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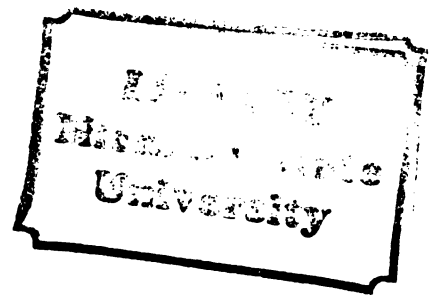

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THE BIOLOGICAL EFFECTS OF AFLATOXIN B_1
AND AFLATOXIN B_1-DICHLORIDE
IN HUMAN CELLS IN CULTURE

presented by

EILEEN MAHONEY-LEO

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THE BIOLOGICAL EFFECTS OF AFLATOXIN B_1 AND AFLATOXIN B_1-DICHLORIDE IN HUMAN CELLS IN CULTURE

By

Eileen Mahoney-Leo

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ABSTRACT

THE BIOLOGICAL EFFECTS OF AFLATOXIN B_1
AND AFLATOXIN B_1-DICHLORIDE
IN HUMAN CELLS IN CULTURE

By

Eileen Mahoney-Leo

Aflatoxin B_1 is one of the most powerful hepatocarcinogens known, however it requires metabolic activation before it can exert its biological effects. A human cell-mediated system was used to study the cytotoxicity of AFB_1 in human cells in culture. Cytotoxicity was observed in normal human fibroblasts at concentrations as low as 1μM.

Aflatoxin B_1-dichloride (AFB_1-Cl_2) is a direct-acting carcinogen which is a model compound for the proposed ultimate metabolite, aflatoxin B_1-2,3-oxide. The cytotoxic and mutagenic effects of AFB_1-Cl_2 were studied in repair-proficient human fibroblasts (NF) and in excision repair-deficient fibroblasts derived from xeroderma pigmentosum (XP) patients. XP cells were more

sensitive than NF cells to the cytotoxic and mutagenic effects of AFB_1-Cl_2. NF cells were able to recover from the potential cytotoxicity and potential mutagenicity of AFB_1-Cl_2 when held in a non-replicating state for up to 10 days following treatment. XP cells showed no ability to recover from the cytotoxic effects of AFB_1-Cl_2.

The loss of DNA-bound AFB_1-Cl_2 residues was monitored in NF and XP cells held in the non-replicating state for up to eight days following treatment. It was observed that more than 60% of the total initially bound AFB_1-Cl_2 residues were lost from the DNA of NF and XP cells by day 7. More than 60% of the total initially bound adducts were also observed to be lost from calf thymus DNA reacted <u>in vitro</u> with radioactive AFB_1-Cl_2 and incubated for eight days. HPLC analyses of DNA adducts revealed the formation of seven adducts in calf thymus DNA and in the DNA of NF and XP cells. The major product was lost from calf thymus DNA by day 8, and all adducts diminished in size over time. The three major products were identified as guanine adducts. The same DNA adducts were formed initially in NF DNA as were seen in calf thymus DNA.

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I would like to thank my family and friends for their encouragement and support throughout this endeavor.

I acknowledge Mr. Ted Van Noord for carrying out the calf thymus DNA experiment and for performing the HPLC analyses.

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LIST OF ABBREVIATIONS

AFB_1	: aflatoxin B_1
AFB_1-Cl_2	: aflatoxin B_1-dichloride
AFG_1	: aflatoxin G_1
AHH	: aryl hydrocarbon hydroxylase
AP	: apurinic/apyrimidinic
BS	: Bloom's syndrome
BP	: benzo(a)pyrene
BPDE	: 7,8-diol-9,10-epoxide of benzo(a)pyrene
C	: centigrade
cpm	: counts per minute
Ci	: curie
DNA	: deoxyribonucleic acid
dpm	: disintegrations per minute
g	: gram
HPLC	: high pressure liquid chromatography
kg	: kilogram
µg	: microgram
µl	: microliter
µM	: micromolar

mg	: milligram
ml	: milliliter
mm	: millimeter
mM	: millimolar
M	: molar
nm	: nanometer
nM	: nanomolar
N	: normal
NF	: normal diploid human fibroblasts
ppb	: parts per billion
RNA	: ribonucleic acid
TG	: thioguanine
UV	: ultraviolet
XP	: xeroderma pigmentosum

INTRODUCTION

It has been well documented that aflatoxins, and in particular aflatoxin B_1 (AFB_1), are very powerful carcinogens in many mammalian species by several different routes of administration. For example, aflatoxin B_1 induced liver carcinomas in male Fischer rats at dietary levels of 1-15 ppb (Wogan *et al.*, 1964). Although the liver is the major target organ for aflatoxin carcinogenesis, other organs are susceptible, including the kidneys, stomach and colon, depending on the route of exposure (Wogan and Newberne, 1967). Epidemiological studies show a positive correlation between the level of dietary intake of AFB_1 and the incidence of human liver cancer in Asia and Africa (Alpert *et al.*, 1974), suggesting that aflatoxins are hepatocarcinogens in man.

The somatic cell mutation theory of the origin of cancer argues that carcinogens are mutagens, and therefore, that DNA is the target macromolecule for the majority of chemical carcinogens. A good deal of evidence supports this theory. It was therefore of interest to study the mutagenic potency of aflatoxin to determine whether it parallels its

carcinogenic potency. One modulating factor in mutagenesis and carcinogenesis is DNA repair. Cells are known to be capable of enzymically removing DNA-bound carcinogen residues, and it has been shown in human cells, that if DNA repair is carried out before the DNA is replicated, it acts to reduce the carcinogenic and mutagenic effects of these carcinogens (Maher *et al.*, 1979; Heflich *et al.*, 1980; Yang *et al.*, 1980). We, therefore, chose to study the effect of DNA repair on the biological effects of aflatoxin B_1-dichloride (AFB_1-Cl_2), a reactive derivative of AFB_1, in repair-proficient and repair-deficient human cells in culture. We carried out comparative studies on the loss of aflatoxin B_1-dichloride-induced adducts from the DNA of normal human cells and of repair-deficient fibroblasts derived from xeroderma pigmentosum patients and the corresponding recovery of these cells from the potentially mutagenic and/or cytotoxic effects of this agent, in an attempt to provide insight into the nature of the potentially mutagenic and cytotoxic lesion(s). Our purpose was to provide some insight into the mechanism(s) by which aflatoxin induces mutations and cytotoxicity in cells in culture, in order, possibly, to gain a better understanding of the mechanism(s) by which aflatoxin induces cancer *in vivo*.

LITERATURE REVIEW

Aflatoxins

Aflatoxins are a group of naturally occuring carcinogens, produced as metabolic by-products of the mold Aspergillus flavus, which grows well on grains and nuts stored in damp conditions. They were first recognized as a problem contaminant in foodstuffs in 1960, when over 100,000 turkeys died due to contamination of their food (Blount, 1961). At the same time, toxicity was seen in cattle(Loosmore,1961a), pigs (Loosmore,1961b), and ducklings (Asplin,1961). The cause of the toxicity was traced to the common factor of Brazilian groundnut meal in the animal food. The toxic factor was extracted from the groundnut meal and discovered to be composed of metabolic by-products of the mold Aspergillus flavus (Sargeant,1961).

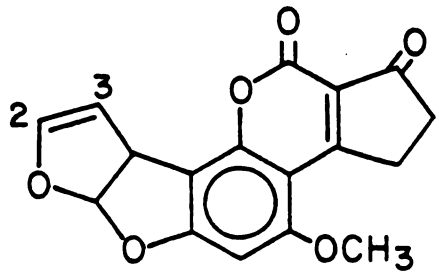
From this mixture of products, four major fractions were isolated. They were named aflatoxin B_1, B_2, G_1 and G_2, based on whether they fluoresced green or blue in ultraviolet light, and on their relative chromatographic mobilities (Nesbitt,1962). The relative toxicities of these

four components vary greatly. In human embryonic cells in culture, the order of toxicity is B_1 > G_1 > G_2 > B_2 (Zuckerman, 1968). This order can differ from one species to another, but in all species studied, AFB_1 (Figure 1) is by far the most toxic of all (Garner and Martin, 1979).

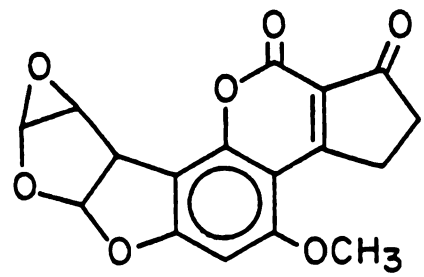
Metabolism of AFB_1

Because it has been determined to be the most toxic, AFB_1 has been the most widely studied of the aflatoxins.

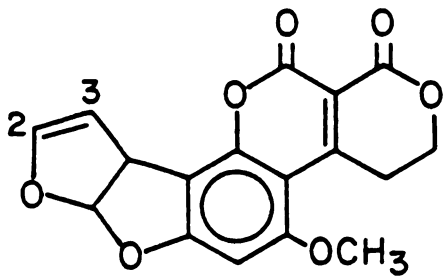
AFB_1 was first shown to require metabolic activation in 1971 when Garner *et al.* showed that AFB_1 was toxic in *Salmonella typhimurium* TA 1530 and TA 1531 only in the presence of rat liver microsomes and a NADPH-generating system. In the absence of the activation system no toxicity was observed. Garner, Miller, and Miller reported in 1972 that, in addition to AFB_1, AFG_1 and sterigmatocystin, a product of *Aspergillus versicolor*, were also toxic to *S. typhimurium* TA 1530 and TA 1531 in the presence of an activation system, while several other metabolites were not. They also examined the extent of AFB_1-binding to commercial RNA in the presence of a rat liver microsome system. They suggested that the toxic metabolite of AFB_1 was the 2,3-oxide (Figure 1) based on their data that AFB_1, AFG_1, and sterigmatocystin were



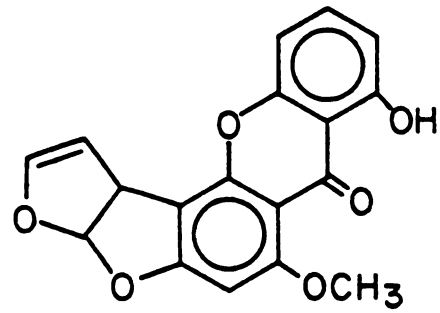
Aflatoxin B_1



Aflatoxin B_1-2,3-oxide



Aflatoxin G_1



Sterigmatocystin

Figure 1. Structures of AFB_1 and related compounds

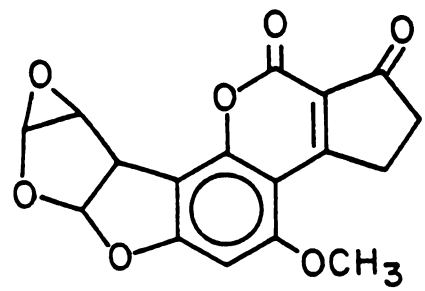
the three most active aflatoxin compounds in the microsome-mediated system, and based on structural data showing that all three had a 2,3 double bond in common, while the distal portion of these molecules differed. They cited as evidence the polycyclic aromatic hydrocarbons which, when activated by the mixed-function oxygenases of liver microsomes produce epoxides, are highly reactive, toxic, and mutagenic in mammalian cells (Grover *et al.*, 1971; Huberman *et al.*, 1971). They also observed that the factor which was toxic to the bacteria and the level of nucleic acid binding *in vitro* were dependent upon the metabolizing system. They, therefore, suggested that the toxic derivative and the derivative that reacted with nucleic acids were one and the same. Since AFB_1, AFG_1, and sterigmatocystin were known to be the most carcinogenic of the aflatoxin derivatives studied, they postulated that the toxic metabolites of these three compounds, which they proposed as the 2,3-oxides, might also be involved in the induction of liver tumors *in vivo*.

All attempts to isolate the toxic derivative of AFB_1 have been unsuccessful. The first biochemical evidence in support of the proposal that the 2,3-oxide was the toxic derivative was obtained by Swenson *et al.* in 1973. Swenson isolated an hydrolysis product of an AFB_1-RNA adduct formed *in vitro* in the presence of rat liver microsomes. Mild acid hydrolysis of the adduct yielded a

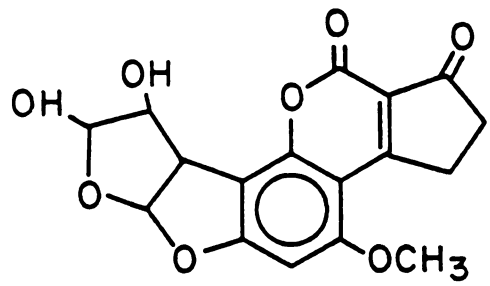
product indistinguishable from synthetic 2,3-dihydro-2,3-dihydroxy-AFB_1 (Figure 2). These data provided evidence that the reactive species of AFB_1 was the 2,3-oxide. This epoxide would be expected to have a strongly electrophilic carbon 2, at which to bind RNA or DNA, and would yield the 2,3-dihydro-2,3-dihydroxy-AFB_1 on mild acid hydrolysis. In 1975 when Swenson *et al.* isolated the hydrolysis products of AFB_1-RNA adducts isolated from rat liver *in vivo* the major product obtained was identical to the 2,3-dihydro-2,3-dihydroxy-AFB_1 obtained *in vitro*. This suggested that AFB_1-2,3-oxide was responsible for a major portion of covalent binding of AFB_1 to nucleic acids in rat liver *in vivo*, and that the 2,3-oxide was probably the ultimate carcinogenic metabolite of AFB_1. Binding of this metabolite to rat liver DNA *in vivo* has since been correlated with the induction of AFB_1-induced liver tumors (Swenson *et al.*,1977).

Toxicity of AFB_1

AFB_1 has been shown to be toxic in a wide range of animal species including rats, pigs, ducklings, turkeys, chickens, and trout (Loosmore,1961a,b; Asplin, 1961). In all cases the primary target organ is the liver (Garner and Martin,1979). Most of the damage is exhibited as liver cell necrosis. There are a wide range of factors influencing toxicity which include diet, age, and sex of the animal



Aflatoxin B_1-2,3-oxide



2,3-dihydro-
2,3-dihydroxy aflatoxin B_1

Figure 2. Structures of AFB_1-2,3-oxide and 2,3-dihydro-2,3-dihydroxy AFB_1

(Wogan, 1968). Since AFB_1 requires metabolic activation in order to exert any biological effects on cells or animals, the level of metabolizing enzymes present in the tissues of different species largely determines the extent to which these species will be affected by AFB_1 (Newberne, 1973). The liver has the highest concentration of the mixed function oxidase systems needed to metabolically activate AFB_1 to the reactive 2,3-oxide. Because it is the primary purpose of these enzyme systems to metabolically convert compounds that cannot be utilized by the body into forms that can be excreted from the body (usually water soluble), the liver is considered to be the primary site for "detoxification." This is accomplished primarily by converting compounds into more polar (water soluble) and less toxic forms. Aflatoxins are highly toxic. Because they are lipid soluble, they partition into the endoplasmic reticulum of cells, which is where they are metabolized. During the detoxification process, compounds may be converted into forms which are actually more toxic than the parent compound. This is the process known as activation, and is essentially what happens in the case of AFB_1. Thus, besides being the major site for detoxification, the liver is often the target organ for activated species generated by this system.

At the cellular level AFB_1 may induce toxicity by inhibiting DNA, RNA and protein synthesis (Wogan, 1968). The

inhibition of protein synthesis is a result of AFB_1 binding at the polysomes (Garvican et al., 1973). AFB_1 has been shown to greatly inhibit hepatic RNA synthesis which may in part be responsible for the hepatic damage observed (Akao et al., 1971). AFB_1 also inhibits rat liver mitochondrial electron transport in vitro (Doherty et al., 1973).

In the presence of an activation system, AFB_1 is toxic to several strains of Salmonella typhimurium and Escherichia coli (Garner and Wright, 1973). AFB_1 has induced toxicity in V79 Chinese hamster cells (Langenbach et al., 1978) and in C3H10T½ mouse embryo cells (Krahn and Heidelberger, 1977) when an activating system was present. Cultured epithelioid human tumor cells from (A549) lung are capable of metabolizing AFB_1, and these cells exhibited sensitivity to the toxicity of AFB_1 as measured by colony forming ability (Wang and Cerutti, 1979).

Carcinogenicity of AFB_1

AFB_1 is one of the most potent hepatocarcinogens known. It was first shown to be carcinogenic in 1961 when Lancaster reported that rats fed on peanut meal contaminated with aflatoxins developed liver tumors. A linear dose response relationship was later observed by Newberne in 1965 when hepatomas were induced in rats fed varying doses

of AFB_1 in their diets. Liver tumors have been induced by AFB_1 in a series of laboratory animals, including mice (Weider et al., 1968), ducks (Adamson, 1973), and monkeys (Carnaghan et al., 1965). It induces liver tumors in the rat or trout at continuous dietary levels of 1 to 15 ppb (μg/kg) and in rats at a single sublethal dose of 7.65 mg/kg body weight (Carnaghan et al., 1967). As indicated above, epidemiological evidence suggests that AFB_1 may be responsible for the high incidence of liver cancer in localized human populations. A typical report is that of Alpert et al., (1971), who showed a positive correlation between the estimated aflatoxin intake and the incidence of primary liver cancer among Ugandans.

Mutagenicity of AFB_1

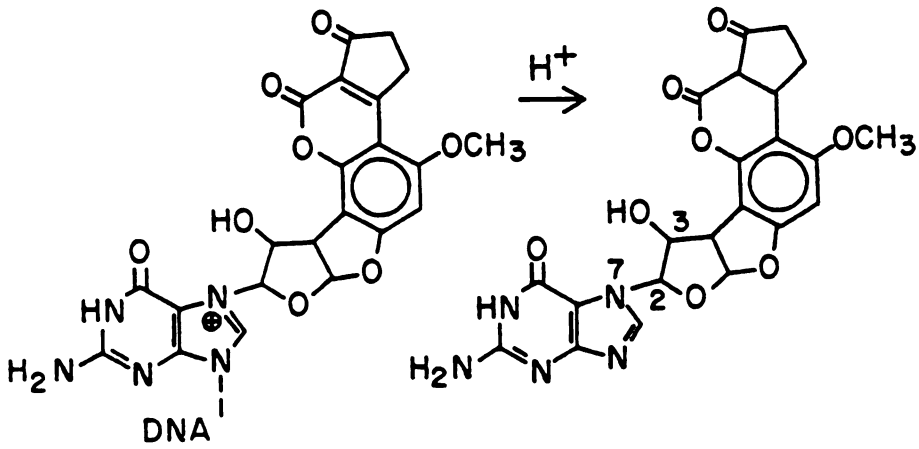
The induction of mutations by AFB_1 has been studied in many organisms. In the presence of rat liver microsome fractions and NADPH, AFB_1 is mutagenic in Salmonella typhimurium (Ames et al., 1973). Mutations are induced in vegetative cultures of Neurospora crassa in the absence of a metabolizing system, but, in resting conidia of N. crassa, mutations are induced only in the presence of a metabolizing system (Ong, 1971), suggesting that the vegetative cultures are capable of metabolism of AFB_1 while conidia are not. In Drosophila, AFB_1 was found to be mutagenic in males treated by injection (Lamb and Lilly,

1971).

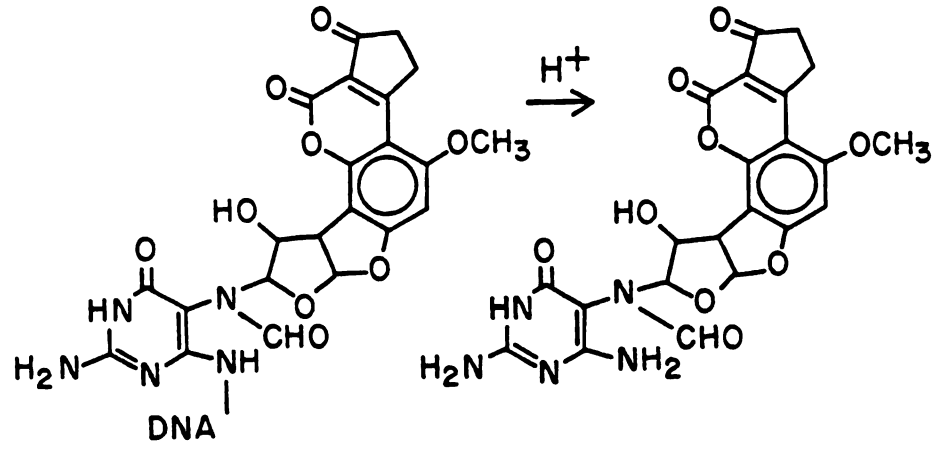
Krahn and Heidelberger (1977) have shown that, in the presence of a rat liver activation system, AFB_1 induced 6-thioguanine-resistant mutants in V79 cells. Langenbach *et al.* (1978), have used a rat liver cell-mediated system to activate AFB_1. In this system, AFB_1 induced ouabain-resistant mutants in V79 Chinese hamster cells. In both these systems, AFB_1 induced lower frequencies of mutations than did benzo(a)pyrene for the same survival level.

AFB_1-induced DNA adducts

In 1977 Essigman *et al.* first isolated the major DNA adduct formed by AFB_1 *in vitro*. In the presence of rat liver microsomes, AFB_1 bound covalently to calf thymus DNA, and on mild acid hydrolysis, yielded a guanine adduct. By means of high pressure liquid chromatography (HPLC), this adduct was isolated and was then characterized by spectral and chemical means. The major adduct was identified as 2,3-dihydro-2-(N^7-guanyl)-3-hydroxyaflatoxin B_1 (AFB_1-N-7-guanine) (Figure 3). This DNA adduct comprised 90% of the total carcinogen bound to DNA, and was formed by reaction of the C-2 atom of AFB_1 with the N-7 atom of guanine. At that time, Essigman noted that this N-7 adduct resembled that of the 7-alkyl-guanines in charge and proposed that apurinic sites might be formed spontaneously in



AFB_1-N-7-guanine



AFB_1-N-7-guanine
(ring-opened form)

Figure 3. Structures of the major AFB_1 DNA adducts and their hydrolysis products

AFB_1-modified DNA, as is seen in alkylated DNA. Later studies by Martin and Garner (1977) revealed that when calf thymus DNA was reacted with AFB_1 in the presence of rat liver microsomes, there were four DNA adducts formed. All of these adducts co-chromatographed with adducts formed with poly dG in the same system. Of the four guanine peaks, there were two major products, both of which were formed through N-7 guanine substitution. D'Andrea and Haseltine (1978) showed that when AFB_1 was reacted with E. coli DNA in the presence of rat liver microsomes, it resulted in alkali-labile sites in the DNA. This data lent support to the earlier suggestion by Essigman that the N-7 adduct might spontaneously depurinate as a result of labilization of the N-glycosylic linkage at the N-7 atom of guanine. The report by D'Andrea and Haseltine also revealed the formation of an adenine adduct in the system they used.

When AFB_1-DNA adducts were isolated by Autrup et al. (1979) from cultured human bronchus exposed to AFB_1, four distinct products were seen. The two major adducts observed were identical to the two major adducts previously reported to be formed in vitro. In the human bronchus system, the two major adducts comprised 70% of the total bound carcinogen, and the ratio of the two products to one another was approximately 1. The second major guanine peak was tentatively identified as 2,3-dihydro-2-(N^5-formyl-2',5',6'-triamino-4'-oxo-N^5-pyrimidyl)-3-hydroxyaflatoxin

B_1 (Figure 3). This adduct is formed from the N-7 guanine adduct as a result of fission of the imidazole ring of guanine. Therefore, it is a ring opened form of the N-7 guanine adduct. The adduct studies of Autrup et al. (1979) in cultured human bronchus revealed that the AFB_1-DNA adducts formed in vitro were qualitatively similar, but differed quantitatively from the AFB_1-DNA adducts found in the in vitro systems previously studied.

Wang and Cerutti (1979) compared the loss of AFB_1-DNA adducts from the DNA of epithelioid human tumor cells (A549). The kinetics of loss of AFB_1 adducts was monitored from the DNA of intact cells and from free DNA which had been isolated from A549 cells immediately after treatment and incubated in physiological conditions over five days. Their results showed a greater loss of AFB_1-DNA adducts from whole cells than from DNA in vitro. They also showed that the percentage of the total adducts which the ring-opened N-7 guanine product comprised increased with time. They suggested that because of its persistence, this adduct might play an important role in the induction of mutations and cancers by AFB_1. Hertzog et al. (1980) studied the kinetics of loss of DNA-bound AFB_1 in rat liver and in vitro. Following intraperitoneal administration of AFB_1 to male rats, the AFB_1-DNA adducts were monitored over 48 hours in rat liver DNA in vivo, and

from rat liver DNA *in vitro*, isolated from rat liver two hours after treatment of the rats with AFB_1. Their studies showed that the half-life of the N-7 guanine adduct, both *in vivo* and *in vitro*, was approximately 24 hours. Based on these data, the authors concluded that a major portion of AFB_1-DNA adducts lost *in vivo* are lost non-enzymically. These findings are in contrast to those reported earlier by Wang and Cerutti (1979). The differences in these reports is probably attributable to differences in the pH of the *in vitro* incubation mixtures. The studies of Hertzog *et al.* (1980) also showed that the ring-opened guanine derivative of AFB_1 was a more persistent lesion than the N-7 guanine adduct *in vivo* and *in vitro*. A recent report by Groopman *et al.* (1981) on *in vitro* reactions of AFB_1 with DNA support the suggestion put forth by Hertzog, that a major portion of AFB_1-DNA adducts lost *in vivo* are lost by chemical rather than by enzymic means. In this study AFB_1 was reacted with calf thymus DNA in the presence of a rat liver activation system. The loss of the major N-7 guanine adduct from DNA was monitored for up to 48 hours under different incubation conditions. They found that the kinetics of loss of the N-7 guanine adduct varied, depending on the pH of the mixture and the level of DNA modification. However, under all conditions studied, spontaneous depurination was observed.

Evidence of repair of the cytotoxic and mutagenic DNA lesions induced by AFB_1

There is strong evidence for the involvement of DNA repair enzymes in removing AFB_1-induced DNA damage in several systems. AFB_1 induced toxicity in *E. coli* in the presence of a rat liver fraction (Garner and Wright, 1973). However, the degree of toxicity varied depending on the genetic markers carried. Mutants deficient in the excision repair pathway (uvr^-) were more sensitive to AFB_1 than wild type strains, but were less sensitive than mutants deficient in recombination repair (recA). The double mutant (uvr^-, recA) was the most sensitive. This suggests that both these repair pathways are involved in repairing AFB_1-induced cytotoxic damage in *E. coli*, but that excision repair appears to be more necessary than recombination repair in surviving this type of damage. Similarly, in the presence of a rat liver system, AFB_1 has been shown to induce toxicity in strains of *Salmonella typhimurium* which have a deletion in the genes coding for repair of UV-light-induced damage (Garner *et al.*,1972). In strains of *Salmonella typhimurium* without this deletion, no toxicity was observed. When AFB_1 was activated by rat liver microsomes in the presence of human cells in culture, repair synthesis was induced in normal cells, but not in repair-deficient XP cells (Stich and Laishes, 1975; Sarasin *et al.*, 1977). In the presence of rat liver microsomes,

AFB_1 has also been shown to induce greater frequencies of mutations in strains of Salmonella typhimurium deficient in excision repair than in wild type strains (Garner and Wright, 1973). All this evidence implicates the involvement of DNA lesions in AFB_1-induced toxicity and mutations.

