (1973), described two cloned human lymphoblastoid lines which, over a period of several years in culture, showed phenotypic changes in expression of peptidase D. They did not find any spontaneous changes in gene expression in lines and sublines from 41 individuals assessed at 26 structural loci. However, treatment of the lines with chemical mutagens or ultraviolet radiation resulted in the identification of 3 changes in phenotype out of 5150 alleles tested. Of these, one appeared to be the result of a structural gene alteration, and two the result of changes at regulatory loci.

In the Chinese hamster ovary cell line, CHO, Siciliano et al., (1983) screened approximately 40 enzyme loci and found the frequency of spontaneous mutation in 383 clones to be 0.6×10^{-4} per locus tested. After u.v. mutagenesis, the mutation frequency increased to 7.3×10^{-4} (a significant difference). In fact, of the loci tested, three were apparent "hot-spots" for mutagenesis, having a mutation frequency of 4.2×10^{-3} after radiation.

It seems unlikely that spontaneous mutation occurring at frequencies such as those described above could account for the high degree of variation seen in the Hepa-1c1 subclones. High levels of variation found in other cell lines for expression of AHH induction and other phenotypes indicates, however, that epigenetic factors may play an important role in determining the phenotype of cells in culture.

Epigenetic change has been described as a heritable alteration in phenotype that is not the result of a genotypic alteration (Lewin, 1980). The process of differentiation is commonly cited as an example of this kind of change. While rates of spontaneous mutation are relatively low (as described above), rates for epigenetic change have been estimated to be approximately 100 fold higher. Peterson (1979), in analyzing the variation in albumin production by clones of H4IIE-C3, calculated a rate of phenotypic variation of 0.5 to 1.4 x 10^{-2} per cell per generation for five hepatoma clones, sequentially subcloned. Furthermore, Baumal, et al. (1973), estimated a rate of spontaneous variation for loss of capacity to synthesize immunoglobulin heavy chains in cloned mouse myeloma cell lines at approximately 10^{-3} per cell per generation. They suggest an epigenetic mechanism may be responsible for this high rate of variation.

With respect to variation in expression of AHH induction, Whitlock, et al. (1976) described a heterogeneity in subclones of an established Buffalo rat liver cell line designated BRL 3C4. The variants were stable in their relative induction capacities over five to six months in continuous culture. Hankinson (1980) described poorly inducible,

BP resistant variants of H4IIE-C3 cells. The variants arose at a spontaneous frequency of 3×10^{-5} events per cell generation. However, the inducibility of the variants was not stable.

The Hepa line, Hepa-1a - from which Hepa-1c1 was derived - produces phenotypic variants in the synthesis and secretion of alpha-fetoprotein, transferrin and albumin (Darlington, et al., 1982). In clones of Hepa-1a, tested for variation in alpha-fetoprotein and transferrin phenotypes, 6 and 10 fold variations in expression were observed. When albumin synthesis was assessed, 3 out of 232 unmutagenized colonies were found to be negative for albumin synthesis, although albumin synthesis in the progenitor population (Hepa-1a) had previously been shown to be stable.

In 1979, Hankinson assessed a subclone of Hepa-1c1 (Hepa 1c1c7) for production of BP resistant - and thus AHH deficient - variants. He described the spontaneous variants as arising at a frequency of 2×10^{-7} events per cell per generation - indicating a mutational, rather than epigenetic origin for the variants. A number of major differences exist between the Hankinson study and the data reported here.

As indicated by Hankinson, the spontaneous Hepa-1c1c7 variants were obtained by selecting for BP resistant cells. The variants so obtained were considerably more resistant to BP than Hs-1 cells described herein. This was indicated by the finding that the ratio of: the EOP of Hankinson's variants grown in the presence of BP compared to their EOP when grown in the absence of BP, was approximately 0.9 (initial plating density of 500 to 5000 cells per 100 mm dish). Hankinson found that increasing the initial plating density resulted in increased

survival in the presence of BP. Thus a finding of a relative EOP of 0.18 for Hs-1 grown in the presence of BP compared to Hs-1 grown in the absence of BP (initial plating density 10^5 cells per 60 mm plate), indicates that Hs-1 cells are poorly resistant to BP in comparison to Hankinson's variants. Furthermore, the relative induced activities of Hankinson's variants compared to their respective progenitor populations was approximately 10 fold lower than those for Hs-1 compared to Hepa-1c1. One variant was uninducible.

