DNA REPAIR SYNTHESIS IN CULTURED HUMAN FIBROBLASTS AS A BIOASSAY FOR CHEMICAL CARCINOGENS

by

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ABSTRACT

The suggestion from epidemiological studies that 80 to 90 per cent of all human cancers may have an environmental factor in its etiology, coupled with the wide use of chemicals in a modern society calls for a simple, rapid and economic prescreening programme to identify chemical carcinogens in the environment. Measures can then be taken to prevent or effectively reduce the exposure of human beings to these agents.

The standard "rodent painting and feeding" test for carcinogenicity of a chemical compound (endpoint being tumour production) is unsuitable for a large screening programme. The cost and logistics of handling thousands of rats or mice (200 - 500 rodents per chemical) is staggering. Besides, the completion of this test requires a relatively long time (up to 2 years).

Most, if not all, chemical carcinogens bind to DNA. Furthermore, almost all DNA-damaging agents, whether physical or chemical, that have been investigated in the proper test system show evidence of a repair effect. This observation raises the possibility of monitoring carcinogen-induced DNA damage and repair as a screening procedure for identifying chemical carcinogens.

Previously, the extent of DNA repair (autoradiographic detection of unscheduled ^{3}HTdR incorporation) in hamster and human cells following exposure to strongly, weakly

and non-oncogenic isomers and derivatives of 4-nitroquinoline 1-oxide (4NQO) was examined. A good correlation was observed between the oncogenicity of a compound and the level of DNA repair synthesis.

In the present study, 64 compounds representing key groups of carcinogens of different molecular structures were examined for the capacity to evoke an unscheduled DNA synthesis in cultured human fibroblasts. This includes 29 directly active proximate or ultimate carcinogens, 15 precarcinogens that require metabolic activation, 16 non-oncogenic compounds and 4 chemicals of unknown carcinogenicity. All directly acting carcinogens triggered a DNA repair synthesis, whereas no unscheduled ^{3}HTdR incorporation was observed following the application of the 16 non-oncogenic compounds. As a rule, the precarcinogens (without metabolic activation) did not elicit DNA repair synthesis. However, longer exposures and higher concentrations of the precarcinogens 2-acetylaminofluorene, aflatoxin B_1 and sterigmatocystin evoked an unscheduled ^{3}HTdR uptake. The results suggest the suitability of using DNA repair synthesis as endpoint, and cultured human cells as subjects in a prescreening programme for chemical carcinogens.

As a probe into possible variations in sensitivity within the human population towards chemical carcinogens, cells from Xeroderma pigmentosum patients (known to be deficient in correcting UV-induced DNA damage) and normal persons were examined for their DNA repair capacity,

frequency of chromosome aberrations and clone forming efficiency following exposure to chemical carcinogens. The XP cells show a considerably reduced DNA repair synthesis when exposed to some but not all chemical carcinogens. With chemicals for which the XP cells exhibited a deficiency in DNA repair they also elicited a higher frequency of chromosome aberrations and lower clone forming capacity than in normal persons.

The advantages, limitations and possible modifications of the DNA repair bioassay for chemical carcinogens are discussed.

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ABBREVIATIONS

Chemical Carcinogens and Mutagens

4-AABP	4-Acetylaminobiphenyl
2-AAF	2-Acetylaminofluorene
2-AAP	2-Acetylaminophenanthrene
4-AAS	4-Acetylaminostilbene
4AQO	4-Aminoquinoline 1-oxide
BA	Benz(a)anthracene
DMN	Dimethylnitrosamine
EMS	Ethylmethanesulfonate
FANFT	N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide
4HAQO	4-Hydroxyaminoquinoline 1-oxide
HN_2	Nitrogen Mustard
ICR-191	Institute for Cancer Research (Philadelphia), Compound #191
MCA	20-Methylcholanthrene
2-Me-4NQO	2-Methyl-4NQO
7-Me-4NQO	7-Methyl-4NQO
MMS	Methylmethanesulfonate
MNNG	N-Methyl-N'-nitro-N-nitrosoguanidine
NMU	Nitrosomethylurea
4NQO	4-Nitroquinoline 1-oxide
6NQO	6-Nitroquinoline 1-oxide

Miscellaneous Terms

ADM	Arginine Deficient Medium
ASG	Alkaline Sucrose Gradient
FCS	Fetal Calf Serum
G6P	Glucose-6-phosphate
^{3}HTdR	Tritiated Thymidine (Thymidine-methyl-^{3}H)
MEM	Eagle's Minimal Essential Medium
NADP	Nicotinamide Adenine Dinucleotide Phosphate (Oxidized Form)
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form)
PBS	Dulbecco's Phosphate Buffered Saline
PBS/Sucrose	0.25M Sucrose in Phosphate Buffered Saline, pH 7.5
S9	Post-mitochondrial (9,000 x g) Supernatant, "Crude Microsomes"
UV	Ultraviolet
XP	Xeroderma Pigmentosum
XP_C	Xeroderma Pigmentosum, Calgary Patient
XP_E	Xeroderma Pigmentosum, Edmonton Patient
XP_H	Xeroderma Pigmentosum, Hamilton Patient
XP_K	Xeroderma Pigmentosum, Kamloops Patient
XP_V	Xeroderma Pigmentosum, Vancouver Patient

INTRODUCTION

The idea that chemicals in man's environment can induce cancers is not a new one. The high frequency of scrotal skin cancer among chimney sweeps in England, as observed by Percival Pott (1775), was believed to be associated with the heavy exposure to soot in that occupation. Studies by Rehn in 1895 (Clayson, 1962) on the elevated risk of workers in the aniline dye industry to develop urinary bladder tumours reinforced the belief that chemicals could act as carcinogens. As a result of these and many subsequent studies, many pure compounds and mixtures have been identified as carcinogens for man (Miller, 1970).

It is difficult to ascertain what proportion of malignant diseases in human beings develop as a consequence of exposure to chemicals. However, epidemiological data suggest that 80 to 90 per cent of all human cancers may be environmentally induced (Higginson, 1969, 1971). Physical, viral or chemical agents (or a combination of two or more of these) are invariably involved as an etiologic factor in carcinogenesis. Considering the wide use of chemicals in a modern society, (e.g. food additives, drugs, cosmetic products, pesticides), it is conceivable that an uncontrolled introduction of chemicals into man's environment does pose a serious health hazard. A major issue now is to identify carcinogens in the environment, and prevent or effectively reduce the exposure of human

beings to these agents.

The conventional method of testing a chemical compound for carcinogenic activity is conducted in rodents. One advantage of this test system is that the endpoint - the development of tumour - is highly relevant. However, since the usual number of test animals employed in testing one compound is about 200 to 500, weak carcinogens or precarcinogens that may induce one tumour in 1,000 to 10,000 animals - tumour frequencies which are totally unacceptable for man - could be easily missed. Such a compound would be considered non-carcinogenic, and therefore a "safe" dose for this compound could be proposed. The issue of calculating "safe" levels in carcinogenicity tests have been adequately discussed by Mantel et al. (1961, 1975a, 1975b).

Another problem besetting the standard "rodent painting and feeding" tests is the prohibitive cost incurred - up to $150,000 for testing a single compound on 200 mice and 200 rats (Weisburger, personal communication). It is estimated that 1,000 new man-made compounds are placed on the market annually. This does not include the multitude of naturally-occurring chemicals that enter man's immediate environment. The cost of testing these compounds for carcinogenicity will be astronomical. Furthermore, these tests require a relatively long time (up to 2 years) to complete. The handling and screening of millions of mice or rats would be a herculean task. There is thus an urgent need for the development of simple, rapid and

economic bioassays for chemical carcinogens.

Most, if not all, chemical carcinogens bind to DNA, but it is not proven whether DNA is the prime target for carcinogenic action. In those cases which have been adequately studied, chemical carcinogens have been found to bind covalently with DNA, RNA and protein in the target tissues (Miller and Miller, 1966 ; Matsushima and Weisburger, 1969; Colburn and Boutwell, 1968; Brookes, 1971; Grover et al., 1971a; Jungmann and Schweppe , 1972). The role of RNA and proteins in carcinogenesis cannot be dismissed. Furthermore, since most, if not all, carcinogens are also mutagens and DNA is well accepted to be the target for mutagenic action, it is reasonable to assume (though it is by no means proven) that DNA is the target molecule for carcinogenic action (Malling and de Serres, 1969; Cookson et al., 1971; Miller and Miller, 1971a; Ames et al., 1972; Brusick, 1972; Fahmy and Fahmy, 1972a, 1972b; Ong and de Serres, 1972; Matter et al., 1972; Trosko and Chu, 1975). With most chemical DNA-damaging agents, the nature of the primary lesion is obscure. However, almost all DNA-damaging agents, whether physical or chemical, that have been investigated in the proper test system show evidence of a repair effect (Hahn et al., 1968; Ball and Roberts, 1970; Horikawa et al., 1972; Stich and San, 1970, 1973; Fox and Ayad, 1971; Elkind and Chang-Liu, 1972; Goodman and Potter, 1972; Lieberman and Dipple, 1972; Stich et al., 1972b; Laishes and Stich, 1973; Hennings et al., 1974; Regan and Setlow, 1974; Stich

and Laishes, 1975). This observation raises the question whether the monitoring of carcinogen-induced DNA damage and repair could be adapted as a screening procedure for carcinogenic compounds (see Appendix 1 for cost analysis).

There are several methods by which the repair of carcinogen-induced DNA damage can be monitored:-

1. One approach to estimate DNA repair is to measure the rate of disappearance of the altered nucleotides, e.g. thymine dimers following UV-irradiation (Setlow and Carrier, 1964; Regan et al., 1968); alkylated DNA bases following exposure to an alkylating agent (Crathorn and Roberts, 1966; Lieberman and Dipple, 1972). This procedure usually requires the isolation of DNA, followed by hydrolysis and chromatographic separation of the abnormal nucleotides. This technique reveals the type of molecular changes induced by a carcinogen, but it is too cumbersome to be used on a large scale.

2. Another sensitive method to monitor DNA repair is based on the observation that following X-irradiation new groups (e.g. phosphate) become exposed and are susceptible to enzymatic attack upon breakage of a DNA strand (Chu and Malling, 1968). The more single-strand breaks that occur in a given DNA sample, the more exposed ends exist. The rate of disappearance of the exposed DNA end-groups, which can be measured at various times following exposure to a carcinogen, will reveal the rate of DNA repair. This sophisticated method remains to be exploited before

it can be applied in a pre-screening programme.

3. Sedimentation of DNA through alkaline sucrose gradients provides a direct measure of single-stranded DNA breakage. Originally designed by McGrath and Williams (1966), this technique involves lysing intact cells directly on top of an alkaline sucrose gradient, whereby mechanical shearing of DNA is kept to a minimum. The idea is based on the assumption that DNA with carcinogen-induced single-strand breaks will not sediment as fast as intact DNA strands and that the return of the broken DNA strands to the original molecular weight following a carcinogen application is a measure of DNA repair. This technique has been successfully employed in measuring DNA damage and repair in vivo and in vitro (Cox et al., 1973; Horikawa et al., 1972; Laishes and Stich, 1973; Laishes, 1974).

4. The most widely used methods of estimating DNA repair synthesis involve the 'resynthesis' of short sections of the DNA molecule which were eliminated by endo- and exo-nuclease enzymes following exposure to exogenous DNA damaging agents (Rasmussen and Painter, 1966). The incorporation of 5-bromo-deoxyuridine (BrdU) into nuclear DNA of carcinogen-treated cells followed by buoyant density centrifugation through a cesium chloride gradient provides an elegant means to distinguish DNA replication from DNA repair (Cleaver, 1970, 1973). However, this procedure is not applicable as a rapid analytical method to screen large numbers of carcinogenic agents.

Incorporation of BrdU into DNA regions undergoing repair coupled with long-wavelength ultraviolet radiation produces a photosensitizing effect which can be used to estimate the extent of DNA repair (Regan et al., 1971; Kimble et al., 1974). Cultured mammalian cells are treated with a chemical carcinogen, allowed to undergo repair replication in the presence of BrdU and thereafter exposed to a pulse of 313 nm light. Repair-replicated regions containing BrdU are rendered alkaline-labile by the irradiation. The extent of DNA breakage and repair is deduced by the degree of shift in the DNA peak following centrifugation in an alkaline sucrose gradient. Only recently introduced, this approach requires further evaluation before it can be considered in a large-scale prescreening programme.

The technique of choice appears to be the autoradiographic detection of radioactive precursors applied during the period of DNA repair synthesis. By exposing non-proliferating cells to exogenous physical (Cleaver, 1970) or chemical agents (Cleaver, 1973 ; Stich and San, 1970, 1973; Stich et al., 1971, 1972a, 1972b) and then allowing them to engage in DNA repair synthesis in the presence of ^{3}HTdR, a measure of unscheduled repair synthesis can be readily obtained. Since hundreds of autoradiographic preparations can be handled simultaneously, and the evaluation of the preparations can be semi-mechanized, this procedure can be applied in large-scale screening tests (Bootsma et al., 1970).

The idea of using DNA repair synthesis of mammalian cells as an indicator for a carcinogenic activity of a compound is based on the assumption that chemical carcinogens interact with DNA and that the ensuing DNA changes will result in a DNA repair synthesis. The working hypothesis was previously examined by comparing the extent of DNA repair in hamster and human cells following exposure to strongly, weakly and non-oncogenic isomers and derivatives of 4-nitroquinoline 1-oxide (4NQO) (Stich et al., 1971). A good correlation was obtained between the oncogenicity of a compound and the level of DNA repair synthesis. Cells respond to highly and weakly oncogenic derivatives of 4NQO with a high and low level of unscheduled DNA synthesis respectively. The non-oncogenic 4NQO derivatives lack the capacity to evoke a detectable DNA repair synthesis.

One of the objectives of this thesis was to evaluate the feasibility of using DNA repair synthesis in cultured human fibroblasts as a bioassay for chemical carcinogens. An attempt is made to investigate the following problems:-

1. Whether the correlation between the carcinogenicity of a chemical compound and the induction of DNA repair synthesis in cultured human fibroblasts can be established for chemicals of different molecular structures.

2. Whether the precarcinogenic, proximate and/or ultimate carcinogenic forms of a chemical carcinogen vary in their capacity to elicit unscheduled DNA synthesis in cultured human fibroblasts.

A second objective in the present study concerned the possible variation in sensitivity within the human population towards chemical carcinogens. Such differences must be considered when deciding on "permissible" or "safe" levels of a chemical compound. To illustrate whether differences in susceptibility to chemical carcinogens do exist in man, cells from Xeroderma pigmentosum patients (who are deficient in repairing UV-induced DNA damage) and normal persons are compared with respect to their DNA repair capacity, frequency of chromosome aberrations and clone forming efficiency following exposure to a chemical carcinogen.

MATERIALS AND METHODS

1. Tissue Culture Techniques

1.1. Culture Media

Eagle's Minimal Essential Medium (MEM). Eagle's minimal essential medium was purchased in powder form from Grand Island Biological Company, Berkeley, California. The powder was reconstituted with distilled water and sterilised by passage through a millipore filter (pore size: 0.22 microns; Millipore Filter Corporation, Mass., U.S.A.).

Arginine Deficient Medium (ADM). Arginine deficient medium was prepared according to the standard formula for Eagle's minimal essential medium (Eagle, 1959; Merchant et al., 1964). The various essential amino acids, with the exception of arginine, were weighed out and dissolved in the manner as directed (see Appendix 3). The non-essential amino acids (in the form of 100x concentrated mixture), as well as the vitamins, were obtained from Flow Laboratories, Inc. (Inglewood, California) and added to the culture medium.

Antibiotics. Culture media were routinely supplemented with the following antibiotics: Penicillin G (General Biochemicals, Chagrin Falls, Ohio; 204 units/ml., final concentration), Streptomycin sulfate (General Biochemicals; 29.6 µg/ml.), Kanamycin (Grand Island Biological Co.; 100 µg/ml.), and Fungizone (Grand Island Biological Co.; 2.5 µg/ml.).

Sodium Bicarbonate. A 7.5% solution, sterilised by

filtration, was prepared as a standard stock. For cultures maintained in Leighton tubes, the culture medium was supplemented with 4 ml. of the 7.5% stock solution (for 800 ml. of culture medium). In the case of petri plate cultures, 16 ml. of sodium bicarbonate was added to 800 ml. of medium.

Fetal Calf Serum (FCS). Fetal calf serum was purchased from Grand Island Biological Company and stored at -20°C. It was inactivated by heating at 56°C for 30 minutes before use. MEM was supplemented with 10% FCS whereas ADM was supplemented with only 5% FCS.

1.2. Cultured Cells

Embryonal Hamster Cells. Primary cultures of 12-day-old Syrian-hamster embryos were initiated as described by Cooper (1968). Diploid embryonal cells from the first to fourth passage were used in the present investigation.

Human Skin Fibroblasts. Human skin fibroblasts were grown from explants of skin punch biopsies from Xeroderma pigmentosum patients (age: 11-22) and normal caucasian donors (age: 18-24):-

XP_{C1} (male, 12 years old, J.T., Calgary)

XP_{C2} (female, 11 years old, M.T., Calgary)

XP_{E} (female, 18 years old, N/A, Edmonton)

XP_{H1} (female, 20 years old, N/A, Hamilton)

XP_{H2} (male, 21 years old, N/A, Hamilton)

XP_{K} (male, 22 years old, C.F., Kamloops)

XP_{V} (female, 21 years old, D.S., Vancouver)

Fibroblast cultures of XP_{H1} and XP_{H2} were kindly provided by Drs. L. Skarsgard (University of British Columbia, Canada) and S. Mak (McMaster University, Canada). Cultures of fibroblasts from the other XP patients were initiated in this laboratory.

A 3 to 4 $mm.^2$ skin-punch biopsy was taken from the forearm of the donor, teased into tiny fragments, sandwiched between two cover glasses and incubated in MEM (15% fetal calf serum) for 2 - 3 weeks at 37°C in a CO_2 incubator. Growth medium was changed at weekly intervals. When fibroblasts migrated from the tissue fragments, the "sandwich" was opened and the tissue fragments were removed, leaving a partial monolayer of fibroblasts on the cover glasses. Upon further incubation, the fibroblasts on individual cover glass were allowed to grow up into a monolayer which could then be subcultured by standard techniques. A detailed description of the biopsy and explant culture techniques are given in Appendix 2.

Stock Cultures. Stock cultures were grown in monolayers in 10 cm. diameter petri plates (Falcon plastic) and maintained in Eagle's Minimal Essential Medium (MEM) supplemented with antibiotics and 10% fetal calf serum. The cultures were kept in a humidified incubator (37°C) staffed with 5% CO_2 and 95% air.

2. Ultraviolet Irradiation

A Sylvania germicidal lamp (G15 T8) was employed

as a source for UV light. At a distance of seven inches, it gave a dose of 30 ergs/mm^2/sec. as measured by a UV light meter (Ultraviolet Products, Inc., San Gabriel, California). The UV meter was calibrated against E. coli survival rates following UV irradiation by Dr. J. Kemp (Simon Fraser University, Burnaby, B. C.).

Cell cultures on cover-slips to be irradiated were taken out of the Leighton tube, dipped three times in sterile phosphate buffer saline (see Appendix 3) to remove serum in the culture medium adhering to the cells. Serum and phenol red (the pH indicator used in culture medium) are known to absorb UV irradiation. Half of each cover-slip was shielded by a tin plate, so that these cells protected from UV light could serve as controls. After irradiation, ^{3}HTdR labelling and the autoradiographic procedures were carried out as for cell cultures exposed to chemical carcinogens.

3. Chemicals

3.1. Source of Chemicals

4-Nitroquinoline 1-oxide, its isomers and derivatives as well as nitrosomethylurea (NMU), were the generous gift of Dr. Y. Kawazoe (National Cancer Centre Research Institute, Tokyo, Japan).

Benz(a)anthracene, 20-methylcholanthrene and derivatives were kindly provided by Dr. C. Heidelberger (McArdle Laboratory for Cancer Research, University of Wisconsin, U.S.A.).

The aromatic amines (2-acetylaminofluorene, 4-acetylaminostilbene, 4-acetylaminobiphenyl, 2-acetylaminophenanthrene and the corresponding N-hydroxy- and N-acetoxy- derivatives, N-myristoyloxy-2-AAF, N-acetoxy-2-myristoyl-AF, and N-myristoyloxy-2-myristoyl-AF), diphenylcarbinols and safrole derivatives were kindly provided by Drs. J.A. Miller, E.C. Miller (McArdle Laboratory for Cancer Research, University of Wisconsin, U.S.A.) and H. Bartsch (IARC, Lyons, France).

N-Methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin) was kindly provided by Dr. C.T. Beer (University of British Columbia, Canada).

Ethylmethanesulfonate and methylmethanesulfonate (Eastman Organic Chemicals, Rochester, New York) were kindly provided by Dr. D.T. Suzuki (University of British Columbia, Canada).

ICR-191 was kindly provided by Dr. H.J. Creech (Institute for Cancer Research, Philadelphia, U.S.A.).

Streptonygrin was kindly provided by Dr. J.W. Lown (University of Alberta, Canada).

Luteoskyrin and rugulosin were kindly provided by Dr. S. Shibata (University of Tokyo, Japan).

The nitrofuran, N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) was kindly provided by Dr. D.R. McCalla (McMaster University, Canada).

Nitrogen mustard was obtained as Mustargen (mechlorethamine hydrochloride) from Merck, Sharpe and Dohme Canada

Ltd. (Kirkland, Que.).

Aflatoxins B_1, B_2, G_1, G_2 and sterigmatocystin were purchased from Makor Chemicals, Jerusalem, Israel.

Dimethylnitrosamine (DMN) was purchased from K and K Laboratories, Plainview, N.Y.

Anhydrous methylguanidine hydrochloride and ethidium bromide were purchased from Sigma Chemical Co., St. Louis, Mo.

Acriflavine Neutral was purchased from Mann Research Laboratories, New York N.Y.

α-Terthienyl and cow parsnip were kindly provided by Drs. G.F.Q. Chan, G.H.N. Towers and E. Camm (University of British Columbia, Canada).

Daunomycin was obtained as Cerubidine from Specia, Paris.

3.2. Solutions of Chemicals

Several of the chemicals tested are readily soluble in water. These include MNNG, NMU, ICR-191, HN_2, acriflavine neutral, daunomycin, ethidium bromide and methylguanidine. An appropriate amount of the test compound was dissolved in tissue culture medium (MEM or ADM as required).

EMS, MMS and DMN are liquids at room temperature. They are readily miscible with water and dilutions were made directly with culture medium.

4NQO and its derivatives are not readily soluble in water. To prepare a 10^{-3}M solution (the standard stock used throughout this study), 0.4 ml. of 100% ethanol was added to an appropriate amount of solid in a disposable plastic tube, followed by addition of 9.6 ml.

of tissue culture medium. The final concentration of ethanol did not exceed 5%.

The polycyclic hydrocarbons (BA, MCA and derivatives), the aromatic amines (2-AAF, 4-AAS, 4-AABP, 2-AAP and derivatives), safrole (and derivatives), diphenylcarbinols, aflatoxins, sterimatocystin,luteoskyrin and rugulosin were dissolved in dimethyl sulfoxide (DMSO) prior to dilution with culture medium to the desired concentrations. The final concentration of DMSO did not exceed 1%.

Unless higher concentrations were required, a 10^{-3}M stock solution was usually prepared immediately before use. Serial dilutions were then made to obtain the desired final concentrations. If ADM was used throughout the entire experiment, then the solution was prepared in ADM, otherwise in MEM.

4. In Vitro Activation of Precarcinogens

4.1. Preparation of Post-Mitochondrial Fraction (S9 Fraction) of Tissue Homogenates

Young adult Swiss mice (males with average body weight of 20 gm.) obtained from the Animal Unit, Faculty of Medicine, University of British Columbia, were killed by cervical dislocation and were immediately dissected. Livers were removed, trimmed of adhering connective tissue and placed into a beaker containing PBS/sucrose buffer at 4°C (PBS, with calcium and magnesium ions, containing 0.25M sucrose, pH 7.5). All subsequent steps were conducted

with solutions and containers maintained at 4°C. Liver from several mice were pooled, washed with PBS/sucrose buffer, dabbed dry on absorbent tissue, weighed, and quickly transferred to separate beakers of fresh PBS/ sucrose buffer such that 6 gm. of liver were contained in 10 ml. buffer. The livers were minced with scissors and the contents of each beaker were transferred to homogenization vessels. The tissues were then homogenized by 10 up-and-down strokes of a loose-fitting Potter-Elvejhem Homogenizer operating at 1,000 rev./min. The homogenate was centrifuged at 9,000 x g for 10 min. (at 4°C) in a pre-cooled rotor (Beckman, Type 40) and nitrocellulose centrifuge tubes. The resulting post-mitochondrial supernatant fractions (sometimes referred to as S9 fractions) were pooled, mixed thoroughly to ensure homogeneity of each batch, and then distributed in measured aliquots to polypropylene tubes, capped, immediately frozen in liquid nitrogen and stored in a Revco freezer at -70°C (Laishes, 1974; Stich and Laishes, 1975).

4.2. Preparation of Activation Mixture and Treatment of Cell Cultures

The activation system was prepared by dissolving, for each cell culture to be treated, 4.0 μmoles NADPH or NADP, 25 μmoles $MgCl_2$, and 20 μmoles G6P in 0.4 ml. of S9 fraction (thawed immediately before use) and adjusting the pH to 7.2 with 0.1 N NaOH.

Equal volumes of precarcinogen solution (at twice the desired final concentration) and activation system (usually 0.5 ml. each) were mixed, vortexed quickly, and the resulting activation mixture was added to cell cultures within 5 seconds of mixing. The cultures were incubated at 37°. At the end of the treatment, the cells were washed three times with culture medium and normal culture medium (MEM or ADM as required) was replaced.

5. Measurement of DNA Repair Synthesis (Autoradiography)

5.1. Preparation of Cell Cultures and Exposure to Test Compound

Cells were seeded onto 10 x 35 mm. cover glasses (Clay Adams) kept in 16 x 85 mm. Leighton tissue culture tubes (Bellco Glass, Inc., Vineland, New Jersey) at approximately 5×10^4 cells per tube and covered with MEM (supplemented with 15 % fetal calf serum). The cell cultures were always used for experimentation before the monolayer has reached confluency. This permits better cytologic preparation, better cell exposure to carcinogens and, in the case of cultures processed for autoradiography, a lower background count.

To distinguish between DNA replication at S-phase and DNA repair synthesis, modification of the procedure developed by Freed and Schatz (1969) was employed. By depriving the cell culture of the essential amino acid arginine, it was observed that DNA synthesis associated with chromosome

replication was drastically reduced (Stich and San, 1970). Upon reaching 80% confluency (2 - 3 days after seeding), the cells were placed into an arginine-deficient medium (5% FCS). This was done by dipping the coverslips about five times into each of two beakers of ADM (no serum), and then transferring them to new Leighton tubes containing 1 ml. of ADM (5% FCS). After $2\frac{1}{2}$ to 3 days in ADM, more than 90% of the cells would be arrested at G_1.

Chemical treatment was carried out by replacing the medium in the Leighton tube culture with a solution of the test compound at the desired concentration. If radioactive-labelling was done immediately after exposure to the test compound, the chemical solution was decanted, and ^{3}HTdR (in ADM, 5% FCS) was added after the cell culture had been rinsed three times with ADM (no serum). Otherwise, the cell culture was put back into ADM following chemical treatment.

5.2. Radioactive-Label Incorporation

Tritiated thymidine (specific activity 20Ci/mmol), obtained as thymidine (methyl-^{3}H) in aqueous solution from New England Nuclear (Dorval, Que.), was used in all pulse-label experiments. Dilution down to a final concentration of 10 µci/ml. was made by mixing with ADM (5% FCS).

Unless otherwise specified, chemically-treated or UV-irradiated cells cultures were pulse-labelled with ^{3}HTdR for 1.5 hours. The ^{3}HTdR-containing medium was then

poured off and any unincorporated radioisotope removed by rinsing two or three times with Hank's balanced salt solution (see Appendix 3). The coverslips were then taken out of the Leighton tubes, immersed in 1% sodium citrate for 12-15 minutes, followed by fixation in acetic acid/ethanol (1:3, V/V), and allowed to air dry.

5.3. Coating with Photographic Emulsion

Coverslips are very fragile and liable to breakage. To facilitate their manipulation, they were mounted on glass slides. A small quantity of paraffin wax was placed on a glass slide and warmed over a flame until it began to melt. An air-dried coverslip was placed on top of the wax with the cell monolayer facing upwards. Once the wax resolidified, the coverslip became anchored to the glass slide.

Acetic Acid has been reported to react with photographic emulsions, resulting in an increase in background grain count (Stocker and Muller, 1967). As a precaution, excess acetic acid was removed by passing the slides through a graded alcohol series, 95% EtOH, 70% EtOH, 20% EtOH (10 minutes each), two changes of distilled water, one change of PBS, two more changes of distilled water (10 minutes each) and were then left to air dry.