Possible mechanisms of mutagenesis by AFB_1

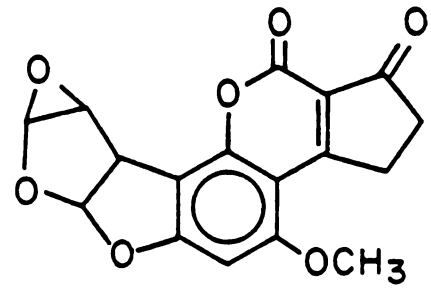
There have been several mechanisms proposed to explain how AFB_1-induced DNA lesions might induce mutations. One of these involves the major adduct which is formed as a result of binding between the C-2 of AFB_1 and N^7 of guanine. This proposed mechanism suggests that the 3-hydroxy group of AFB_1 can hydrogen bond with the oxygen at C-6 of guanine and disrupt normal base pairing, resulting in a point mutation. However, when AFB_1-Cl_2 binds DNA, the 3-chloro group is retained and thus could not undergo similar hydrogen bonding. Since AFB_1-Cl_2 is as potent a mutagen as AFB_1 when normalized for amount bound to DNA, this mechanism seems unlikely (Swenson et al., 1977). Another possible mechanism is that the large group introduced into DNA by AFB_1 causes steric hindrance of the polymerase. This might result in a failure to replicate a section of DNA which would lead to a deletion mutation. It must be remembered that minor unidentified DNA adducts, rather than the major adducts, may be responsible

for producing the biological consequences of AFB_1. For alkylating agents, it appears that minor adducts (for example, 0-6 alkyl guanine) are important in the induction of mutations in mammalian cells, rather than more predominant lesions (e.g. ethylated phosphotriesters for ENU and 0-6 methyl guanine for MNU (Newbold et al.,1980; Peterson et al.,1979).

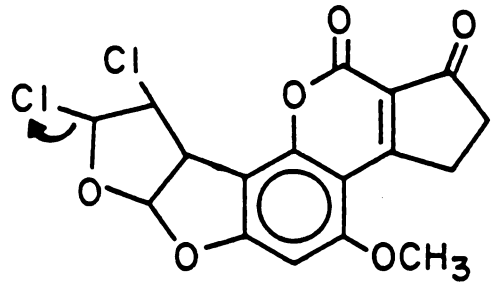
AFB_1-Cl_2 as a model compound for the ultimate metabolite AFB_1-2,3-oxide

As stated above, attempts to isolate the reactive metabolite, AFB_1-2,3-oxide, or to synthesize it were unsuccessful, probably because of its highly reactive nature (Swenson et al.,1973, 1975, 1977). AFB_1-Cl_2 was therefore synthesized by Swenson et al. (1977) as a model compound for the 2,3-oxide (Figure 4). They showed that AFB_1-Cl_2 is similar in chemical properties to AFB_1-2,3-oxide. It has an electrophilic carbon 2 and binds covalently to DNA and RNA at this position, as does AFB_1-2,3-oxide. It reacts predominantly with guanine. Its hydrolysis products react only to a very small extent with nucleic acids.

AFB_1-Cl_2 is a direct-acting mutagen and carcinogen. It causes tumors at the site of injection in rats and induces skin papillomas in mice, whereas AFB_1 does not



Aflatoxin B_1-2,3-oxide



Aflatoxin B_1- dichloride

Figure 4. Structures of AFB_1-dichloride and AFB_1-2,3-oxide

(Swenson et al., 1977). It is mutagenic in Salmonella typhimurium, and when the mutagenicities of the dichloride and AFB_1 were compared in Drosophila melanogaster (Fahmy et al., 1978), it was observed that the two compounds induced the same mutational spectra, but with higher activity observed for the dichloride. This would be expected since the reactive species would be generated more readily from the dichloride than from AFB_1, which must first be metabolized. These studies did not include comparisons of levels of DNA binding for the two carcinogens. We would expect that the greater mutagenic activity observed for AFB_1-Cl_2 was the result of a greater level of DNA binding for this compound in the target cells.

The general toxicities induced in Drosophila by AFB_1 and AFB_1-Cl_2 were virtually identical. That is, for males treated by injection, the LD_{50} levels were reached at the same concentration for both chemicals. However, when the cytotoxicity of AFB_1 and the dichloride was assayed in cells at various stages in the germ cell line, there were marked differences observed. In all cases, AFB_1-Cl_2 induced greater cytotoxicity in these cells at concentrations equal to AFB_1. The difference in the cytotoxicities induced was the greatest for the mature sperm. The difference was less for metabolically active cells in the earlier stages of spermatogenesis. This suggests that the lower levels of toxicity by AFB_1 seen

in these cells is the result of the extent of metabolic activation taking place.

Somatic Cell Mutation Theory of Cancer

Our interest in aflatoxin was based on the evidence for its nature as a powerful carcinogen. The somatic cell mutation theory of the origin of cancer argues that cancers are caused as a result of somatic cell mutations. We, therefore, chose to study the induction of mutations by aflatoxin B_1-dichloride in human cells in culture in an attempt to further our understanding of the mechanism(s) by which chemicals induce mutations, and possibly cancers, in human cells.

The somatic cell mutation theory of the origin of cancer was first proposed by Boveri in 1914 in an attempt to explain the observation that the cancer cell phenotype appeared to be inherited, that is, daughter cells retained the transformed phenotype of their parent cancer cells. At the time that this theory was proposed, DNA was not yet understood to be the genetic material. In its present form, the somatic cell mutation theory states that cancer is a result of mutational events in somatic cells and, therefore, that DNA is the target macromolecule for most chemical carcinogens and carcinogenic radiations. This

theory was unsupported for many years by a lack of correlation between carcinogenesis and mutagenesis. As a result, there were other theories put forth to explain the mechanism of chemical carcinogenesis. For example, Miller et al. (1963) proposed that protein was the target macromolecule for chemical carcinogens when they found that some polycyclic aromatic hydrocarbons showed preferential binding to a particular protein fraction. A similar hypothesis was held by Heidelberger (1964). In an attempt to explain the heritable nature of the change resulting from loss of a protein from a cell, Pitot and Heidelberger (1963) proposed that reaction of a carcinogen with a growth-controlling protein resulted in inactivation of this protein, and they employed the Jacob-Monod model of gene repression to explain how a protein deletion could result in a heritable change in the cell. However, when Brookes and Lawley (1964) demonstrated a positive correlation between binding of polycyclic aromatic hydrocarbons to mouse skin DNA and their carcinogenic potency, it revived interest in the somatic cell mutation theory, with particular emphasis on DNA as the target macromolecule. Further interest in this theory was generated as a result of the work of Miller and Miller (1968) who demonstrated the requirement for many chemical carcinogens to be metabolically activated before they can exert their biological effects. Since the mutation assays used until this time did

not include means to metabolically activate the carcinogens, the failure to find a correlation between mutagenicity and carcinogenicity may be related to metabolic activation. This realization spurred development of activation systems suitable for use with mutational assays. Since this development correlations between mutagenicity and carcinogenicity as high as 90% have been found (McCann et al., 1975).

Evidence in support of the somatic cell mutation theory

a) The majority of carcinogens tested have proved to be mutagens

If cancers arise as a result of mutational events, then carcinogens should prove to be mutagens in one or more test system. Furthermore, there should be a correlation between the carcinogenic potency and the mutagenic potency of carcinogens. In support of this, approximately 90% of the chemical carcinogens tested to date in the Ames' Salmonella assay have been shown to be mutagens (McCann et al., 1975), although strong carcinogens have not always been found to be strong mutagens and strong mutagens are not always strong carcinogens. This is not surprising, since mutagenesis test systems utilize a different activation system than carcinogenesis test systems, and therefore, differences could be expected. Furthermore, carcinogenesis

tests involve animal studies, whereas mutagenesis tests usually involve bacteria, fungi or cell cultures. Considering the enormous biological differences between these test systems, it is not surprising to find that the correlation between mutagenicity and carcinogenicity is not perfect.

b) Clonal nature of tumors

There is considerable evidence that human tumors arise as a clone from a single cell of origin. This evidence includes cytogenetic studies which show the same abnormal karyotype for all cells derived from a single tumor (Sandberg and Hossfield, 1970) and studies on the level of glucose-6-phosphate dehydrogenase, which showed that cells derived from the same tumor all had the same levels of this enzyme (Linder and Gartler, 1965).

c) DNA excision repair-deficient xeroderma pigmentosum patients

1. Characterization

Perhaps the best evidence in support of the somatic cell mutation theory of cancer is the clinical and experimental data available on patients with xeroderma pigmentosum.

Xeroderma pigmentosum (XP) is a human syndrome, inherited as an autosomal recessive trait, which is characterized primarily by an increased sensitivity of the skin to sunlight, causing patients to develop abnormal pigmentation and skin tumors in sun-exposed areas at a very young age (Robbins et al., 1974). Mental retardation, areflexia, and other neurological disorders can also be associated with the disease (Robbins et al., 1974). Affected individuals develop sunlight-induced skin cancer with a prevalence of approximately 100%-compared to an annual rate in the U.S. of 1 to 2 per 10^3 for the general population (Setlow,1978). This frequency has been markedly reduced in patients protected from sunlight from an early age (Robbins et al.,1974).

In 1968 Cleaver observed that, compared to skin fibroblasts derived from normal persons, fibroblasts derived from XP patients showed decreased ability to carry out excision repair of ultraviolet (UV) radiation-induced DNA lesions. It is now recognized that there are at least two different modes of excision repair operating in mammalian cells. One is designated nucleotide excision repair because the damaged base residue is excised within an oligonucleotide by two sequential single strand nicks. The first nick is introduced by an endonuclease and the second by an exonuclease. The correct nucleotides are inserted by a DNA polymerase, and the ends are sealed by a polynucleotide

ligase. (Grossman, 1979). The second form of excision repair is called base excision repair. In this mode a modified base is released as a free base by a DNA glycosylase or leaves spontaneously, resulting in an apurinic/apyrimidinic (AP) site. The AP site is then removed in the same way as described above for nucleotide excision repair, with an AP-specific endonuclease and exonuclease responsible for cleaving the damaged oligonucleotide. A polymerase and ligase act to synthesize and join the new DNA segment as described. There is evidence of a third method of removing damage from DNA, but it cannot be considered excision repair because the DNA remains intact. This mode of repair results in the insertion of a free purine residue (Chetsanga and Lindahl, 1979). Until 1980, excision repair of UV-induced DNA damage had been considered to be a classical example of nucleotide excision. However, Haseltine et al. (1980) found a dimer-specific endonuclease in M. luteus with two enzymic activities. A pyrimidine dimer-specific DNA glycosylase cleaves the glycosylic bond between the one thymine base and its sugar, leaving an AP site, but the thymine dimer is still intact. An AP endonuclease cleaves the DNA adjacent to the AP site, and exonuclease cleaves the oligonucleotide containing the AP site from the DNA. A polymerase and ligase act in the same manner as described for nucleotide excision.

When Cleaver tested the XP cells for their ability to

carry on excision repair following exposure to UV light, he measured the extent of repair replication using ^{3}H-BUdR. By use of buoyant density gradients, density-labeled, newly synthesized DNA was separated from DNA in which repair had resulted in insertion of ^{3}H-BUdR in small patches. The XP cells proved to be very deficient in this process. These results suggested that the deficiency of XP cells in this DNA repair pathway was, at least in part, responsible for their increased incidence of sunlight-induced skin cancers. Cleaver further suggested that the mechanism by which this DNA repair defect resulted in tumors might be by causing enhanced frequencies of somatic mutations as a result of unrepaired DNA damage.

XP strains that are deficient in excision repair have been subdivided into groups based on complementation analysis by means of cell fusion (Kraemer et al., 1975). If, upon cell fusion of two strains, the heterokaryon exhibited normal levels of repair synthesis, the cells were classified as being in separate complementation groups. If two strains showed low levels of repair synthesis upon fusion, they were categorized as being in the same complementation group. Seven complementation groups have been identified to date (Kraemer et al., 1975 ; Takebe, 1978). Therefore, mutations in at least seven different loci can give rise to the XP phenotype. It is not known whether these seven loci represent seven distinct cistrons. There

are different rates of excision repair observed among the different complementation groups.

Although there are variations in the levels of excision repair seen in the seven complementation groups of XP cells, all excision repair deficient XP strains appear to have a defect in the incision step. The introduction of T4 endonuclease into these XP cells restores normal repair replication (Tanaka et al., 1977). Cell extracts from groups A, C, and D are capable of excising dimers from UV-irradiated naked DNA (Mortelmans et al., 1976), but group A cells are unable to remove dimers from UV-damaged chromatin, suggesting that the defect may be in the recognition of the damage, or in a cofactor involved in the repair of the chromatin.

2. Evidence that DNA excision repair protects cells from the potentially harmful effects of carcinogens

A large variety of physical and chemical agents induce DNA damage in human cells. The majority of agents studied appear to be handled by base excision or nucleotide excision. Regan and Setlow (1974) have divided a series of carcinogens into two groups, according to the size of the excised oligonucleotides. One group causes large oligonucleotides to be excised resulting in long or "UV-like" repair.

This group included the bulky chemicals N-acetoxy-2-acetylaminofluorene and 4-nitroquinoline-oxide. The other group of agents induces short or "X-ray-like" repair.

There is evidence that excision repair in human cells can reduce the potentially cytotoxic and mutagenic effects of carcinogenic agents, including UV-light and chemicals. When excision repair-proficient normal human fibroblasts and excision repair-deficient fibroblasts derived from several different XP patients were exposed to equal doses of UV light, the XP cells exhibited a higher frequency of induced mutations than did the normal cells (Maher et al, 1976). More importantly, when a series of XP-derived skin fibroblasts, representing different complementation groups, was tested for their sensitivity to the mutagenic effects of UV light, cells with the lowest rate of excision repair (XP12BE with less than 1% of normal) showed the greatest sensitivity to the mutagenic effects of UV. Other XP cells tested showed a positive correlation between the rate of excision repair and level of resistance to UV-induced mutagenicity (Maher et al.,1979). XP cells have also been shown to be more sensitive than normal cells to the mutagenic effects of several polycyclic aromatic hydrocarbons and aromatic amides (Maher et al., 1976 and Heflich et al., unpublished). When UV-irradiated normal and XP cells were held in a non-dividing state, in order to allow time for excision repair, normal cells showed a

decrease in mutation frequency over time. XP12BE cells showed no decrease, and XP2BE cells, with intermediate repair, showed an intermediate rate of decrease over time. Normal cells have also shown the ability to recover from the potentially mutagenic effects of some aromatic amides (Heflich et al., 1980) and from BPDE (Yang et al., 1980). XP12BE cells did not. These data suggest that excision repair processes act to reduce the mutagenic effects of UV light and these chemical carcinogens.

Although the majority of investigators used UV radiation of wavelength 254 nm, Maher and McCormick and coworkers have observed similar effects with simulated sunlight (unpublished studies). Furthermore, Trosko et al. (1970) have shown that, when human cells in culture were exposed to two hours of midsummer sunlight in mid-afternoon, there was 0.07% thymine dimer formation. Thymine is the major lesion induced by 254 nm light. Therefore, studies using 254 nm are indicative of the kinds of biological effects occurring in the skin of persons exposed to sunlight. Takebe et al. (1977) have shown a direct correlation between the extent of excision repair capacity and the age at which XP patients suffer the onset of skin cancers. They studied 50 XP patients and found that all those who developed cancers before the age of eight were from the group which exhibited the lowest levels of excision repair. Only one patient from this group reached the age of

12 without developing skin tumors. In contrast, patients with intermediate levels of excision repair did not, on average, develop tumors until after the age of 17. These data suggest that susceptibility to sunlight-induced skin cancers is dependent on the excision repair capacity of the cells. The validity of this conclusion depends on the assumption that all patients had equal sunlight exposure. Taken together, these data suggest that the greatly elevated frequency of sunlight-induced skin carcinomas in XP patients is a consequence of elevated frequencies of sunlight-induced mutations in the target cells. The evidence suggests that the increased frequencies of mutations and cancer are a direct result of the reduced ability of XP cells to repair the sunlight-induced DNA damage compared to normal cells.

d) A second form of xeroderma pigmentosum disease, XP variants

There is one group of XP patients, referred to as XP variants, who exhibit the same clinical manifestations as the classical XP patients, but who have approximately normal rates of excision repair following UV-irradiation (Cleaver, 1972). These cells appear to have a defect in cellular processes involved in replicating DNA using a template containing unexcised lesions (Park and Cleaver, 1979). The results of Park and Cleaver indicated that in

the first few hours after irradiation, XP variant cells synthesize smaller fragments of DNA than do normal cells. However, the DNA increased in size at the same rate in normal and variant cells. They concluded that UV-damaged sites interrupt DNA replication in variant cells more often than in normal cells. When Maher et al. (1976) investigated the frequencies of 8-azaguanine-resistant mutants induced by ultraviolet light, they found that higher frequencies of mutations were induced in XP variant-derived fibroblasts than in normal cells at equal doses of UV-light. When mutation frequencies per surviving cell induced by UV-light were plotted against the cytotoxic effect of the exposure, the XP variant cells exhibited a higher number of mutations per lethal event than did the normal cells or excision repair-deficient XP cells. These results suggest that XP variant cells employ an abnormally error-prone mechanism to deal with unexcised DNA lesions. The fact that cancer-prone XP variants have a defect which differs from that of classical XP patients, and yet which results in XP variant-derived cells being hypermutable by UV light is in support of the somatic cell mutation theory of the origin of cancer.

e) Bloom's syndrome

Another human syndrome which exhibits a high frequency of cancer is Bloom's syndrome (BS). These patients show a

marked increase in frequency of leukemias and lymphomas over the normal population (Bloom, 1966). Fibroblasts derived from BS patients exhibit increased spontaneous levels of sister chromatid exchanges and chromosome instability (Chaganti et al., 1974), including breakage and rearrangements. It has recently been reported by Warren et al. (1981) that fibroblasts derived from two BS patients exhibited increased rates of spontaneous mutation to 6-thioguanine-resistance, when compared to cells from normal human donors. In these studies, untreated skin fibroblasts from both normal and BS patients were assayed for background levels of $HPRT^-$ mutants after 15 days of growth in non-selective medium. These data also support the somatic mutation theory of the origin of cancer because they show a correlation between a high incidence of somatic cell mutations and the elevated frequency of cancers observed in Bloom syndrome patients. The mechanism of the hypermutability in these cells is unknown.

Evidence for other mechanisms of carcinogenesis

a) Teratocarcinomas

The most impressive evidence which argues against the somatic mutation theory of cancer is work on mouse teratocarcinomas, which are malignant epithelial tumors which can be induced to arise with high frequency when inbred mouse

embryos are transplated to an extra-uterine site. Mintz and Illmensee (1975) found that when cells from a mouse teratocarcinoma were injected into normal mouse blastulas, normal mice developed containing tumor-free tissues derived from the teratocarcinoma. Upon autopsy, analysis of tissue samples from these embryos revealed teratocarcinoma-derived cells in all of its tissues. This evidence challenges the concept implied by the somatic cell mutation theory that malignant transformation is irreversible. In contrast, it suggests that some tumor cells can be restored to normal behavior. These data argue in favor of an epigenetic mechanism of carcinogenesis. However, it is possible that teratocarcinomas arise in response to stimuli from the foreign environment of the kidney capsule (where mouse blastocyst cells gave rise to these tumors) and when such apparently induced tumor cells are returned to a normal environment, i.e. the blastula, they then receive normal stimuli and develop normally (Martin, 1980).

b) Studies with plastic film

When sheets of plastic film were inserted into the tissues of rats, tumors were induced (Alexander et al., 1958). However, when perforations were made in the film, or when the film was ground and injected into the animals, no tumors were induced. It is difficult to explain these results in terms of the somatic cell mutation theory, since,

if the intact film caused mutations, e.g. by introducing a mutagenic chemical, the same chemical should have been contained on the perforated or ground plastic and these should also have caused tumors. These data suggest that tumors were induced by interruption of cell-cell communication in rat tissues as a result of the plastic film. One possible mechanism is that initiated cells are suppressed in some way by neighboring cells, and that when cell-cell communication is interrupted, these initiated cells are allowed to be expressed.

c) Arguments against the XP story

Although the fact that cells from persons with some cancer prone syndromes are deficient in excision repair and hypermutable by several carcinogens provides the best support for the somatic mutation theory of cancer, questions regarding the extrapolation of these data to the mechanism of cancer *in vivo* have been raised. Cairns argued recently (1981) that if the increased incidence of skin tumors in XP patients is due to mutations induced by exposure to sunlight, as assumed by the somatic cell mutation theory, and if XP cells are also hypermutable by other environmental carcinogens, these patients should show an increased risk of developing internal cancers as a result of exposure to environmental chemicals. In support

of this, Kraemer has reported at least thirteen XP patients with primary non-skin, non-eye neoplasms (1978). These include several neoplasms of the oral cavity (which may be the result of sunlight exposure), two primary brain tumors, a single testicular sarcoma, breast carcinoma, and a benign thyroid tumor. Cairns (1981) has argued that the incidence of internal cancers should be much higher than this based on the fact that several groups have shown that XP cells are deficient in the rate of excision repair of lesions induced by certain chemical carcinogens (Maher et al., 1976; Heflich et al.,1980). However, in discussing the question, Cairns assumes that cells from XP patients are equally as deficient in rate of repair of such lesions as they are in removing UV-induced damage. The data does not support this view. For example, studies with polycyclic aromatic hydrocarbon derivatives (Maher et al., 1976) indicate that the differential in rate of repair of these lesions between normal and XP cells is three to six-fold. For repair of UV-induced DNA damage, the differential is ten to fifty-fold. Therefore, one would not expect environmental mutagens to be present at high enough levels to induce significant numbers of mutations even in the excision repair-deficient XP cells. Furthermore, there is no question but that exposure of the population to sunlight is greater than to chemical carcinogens in the environment because the annual incidence of new cases of sunlight-induced skin cancers in the general population is much

higher than of any other form of cancer (Braun, 1977).

Nevertheless, the fact that skin cancer is common in patients given strong immuno-suppressive therapy (Kinlen, 1979) suggests that there are potentially malignant cells present in the cells of normal patients which are allowed to express only when their immune system is repressed. Dupuy and Lafforet (1964) have published evidence that XP patients exhibit a defect in cell-mediated immunity, determined by their inability to develop sensitization to dinitrochlorobenzene. This evidence suggests that the increased incidence of skin tumors observed in XP patients may, in part, be due to an immune deficiency. Thus, there may be more involved in the hypersensitivity of XP patients to developing skin tumors than merely increased sensitivity to somatic cell mutations caused by deficient rates of DNA repair.

Taken together, these three examples suggest that the evidence available today, which argues in favor of the involvement of somatic cell mutations in the initiation of tumors, must always be tempered by that which cannot be explained by this theory. They remind one that the process of neoplastic transformation is very complex, and that in all likelihood there is more than one possible mechanism by which normal cells can become malignant.

Concluding remarks

Aflatoxin B_1 is a very potent carcinogen in several mammalian species, and has been indicated in epidemiological studies as a possible hepatocarcinogen in man. The mechanisms responsible for cancer induction are not fully understood, but the theory which best correlates the facts known is the somatic cell mutation theory. We, therefore, attempted to study the induction of mutations by AFB_1 in human cells, in an effort to further our understanding of the mechanism(s) by which chemicals induce mutations, and possibly cancers, in human cells. The availability of excision repair-deficient strains of human cells (XP-derived) provides a system in which the role of DNA repair in the induction of cytotoxicity and mutations in human cells can be studied. This system also provides a means by which the DNA adducts responsible for the biological effects of chemical carcinogens in human cells can be studied. In this study, we have attempted to study the biological effects of AFB_1 and AFB_1-Cl_2 in human cells in culture, in particular, the cytotoxicity and mutagenicity of these carcinogens, the effect of DNA repair on these biological effects, and the nature of the DNA adducts responsible for these biological consequences.

MATERIALS AND METHODS

Chemicals

AFB_1 was purchased from Calbiochem, Los Angeles, Ca. Unlabeled and generally tritiated AFB_1-Cl_2 (0.78 Ci/mmole) were a gift from Dr. David Swenson, the Upjohn Company, Kalamazoo, MI. Calf thymus DNA, dGMP, dAMP, dTMP, 6-thioguanine, sodium-lauroylsarcosine, T_1 and pancreatic RNases, trichloroacetic acid, and bovine serum albumin were obtained from Sigma Chemical Co, St. Louis, Mo. Glass distilled acetone, methanol, dimethylsulfoxide, chloroform, butanol, and hexane were purchased from Burdick and Jackson Laboratories, Muskegon, MI. Cesium chloride was purchased from Gallard-Schlesinger Chemical Manufacturing Corporation, Carle Place, NY. Creosol was obtained from Matheson, Coleman and Bell, Northwood, OH. Phenol, Folincicolteau reagent, and hydroxyquinolene were obtained from Fischer Scientific Co, Pittsburgh, PA. Trypsin was obtained from Grand Island Biological Co, Grand Island, NY. Fetal calf serum was bought from KC Biologicals, Lenexa, KA. or from Grand Island Biological Co. Ham's F10 medium lacking hypoxanthine, and Eagles' minimal essential medium (MEM) was

obtained from KC Biologicals. Penicillin, streptomycin sulfate, and gentamycin were obtained from Schering Corp., Kenilworth, NJ.

Preparation of Solutions

Phosphate buffered saline (PBS) consisted of 8.0 g NaCl, 0.2 g KCl, 1.5 g Na_2HPO_4, 0.2 g KH_2PO_4 in one liter of distilled water. The solution was brought to a pH of 7.2 with 1 N HCl.

Stock solutions of 6-thioguanine were prepared by dissolving 134 mg of 6-thioguanine in 2 ml 1 N NaOH. 98 ml of distilled water was added, and the solution was then filter sterilized. This yielded an 8.0 mM solution which was used at a 1:200 dilution for a final concentration of 40 μM.

Antibiotics 5 g of streptomycin sulfate and $5x10^6$ units of penicillin were dissolved in 166.7 ml distilled water and then filter sterilized. This stock solution was used at 1:250 dilution to yield a final concentration of 100 units penicillin/ml and 100 μg streptomycin/ml. Stock solutions of gentamycin were prepared by dissolving 1 g in 100 ml distilled water. The solution was then filter sterilized and used at a 1:200 dilution to yield a final concentration of 50 μg/ml.

Versene trypsin was made with 8.0 g NaCl, 0.4 g KCl, 0.58 g $NaHCO_3$, 0.50 g trypsin (1:250 dilution of stock solution obtained from Difco, Detroit, MI.), and 0.50 g versene ethylene diamine tetraacetic acid (EDTA) in one liter of distilled water.

Standard saline citrate (1X SSC) (0.15 M NaCl, 0.015 M Na_3Citrate) consists of 8.8 g NaCl, 4.4 g of Na_3Citrate in one liter of distilled water. It was brought to a pH of 7.0 with 1 N HCl for these studies.

Kirby phenol 11 ml distilled water was added to 100 g distilled phenol. 14 ml M-Creosol was then added and the mixture was heated at 60°C to allow solutions to mix thoroughly. 0.1 g 8-hydroxyquinoline was added and the solution was stored at -20°C.

Trichloroacetic acid (TCA) To make a 100% solution of TCA, 453 g TCA was dissolved in 125 ml distilled water. The volume was brought to 453 ml with distilled water. The solution was then refrigerated. To make 5% or 10%solutions the proper dilutions were made with distilled water.

Preparation of Medium

To make 20 liters of Eagles' MEM, 44 g $NaHCO_3$ was added to two packets of powdered Eagles' medium and the

volume was brought to 20 liters with glass distilled water. Medium was then filter sterilized, and a contamination check was done by incubating several bottles from each batch for one week at 37°C.