The protocol used by Hankinson detects AHH deficient variants. However, variants with inducible activity reduced only to the level found for Hs-1, or ranging from that level to levels the same as, or higher than, that found in Hepa-1c1 will not be detected due to their sensitivity to the presence of BP in the selective medium. It is suggested, therefore, that while the variants obtained by Hankinson are mutational in origin, the variants described herein may represent variants formed through epigenetic rather than genetic change.

A review by Fidler and Hart (1982) of the biological diversity of metastatic tumours and tumour cell lines, indicates that very high levels of variation exist in the metastatic potential of cloned populations. For example, a melanoma - K-1735 - established in culture and cloned after the fifth passage in vitro, produced 20 out of 22 clones that differed significantly from the parent tumour with respect to their metastatic capacity. Similarly, 15 of 17 clones of the B16 melanoma line, and 15 of 21 clones from a recently induced fibrosarcoma differed significantly from the parent tumour in this regard.

Furthermore, it was found that the metastatic potential of parent lines often remains stable in culture for years, and clonal populations grown together produce a stable metastatic phenotype. The latter is lost when cells are recloned. That is, subcloning after as few as 20 passages of a clone by itself results in the procurement of "heterogeneous" clones. Heterogeneity is not limited to metastatic potential, but can include phenotypic expression of hormone receptors, marker enzymes, antigenicity, or immunogenicity.

If one considers the examples of high rates of spontaneous variation cited above, and in this report, it is clear that such variation is common in cells originating from a tumour. Thus the high rate of variation seen in AHH induction in Hepa-1c1 subclones and stability of inducing capacity in Hepa-1c1, Hs-1 and Hs-1 may, in fact, be dependent on their tumour cell origin.

One is still left, however, attempting to understand the cause of the variation at the molecular level. In order to look for specific or general changes, an analysis of chromosome constitution, growth parameters and Ah receptor characteristics in Hepa-1c1, Hs-1 and Hs-9 was undertaken.

Chromosome Constitution and Growth Parameters

Hepa-1c1, Hs-1 and Hs-9 populations were assessed for their chromosome constitution, generation time, growth in the absence of low molecular weight serum factors (i.e. in dialyzed fetal calf serum), and growth in the presence of the cytochrome P_1-450 inducer - BP.

i) Chromosome Constitution

Major differences were not seen between the populations with respect to generation time. Differences in modal chromosome numbers were seen, but variations about the means were similar, suggesting that the differences may not be significant. Specific chromosome identification (eg. by banding techniques) would have to be undertaken in order to determine whether Hs-1 cells have a chromosomal rearrangement, or to identify a chromosome that could be associated with inhibition of AHH induction.

As described in "Results", Hs-1 does not produce decreased levels of Ah receptor and therefore retains at least one gene coding for receptor. Inhibition of induced AHH activity by 7,8-benzoflavone indicates that Hs-1 also has retained at least one gene coding for cytochrome P_1-450 synthesis. This information, combined with the finding that the modal chromosome number is higher for Hs-1 (60) than Hepa-1c1 (58) suggest that chromosome loss is unlikely to account for the altered phenotype of Hs-1. Rather, if a genetic change has occurred it is more likely to be a rearrangement or point mutation.

ii) Growth of Hs-1 in Dialyzed Fetal Calf Serum

Growth of Hs-1 in dialyzed fetal calf serum was assessed due to an apparent change in phenotype observed during interspecies hybridization experiments. There was an apparently spontaneous increase (greater than 6 fold) in EOP of these cells in dialyzed fetal calf serum between generations 60 and 117.