The slides were coated with photographic emulsion in the dark-room. A Kodak Wratten Series 2 red filter was used. Kodak NTB-3 nuclear-track emulsion was thawed

at 43°C in a water-bath in the dark, and diluted with an equal volume of distilled water. Glass slides were then individually dipped in the emulsion, air-dried in a vertical position, and then stored at 4°C in light-tight boxes for 14 days.

5.4. Processing and Staining of Autoradiograms

Autoradiograms were brought back to room-temperature after 14 days of exposure. Processing was done at 18°C in Kodak D-19 developer (3 minutes), stop bath (30 seconds), Kodak fixer (10 minutes) and hypoclearing agent (1 minute). After a 30-minute rinse in running water (18°C), the slides were stained with 2% aceto-orcein for 10 minutes, dehydrated through successive immersion (1.5 minutes each) in 95% ethanol, butanol, butanol/xylol, two changes of xylol and mounted in Permount (Fisher Scientific Co.) by superimposing another coverslip over that bearing the cell monolayer.

5.5. Analysis of Autoradiograms

The amount of DNA repair synthesis was estimated by counting the number of grains over each nucleus. Care was taken that nuclei of comparable size were selected so that only cells of the same ploidy were used. Routinely, grain counts were made on small interphase nuclei. Background count was taken into consideration by reckoning the number of grains over an area equal in size to that of the

nucleus. At least 30 nuclei, at random locations throughout the entire coverslip culture, were analysed for grain number. When the grain number appeared below 10 grains/nucleus, at least 100 nuclei were scored. Based on statistical calculations, Rogers and England (1973) have demonstrated that the accuracy of estimating the radioactivity per nucleus will depend, not on the number of nuclei counted, nor on the total area of emulsion scanned, but on the total number of silver grains counted in one sampling of the population. A more detailed consideration of the statistical analysis of autoradiograms is included as an appendix in this thesis (see Appendix 4).

6. Chromosome Studies

6.1. Preparation of Cell Cultures

Cultured human fibroblasts were seeded onto 20 mm^2. coverslips (Corning) in 35 mm. petri dishes at approximately 5×10^4 cells per dish and covered with MEM (15% FCS). In order to obtain well spread metaphase plates, the cells were used before they reached 80% confluency.

6.2. Exposure to Test Compound

Exposure of petri plate cultures to the test chemicals was performed in the following manner. The medium was removed with a sterile pasteur pipette connected to a suction device. One ml. of the chemical solution was added for the time period desired (maintained at 37°C

in a CO_2 incubator) and then removed by suction. After rinsing twice with MEM (no serum), the cells were covered with 2 ml. of MEM (15% FCS) and returned to the CO_2 incubator.

6.3. Cytologic Preparations

6.3.1. Reagents

Colchicine (BDH Chemicals Ltd., Poole, England). A 1% stock solution in distilled water was prepared from which appropriate dilution to a 0.01% solution could be made. Human cells were treated with 0.1 ml. of the 0.01% solution per ml. of medium (i.e. a final colchicine concentration of 10 µg/ml.).

Sodium Citrate. Sodium citrate (Fisher Scientific Co.) was used as a 1% solution (W/V) in distilled water.

Fixative. A mixture of three parts of 100% ethanol with one part of glacial acetic acid (V/V) was used as a fixative. It was usually prepared 1 to 2 hours before use.

Aceto-Orcein. Aceto-orcein, used as a 2% solution, was prepared by refluxing for 6 hours the appropriate amount of orcein (BDH Chemicals Ltd., Poole, England) in 45% acetic acid. The solution so obtained was always filtered (with Whatman No. 1 paper) before use.

6.3.2. Chromosome Preparations

Once cell divisions were detected (by observation under an inverted microscope) following exposure to a test compound,

0.2 ml. of a 0.01% solution of colchicine was added to coverslip cultures (containing approximately 2 ml. culture medium) for 4-5 hours (final concentration of colchicine: 10 µg/ml.). The coverslips were then transferred to petri dishes containing a 1% sodium citrate solution. This hypotonic treatment (20 minutes) enables the cells to increase in volume and permits a nice spread of the metaphase chromosomes. Fixation of the cells was carried out in a 3:1 mixture (V/V) of 100% ethanol and glacial acetic acid (10 minutes). Once air dried, the cells were stained with 2% aceto-orcein, dehydrated through immersion in 100% EtOH, butanol, butanol/xylol, two changes of xylol and mounted in Permount (Fisher Scientific Co.).

6.4. Analysis of Metaphase Plates for Chromosome Aberrations

For each sample about 30 to 50 well-spread metaphase plates were analyzed for chromatid breaks, exchanges and fragmentation. When the frequency of metaphase plates with chromosome aberrations appeared to be below 5%, at least 150 metaphases were scored.

7. Survival Studies

About 2,000 human diploid fibroblasts were seeded in 5 cm. sterile disposable petri plates (Falcon Plastic), covered with 4 ml. of MEM (10% FCS) and allowed to settle down as single cells overnight (16-20 hours) before chemical treatment. It was observed that cell division did not

usually occur within this period of time.

Exposure to the test chemical was performed by suctioning off the culture medium and replacing it with 3 ml. of the chemical solution in MEM (5% FCS). Following chemical treatment, the cultures were rinsed twice with MEM (no serum) and incubated in fresh MEM (15% FCS) which was changed after 7 days.

When the clones from surviving cells had reached the 50 - 60 cell stage (approximately 10 - 14 days post-treatment) the cultures were fixed with ethanol-acetic acid (3:1, V/V) for 10 minutes, washed in 70% ethanol, air dried and stained with a 2% aqueous solution of Toluidine Blue (Fisher Scientific Co.). Excess dye was removed by rinsing in distilled water.

The clones containing 50 or more cells were counted under a regular dissecting microscope. Usually, counts from three to six cultures were averaged for each concentration of the test chemical. The data were expressed as a percentage of the clone count in the untreated control.

RESULTS

1. Unscheduled Incorporation of Tritiated Thymidine (^{3}HTdR) in Mammalian Cells as a Measure of Repair of DNA Damage Following Exposure to Chemical Carcinogens

One of the objectives of this thesis was to evaluate the feasibility of using DNA repair synthesis in cultured human fibroblasts as a bioassay for chemical carcinogens. An important question that arises is whether the unscheduled incorporation of tritiated thymidine in carcinogen-treated fibroblasts can be taken as a measure of the ensuing DNA repair. Furthermore, in order to design a protocol to monitor the repair of carcinogen-induced DNA damage, the following problems must be considered:

- whether a distinction can be made between DNA repair synthesis and DNA synthesis associated with chromosome replication.
- whether all or only a fraction of the carcinogen-treated cells exhibit a DNA repair synthesis.
- whether different carcinogen doses (in terms of exposure time or concentration) have any effect on the level and duration of the ensuing DNA repair synthesis.
- whether the capacity of cultured cells to repair DNA damage can be temporarily reduced or blocked by chemicals.

These questions will be examined in the following three chapters:

1.1. Dose Response

1.2. Duration of DNA Repair

1.3. DNA Repair Inhibition

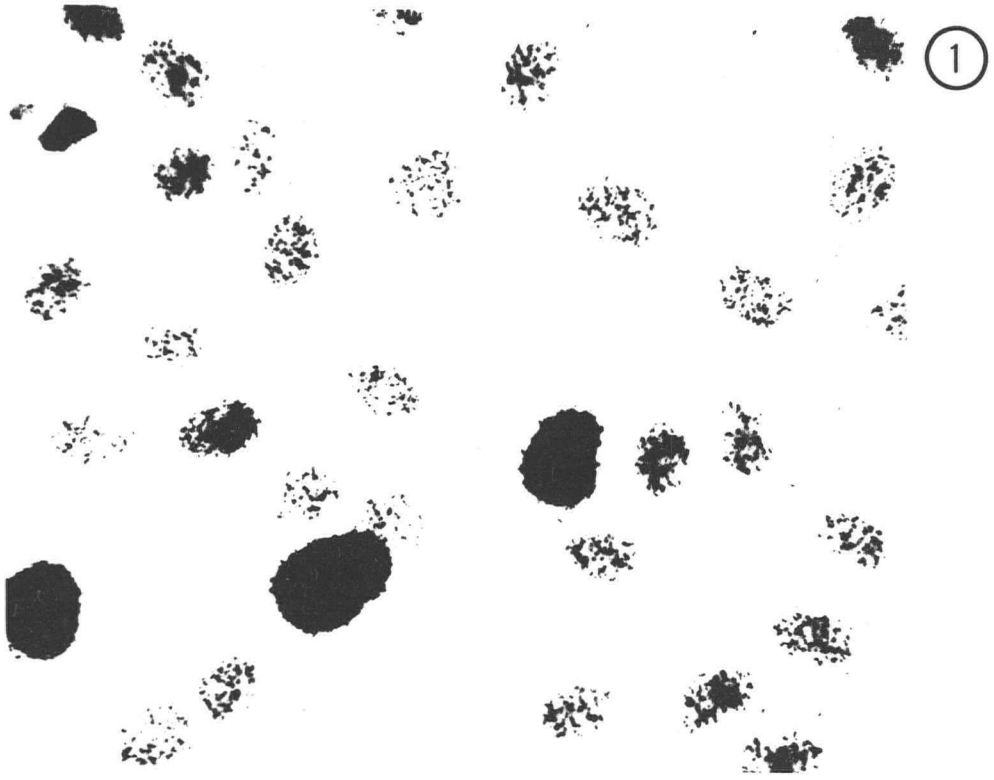
1.1. Dose Response

To separate DNA-repair synthesis (unscheduled incorporation of ^{3}HTdR) from DNA-replication synthesis associated with chromosome replication at S-phase, cultures of mammalian cells were kept in arginine-deficient medium (ADM) for 3 days prior to their exposure to carcinogens. The ADM suppresses the flow of cells from G_1 into S-phase. Thus the DNA-repair synthesis can be examined in non-dividing cells without any interference by a DNA synthesis associated with chromosome replication (Fig. 1 and 2).

At first we examined the proportion of cultured cells that showed a DNA repair synthesis following exposure to carcinogens. About $2x10^6$ Syrian-hamster cells from primary to tertiary cultures, 10^6 cells from a diploid human embryo and $5x10^5$ cells from a triploid therapeutically aborted embryo and 10^6 cultured cells from a 22 year old female were screened for cells lacking the capacity of DNA repair. Less than 1 in 100,000 nuclei showed no incorporation of ^{3}HTdR following exposure to $4x10^{-6}$M 4NQO for 1.5 h, or UV-irradiation (900 ergs/mm^2). Nuclei that show no unscheduled incorporation of ^{3}HTdR were small and stained heavily with orcein. They probably represent pycnotic nuclei of dying or dead cells. If viable mammalian cells lacking DNA-repair synthesis exist at all, their frequency in a population of

Figure 1 Embryonal Syrian-hamster cells cultured in MEM and exposed to 4NQO ($4x10^{-6}$M) and ^{3}HTdR for 1.5 hours. Autoradiograph. The S-phase nuclei are heavily labelled, whereas all other nuclei show a restricted number of grains.

Figure 2 Embryonal Syrian-hamster cells kept for 3 days in arginine-deficient medium (ADM) and exposed to 4NQO ($4x10^{-6}$M) and ^{3}HTdR for 1.5 hours. Autoradiograph. Note the absence of heavily labelled S-phase nuclei and the ^{3}HTdR incorporation in all nuclei of the non-proliferating cells.



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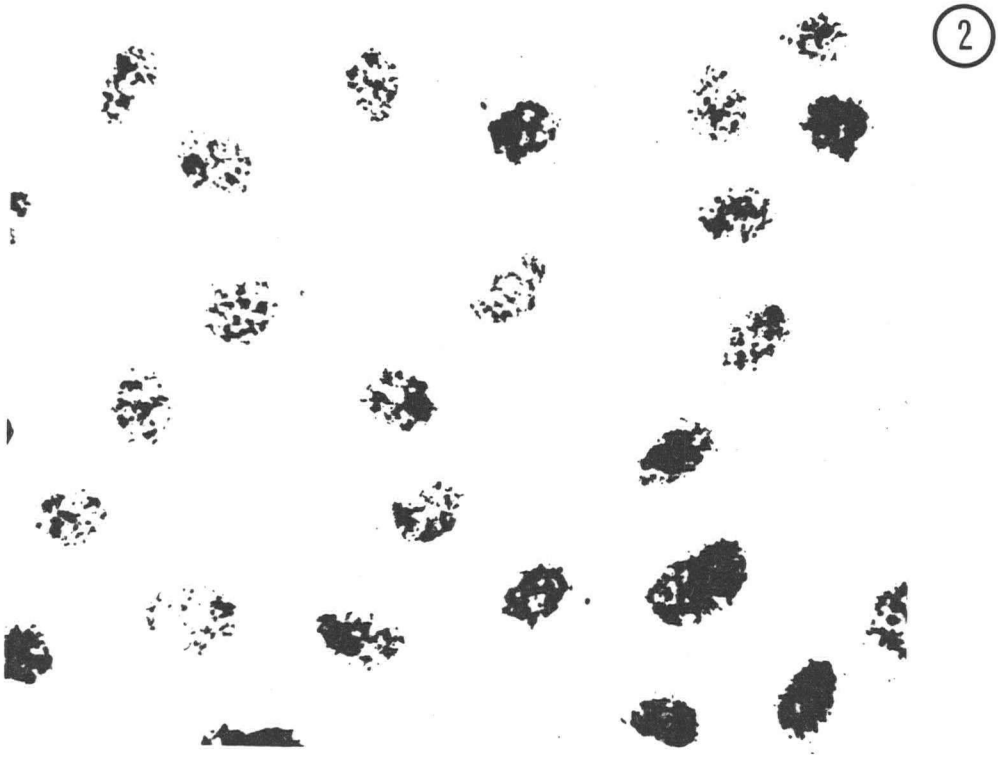

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of normal human cells must be extremely low.

The amount of ^{3}HTdR incorporation into nuclear DNA of cells exposed to active carcinogens depends among many other factors on the ploidy of cells or more precisely expressed on the DNA content of a nucleus (Fig. 3a). The histogram of Fig. 3b shows the distribution of grains per nucleus of cells with a 2C, 4C or 8C amount of DNA. This relationship between DNA content per nucleus and amount of ^{3}HTdR incorporation must be kept in mind when autoradiographs of cell stages with various DNA levels (e.g. G1 versus G2 nuclei; metaphase plates versus telophase nuclei) or cells with different chromosome numbers are compared with each other.

The effect of various carcinogens on DNA repair synthesis was examined by following an initial carcinogen treatment with 1.5 hours of ^{3}HTdR (Fig. 4). The average number of grains per nucleus in cells exposed to a single dose of various proximate or ultimate carcinogens are shown in Figs. 5 and 7. The results shown examplify the most common types of dose response curves.

Three distinctive features can be observed:-

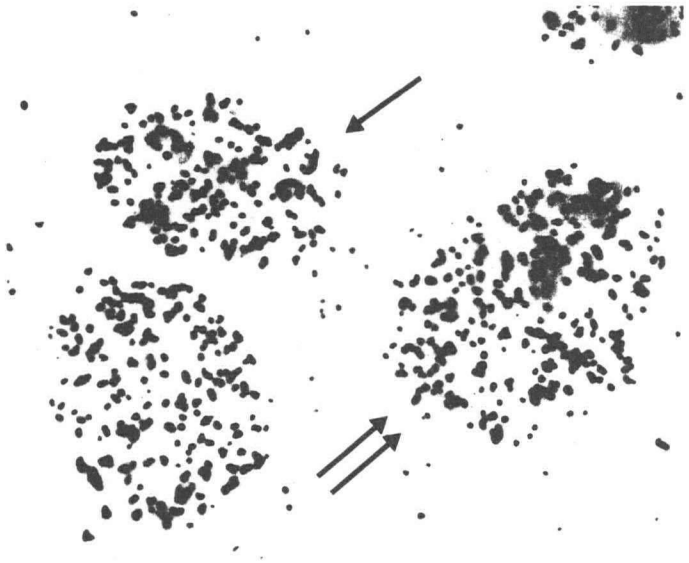
(1) The range of concentrations of various carcinogens that trigger detectable levels of DNA repair synthesis in cultured human fibroblasts varies greatly (Fig. 5).

(2) The lethal dose of various carcinogens also differ ranging from about 10^{-8}M to 10^{-2}M (Fig. 6).

(3) Previously, we pointed out a good correlation between the level of DNA repair evoked by a carcinogen and the

Figure 3(a) Unscheduled ^{3}HTdR incorporation in cultured Syrian-hamster cells following exposure to a carcinogen. Autoradiograph. The small nucleus is probably in the G_1 phase (one arrow) and the larger one in G_2 phase (two arrows).

Figure 3(b) Frequency distribution of cells with various numbers of grains above their nuclei. Cultured Syrian-hamster cells were pretreated for 3 days with ADM to block DNA replication and mitosis, thereupon exposed to 4NQO (4×10^{-6}M) and ^{3}HTdR for 1.5 hours. Autoradiograph, grain counts. 2C, 4C and 8C refer to diploid, tetraploid and octoploid nuclei.



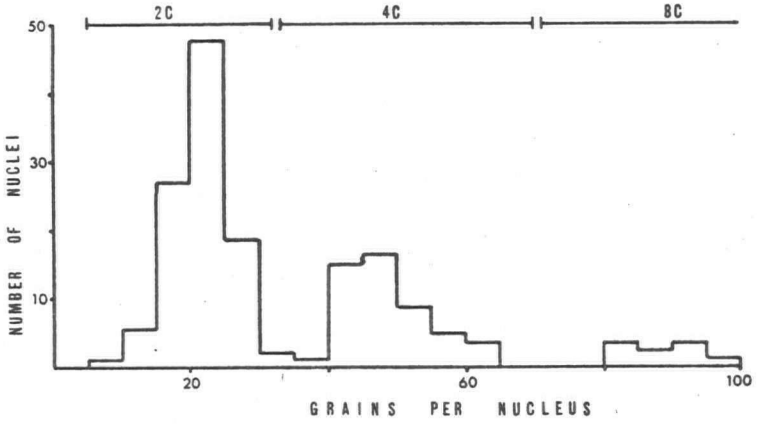
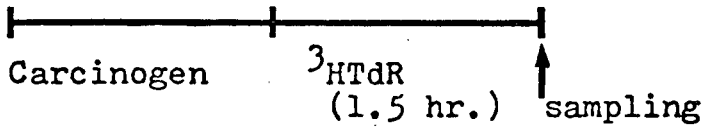

2C
4C
8C
50
30
10
NUMBER OF NUCLEI
20
60
100
GRAINS PER NUCLEUS

Figure 4 Experimental design: Unscheduled ^{3}HTdR incorporation in cultured human fibroblasts exposed to one dose of a chemical carcinogen followed by ^{3}HTdR (10 μCi/ml., 1.5 hours).

Figure 5 Variations in concentration of three carcinogens that trigger a DNA repair synthesis in cultured human fibroblasts. Exposure to carcinogen was followed by ^{3}HTdR (1.5 hours).

Figure 6 Variations in concentration of three carcinogens that affect the clone forming capacity of cultured human fibroblasts.

(4) Experimental Design:



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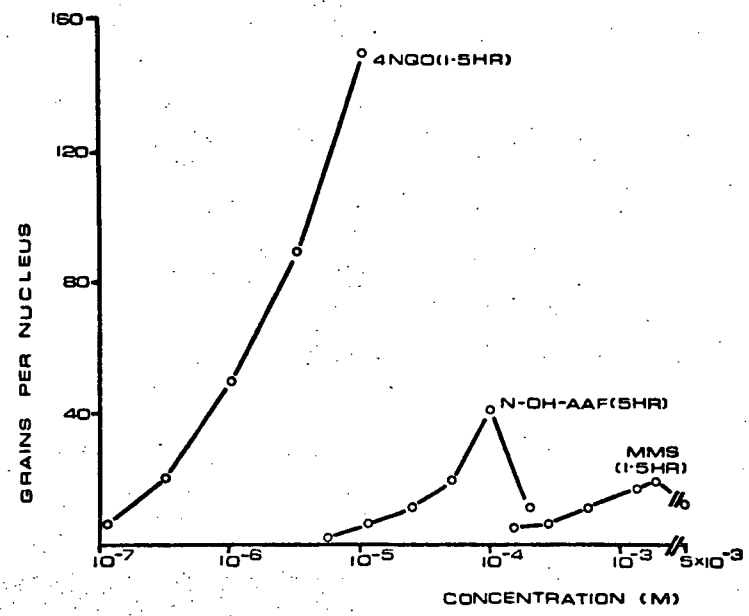

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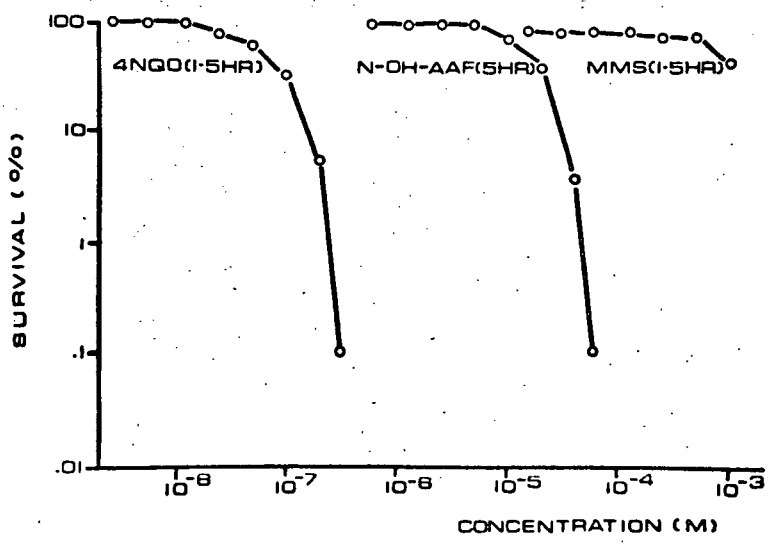


degree of its carcinogenic potential when the action of strong and weak carcinogenic 4NQO isomers and derivatives was compared (Stich et al., 1971). However, no such correlation became obvious when carcinogens of different molecular structures were included in the comparative study. For example, 4NQO elicited a high level of unscheduled ^{3}HTdR incorporation at relatively low doses, while the 6,7-epoxide of MCA required about 1,000 times higher concentrations to trigger a relatively low level of DNA repair synthesis (Fig. 7). Obviously 4NQO is not 1,000 times more carcinogenic than MCA or its epoxide.

A different type of dose-response study concerns the effect of different exposure times to a carcinogen on DNA-repair synthesis (Table I). With several carcinogens (e.g. N-OH-AAF), little or no DNA repair synthesis was observed after a short exposure time. One explanation is that these chemicals may require more time for interaction with DNA to come about. With some carcinogens (e.g. 4NQO, N-Ac-AAF) a longer exposure time reveals little or no increase in the level of ^{3}HTdR incorporation. One explanation is that the carcinogen remains active only for a short time and no further DNA damage is inflicted upon prolonged exposure. Another possibility is that once the repair of the initial DNA damage is underway, further DNA damage will not be catered to because the repair system is overtaxed. This aspect will be considered in some detail in connection with the duration of DNA repair and the effect of carcinogens

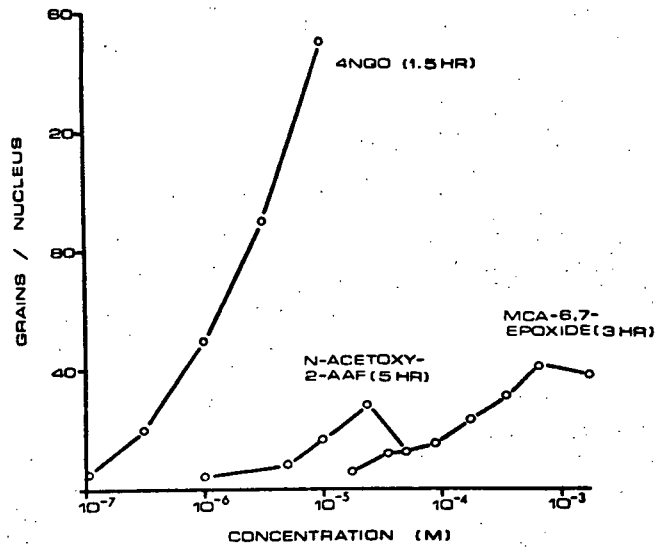


Figure 7 Different levels of unscheduled DNA synthesis in cultured human fibroblasts elicited by carcinogens of different molecular structures.

TABLE I Effect of Exposure Time to a Chemical Carcinogen on the Level of Unscheduled ^{3}HTdR Incorporation in Cultured Human Fibroblasts*

	N-Hydroxy-2-AAF 5×10^{-5}M	N-Acetoxy-2-AAF 2.5×10^{-5}M	4NQO 5×10^{-6}M
Exposure Time	Grains Per Nucleus		
1.5 Hours	3	33	90
3 Hours	12	28	72
5 Hours	20	25	58

* Exposure to carcinogen was followed by 1.5 hour ^{3}HTdR (10 μCi/ml.).

on chromosome aberrations and cell survival.

1.2. Duration of DNA Repair

Cultured human fibroblasts were kept in ADM prior to and throughout the entire experiment. In this way it was possible to estimate the duration of DNA repair synthesis in non-dividing cells. The duration of DNA repair synthesis was examined by exposing non-dividing cells to 1.5 h pulses of ^{3}HTdR at various time intervals after the application of carcinogen (Fig. 8). Samples for the autoradiographs were taken at the end of each ^{3}HTdR pulse. For the first sample carcinogen and ^{3}HTdR were applied simultaneously for 1.5 h. The time course of repair synthesis following exposure to different chemical carcinogens is shown in Fig. 9. The major part of repair synthesis appears to be completed by about 8 to 10 h post-treatment. However, a low but significant uptake of ^{3}HTdR into nuclear DNA can be detected for a prolonged period. Repair synthesis follows a similar time course in cells whether they are exposed to higher or lower concentrations of a carcinogen (Fig. 10). For comparative purposes the course of repair synthesis following a single dose of UV (900 ergs/mm^{2}) was added in Fig. 11. The design of experiments was similar to the previous ones in which a chemical carcinogen was used to induce repair synthesis. The duration of the main phase of repair synthesis is similar in cells irradiated with UV (100-900 ergs/mm^{2}) or exposed to various chemical

Figure 8 Experimental Design. Duration of unscheduled DNA synthesis induced in cultured human fibroblasts by a single dose of UV radiation or chemical carcinogen.

Figure 9 - 11 Duration of unscheduled DNA synthesis in cultured human fibroblasts following exposure to a single dose of 4NQO, MNNG or UV radiation. Each point denotes the time when the sample was taken and the uptake of ^{3}HTdR over a 1.5-hour period prior to sampling (represented by grains per nucleus).

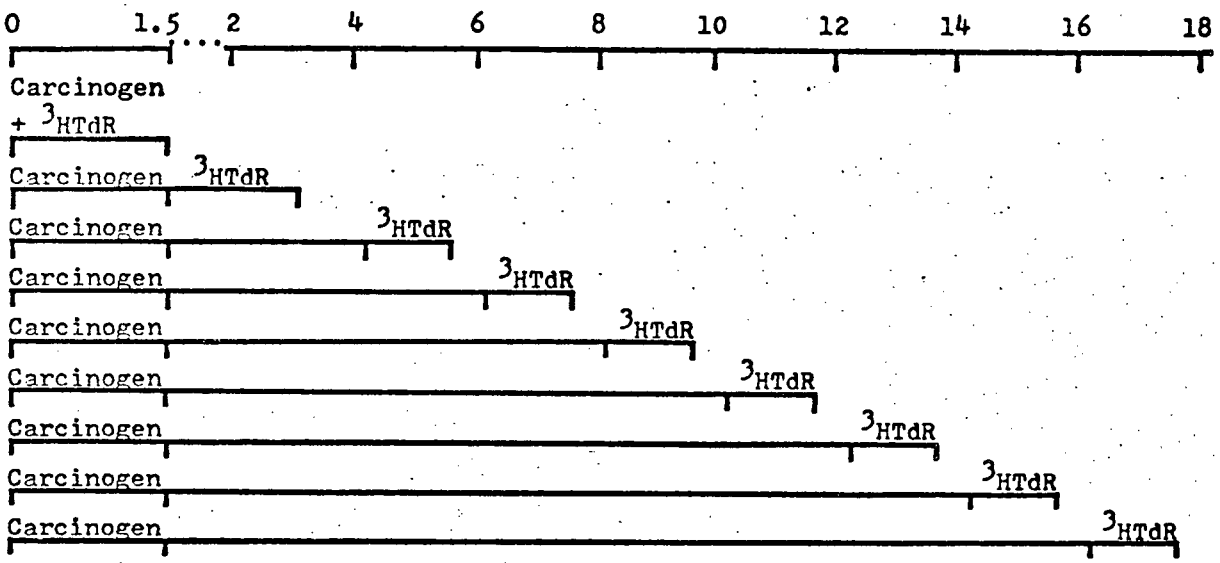
(Figure 9) 4NQO (8×10^{-7}M, 1.5 hours), □, or MNNG (5×10^{-5}M, 1.5 hours), ●.
⊢⊣ = Exposure to carcinogen.