To make 20 liters of Ham's F-10, 24 g $NaHCO_3$ and 0.012 g phenol red were added to two packets of powdered Ham's medium, and the volume was brought to 20 liters with distilled water. It was filter sterilized and a contamination check was run as described.

Cell Cultures

Stocks of normal diploid human fibroblasts derived from foreskins were established, cultured and stored until use in liquid nitrogen. Normal fibroblast cultures were prepared from foreskin material. Tissue samples received in test tubes containing serum-free medium supplemented with antibiotics were sliced into small fragments with a sterile scalpel. Individual fragments were then placed into 35-mm glass culture dishes and a few drops of serum were added. After 24 hours, the cells had attached to the culture dish and fresh serum-containing medium was added. Cells derived from skin biopsies taken from XP patients XP 12BE (CRL1223), XP2BE (CRL 1166), XP4BE (CRL 1162), XP7BE (CRL1200) and cells from Lesch-Nyhan patients (CRL 1112) were obtained from the American Type Culture Collection,

Rockville, MD. Cells from patient XP2BI were obtained from Dr. Colin Arlett, Brighton, England. Cells derived from malignant human tumors which developed in various organs of the body and non-malignant human cells (see Table 2 in Results section) were obtained from the Naval Biosciences Laboratory, Naval Supply Center, Oakland, California. All cultures were tested for the presence of mycoplasma a number of times during the experiments, as described below, and determined to be free of contamination.

Storage of Cells

For the purpose of storing cells, a 1:10 mixture of dimethylsulfoxide (DMSO) and freezing medium (Ham's F-10 with 17% fetal calf serum, and 50 µg/ml gentamycin) was prepared. Cells were enzymically detached from the culture vessel with trypsin, suspended in a small volume of this freezing mixture and placed in freezing vials. The vials were transferred to a -80°C freezer in an insulated styrofoam carton, and left there for 1.5 to 2 hours, to allow for cells to undergo freezing at approximately 1°C per minute. Vials were then placed in a liquid nitrogen freezer and stored indefinitely. In order to obtain the greatest recovery and viability of cells when thawing cells, the cells were warmed in a 37°C water bath as quickly as possible and then pipetted directly into a culture flask which contained at least 15 ml of media which

had been pre-warmed and pre-equilibrated in a 37°C incubator, with 5% CO_2, 99% humidity.

Culture Medium

Cells were cultured in a humid atmosphere of 5% CO_2 and air at 37°C. Normal cells were cultured in HAM's F-10 medium lacking hypoxanthine and supplemented with 10-15% fetal calf serum. XP and LN cells were supplemented with 15% fetal calf serum. Transformed cells derived from human tumors were cultured in Eagles' minimal essential medium, supplemented with 10% fetal calf serum. All culture medium contained antibiotics (100 units penicillin/ml and 100 µg streptomycin/ml or 50 µg/ml gentamycin).

Spent Medium

The spent medium used in biological recovery experiments was medium which had been on exponentially growing stock cultures for 48 hours.

Selection Medium

For the selection of 6-thioguanine-resistant mutants, Ham's F-10 medium, supplemented with 10% fetal calf serum and 40 µM thioguanine, was used. 40 µM was chosen because it was shown to prevent normal cells from doubling (see

Figure 5) and to yield the frequency of resistant cells shown in Figure 6.

Testing of Serum

Because batches of fetal calf serum differ significantly, it was necessary to test each new lot for ability to support clonal growth and growth at high density. It was also necessary to test serum to insure that it was not so rich in purines that it competed with the thioguanine in the selection medium and permited non-resistant cells to survive.

Testing serum for ability to support cloning involved plating untreated cells (normal and/or XP) into 60-mm-diameter dishes with medium containing the serum to be tested and dishes with medium containing control serum, whose ability to support cloning was already known. Cells were plated into these dishes at cloning density (100-300/dish), fed, stained, and scored as described (see in situ cytotoxicity assay). The cloning efficiency of cells grown with the test serum was compared with that of the control serum and a determination was made as to whether the test serum is adequate to support cloning.

In order to test serum for growth at high density, cells were plated into a series 60-mm-diameter dishes with

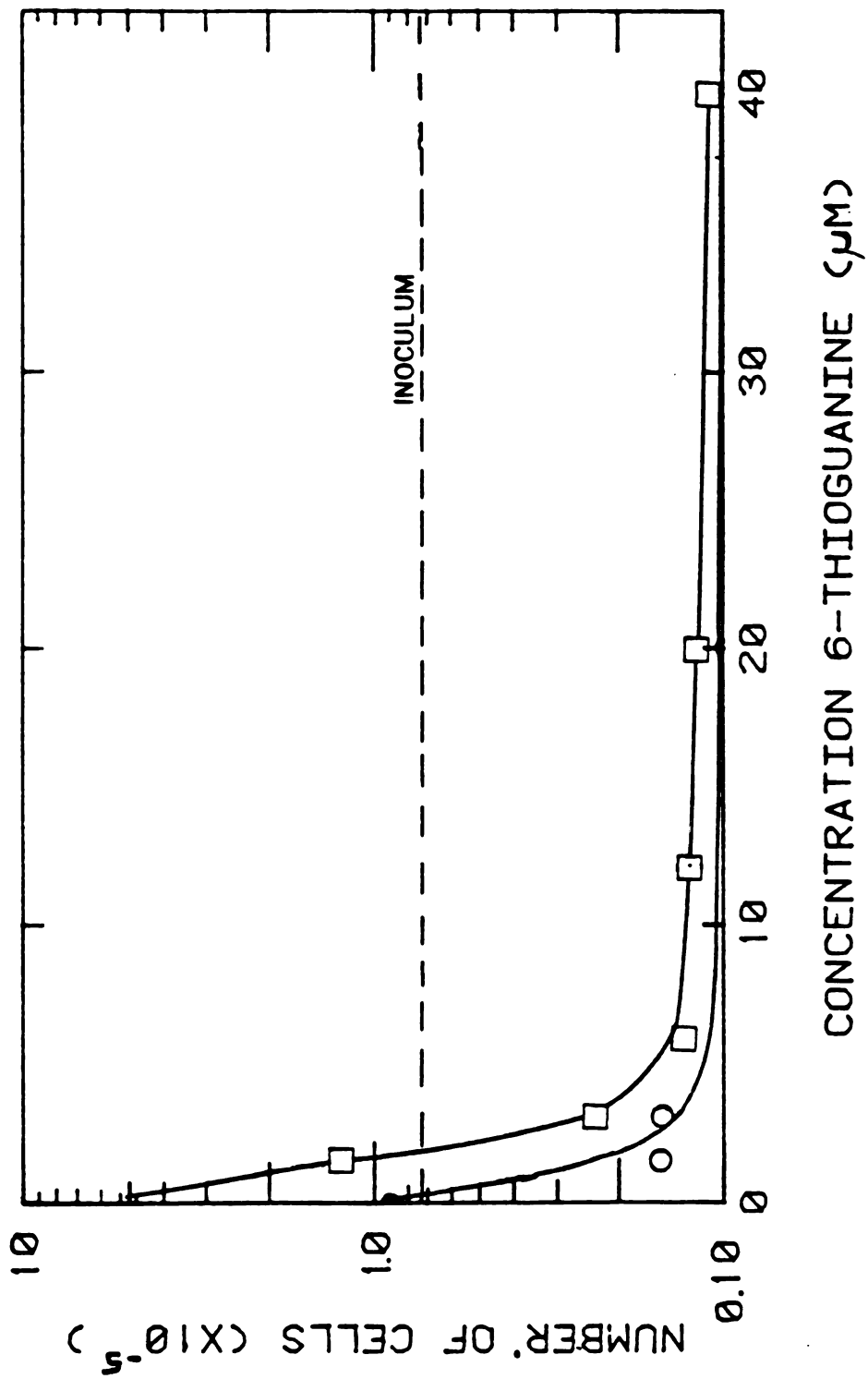


Figure 5. Serum test: growth of cells in thioguanine after seven days. Inoculum was 75,000 cells plated on day 0. (○) test serum; (□) approved serum

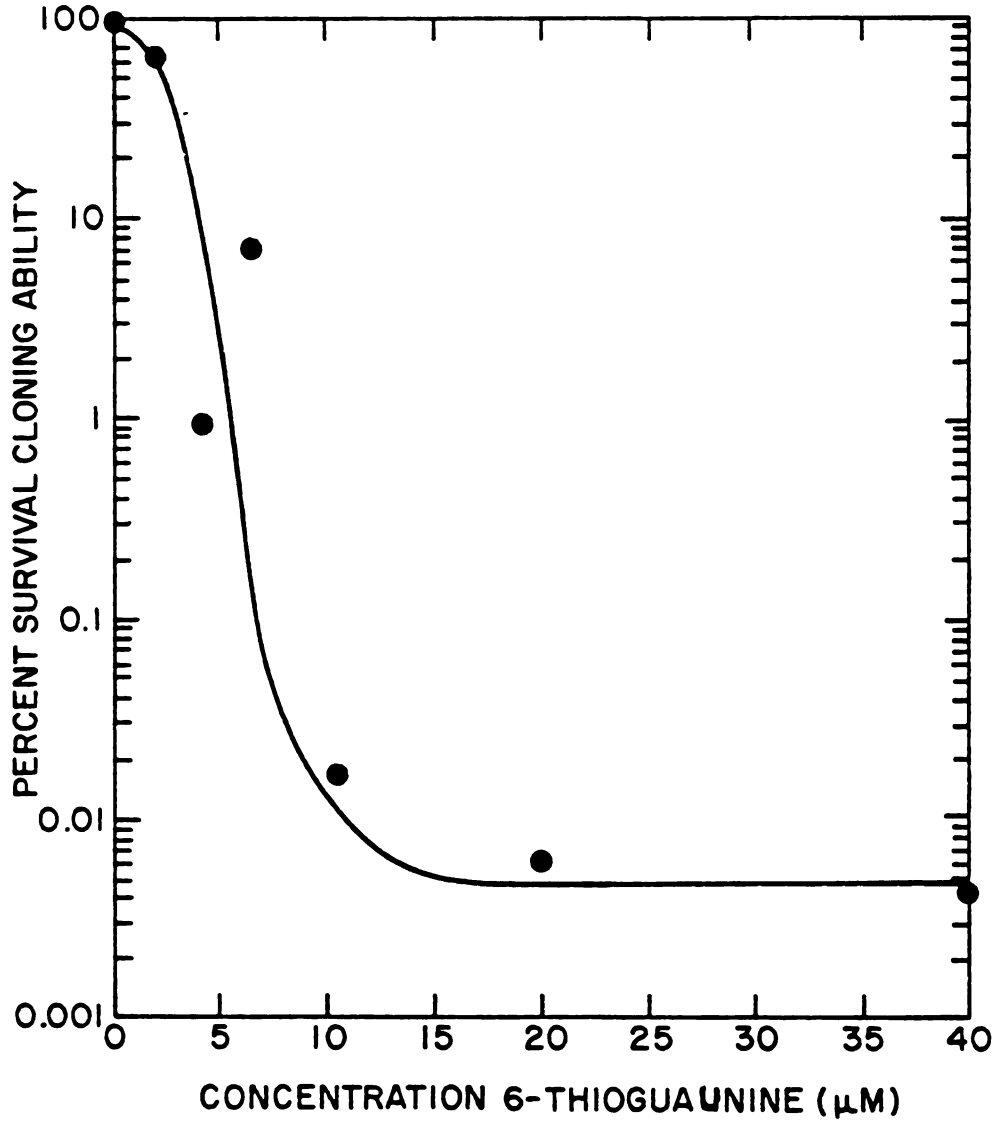


Figure 6. Serum test: colony-forming ability in thioguanine

medium containing test serum or control serum, at a density of 20,000/dish. Cells were allowed to grow and their density monitored over 14 days by means of an electronic counter (Coulter) (Figure 7). By comparing the density the cells reached in the test serum with the density they reached in the control serum, it was determined whether the new serum was adequate to support growth at high density. The ability of cells to clone well and to grow to high density are two factors which determine whether cultures are healthy. It is important to use healthy cultures in these experiments to insure good cloning efficiency for the most reliable results.

Before a new lot of serum could be used for mutation experiments, it had first to be tested to see whether growth of non-resistant cells was inhibited by thioguanine. Cells were plated into 60-mm-diameter dishes at a density of 40,000/dish. Thioguanine was added to the dishes at various concentrations (ranging from 0-40 μM) and cell numbers monitored for seven days. Figure 6 shows a typical curve obtained in this test. Cell numbers decreased with increasing thioguanine concentrations until they leveled off near 10 μM. If the serum contained high levels of purines, these would compete with the thioguanine for incorporation into nucleic acids and allow cells to grow at higher thioguanine concentrations. This would not be suitable to use for mutation experiments.

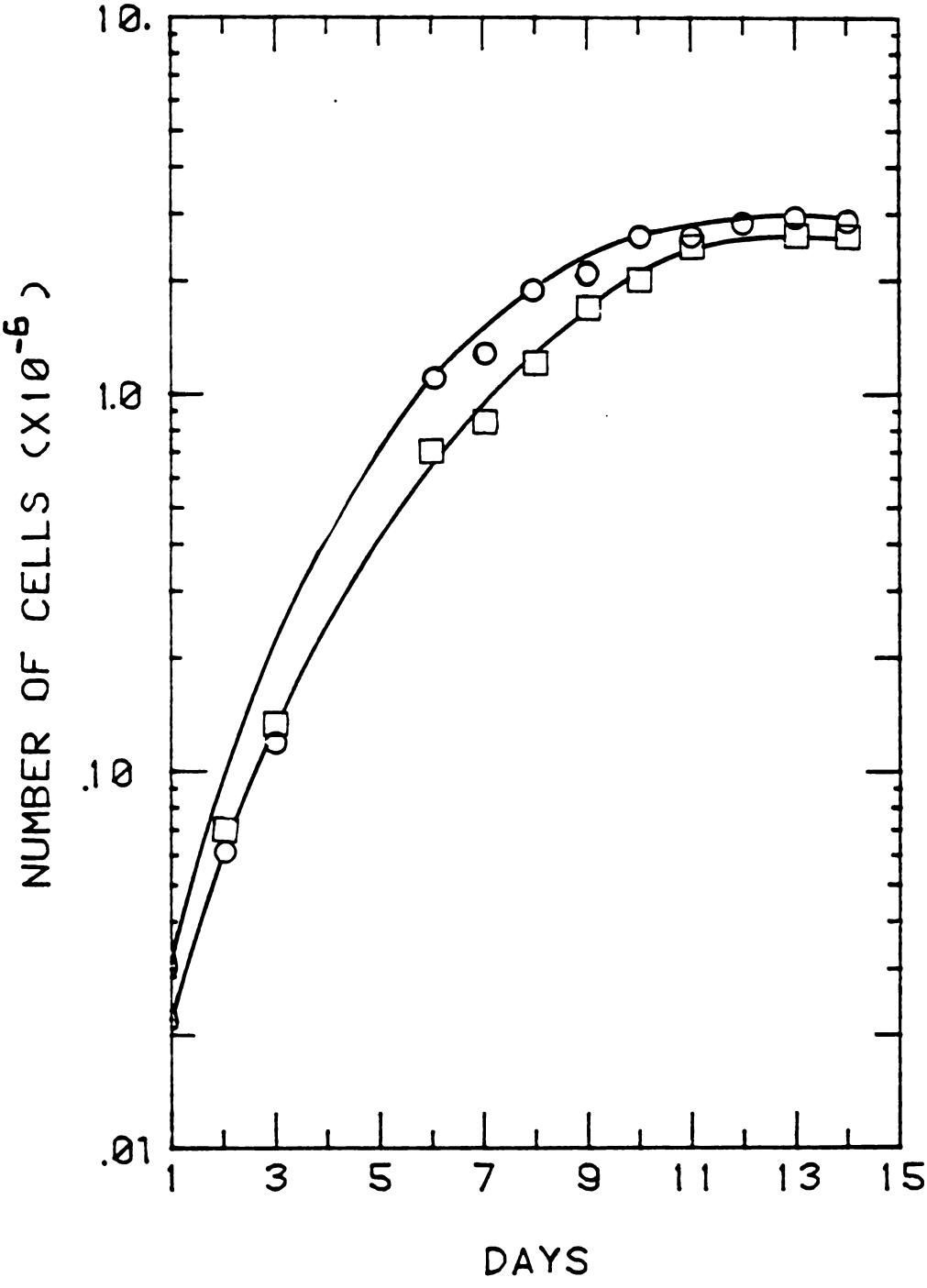


Figure 7. Serum test: growth of cells to high density. (○) NFSL18; (□) NF812

Serum was also tested to determine whether it would yield a low enough frequency of thioguanine-resistant mutants from an untreated population to make it acceptable for selection of induced 6-thioguanine-resistant mutants (see Figure 6). Untreated cells were plated into dishes at different cell densities (200-72,600) and thioguanine concentrations (0-40 μm), and were fed, stained, and scored as described. One population of treated cells, which were ready to be selected in an experiment using serum which had already been tested and was in use for mutation experiments, was also plated into dishes at a cell density of 72,600 (500/mm^2) and thioguanine concentration of 40 μM. The number of induced mutants obtained in the test serum and the control serum were then compared as an indication of the ability of the new batch to support growth of newly induced mutants.

Testing for Mycoplasma Contamination

50 μl ^3H uridine (100 μCi/ml) and 50 μl ^{14}C uracil (10 μCi/ml) were added to cell cultures in exponential growth and the cells were allowed to incubate 4 hours or longer. The supernatant was poured off and the cells in each flask were washed twice with PBS. To each flask 5 ml of 0.1% sodium lauroylsarcosine (SLS) was added for 5 minutes. The SLS was poured off and added to 10 ml 20% TCA. Another 5 ml of 0.1% SLS was used to wash each flask and

this was also added to the TCA. This solution was left on ice for 30 minutes. Solutions were filtered individually, using Whatman GF/A paper,and the filters were washed with 10% TCA. Filters were dried, and placed into individual scintillation vials, and then 0.2 ml of 0.2 M HCl was added to each. 10 ml of scintillation fluid was added and the samples were counted on a Beckman 9000 LS with dpm capability. If a ratio of ^{3}H to ^{14}C greater than 20:1 was seen, it was assumed that the cells were mycoplasma-free, since only mycoplasma will incorporate the ^{14}C-labeled uracil. Human cells will incorporate uridine, but not uracil.

Benzo(a)pyrene (BP) Metabolism Assay

In order to assay for BP metabolism, exponentially growing cells were treated in 25 cm^2 T flasks with tritiated benzo(a)pyrene (BP) in serum-containing medium, and allowed to incubate for 24 hours. The media was then taken off the cells, extracted twice with hexane, and the aqueous fraction was then separated and assayed for radioactivity by adding 1 ml to 10 ml aqueous scintillant and counting the radioactivity in the solution on a Hewlett Packard Tricarb Scintillation Spectrometer. Since many of the cellular metabolites of BP are water soluble, while BP is not, the amount of radioactivity present in the water fraction was taken as a measure of the extent of BP metabolism that had taken place in the cells.

Protein Assay

In order to normalize the results of the many different cell samples assayed for metabolism of BP, a protein determination was made on each sample assayed. The specific activity for each sample was then calculated (water soluble metabolites produced per milligram of protein present in the cell sample). The protein assay used was that of Lowry modified by Oyama and Eagle (1956). Three solutions were used: A consisted of 20 g Na_2CO_3, 4 g NaOH, 0.2 g NaK tartrate in 1 liter distilled water; B consisted of 5 g $CuSo_4 \cdot 5H_2O$ in 1 liter distilled water; C consisted of 50 parts A to 1 part B prepared fresh daily. Folin-Ciocalteu phenol reagent was also used. After the medium was removed from the monolayer of cells for hexane extraction, the cells remaining in the flasks were lysed by incubating them in 3 ml Lowry solution A for 15 minutes. A 0.5 ml aliquot of the resulting solution was added to 1 ml distilled water and the total volume was brought to 6.0 ml with solution C. Phenol reagent (0.5 ml) was added and the mixture was incubated for an additional 30 minutes. The absorbance at 650 nm of each sample was read, using a Beckman spectrophotometer, and compared to those of protein standards run with each assay to determine the amount of protein present in each sample. The protein standards consisted of bovine serum albumin (BSA) samples at three different concentrations (20, 40, 60 μg/ml) which had been

incubated in 3 ml of solution A for 15 minutes, after which the volume was brought to 6 ml with solution C and the phenol reagent was added as described. The absorbance at 650 nm was determined and the standard curve was constructed from which to determine the protein concentration of the experimental samples. Figure 8 represents the results of protein assays determined for a series of cell strains which had been assayed for metabolism at the same time. The curve was constructed from BSA standards (illustrated by closed circles). The protein concentrations of the experimental samples were determined by correlating the absorbance values obtained at 650 nm to the protein concentration, according to the standard curve.

In Situ Cytotoxicity Assay

Between 100 and 3000 cells were plated into 60-mm-diameter dishes (6-12 dishes per treatment dose) and allowed to attach for 15-18 hours. No more than 3000 cells were plated into this size dish because there appears to be an effect on cloning efficiency, such that cells plated out at higher density show an increased cloning ability than those plated at less than 3000. This tends to give an artificially increased survival result when cells are plated at higher density. There are also reports that at higher densities than these, toxins released from dead cells may reduce the

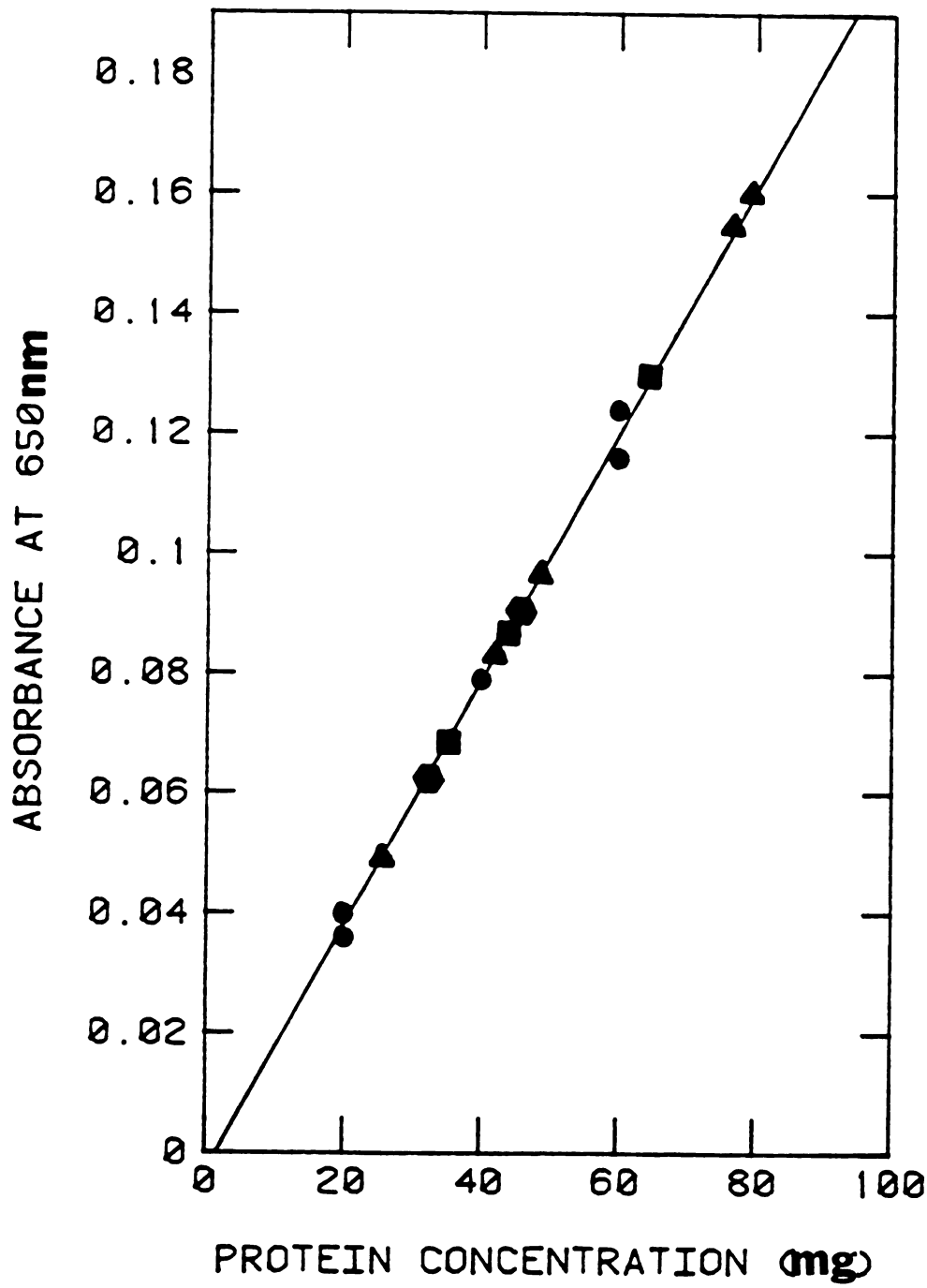


Figure 8. Determination of protein content in cellular samples. (●) BSA control; (▲) PC-3; (■) Hs835T; (⬟) Hs703T

survival. The culture medium was then replaced with serum-free medium. (The particular medium used was determined by the cell type and is designated in the text.) The carcinogen, dissolved in 100% glass distilled acetone dried over a molecular sieve, was introduced into the individual dishes by micropipette. The final concentration of acetone in medium was not more than 0.5%. After two hours, the medium containing carcinogen was removed and replaced with fresh culture medium. Cells were fed after one week and stained when clones were of macroscopic size (at approximately 2 weeks). At this time they were rinsed in saline, fixed in methanol, stained with 0.2% methylene blue, and counted. The cloning efficiency of the treated cells, divided by the cloning efficiency of the control cells, which received only solvent, determined the cytotoxicity of the compound and was expressed as a percent.

Replating Cytotoxicity Assay

Cells in exponential growth were trypsinized and plated into 60-mm-diameter dishes. The desired densities (0.5×10^5 - 0.2×10^6) depended on the expected survival levels and corresponded to the cell densities at which cells were treated for mutation experiments (see below). Cells were allowed to attach for 15-18 hours. The medium was then changed and the carcinogen was administered in the

same way as has been described above for *in situ* experiments. After a treatment period of two hours, the medium was removed and the cells were rinsed with PBS, trypsinized, resuspendended in fresh culture medium and plated into 60-mm-diameter dishes (6-12 per treatment dose) at cloning densities (100-3000/dish). Colonies were fed, stained, and scored as already described.

Assay of Biological Recovery from Potentially Cytotoxic Effects

Cells ($\sim 0.5 \times 10^4$) were seeded into 60-mm-diameter culture dishes, fed every 2-3 days with fresh culture medium and allowed to grow to confluence. Cells were fed with fresh medium on the day they reached confluence, then kept at confluence for 3 days without feeding before they were treated with carcinogen. (For example, cells which were fully confluent on Sunday were treated on Wednesday). Administration of carcinogen has already been described. Following 2 hour treatment, cells to be assayed for survival immediately (day 0) were trypsinized, resuspended, and plated into 60-mm-diameter dishes at cloning density. Those cultures to be assayed for survival at later times had the treatment medium replaced with the spent medium which had been removed from the dishes at the time they received serum-free medium. If they were maintained in confluence for more than 24 hours, they were fed daily with spent

medium. At the designated times, they were trypsinized, and plated out for assay of survival of colony-forming ability.