The increase is described as spontaneous since it appears to have occurred in the absence of selection. However, it may be the

consequence of overgrowth of Hs-1 by a clonal population of cells selected for by some as yet undefined parameter. In that case, the change would have been a progressive one, occurring through generations 60 to 117. (An example of such a progressive change has been described by Wojtyk and Goldstein (1982) with respect to protein synthetic errors in cultured human fibroblasts.) The change observed in Hs-1 did not appear to alter the stability of the AHH phenotype of the Hs-1 population.

The phenotypic change could be attributable to mutation (eg. such as that described by Povey, et al., 1973) or epigenetic change. Its main importance, here, however, is to illustrate that the Hs-1 population, while stable with respect to its relative level of induced AHH activity, may represent an evolving population, with at least transient heterogeneity in one, or some of its characteristics. Hs-1 may, therefore, represent a population similar to, for example, the previously described metastatic tumour populations, or H4IIE-C3 clones showing almost immediate phenotypic variation in albumin synthesis upon subcloning (Peterson, 1979). Further sequential subcloning of Hs-1 and the other Hepa-1c1 subclones obtained, with assessment of their AHH induction characteristics would serve to clarify this point.

iii), Growth in Benzo(a)pyrene

Hs-1 cells showed an increased resistance to the presence of BP in growth medium, compared to Hepa-1c1 or Hs-9 cells. The latter populations resembled each other in their level of resistance. These results were as expected, since the poorly inducible Hs-1 cells would be less efficient in metabolizing BP to its toxic products than Hs-9 or

Hepa-1c1. Consequently, Hs-1 should be less sensitive to the presence of BP in the growth medium. Environmental exposure of these populations to BP (or other PAHs that are metabolized by AHH to toxic products) would presumably result in selection for growth of Hs-1 cells, and against Hepa-1c1 and Hs-9 cells. The implications of such selection are discussed below ("Practical Implications").

Ah Receptor Characteristics

i) Quantification of Ah Receptor

Receptor was quantified by incubating cells in culture in the presence of 1 nM [^{3}H] TCDD or incubating cytosol *in vitro* with different concentrations of [^{3}H] TCDD for a modified Scatchard analysis. For either method, the number of fmol of specifically bound [^{3}H] TCDD per mg of protein was determined. Scatchard analysis was also used to determine values for affinity of binding (Kd) and number of receptor molecules per cell (Clark and Peck, 1977).

The mean quantity of receptor detected as fmol [^{3}H] TCDD specifically bound per mg protein appeared to increase from the Hs-1 to Hepa-1c1 to Hs-9 populations. However, there was no significant difference (at $p = 0.05$) between Hs-1 and Hepa-1c1, or between Hs-9 and Hepa-1c1. The quantity of total receptor present in cell extracts could not, therefore, be directly associated with the relative levels of induced AHH activity in each subclone.

The per cent of total binding represented in the nuclear extract of

Hepa-1c1 and Hs-1 was not significantly different (at p 0.05). The differences between Hs-9 and Hepa-1c1 were statistically significant. The values for fmols specifically bound [^{3}H] TCDD in nuclear extracts differed significantly only between Hepa-1c1 and Hs-9.

The data indicate that a larger quantity of receptor reaches the nucleus in Hs-9 cells compared to Hepa-1c1 cells. However, a consistently higher level of induced AHH activity in Hs-9 cells compared to Hepa-1c1 was not observed. It would therefore appear that the increase in nuclear receptor does not stimulate increased cytochrome P_1-450 transcription.

The data for Hs-1 cells can be interpreted in a number of ways. Since the Ah receptor can translocate to the nucleus of these cells, the low level of induced AHH activity in this subclone could be the result of, for example, a block in receptor-TCDD complex-mediated induction of cytochrome P_1-450 transcription, an alteration in the processing of cytochrome P_1-450 mRNA resulting in a decrease in cytochrome P_1-450 translation, or an alteration in the cytochrome P_1-450 genetic code resulting in the production of cytochrome P_1-450 possessing reduced catalytic activity. All but the last possibility could result from genetic or epigenetic change.