(Figure 10) Different concentrations of 4NQO, 5×10^{-6}M (○), 8×10^{-7}M (●).

(Figure 11) UV radiation (260 nm, 900 ergs/mm^2).

Figure 12 Time course of DNA repair synthesis in cultured human fibroblasts following short term exposure to 4NQO (5×10^{-6}M, 1.5 hours) and measured as unscheduled incorporation of ^{3}HTdR (autoradiography, ▽) or as a shift in the sedimentation rate in an alkaline sucrose gradient (■). By about 12 hours, post-treatment DNA repair synthesis ceases and the sedimentation profile approaches the original pattern. (Sedimentation: ordinate denotes fraction number corresponding to peak).

(8) Protocol:



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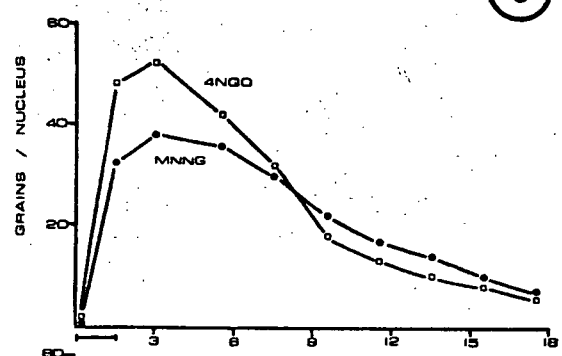

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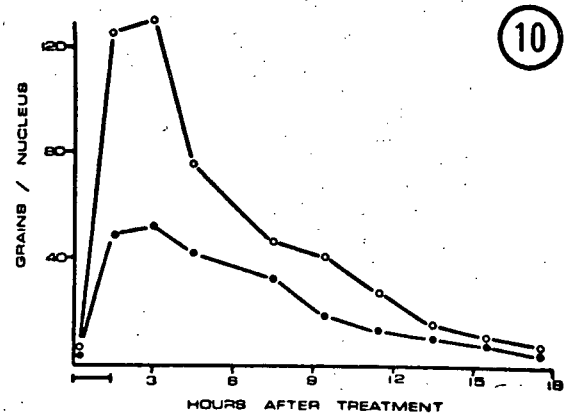

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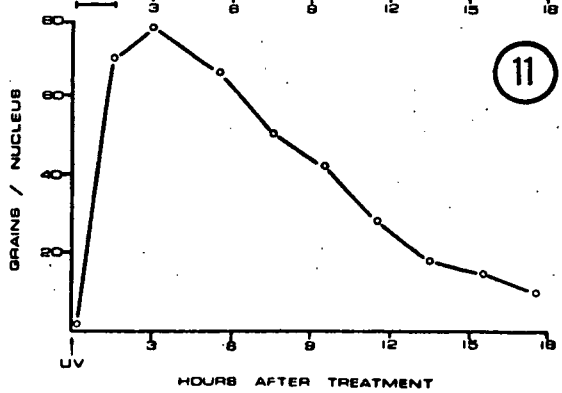

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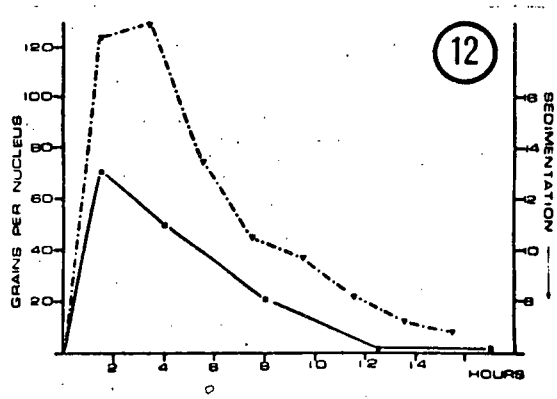


carcinogens.

One criticism of the autoradiographic technique is whether the unscheduled ^{3}HTdR uptake could be equated to DNA repair synthesis. The unscheduled incorporation of ^{3}HTdR indicated DNA synthetic activity but does not provide any evidence that the newly synthesised DNA segments are joined at all. To answer this question, one can resort to the alkaline sucrose gradient (ASG) technique. In this procedure, a shift in the sedimentation rate indicates that the DNA has been fragmented whereas a return to the normal sedimentation rate implies that the DNA molecule has been restored. For comparison, an ASG analysis of DNA from 4NQO-treated human fibroblasts has been included (Fig. 12). It is of interest to note that the time course of DNA repair (measured as unscheduled incorporation of ^{3}HTdR) resembles the period of rejoining of the fragmented DNA to its original size (measured as shift in sedimentation rate in an alkaline sucrose gradient).

1.3. DNA Repair Inhibition

There is a plethora of evidence showing that a genetically impaired DNA repair mechanism increases the sensitivity of bacterial and mammalian cells towards the lethal and mutagenic effect of physical and chemical agents. The question must be raised whether the capacity of cultured cells to repair DNA can be temporarily reduced or blocked by chemicals and whether exposure to one carcinogen would sensitize cells

towards the effect of other chemical carcinogens.

In this chapter the dual action of carcinogens is examined: namely their capacity to induce DNA lesions and their capacity to affect a normal DNA repair synthesis.

The following experimental protocol was used: non-dividing human cells were either UV-irradiated and thereafter exposed to chemical carcinogens, or treated with the chemical carcinogen prior to, as well as after, UV-irradiation (Fig. 13). Following this combined application of UV-irradiation and a chemical carcinogen, the cells were placed into culture medium with ^{3}HTdR to measure DNA repair synthesis (Fig. 14, 15 and 16) or maintained in MEM (15% FCS) to estimate the clone-forming capacity (Fig. 17 and 18).

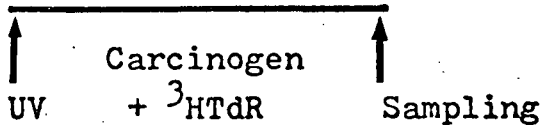
The results show that all the active chemical carcinogens examined inhibit DNA repair synthesis and that various carcinogenic compounds greatly differ in their capacity to induce DNA lesions and to inhibit DNA repair. Unfortunately, it is somewhat difficult to assess the results of the combination experiments, because the chemical carcinogens add new DNA lesions to those induced by UV-irradiation. Thus the ensuing unscheduled DNA synthesis is a product of the DNA lesions induced by UV, DNA lesions induced by the chemical, and the extent to which DNA repair synthesis is adversely affected by UV and the chemical carcinogen. In spite of these technical restrictions, one can grade carcinogens as strong or weak inducers of DNA lesions and strong or weak inhibitors of DNA repair.

Figure 13 Experimental Design. Inhibition of DNA repair synthesis in cultured human fibroblasts following the combined application of UV radiation and a chemical carcinogen.

Figures 14-16 Inhibition of DNA repair synthesis in cultured human fibroblasts following the combined application of UV radiation and a chemical carcinogen.

- □ = unscheduled ^{3}HTdR incorporation following 1.5 hours of N-acetoxy-4-AAS (Figure 14), nitrogen mustard (Figure 15), or FANFT (Figure 16).
- ● = unscheduled ^{3}HTdR incorporation following UV irradiation (900 ergs/mm^{2}) and one of the three compounds.
- ▲ = theoretical curves showing a strict additive effect of UV and one of the three compounds (an additive effect occurs at the lowest concentrations employed).

(13) Protocol:



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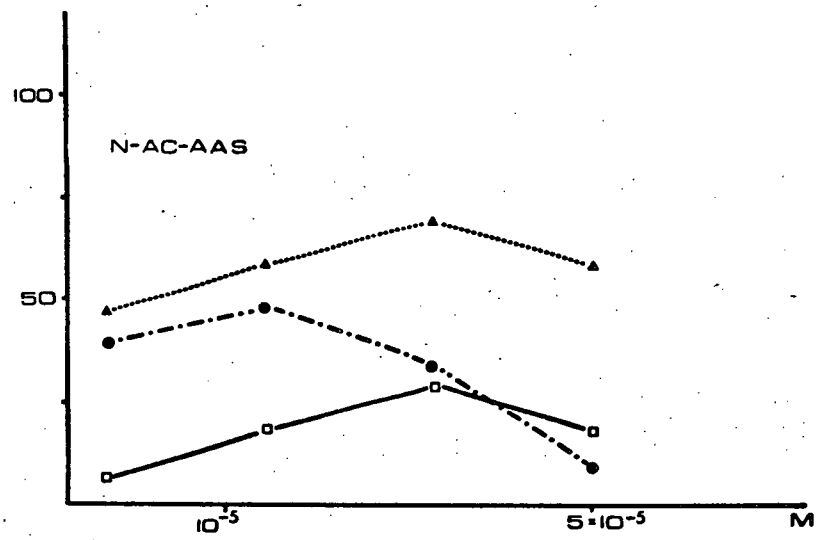

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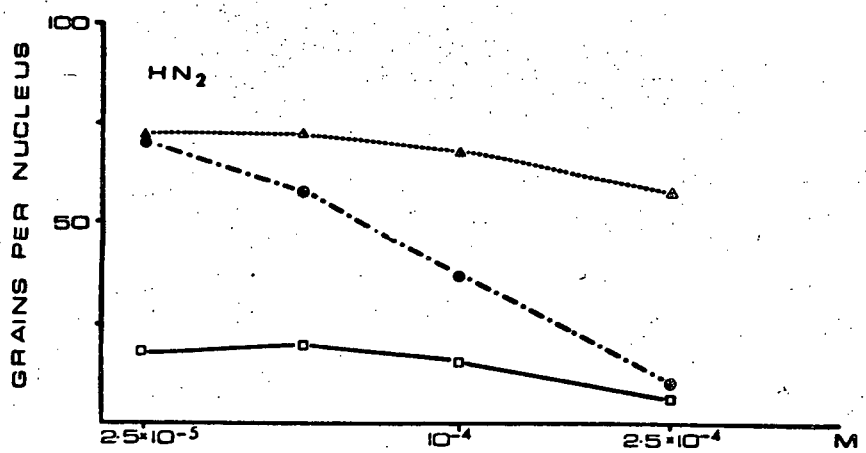

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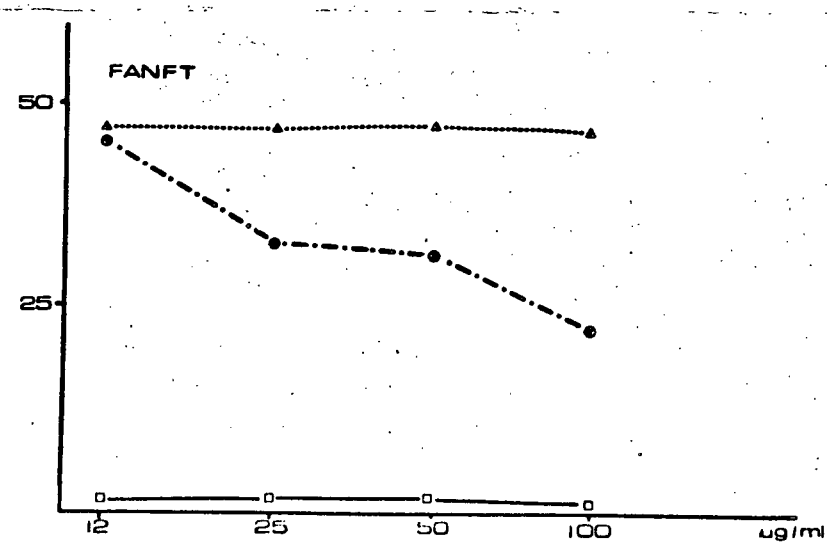
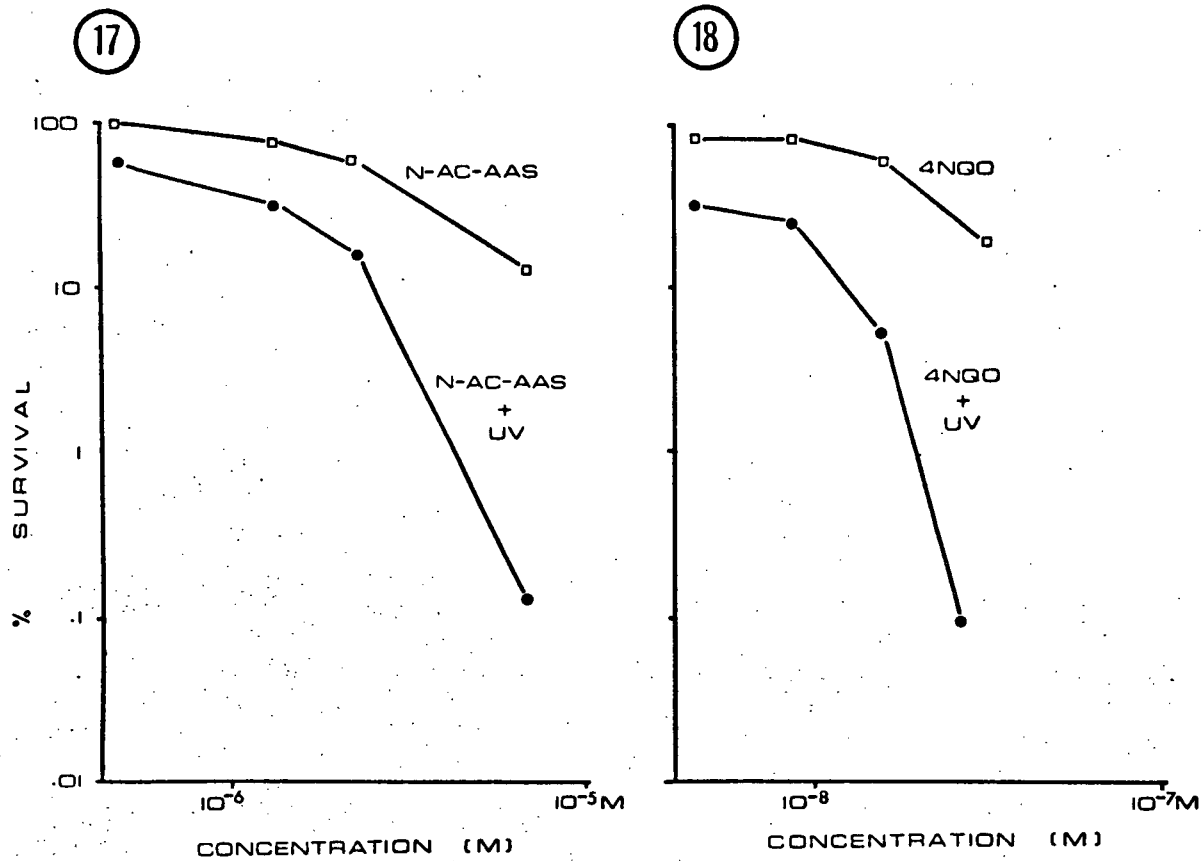




Figures 17-18 Effect of a combined treatment of UV radiation and a chemical carcinogen on the clone-forming capacity of cultured human fibroblasts.

(Figure 17) UV radiation (16 ergs/mm^2) followed by N-acetoxy-4-AAS (5 hours)

(Figure 18) UV radiation (16 ergs/mm^2) followed by 4NQO (1.5 hours)

The absence of a DNA repair synthesis following the administration of a compound could be due to the lack of its interaction with DNA (e.g. non-oncogenic compounds) or due to a blockage of repair processes by a chemical with a strong inhibitory property (e.g. acriflavine, daunomycin, mitomycin C). An unanswered question remains as to whether a chemical could have a carcinogenic effect by merely inhibiting repair of 'spontaneously' occurring DNA lesions and in this manner contribute to genetic anomalies and neoplastic transformation. Extensive studies by Gaudin et al. (1972a, 1972b) have revealed that all co-carcinogens examined inhibit DNA repair synthesis.

2. DNA Repair Deficiency in Xeroderma Pigmentosum Cells

Numerous studies on microbial systems have revealed that an elevated sensitivity to UV-irradiation is due to a defect in one of the steps in the DNA excision-repair or 'cut and patch' procedure (Witkin, 1969; Kondo et al., 1970; Ganesan and Smith, 1971). The excision-deficient bacterial mutants and UV-sensitive yeast cells also show an increased sensitivity to the lethal action of exogenous chemicals and an elevated mutation rate when exposed to UV-radiation or challenged with several chemical mutagens (Haynes et al., 1968; Kondo and Kato, 1968). These microbial systems have proven to be good models for normal and repair-deficient human cells in which the role of DNA repair and its genetic control are only now being slowly unravelled. For example,

homozygous recessive Xeroderma pigmentosum (XP) cells are known to be deficient in correcting UV-induced thymine dimers and possibly cytosine dimers in the DNA molecules (Cleaver, 1968; Setlow et al., 1969).

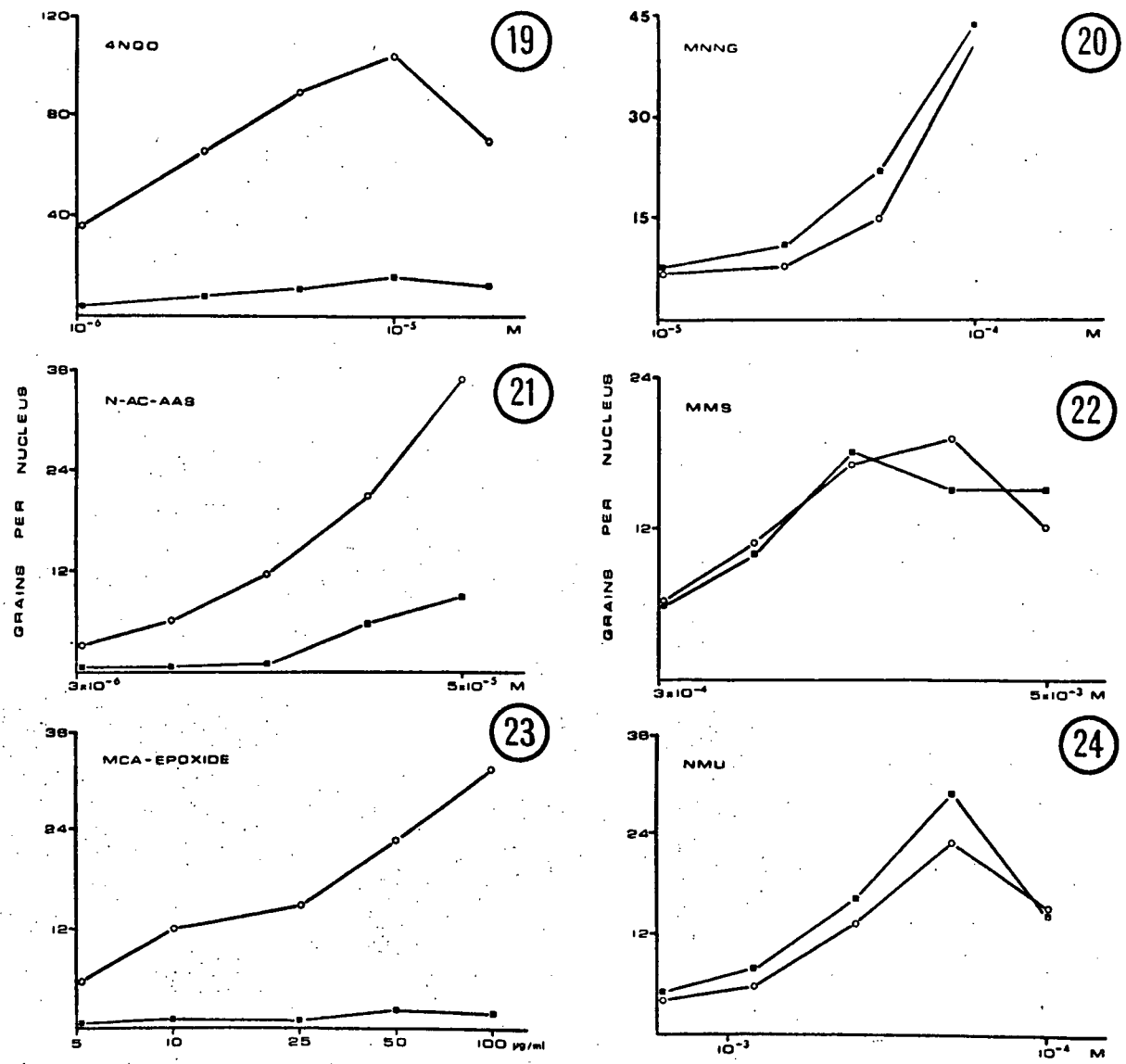
In this chapter, an attempt is made to answer three questions concerning the sensitivity of Xeroderma pigmentosum cells to chemical carcinogens. The first question is whether XP cells respond to chemical carcinogens with a reduced DNA repair capacity. A second point of interest is the wide spectrum of DNA repair deficiencies found in cells of unrelated patients. The question is therefore whether XP cells, which greatly differ in their capacity to repair UV-induced DNA changes, also differ in their response to chemically induced DNA alterations. Thirdly, the question must be raised as to the DNA repair capacity of XP-heterozygous persons. The ultimate goal is to explore the possible use of the DNA repair bioassay in the identification of persons hypersensitive towards chemical carcinogens. Unless otherwise stated, XP_E cells were used in comparative studies with cells from control persons.

2.1. Response of XP Cells to Different Carcinogens

The response of XP cells towards chemical or viral carcinogens has remained unknown until recently (Cleaver, 1971a; Setlow and Regan, 1972; Stich and San, 1971; Stich et al., 1972a, 1972b). To gain information on this important question, key carcinogens of diverse molecular structures were selected and added to cultured XP fibroblasts and

normal human cells for short time periods (1.5 - 5 hours). The unscheduled incorporation of ^{3}HTdR into nuclear DNA of non-dividing cells was then estimated by the previously described autoradiographic technique. The results are summarized in Figs. 19 - 24. The XP cells show a considerably reduced DNA repair synthesis when exposed to some but not all chemical carcinogens (e.g. 4NQO, N-Ac-AAS, MCA-Epoxide) (Fig. 19, 21, 23). Thus, it is misleading to refer to XP cells as deficient in DNA repair without mentioning the 'agent provocateur'. As can be seen from Fig. 20, 22, 24 and Table II, XP cells can cope normally with a DNA damage induced by typical alkylating mutagens and carcinogens, including MNNG, MMS and NMU.

This cell-controlled degree of response to various chemical agents is difficult to interpret at present. Although the reaction between DNA and the various agents used are not fully known the ensuing molecular changes will certainly differ: thymidine dimers and, to a lesser degree, cytosine-thymidine dimers, are formed by ultraviolet irradiation (Smith and Hanawalt, 1969), arylamidation of guanine and, to a lesser extent, of adenine, will follow exposure to N-hydroxy and N-acetoxy-AAF (Miller et al., 1966a; Kriek et al., 1967), and the binding between 4NQO or 4HAQO and DNA may lead to charge transfer complexes involving purines (Okano et al., 1969; Paul et al., 1971; Okano et al., 1972), or to an electrophilic attack of nucleic acid bases (Enomoto et al., 1968). By contrast, X-ray and MNNG seem



Figures 19-24 Unscheduled ^{3}HTdR incorporation in cultured fibroblasts of Xeroderma pigmentosum patients (■) and control persons (O) following short term exposures to carcinogens and mutagens:

(Figure 19) 4NQO (1.5 hours)
(Figure 20) MNNG (3 hours)
(Figure 21) N-Acetoxy-4-AAS (5 hours)
(Figure 22) MMS (1.5 hours)
(Figure 23) MCA Epoxide (3 hours)
(Figure 24) NMU (1 hour)

TABLE II Unscheduled DNA Synthesis in Xeroderma Pigmentosum Fibroblasts Following Exposure to Chemical Carcinogens

Normal Repair Capacity*	Reduced Repair Capacity
MNNG	4NQO & carcinogenic derivatives
MMS	3-Me-4NPO
EMS	MCA-6,7-Epoxide (K-region)
NMU	BA-5,6-Epoxide (K-region)
HN_2	N-Acetoxy-2-AAF
ICR-191	N-Hydroxy-2-AAF
Streptonigrin	N-Acetoxy-4-AAS
Methylguanidine (Nitrosation)	N-Hydroxy-4-AAS
	N-Acetoxy-4-AABP
	N-Hydroxy-4-AABP
	N-Acetoxy-2-AAP
	N-Hydroxy-2-AAP
	N-Myristoyloxy-2-AAF
	N-Acetoxy-3-AAF
	1'-Hydroxy-Safrole
	3'-Hydroxy-Safrole
	3'-Acetoxy-Safrole
	1,1-Diphenyl-2-propynyl-N-cyclohexylcarbinol
	1-Phenyl-1-(3,4-xylyl)-2-propynyl cyclohexylcarbamate
	DMN (activation)
	Aflatoxin B_1 (activation)
X-ray	UV

* Compared to level of unscheduled ^{3}HTdR incorporation in fibroblasts from control persons.

to elicit a normal degree of DNA repair synthesis in XP cells (Cleaver, 1971a; Stich et al., 1972b). It seems likely that the two groups of agents differ in the way in which they cause single-strand breaks (Regan and Setlow, 1974). An incision-producing enzyme seems to be required to induce strand breakage and initiation of repair processes following ultraviolet, nitroquinoline oxides and the aromatic amide derivatives, whereas its presence seems unnecessary to produce DNA strand breaks and repair synthesis following the agents of the second group.

2.2. Variation in DNA Repair Capacity

With respect to UV-induced DNA alterations, various levels of DNA repair deficiencies have been found in cultured cells obtained from several unrelated XP patients (Bootsma et al., 1970; Cleaver, 1970 , 1972 ; Stich et al., 1972b; Robbins et al., 1972, 1974). Despite the fact that slightly different techniques were used in different laboratories to estimate the degree of deficiency and that the results may not be strictly comparable, the data clearly demonstrate that among the different XP patients examined the DNA repair synthesis varies from an undetectable level to 100% of that found in cells of non-afflicted control persons following UV-irradiation. Complementation studies (somatic cell hybridisation between cells of two unrelated XP patients) suggest the presence of different gene mutations among XP patients (De Weerd-Kastelein et al., 1972, 1973, 1974).

The question then arises as to whether the XP cells, which greatly differ in their capacity to repair UV-induced DNA changes, also differ in their response to chemically induced DNA alterations. Cell cultures of six XP patients were compared for the degree of DNA repair synthesis following exposure to UV and a selected group of chemical carcinogens. Each compound was used at a concentration which results in a very high degree, if not the maximum, of unscheduled DNA synthesis. The results are expressed in percentages of DNA repair synthesis found in cultured cells of twelve unaffected control persons (Table III). The levels of DNA repair synthesis among the unrelated XP patients differed significantly and were barely affected by the type of initiating agent used. Sister (XP_{H1}) and brother (XP_{H2}), however, showed a comparable degree of deficiency (Fig. 25). The similar response of sib members (XP_{H1}, and XP_{H2}, XP_{C1} and XP_{C2}) following such diverse obnoxious agents as N-hydroxy-AAF, N-acetoxy-AAF, 4-nitroquinoline 1-oxide and ultraviolet indicates genetic control of the degree of impairment.

2.3. XP with Normal DNA Repair Capacity

It has been suggested that the clinical manifestations of XP (hypersensitivity to UV and multiple actinic skin lesions) could be attributed to the defective or deficient DNA repair mechanism in these patients. Whether the degree of DNA repair deficiency varies with the severity of the

TABLE III Comparative Levels of DNA Repair Synthesis in Xeroderma Pigmentosum Fibroblasts Following Exposure to UV, 4NQO, N-Acetoxy-2-AAF and MNNG

Patient	Sex	Ultraviolet 1000 ergs per mm^2	4NQO $2x10^{-6}M$ 1.5 Hr	N-Acetoxy-2-AAF $5x10^{-5}M$ 5 Hr	MNNG $2x10^{-4}M$ 1.5 Hr
XP_{H1}	F	9.8	11.5	10.4	103
XP_{H2}	M	12.1	13.1	16.0	103
XP_{E}	F	21.5	26.9	22.9	102
XP_{C1}	M	33.3	32.6	31.1	110
XP_{C2}	F	29.4	31.9	27.4	108
XP_{V}	F	36.0	33.6	29.1	105
XP_{K}	M	56.6	53.8	60.7	107
Heterozygotes					
XP_{E} Father		99.8	102.9	101.1	104
XP_{E} Mother		102	111.5	106	101
XP_{C} Father		104	120.2	98.6	112
XP_{C} Mother		102	122.1	102	106
XP_{V} Mother		101	108.5	100	102
XP_{K} Father		123.5	94.2	104	106
XP_{K} Mother		116.1	95.2	106	109
Control*	M+F	100%	100%	100%	100%

* Fibroblasts of twelve unaffected subjects (six males and six females) were used to obtain a control level of DNA repair synthesis. The unscheduled incorporation of ^{3}HTdR varied within 11% among the 12 individuals examined. The average number of grains above nuclei of cells which were irradiated or exposed to various chemical agents was put at 100%.