Cell-mediated Cytotoxicity Assay

Transformed human epithelial cells (Hs703T, Hs835T, see Table 2) were grown to high density in 250 ml flasks (75 cm^2) (Corning, NY), trypsinized, suspended in culture medium, and X-irradiated in suspension on ice with 3000 rads using a GE Maxitron 300 kvp X-ray unit (300 rads/minute for 10 minutes). Irradiated cells were used immediately or were frozen as described for later use. Cells were shown to retain their ability to attach to the plastic dishes following freezing and thawing by monitoring the numbers of cells which attached to plastic determined over several days post-irradiation. They were tested for their ability to metabolize carcinogens by comparing survival in target cells treated in the presence of metabolizing cells before and after X-raying.

The cytotoxicity assay itself involved plating target cells (normal diploid or xeroderma pigmentosum-derived human fibroblasts (NF or XP)) into 60-mm-diameter dishes at desired densities ($0.3-2.6x10^5$/dish). After allowing 4 hours for the cells to attach, the transformed metabolizing cells were seeded on top of the target cell cultures at various densities ($0.3-1.2x10^6$), depending upon the

purpose of the experiment. (See Results section) Cells were then treated with AFB_1 which was dissolved in DMSO and administered in serum-containg medium because a treatment time of 24-48 hours was needed in order to allow time for the carcinogen to be metabolized into a reactive form. Keeping cells in serum-free medium for 24 hours was shown to have a deleterious effect on cloning ability. Following treatment, the medium was removed, and the cells were rinsed and trypsinized from the culture dishes. Because the transformed cells detached from the plastic more quickly than did the untransformed fibroblast target cells, following addition of trypsin to the dishes, nearly all the transformed cells could be seen to be floating in the trypsin while the untransformed cells were still attached. It was thus possible to separate the two different cell types from each other. When the majority of the epithelial cells had been removed, the target cells were trypsinized, counted with a hemacytometer, resuspended in fresh culture medium and plated into dishes at cloning density. They were then fed, and when the colonies had developed, were stained and scored as described.

Replating Mutagenicity Assay

Cells were grown asynchronously for three days, then trypsinized and plated into dishes at the desired densities. For each dose, a population of 0.33 - 1.3×10^6 cells

was plated into 150-mm-diameter dishes. The number of dishes plated was determined to furnish a surviving population of at least 1.0×10^6 cells for each dose following treatment. Cells were allowed 15 to 18 hours to attach. Culture medium was replaced with serum free medium and the cells were exposed to carcinogen as described. Cells were allowed to replicate and undergo phenotypic expression in their dishes for approximately two cell divisions, after which each treatment population was trypsinized, pooled and replated at lower density as necessary ($0.3\text{-}1.0 \times 10^6$/dish; 3-5 dishes per treatment) in order to maintain the cells in exponential growth during the expression period. The length of the expression period was adjusted for each determination to allow the cells to undergo between 4 and 6 population doublings before selection of 6-thioguanine-resistant mutant cells.

In order to monitor the number of population doublings, it was necessary to estimate the percent survival for each treatment based on the dose administered and data from previous cytotoxicity experiments. From this we can estimate the number of cells which had been killed and the number of cells remaining alive. The increase in cell numbers during the expression period was monitored with an electronic cell counter. From the data, we estimated how many divisions the living cells had gone through, taking into account what fraction of the population is represented as

non-dividing dead cells. However, only after the results of the accompanying cytotoxicity experiments became available (approximately 14 days after treatment, which is usually several days after the cells have been selected) were the actual survival levels known and the number of cell divisions accurately determined in retrospect.

From related studies carried out in this laboratory, it appears that at least four to six doublings are required before the cell is resistant to thioguanine (Maher et al., 1979). Cells are resistant to thioguanine when they do not have a functional hypoxanthine/guanine phosphoribosyl transferase (HPRT) enzyme activity. This number of divisions is probably necessary because the cell must dilute out the residual number of HPRT enzyme molecules present in the cell before the mutational event occurred. At the end of the expression period, the cells were trypsinized, pooled, and plated into plastic 100-mm-dishes (64 dishes per point) in selective medium at a density of 300-500 cells/cm^2 (16,500-27,500 cells/dish). Normal diploid and XP-derived human fibroblasts have been demonstrated to be capable of transferring the phosphorylated nucleoside of 6-thioguanine (Corsaro and Midgeon, 1977). This process is referred to as metabolic cooperation. Passage of these nucleosides from $HPRT^+$ cells to $HPRT^-$ cells results in cell death for the $HPRT^-$ mutant. Therefore, the cells had to be plated out at low enough density (300-500/mm^2) for selection of

6-thioguanine-resistant mutants, in order to minimize the loss of mutants from metabolic cooperation.

Thioguanine was present in the selective medium at a final concentration of 40 μM in order to prevent non-mutant colonies from developing. Cells were refed with selective medium after seven days and stained after two weeks. A control population of $1.0\text{-}1.5\text{x}10^6$ cells was included with every experiment to correct for background mutation frequency. At the time of selection, cells from each treatment were also plated at cloning densities in non-selective medium in order to determine cloning efficiency at the time of selection. This number is needed in order to calculate the number of mutants induced from the number of mutant colonies observed because the cells plated in the selection dishes are plated at very low (nearly cloning) densities, and therefore corrections must be made for the cloning efficiencies of the cells.

A cytotoxicity experiment (in some cases using the in situ technique, in others the replating technique) was carried out along with each mutation experiment to determine the percent survival. However, since the cell density at which cells were treated in situ was considerably lower than the density at which cells were treated for induction of mutations, the cytotoxicity results could not be taken to represent exactly the amount of cell

killing which was happening in the mutation experiment. Therefore, in some cases, a replating cytotoxicity accompanied the mutation experiment. However, although these cells were treated at the same density as the cells in the mutation experiment, unlike the latter they were trypsinized directly after treatment, and therefore, they did not represent the same conditions under which the mutation experiments were carried out either. However, since it was not possible to carry out cytotoxicity assays under exactly the same conditions as the mutation assays, these two types of cytotoxicity assays were taken to represent the range of killing values produced. Fortunately, the variations between the results from the two types of cytotoxicity experiments was not very great, and therefore the cytotoxicity experiments were assumed to give a reasonable approximation of the actual cell killing.

Assay of Biological Recovery from Potentially Mutagenic Effects

Cells were seeded into 250 ml flasks (75 cm^2), fed regularly with fresh culture medium, and allowed to grow to confluence. Cells were fed with fresh medium on the day they reached confluence, then kept at confluence for three days without refeeding (as described in the assay of biological recovery from cytotoxicity). Cells were given a two hour treatment with AFB_1-Cl_2. Those cultures to be

assayed immediately for mutations (day 0) were trypsinized, pooled, resuspended and plated out at expression densities. Those cells to be assayed at later times (day 2, 4, 8) were fed with spent medium as described and assayed at the designated times in the same way as day 0 samples. Determination of cell numbers, numbers of plates needed, population doubling, selection densities etc. were made in the same way as in replating mutation experiments. Cytotoxicity experiments, and replating efficiency dishes were included for all experiments as described.

Reconstruction Experiments

As explained above, mutants can be lost as a result of metabolic cooperation. In order to determine the efficiency of recovery of induced mutations under the selection conditions used, and to detect the effect of metabolic cooperation, a known number of $HPRT^-$ Lesch-Nyhan (LN) cells were seeded at the time of selection into a series of control and experimental dishes. LN cells are derived from human patients with an inherited defect in HPRT activity. The control dishes contained only Lesch-Nyhan cells, while the experimental dishes contained cells from each treatment population and one untreated control population at the same density at which they have been seeded for selection. The number of Lesch-Nyhan clones observed for each treatment

group, divided by the number of Lesch-Nyhan clones seen in the control dishes, indicated the fraction of 6-thioguanine-resistant mutants recovered. This number was a factor used in calculating the number of induced mutations from the number of mutant colonies observed. Reconstruction experiments accompanied all mutation experiments. The results indicated a recovery of 75-100% of the LN colonies in our experiments.

Isolation of DNA from Human Cells

Cells were rinsed with PBS, then harvested in trypsin-versene, buffered at pH 7.0 with potassium phosphate. Cells were lysed in 0.5% sodium lauroylsarcosine and incubated at 37°C for 30 minutes. (At this point cells were frozen at -20°C for future analysis) Pancreatic RNase (25 µg/ml) and T_1 RNase (20 units/ml) were added and the solution was allowed to incubate for 30 minutes at 37°C. Pronase (0.1 µg/ml) was added and the solution was incubated for 30 minutes at 37°C. The solution was then extracted twice with a 24:23:1 mixture of Kirby phenol, chloroform and butanol. The two phases were separated by centrifugation at 12,000 x g for 10 minutes. The DNA in the aqueous phase was collected and dialyzed 3 times against 4 liters of SSC, pH 7.0.

Purification of DNA Samples

A 4.5 ml aliquot of the dialyzed DNA solution was added to 5.46 g CsCl for each gradient, then centrifuged to equilibrium in a Beckman ultracentrafuge at 105,000 x g for 18 hours. The absorbance profile at 260 nm of each fraction of gradient was determined (Figure 9) and the DNA fractions were pooled. The DNA gradient was fractionated with a DENSI-FLOW fractionator, Searle, Fort Lee, NJ. In those cases where DNA samples were used for determining the level of carcinogen binding to DNA, the purified DNA solution was then dialyzed against 4 liters of SSC. For high pressure liquid chromatography (HPLC) studies, the DNA fractions were dialyzed once against SSC, then once against 0.1x SSC pH 7.0 and finally once against 0.0015 M sodium citrate pH 7.0, in order to minimize the salt concentration present in the DNA solution.

HPLC Analysis of DNA Adducts

Cellular DNA samples were lyophilized to reduce the volume 10-fold before acid hydrolysis. The DNA samples were hydrolyzed under mild acid conditions (0.15 N HCl at 100^{o}C for 15 minutes). After hydrolysis, all DNA samples (cellular and calf thymus DNA) were lyophilized and redissolved in 0.5 ml methanol. Potassium acetate (0.1M) was added to bring the pH to 5.5, and these samples (0.5-0.7

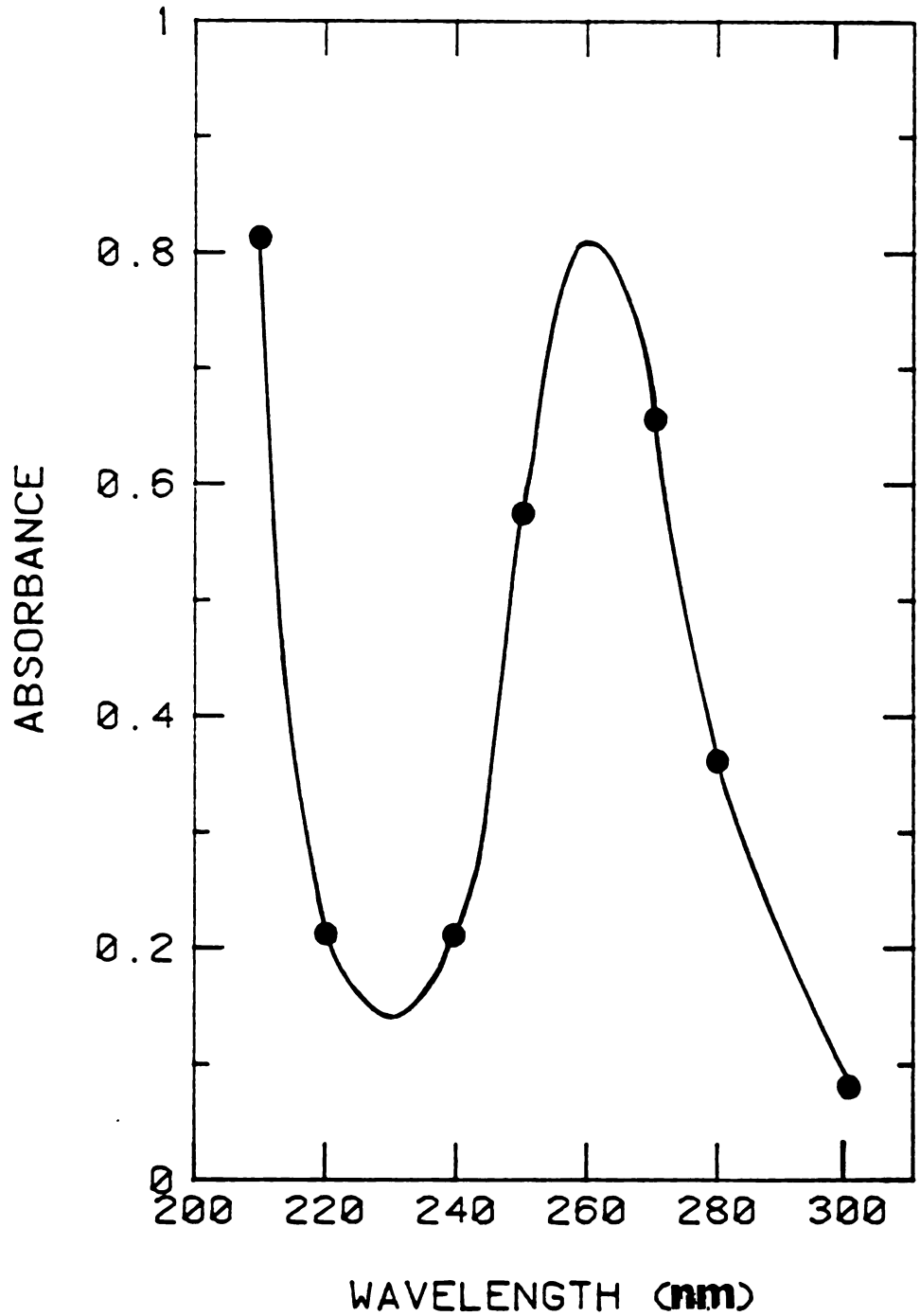


Figure 9. DNA absorption spectrum

ml) were then subject to high pressure liquid chromatography, using a Spectra Physics (Sunnyvale, CA) SP8000 instrument equipped with a Waters Associates (Milford, MA) reverse phase μ Bondapak C^{18} column (3.9 x 300 mm). A 70 minute methanol/water gradient (concave see Table 1) was applied to the column which was maintained at 50°C with a flow rate of 1 ml/minute. Fractions were collected at 5 minute intervals and mixed with 10 ml of aqueous counting scintillant (Amersham, Arlington Heights, IL.) and radioactivity was measured on a Beckman LS9000 Liquid Scintillation Spectrometer equipped with dpm calculation capacity. For all HPLC profiles of cellular and calf thymus DNA, the time (ie. day 0, 2, 8) reflects the time at which the DNA isolation procedure was started. This does not take into account the time required to isolate and purify the DNA, which was 24-72 hours. Therefore, the HPLC profiles reflect the DNA adducts present in the DNA following these processes. (See Discussion Section).

Studies with Calf Thymus DNA

For studies with calf thymus DNA, 5 μCi of ^{3}H-AFB_1-Cl_2 (2.5 μg) were reacted with 10 mg calf thymus DNA in 7.5 ml 0.02 M potassium phosphate buffer, pH 6.7, and incubated at 37°C. Samples were taken at various times up to 8 days after treatment. The DNA was isolated,

Table 1. HPLC gradients used

	Time	% methanol	% water
Gradient 1.	0.0	10	90
	24.0	52	48
	56.0	80	20
	58.0	100	0
	70.0	100	0
Gradient 2.	0.0	10	90
	20.0	45	55
	50.0	45	55
	70.0	100	0

purified, and its specific activity determined as described.

The optical standards used for co-chromatographic comparison with the experimental (cellular and calf thymus) DNA samples were synthesized by reacting 25 μg cold AFB_1-Cl_2 with 1 mg dGMP in 2 ml 0.02 M potassium phosphate buffer pH 6.7, or with 2 mg calf thymus DNA in 5 ml buffer for 30 minutes at 37°C. Unreacted compound was extracted three times with ethyl acetate. Standards made with calf thymus DNA were acid hydrolyzed as described and both dGMP and calf thymus standards were lyophilized and redissolved in buffer as described for DNA adducts.

RESULTS

STUDIES WITH AFB_1

Identification of a human cell strain capable of metabolizing aflatoxin B_1

AFB_1 requires metabolic activation before it can exert its biological effects (Garner et al.,1972). Since these cells are unable to metabolize AFB_1, it was necessary to employ an activating system in order to study its effects in human fibroblasts in culture. Because benzo(a)pyrene (BP) and AFB_1 are both metabolized by the cytochrome P450 mixed function oxidase enzyme systems present in microsome fractions, it was reasoned that cells retaining the ability to metabolize BP would be likely to have AFB_1 metabolism ability.

Table 2 shows the results of screening eighteen different cell strains, including sixteen cell lines derived from human tumors, and two normal human strains for their ability to metabolize BP into water soluble metabolites. The ability of Hs835T cells to produce such metabolites was

Table 2. The level of benzo(a)pyrene metabolism in several human cell strains.

Cell type	Description	total protein (mg)	cpm	Specific activity: nanomoles of BP converted/mg protein
Hs700T	Metastatic adenocarcinoma	.312	1731	27.8 ± 6
		.312	2619	
Det562	Pharyngeal carcinoma	.264	1873	24.2 ± 5
		.186	1769	
Hs703T	Liver carcinoma	2.232	14983	18.3
Hs835T	Kidney carcinoma	.936	4214	12.3 ± 3.6
		.177	505	
		.800	2915	
		.684	2456	
A498	Kidney carcinoma	.480	1523	10.7 ± 0.4
		.456	1590	
A253	Epidermoid carcinoma of neck	.444	1778	8.7 ± 4.4
		.426	578	
A549	Lung carcinoma	.714	682	3.6 ± 0.4
		.516	626	
Hs696T	Metastatic adenocarcinoma	.174	148	3.2 ± 0.4
		.156	113	

Table 2. (cont'd)

Cell type	Description	total protein (mg)	cpm	Specific activity: nanomoles of BP converted/mg protein
Hs766	Metastatic carcinoma of pancreas	.516 .456	481 458	3.2 ± 0.4
Hs683T	Glioma	.576 .144	127 135	2.4 ± 1.6
HT1080	Fibrosarcoma	1.068 .864	149 146	1.6 ± 0.8
JAR	Choriocarcinoma	.192	39	0.8
Hs695T	Melanoma	.264 .279	69 42	0.8 ± 0.4
A172	Glioblastoma	.768 .684	114 90	0.8 ± 0.4
FHs74Int	Normal intestine	.734	364	2.4 ± 1.2
PC-3	Bone marrow from prostate cancer patient	1.032 .816	279 192	0.8 ± 0.4
NF801.5	Normal skin fibroblast	.333	32	0.4

assayed as a positive control with each series of cells screened to insure consistency in the test system. The results indicated that six of the strains tested (all of which were transformed cell lines) exhibited a consistently greater level of metabolism than the other twelve strains tested. The data were expressed in terms of activity per mg of protein as determined by the Lowry protein assay. This assay was used to facilitate comparison of different cell lines rather than cell numbers because the amount of protein was found to vary from one cell type to another. For example, the data in Figure 10 indicated that PC-3 cells have a higher cellular protein content than do NF811.

The effect of cell number and exposure time on the extent of metabolism

Aryl hydrocarbon hydroxylase (AHH) activity was measured as a function of cell number and time of exposure to BP. Figure 11 illustrates that, in PC-3 cells treated with BP, the level of water soluble metabolites produced increased linearly with the number of cells treated. The data in Table 3 indicate that increasing the exposure period from 24 to 48 hrs for PC-3 cells treated with BP resulted in a significant increase in the level of metabolic conversion of BP.

Langenbach et al. (1979) have shown that primary

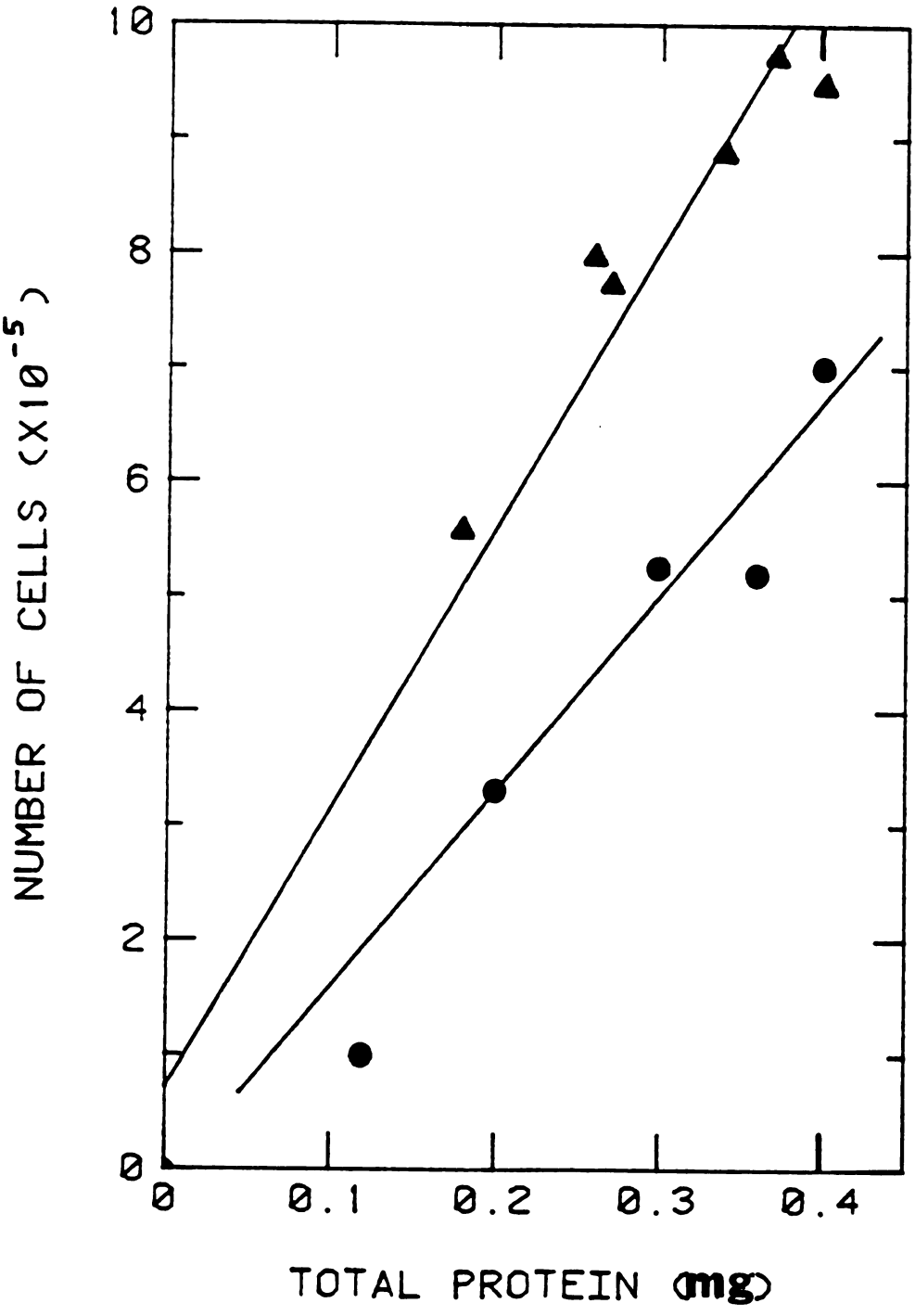


Figure 10. The relationship between cell number and protein content in PC-3 and normal cells. (▲) NF; (●) PC-3

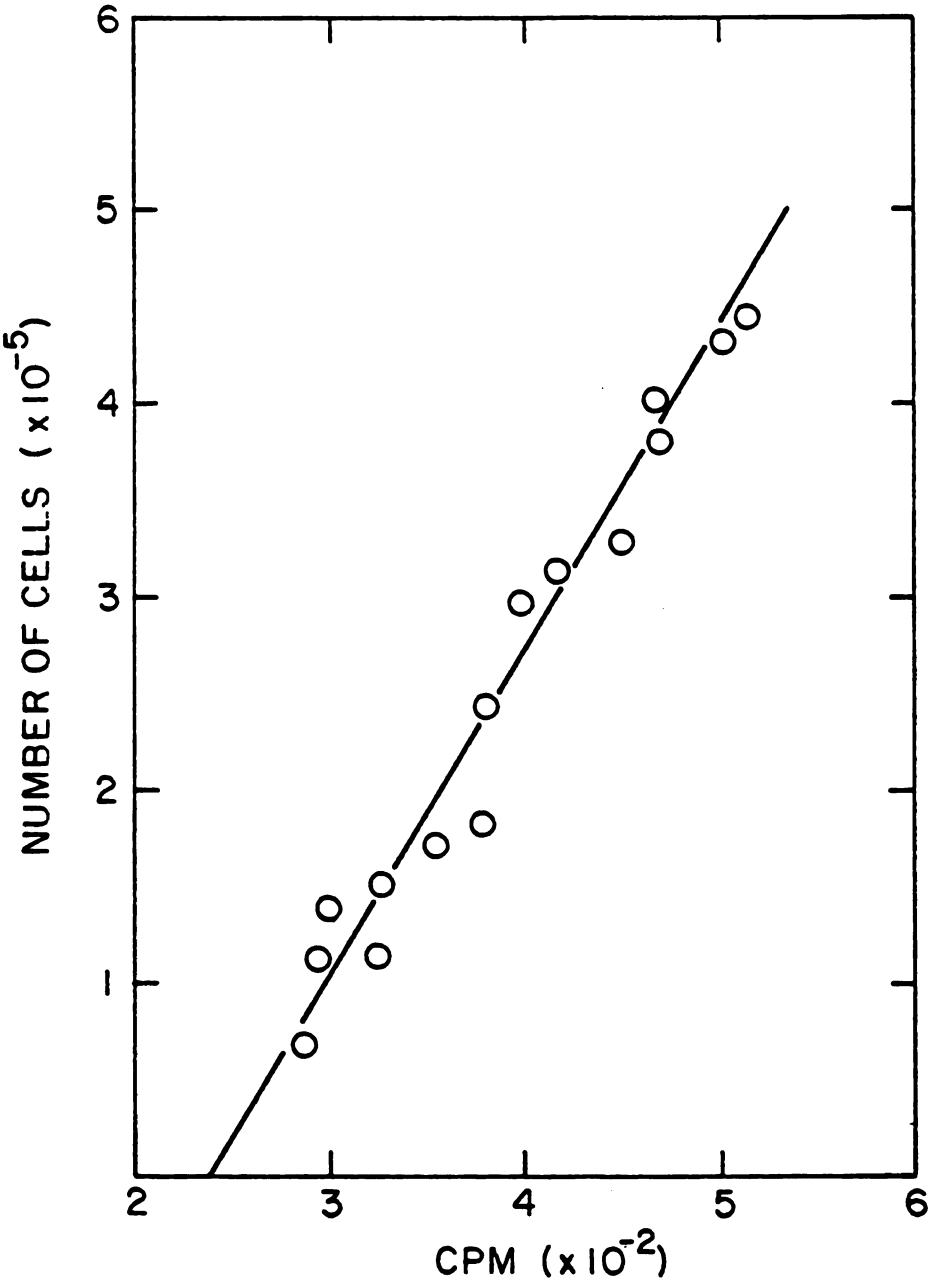


Figure 11. The relationship between number of cells assayed and the level of water soluble metabolites produced. PC-3 cells

Table 3. Level of BP metabolism in PC-3 cells following 24 and 48 hour exposures to BP

	Specific activity: nanomoles of BP converted/mg. protein
24 hr. exposure	0.254
	0.286
	0.368
	0.269
	average = 0.294 $\pm$ .051
48 hr. exposure	0.568
	0.771
	0.856
	0.856
	average = 0.763 $\pm$ .136

rat kidney cultures exhibit a higher level of AFB_1 metabolism than do rat skin fibroblasts, suggesting cell specificity in carcinogen metabolism. We, therefore, chose to use the transformed human liver cell line (Hs703T) in our cell-mediated system for activation of AFB_1. We also chose to test the transformed human kidney line (Hs835T) because it exhibited properties which made it easier to culture and it also exhibited high levels of BP metabolism.