A quantitative association between Ah receptor reaching the nucleus and amount of cytochrome P_1-450 mRNA induced has been described by Tukey, et al. (1982). These workers were investigating the differences among inbred strains of mice in which AHH responsiveness segregates in an autosomal dominant manner. Mice with genotypes designated Ah^b/Ah^b, or Ah^b/Ah^d, both have AHH activity that is highly inducible with TCDD

compared to the $\underline{Ah}^{d}/\underline{Ah}^{d}$ mouse, which requires approximately 10 fold more TCDD to induce a level of maximal AHH activity similar to that in $\underline{Ah}^{b}/\underline{Ah}^{b}$, or $\underline{Ah}^{b}/\underline{Ah}^{d}$ mice. When mice of each genotype were tested, they were found to have levels of translocated nuclear Ah receptor that correlated strongly with their levels of induced cytochrome P_1-450 mRNA.

ii) Subcellular Distribution of [^{3}H] TCDD

The total quantity of [^{3}H] TCDD within the cells of all three populations was similarly distributed, as indicated by measurements of radioactivity in cytosol, washed nuclei and microsomal homogenates. The extent of uptake of [^{3}H] TCDD into each of these compartments appeared to be the same in all three populations and could not be associated with differences in level of induced activity. Furthermore, no differences were detected in the proportions of [^{3}H] TCDD of the total cell homogenate that were represented by specifically bound [^{3}H] TCDD in each population.

iii) Translocation of Specifically Bound [^{3}H] TCDD

Hepa-1c1, Hs-1 and Hs-9 were all capable of a time- and temperature-dependent translocation of specifically bound [^{3}H] TCDD from the cytosol to the nucleus. Translocation in Hepa-1c1 and Hs-1 followed a similar time course. The proportion of total specifically bound [^{3}H] TCDD that became detectable in nuclear extracts appeared to increase more rapidly in Hs-9, compared to Hepa-1c1 or Hs-1 cells. Thus a more efficient translocation process in Hs-9 cells may be the

reason for finding a higher proportion of total binding and significantly more specifically bound [^{3}H] TCDD in the nucleus of Hs-9 cells compared to Hepa-1c1 cells.

iv) Co-ordinate Measurement of Ah Receptor and Induction of Aryl Hydrocarbon Hydroxylase Activity

Co-ordinate measurement of Ah receptor and induced AHH activity was accomplished by quantifying the amount of receptor present in cells at the time that inducing agent was added to sister cultures. There was a correlation of 0.62 between the amount of nuclear receptor measured and the level of induced AHH activity. Lack of complete correlation indicates that factors other than the quantity of receptor present in the nucleus are involved in the control of the level of AHH activity induced. The lack of correlation between separately measured mean nuclear receptor quantity and mean relative levels of induction for Hs-1, or Hs-9 compared to Hepa-1c1 (see "Discussion - Ah Receptor Characteristics - i") should be taken into consideration when assessing this information. These data indicate that the relative importance of the relationship between nuclear receptor quantity and AHH induction may differ among populations.

v) Specificity of [^{3}H] TCDD Binding

The specific nature of Ah receptor binding can be demonstrated by the competetive elimination of the [^{3}H] TCDD binding by cytochrome P_1-450, but not cytochrome P-450, inducers. Specificity of [^{3}H] TCDD

binding to the Ah receptor was shown for Hepa-1c1, Hs-1 and Hs-9 by loss of detectable binding in the presence of excess unlabelled TCDD, or MC, reduced binding in the presence of excess BA, and little change in binding in the presence of excess phenobarbital. The results indicate that Ah receptor in each population has retained a comparable specificity of binding for PAH's. Furthermore, due to the essentially complete nature of the competition with unlabelled TCDD, or MC, the receptor binding can be said to be saturable and of a non-covalent nature for all three populations.