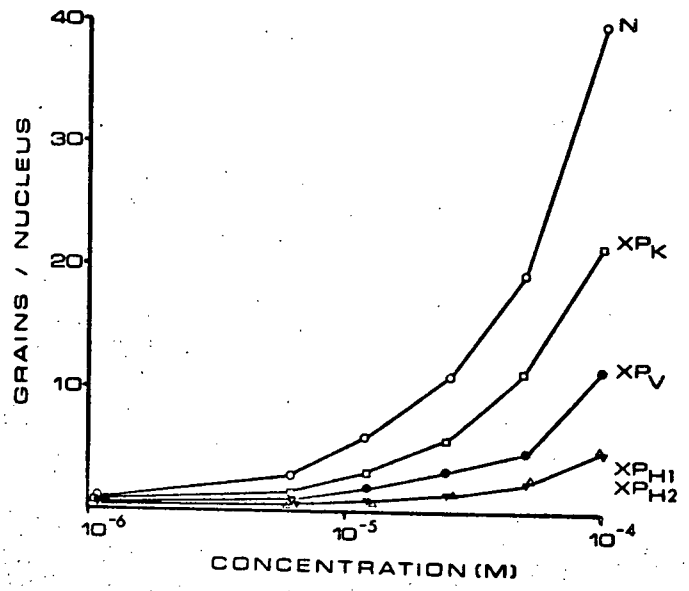


Figure 25 Unscheduled incorporation of ^{3}HTdR into nuclei of normal and four XP fibroblasts exposed for 5 hours to N-hydroxy-AAF. Autoradiography.

- ○ Normal person
- □ XP$_K$ (Kamloops)
- ● XP$_V$ (Vancouver)
- △ XP$_{H1}$ (Hamilton)
- ▼ XP$_{H2}$ (Hamilton)

clinical picture is difficult to assess at the moment. The fact that a few cases of patients with deficient XP symptoms have a normal DNA repair capacity argues against a causal relationship between the clinical manifestations and an incision defect at the molecular level (Robbins et al., 1972; Cleaver, 1972 ; Robbins and Burk, 1973).

2.4. DNA Repair Capacity in XP Heterozygotes Following Exposure to Chemical Carcinogens

Cultured cells from XP patients have been shown to exhibit DNA repair deficiency following exposure to some but not all chemical carcinogens (Section 2.1.). Furthermore, with respect to UV or chemical-induced DNA alterations, various levels of DNA repair deficiency have been found in cultured cells obtained from several unrelated XP patients. The question follows whether the parents of XP patients, being obligate heterozygotes, would manifest a variation in DNA repair capacity. The level of unscheduled ^{3}HTdR incorporation in cultured cells from different XP heterozygotes following exposure to several chemical carcinogens are not significantly different from that in the control cells (Section 2.2., Table III). Other heterozygote studies have revealed variable results with repair of UV-induced DNA damage ranging from 50 to 100 percent of normal (Cleaver, 1969, 1971b, 1972 ; Bootsma et al., 1970; Kleijer et al., 1973). However, since some of the techniques employed did not measure repair activity directly, the findings are only

suggestive of differences among the various heterozygotes.

3. DNA Repair, Chromosome Aberrations and Clone Forming Ability in Normal and Xeroderma Pigmentosum Cells

Variations in sensitivity within the human population should be considered when deciding on "permissible" or "safe" levels of carcinogenic or mutagenic agents. At present this aspect of environmental carcinogenesis and mutagenesis is somewhat neglected because of the scarcity of reliable information about the range of sensitivity within the human population and the response of sensitive cells towards particular chemical compounds. In the preceding section the repair of DNA damage in XP cells exposed to various chemical carcinogens was examined. This chapter reviews the frequency of chromosome aberrations in cultured cells of patients with Xeroderma pigmentosum and of control persons following exposure to various chemical carcinogens.

Chromatid breaks, isochromatid breaks and single and multiple exchanges which can be readily quantified were employed as a sensitive indicator of an induced damage to the genome of normal and XP cells.

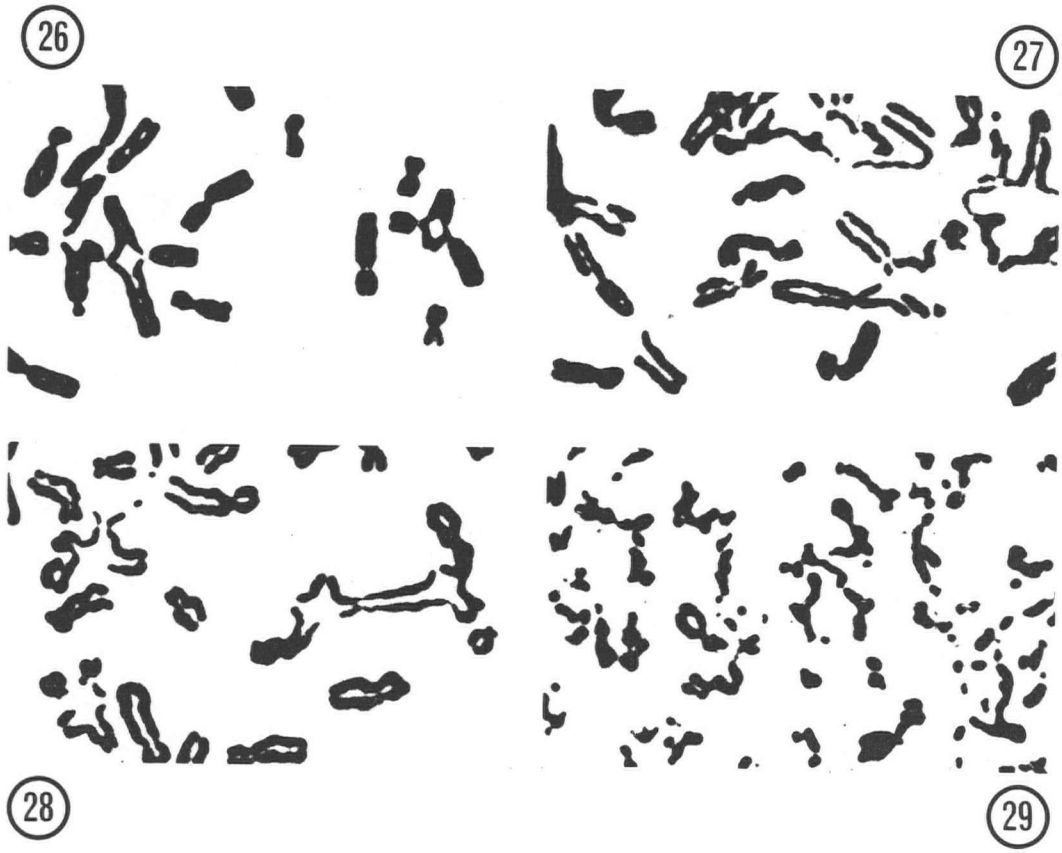
The second question raised is whether any differences between normal and XP cells in response to chemical carcinogens can also be detected at the cellular level. Clone forming capacity of normal and XP cells following exposure to various chemical carcinogens was used as one endpoint.

As previously described, XP cells with varying degree of DNA repair deficiency respond to certain chemical carcinogens differently (Section 2.1.). The question then arises as to whether such differences in DNA repair capacity are reflected in the frequency of chromosome aberrations and clone forming efficiency after exposure to chemical carcinogens.

3.1. Chromosome Aberrations in Normal and XP Cells Following Exposure to Chemical Carcinogens

The frequency of metaphase plates with chromosome aberrations in populations of cultured normal and XP cells following exposure to various chemical carcinogens is shown in Fig. 30 - 35. The types of chromosome aberrations observed include single or multiple chromatid breaks, single or multiple chromatid exchanges and chromatid fragmentation (Fig. 26 -29). Two types of chemicals were examined: (1) those that induce DNA changes that can be normally repaired by XP cells and (2) compounds that evoke DNA alteration that cannot be corrected at a normal rate by the repair deficient XP cells.

Among the compounds that lead to relatively high levels of DNA repair synthesis in control cells but evoke only low levels of unscheduled ^{3}HTdR uptake in XP cells, the following are included: 4NQO, BA-epoxide, N-hydroxy-2-AAF, N-acetoxy-2-AAF, N-hydroxy-4-AAS, and N-acetoxy-4-AAS. In the XP cells, a striking increase in chromatid aberrations occurred at doses of carcinogens which did not significantly



Figures 26-29 Part of metaphase plates of cultured XP cells exposed for 1.5 hours to 2.5 x 10^{-7}M 4NQO and sampled 20 hours post treatment.

(Figure 26) Two translocation figures.

(Figures 27-28) Chromatid breaks, multiple chromatid exchanges and chromatid fragments.

(Figure 29) Severe fragmentation of the entire chromosome complement.

Figures 30-35 Frequency of metaphase plates with chromosome aberrations in XP (■) and normal (O) cell cultures following a single dose of a carcinogen:-

(Figure 30) 4NQO (1.5 hours)

(Figure 31) BA Epoxide (3 hours)

(Figure 32) N-Hydroxy-2-AAF (5 hours)

(Figure 33) N-Acetoxy-2-AAF (5 hours)

(Figure 34) N-Hydroxy-4-AAS (5 hours)

(Figure 35) N-Acetoxy-4-AAS (5 hours)

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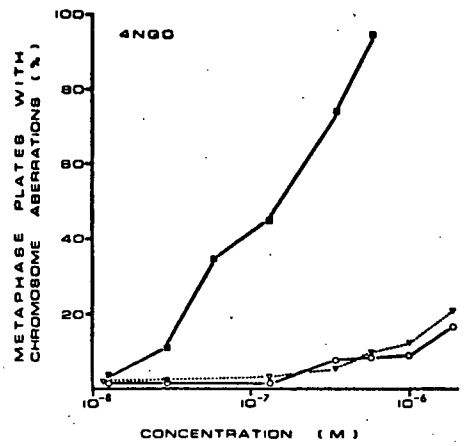

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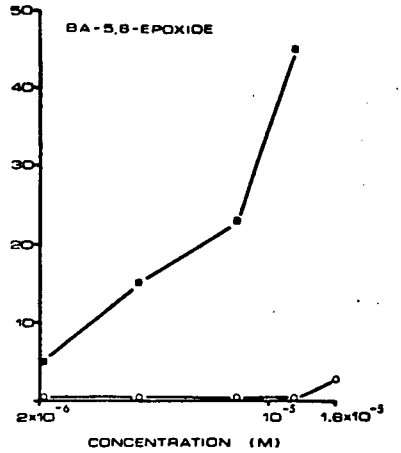

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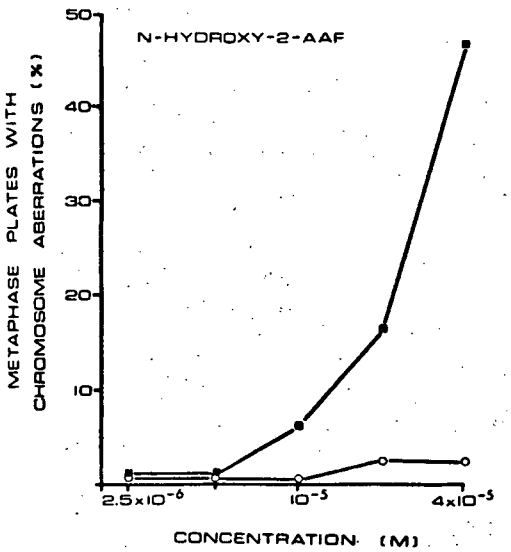

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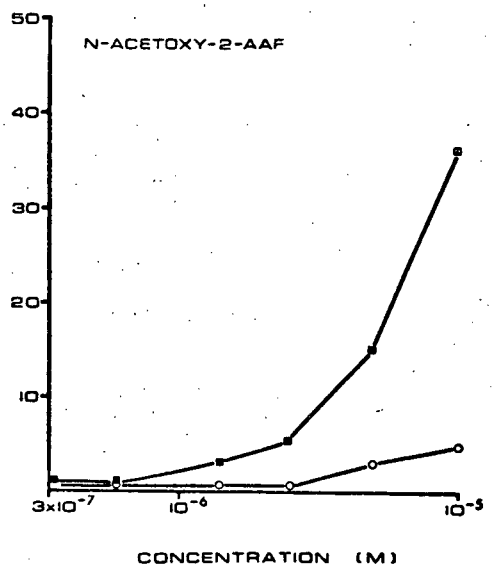

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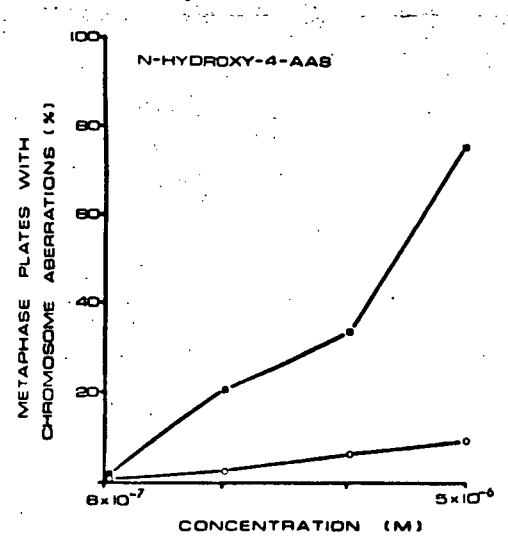

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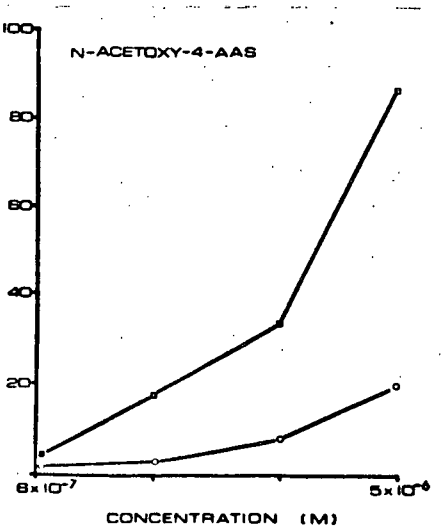

elevate the frequency of chromosome aberrations in the control fibroblasts (Fig. 30-35). About 100 to 1,000 times higher concentration of these carcinogens must be applied to normal cells in order to obtain frequencies of aberrations comparable to those induced in the sensitive XP cells.

Since an exclusive reliance on one sampling period could easily lead to erroneous conclusions, a time study has been performed with 4NQO (Fig. 36) and BA-6,7-epoxide (Fig. 37). For a two-day period the frequency of XP metaphase plates with chromosome aberrations was above that seen in equally treated normal cells. Thereafter the level of chromosome aberrations of the 4NQO or BA-6,7-epoxide exposed normal and XP cells was in the range of untreated cells. Thus the 4NQO or BA-6,7-epoxide concentration used induced a wave of chromosome aberrations in the XP cells.

To answer the question whether the length of exposure to a chemical carcinogen has any effect on the frequency of chromosome aberrations in the treated cells, the data on two examples are presented here. Cultured normal XP cells exposed to N-acetoxy-2-AAF ($5x10^{-6}M$) or N-acetoxy-4-AAS ($5x10^{-6}M$) showed an increase in the frequency of chromosome aberrations with exposure time (Fig. 38). A particular exposure time (5 h. in this instance) was arbitrarily chosen for the comparative study between XP and control cells.

MNNG was chosen as a representative of the group of chemical carcinogens that elicit comparable levels of DNA

Figure 36 Frequency of metaphase plates with chromosome aberrations in XP (■) and normal (O) cell cultures at various times following a single dose of 4NQO ($1x10^{-7}$M, 1.5 hours). C= the frequency of metaphase plates with chromosome aberrations in untreated cultures.

Figure 37 Frequency of metaphase plates with chromosome aberrations in XP (■) and normal (O) cell cultures at various times following a single dose of BA Epoxide ($4x10^{-6}$M, 3 hours). C = the frequency of metaphase plates with chromosome aberrations in untreated cultures.

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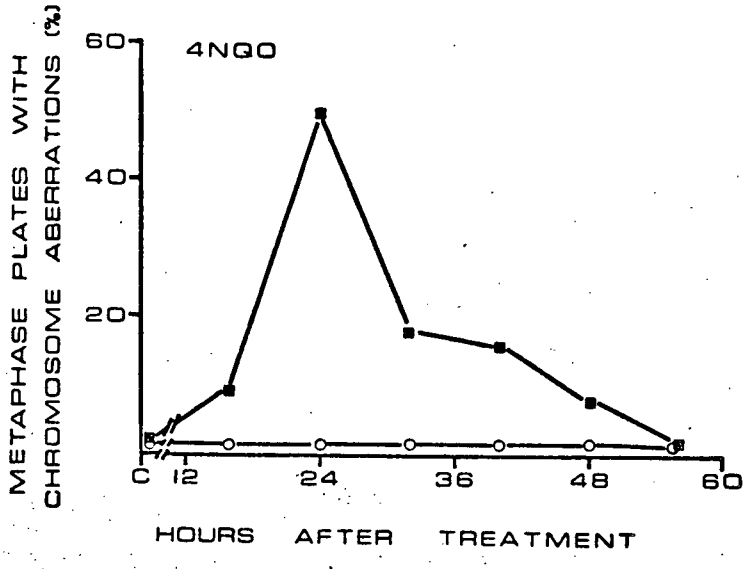
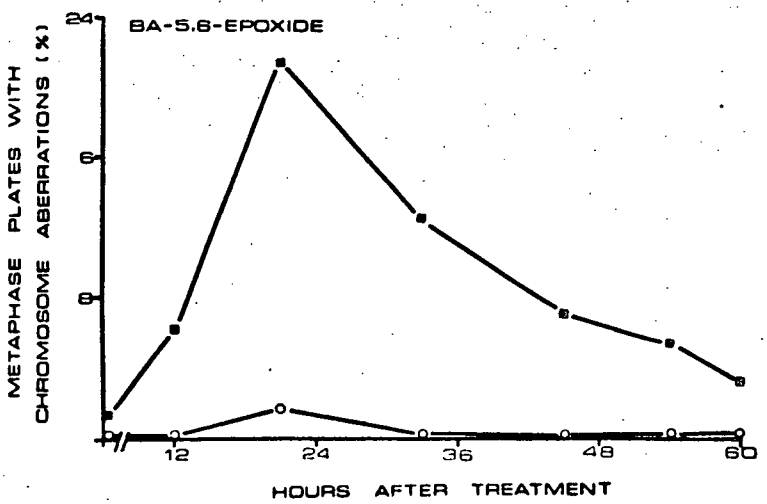

4NQO
METAPHASE PLATES WITH CHROMOSOME ABERRATIONS (%)
60
40
20
0
12
24
36
48
60
HOURS AFTER TREATMENT

37


BA-5,6-EPOXIDE
METAPHASE PLATES WITH CHROMOSOME ABERRATIONS (%)
24
6
0
12
24
36
48
60
HOURS AFTER TREATMENT

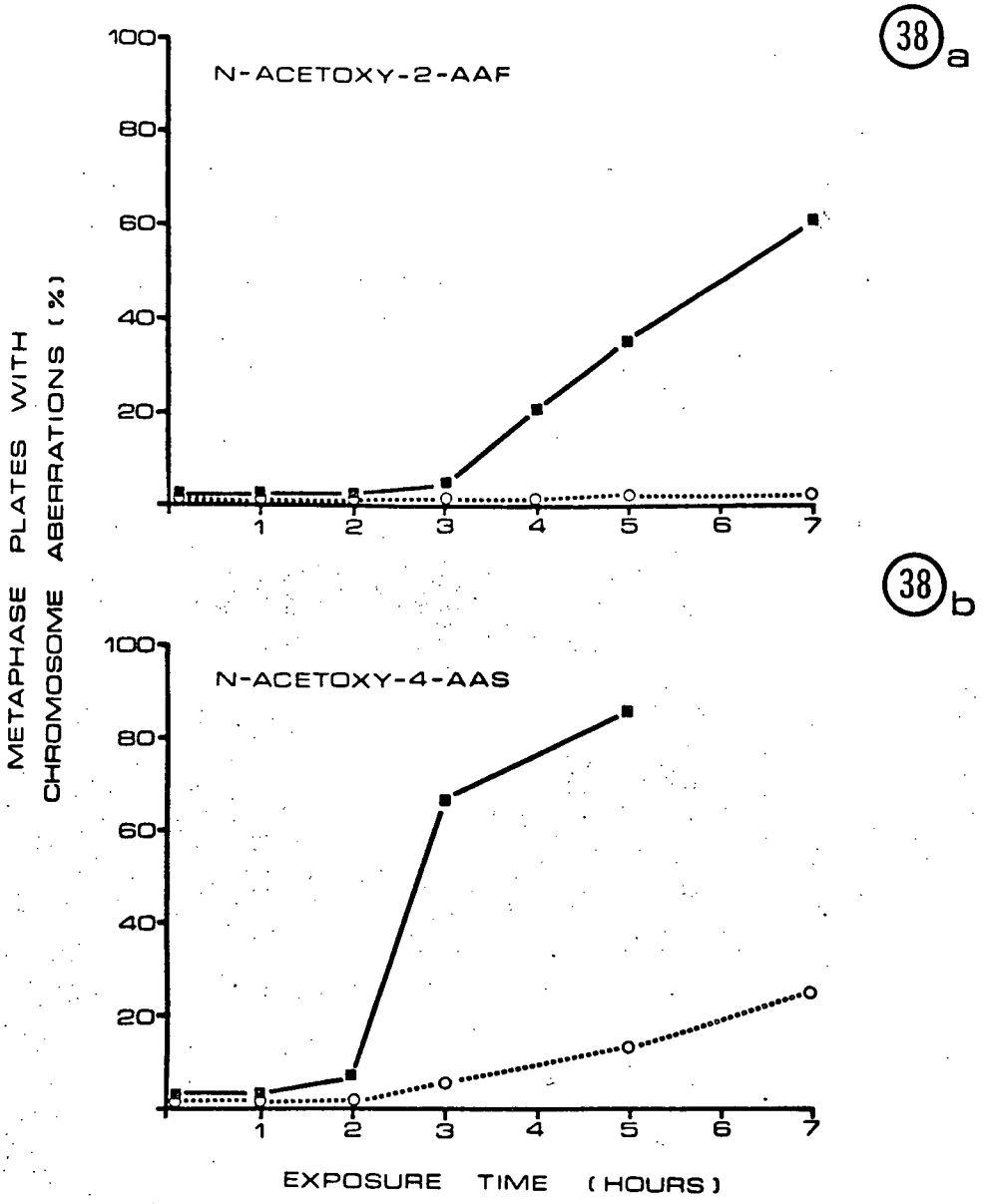


Figure 38 Effect of variation in exposure time to a chemical carcinogen on the frequency of metaphase plates with chromosome aberrations in XP (■) and normal (O) cell cultures:-

(a) N-Acetoxy-2-AAF (5×10^{-6}M)

(b) N-Acetoxy-4-AAS (5×10^{-6}M)

repair synthesis in XP cells and controls. The frequency of metaphase plates with chromatid breaks and chromatid exchanges in the cultured XP cells resembles that found in control fibroblasts treated with equimolar concentrations of MNNG (Figs. 39 and 40).

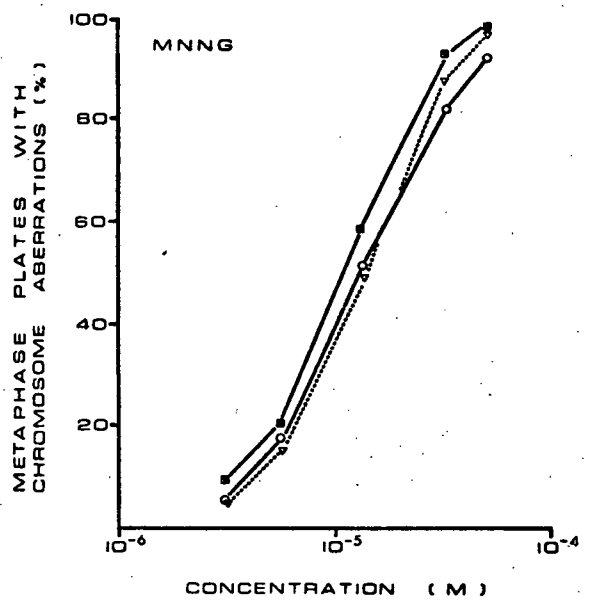
3.2. Clone-Forming Capacity of Normal and XP Cells Following Exposure to Chemical Carcinogens

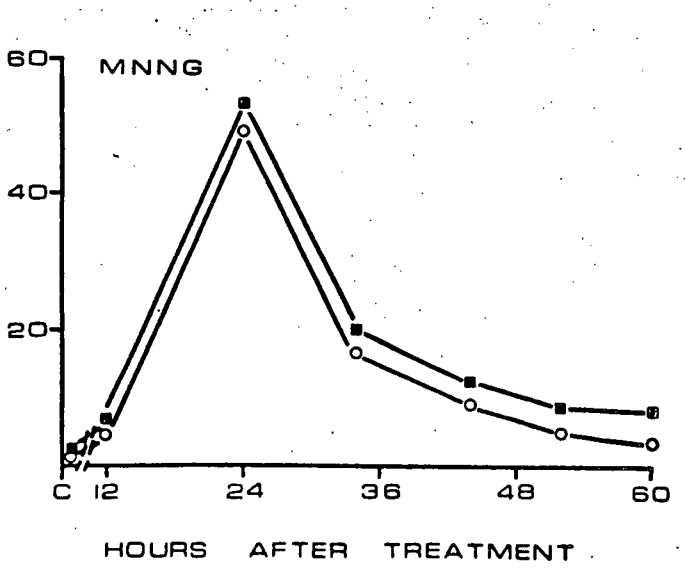
The response of cells to damaging agents is to a large extent under genetic control. Bacteria deficient in the excision mechanism of pyrimidine dimers or in the recombination process show an increased susceptibility to physical and chemical mutagens (Haynes et al., 1968; Kondo and Kato, 1968). The elevated sensitivity was demonstrated by comparing the clone-forming capacity of repair-deficient mutants with that of normal bacteria. This procedure was successfully applied to cultured cells of patients with XP. The repair-deficient cells proved to be more sensitive to the lethal effect of UV-radiation than fibroblasts from non-afflicted persons (Stich et al., 1972a; Maher et al., 1975b).

Somatic cells of XP patients are also defective in the repair of DNA alterations induced by various chemical carcinogens (Section 2.1.). The question must be raised whether the reduced repair level of chemically induced DNA lesions in the XP cells is reflected in an increased sensitivity to the inducing agents.

Figure 39 Frequency of metaphase plates with chromosome aberrations in cultured XP cells (■) and normal cells (O , ▽) exposed to various concentrations of MNNG (3 hours).

Figure 40 Frequency of metaphase plates with chromosome aberrations in cultured XP cells (■) and normal cells (O) at various times following a single dose of MNNG (1.2×10^{-5}M, 3 hours).


39
MNNG
METAPHASE PLATES WITH CHROMOSOME ABERRATIONS (%)
100
80
60
40
20
10^{-6}
10^{-5}
10^{-4}
CONCENTRATION (M)


40
MNNG
60
40
20
C
12
24
36
48
60
HOURS AFTER TREATMENT

In this chapter the clone-forming capacity and level of DNA repair of XP and normal cells exposed to various chemical carcinogens is reported.

As representative examples of the group of chemical carcinogens which elicited a reduced level of DNA repair synthesis in XP cells, the following have been included: 4NQO, 4HAQO, 2-Me-4NQO, 7-Me-4NQO, BA-Epoxide, N-hydroxy-2-AAF, N-acetoxy-2-AAF, N-hydroxy-4-AAS and N-acetoxy-4-AAS. The effect of these compounds on the capacity of normal and XP cells to form clones is shown in Figs 41 - 49. The XP cells are highly sensitive to the lethal effect of these carcinogens and their response is comparable to that following UV-irradiation (Fig. 50). A similar killing effect in control cells was apparent only at much higher concentrations of the carcinogens used.

With respect to MNNG and MMS, which induced a comparable level of unscheduled ^{3}HTdR uptake in both XP and control cells, there was only a small difference in clone-forming capacity between the XP cells and normal ones (Fig. 51, 52).

3.3. Effect of Chemical Carcinogens on the Frequency of Chromosome Aberrations and Clone-Forming Capacity in XP Cells with Different DNA Repair Deficiencies

It has been found that fibroblasts of unrelated XP patients do exhibit different DNA repair capacities (Section 2.2.). For example, with respect to the repair of UV-induced

Figures 41-46 Clone-forming capacity of normal human fibroblasts (O) and XP cells (■) following exposure to a single dose of a carcinogen (expressed as percentage of survival in each cell line's untreated controls).