Cell-mediated cytotoxicity studies with AFB_1

Figures 12 and 13 show the cytotoxic response induced by AFB_1 in XP target cells in the presence of Hs835T (Figure 12) or Hs703T (Figure 13) metabolizing cells. In the presence of either of these metabolizing systems, concentrations of AFB_1 as low as 1 μM were cytotoxic to the XP target cells. XP cells showed no cytotoxic response in the absence of an activation system. These data present evidence of metabolic conversion of AFB_1 to a toxic metabolites in this cell-mediated system. Figure 14 compares the cytotoxic response of XP cells in the presence of the two different metabolizing cell lines, viz. Hs703T and Hs835T when seeded at the same density. The results showed there was more killing in the presence of Hs703T cells than in the presence of Hs835T cells, suggesting that metabolic conversion of AFB_1 was more efficient in Hs703T cells than in Hs835T cells.

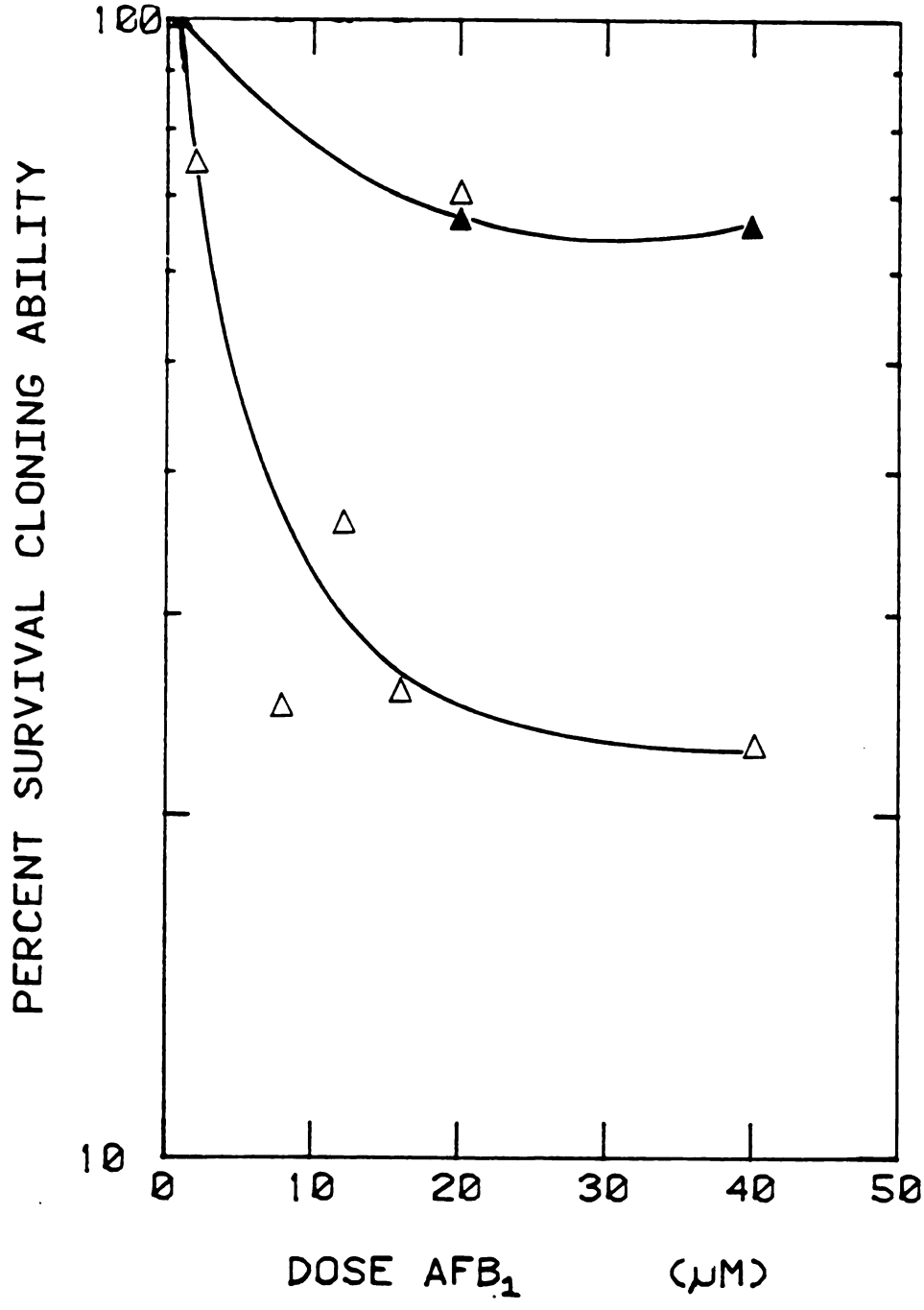


Figure 12. Cell-mediated cytotoxicity of AFB_1 in XP cells using two concentrations of $Hs835T$ cells to activate AFB_1. (▲) 0.6×10^6; (△) 1.2×10^6

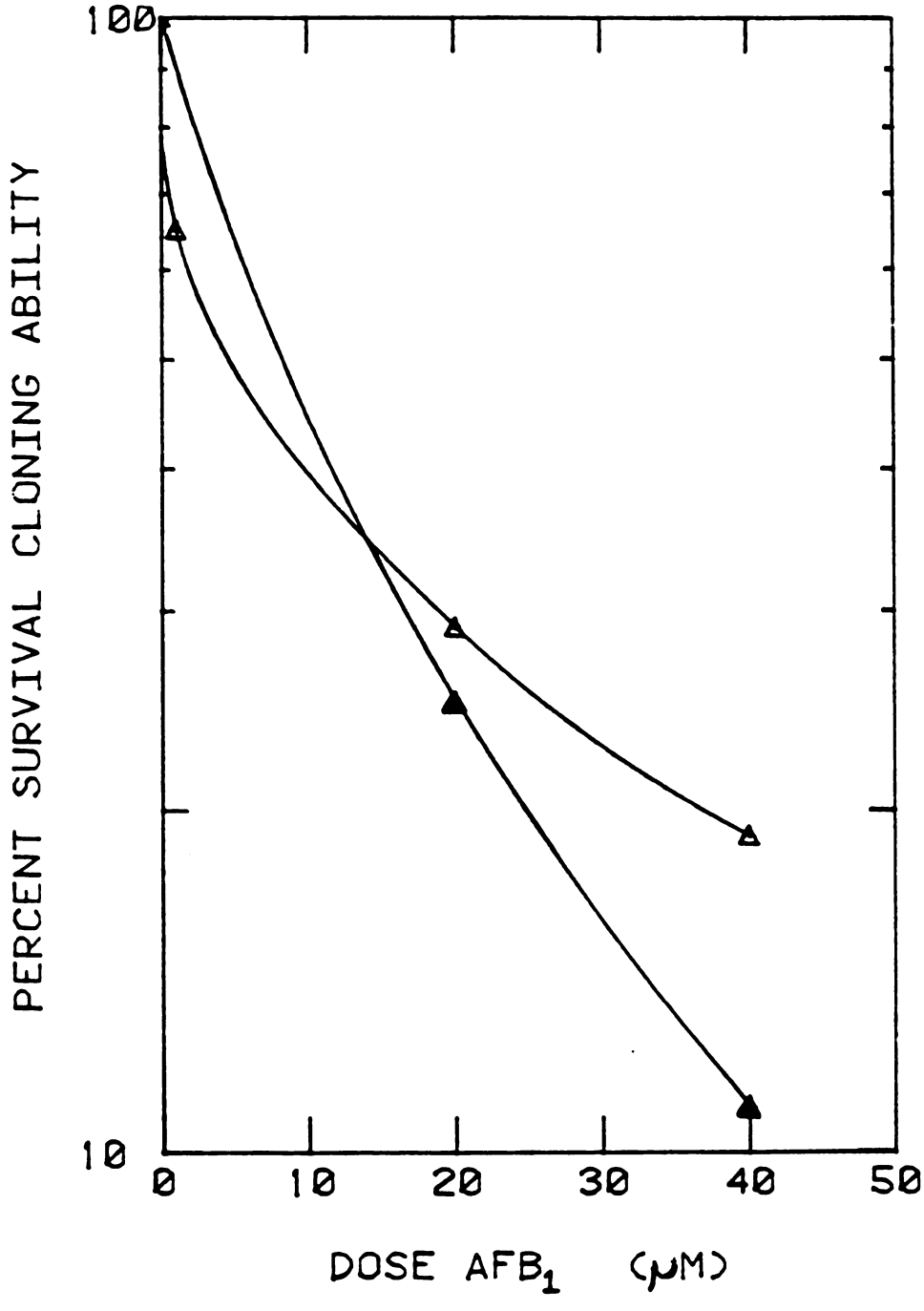


Figure 13. Cell-mediated cytotoxicity of AFB_1 in XP cells using two concentrations of Hs703T cells to activate AFB_1. (▲) 0.4×10^6; (△) 0.6×10^6

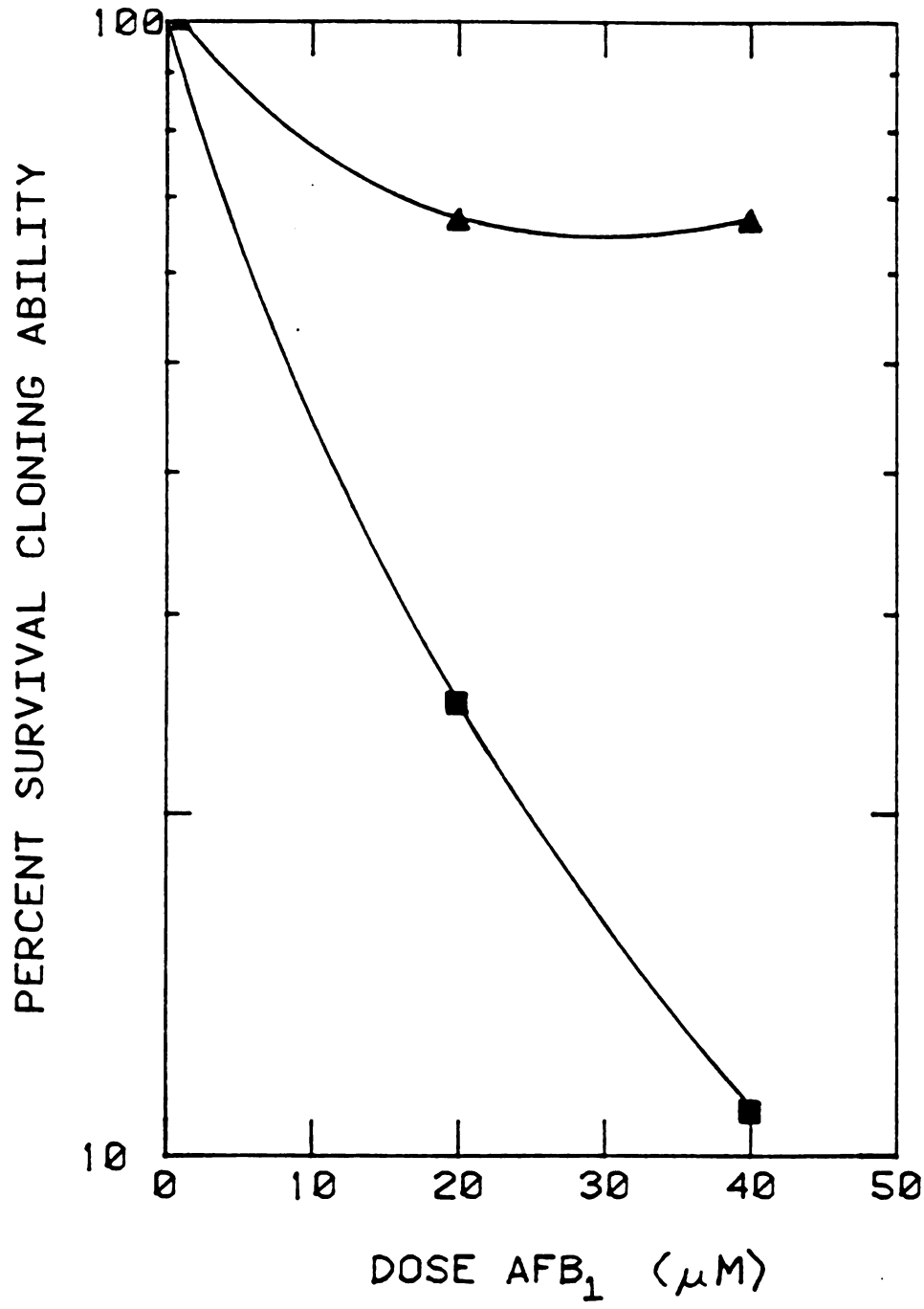


Figure 14. Comparison of the levels of cytotoxicity induced by AFB_1 in XP cells in the presence of equal numbers of Hs835T and Hs703T cells. (▲) Hs835T; (■) Hs703T

The effect of varying the number of metabolizing cells on the cytotoxic response in target cells

Figure 12 shows the effect that varying the number of metabolizing cells present during AFB_1 treatment had on the cytotoxicity observed in target XP cells. When XP cells were treated with AFB_1 in the presence of two different ratios of Hs835T cells to XP cells, the cytotoxic response was greater when the metabolizing cells were present at the higher ratio. The exposure time in both cases was 24 hours. The same effect was observed when the cells were treated in the presence of two different ratios of Hs703T cells to XP cells, as shown in Figure 13. This is probably because more of the AFB_1 was converted into the reactive metabolite when more metabolizing cells were present. We also noticed a distinct leveling off in both of the XP survival curves obtained with AFB_1 in the presence of the Hs835T cells. This is probably caused by saturation of metabolizing enzymes in the cells at that concentration, or by some sort of feedback inhibition.

It should be noted that this bend in the survival curve was not as evident when Hs703T cells were used as the source of metabolism. This suggests that the enzymes in the Hs703T cells were not saturated by the concentrations of AFB_1 administered. Further evidence that the Hs703T cells have a higher capacity for metabolism of AFB_1 than

do Hs835T cells can be seen in Figure 14. In the presence of the same number of metabolizing cells, XP cells showed more cell killing in the presence of Hs703T cells than with Hs835T cells. A concentration of 20 μM resulted in 70% survival with Hs835T cells compared to 25% survival with Hs703T cells. This is further evidence that the Hs703T cells have a higher capacity for metabolism of AFB_1. This is consistent with in vivo data which has shown that rat liver carries out greater levels of AFB_1 metabolism than does kidney (Wogan, 1968). Based on these results, Hs703T cells were determined to be a more desirable system with which to study the effect of AFB_1 in human fibroblasts.

Optimal ratios of metabolizing cells to target cells

To further optimize the system, we wished to determine the ratio of Hs703T:XP cells at which the maximum conversion of AFB_1 into cytotoxic metabolites was observed. It would be desirable to use cells at this ratio to ensure that consistent results were obtained. Figure 15 shows the effect on the survival in XP cells that varying the ratio of Hs703T cells to XP cells had while the concentration was kept constant at 10 μM. The results showed that cytotoxicity increased with increasing ratios until it leveled off at a ratio of Hs703T cells to XP cells of around 5:1. It

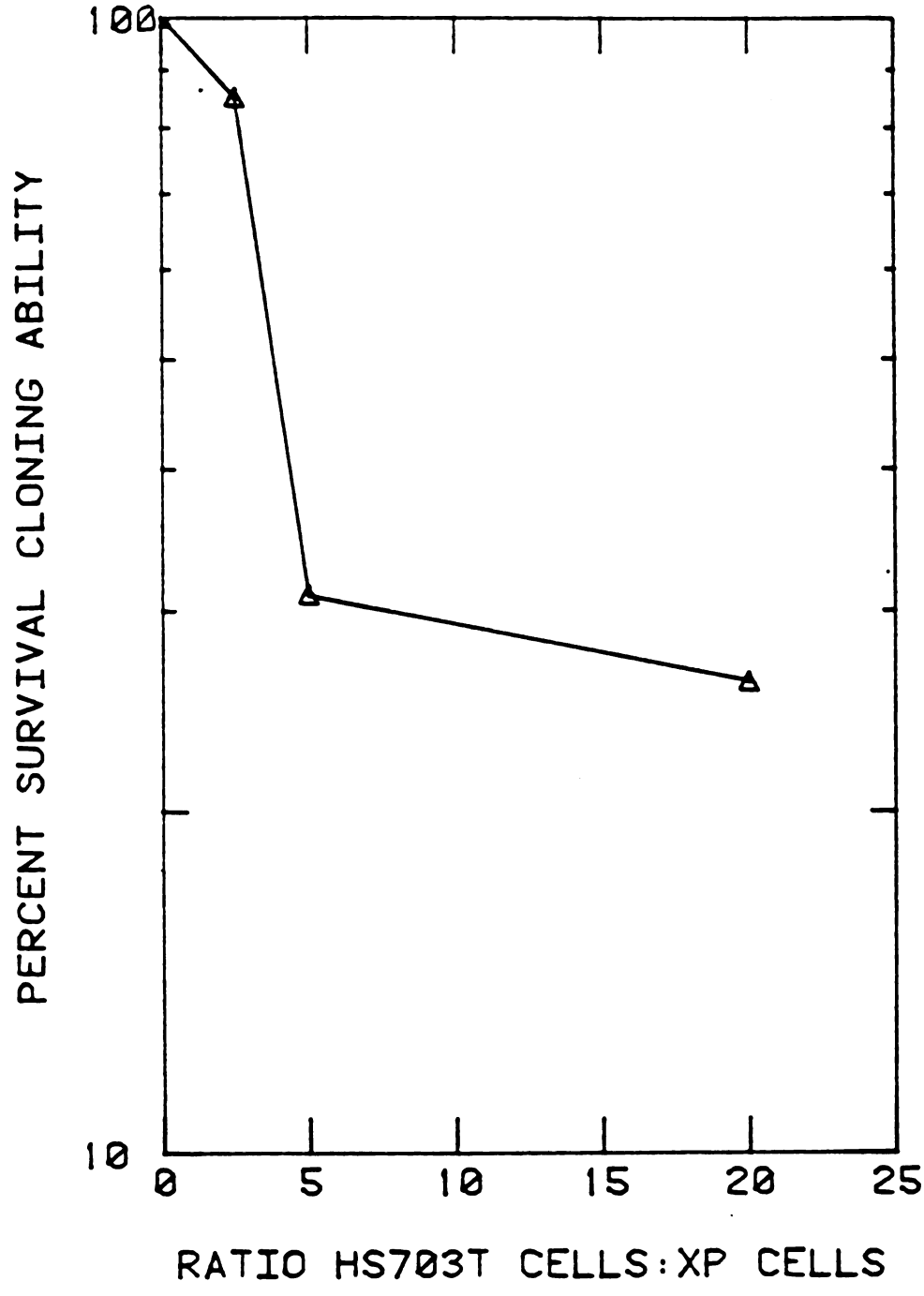


Figure 15. The effect of varying the ratio of Hs703T cells to XP cells on the cytotoxicity induced by AFB_1 in XP cells.

appears that this ratio allows for the maximum level of metabolic conversion possible in this system at this particular concentration of AFB_1.

AFB_1-induced cytotoxicity in Hs703T cells as targets

Hs703T cells, themselves, were treated with varying concentrations of AFB_1, allowed to incubate for 24 or 48 hours, and then assayed for survival. Figure 16 shows the results obtained. AFB_1 induced cytotoxicity in Hs703T cells after both incubation times, however the level of cytotoxicity was much greater after a 48 hour exposure than after 24 hours. For a 1.25 μM concentration, the survival after 24 hours was 60% and after 48 hours, survival was 1%. This may represent an induction of the enzymes involved, since a two-fold difference would be expected if this was simply an additive effect caused by the longer exposure time.

Comparison of the cytotoxic response to AFB_1 in three different target cells

The cytotoxic responses of NF, XP, and Hs703T target cells to AFB_1 are compared in Figure 17. It is interesting to note that, while XP cells proved more sensitive than NF cells, Hs703T cells appeared to be more sensitive than XP cells. These data suggested that either Hs703T cells

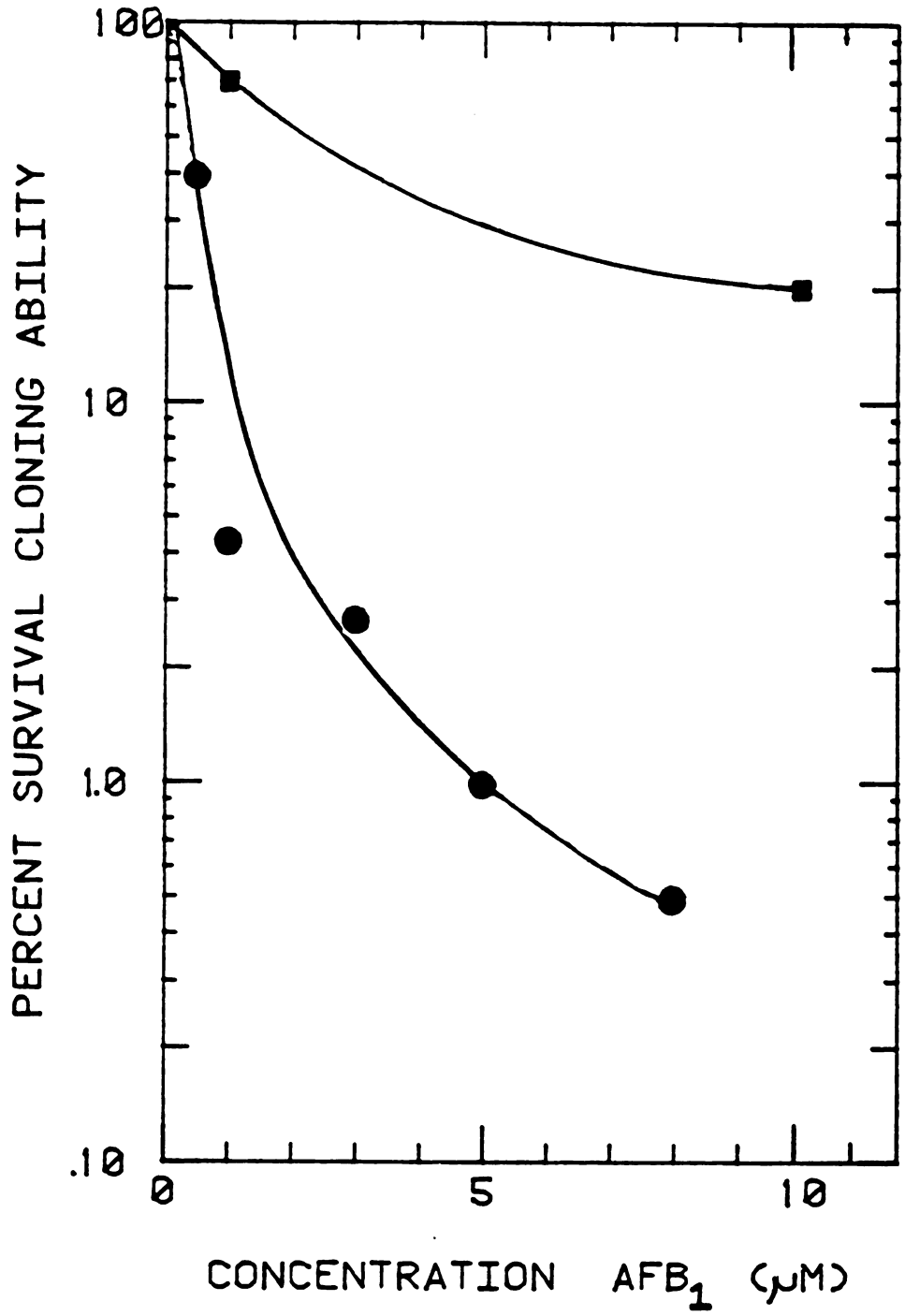


Figure 16. Cytotoxicity of AFB_1 in Hs703T cells following 24 and 48 hour exposure times.
(■) 24 hours; (●) 48 hours

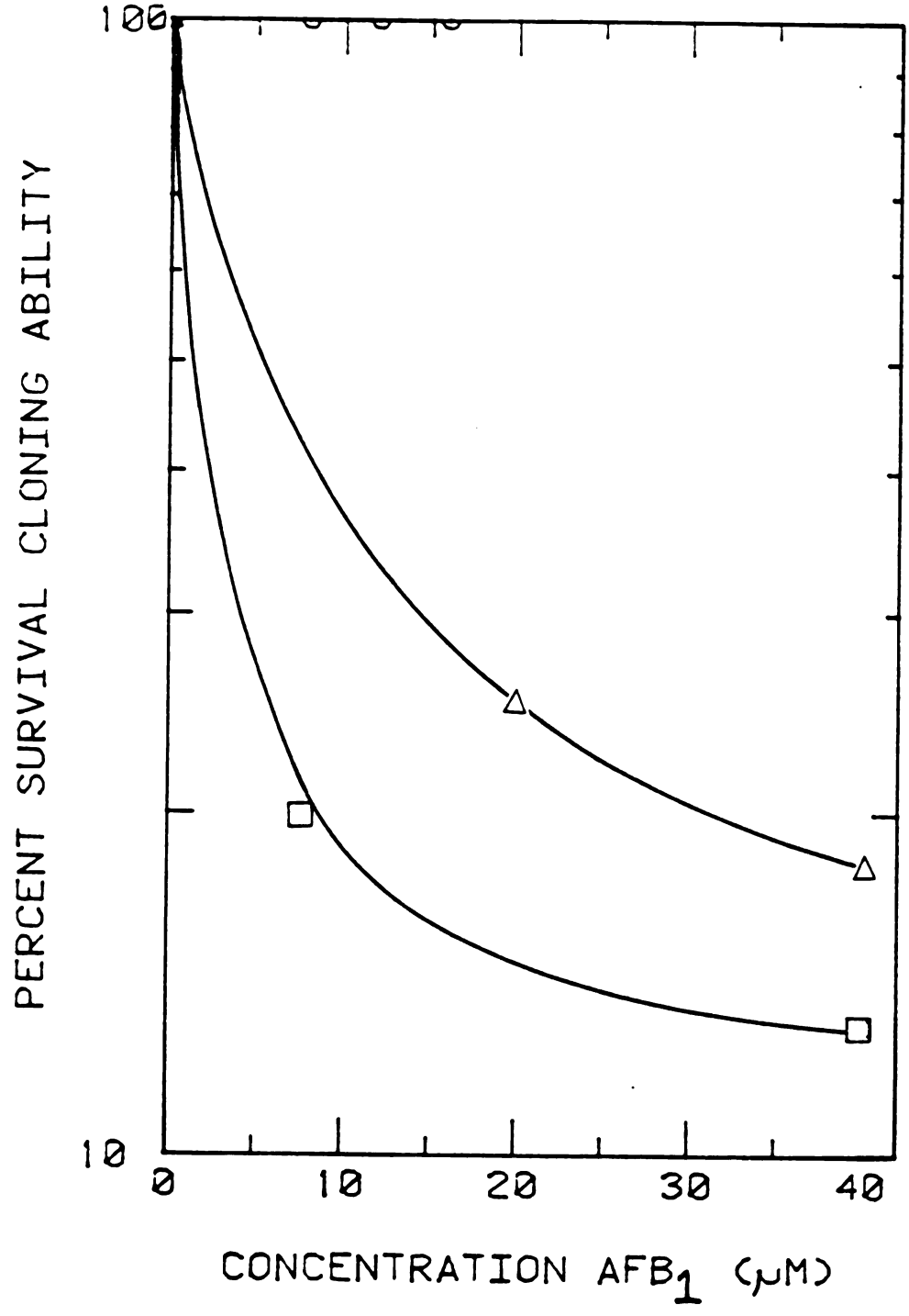


Figure 17. Comparison of the levels of cytotoxicity induced by AFB_1 in NF, XP and Hs703T target cells. (○) NF; (△) XP; (□) Hs703T

were deficient in the repair of AFB_1-induced cytotoxic lesions, or that the Hs703T were receiving a higher effective concentration of AFB_1. The latter is a more likely explanation since metabolism is taking place within the target cell itself. The reactive metabolite of AFB_1, the 2,3-epoxide, is very unstable, and is believed to have a half-life of only a few seconds in water. Therefore, it would not be expected to reach the target cells as readily as it would reach the metabolizing cells themselves. Since AFB_1 metabolism is probably taking place in the nuclear membrane of the Hs703T cells, more of the metabolites produced at a given concentration will reach the DNA in Hs703T cells than in XPs , where the highly unstable metabolite must be transported from Hs703T cells to XP cells and then into the nucleus. Thus, one would expect the actual amount of the reactive metabolite reaching the XP cell DNA to be much lower than that reaching Hs703T DNA.