Incomplete competition in the presence of even a 1000 fold excess of BA suggests a difference in receptor affinity between TCDD and BA may exist. This may correlate with their respective capacities for AHH induction. In all three populations, TCDD induces a higher level of AHH activity at an approximately 6×10^3 fold lower concentration than BA. This type of relationship has been previously described in mouse liver (Bigelow and Nebert, 1982).

Detectable binding of [^{3}H] TCDD in nuclear extracts of all three populations, in the presence of excess BA was consistently decreased to a greater extent than was cytosol binding. The reason for this is unknown.

vi) Effect of Benzo(a)anthracene Pre-treatment on Ah Receptor Quantity

Treatment of Hepa-1c1, or Hs-9 cells for 18 with 6.5 M BA in 0.1% DMSO prior to incubation in [^{3}H] TCDD had little effect on

receptor quantity compared to cells treated for 18 hours with DMSO alone. No induction of Ah receptor was apparent. Hs-1 cells tended to have fewer receptors after exposure to BA. This can be explained by the existence of competition between residual BA - metabolized poorly in Hs-1 compared to Hepa-1c1 or Hs-9 - and [^{3}H] TCDD.

Vero Subclones

No variation was seen among Vero progenitor, Vs-1 and Vs-2 populations with respect to inducibility of AHH activity. Neither the Vero progenitor population, nor Vs-1 had detectable specific TCDD binding in cytosol or nuclear extracts.

It was not surprising to find that Vs-1 was hypotetraploid since such cells were frequently observed in the progenitor population. This finding does serve, however, to suggest that lack of induction capacity in Vero is unlikely to be the result of reduced gene dosage for Ah receptor itself, or other genetic components of the induction process.

Practical Implications for Cancer Risk Assessment

The correlation between induced AHH activity and Ah receptor characteristics was investigated, in part, so that further genetic analysis of the AHH induction system might be undertaken. This was particularly true in light of the hopes that receptor detection might be a useful tool in assessing degree of response to PAHs and consequent risk for the development of certain forms (eg. bronchopulmonary) of

cancer. However, the detection of nuclear receptor in the uninducible HTC cell line, the poorly inducible Hepa subclone 1 (described herein), and BP resistant clones of mutagenized Hepa 1c1c7 cells (Legraverend, et al., 1982), reduces the probability that Ah receptor measurement will be useful in this regard. Furthermore, attempts to correlate the quantity of nuclear receptor with level of induced AHH activity may have limited success in light of the data obtained for Hs-9 compared to Hepa-1c1. That is, a larger quantity of nuclear receptor in one population of cells compared to another may not be indicative of a higher level of inducibility of AHH activity. In light of these findings, it would appear that quantification of Ah receptor cannot replace the AHH induction assay in predicting risk for the develoment of lung cancer. It is therefore all the more important to reduce variability in the AHH assay of lymphocytes.

The spontaneous occurrence of clonal variants in Hepa-1c1 underscores a possible cause for some of the difficulties encountered in standardizing an AHH assay for human lymphocyte samples. Lymphocytes are a rapidly dividing population with turn-over times of 3 to 4 days (Osmond, 1976). Spontaneous variations (whether due to epigenetic or genetic change), followed by changes in the clonal constitution of a lymphocyte population could alter an individual's AHH induction phenotype. The predominance of any clone(s) over others would be enhanced if selection pressures increased the survival of clones with variant AHH phenotpyes. Selection would result from exposure to changing levels of PAHs (such as BP) in the individual's

environment. Variation in AHH induction phenotype occurring between different tissues in the same individual (eg. lymphocytes and macrophages, as reported by McLemore, et al., 1981) may also be attributable, at least in part, to clonal variation.

In order to test these possibilities, it would be useful to incorporate the finding of stable relative induction capacities reported herein in the application of the AHH assay. Incorporation of an inducible cell line as a comparison control, when lymphocytes are assayed for induction of AHH, could reduce the variability that appears to be an inherent part of the assay. Obviously, understanding the balance between variable and stable elements in control of the induction process is critical - first to elucidate the cause, or effect, relationship between AHH induction and lung cancer; and second, to determine the appropriateness of using AHH phenotypes in the estimation of cancer risk.