(Figure 41) 4NQO (1.5 hours)

(Figure 42) 4HAQO (1.5 hours)

(Figure 43) 2-Methyl-4NQO (1.5 hours)

(Figure 44) 7-Methyl-4NQO (1.5 hours)

(Figure 45) N-Hydroxy-2-AAF (5 hours)

(Figure 46) N-Acetoxy-2-AAF (5 hours)

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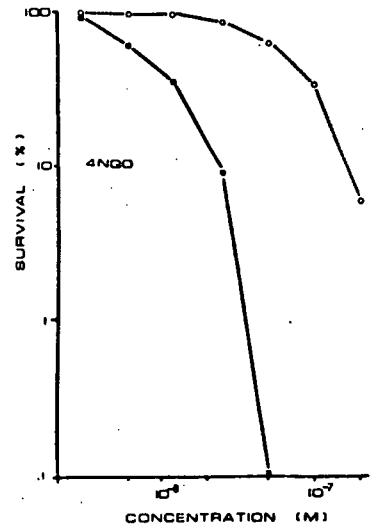

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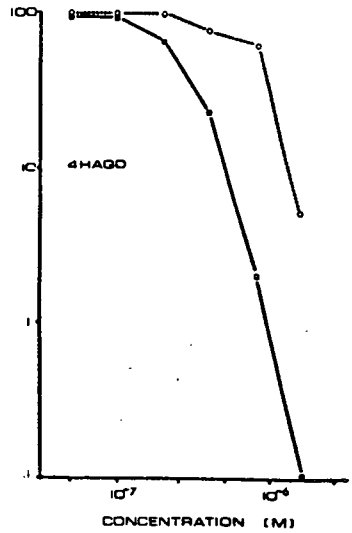

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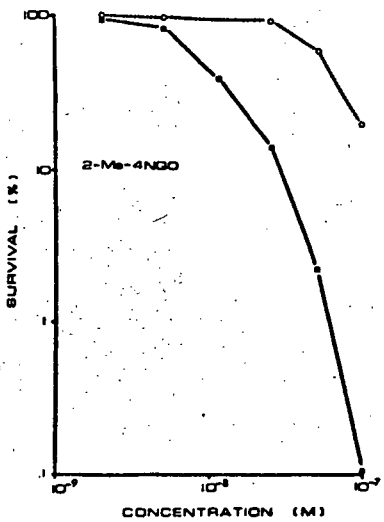

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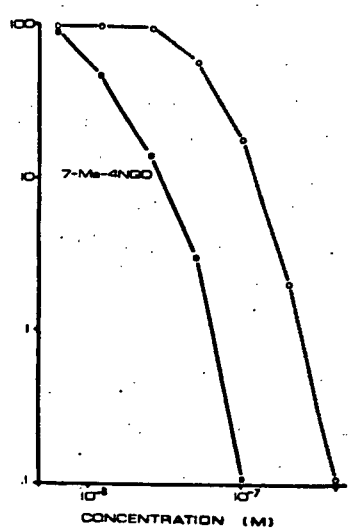

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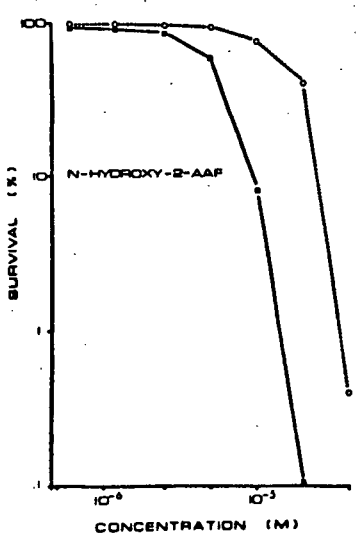

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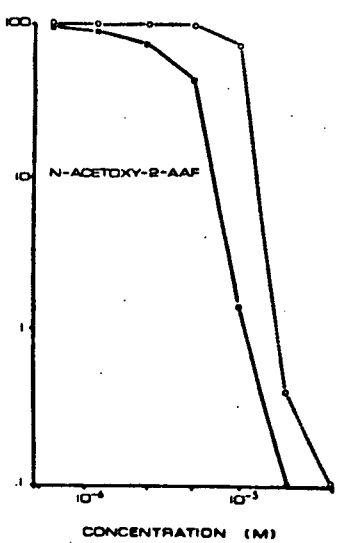

Figures 47-52 Clone-forming capacity of normal human fibroblasts (O) and XP cells (■) following exposure to a single dose of a carcinogen.

(Figure 47) N-Hydroxy-4-AAS (5 hours)

(Figure 48) N-Acetoxy-4-AAS (5 hours)

(Figure 49) BA-5,6-Epoxide (5 hours)

(Figure 50) UV radiation (260nm)

(Figure 51) MNNG (3 hours)

(Figure 52) MMS (1.5 hours)

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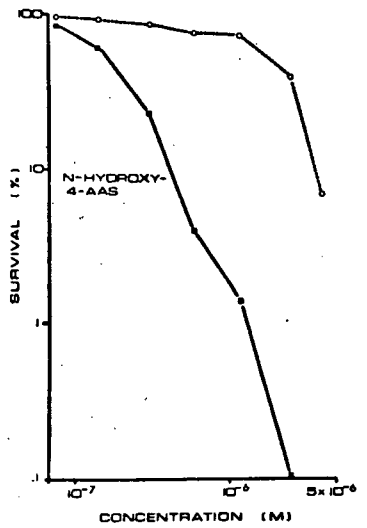

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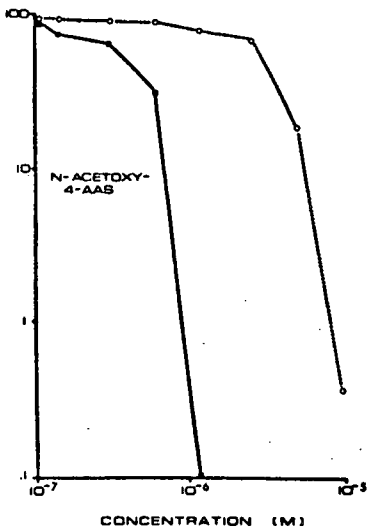

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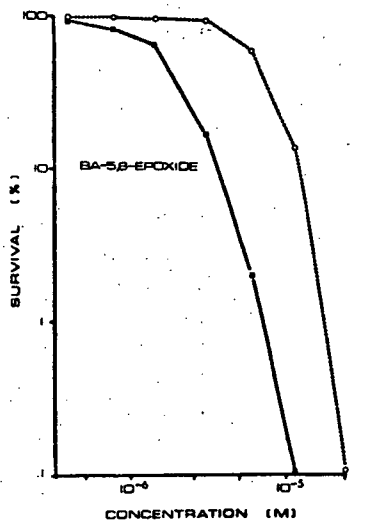

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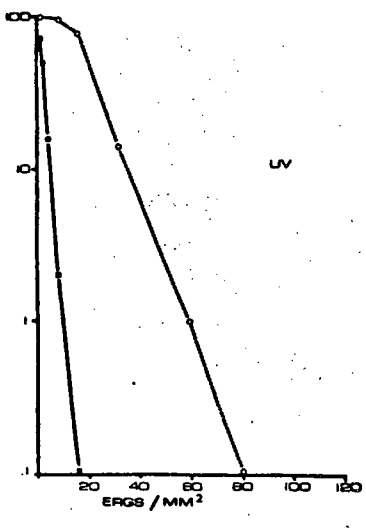

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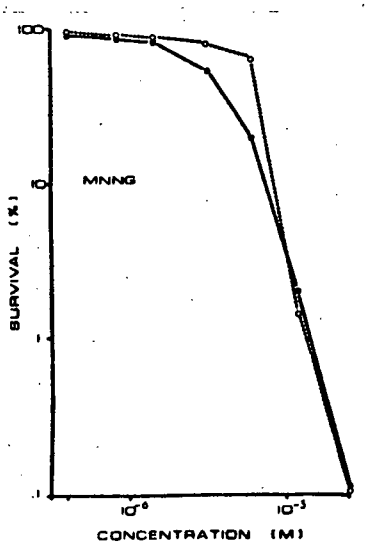

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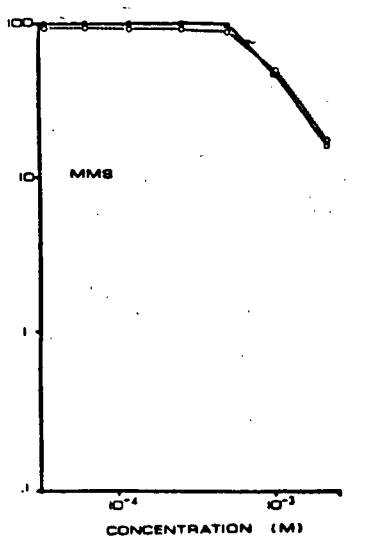


DNA damage, the level of unscheduled ^{3}HTdR uptake in various XPs (as compared to unafflicted controls) were found to be as follows: XP-H_1 9.8%, XP-H_2 12.1%, XP-E 21%, XP-C 34%, XP-V 36% and XP-K 56% (Table III and Section 2.2.). Furthermore, the different levels of DNA repair synthesis among the unrelated XP patients were not affected by the type of initiating agent used (Table III and Section 2.2.). The question which arises from this observation is whether such differences in DNA repair capacity is reflected at the chromosome or cellular level. A comparative study was then performed with cells from four unrelated XP patients. Again, a representative compound was selected from the group of chemical carcinogens which elicited a reduced level of unscheduled ^{3}HTdR uptake in XP cells (e.g. 4NQO, N-acetoxy-2-AAF) and those which provoked a comparable level of DNA repair synthesis in both XP and control cells (e.g. MNNG). In each instance, the level of unscheduled ^{3}HTdR incorporation, the frequency of metaphase plates with chromosome aberrations and the clone-forming capacity were examined. The results are shown in Tables IV to VI. It must be pointed out that, for technical reasons, the same concentration of a carcinogen cannot be used if observable results are to be obtained in the three assay systems. In general, the concentrations required to produce a detectable level of unscheduled ^{3}HTdR uptake are higher than those used for chromosome aberrations and cloning studies.

With reference to the different XPs tested, it appears

that the level of DNA repair deficiency correlates with the frequency of chromosome aberrations and clone forming capacity. As an example, one can compare the response of XP-K and XP-E to 4NQO (Table IV). With respect to DNA repair capacity, XP-K is approximately 50% of normal whereas XP-E is 25%. The percentage of metaphase plates with chromosome aberrations is 12.8% in XP-K but it is more than four fold higher (57.1%) in XP-E (which is more DNA-repair deficient). The clone-forming capacity after exposure to 4NQO in XP-K is 38%, about two fold higher than the 16% in the more repair deficient XP-E. The response of cells from different XP patients following exposure to N-acetoxy-2-AAF resembles that after 4NQO (Table V).

There is no significant difference following exposure to MNNG in the frequency of chromosome aberrations and clone-forming capacity among cells from XP patients with different DNA repair capacities (Table VI). Moreover, the percentage of metaphase plates with chromosome aberrations and the clone-forming capacity in XP cells with different DNA repair deficiency resembles that found in control fibroblasts treated with equimolar concentrations of MNNG.

3.4. Chromosome Aberrations and Clone-Forming Capacity in XP Heterozygotes Following Exposure to Chemical Carcinogens

In the preceding section (3.3.), it has been reported that cells from XP patients with different levels of DNA

TABLE IV Level of Unscheduled ^{3}HTdR Incorporation, Frequency of Chromosome Aberrations and Clone Forming Capacity of Cultured Fibroblasts From Xeroderma Pigmentosum Patients and Control Persons Following Exposure to 4-Nitroquinoline 1-Oxide.

	4-Nitroquinoline 1-Oxide (4NQO)		
	DNA Repair Grains/Nucleus[1]	Metaphase Plates With Chromosome Aberrations (%)[2]	Clone-Forming Capacity[3]
XP_{H1}	12	68.5	--
XP_{E}	28	57.1	16
XP_{C1}	34	20.3	33
XP_{K}	56	12.8	38
Heterozygotes			
XP_{E} Father	107	--	--
XP_{E} Mother	116	3.8	71
XP_{C} Father	125	--	--
XP_{C} Mother	127	--	86
XP_{K} Father	98	1.5	72
XP_{K} Mother	99	1.9	80
Control	104	0.7	79
Control	102	0.0	72

1. Unscheduled incorporation of ^{3}HTdR was measured following 1.5-hr. exposure to 2×10^{-6}M 4NQO. Autoradiography.
2. Chromosome aberrations (breaks and exchanges) were counted on fibroblast cultures exposed to 5×10^{-7}M 4NQO (1.5 hr.).
3. Clone-forming capacity after 1.5-hr. exposure to 10^{-8}M 4NQO was expressed as % of each cell line's untreated control.

TABLE V Level of Unscheduled ^{3}HTdR Incorporation, Frequency of Chromosome Aberrations and Clone Forming Capacity of Cultured Fibroblasts From Xeroderma Pigmentosum Patients and Control Persons Following Exposure to N-Acetoxy-2-Acetylaminofluorene.

	N-Acetoxy-2-Acetylaminofluorene		
	DNA Repair Grains/Nucleus[1]	Metaphase Plates With Chromosome Aberrations (%)[2]	Clone-Forming Capacity[3]
XP_{H1}	3	75.9	--
XP_{E}	6	47.9	29
XP_{C1}	9	26.2	17
XP_{K}	17	17.6	--
Heterozygotes			
XP_{E} Father	28	--	--
XP_{E} Mother	33	2.0	76
XP_{C} Father	28	--	--
XP_{C} Mother	29	2.8	80
XP_{K} Father	29	3.5	--
XP_{K} Mother	30	--	--
Control	27	4.7	86
Control	29	3.2	81

1. Unscheduled incorporation of ^{3}HTdR was measured following 5-hr. exposure to $5x10^{-5}$M N-acetoxy-2-AAF. Autoradiography.
2. Chromosome aberrations (breaks and exchanges) were scored on fibroblast cultures exposed to $5x10^{-6}$M N-acetoxy-2-AAF (5 hr.).
3. Clone-forming capacity after 5-hr. exposure to 10^{-5}M N-acetoxy-2-AAF was expressed as % of each cell line's untreated control.

TABLE VI Level of Unscheduled ^{3}HTdR Incorporation, Frequency of Chromosome Aberrations and Clone Forming Capacity of Cultured Fibroblasts From Xeroderma Pigmentosum Patients and Control Persons Following Exposure to N-Methyl-N'-Nitro-N-Nitrosoguanidine

	N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG)		
	DNA Repair Grains/Nucleus[1]	Metaphase Plates With Chromosome Aberrations (%)[2]	Clone-Forming Capacity[3]
XP_{H1}	41	--	--
XP_{E}	41	56.2	69
XP_{C1}	44	46.6	73
XP_{K}	43	48.2	--
Heterozygotes			
XP_{E} Father	42	--	--
XP_{E} Mother	40	46.2	70
XP_{C} Father	45	--	--
XP_{C} Mother	42	42.0	58
XP_{K} Father	42	--	--
XP_{K} Mother	44	--	--
Control	39	50.0	63
Control	41	47.2	68

1. Unscheduled incorporation of ^{3}HTdR was measured following 3-hr. exposure to 2×10^{-4}M MNNG. Autoradiography.
2. Chromosome aberrations (breaks and exchanges) were scored on fibroblast cultures exposed to 2.5×10^{-5}M MNNG (3 hr.).
3. Clone-forming capacity after 3-hr. exposure to 5×10^{-6}M MNNG was expressed as % of each cell line's untreated control.

repair capacity show different sensitivities towards 4NQO and N-acetoxy-2-AAF with respect to chromosome aberrations and clone-forming capacity. The question follows whether the parents of XP patients, being obligate heterozygotes, would manifest an increased sensitivity towards chemical carcinogens. The level of unscheduled ^{3}HTdR uptake in different XP heterozygotes following exposure to several chemical carcinogens resembles that in control cells (Section 2.2., Table III). The response of several XP heterozygotes towards 4NQO, N-acetoxy-2-AAF and MNNG are shown in Table IV - VI, Section 3.3.. The frequency of chromosome aberrations and clone-forming capacity in the XP heterozygotes are not significantly different from that in the control cells.

4. Precarcinogens and Ultimate Carcinogens

Many chemical carcinogens are not active at the site of administration but they preferentially cause tumours in specific target organs. For instance, 2-acetylamino-fluorene (2-AAF), whether introduced topically or by subcutaneous injection, causes predominantly liver tumours (Miller, 1970). This organotropic phenomenon suggests that the carcinogen requires metabolic activation by enzymatic systems in the target organs. Further support for the presence of such activating metabolism is derived from the fact that metabolites isolated from animals exposed to a chemical carcinogen are more potent than

the parent compound (Miller and Miller, 1969). These metabolites act on local tissue targets as well as the usual remote target organs. Chemical carcinogens which require metabolic activation are described as precarcinogens while the metabolites come to be known as proximate (if further metabolism is required) or ultimate carcinogens. It now seems clear that the carcinogenic alkylating agents are ultimate carcinogens in the form administered (Miller and Miller, 1971b). They are strong electrophiles and react readily and directly with cell constituents. Most other chemical carcinogens apparently require metabolic activation, usually enzymatic, to become ultimate carcinogens (Miller and Miller, 1971b).

From the foregoing discussion, it appears likely that many of the chemical carcinogens the human population comes into contact with are in the precarcinogenic form. In order to consider the use of DNA repair synthesis in cultured human fibroblasts as a pre-screening bioassay for environmental chemical carcinogens, it is therefore of paramount importance to determine whether this system responds to precarcinogens as well as to proximate and ultimate carcinogens.

In this chapter, the pre-carcinogenic, proximate and/or ultimate carcinogenic forms of key compounds from a few representative groups of chemical carcinogens are examined with respect to their capacity to elicit unscheduled DNA synthesis, chromosome aberrations and reduction in

colony formation in cultured human skin fibroblasts. These compounds include polycyclic aromatic hydrocarbons, aromatic amines, N-oxides and nitroso compounds.

4.1. Polycyclic Aromatic Hydrocarbons

The idea that epoxides might be metabolic intermediates in the oxidation of polycyclic hydrocarbons was suggested about 25 years ago by Boyland (1950). Based on theoretical calculations it is concluded that the K-region (formerly often called the meso-phenanthrenic region) is particularly apt to undergo addition reactions and the chemical reactivity of this double bond has been demonstrated experimentally (Arcos and Argus, 1974). Probably because of their instability, direct evidence for the formation of the epoxides of carcinogenic polycyclic hydrocarbons is not available. However, the finding by Grover et al. (1971b) that the K-region epoxides of benz(a)anthracene and dibenz(a,h)anthracene transform hamster fibroblasts and mouse prostate cell lines with greater efficiency than either the parent hydrocarbons, the dihydrodiols, or the phenols (which could be derived from these epoxides) is consistent with the epoxide intermediate hypothesis.

In this chapter, the capacity of benz(a)anthracene (BA), 20-methylcholanthrene (MCA), their K-region epoxide and dihydrodiol to elicit a DNA repair synthesis is examined. The structural formulae of benz(a)anthracene and 20-methylcholanthrene, their K-region epoxides and

the corresponding dihydrodiol are depicted in Fig. 53. An unscheduled incorporation of ^{3}HTdR into nuclear DNA was observed in non-dividing cells exposed for 5 h. to BA-5,6-epoxide at concentrations ranging from 10^{-5} to $5x10^{-4}$M (Fig. 54). At these doses the precarcinogen BA and the metabolite BA-cis-5,6-dihydrodiol did not trigger a detectable amount of DNA repair synthesis. A similar pattern was observed with 20-methylcholanthrene (MCA), its active K-region epoxide and inactive dihydrodiol (Fig. 55).

4.2. Aromatic Amines

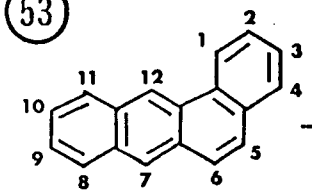
Within the class of fully aromatic non-substituted ring systems, it is among the penta- and hexacyclic hydrocarbons that the most potent carcinogens are found. Except for two compounds no unsubstituted polycyclic hydrocarbon below four condensed rings possess detectable carcinogenic activity (Arcos and Argus, 1974). However, if an amino group is introduced at specific positions, these ring systems often acquire a very high level and multi-target carcinogenic activity. Furthermore, metabolic interconversion of the amino group with the hydroxylamino and nitroso substituents produces derivatives which are substantially more active and ubiquitously carcinogenic than the amines themselves. The N-hydroxy metabolites of several aromatic amines have been demonstrated to be proximate carcinogens. N-hydroxylation is a critical

Figure 53 Structural formula of the precarcinogens BA and 20-methylcholanthrene, the highly reactive K-region expoxide (proximate carcinogen) and the corresponding dihydrodiol (inactive metabolite).

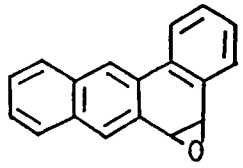
Figure 54 Unscheduled DNA synthesis in normal human cells exposed for 3 hours to BA (□), BA-5,6-epoxide (▽) or BA-cis-5,6-dihydrodiol (●).

Figure 55 Unscheduled DNA synthesis in normal human cells exposed for 3 hours to 20-methylcholanthrene (□), MCA-6,7-epoxide (▽) or MCA-6,7-dihydrodiol (●).

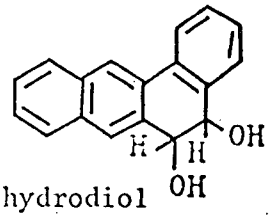
(53)



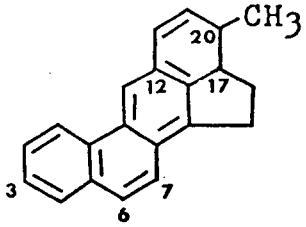
Benz(a)anthracene



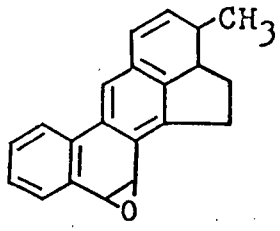
BA-5,6-Epoxide



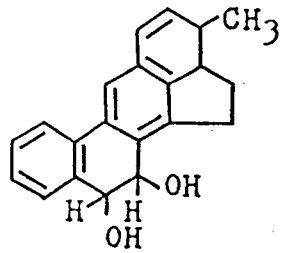
BA-5,6- Dihydrodiol



20-Methylcholanthrene

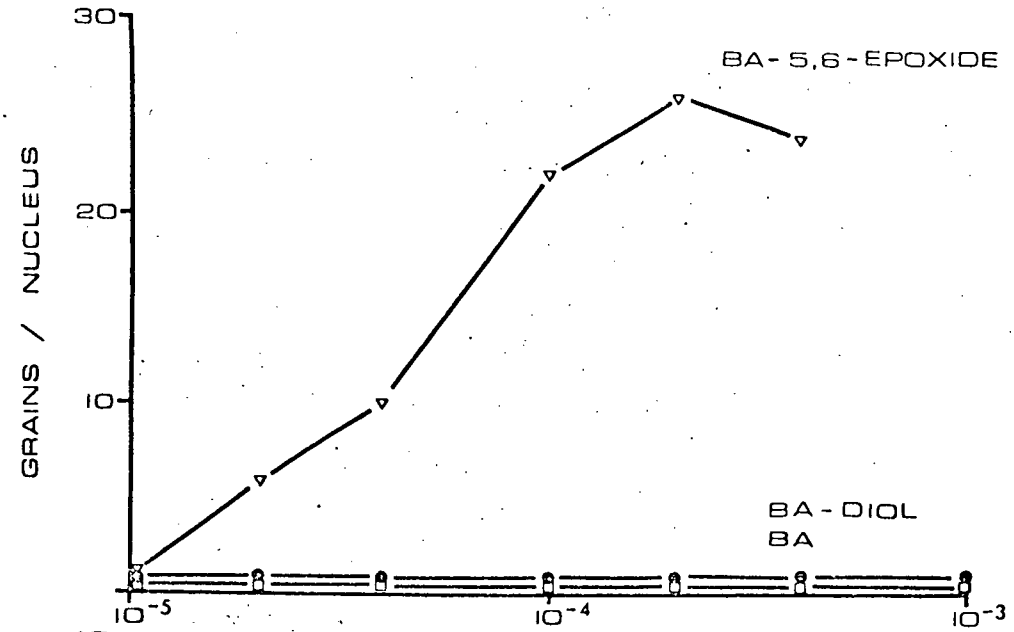


MCA-6,7-Epoxide



MCA-6,7-Dihydrodiol

(54)



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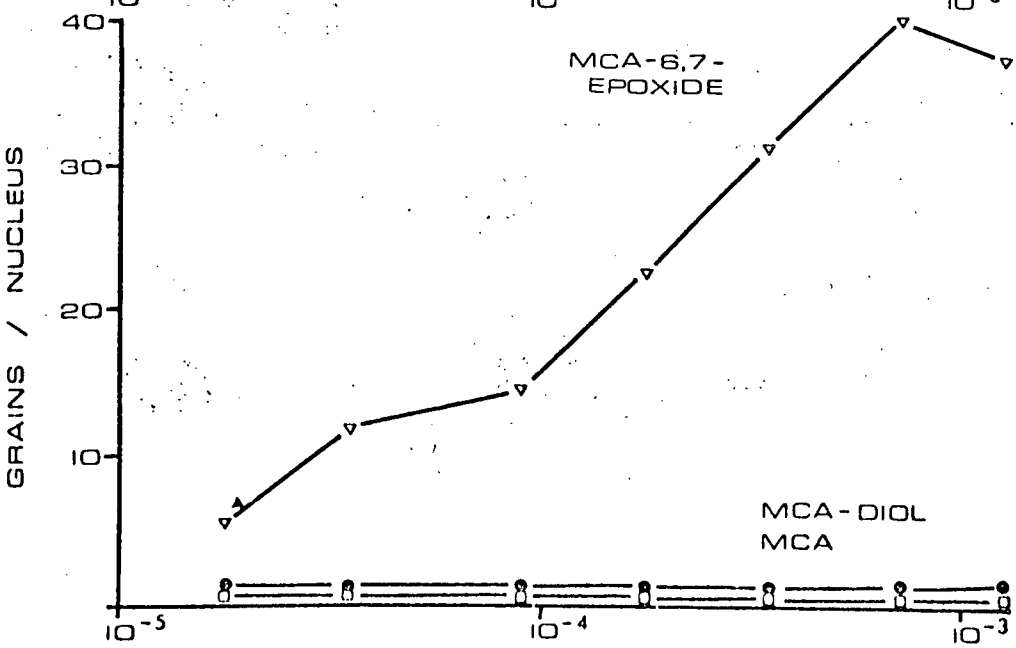
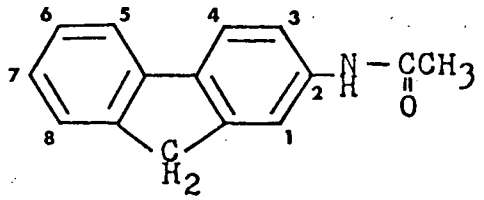


metabolic activation step of aromatic amine carcinogens and has been found to take place in virtually every tissue and species in which these agents exert a carcinogenic effect (Arcos and Argus, 1974). A second activation by esterification yields derivatives (e.g. N-acetoxy and N-sulfate) which are more active than the N-hydroxy compounds (Miller and Miller, 1969).

In this chapter, the following aromatic amines (pre-carcinogens) and their corresponding N-hydroxy and N-acetoxy derivatives (proximate and ultimate carcinogens respectively) are examined for their capacity to elicit DNA repair synthesis in cultured human fibroblasts: 2-acetylamino-fluorene (2-AAF), 2-acetylaminophenanthrene, 4-acetylaminobi-phenyl and 4-acetylaminostilbene(Fig. 56).

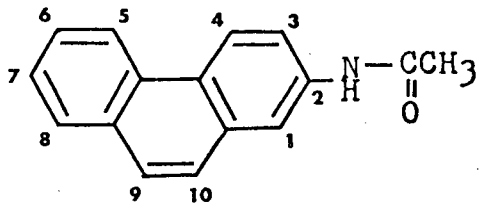
4.2.1. 2-Acetylaminofluorene

2-Acetylaminofluorene (or 2-fluorenylacetamide) was used as a highly effective insecticide until Wilson et al. (1941) discovered that rats administered 2-AAF developed tumours in the liver and in various tissues and internal organs. Furthermore, it was found to be metabolized in part to N-hydroxy-2-AAF (Cramer et al., 1960). This N-hydroxy metabolite is a more active and versatile carcinogen than the parent compound. Subsequent studies strongly indicate that the in vivo reactivity of the N-hydroxy compounds is dependent, at least in part, on the formation of esters of these compounds (Miller and Miller, 1969).