One way in which to test this hypothesis was to expose XP, NF and Hs703T cells to a carcinogen which does not require metabolic activation, and assay for survival. We, therefore, exposed all three cell strains to AFB_1-Cl_2, the direct-acting analog of AFB_1-2,3-oxide. The cells were treated <u>in situ</u> with the same carcinogen solution all at the same time. The results (Figure 18) showed that XP cells were more sensitive to the direct-acting carcinogen, AFB_1-Cl_2, than were NF and Hs703T cells, which

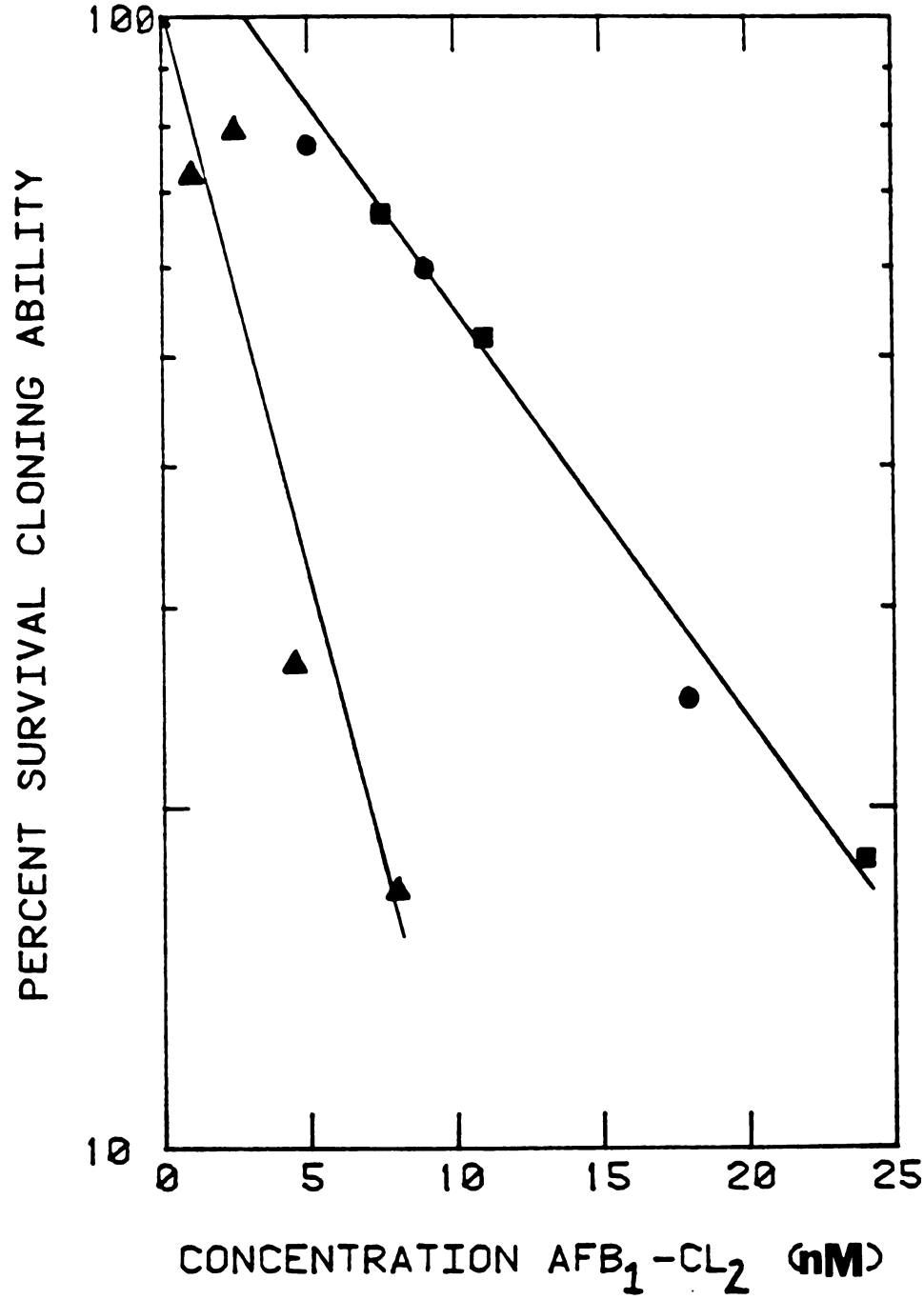


Figure 18. Cytotoxicity of AFB_1-Cl_2 in NF, XP, and Hs703T cells. (●) NF; (▲) XP; (■) Hs703T

appeared to be equally sensitive. These results suggest that Hs703T cells have normal DNA repair and that the greater sensitivity that Hs703T cells showed for AFB_1 was probably the result of a higher effective concentration.

Use of AFB_1-Cl_2 as a model for AFB_1 in studying the biological effects of AFB_1 in human cells in culture

In order to investigate the role of excision repair on the cytotoxic and mutagenic effects of AFB_1 in human cells, it was necessary to use repair-proficient cells. However, it became evident from these preliminary studies that if cytotoxicity and mutations in normal cells were to be induced with the cell-mediated system it would require very high concentrations of AFB_1. In fact, with these cells, we saw no toxicity for the concentrations we administered. This is probably because during the length of exposure time needed for metabolism (24 or 48 hours) normal cells are continually carrying out excision repair of the potentially cytotoxic DNA lesions induced by AFB_1.

Because of the high concentrations of AFB_1 required for normal cells, the need to treat large number of cells for the binding and adduct studies planned, and the high cost of radioactive AFB_1, it became apparent that these studies would be very difficult and very expensive. Although Hs703T cells required much lower concentrations than

normal cells before cytotoxcity was induced, they could not provide a system in which to study the biological effects of AFB_1 because the recovery experiments proposed (see below) required that cells be held in a non-dividing state by means of contact inhibition. Hs703T cells, like most transformed cell lines, are not contact inhibited.

As an alternative, we chose to use AFB_1-Cl_2, which has been synthesized as a model compound for AFB_1-2,3-oxide, the probable reactive metabolite of AFB_1. AFB_1-Cl_2 is a direct-acting carcinogen, which binds to DNA and induces mutations in Salmonella typhimurium in the absence of any activating system, and produces sarcomas at the site of injection in rats. As expected for AFB_1-2,3-oxide, it has an electrophilic carbon 2, and the major DNA adducts are formed between the carbon 2 and nucleophilic sites in DNA. (Swenson et al, 1975)

STUDIES WITH AFB_1-Cl_2

Comparative sensitivity to the cytotoxic effects of AFB_1-Cl_2

Figure 19 shows the cytotoxic response of normal and XP cells to AFB_1-Cl_2. For these studies, NF and XP cells were seeded and treated in situ at the same time with the same carcinogen solutions to insure uniformity of

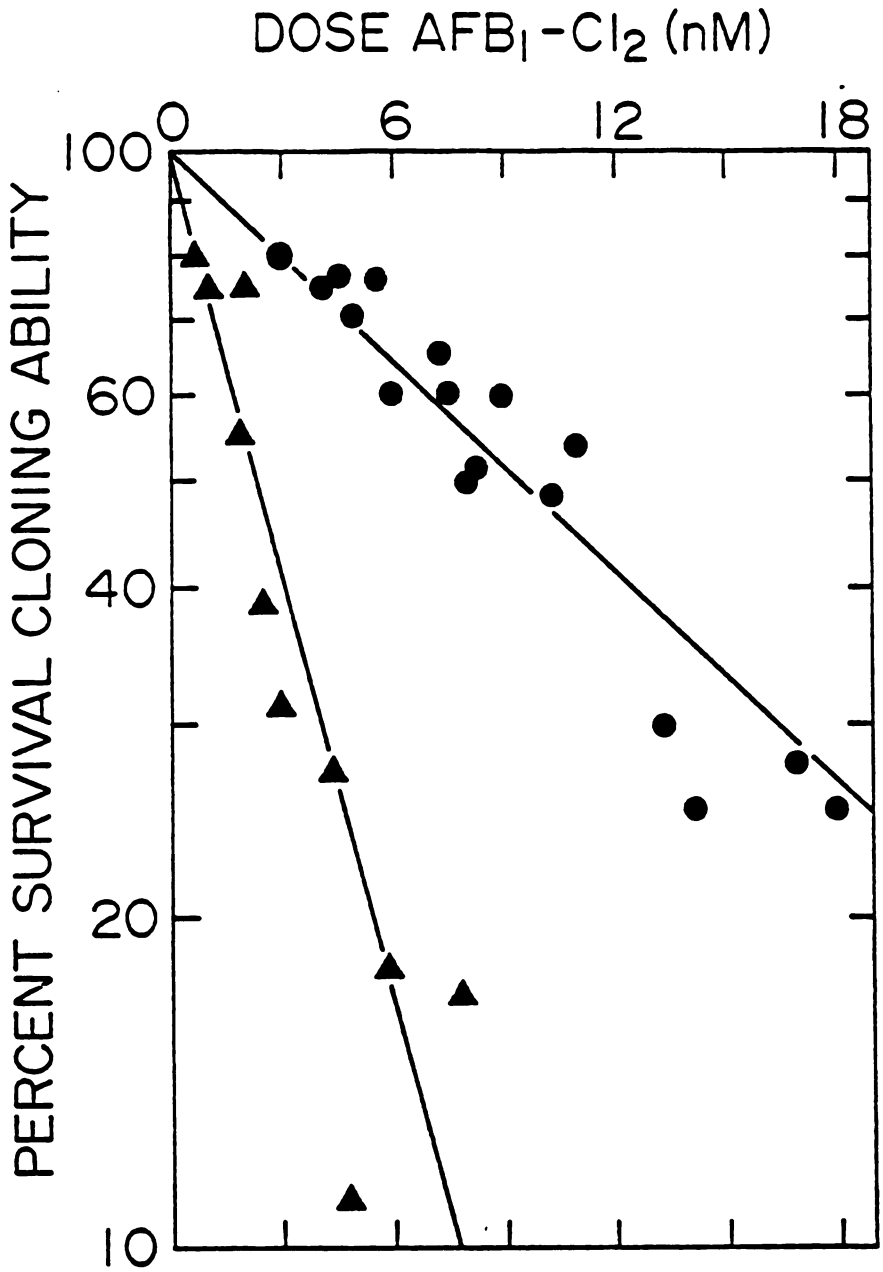


Figure 19. Cytotoxicity of AFB_1-Cl_2 in NF and XP cells. Cells were treated *in situ*. (●) NF; (▲) XP

concentration administered. The results showed that normal diploid human fibroblasts were less sensitive than XP12BE cells to the cytotoxic effects of AFB_1-Cl_2. These data suggest that normal cells are capable of excising more of the potentially cytotoxic lesions induced by AFB_1-Cl_2 than are XP cells. To test this hypothesis, we compared the sensitivity of a series of XP-derived cells to the toxicity induced by AFB_1-Cl_2. Since each of the XP cell strains we examined had been shown to possess a different level of excision repair capacity for UV-induced DNA (Robbins et al.,1973), a correlation between their rate of excision repair of UV damage and their resistance to AFB_1-Cl_2-induced toxicity would support the hypotheses that excision repair of the potentially cytotoxic lesion by NF cells was responsible for their greater resistance to AFB_1-Cl_2-induced toxicity. It would also indicate that one or more steps were common to the repair of both kinds of lesions.

In this study, normal cells and a series of XP cells derived from different complementation groups were exposed to varying concentrations of AFB_1-Cl_2 and assayed for survival in situ. The results are shown in Figure 20. XP12BE cells, with less than 2% the normal rate of excision repair, showed the greatest sensitivity to AFB_1-Cl_2 of all cells tested. XP7BE cells from Group D, with an intermediate excision repair rate, were less sensitive than XP12BE

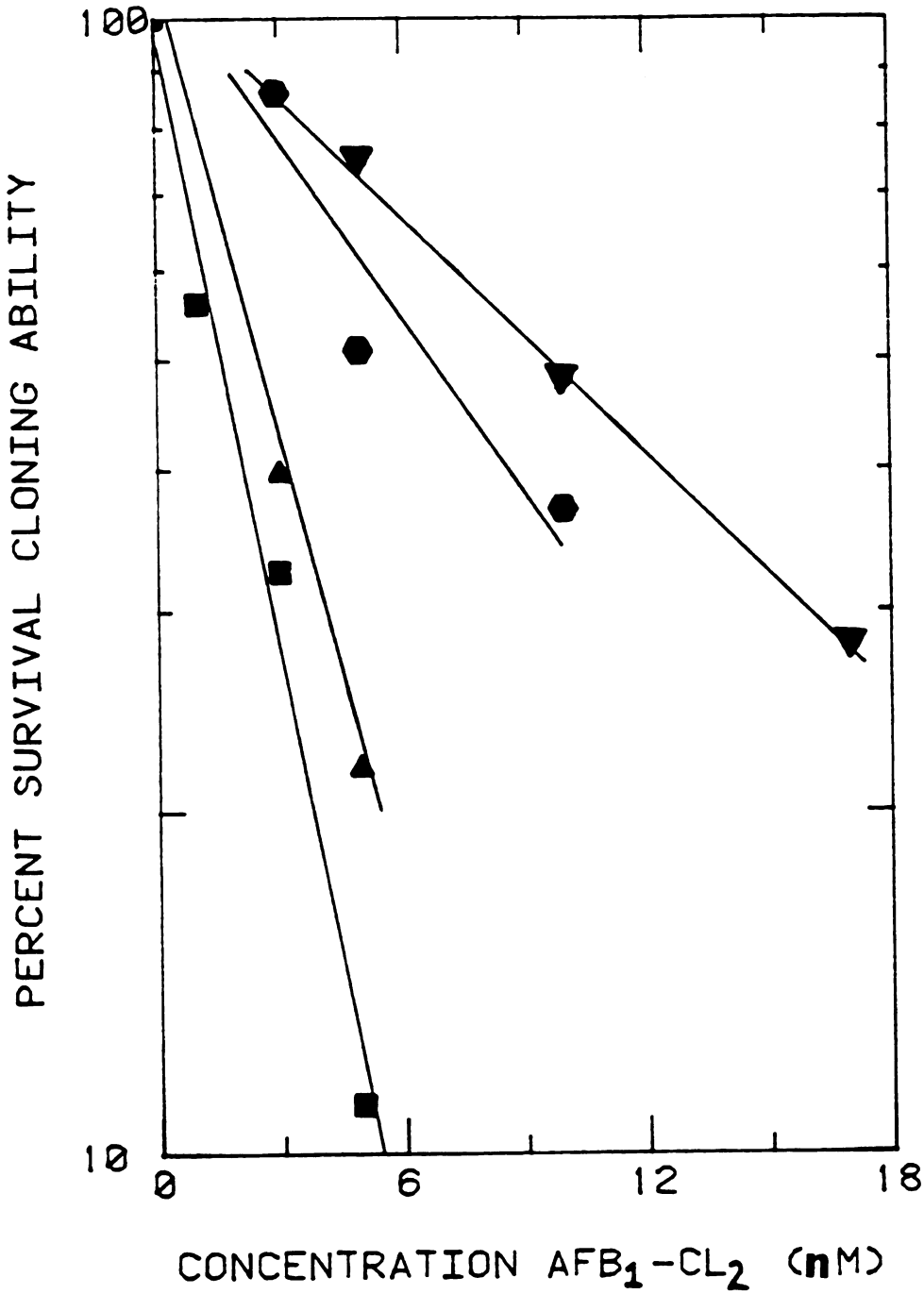


Figure 20. Cytotoxicity of AFB_1-Cl_2 in three different XP strains and normal cells.
(■) XP12BE; ▲ XP7BE; (●) XP4BE; (▼) NF

cells, but more sensitive than XP4BE cells, which have a normal rate of excision repair, but have abnormal replication past DNA lesions. All three of the XP strains tested showed a greater sensitivity to AFB_1-Cl_2 than NF cells. It appears from these data that the degree to which a cell strain is capable of repair of UV-induced lesions correlates with the ability of cells to survive AFB_1-Cl_2 treatment.

The relationship between level of DNA binding and survival

If normal cells are capable of excising potentially cytotoxic DNA lesions induced by AFB_1-Cl_2 more rapidly than are XP cells, we would expect that, if both sets of cells received equal initial binding levels, normal cells would exhibit a higher survival than XP cells because they would have been able to excise some of these lesions before the potentially lethal effect was fixed in the cell. To test this hypothesis, a series of confluent NF and XP cultures were treated with varying concentrations of tritiated AFB_1-Cl_2 and were immediately assayed for percent survival and for the number of AFB_1-Cl_2 residues bound to DNA. Figure 21 shows the relationship between survival and the number of AFB_1-Cl_2 residues initially bound to DNA in NF and XP cells. These data showed that for equal levels of initial DNA binding, NF cells showed a greater survival.

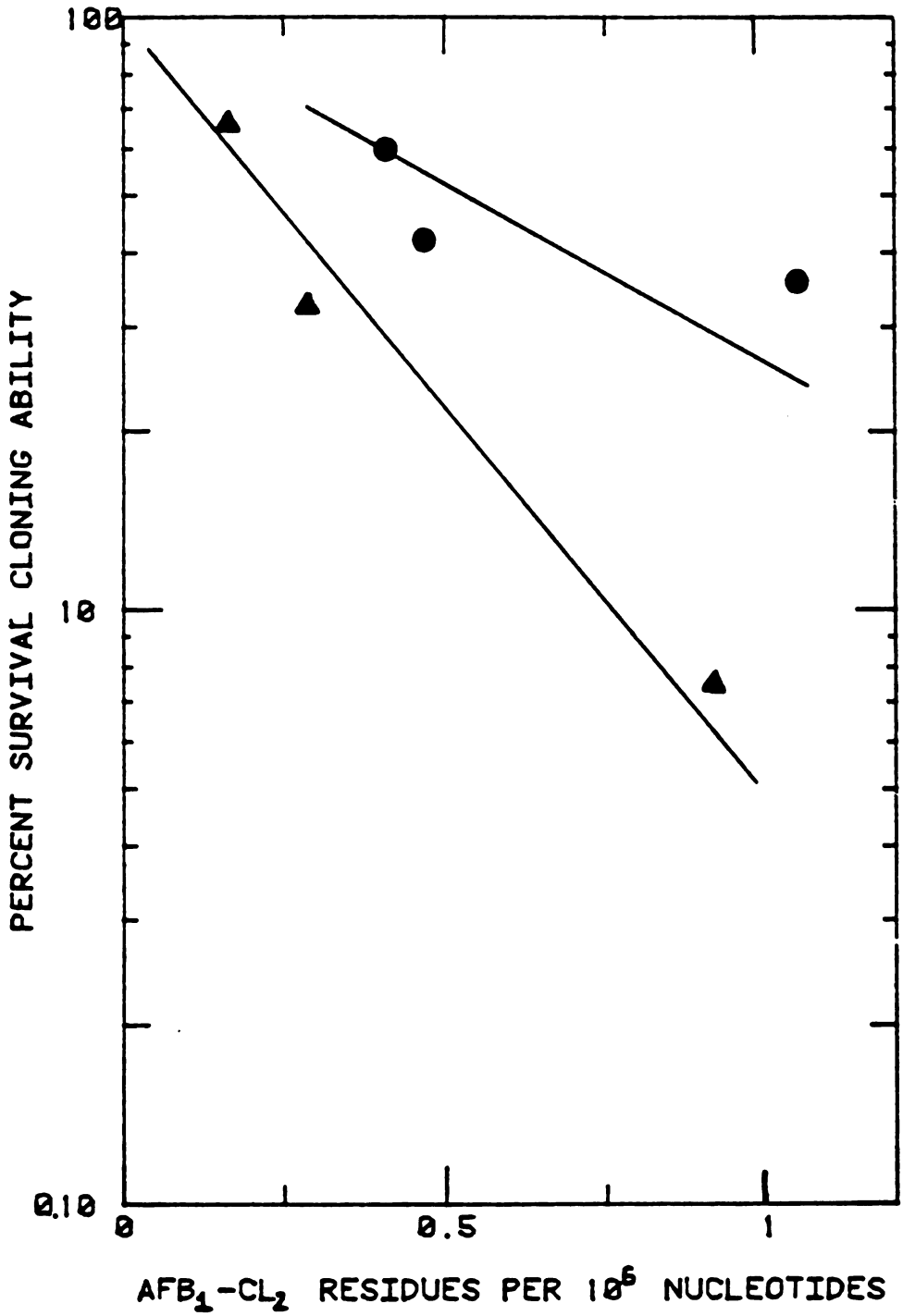


Figure 21. Relationship between levels of cytotoxicity and DNA binding induced by AFB_1-Cl_2 in normal and XP cells. (●) NF; (▲) XP

Although binding is measured immediately after treatment, survival is dependent on events in the cell cycle and is determined at some time later than initial carcinogen binding. The data suggest that the reason NF cells exhibit a higher survival than XP cells when their initial DNA binding levels are equal is because, before the critical time for cell killing is reached, NF cells can excise a portion of the lesions, thus lowering the toxicity, while XP cells cannot.

Rate of recovery of confluent cultures from the potential cytotoxicity induced by AFB_1-Cl_2 lesion(s)

If normal cells are more resistant than XP12BE cells to the killing effects of AFB_1-Cl_2 because of their ability to repair the cytotoxic lesion more rapidly than XP cells, they should be able to recover from the potentially cytotoxic damage of AFB_1-Cl_2 if they are given time to carry out the repair processes needed before the critical time in the cell cycle, which determines cell killing, is reached. To test this hypothesis, a series of NF and XP cells, which were in the G_0 state after being grown to confluence, were treated with various concentrations of carcinogen and assayed for survival at various times over a 10 day period following treatment. The results are shown in Figures 22a and 22b. Cultures of normal fibroblasts revealed the ability to recover to a survival of up to 85% under

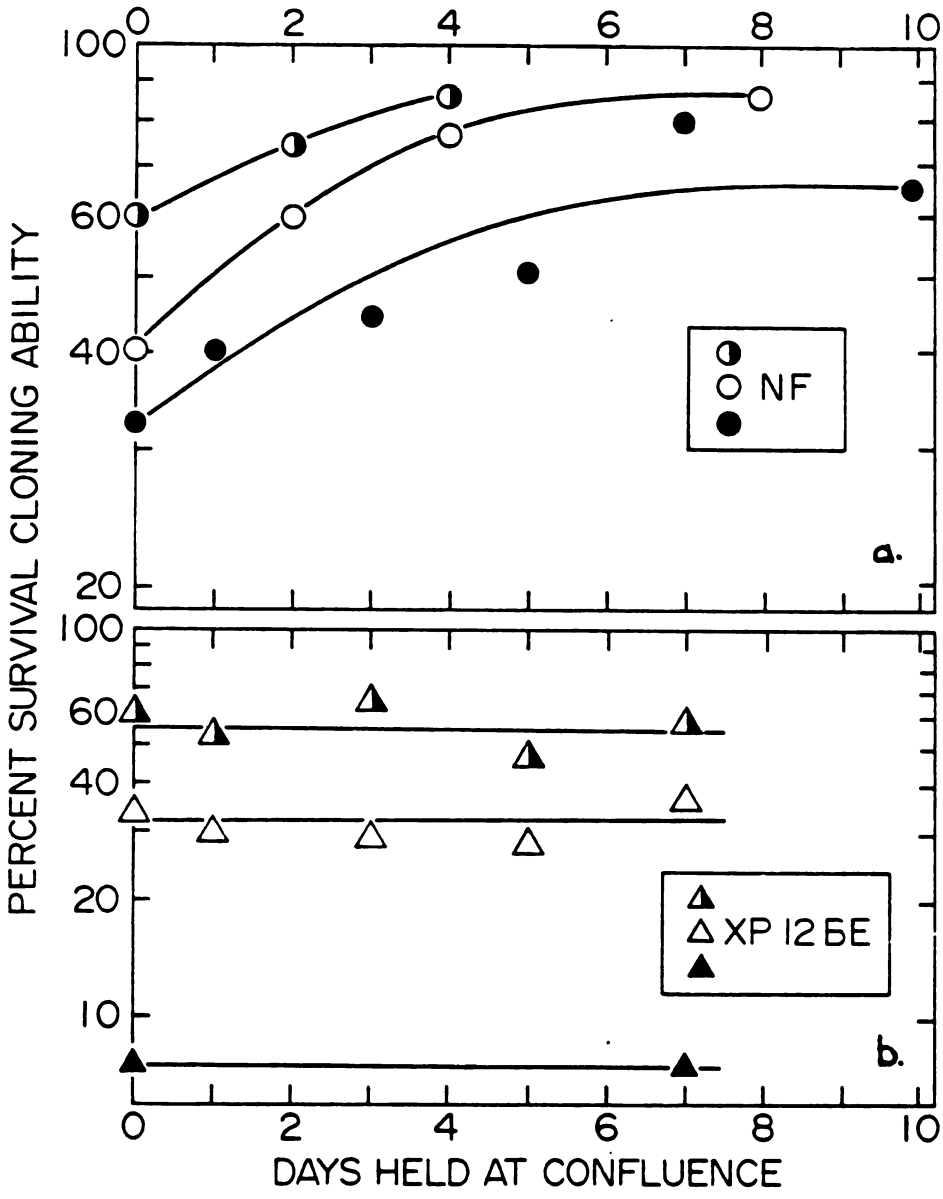


Figure 22. Recovery of confluent cells from the potential cytotoxicity of AFB_1-Cl_2.

these conditions. Cells treated with a concentration of carcinogen which gave an initial survival of ~ 60% exhibited 85% survival by 4 days, while cells with an initial survival of ~ 40% required 8 days to reach 85% survival. Cells with an initial survival of ~ 35% showed a survival of only 70% after 10 days at confluence (Figure 22a). In contrast, XP cells exposed to three different concentrations of carcinogen showed no significant change in survival over seven days (Figure 22b). These data strongly suggest that normal cells are capable of excising the potentially cytotoxic lesion(s) while XP cells are not.