APPENDIX

Hybridization of Hs-1 and Vs-1

Hybridization experiments were undertaken in order to assess the nature of the genetic control of AHH induction in the Hs-1 and Vs-1 populations. The expression, or lack thereof, of AHH induction and associated Ah receptor characteristics in hybrid cells could be used to determine the complexity of the genetic control of induction (i.e. monogenic vs. polygenic) and its nature (i.e. dominant, co-dominant, or recessive), or serve to assess the possible role of epigenesis in altering induction phenotypes.

Selective Medium

A growth medium that would select for growth of hybrids and against Hs-1 and Vs-1 growth was designed. It contained alpha minimal essential medium plus dialyzed fetal calf serum and 3.4×10^{-5} M ouabain. Hs-1 cells were ouabain resistant, but grew poorly in the presence of dialyzed fetal calf serum. Vs-1 cells grew well in the presence of dialyzed fetal calf serum, but did not grow in the presence of ouabain at the above-specified concentration.

Cells that grew in the selective medium, after treatment with a fusogen (described below), were considered to be putative hybrids. Their phenotypes, with respect to the characteristics described below as "Phenotypic Markers", were then assessed to determine whether or not they were true hybrids. Modal chromosome numbers approximating the sum of Hs-1 and Vs-1 modal chromosome numbers, and the presence of at least

one phenotype unique to Hs-1 cells and one unique to Vs-1 cells in putative hybrid cells would identify selected cells as true hybrids.

Phenotypic Markers

Chromosome number, representation of metacentric and telocentric chromosomes in a typical metaphase spread, marker chromosome presence, phosphoglucomutase isozyme patterns on starch gels (Spencer, et al., 1964) and patterns of microsomal polypeptide migration on SDS-polyacrylamide gels (Maizel, 1971) were determined.

Modal chromosome numbers were 60 for Hs-1 and 55 for Vs-1. Hs-1 cells characteristically had 1 metacentric and 9 submetacentric chromosomes. The remainder were telocentric. Vs-1 had 12 metacentric and 30 submetacentric chromosomes, and a marker chromosome (illustrated in Fig. 1B) morphologically unlike any Hs-1 chromosome. Remaining chromosomes were acrocentric.

Phosphoglucomutase isozyme patterns differed between Hepa-1c1 and Vero as illustrated in Fig. 1A (Hepa-1 represents Hepa-1c1). The banding patterns for Hs-1 and Vs-1 microsomal polypeptides differed in some regions of a polyacrylamide gel with respect to banding densities and presence, or absence of bands. A band representative of protein with an approximate molecular weight of 15,000 was present in Vs-1, but absent from Hs-1. Hs-1 had four bands (approximate MW 36,000; 38,000; 85,000 and 88,000) that did not appear in the Vs-1 banding pattern.

Results of Hybridization Experiments

Eight separate hybridization experiments (each taking approximately two months) were carried out. The number of cells initially plated, ratio of Hs-1 to Vs-1 cells plated, length of time in selective medium