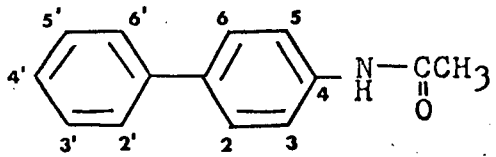
2-Acetylaminofluorene

(2-AAF)



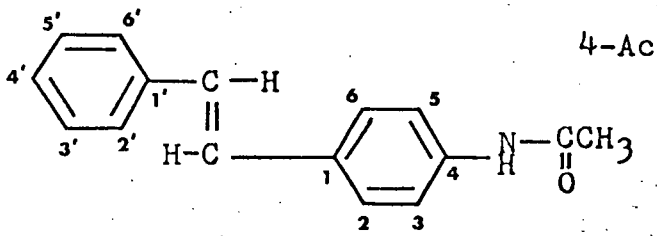
2-Acetylaminophenanthrene

(2-AAP)



4-Acetylaminobiphenyl

(4-AABP)



4-Acetylaminostilbene

(4-AAS)

Figure 56 Structure of the carcinogenic aromatic amines:

2-Acetylaminofluorene (2-AAF)

2-Acetylaminophenanthrene (2-AAP)

4-Acetylaminobiphenyl (4-AABP)

4-Acetylaminostilbene (4-AAS)

The data also suggest that the esters are more proximate carcinogens than the parent N-hydroxy derivatives. This led to the postulation the N-acetoxy and N-hydroxy-2-AAF are the ultimate and proximate carcinogenic forms of 2-AAF respectively (Fig. 57).

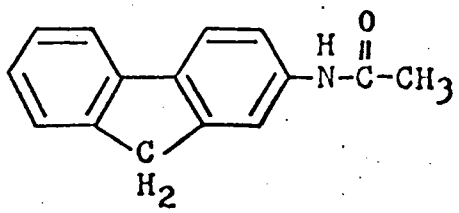
In this chapter, DNA repair synthesis was examined on normal human fibroblasts exposed to the precarcinogen 2-AAF and its proximate and ultimate carcinogenic forms, N-hydroxy-2-AAF and N-acetoxy-2-AAF respectively. The results are shown in Fig. 57. At equimolarity, the chemically reactive N-acetoxy-2-AAF is more active than N-hydroxy-2-AAF in producing DNA lesions resulting in a repair synthesis. At higher concentrations, N-hydroxy-2-AAF may be activated by enzymatic esterification. This response of cultured fibroblasts is similar to that observed in human peripheral lymphocytes (Lieberman et al., 1971a). 2-AAF shows a very weak capacity for eliciting a DNA repair synthesis and this only at very high concentrations.

Inactive metabolites of N-hydroxy-2-AAF which were found in the urine of treated rats have been identified as 1-, 3-, 5- and 7-hydroxy-2-AAF (Arcos and Argus, 1974). These non-carcinogenic metabolites were included in the present investigation. No unscheduled DNA synthesis was elicited in cultured human fibroblasts by these compounds (Table VII).

4.2.2. 2-Acetylaminophenanthrene

2-Aminophenanthrene is a very potent carcinogen in the

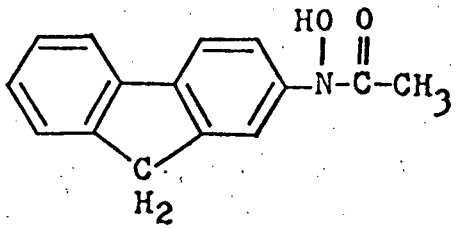
Figure 57 Unscheduled incorporation of ^{3}HTdR into nuclei of normal human fibroblasts exposed for 5 hours to the precarcinogen 2-AAF (□), proximate carcinogen - N-hydroxy-2-AAF (▽) or ultimate carcinogen - N-acetoxy-2-AAF (○). Autoradiography.



2-Acetylaminofluorene (2-AAF)

(Precarcinogen)

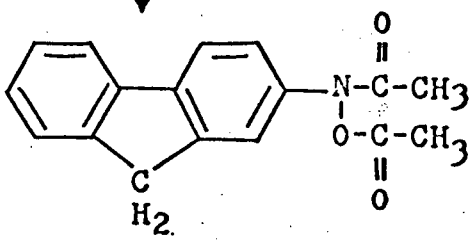
Hydroxylation



N-Hydroxy-2-AAF

(Proximate Carcinogen)

Esterification



N-Acetoxy-2-AAF

(Ultimate Carcinogen)

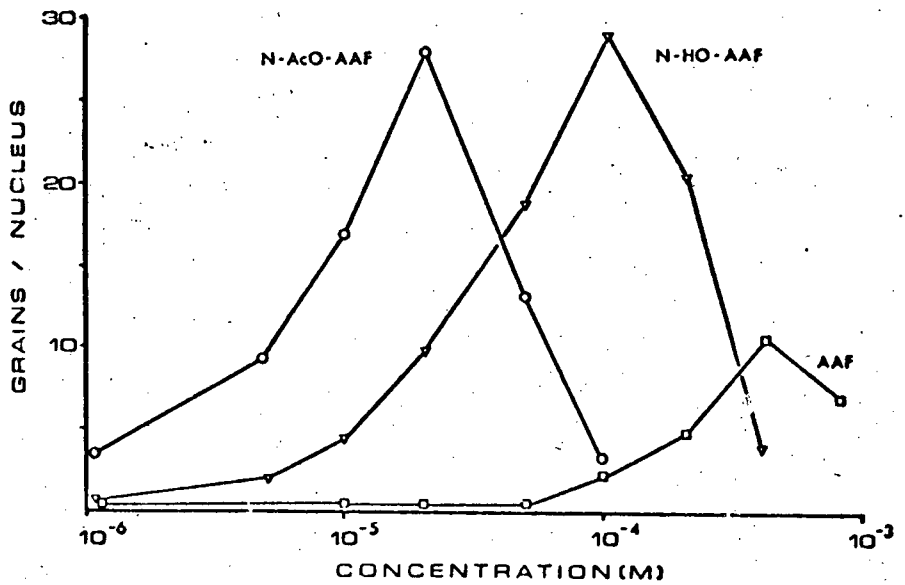


TABLE VII Unscheduled DNA synthesis in Cultured Human Fibroblasts Following 5-Hour Exposure to Active and Inactive Metabolites of 2-Acetylaminofluorene (2-AAF)

	Grains Per Nucleus*							
Concentration (M)	10^{-3}	3.3×10^{-4}	10^{-4}	5×10^{-5}	2.5×10^{-5}	1.2×10^{-5}	6×10^{-6}	10^{-6}
2-AAF	0.1	0.6	0.3	0.4	0.2	-	-	-
N-Hydroxy-2-AAF	-	-	18.0	13.0	10.0	5	3	3
N-Acetoxy-2-AAF	-	-	27.0	21.0	17.0	8	4	2
1-Hydroxy-2-AAF	1.8	0.5	0.2	0.2	0.1	-	-	-
3-Hydroxy-2-AAF	0.6	0.2	0.4	0	0.6	-	-	-
5-Hydroxy-2-AAF	0	0	0.1	0.1	0.1	-	-	-
7-Hydroxy-2-AAF	0.5	0.1	0.3	0.1	0	-	-	-

* "-" denotes concentration not tested.

rat when administered orally (Huggins and Yang, 1962; Dannenberg and Huggins, 1969). The N-acetylated form, 2-acetylaminophenanthrene, appears slightly less active than the free amine (Hartman et al., 1959; Dannenberg and Huggins, 1969). Tumours induced by these compounds include that of the mammary gland (in females) and of the ear duct and gastrointestinal tract, and leukemia.

N-hydroxy-2-acetylaminophenanthrene has been demonstrated to be the proximate carcinogen of the parent amide. Rats fed 2-acetylaminophenanthrene excrete substantial amounts of the N-hydroxy metabolite, mostly as glucuronides (Miller et al., 1966b). N-hydroxy-2-acetylaminophenanthrene is a more potent carcinogen than the parent amide. The N-acetoxy-2-acetylaminophenanthrene, an activation product by esterification, was shown to be more active than the N-hydroxy derivative (Miller and Miller, 1969).

In this chapter, the capacity of 2-acetylaminophenanthrene (2-AAP) and its proximate (N-OH) and ultimate(N-Ac) carcinogenic forms to elicit a DNA repair synthesis in cultured human fibroblasts is examined. The results are shown in Fig. 58. At equimolar concentrations, N-acetoxy-2-AAP is more active than N-hydroxy-2-AAP in inflicting DNA damage resulting in a repair synthesis. The parent compound 2-AAP failed to elicit any unscheduled DNA synthesis even at much higher concentrations.

4.2.3. 4-Aminostilbene

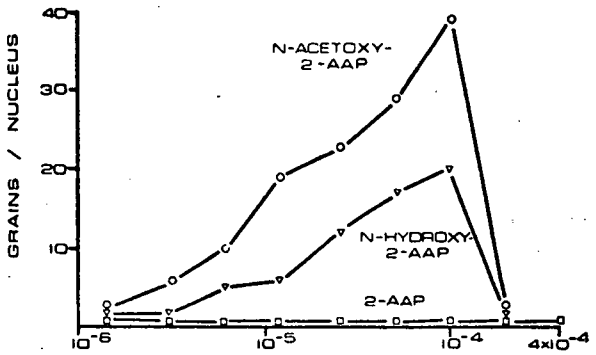
Figures 58-60 Unscheduled incorporation of ^{3}HTdR into nuclei of normal human fibroblasts exposed for 5 hours to the precarcinogenic, proximate or ultimate carcinogenic forms of aromatic amines. Autoradiography.

(Figure 58) 2-Acetylaminophenanthrene (□) - precarcinogen.
N-Hydroxy-2-AAP (▽) - proximate carcinogen.
N-Acetoxy-2-AAP (○) - ultimate carcinogen.

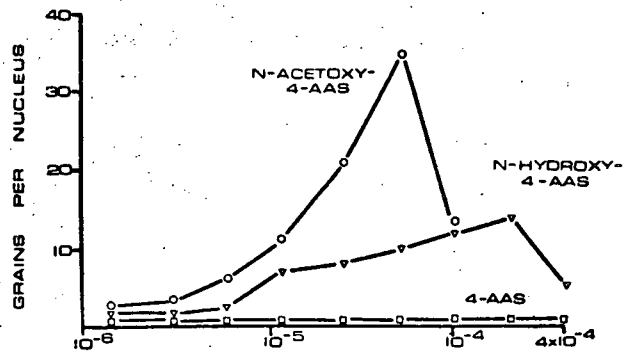
(Figure 59) 4-Acetylaminostilbene (□) - precarcinogen.
N-Hydroxy-4-AAS (▽) - proximate carcinogen.
N-Acetoxy-4-AAS (○) - ultimate carcinogen.

(Figure 60) 4-Acetylaminobiphenyl (□) - precarcinogen.
N-Hydroxy-4-AABP (▽) - proximate carcinogen.
N-Acetoxy-4-AABP (○) - ultimate carcinogen.

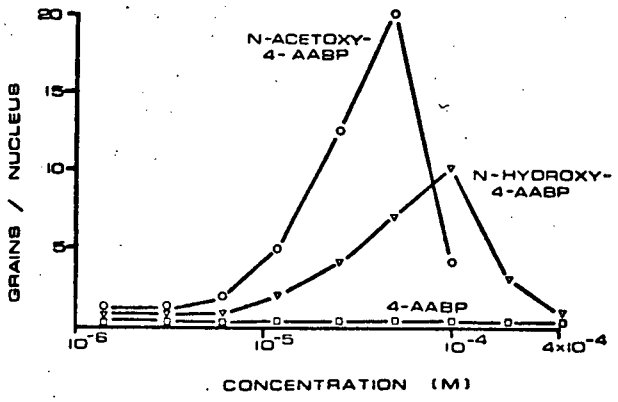
58


GRAINS / NUCLEUS
40
30
20
10
N-ACETOXY-
2-AAP
N-HYDROXY-
2-AAP
2-AAP
10⁻⁶
10⁻⁵
10⁻⁴
4x10⁻⁴

59


GRAINS PER NUCLEUS
40
30
20
10
N-ACETOXY-
4-AAS
N-HYDROXY-
4-AAS
4-AAS
10⁻⁶
10⁻⁵
10⁻⁴
4x10⁻⁴

60


GRAINS / NUCLEUS
20
15
10
5
N-ACETOXY-
4-AABP
N-HYDROXY-
4-AABP
4-AABP
10⁻⁶
10⁻⁵
10⁻⁴
4x10⁻⁴
CONCENTRATION (M)

In the search for tumour inhibitory substances, Haddow et al., (1948) discovered 4-aminostilbene. The compound as well as the N-acetylated derivative, 4-AAS was later found to be an active carcinogen. Administered subcutaneously or orally, these two compounds induced a whole spectrum of neoplasms in the rat (mammary gland, ear duct, lung, kidney, intestines). The nature and distribution of these tumours are very similar to those produced by 2-AAF (Haddow, 1953).

N-hydroxy-4-AAS is excreted in the urine, mainly conjugated with glucuronic acid, in rats fed 4-AAS (Baldwin and Romerii, 1965). In the Miller's group, the N-hydroxy derivative was found to be a definitely stronger carcinogen than either 4-amino or 4-acetylaminostilbene toward the mammary gland, forestomach, subcutaneous tissue and small intestine in the rat, but equally carcinogenic toward the ear duct glands (Andersen et al., 1964).

In this chapter, the parent amide, 4-aminostilbene, its N-acetylated derivative, 4-acetylaminostilbene, and the presumed proximate and ultimate carcinogenic forms (N-hydroxy-4-AAS and N-acetoxy-4-AAS) are examined for their capacity to inflict DNA damage in cultured human fibroblasts. The results are shown in Fig. 59. The presumed proximate and ultimate carcinogenic forms (N-OH-4-AAS and N-Ac-4-AAS) elicited an unscheduled DNA synthesis within the concentration range studied. Exposure to the parent compound did not result in any detectable

level of unscheduled DNA synthesis in cultured human fibroblasts.

4.2.4. 4-Aminobiphenyl

Pure biphenyl is not a carcinogen, but 4-aminobiphenyl (4-ABP), a substantial contaminant in the dye manufacturing industry, has been shown to be a powerful bladder carcinogen in man (Arcos and Argus, 1974). Given by the subcutaneous or oral route to rat, 4-aminobiphenyl induces tumours in the mammary gland, acoustic sebaceous gland, liver and small intestine (Walpole and Williams, 1958). The carcinogenic potencies of 4ABP and 4AABP are roughly comparable (Walpole and Williams, 1958). N-hydroxy-4-acetylaminobiphenyl and N-acetoxy-4-acetylaminobiphenyl elicit a considerable increase in carcinogenic activity and spectrum of tissue targets (Miller and Miller, 1969). This finding is consistent with the assumption that N-hydroxy- and N-acetoxy- derivatives are the proximate and ultimate carcinogenic forms respectively of the parent compound, 4-acetylaminobiphenyl.

In this section, 4-aminobiphenyl, 4-acetylaminobiphenyl and the assumed proximate and ultimate carcinogenic forms (N-hydroxy-4-AABP and N-acetoxy-4-AABP) are examined for their DNA damaging capacity in cultured human fibroblasts. An unscheduled incorporation of ^{3}HTdR is observed following exposure to the N-hydroxy- and N-acetoxy- derivatives (Fig. 60). No unscheduled DNA synthesis was detected in the cases of

4ABP and 4AABP.

4.2.5. Summary on Aromatic Amines

Among all four groups of aromatic amines tested, the N-hydroxy- and N-acetoxy- derivatives (the presumed proximate and ultimate carcinogenic forms of the parent compounds) possess the capacity to induce DNA alterations resulting in an unscheduled DNA synthesis. At equimolar concentrations the parent compounds (precarcinogens) failed to elicit any unscheduled incorporation of ^{3}HTdR. Following exposure to the parent amines, DNA repair synthesis was observed, if at all, only when very high doses of the compounds were used. The simplest interpretation is to assume that cultured human fibroblasts could activate to a limited degree precarcinogenic aromatic amines. The precarcinogens would require metabolic activation before direct interaction with DNA and DNA repair synthesis could occur. The results show that the assay system is suitable for the detection of proximate and ultimate carcinogens only, as it may yield "false" negatives with precarcinogens.

4.3. N-Oxides

The N-oxides are included in this discussion because there is mounting evidence that some of them may require metabolic conversion into proximate carcinogens (Arcos and Argus, 1974). An N-oxide grouping is formed when

the nitrogen in a chemical compound is linked to oxygen by coordinate bonds (usually represented as N→O to distinguish it from an N = O double bond). In this particular situation, the electron density is displaced towards oxygen. The N→O bond is highly polarised and presumably very active.

A notable example is 4-nitroquinoline N-oxide, a powerful antibacterial, fungicidal, mutagenic and carcinogenic agent (Mita, 1971). It is highly toxic toward cancer cells in vivo and in vitro (Fukuoka, 1971). The carcinogenic activity of 4NQO has been demonstrated in various species (mice, rat, hamster, guinea pig, rabbit and fowl). Depending on the route of administration, 4NQO induces tumours in a wide spectrum of target tissues (Arcos and Argus, 1974).

Although the active molecular form remains unsettled, 4NQO is rapidly reduced to 4-hydroxyaminoquinoline N-oxide, which does not differ appreciably from 4NQO in carcinogenic activity (Kawazoe and Araki, 1970). On the basis of similar carcinogenic potency and target tissue spectrum in 4NQO and 4HAQO, it is difficult to envisage 4HAQO as the proximate carcinogen. The assignment of 4HAQO as the proximate carcinogenic form is grounded mainly on metabolic and tissue distribution aspects. A circumstantial piece of evidence in support of this designation may be derived from a consideration of the 3-halogen substituted derivatives of 4NQO.

In 3-chloro-4NQO and 3-fluoro-4NQO, the halogen is

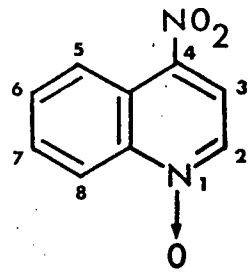
more reactive than the nitro group towards nucleophilic reagents. This reaction is expected to compete with metabolic reduction of the nitro group to a hydroxylamino group with concomitant reductive dehalogenation to the N-hydroxy proximate carcinogen. The halogen in 3-bromo-4NQO is less reactive towards nucleophiles and is actually found to undergo transformation to the proximate carcinogen 4HAQO more readily than the chloro and fluoro derivatives. 3-Bromo-4NQO also proved to be the most potent carcinogen of the three halogen derivatives, thus indirectly lending support for the assignment of 4HAQO as a proximate carcinogen (Kawazoe and Araki, 1970).

In this chapter, an attempt is made to investigate whether the level of DNA repair synthesis elicited in cultured fibroblasts would parallel the ease with which the proximate carcinogen is formed. A comparison is made among the 3-halogen substituted 4NQO derivatives with respect to their capacity to provoke an unscheduled DNA synthesis in cultured human fibroblasts. Following the same rationalisations as in the preceding paragraph, if 3-bromo-4NQO produces a better yield of the proximate carcinogen than the corresponding chloro and fluoro-derivatives, it will also elicit the highest level of DNA repair synthesis. The levels of unscheduled DNA synthesis elicited by 4NQO and 4HAQO have been included for comparison. From Fig. 61 it is evident that both 4NQO and 4HAQO provoked a comparable level of unscheduled

DNA synthesis. The very low level of unscheduled ^{3}HTdR uptake observed with 3-fluoro-4NQO may be explained by the possibility that little of this compound was transformed into the proximate carcinogenic form. Difficulty was experienced with the solubility of 3-bromo-4NQO and 3-chloro-4NQO in culture medium. As a result, no unscheduled DNA synthesis was observed in cultured human fibroblasts exposed to a suspension of these two compounds.

Another piece of indirect evidence supporting the notion that 4HAQO is the proximate carcinogenic form of 4NQO may be provided from 3-methyl-4NQO. The presence of a methyl substituent at the 3-position has rendered this 4NQO derivative at most marginally active as a carcinogen (Arcos and Argus, 1974). In chemical terms, the loss of activity has been attributed to the 3-methyl group presenting steric encumbrance of the access to the nitro group, thereby creating a hindrance to reduction to the proximate carcinogenic hydroxylamine compounds. 3-Methyl-4NQO also exhibits a small capacity to induce DNA repair synthesis in mammalian cells (Stich et al., 1971). Again, the observation may be explained in terms of the difficulty for 3-methyl-4NQO to undergo transformation into 4HAQO.

A possible conclusion from this chapter is that the level of unscheduled DNA synthesis elicited by various precarcinogenic 4NQO derivatives is parallelled by the ease with which the proximate carcinogen (4HAQO) is formed.



4NQO

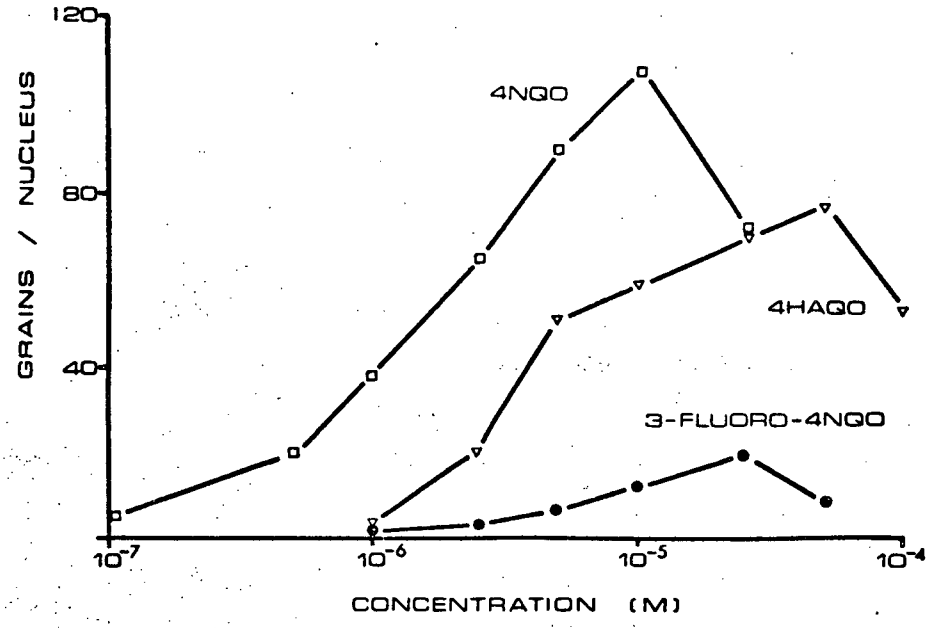


Figure 61 Unscheduled DNA synthesis in cultured human fibroblasts following 1.5-hour exposure to 4NQO (□), 4HAQO (▽) or 3-fluoro-4NQO (●). Autoradiography.

4.4. Nitroso Compounds (Aliphatic Carcinogens)

The three preceding chapters have been concerned with the metabolic activation of aromatic carcinogens and the capacity of the ultimate carcinogenic forms to provoke DNA damage followed by an unscheduled DNA synthesis in cultured human fibroblasts.

In this chapter, an example of an aliphatic carcinogen requiring metabolic activation is presented. Dimethylnitrosamine is a potent carcinogen. Upon injection into mice, it induces liver and lung neoplasma and a few kidney tumours (Toth et al., 1964; Terracini et al., 1966). DMN is converted by the mixed function oxidases of the endoplasmic reticulum to intermediates which decompose readily to yield alkylating species (Magee, 1972). It has been demonstrated by Laishes and Stich (1973) that DMN alone (precarcinogen) did not inflict any DNA damage (as measured by the alkaline sucrose gradient technique) nor elicit any DNA repair synthesis (as evidenced by the unscheduled uptake of ^{3}HTdR) in cultured human fibroblasts. However, when DMN was mixed with the post-mitochondrial fraction of mouse liver homogenate and then added to cultured human fibroblasts, DNA damage and an ensuing unscheduled DNA synthesis was observed. A plausible explanation is that little or no activation of DMN took place with the enzymes present in the cultured fibroblasts.

4.5. Effect of Precarcinogens and Ultimate Carcinogens on Chromosome Aberrations and Clone-Forming Capacity in Cultured Human Fibroblasts

One tentative conclusion from the present section is that an unscheduled DNA synthesis in cultured human fibroblasts could be demonstrated only with proximate and ultimate carcinogens. Precarcinogens as a rule failed to elicit any unscheduled incorporation of ^{3}HTdR unless they are readily activated in the fibroblasts. The question arises as to whether both precarcinogens and ultimate carcinogens could induce chromosome aberrations and reduce the clone-forming capacity of cultured human fibroblasts. The effect of the precarcinogens benz(a)anthracene, 2-acetylaminofluorene, 4-acetylaminostilbene and 4-nitroquinoline 1-oxide and their corresponding proximate and ultimate carcinogenic forms on the frequency of chromosome aberrations in cultured human fibroblasts are shown in Figs. 62 - 65. The precarcinogen benz(a)anthracene and its inactive degradation product benz(a)anthracene-cis-5,6-dihydrodial did not induce any chromosome aberrations in cultured human fibroblasts. Chromosome aberrations were produced only with the ultimate carcinogen. With 2-acetylaminofluorene and 4-acetylaminostilbene, chromosome aberrations were observed in cultured human fibroblasts following exposure to the proximate (N-hydroxy derivative) and ultimate (N-acetoxy derivative) carcinogenic forms but the precarcinogen itself was inactive. In the case of the precarcinogen 4NQO, because

Figures 62-65 Frequency of metaphase plates with chromosome aberrations in cultured fibroblasts from Xeroderma pigmentosum patients following short-term exposure to precarcinogens, proximate or ultimate carcinogens.

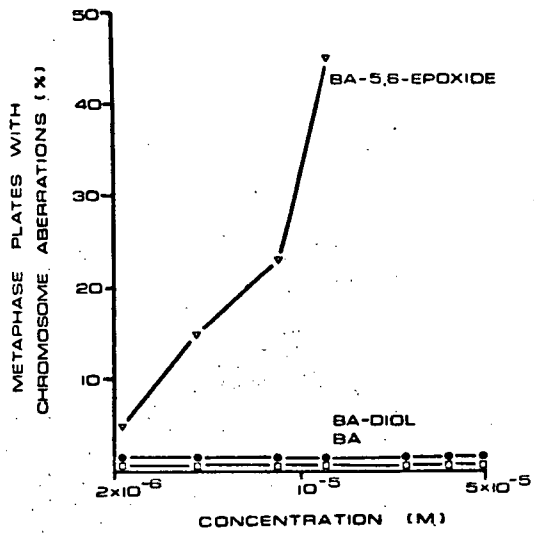
(Figure 62) BA (3 hr.) (□) - precarcinogen.
BA-5,6-Epoxide (▽) - proximate carcinogen.
BA-cis-5,6-dihydrodiol (●) - inactive metabolite.

(Figure 63) 2-AAF (5 hr.) (□) - precarcinogen.
N-Hydroxy-2-AAF (▽) - proximate carcinogen.
N-Acetoxy-2-AAF (○) - ultimate carcinogen.

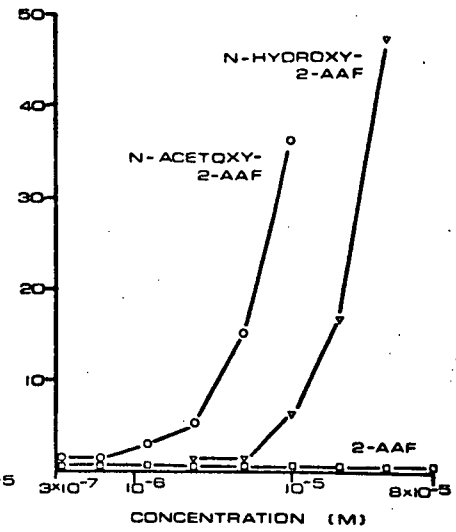
(Figure 64) 4-AAS (5 hr.) (□) - precarcinogen.
N-Hydroxy-4-AAS (▽) - proximate carcinogen.
N-Acetoxy-4-AAS (○) - ultimate carcinogen.

(Figure 65) 4NQO (1.5 hr.) (□) - precarcinogen.
4HAQO (1.5 hr.) (▽) - proximate carcinogen.