Comparative sensitivity to the mutagenic effects of AFB_1-Cl_2

In order to investigate the mutagenic effcts of AFB_1-Cl_2 in human cells in culture, normal and XP12BE cells were treated with varying concentrations of AFB_1-Cl_2 and assayed for the frequency of induction of 6-thioguanine resistant mutants. XP12BE cells showed a markedly greater sensitivity to the mutagenic effects of AFB_1-Cl_2 than did NF cells (Figure 23 and Table 4). These data suggest that it is the ability of NF cells to excise the mutagenic lesion(s) induced by AFB_1-Cl_2 that is responsible for the greater resistance of NF cells to the mutagenic effects of AFB_1-Cl_2.

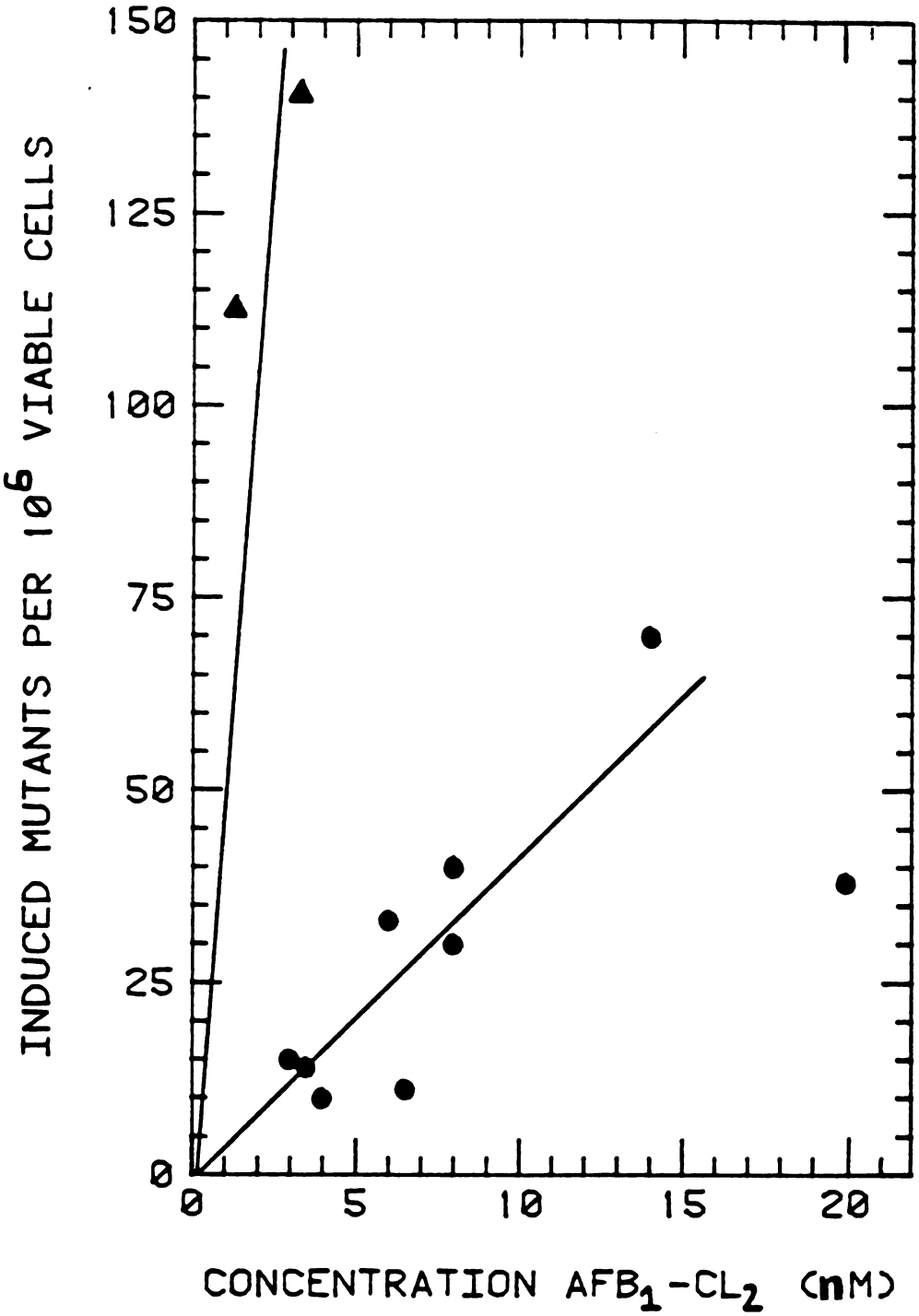


Figure 23. Induction of 6-thioguanine-resistant mutants by AFB_1-Cl_2 in normal and XP cells.
(●) NF; (▲) XP

Table 4. The induction of 6-thioguanine-resistant mutants by AFB_1-Cl_2 in NF and XP cells

Cell strain	concentration AFB_1-Cl_2 (μM)	original percent survival	TG resistant colonies	cells plated ($x10^{-6}$)	Replating efficiency	Efficiency of recovery of mutants	Mutation frequency ($x10^{6}$)
XP12BE	0	100	0	1.76	.057	1.0	0
	1.3	68	21	1.76	.053	1.0	119
	3.0	37	16	1.76	.055	1.0	140
NF*	0	100	0	1.06	.17	1.0	0
	3.7	87	4	1.06	.24	1.0	15
	5.9	47	8	1.06	.20	0.95	33
	8.0	34	9	1.06	.20	1.0	41
NF	0	100	3	1.76	.37	0.85	4.7
	3.0	80	15	1.76	.46	0.88	14
	4.0	67	8	1.76	.31	0.82	10
	6.2	50	12	1.76	.47	0.92	13
NF*	0	100	0	1.76	.12	0.94	0
	8.0	41	4	1.76	.10	0.92	25
	14.	26	13	1.76	.11	0.91	71
	20.	11	4	1.76	.074	0.85	36

* Cytotoxicity experiments were done by replating.

Rate of recovery of confluent cultures from the potentially mutagenic effects of AFB_1-Cl_2

At the same times that survival was assayed in the above biological recovery experiments, normal cells were also assayed for induced mutation frequencies, in order to determine whether they were capable of removing the potentially mutagenic DNA lesions induced by AFB_1-Cl_2. The results of these studies are shown in Figure 24. The normal cells appeared to recover from the potentially mutagenic effects of AFB_1-Cl_2, when held in a nondividing state. These data, together with the differential mutagenicity observed for NF and XP cells, suggest that normal cells are capable of excising the potentially mutagenic DNA lesion induced by AFB_1-Cl_2.

Rate of loss of covalently bound AFB_1-Cl_2 residues from cellular DNA

In the course of the biological recovery experiments described above, we also monitored the rate of loss of the covalently bound radioactive AFB_1-Cl_2 residues from human cell DNA by determining its specific activity at different times after treatment. At the same time that cells were harvested for assaying of survival and mutation frequency, a portion of cells (25-150 $x10^6$) were harvested for isolation of cellular DNA, and the specific activity

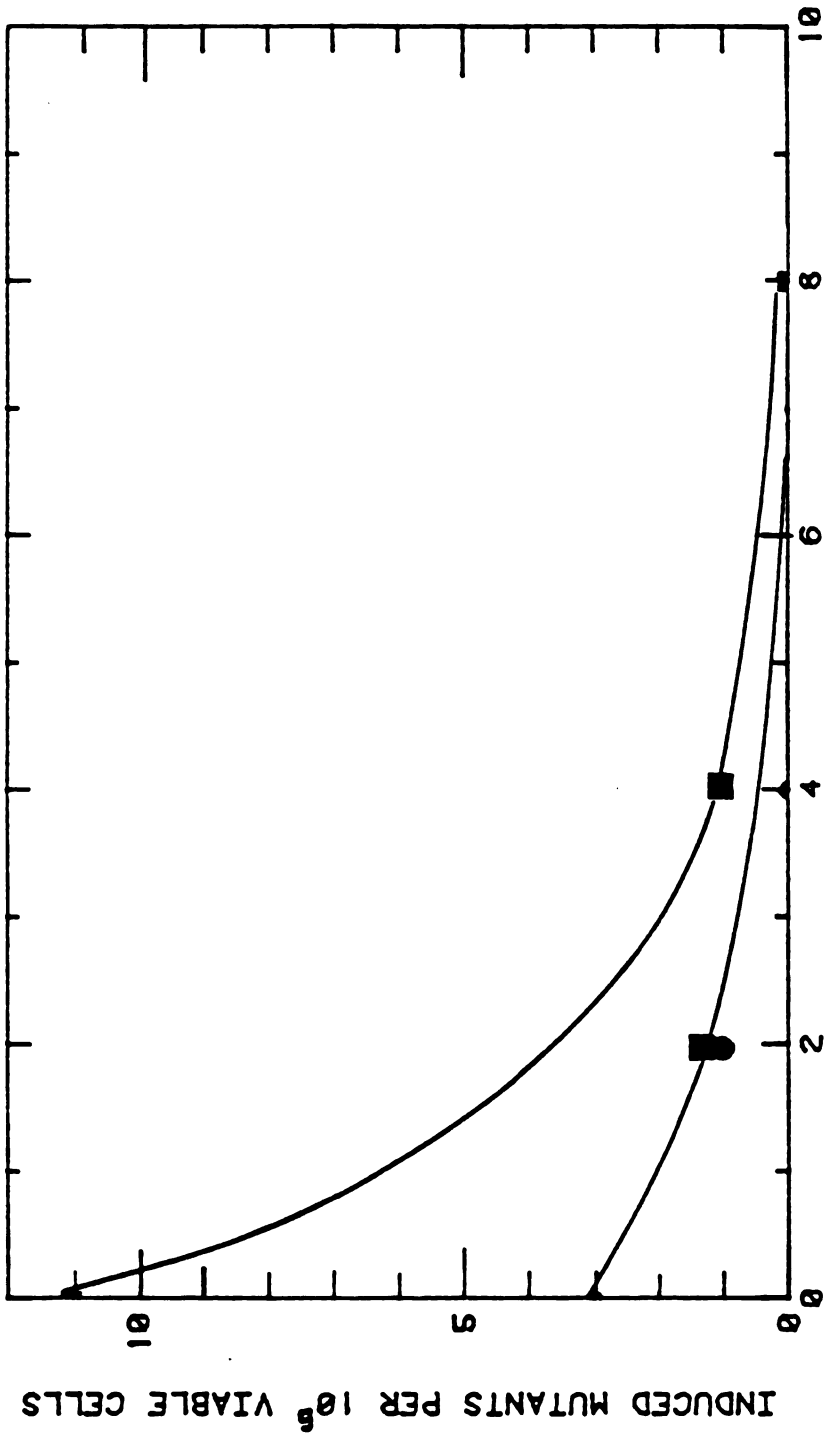


Figure 24. Recovery of normal cells from the potential mutagenicity of AFB_1-Cl_2.

of the DNA was determined. The results are shown in Figure 25. Table 5 shows the survival levels and the corresponding levels of binding (AFB_1-Cl_2 residues bound/10^6 nucleotides) for NF and XP cells in these experiments. At an initial survival level in XPs of ~8%, the level of binding was 0.92. For NF cells with initial survival levels of 60% and 45%, the levels of binding were 0.42 and 0.47 respectively. Unexpectedly, after 4 days at confluence, both normal and XP cells showed a loss of more than 40% of the total initially bound AFB_1-Cl_2 residues, and both cell strains showed approximately 35% remaining after 8 days. Since only the normal cells showed recovery from the potentially cytotoxic effects of AFB_1-Cl_2 (see Figure 22), there was no correlation between loss of total DNA-bound AFB_1-Cl_2 residues and the ability of cells to recover from the potentially cytotoxic effects of AFB_1-Cl_2. An interpretation consistent with these data is that the major DNA adduct(s) (accounting for more than 60% of the total) produced by AFB_1-Cl_2 in human cells in culture is(are) not responsible for producing the cytotoxic effects of AFB_1-Cl_2.

Rate of loss of covalently bound AFB_1-Cl_2 residues from calf thymus DNA

One explanation for our findings, that AFB_1-Cl_2 adducts are lost from the DNA of XP12BE cells, is that the

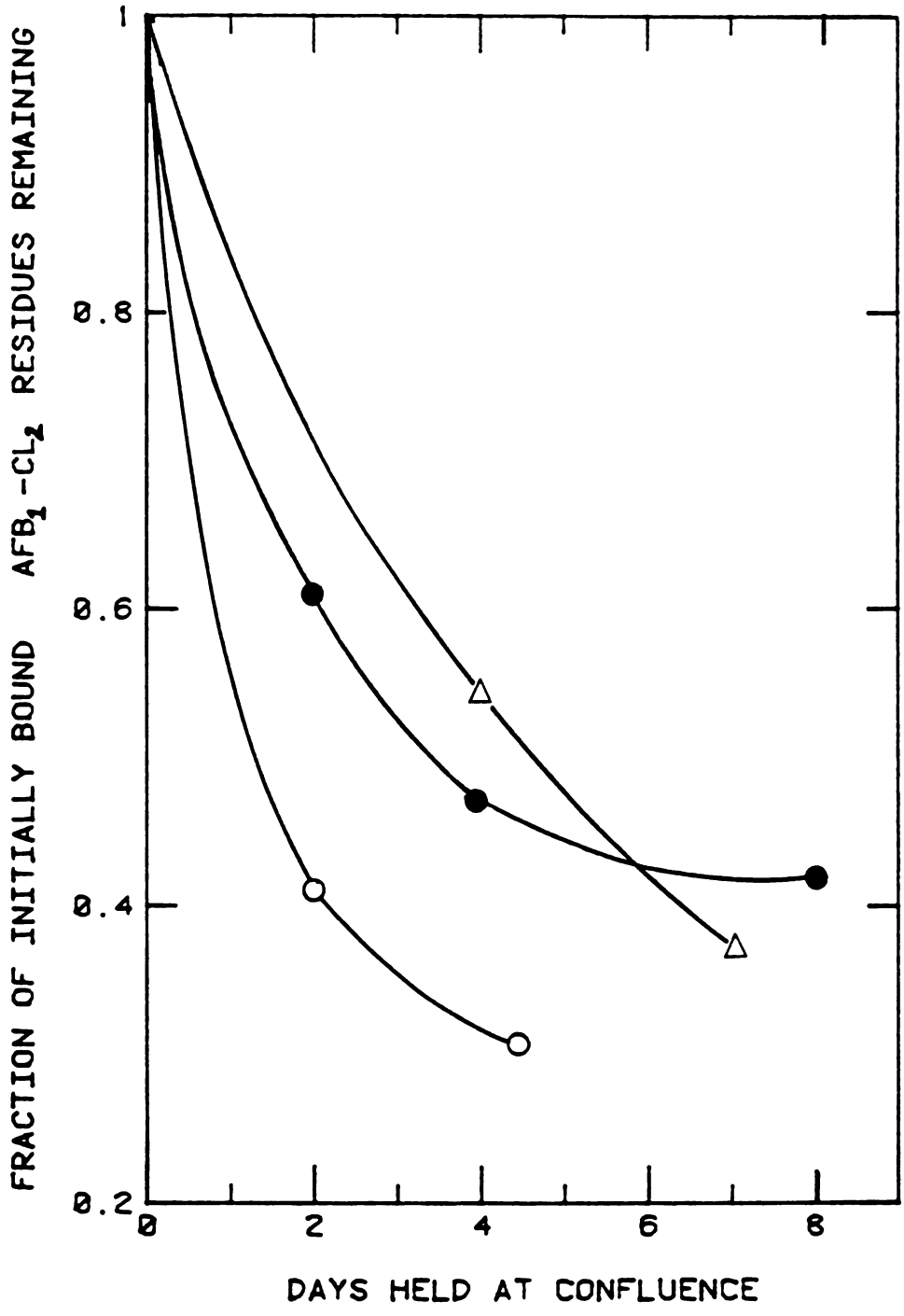


Figure 25. Loss of DNA-bound AFB_1-Cl_2 residues from NF and XP cells over time held in confluence. (●,○) NF; (△) XP.

Table 5. Levels of DNA binding in AFB_1-Cl_2-treated human cells over time.

Cells	Day	% survival	AFB_1-Cl_2 residues bound/10^6 nucleotides	fraction of initially bound residues remaining
NF	0	42	0.47	1.0
	2	58	0.29	0.617
	4	76	0.22	0.468
	8	85	0.20	0.425
NF	0	60	0.41	1.0
	2	73	0.17	0.414
	4	85	0.13	0.317
XP	0	7.6	0.92	1.0
	4	NA*	0.50	0.543
	7	7.9	0.33	0.367

*Data not available

adduct(s) is chemically unstable. Such instability has been reported by Wang and Cerutti (1979) for AFB_1 adducts. We, therefore, chose to study the rate of loss of DNA-bound AFB_1-Cl_2 residues form calf thymus DNA using the particular experimental conditions described in materials and methods. Figure 26 shows the kinetics of loss of total radioactivity from calf thymus DNA over eight days under these conditions. Only 38% of the initially bound residues remained after 4 days and, on day 8, only 31% remained. It appears from these data ,that a major portion of the DNA-bound AFB_1-Cl_2 residues are lost spontaneously from calf thymus DNA as a result of chemical instability. More importantly, the kinetics of loss of the covalently bound AFB_1-Cl_2 residues from calf thymus DNA resembled the kinetics of loss from the cellular DNA of both normal and XP cells (see Figure 25), suggesting that the major portion of the initially bound residues are lost from both kinds of human cells non-enzymically. However, the initial rate of loss by the normal cells appears to be slightly more rapid than that of XP12BE cells.

HPLC analysis of covalently bound AFB_1-Cl_2-induced DNA adducts in calf thymus DNA

Since DNA adducts were lost from calf thymus DNA with kinetics similar to that of cellular DNA, and since use of

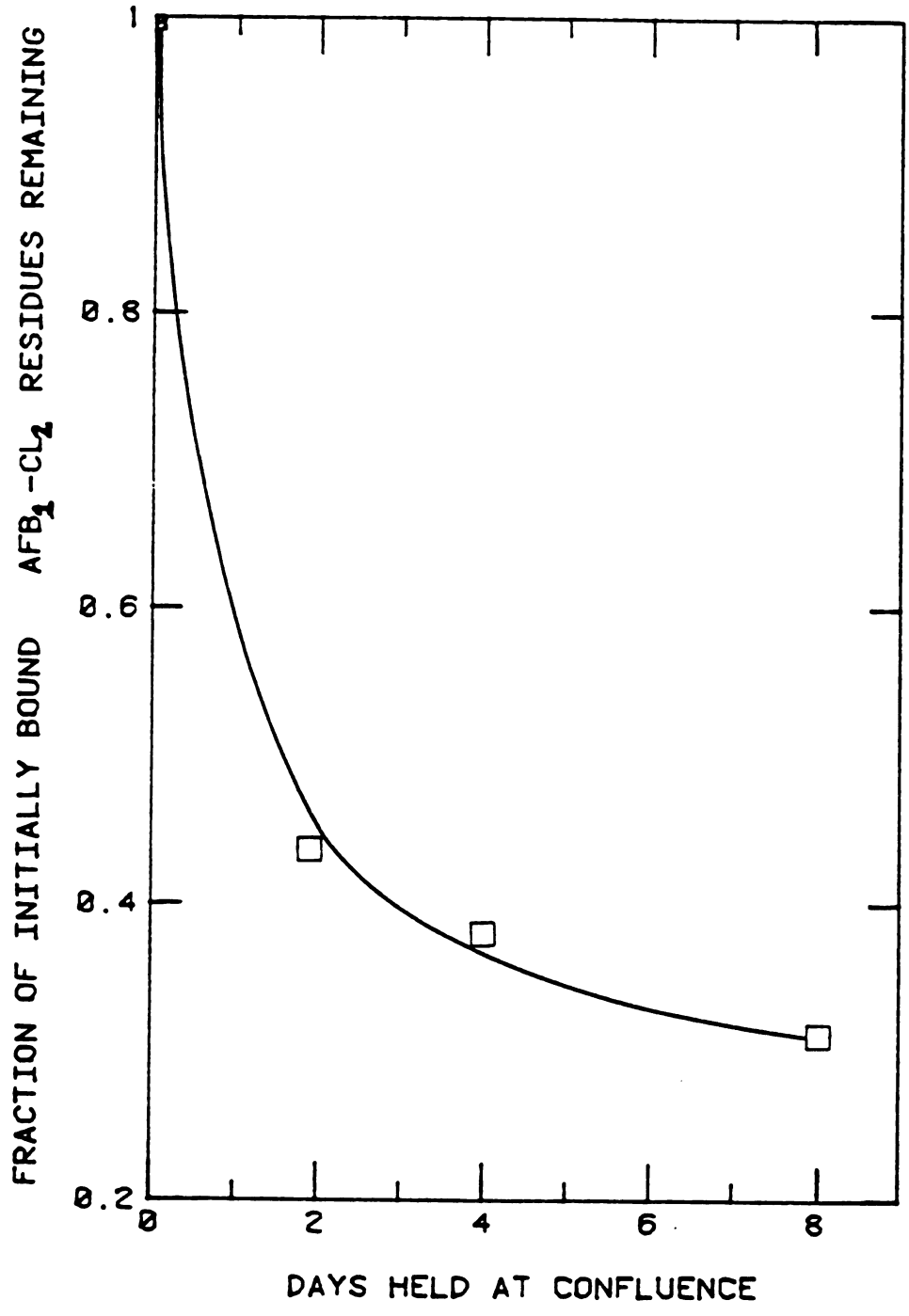


Figure 26. Loss of DNA-bound AFB_1-Cl_2 residues from calf thymus DNA over time.

calf thymus DNA allowed us to work with much greater quantities of DNA than were possible with cellular samples, we chose to study the loss of individual DNA adducts over time from calf thymus DNA. Calf thymus DNA was reacted with ^{3}H-AFB_1-Cl_2, as described, and the HPLC profiles obtained from the various samples of calf thymus DNA are shown in Figure 27. The day 0 profile revealed six distinct peaks, two of which were major peaks, a and b. Analysis of the DNA at later times showed that peak a was greatly diminished by day 2, and could not be detected on day 8. Peak a co-chromatographed with an adduct formed when dGMP was reacted with AFB_1-Cl_2. It appears, therefore, that a guanine adduct accounts for the major loss of DNA-bound AFB_1-Cl_2 residues from calf thymus DNA. Note that peaks b, c, d, e and f also showed a decrease in size over time. Peak b appeared to be the most persistent of the peaks observed. Approximately 50% of peak b is lost by day 2, but the loss appears to be slower from day 2 to day 8. Peaks a, b and c probably represent guanine adducts because they all co-chromatographed with dGMP products. While we did not structurally identify the adducts formed, it seems probable that peak a represents the N-7 guanine adduct of AFB_1-Cl_2, since it is the N-7 guanine adduct of AFB_1 which has been shown to account for most of the loss of AFB_1 residues from DNA through spontaneous depurination (Hertzog et al., 1980).

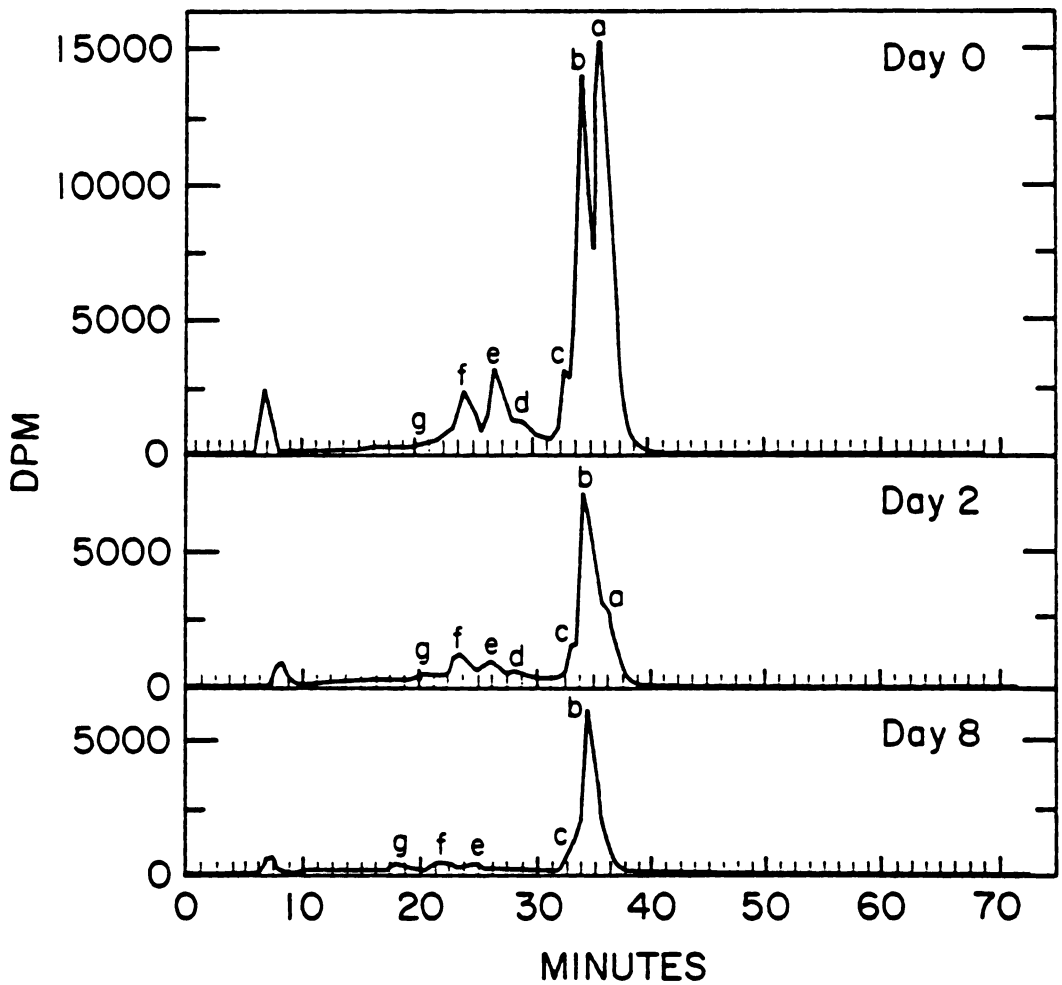


Figure 27. HPLC profiles of AFB_1-Cl_2 DNA adducts in calf thymus DNA on days 0, 2, and 8 after treatment.

HPLC analysis of covalently bound AFB_1-Cl_2-induced DNA adducts in human cellular DNA

Because of the extreme cytotoxicity of AFB_1-Cl_2 and the very low levels of DNA binding found in human cells exposed to biologically meaningful concentrations of this carcinogen, it was not possible to analyze the DNA adducts formed or remaining in such cells to our satisfaction. Furthermore, the low specific activity of these cellular DNA samples did not allow us to detect all the peaks seen in calf thymus DNA. Therefore, we treated a large number of normal human cells (200×10^6) with a very high concentration of ^3H-AFB_1-Cl_2 (about 400 times the concentration required for a 37% survival response) in an attempt to obtain a more complete adduct profile, which could then be compared to the calf thymus profile. The results in Figure 28 showed that the same DNA adducts were formed in human cells treated this way as were formed in calf thymus DNA. Since peaks a and b were the major products formed in both cellular and calf thymus DNA, and since the kinetics of loss of adducts were quite similar in the two systems (Figure 25 and 26), the data suggested that the major loss of DNA-bound AFB_1-Cl_2 residues from human cells in culture was in the form of the product in peak a, which is a guanine adduct and was probably the N-7 guanine adduct.