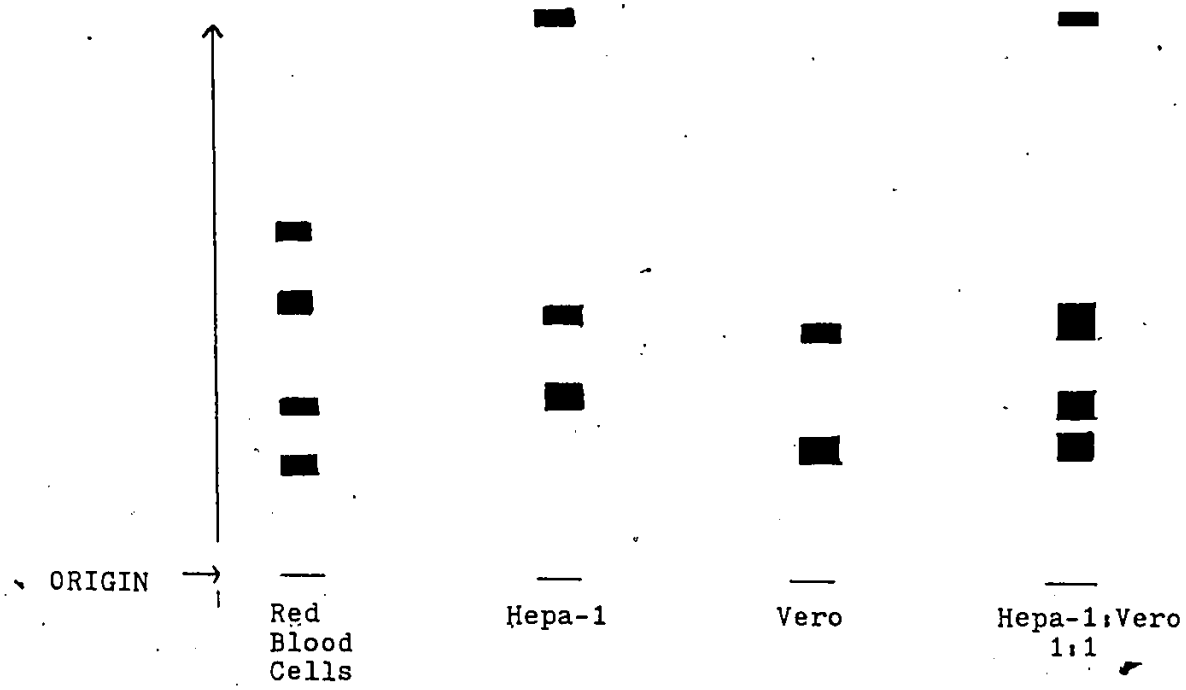

Fig. 1A. Phosphoglucomutase isozyme patterns on starch gel electrophoresis.

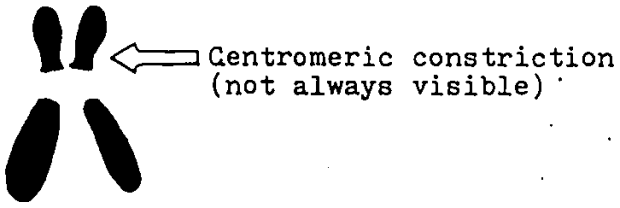

Fig. 1B. Vero marker chromosome.

following fusogen treatment, make-up of selective medium, and application of and type of fusogen (polyethylene glycol 1000 or 6000, or inactivated Sendai virus) were varied in order to optimize the hybridization procedure. Ten putative hybrids were isolated, but could not be identified as true hybrids by the phenotypic criteria described above.

It is postulated that a major reason for lack of success was due to a change in Hs-1 growth requirements. As described in the text, the efficiency of plating of Hs-1 in dialyzed fetal calf serum increased over a number of generations. Therefore the efficacy of the selective medium may have been continually decreasing.

Heterokaryons between Hs-1 and Vs-1 were identified, indicating that the cells could undergo initial fusion. A heterokaryon was identified as a cell containing one radiolabelled nucleus and one unlabelled nucleus in a cytoplasm containing intracellular latex beads. Prior to treatment with a fusogen, the nuclei of 81% of Vs-1 cells were labelled with [^{3}H] TdR and the cytoplasm of 99% of Hs-1 cells was identified by the presence of intracellular latex beads (1μ in diameter). Within 24 hours of fusogen treatment, cells were fixed and treated for autoradiography (see "Experimental Procedures"). The number of Hs-1/Vs-1 heterokaryons was then determined by microscopic examination and found to be higher (2 heterokaryons in 544 cells) with fusogen treatment than without (no heterokaryons in 1197 cells).

For further information regarding the hybridization experiments, the reader is referred to the author, Dr. C. Forster-Gibson, Dept. of Paediatrics, Queen's University, Kingston, Ontario. A project report is also in the hands of Dr. M.J. Dufresne, at the University of Windsor.

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