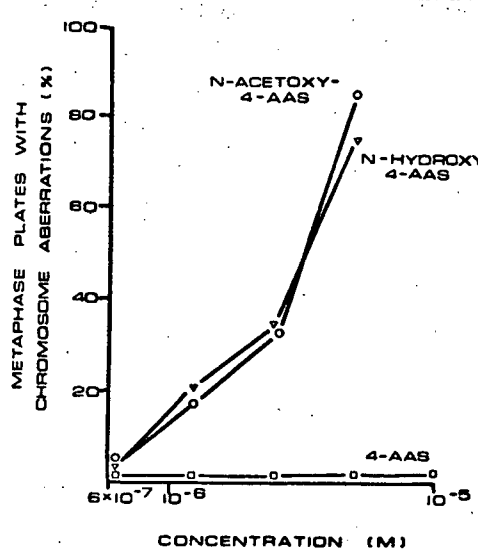
62


METAPHASE PLATES WITH CHROMOSOME ABERRATIONS (%)
50
40
30
20
10
BA-5,6-EPOXIDE
BA-DIOL
BA
2x10⁻⁶
10⁻⁵
5x10⁻⁵
CONCENTRATION (M)

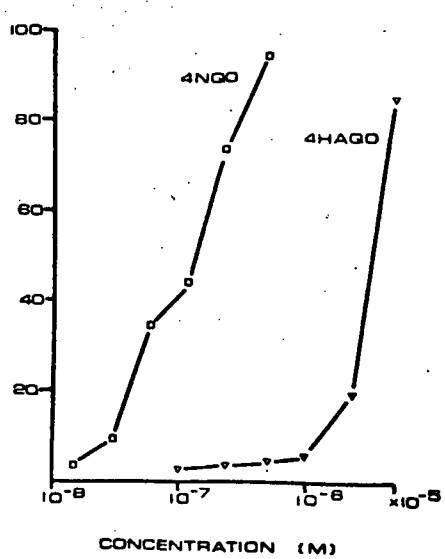
63


50
40
30
20
10
N-HYDROXY-
2-AAF
N-ACETOXY-
2-AAF
2-AAF
3x10⁻⁷
10⁻⁶
10⁻⁵
8x10⁻⁵
CONCENTRATION (M)

64


METAPHASE PLATES WITH CHROMOSOME ABERRATIONS (%)
100
80
60
40
20
N-ACETOXY-
4-AAS
N-HYDROXY-
4-AAS
4-AAS
6x10⁻⁷
10⁻⁶
10⁻⁵
CONCENTRATION (M)

65


100
80
60
40
20
4NQO
4HAQO
10⁻⁸
10⁻⁷
10⁻⁶
x10⁻⁵
CONCENTRATION (M)

of the ease with which it is converted into the proximate carcinogen 4HAQO, both derivatives are equally effective in the production of chromosome aberrations.

The effect of various precarcinogens and their corresponding proximate and ultimate carcinogenic form on the clone-forming capacity of cultured human fibroblasts are depicted in Figs 66 - 69. Following short term exposure to benz(a)anthracene (precarcinogen) and the inactive metabolite (BA-cis-5,6-dihydrodiol), the clone-forming capacity of cultured human fibroblasts did not differ from that in the untreated control. A reduction in the number of clones formed was observed only after the cells were exposed to the ultimate carcinogen, BA-5,6-epoxide. A similar phenomenon was observed with 2-AAF and 4-AAS, with the precarcinogen being ineffective in reducing clone formation. With regard to 4NQO, both the precarcinogen and ultimate carcinogen (4HAQO) affected the clone-forming capacity of cultured human fibroblasts to a similar extent. The almost identical response to 4NQO and its ultimate carcinogen (4HAQO) could probably be explained by the fact that 4NQO is readily transformed into 4HAQO.

From this section, it may be concluded that, as a rule, precarcinogens do not evoke any chromosome aberrations nor affect the clone-forming capacity of cultured human fibroblasts unless they are transformed into ultimate carcinogenic derivatives.

Figures 66-69 Clone-forming capacity of cultured human fibroblasts following short-term exposure to precarcinogens, proximate or ultimate carcinogens (expressed as percentage of survival in untreated controls).

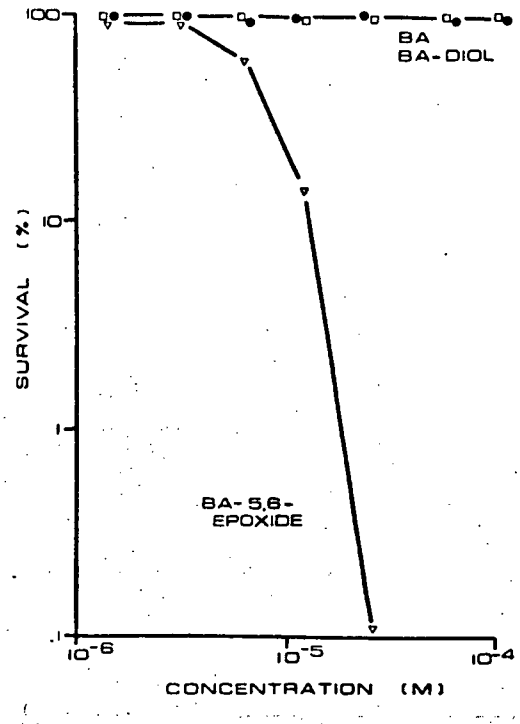
(Figure 66) BA (3 hr.) (□) - precarcinogen.
BA-5,6-epoxide (▽) - proximate carcinogen.
BA-Cis-5,6-dihydrodiol (●) - non-carcinogenic metabolite.

(Figure 67) 2-AAF (5 hr.) (□) - precarcinogen.
N-Hydroxy-2-AAF (▽) - proximate carcinogen.
N-Acetoxy-2-AAF (O) - ultimate carcinogen.

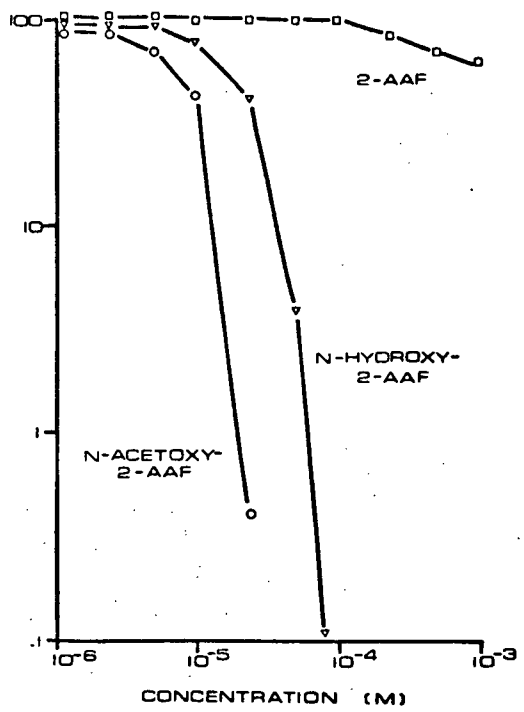
(Figure 68) 4-AAS (5 hr.) (□) - precarcinogen.
N-Hydroxy-4-AAS (▽) - proximate carcinogen.
N-Acetoxy-4-AAS (O) - ultimate carcinogen.

(Figure 69) 4NQO (1.5 hr.) (□) - precarcinogen.
4HAQO (▽) - proximate carcinogen.

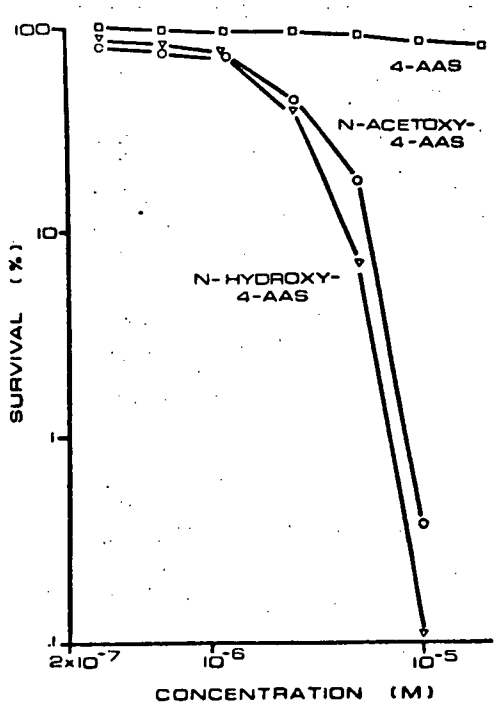
66


100
10
1
.1
SURVIVAL (%)
BA
BA-DIOL
BA-5,6-
EPOXIDE
10⁻⁶
10⁻⁵
10⁻⁴
CONCENTRATION (M)

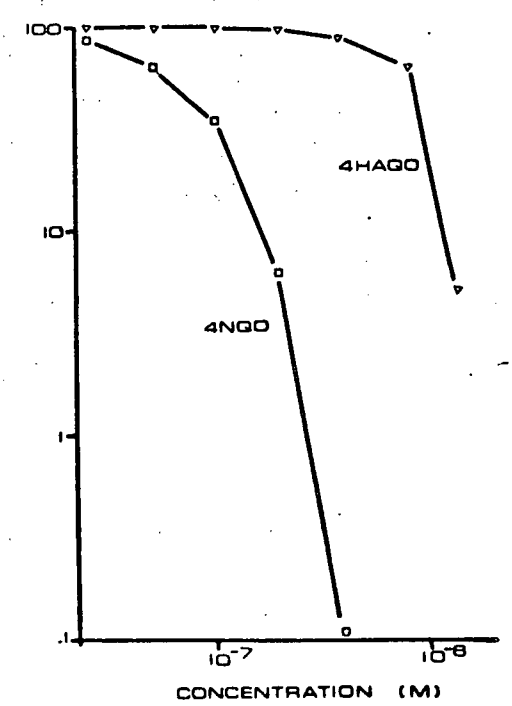
67


100
10
1
.1
2-AAF
N-HYDROXY-
2-AAF
N-ACETOXY-
2-AAF
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
CONCENTRATION (M)

68


100
10
1
.1
SURVIVAL (%)
4-AAS
N-ACETOXY-
4-AAS
N-HYDROXY-
4-AAS
2x10⁻⁷
10⁻⁶
10⁻⁵
CONCENTRATION (M)

69


100
10
1
.1
4HAQO
4NQO
10⁻⁷
10⁻⁸
CONCENTRATION (M)

5. Carcinogenic Capacity and DNA Repair Level

In Chapters 3 and 4, data have been presented to support the notion that the carcinogenicity of a chemical is correlated with its capacity to damage DNA as evidenced by the ensuing unscheduled DNA synthesis. An important but as yet unanswered question concerns the relationship between the level of DNA repair triggered by a carcinogen and the degree of its carcinogenic potential. Previously a good correlation between these two factors has been demonstrated when the action of strong and weak carcinogenic 4NQO isomers and derivatives were compared (Stich et al., 1971, 1974). Highly oncogenic derivatives initiate repair synthesis at considerably lower doses than weakly oncogenic ones. The maximum level of ^{3}HTdR incorporation into cells exposed to weakly oncogenic derivatives is always lower than in those treated with highly oncogenic compounds. The question then arises as to whether such a correlation between carcinogenic potential and level of DNA repair synthesis could be extended to carcinogens of different molecular structures. A few carcinogens have been included in a comparative study. From Fig. 7, it is evident that 4NQO elicited a high level of unscheduled ^{3}HTdR incorporation at relatively low doses, while the 6,7-epoxide of MCA required about 1,000 times higher concentration to trigger a relatively low level of DNA repair synthesis. Obviously 4NQO is not 1,000 times more carcinogenic than MCA or its epoxide. N-Acetoxy-2-AAF,

also a potent carcinogen, initiates a comparatively lower unscheduled DNA synthesis than in the case of 4NQO even though much higher concentrations were used. Thus, the level of unscheduled DNA synthesis elicited by carcinogens of different molecular structures does not reflect their respective carcinogenic activity.

6. Design and Trial of a Rapid In Vitro Bioassay for Chemical Carcinogens

To cope with the carcinogenicity testing of the relatively large number of man-made compounds that are placed on the market annually, and the multitude of naturally-occurring chemicals that enter man's immediate environment, the introduction of fast and economic prescreening procedures has become a necessity. Most of the newly-developed methods that appear suitable for a large-scale prescreening programme depend on the capacity of carcinogens to induce mutations or to affect the DNA of indicator organisms(Stoltz et al., 1974). At present, a great emphasis is being placed on the use of various bacteria (Slater et al., 1971; Kada et al., 1972; Ames et al., 1973a, 1973b; McCalla et al., 1975), yeast (Koske and Stich, 1973; Fahrig, 1974), Neurospora (Ong and de Serres, 1972; De Serres, 1974) or Drosophila (Sobel, 1974) as sensitive, economical indicator organisms. However, inconsistency among the various types of test organisms in their response to a particular carcinogen is not uncommon.

In addition, the feasibility of extrapolating observations from microbial systems to the human situation poses another problem. In view of the limitations of these microbial bioassays, it may be worthwhile to explore the use of cultured human cells as test subjects and the application of DNA repair synthesis estimated by unscheduled ^{3}HTdR incorporation as an endpoint (Rasmussen and Painter, 1966; Stich et al., 1971), to measure the DNA damaging action of chemical carcinogens.

To test the reliability of DNA repair as a bioassay for chemical carcinogens, 64 different compounds were examined. These include precarcinogens that require metabolic activation, proximate and ultimate carcinogens, man-made and naturally-occurring compounds, non-carcinogenic but mutagenic chemicals (e.g. daunomycin, ethidium bromide) and non-carcinogenic as well as non-mutagenic chemicals. The responses of cultured human fibroblasts to these compounds are summarised in the following three sections.

6.1. Concentration of Test Compounds

The range of concentrations of various carcinogens that trigger detectable levels of DNA repair synthesis in cultured human fibroblasts varies greatly (Fig. 5). Similarly, the lethal effect of various carcinogens ranges from about 10^{-8}M to 10^{-2}M (Fig. 6). Therefore one cannot suggest the use of a particular set of concentrations that would be applicable for all chemical

carcinogens. Even the use of the highest tolerable dose could produce misleading results, since inhibition of the DNA repair system by high concentrations of a carcinogen would elicit undetectable levels of an unscheduled ^{3}HTdR incorporation (Fig. 70). The range of concentrations is best selected by first establishing the dose of a carcinogen that would give an obvious toxic effect within the time of the experiment (exposure to test compounds for 1.5 to 5 h. followed by 1.5 h. of ^{3}HTdR), and then starting a dilution series with half this toxic dose.

6.2. Trial of Bioassay

The response of cultured human fibroblasts to the 64 different test compounds (summarised in Table VIII) revealed a consistent pattern: 1. Cells exposed to proximate and ultimate carcinogens responded with a DNA repair synthesis; 2. Precarcinogens either elicited no detectable levels of unscheduled ^{3}HTdR incorporation, or triggered a DNA repair at high concentrations or at longer exposure times (e.g. 2-AAF, Aflatoxin B_1); 3. Non-carcinogenic compounds failed to initiate DNA repair synthesis. In this connection, it is of interest to note the negative response to acriflavine, daunomycin and ethidium bromide which are very potent mutagens but seem to lack a carcinogenic capacity.

6.3. Precarcinogens

In vitro test systems using microbial cells respond

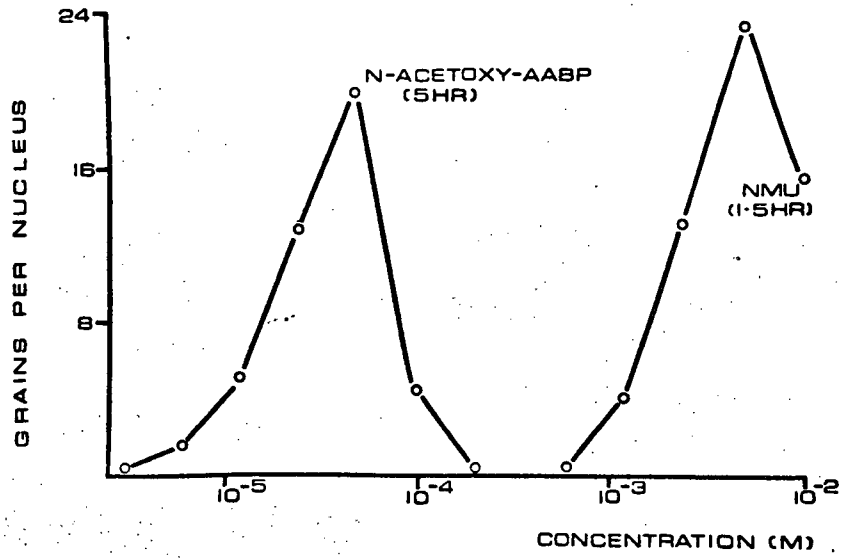


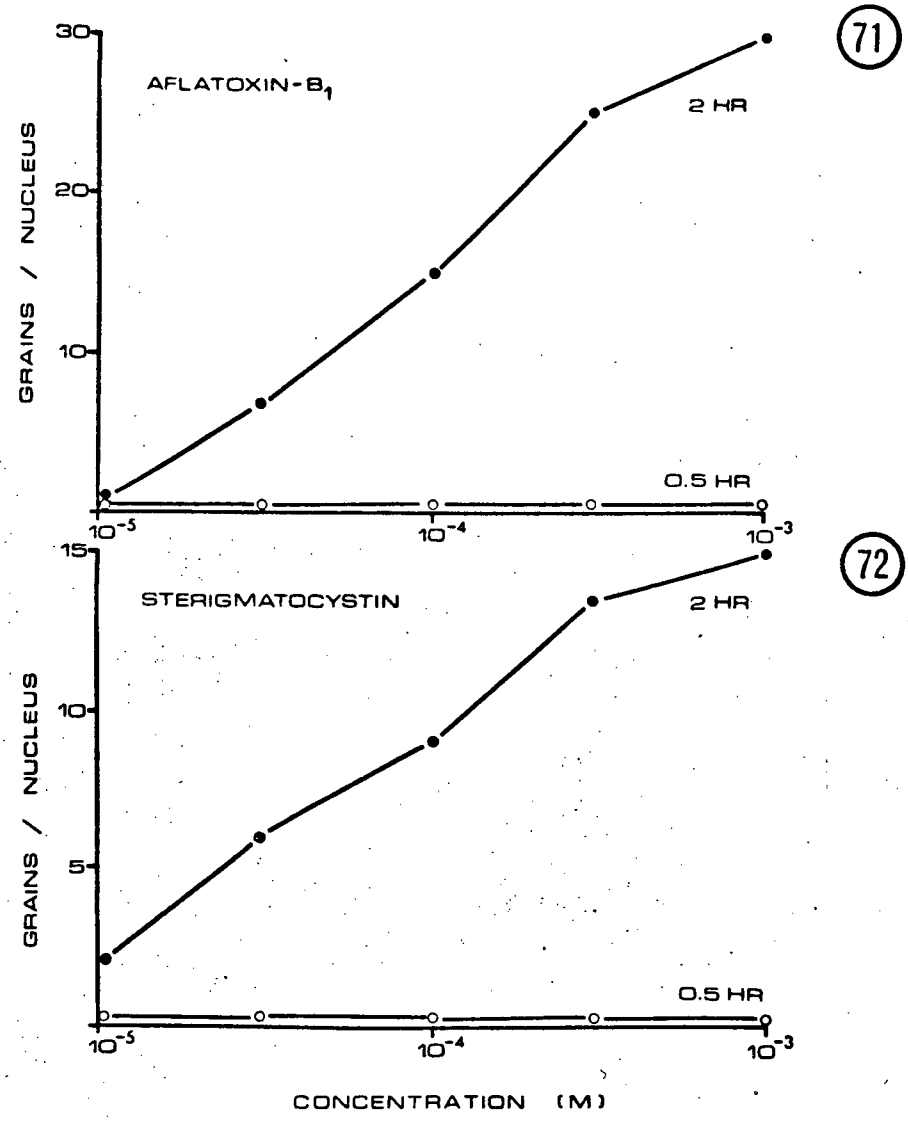
Figure 70 Inhibition of DNA repair in cultured human fibroblasts elicited by high concentrations of a chemical carcinogen.

TABLE VIII DNA REPAIR SYNTHESIS (UNSCHEDULED INCORPORATION OF ^{3}HTdR) OF CULTURED HUMAN FIBROBLASTS EXPOSED TO PRECARCINOGENS, CARCINOGENS, NON-CARCINOGENIC DERIVATIVES AND MUTAGENS

CARCINOGENICITY[1]	COMPOUND	EXPOSURE TIME	CONCENTRATION RANGE WITHIN WHICH UNSCHEDULED ^{3}HTdR UPTAKE DETECTED (M) 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2}	MAXIMUM GRAINS PER NUCLEUS
PC	4NQO	1.5 Hrs		150
UC	4HAQO	1.5 Hrs		78
PC	2-METHYL-4NQO	1.5 Hrs		122
NC	6NQO	1.5 Hrs		0
NC	4AQO	1.5 Hrs		0
PC	BA	3 Hrs		0
UC	BA-5,6-EPOXIDE	3 Hrs		26
NC	BA-cis-5,6-DIHYDRODIOL	3 Hrs		0
PC	MCA	3 Hrs		0
UC	MCA-6,7-EPOXIDE	3 Hrs		40
NC	MCA-6,7-DIHYDRODIOL	3 Hrs		0
PC	2-AAF	5 Hrs		10
UC	N-HYDROXY-2-AAF	5 Hrs		42
UC	N-ACETOXY-2-AAF	5 Hrs		34
NC	1-HYDROXY-2-AAF	5 Hrs		0
NC	3-HYDROXY-2-AAF	5 Hrs		0
NC	5-HYDROXY-2-AAF	5 Hrs		0
NC	7-HYDROXY-2-AAF	5 Hrs		0
PC	4-AAS	5 Hrs		0
UC	N-HYDROXY-4-AAS	5 Hrs		14
UC	N-ACETOXY-4-AAS	5 Hrs		35
PC	4-AABP	5 Hrs		0
UC	N-HYDROXY-4-AABP	5 Hrs		10
UC	N-ACETOXY-4-AABP	5 Hrs		20
PC	2-AAP	5 Hrs		0
UC	N-HYDROXY-2-AAP	5 Hrs		20
UC	N-ACETOXY-2-AAP	5 Hrs		40
UC	N-MYRISTOYLOXY-2-AAF	5 Hrs		13
UC	N-ACETOXY-2-MYRISTOYL-AF	5 Hrs		12
UC	N-MYRISTOYLOXY-2-MYRISTOYL-AF	5 Hrs		14
UC	MNNG	3 Hrs		41
UC	MMS	1.5 Hrs		19
UC	EMS	3 Hrs		20
UC	NMU	1 Hr		23
X	ICR-191	3 Hrs		16
UC	HN_2	3 Hrs		15
PC	SAFROLE	5 Hrs		0
UC	1'-HYDROXY-SAFROLE	5 Hrs		17
X	3'-HYDROXY-SAFROLE	5 Hrs		14
X	3'-ACETOXY-SAFROLE	5 Hrs		26
NC	1'-KETO-SAFROLE	5 Hrs		0
UC	1,1-DIPHENYL-2-PROPYNYL-N-CYCLOHEXYLCARBINOL	1.5 Hrs		38
UC	1-PHENYL-1-(3,4-XYLYL)-2-PROPYNYL-CYCLOHEXYLCARBAMATE	1.5 Hrs		41
NC	1,1-DIPHENYL-2-BUTYNYL-N-CYCLOHEXYLCARBAMATE	1.5-5 Hrs		0
NC	DIPHENYLCARBINOL	1.5-5 Hrs		0
X	STREPTONIGRIN	1.5 Hrs		30
PC	AFLATOXIN B_1	0.5 Hr		0
PC	AFLATOXIN B_1	2 Hrs		30
UC	AFLATOXIN B_1 (Activation)	0.5 Hr		16
PC	AFLATOXIN G_1	0.5 Hr		0
UC	AFLATOXIN G_1 (Activation)	0.5 Hr		15
NC	AFLATOXIN G_2	0.5 Hr		0
NC	AFLATOXIN G_2 (Activation)	0.5 Hr		0
PC	STERIGMATOCYSTIN	0.5 Hr		0
PC	STERIGMATOCYSTIN	2 Hrs		15
UC	STERIGMATOCYSTIN (Activation)	0.5 Hr		40
PC	DIMETHYLNITROSAMINE	1 Hr		0
UC	DIMETHYLNITROSAMINE (Activation)	1 Hr		23
NC	METHYLGUANIDINE	2-5 Hrs		0
UC	METHYLGUANIDINE (Nitrosation)	4 Hrs		52
NC	ACRIFLAVINE NEUTRAL	3-6 Hrs		0
NC	DAUNOMYCIN	3-6 Hrs		0
NC	ETHIDIUM BROMIDE	3-6 Hrs		0
PC	LUTEOSKYRIN	1.5-5 Hrs		0
UC	LUTEOSKYRIN (Activation)	1.5-5 Hrs		0
PC	RUGULOSIN	1.5-5 Hrs		0
UC	RUGULOSIN (Activation)	1.5-5 Hrs		0

[1] PC = precarcinogen ; UC = ultimate carcinogen including proximate carcinogen ; NC = non-carcinogen ; X = unknown carcinogenicity

to reactive forms of carcinogens, but usually fail to detect precarcinogens that require metabolic activation. Cultured human fibroblasts behave similarly. For example, short-term exposures to MCA, 4-AAS, 4-AABP and 2-AAP and safrole did not result in detectable unscheduled incorporation of ^{3}HTdR, whereas the hydroxy and acetoxy forms (representing the proximate or ultimate carcinogens) did trigger DNA repair synthesis. There were exceptions to this pattern. Relatively high doses of 2-AAF (Fig. 57) or longer exposures to aflatoxin B_1 and sterigmatocystin induced a DNA repair synthesis (Fig. 71 and 72), although these three compounds required activation (Cramer et al., 1960; Stich and Laishes, 1975). The simplest explanation is that cultured fibroblasts carried low levels of activation enzymes, even though this activation capacity of fibroblasts was restricted. In the case of DMN, even concentrations exceeding 10^{-2}M did not elicit a DNA repair synthesis (San, unpublished data). Since the activation potential of human fibroblasts cannot be predicted from the molecular structure of a precarcinogen, inclusion of an activation procedure in the bioassay in mandatory. Nevertheless, there remains a certain drawback to this approach, because the activation of a compound may easily be missed by applying the wrong activating system or source of activation enzymes. The carcinogens luteoskyrin and rugulosin [which gave a negative response when applied directly or added in combination with the S9 activation system (Garner and Hanson, 1971; Malling, 1971; Ames et al., 1973b)] may belong in this category.



Figures 71-72 Unscheduled DNA synthesis in cultured human fibroblasts evoked by the precarcinogens aflatoxin B_1. (Figure 71) and sterigmatocystin (Figure 72). Exposure to carcinogen was followed by ^{3}HTdR (3 hours).

● 2-hour exposure to carcinogen

○ 0.5-hour exposure to carcinogen

DISCUSSION

One of the objectives of this thesis was to evaluate the use of DNA repair synthesis in cultured human fibroblasts as a bioassay for chemical carcinogens. A second objective concerned the possible variation in sensitivity within the human population towards chemical carcinogens. In particular, cultured cells from Xeroderma pigmentosum patients (known to be deficient in repairing UV-induced DNA damage) and normal persons were compared with respect to their DNA repair capacity, frequency of chromosome aberrations and clone forming efficiency following exposure to a chemical carcinogen. Since the different questions asked are quite independent of one another, it seems appropriate to discuss each issue in a separate section. The discussion has therefore been subdivided into the following chapters:-

1. The use of unscheduled DNA synthesis in the identification of chemical carcinogens.

2. The use of DNA repair in the identification of sensitive cells.

3. DNA damage, chromosome aberrations and carcinogenesis.

4. Perspectives.

1. The Use of Unscheduled DNA Synthesis in the Identification of Chemical Carcinogens

The results of the present study show the feasibility of introducing DNA repair synthesis of cultured human

fibroblasts as a sensitive system for the detection of chemical carcinogens. Of 64 chemical examined, 29 were directly active proximate or ultimate carcinogens, 15 would be classified as precarcinogens and 16 were non-oncogenic compounds. The carcinogenic capacity of four chemicals - streptonigrin, ICR-191, 3'-hydroxy-safrole and 3'-acetoxy-safrole - is unknown at present. All the proximate and ultimate carcinogens triggered DNA repair synthesis, while no detectable level of unscheduled ^{3}HTdR incorporation was observed following treatment with the 16 non-oncogenic compounds. Of the 15 so-called precarcinogens, 5 responded positively, while the remainder did not elicit DNA repair synthesis at the concentrations and exposure times applied in the present experimental series. It is very likely that fibroblasts can activate the five precarcinogens, 4NQO, 2-methyl-4NQO, 2-AAF, aflatoxin B_1, and sterigmatocystin to a limited degree.

In addition to the 64 compounds examined during the present study, we previously tested 12 carcinogenic and 8 non-carcinogenic isomers and derivatives of 4NQO using Syrian hamster cells as subjects and unscheduled ^{3}HTdR incorporation as endpoint (Stich et al., 1971). Only the 12 carcinogens triggered detectable levels of DNA repair synthesis.

Results from the trial of the DNA repair bioassay show that this test system is suitable for the detection of proximate and ultimate carcinogens only, as it may

yield "false" negatives with precarcinogens. This difficulty can be overcome by mixing the precarcinogens with a post-mitochondrial fraction from a liver homogenate (S9 mix). Using this procedure, it has been possible to activate several precarcinogens in vitro, e.g. dimethylnitrosamine (Malling, 1971; Laishes and Stich, 1973), aflatoxin B_1 (Garner and Hanson, 1971; Stich and Laishes, 1975) and sterigmatocystin (Stich and Laishes, 1975).