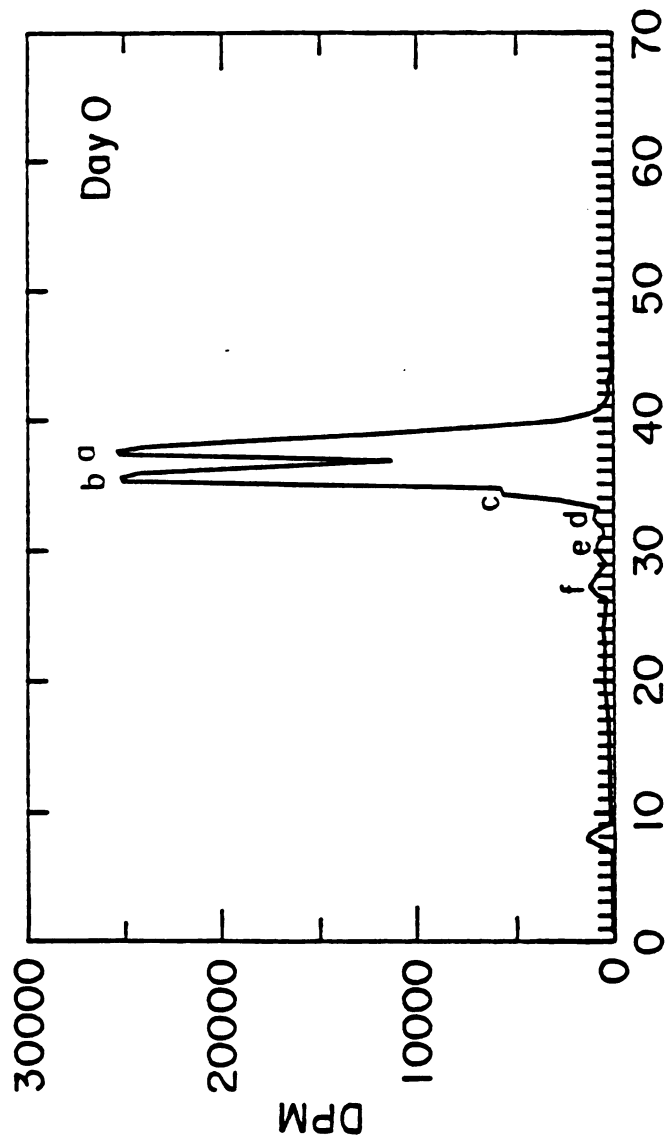


Figure 28. HPLC profile of AFB_1-Cl_2 DNA adducts in human cellular DNA on day 0.

The six peaks which were observed in calf thymus DNA at the earliest times examined (day 0) were also present in the human cell DNA on day 0. There were, however, some differences observed in the ratios of the peaks obtained. While peaks a and b appeared approximately equal to each other in size in both calf thymus and cellular DNA, the proportion of the total which the smaller peaks d, e and f appeared to represent in human cell DNA appeared smaller than in calf thymus DNA on day 0. It is possible that these differences are the result of slight differences in the experimental conditions, or that different adduct ratios are produced *in vivo* and *in vitro* as has been reported for AFB_1 (Garner and Martin, 1977).

DISCUSSION

Cell-mediated activation of AFB_1

The results of our studies, involving human carcinoma-derived cells (Figures 12 and 13), have shown that a human cell-mediated system can be used to activate AFB_1 into a form capable of damaging human target cells. This system makes it possible to study the biological effects of AFB_1 in human fibroblasts.

As pointed out above, Langenbach *et al.* (1978) have shown that AFB_1, which is a potent liver carcinogen, is metabolized more extensively in primary rat liver cells than in rat skin fibroblasts to a component which can induce mutations in V79 cells. Benzo(a)pyrene (BP), which is a powerful skin and lung carcinogen, but not an hepatic carcinogen is more extensively converted by rat skin fibroblasts than by rat liver cells to a metabolite which induced mutations in V79 cells (Autrup *et al.*, 1979). This provides evidence for cell specificity in the metabolism of chemical carcinogens.

Results from our studies indicate that the liver carcinoma-derived human cell line metabolized AFB_1 to a greater extent than did the kidney carcinoma-derived cell line (Figure 14), supporting the observations of Langenbach et al. (1978) and other data obtained from studies with whole animals, indicating that greater metabolic activation of AFB_1 occurs in rat liver cells than in rat kidney cells (Wogan et al., 1971). However, tumor-derived transformed cell lines which have been grown in culture for a long period of time have properties that differ in many ways from the diploid cells of the organ in which the tumor originated. It has been demonstrated that the human liver carcinoma-derived cell line used in our studies is more efficient at converting BP into its toxic metabolite than are cell lines derived from human lung or kidney tumors (Aust et al., 1980). This differs from the situation in vivo for this chemical (Autrup et al., 1979). Our data with AFB_1 metabolism support the idea that there is cell specificity in the metabolism of carcinogens in vivo, but we recognize that this specificity may be fortuitous.

Our initial studies, showing that at equal concentrations of AFB_1, Hs703T cells were more sensitive than XP cells (Figure 17), suggested to us that Hs703T cells were either deficient in excision repair or that their greater cytotoxic response was the result of more of the reactive metabolite of AFB_1 reaching the target DNA in

these cells. The results of our comparative studies using the direct acting carcinogen, AFB_1-Cl_2, in which the survival of Hs703T cells proved equal to that of normally repairing cells (Figure 18), supported the latter hypothesis. The hypothesis that, because AFB_1 metabolism was taking place in these cells, they received a higher effective dose than the XP cells predicts a greater initial binding of AFB_1 to DNA in Hs703T cells than in XP cells, and a lower level of DNA repair synthesis in XP cells than in Hs703T cells following AFB_1 treatment. However, such studies have not yet been carried out.

AFB_1-induced DNA lesions are "UV-like"

In the presence of rat liver microsomes, AFB_1 has been shown to induce cytotoxicity in *Salmonella typhimurium* TA 1530 and TA 1531, both of which have a deletion in the genes coding for repair of ultraviolet light-induced DNA damage (Garner, Miller, and Miller, 1972). Strains of *S. typhimurium* without this deletion are resistant to AFB_1. AFB_1 is toxic in mutant strains of *E. coli*. Although the excision repair mutants are more sensitive than recombination repair mutants, the double mutant (uvr^- rec^-) is the most sensitive (Garner and Wright, 1973). This is the same order of sensitivity observed for UV light-induced toxicity. Stich and Laishes (1975) and Sarasin *et al*. (1977) have reported that AFB_1 induces

DNA repair synthesis in normal human cells when the cells are treated in the presence of a rat liver activation system. No repair synthesis was induced in XP cells. The results of our studies, using a human cell mediated activation system, indicate that excision repair-deficient XP cells are more sensitive to the cytotoxicity induced by AFB_1 than are normal human cells for all concentrations tested (up to 12 μM) (Figure 17). The resistance observed in the normal cells is probably reflecting excision repair of the majority of the cytotoxic lesion(s) during the 24 hour exposure time. Taken together, these data strongly suggest that the cytotoxic lesion(s) induced by AFB_1 is "UV-like" (i.e. repaired by a process which has one or more steps in common with excision repair of UV-induced lesions), and that the ability to carry out excision repair protects cells from the cytotoxic effects of AFB_1.

AFB_1-Cl_2-induced DNA lesions are "UV-like"

We have shown in the present studies that XP cells were more sensitive than normal cells to the cytotoxic (Figure 19) and mutagenic effects (Figure 23) of AFB_1-Cl_2, and that the degree of resistance of a series of XP cell strains to the toxic effects of AFB_1-Cl_2 correlated with their ability to carry out excision repair of UV-induced DNA damage (Figure 20). Furthermore, we showed that XP12BE cells exhibited a lower survival than normal cells

when initial DNA binding levels for AFB_1-Cl_2 were equal (Figure 21). XP cells are deficient in the exicision repair of UV light-induced DNA damage (Cleaver et al., 1968). We, therefore, propose that excision repair of lesions induced by AFB_1-Cl_2 is "UV-like".

Relationship between time for repair and the ultimate biological effects of carcinogen

It is important to note that binding is measured immediately after treatment, whereas survival cannot be measured until a later time, since it is dependent upon a cell being able to carry out all the steps necessary for repeated cell replication, so that a colony will be formed. If we assume that a cell's chance of survival is determined by the average number of potentially toxic carcinogen residues remaining bound to DNA at some later time, as has been suggested for UV (Konze-Thomas et al., in press) and benzo(a)pyrene diol epoxide (BPDE) (Yang et al., in press), and, that at equal concentrations administered an equal amount is bound to DNA in NF and XP cells, then the in situ survival data suggest that NF cells are excising a portion of the initially bound carcinogen residues so that an increased number of cells are able to replicate and form colonies. They further suggest that if individual cells in the population could be assayed for the level of DNA binding at the precise time that their survivals were

determined, they would exhibit the same average level of binding as XP cells with the same survival. Results from our mutagenicity studies (Figure 23) showed that XP cells were more sensitive than NF cells to the mutagenicity of AFB_1-Cl_2, suggesting that normal cells are removing some of the potentially mutagenic lesions before mutations are "fixed" in the cell.

Normal cells showed the ability to recover from the potentially cytotoxic effects of AFB_1-Cl_2, (Figure 22), and also showed the ability to recover from the potentially mutagenic effects of AFB_1-Cl_2 (Figure 24). We also noticed that the initial frequency of mutations observed in these cells treated in confluence and released was lower than would be expected for cells treated while in exponential growth. Autoradiography studies (Konze-Thomas et al., 1979) and studies measuring shift in buoyant density of DNA replicating in the presence of BudR (Yang et al., in press) showed that cells released from confluence have more time before DNA replication than do exponentially growing cells. The population taken from confluence is in the G_o state and does not begin DNA synthesis for ~20 hours. The reduced mutation frequency observed in the cells released from confluence is probably the result of these cells excising more of the potentially mutagenic lesions than do the dividing cells before DNA replication.

Experiments by a number of workers from this laboratory showed that normal cells are capable of excising the potentially cytotoxic and mutagenic DNA adducts induced by a series of aromatic amides (Heflich et al., 1980) and by the anti isomer of the 7,8-diol, 9,10-epoxide of benzo(a)pyrene (BPDE) (Yang et al., 1980), which is the ultimate carcinogen of BP (Sims et al., 1974). The data from the present study, showing a three-fold greater than normal sensitivity of XP cells to the killing action of AFB_1-Cl_2 (Figure 19) and approximately seven-fold greater sensitivity to its mutagenic action (Figure 23), strongly suggest that normal cells are capable of excising the potentially cytotoxic and mutagenic DNA lesion(s) induced by AFB_1-Cl_2, and that the ability to carry out excision repair of this lesion(s) protects the normal cells from the potential cytotoxicity and mutagenicity of AFB_1-Cl_2. In contrast, XP12BE cells apparently cannot carry out this type of repair.

Lack of correlation between rate of loss of AFB_1-Cl_2 DNA adducts and recovery of cells from the potential biological effects

Normal cells held in a non-replicating state have been shown to excise the DNA adduct induced by various aromatic amides (Heflich et al., 1980) and that formed by BPDE (Yang et al., 1980) at the same rate that cells recover

from the potentially cytotoxic effect of these agents. In contrast, XP cells show no increase in survival and no loss of DNA adducts with time held in confluence. The data from the present study also showed that the rate of loss of DNA-bound AFB_1-Cl_2 residues from normal cells corresponded to the rate of recovery observed for these cells from the cytotoxic effects of AFB_1-Cl_2 (Figures 22 and 25). However, although XP cells showed no ability to recover from the cytotoxic effects of AFB_1-Cl_2, they showed a loss of DNA-bound residues over time (total counts) at a rate only slightly slower than that seen in the normal cells (Figure 25). More than 60% of the total DNA bound carcinogen residues were lost from the DNA of both normal and XP cells over seven or eight days. These data indicate that the adduct(s) responsible for the cytotoxic effect of AFB_1-Cl_2 must be minor in number. Otherwise, their continued presence in XP cells would have been manifested in the data of Figure 25.

Since XP cells are much more sensitive to the mutagenic effects of AFB_1-Cl_2, the DNA adduct(s) responsible for the mutagenicity induced by AFB_1-Cl_2 would not appear to be the major adduct(s) lost from NF and XP cell DNA. These data suggest that the potentially cytotoxic and mutagenic adduct(s) formed by AFB_1-Cl_2 is minor in amount. It was surprising to find that there was a major DNA adduct, which in normal cells was lost with the same

kinetics as that of the decrease in the mutations and cell killing, but which was not responsible for induction of toxicity or mutations in human cells in culture. However, there is precedent in the literature for the idea that minor adducts can be more significant than the major adducts. For example, methylnitrosourea (MNU) produces three major DNA adducts. The most predominant of these is the N-7 guanine adduct, which makes up about 70% of the total initially bound residues (Lawley, 1966). Reports in the literature indicate that this adduct is not involved in the induction of mutations in V79 cells by MNU, but that a minor adduct, 0-6 methyl guanine, is the mutagenic adduct (Newbold et al., 1980). The 0-6 methyl guanine adduct comprises approximately 7% of the total DNA adducts. It has not been clearly demonstrated which adduct(s) is responsible for cytotoxicity, but there is evidence to suggest the N-3 adduct of adenine is involved (Medcalf and Connell, in press).

There are considerable differences between the types of DNA lesions produced by MNU and those produced by AFB_1-Cl_2. For example, MNU results in a methyl group binding to the DNA base, while AFB_1-Cl_2 results in a bulky five ring structure binding to DNA. MNU is not repaired by an excision repair process which has a step in common with repair of UV-induced lesions, (Lawley, 1981) whereas from our work, it appears that AFB_1-Cl_2-induced DNA

lesions are repaired by such a system. Nevertheless, MNU offers a good example of a carcinogen for which the major DNA adduct produced is not responsible for the biological effects.

In contrast to our results with AFB_1-Cl_2, studies in this laboratory by Yang et al. (1980) have shown a high correlation between the rate of loss of the N-2 guanine adduct and the rate of recovery of normal cells from the cytotoxic and mutagenic effects of the 7,8-diol-9,10-epoxide of benzo(a)pyrene (BPDE).

The N-2 guanine adduct, which is stable, was the only DNA adduct detected in those studies. It is, of course, possible that the N-2 guanine adduct observed in these studies is actually not cytotoxic or mutagenic, and that the enzymic loss of this adduct is not important in effecting the biological recovery observed in normal cells. The possibility exists that there was an unstable adduct which was lost from the DNA during the isolation and purification procedure, which included a 40 hour cesium chloride gradient and the lysing of cells at 60°C for 30 minutes, or that there was an adduct too minor to be detected in these samples, which is also lost enzymically from normal cells, and which is responsible for the cytotoxic and mutagenic effects of BPDE. However, to account for the data these hypothetical adducts would have to be lost with

exactly the same kinetics as the N-2 guanine adduct.

Loss of the major DNA adduct induced by AFB_1-Cl_2 is non-enzymic

There have been reports in the literature concerning the loss of DNA-bound AFB_1 residues *in vivo* and *in vitro*. Wang and Cerutti (1979) reported that loss of total AFB_1 was faster in intact human cells in culture than from human cell DNA *in vitro*. They therefore concluded that at least a portion of the total DNA-bound AFB_1 residues was removed by a cellular process. Hertzog, Smith, and Garner (1980) monitored the loss of product from rat liver DNA following intraperitoneal administration of ^3H-AFB_1. They reported that the rate of loss of AFB_1-guanine, which accounts for more than 80% of the initially bound residues, is the same *in vivo* and *in vitro*. They attributed the difference between their results and Wang and Cerutti's results to differences in the pH of the incubation mixture and conclude that a considerable portion of the initially bound AFB_1 residues is lost from DNA *in vivo* due to chemical rather than enzymic loss. A recent report by Groopman *et al.* (1981) has shown that the kinetics of loss of AFB_1-guanine from calf thymus DNA varies considerably under various experimental conditions. Both the pH of the incubation mixture and the level of substitution affected the kinetics of loss in these

studies. However, there was spontaneous depurination of AFB_1-modified calf thymus DNA observed under all conditions studied. It appears from these reports that AFB_1-guanine is lost spontaneously from calf thymus DNA. However, the kinetics of chemical loss occurring within intact cells is not clear, since the conditions in the nucleus are not known, and may in fact, vary with different stages of the cell cycle. Our studies showed that the kinetics of loss of DNA-bound residues was similar in normal cell DNA and XP cell DNA, although the rate appears to be slightly faster in normal cells than in XP cells (Figure 25). The rate of loss of residues from calf thymus DNA is similar to that seen in cellular DNA, however this rate is dependent on the incubation conditions. While this loss indicates that there is spontaneous loss of AFB_1-Cl_2 residues from calf thymus DNA, we cannot know that our incubation conditions reflect the conditions present in our cells, and therefore the exact kinetics of chemical loss in cells is probably best represented in the XP cell profile. These data suggest that the loss of these DNA lesions is non-enzymic. Although one could explain a similar rate in normal and XP cells by invoking a common enzymic repair process other than that involved in the repair of UV-like lesions, it is difficult to explain the loss from calf thymus DNA from anything other than chemical loss. However, we did not do repair replication studies to see if XP cells were excising DNA lesions.

Isolation of the major DNA adducts induced by AFB_1-Cl_2

It has been shown by several groups that the major DNA adduct formed by AFB_1 *in vivo* and *in vitro* is the N-7 guanine adduct, and that this adduct makes up between 80-95% of the total initially bound residues (Essigman *et al.*,1977; Garner and Martin, 1977; Hertzog *et al.*,1980; Groopman *et al.*,1981). It has also been shown that the N-7 guanine adduct is lost *in vivo* and *in vitro* with a half-life between 7.5 and 100 hr. depending upon the conditions of the study (Wang and Cerutti, 1979; Hertzog *et al.*,1980). These studies have also suggested that a fraction of the N-7 guanine lost during this period is transformed into a more stable ring-opened form, which appears to be a more persistent lesion in the DNA. This transformation takes place quickly with AFB_1, and in calf thymus DNA, the presence of this fission product is at its maximum 24 hours after treatment (Croy and Wogan, 1981; Groopman *et al.*, 1981). There have been thirteen DNA adducts identified for AFB_1, all of which are guanine and adenine adducts. As pointed out above, the HPLC profiles of DNA adducts which we obtained from calf thymus DNA and normal cells treated with AFB_1-Cl_2 initially revealed six distinct peaks on day 0 (Figures 27 and 28). Peaks *a* and *b* were predominant. We observed that peak *a* was eventually lost completely from calf thymus DNA, and that

the other five peaks were also decreased to some extent over time.

Because of the unstable nature of the major DNA adduct produced by AFB_1-Cl_2 (peak a) it is very probable that a portion of this adduct is being lost from DNA during the DNA isolation procedure which we used. The small amount of DNA obtained from cultured cells makes it impossible to precipitate the DNA with ethanol, as can be done with DNA from animal material or commercial calf thymus DNA. It was, therefore, necessary to purify our DNA by means of a cesium chloride gradient, which was run at 25°C for 18 hours. This also insured removal of any non-covalently bound carcinogen (intercalated). Since this procedure was necessary for our cellular samples, we purified the calf thymus DNA on cesium chloride as well, in order to allow comparison between the two profiles. Because of this procedure, our samples were at 25°C for nearly one day before they were actually assayed for adducts. This contrasts with studies in the literature, in which this lag time was only a few hours. It is probable, therefore, that if our adduct profiles were taken immediately after treatment, peak a and, possibly, peak b would be considerably larger than we observed. In support of this prediction, we found that when a cellular DNA sample was run on cesium chloride for 60 hours, peak a had been lost from the DNA before the HPLC profile was obtained. This latter profile,

shown in Figure 29, indicates the care that must be taken to process all DNA samples consistently when working with carcinogen adducts as unstable as AFB_1-Cl_2.

It is also interesting to note that there appear to be some quantitative differences between the cellular and calf thymus samples in the proportion of the total that each adduct comprises. For example, peaks d, e, and f appear to be in smaller percentages in the cellular DNA than in the calf thymus DNA. Garner and Martin (1977) have reported for AFB_1, that although the same DNA adducts are formed in vivo and in vitro, the ratios of those adducts differ.

Possible mechanisms of carcinogenesis by AFB_1 and AFB_1-Cl_2

It has been reported in the literature that AFB_1 is the most powerful rat hepatocarcinogen known. This is based on the very low concentrations of AFB_1 required to induce liver tumors in these animals. AFB_1-Cl_2 also has been shown to be a powerful carcinogen in rats, and also requires very low doses to induce tumors (Swenson et al., 1977). In our present studies, we have observed that both AFB_1 and AFB_1-Cl_2 required very low doses to induce mutations and/or cytotoxicity in human cells in culture.

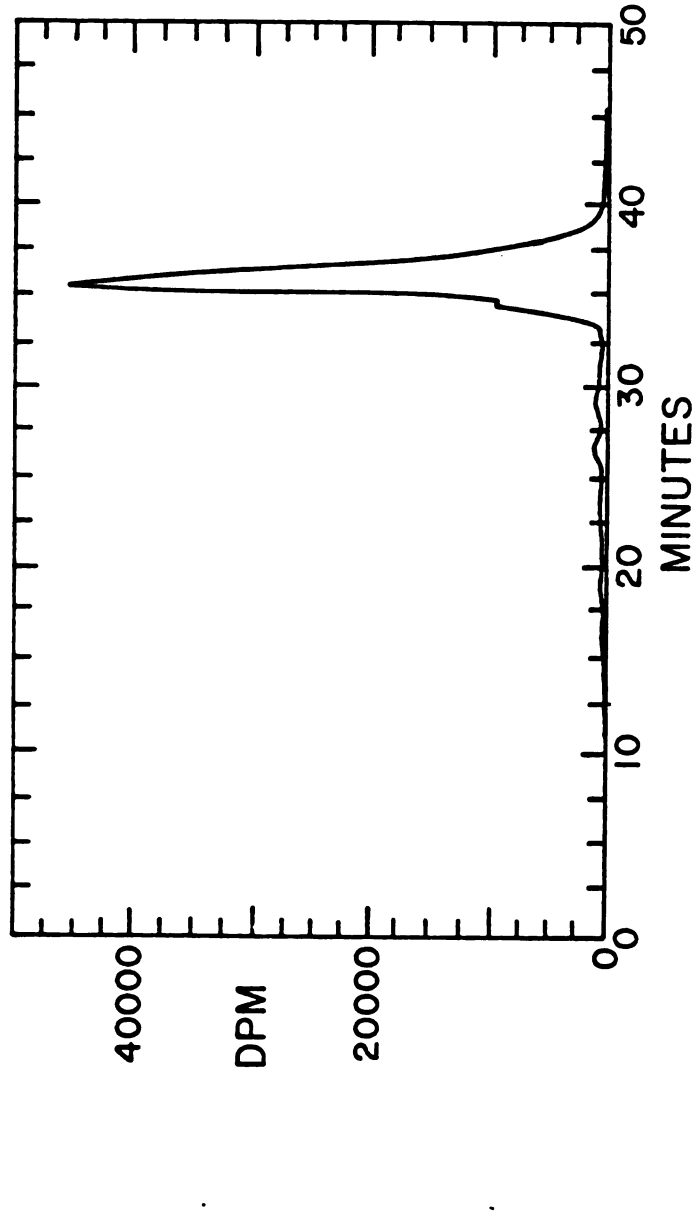


Figure 29. HPLC profiles of AFB_1-Cl_2 DNA adducts in human cellular DNA following a 60 hour cesium chloride gradient.

AFB_1-Cl_2 appears to have a very high mutagenic efficiency. In Drosophila, at equal doses, AFB_1-Cl_2 was 100 times more efficient in inducing mutations than was AFB_1, probably because of the need for activation of AFB_1. Our studies with AFB_1-Cl_2 revealed that, when compared with other mutagens on the basis of mutation frequency per DNA adduct, AFB_1-Cl_2 was the most powerful mutagen ever studied in this system. We also showed that AFB_1-Cl_2 was highly toxic to human cells at very low concentrations. This extreme cytotoxicity may contribute to its carcinogenicity in vivo by causing the replication of the surviving cells to repopulate the liver. This would allow some initiated (mutated) cells to begin the clonal growth necessary for tumor formation. This has been suggested as a possible mechanism for the induction of tumors by other carcinogens (Craddock, 1975).

The nature of the potentially mutagenic lesion(s) induced by AFB_1 or AFB_1-Cl_2 is still unclear. A report published by Wang and Cerutti (1979) proposed that for AFB_1, the initially formed N-7 guanine adduct is not mutagenic, but that it undergoes a fission of the imidazole ring of guanine, which stabilizes the adduct. They propose that since this form of the adduct is more persistent in DNA, it may contribute to the mutagenicity of AFB_1. It is important to note, however, that while the ring-opened form is the predominant lesion found in rat liver DNA after 48

hours, the number of these lesions (residues bound/10^6 nucleotides) peaks at 24 hours, and at the peak, the total is less than 19% of the initial concentration of AFB_1-N-7 guanine. Although another report by Croy and Wogan (1981) states that the level of the ring opened form (expressed as percent of total bound residues) is relatively stable at 70% after 24 hours, it is important to note that the actual number of these residues bound to DNA in rat liver DNA *in vivo* decreases from its maximum at 24 hours to approximately 41% of that maximum by 72 hours. However, it is apparent from these studies that the ring-opened product, once it is formed, is being lost more slowly from rat liver DNA *in vivo* than is the N-7 guanine adduct.

In our HPLC profiles of AFB_1-Cl_2- modified calf thymus DNA, we did not detect any such transformation product. However, if the transformation were completed by 24 hours, as has been reported for AFB_1, any transformation in our system would have taken place before our first profile was obtained, since our isolation procedure required more than 24 hours. It would be important in future studies to analyze an adduct profile of AFB_1-Cl_2-treated calf thymus DNA which was isolated by ethanol precipitation, in order to avoid the 20 hour delay in the present studies. Besides telling us how much of peak *a* was lost during this time, it would also tell us whether there is a transformation of products, as has been reported for AFB_1. If

any of the peaks present in our initial profile were missing in the new 0 time profile, it would suggest that this peak was formed by transformation of one of the other adducts. If such a transformation in DNA adducts were seen, it would then be interesting to investigate whether it is this product which is responsible for the biological effects of AFB_1-Cl_2. This could possibly be done by synchronizing cells (especially XP cells since there would then be no repair going on to interfere with interpretation of results) and treating them with carcinogen at different times in the cell cycle prior to S. If we assume that DNA replication is the critical event responsible for fixing mutations , as has been indicated with BPDE (Yang et al., in press) and UV light (Konze-Thomas et al.,in press), then we would expect to see a lower frequency of mutations in cells treated just before S if the initial DNA adduct is not mutagenic, but must first undergo a stabilization process. Our preliminary results from one experiment indicated that cells treated just prior to S showed a lower frequency of mutations than cells treated asynchronously at the same concentrations of AFB_1-Cl_2. Since the cell synchrony method previously used in this laboratory results in an extended S period, a different method for synchronizing cells (presently being developed) may be necessary for these studies.

Conclusion

In summary, our results indicate that AFB_1-Cl_2 is a good model compound with which to study the biological effects of AFB_1 in human cells, since it is highly toxic and mutagenic in human cells as is AFB_1. AFB_1-Cl_2-induced DNA lesions appear to be "UV-like", that is, they are repaired by a process which has one or more steps in common with excision repair of UV-induced lesions. It appears that excision repair can reduce the potentially cytotoxic effects of both compounds and that it can reduce the potentially mutagenic effect of AFB_1-Cl_2. The major DNA adducts induced by AFB_1-Cl_2 appear not to be responsible for the potential cytotoxicity and mutagenicity induced in human cells in culture. We speculate that the extreme cytotoxicity, in conjunction with the mutagenic effects of AFB_1 and AFB_1-Cl_2 may be responsible for their carcinogenic potency *in vivo*.

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