Considering that the aim of the DNA repair assay is to identify environmental carcinogens and mutagens and prevent human populations from being exposed to these agents, the relevance of using human cells as test subjects is an advantage that cannot be ignored. In addition, since the unscheduled DNA synthesis triggered by a carcinogen occurs in more than 99% of the treated cells, only a small number of cells are required. Furthermore, human fibroblasts can be readily obtained from skin biopsies of "normal" and cancer predisposed persons. The technique can also be adapted for peripheral lymphocytes. Minute quantities of human blood are adequate to provide sufficient numbers of lymphocytes for short term cultures (48 - 72 hours) during which time a DNA repair assay can be performed (Burk et al., 1971; Lieberman et al., 1971a, 1971b; Clarkson and Evans, 1972; Jacobs et al., 1972).

DNA repair synthesis can be detected in human fibroblasts following exposure to carcinogens of various chemical structures. The DNA repair assay has the advantage that

the end-point (unscheduled ^{3}HTdR incorporation) is a general phenomenon not limited to one specific type of DNA damage but encompasses a variety of alterations or lesions in the DNA molecules.

In spite of its many advantages and its high relevance to man, the DNA repair bioassay is not without its limitations. The technique suffers from the fact that it does not throw any light on the precise DNA-carcinogen interaction that triggers DNA repair synthesis. For example, alkylation of DNA may occur at N1, N3 or N7 of adenine, N3, N7, O6 of guanine, N3 of cytosine and N3 and O4 of thymine (Lawley and Brookes, 1963; Lawley and Thatcher, 1970; Lawley et al., 1971/1972, 1973; O'Connor et al., 1972; Sarma et al., 1974). Which of these events initiates repair and whether all alkylation products are removed by excision repair and at what rate remains to be elucidated.

There are several unsolved questions worthy of attention. The level of DNA repair synthesis depends on the concentration of carcinogen, duration of application and type of carcinogen. For successful application of the DNA repair technique in a large scale prescreening programme, standardized treatment and measuring procedures should be introduced. However, a too rigid regime could produce misleading results. The toxicity of the test compound and its capacity to inhibit a DNA repair system must be taken into account when the range of concentrations and the time of exposure are determined (Stich et al., 1974). Therefore a toxicity

test must precede the actual DNA repair study.

The level of DNA repair synthesis may be influenced by the levels of ^{3}HTdR uptake into cells and the availability of precursors. Furthermore, a DNA repair synthesis may require deoxyadenosine or deoxyguanosine rather than thymidine or cytosine as precursors (Cleaver, 1973; Lieberman and Poirier, 1973). For instance, cells exposed to the carcinogen β-propiolactone which interact with purines insert only purine precursors during DNA repair (Hennings et al., 1974).

The range of concentrations of various carcinogens that trigger detectable levels of DNA repair synthesis in cultured human fibroblasts varies greatly. There is also a lack of uniformity in the exposure times (0.5 to 5 hours) required for different chemical carcinogens to elicit a detectable level of unscheduled ^{3}HTdR incorporation. In the case of precarcinogens, this may be linked to the time period within which active intermediates or metabolites are produced, or (in the case of both precarcinogens and ultimate carcinogens) to the time required for the formation of DNA-carcinogen complexes to bring about an alteration in the DNA structure.

The type of exposure poses another problem in comparing the level of unscheduled DNA synthesis triggered by different chemical carcinogens. Warren and Stich (1975) showed that if a second dose of 4NQO is given within 3 hours of the first one, the DNA repair capacity of the

human fibroblasts is severely curtailed. However, if the second dose is administered more than 5 hours after the first one, the unscheduled incorporation of ^{3}HTdR appears normal. In this 3-hour period the cultured cells show an increased sensitivity to the lethal effect and chromosome-damaging action of the second 4NQO dose. The period of reduced DNA repair capacity seems to increase the mutagenic effect of the chemical carcinogen. Whether a 5-hour continuous exposure to a chemical carcinogen is equivalent to a double-dose exposure within the "refractive period" as described by Warren and Stich remains to be assessed.

The stability of a chemical carcinogen in aqueous solution also deserves some consideration. The routine procedure in the DNA repair assay is to label with ^{3}HTdR following exposure to a carcinogen. Concomitant exposure of cultured human fibroblasts to both carcinogen and ^{3}HTdR may pick up some unscheduled DNA synthetic activity which would have been missed by the post-carcinogen labelling procedure. This is one aspect that warrants further investigation.

Another as yet unanswered question concerns the relationship between the level of DNA repair triggered by a carcinogen, and the degree of its carcinogenic potential. Previously we pointed out a good correlation between these two factors when the action of strong and weak carcinogenic 4NQO isomers and derivatives were compared

(Stich et al., 1971, 1974). However, no such correlation became obvious when carcinogens of different molecular structures were included in the comparative study. For example, 4NQO elicited a high level of unscheduled ^{3}HTdR incorporation at relatively low doses, while MMS, EMS and the 6,7-epoxide of MCA required about 1,000 times higher concentrations to trigger a relatively low level of DNA repair synthesis. Obviously 4NQO is not 1,000 times more carcinogenic than MCA epoxide, MMS or EMS. The absence of a good correlation between the level of DNA repair triggered by a carcinogen and the degree of its carcinogenic potential could be due to the difficulty in placing a quantitative value on carcinogenicity (from in vivo rodent assays). Many chemical carcinogens are known to exhibit species and organ specificity. Aflatoxin B_1, for example, could be considered a potent, weak or non-carcinogenic agent, depending on the species examined (Wogan, 1971).

The solubility of chemical carcinogens in an aqueous media may influence their penetration into cells and nuclei, which in turn may affect the extent of DNA alterations induced. With a few exceptions (e.g. 4NQO, MNNG, MMS, EMS, NMN, ICR-191 and HN_2), most carcinogens tested (especially polycyclic aromatic compounds) are hydrophobic. They are usually dissolved in DMSO or 100% EtOH but precipitation occurs upon dilution with tissue culture medium, resulting in a micro-suspension. Whether the solubility problem is one of the causes leading to a

low level of unscheduled DNA synthesis is not known, although the low level of unscheduled ^{3}HTdR incorporation obtained with water soluble carcinogens such as MMS, EMS and HN_2 tend to argue against such a simple correlation.

Fetal bovine serum, when used at 10 - 20% concentration of the total volume of culture medium, has been demonstrated to effectivly reduce the DNA damaging capacity of a chemical carcinogen (Stich and San, unpublished data). Binding of carcinogen by serum protein may prevent the former from interacting with DNA. On the other hand, formation of a carcinogen-protein complex may assist the transport of the carcinogens to the cell nucleus.

The in vitro activation of pre-carcinogens and carcinogen-conjugates is another area that still needs to be properly explored. Results from the trial of the DNA repair bioassay show that this test system is suitable for the detection of proximate and ultimate carcinogens only, as it may yield "false" negatives with precarcinogens (Table IX). The precarcinogens luteoskyrin and rugulosin gave a negative response when applied directly or added in combination with the S9 activation system (San and Stich, 1975; Table VIII and IX). Likewise, the application of the same activation procedure was not successful with the precarcinogens benz(a)anthracene, 20-methylcholanthrene and 2-acetylaminofluorene (Stich and San, unpublished data). In fact, the in vitro activation of 2-acetylaminofluorene has not been demonstrated to date (Heidelberger, 1973).

TABLE IX The application of the DNA repair assay to 60 compounds including 29 carcinogens, 14 precarcinogens and 17 non-carcinogens.

CARCINOGENS		PRECARCINOGENS		NON-CARCINOGENIC DERIVATIVES
POSITIVE[1]	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE
4-Hydroxyaminoquinoline 1-oxide	Luteoskyrin (activation)	4-Nitroquinoline 1-oxide	Benz(a)anthracene	6NQO
BA-5,6-Epoxide	Rugulosin (activation)	2-Methyl-4NQO	20-Methylcholanthrene	4-Aminoquinoline-1-oxide
MCA-6,7-Epoxide		Aflatoxin B_1	2-Acetylaminofluorene	BA-cis-5,6-dihydrodiol
N-Hydroxy-2-AAF		Sterigmatocystin	4-Acetylaminostilbene	MCA-6,7-dihydrodiol
N-Acetoxy-2-AAF			4-Acetylaminobiphenyl	1-Hydroxy-2-AAF
N-Hydroxy-4-AAS			2-Acetylaminophenanthrene	3-Hydroxy-2-AAF
N-Acetoxy-4-AAS			Safrole	5-Hydroxy-2-AAF
N-Hydroxy-4-AABP			Dimethylnitrosamine	7-Hydroxy-2-AAF
N-Acetoxy-4-AABP			Luteoskyrin	1'-Keto-safrole
N-Hydroxy-2-AAP			Rugulosin	1,1-Diphenyl-2-butynyl-N-cyclohexylcarbamate
N-Acetoxy-2-AAP				Diphenylcarbinol
N-Myristoyloxy-2-AAF				Aflatoxin G_2
N-Acetoxy-2-myristoyl-AF				Aflatoxin G_2 (activation)
N-Myristoyloxy-2-myristoyl-AF				Methylguanidine
N-Methyl-N'-nitro-N-nitroso-guanidine				Acriflavine neutral
Methylmethanesulfonate				Daunomycin
Ethylmethanesulfonate				Ethidium bromide
Nitrosomethylurea				
Nitrogen mustard				
1'-Hydroxy-safrole				
1,1-Diphenyl-2-propynyl-N-cyclohexylcarbinol				
1-Phenyl-1-(3,4Xylyl)-2-propynyl-cyclohexyl-carbamate				
Aflatoxin B_1 (activation)				
Aflatoxin G_1 (activation)				
Sterigmatocystin (activation)				
Dimethylnitrosamine (activation)				
Methylguanidine (nitrosation)				

[1]Positive: unscheduled incorporation of ^{3}HTdR into DNA of cultured human fibroblasts that were arrested at G_1 by an arginine deficient, low serum (5%) culture medium.

The standard method of applying the S9 liver fraction obviously has its limitations. For example, nitrofurans need a reductive and not an oxidative metabolic activation (McCalla and Voutsinos, 1974); cycasin requires cleavage by β-glucosidase (Laqueur and Matsumoto, 1966; Spatz, 1968) and β-glucuronidase treatment enhances the mutagenic effect of β-glucuronide conjugates of some carcinogens (Durston and Ames, 1974).

Short term culture of differentiated cells from various tissues and organs can now be maintained (Leffert and Paul, 1972; Saijo, 1972, Lewis et al., 1973; Noyes, 1973; Shapiro and Schrier, 1973; Fujita et al., 1974; Slavinski et al., 1974). This permits the examination of organ-specific activation or inactivation of carcinogens. The use of tissue and organ cultures in a prescreening programme invariably augments the work load, although it will prove invaluable in testing carcinogens which failed to trigger any unscheduled DNA synthesis in cultured human fibroblasts. It is essential, however, to ensure that cells from different organs or tissues do maintain their tissue-specific properties under the conditions prevailing in cultures.

Another approach to tackle the problem of organ specificity of carcinogens is the more complex in vivo/in vitro combination system developed by Stich and Kieser (1974). In this procedure, a carcinogen is administered to the test animal by injection (subcutaneous or intraperitoneal) or force-feeding. The test animal is sacrificed 2 hours later,

tissue pieces from various organs are removed and maintained in ^{3}HTdR-containing culture medium for 3 hours. The tissue pieces are then fixed, embedded, sectioned and processed for autoradiography. Using this technique, it has been shown that with 4-nitroquinoline 1-oxide, unscheduled DNA synthesis was detected only in the lung, which is the site of tumour formation. In contrast, dimethylnitrosamine appears to be mainly activated in the liver of rodents, and to a much smaller extent in other organs including kidney and respiratory tracts. DNA repair synthesis was observed in those tissues which give rise to neoplasms: mainly in cells of the liver and lung and in cell clusters of the convoluted kidney tubules. It must be mentioned that this in vivo/in vitro combination is not suitable in a large scale prescreening programme of chemical carcinogens, because it does not satisfy the requirement for a simple, rapid and inexpensive assay.

2. The Use of DNA Repair in the Identification of Sensitive Cells.

Results from the present series of studies indicated that cultured fibroblasts from Xeroderma pigmentosum patients exhibited a reduced capacity to repair DNA damage induced by some but not all chemical carcinogens. Xeroderma pigmentosum cells also have an elevated frequency of chromosome aberrations and a reduced clone forming capacity following exposure to chemical carcinogens

which elicited in them a reduced level of unscheduled DNA synthesis. If this type of selective response also occurs in cells carrying other cancer predisposing genes, one can predict that the human population must consist of numerous groups, subgroups and families, each being sensitive to a different set of carcinogenic agents. There is evidence in support of such an assumption. For example, cultured fibroblasts of patients with Klinefelter's syndrome (Mukerjee et al., 1970), Fanconi's anemia (Young, 1971a), Down's syndrome (Todaro and Martin, 1967; Young, 1971b), and XY-gonadal dysgenesis (Mukerjee et al., 1972) are highly susceptible to viral (SV40) induced transformation whereas the XP cells do not show this phenomenon (Parrington et al., 1971). Defective DNA repair has been demonstrated in Fanconi's anemia where the deficiency is believed to be in an exonuclease function which removes the damaged strand of DNA after the endonucleocytic strand scission has been made (Poon et al., 1974). Lymphocytes from Fanconi's anemia patients are more susceptible (compared to cells from unafflicted persons) to chromosome damage by DNA cross-linking agents (Sasaki and Tonomura, 1973). A number of cancer-prone and/or sun-sensitive genetic and nongenetic conditions have been examined for their capacity to repair UV-induced DNA damage but no defect was demonstrated (Epstein, 1974). This variation in the type and spectrum of sensitivity among patients with different disease conditions poses a serious problem in calculating "safe"

doses of noxious agents - whether the response of the "average" man or the most sensitive one should be used as basis.

Another complication in setting "safe levels" of a potential health hazard is the genetic heterogeneity within a particular defect, as is the case for Xeroderma pigmentosum. Patients manifesting typical XP clinical symptoms may have a DNA repair level ranging from over 90% deficiency to near normal capacity. Since they show the clinical symptoms, it is conceivable that XP patients with normal excision repair capacity still possess an elevated sensitivity towards UV-irradiation and other DNA-damaging agents. Indeed, recent studies have demonstrated that XP cells with normal levels of DNA excision repair are deficient in post-replication repair of UV-induced damage (Maher et al., 1975a; Lehmann et al., 1975) and have only 70% of the normal capacity to reactivate UV-damaged adenovirus-2 (Day, 1974, 1975).

Rare tumour predisposing genes (a few cases per million) do not seem to present a major health problem. Nevertheless, the number of heterozygous carriers of such genes is by no means negligible. According to Swift's calculation on Fanconi's amenia (FA), there should be about 650,000 FA carriers among the 200×10^6 North Americans (Swift, 1971). If these estimations are extended to other gene mutants and chromosome anomalies, then the proportion of persons carrying at least one cancer predisposing genetic defect must

be considerable. It is of interest to note that the recessive antosomal gene of Fanconi's anemia increases (three fold) the risk of a heterozygote dying from malignancy. Although XP heterozygotes have not been conclusively demonstrated to exhibit any DNA repair deficiency or sensitivity towards chemical carcinogens, it remains to be shown whether they are more prone to tumour formation than normal persons.

The studies on Xeroderma pigmentosum have augmented our perspective of the whole problem of carcinogen screening. Because of the conceivable variation in the type and spectrum of sensitivity towards carcinogenic agents among the human population, an adequate screening programme must encompass test of chemicals in "normal" persons, genetically afflicted patients and high cancer risk groups. The estimation of DNA repair synthesis coupled with enumeration of chromosome aberrations and analysis of the clone forming capacity should yield insight into the spectrum of sensitivity towards carcinogenic agents.

3. DNA Damage, Chromosome Aberrations and Carcinogenesis.

A discussion on the use of DNA repair synthesis in the identification of chemical carcinogens will be incomplete without mentioning the possible role of DNA repair in chemical carcinogenesis. Many if not all carcinogens interact with DNA and show mutagenic properties. It is possible that an unrepaired or misrepaired DNA damage

may lead to mutations which under certain circumstances may result in a neoplastic transformation. The high sensitivity of XP cells to UV and certain chemical carcinogens correlates well with the deficient DNA repair capacity. It is tempting to link the reduced DNA repair capacity (or unrepaired DNA damage) with the common occurrence of skin cancers in XP patients. However, recent reports indicate that the XP phenotype can occur in the absence of a deficient DNA repair capacity. Furthermore, DNA repair deficiency may not be necessary for the development of most mammalian cancers, since cancer cells in general are quite able to repair UV- as well as chemical-induced DNA damage (Stich and San, 1970; Norman et al., 1972; Lieberman and Forbes, 1973; Epstein, 1974; Robbins et al., 1975).

The DNA repair deficiency in XP cells correlates with the frequency of chromosome aberrations following exposure to certain carcinogens. For example, the more deficient the DNA repair capacity of an XP patient, the higher the frequency of chromosome aberrations following exposure to carcinogens such as 4NQO and N-acetoxy-AAF (Tables IV & V). An attractive hypothesis to explain the apparent correlation between the two phenomena is that unrepaired DNA damage (or alterations) somehow leads to abnormalities at the chromosome level. However, a causal relationship between unrepaired DNA damage and chromosome aberrations is difficult to prove at the moment. Furthermore, the role of chromosome aberrations in carcinogenesis is not clear. A preponderant number of human tumours

have been found to be accompanied by chromosomal changes ranging from hypodiploidy to hyperploidy (Sandberg, 1974). Marker chromosomes are found in at least 50% of human cancers. However, no characteristic or specific karyotypic picture has emerged for any tumour and no two tumours with identical karyotypes have been described to-date (Atkin, 1974). The only exception may be the consistent and characteristic presence of the Ph'-chromosome in chronic myelocytic leukemia, but even here there is evidence that does not entirely support the view of the abnormal chromosome playing a role in the development of the leukemia. The occurrence of diploid cancers, though extremely rare, suggests that diploidy and neoplasia are compatible (Sandberg, 1974). Chromosome aberrations may represent mere epiphenomena to neoplastic transformation. Thus, the role of chromosomal changes in the causation of human tumours remains a problem that is far from being settled.

4. Perspectives

Except for individuals who are engaged in a particular industrial concern, the average person is not likely to be exposed to excessive doses of a single chemical compound. The involvement of several mutagenic and carcinogenic agents seems more probable under naturally occurring conditions. In addition, the exposure may not be limited to chemical carcinogens alone; viral or physical agents could also come into play.

One promising feature of the DNA repair bioassay is that it permits *in vitro* simulation of naturally occurring conditions in man. For example, the formation of potent carcinogenic compounds from nitrite (nitrate) and secondary amines, amides, ureas, guanidines, carbamates etc. at appropriate acidic conditions (e.g. in the human stomach) is well substantiated by various studies (e.g. Mirvish, 1972; IARC Scientific Publication No. 3). In view of this observation, the massive consumption of food products and nitrosatable drugs by the human population does pose a potential health hazard. The DNA repair synthesis system provides a procedure for detecting the formation of carcinogenic nitrosations produced under a variety of conditions: various concentrations of nitrite and of nitrosatable compound, various pH levels, various lengths of nitrosation etc. The role of electron scavengers on the nitrosation reaction can also be examined. For example, ascorbate that is consumed daily by man can at the right molar ratio to nitrite suppress the formation of nitrosation products (Mirvish *et al.*, 1972; Greenblatt, 1973; Fan and Tannenbaum, 1973; Archer *et al.*, 1975). Furthermore, ascorbate, upon oxidation, loses its inhibitory effect on the nitrosation reaction. The oxidation process in turn is accelerated by the presence of metal ions such as Cu^{++}, Fe^{+++}. The multitude of complex interactions, which will determine whether or not a carcinogenic compound will be formed, can be simulated *in vitro* by using the DNA repair bioassay (Lo and Stich, 1975).

The interaction between physical and chemical agents can be readily detected by the DNA repair bioassay. This may be illustrated by 8-methoxypsoralen, a photosensitizing chemical commonly found in the plant kingdom (Baden et al., 1972). 8-Methoxypsoralen is a potent mutagen (Igali et al., 1970; Bridges, 1971) and carcinogen (Grube et al., 1975) when combined with long-wave (355 nm) UV radiation. Other photosensitizing chemicals can be extracted from such common plants as marigold (Tagetis erectus) (Fig. 73) and cow parsnip (Heracleum lanatum) (Fig. 74) which is a favourite vegetable among west coast Indians (Turner and Bell, 1973). The possibility of employing the DNA repair assay in detecting mutagenic agents in crude plant extracts will facilitate the screening of environmental carcinogens, or the possible generation of carcinogens from complex mixtures found in spices, herbs, exotic fruits, teas etc.

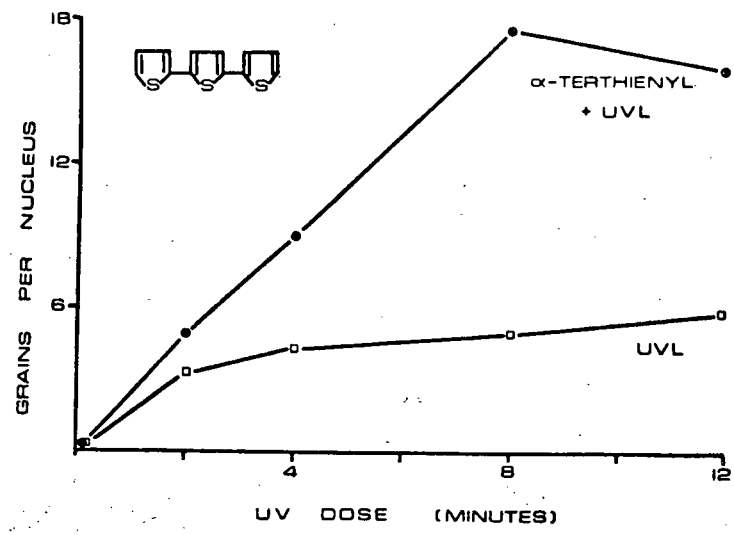
It is estimated that 80% to 90% of all human cancers are environmentally induced (Higginson, 1969, 1971). The possibility of monitoring the environment for carcinogenic agents will be an invaluable step towards cancer prevention.

Adaptation of the DNA repair assay for extracts from human faeces, urine, gastric juices and nasal exudates may permit the identification of groups in the human population that may run a higher risk to develop cancer as a result of industrial or other environmental exposure to obnoxious agents. Several international programmes,

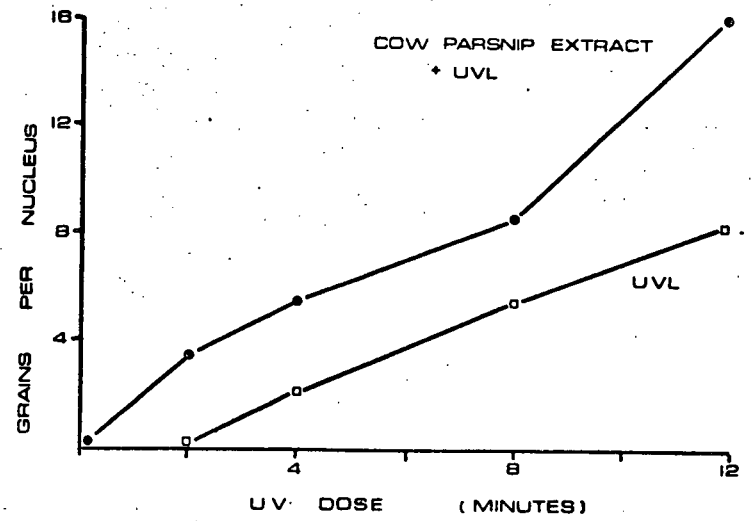
Figures 73-74 DNA repair synthesis of cultured human fibroblasts following exposure (1 hr.) to a photosensitizing chemical and irradiation by long wave (355 nm) UV at 7 inches from an F15T8-BL Sylvania Blacklite. Autoradiography of unscheduled incorporation of ^{3}HTdR (10 μCi/ml.. for 2 hr.).

(Figure 73) α-Terthienyl from marigold (Tagetis erectus).

(Figure 74) Cowparsnip (Heracleum lanatum Michx.) extract.


18
12
6
GRAINS PER NUCLEUS
S
S
S
α-TERTHIENYL
+ UVL
UVL
4
8
12
UV DOSE (MINUTES)

73


18
12
8
4
GRAINS PER NUCLEUS
COW PARSNIP EXTRACT
+ UVL
UVL
4
8
12
UV DOSE (MINUTES)

74

including the U.S.-Japan Cooperative Medical Science Programme and workshops on carcinogenesis assays and rapid screening tests sponsored by the Internations Agency for Research on Cancer have indicated interest in some of these areas.

The DNA repair assay may be modified in a pre-screening programme for chemical carcinogens in the following fashion. In the event of a negative response obtained in the first assay procedure, the next step may be undertaken:-

Step 1 Assay for unscheduled DNA synthesis in cultured human fibroblasts following exposure to test compound or combination of chemical (and/or physical) agents.

Step 2 Repetition of Step 1 with in vitro activation employing liver microsomal enzymes (S9 mix).

Step 3 Repetition of Step 1 coupling exposure to test compound with UV irradiation (prior to or after administration of test compound) to check for inhibitory effect of chemical on UV-induced repair.

Step 4 Administration of test compound to rodents in vivo, followed by monitoring unscheduled DNA synthesis in various organ pieces in vitro to assay for any organotropic effect of test compound.

The DNA repair assay on human cells permits simulation of actual human situations. The inclusion of cells from normal and high cancer risk persons in this test system

may throw some light on the variation in sensitivity towards carcinogens and will be of great value in setting "safe" levels. Additional information on the test compound may be obtained through some of the following procedures:-

- in vitro transformation of rodent or human cells (a highly relevant endpoint since it makes use of a reaction involved in carcinogenesis) (Heidelberger, 1973; Kakunaga, 1973; Sanford, 1974; Evans and DiPaolo, 1975; Pienta, 1975).
- recessive mutant test of Drosophila melanogaster (a genetically well-defined system with high sensitivity and the capacity to activate chemical precarcinogens and premutagens may elucidate the type of genetic changes induced in a multicellular organism; Sobel, 1974).
- Ames' Salmonella test with strains susceptible to frameshift mutations and base-pair substitutions combined with the S9 or other activation mixtures for precarcinogens and premutagens (a rapid, economic and quantitative test system which at the same time enables one to gain an insight into the type of DNA changes induced by the test compound; Ames et al., 1973a, 1973b; McCann et al., 1975).

The average man in an industrial society is exposed to a multitude of chemical, physical and viral oncogens rather than to a single agent. While the identification of individual carcinogenic agents must be continued in the search for effective measures in cancer prevention, it is

not unreasonable to predict that the etiology of neoplastic transformation is a multifactorial one. The total carcinogenic or mutagenic load of our environment is difficult to assess through the use of assay systems for single or even combinations of several carcinogenic agents. An epidemiological approach appears the only one able to cope with such complex naturally occurring situations. The establishment of tumour registries and improved data-collecting procedures will provide adequate information on the patients' ethnic origin, occupation, viral infections, prenatal exposure to drugs, etc. and will greatly help in identifying environments of high cancer risks. The use of "built in" indicator or accumulator organisms for chemical carcinogens provides an "early warning" system that responds to the integrated "carcinogenic load" of a particular environment. For example, the recent international study on the occurrence of skin papillomas in various flatfish species with respect to their distribution in industrially contaminated as well as non-polluted areas offers a promising approach in this direction (Stich and Acton, 1976; Stich et al., 1976).

SUMMARY

1. The primary objective of this study was to evaluate the feasibility of using DNA repair synthesis in cultured human fibroblasts as a simple, rapid and economic bioassay for chemical carcinogens. DNA repair synthesis in human fibroblasts was measured by the autoradiographic detection of unscheduled ^{3}HTdR incorporation following short term exposure to strongly, weakly or non-oncogenic compounds.

2. The effect of various carcinogen doses on DNA repair synthesis was examined. Five distinctive features could be observed:

2.1. The range of concentrations of various carcinogens that triggered detectable levels of DNA repair synthesis in cultured human fibroblasts varied greatly.

2.2. The lethal dose of various carcinogens also differed ranging from about 10^{-8}M to 10^{-2}M.

2.3. A good correlation was observed between the level of DNA repair evoked by a carcinogen and the degree of its carcinogenic potential when the action of strong and weak carcinogenic 4NQO isomers and derivatives were compared. However, no such correlation became obvious when carcinogens of different molecular structures were included in the comparative study.

2.4. The major part of DNA repair synthesis following exposure to different chemical carcinogens appeared to be completed by about 8-10 hours post treatment.

2.5. All the active chemical carcinogens examined inhibited DNA repair synthesis.

2.6. Various carcinogenic compounds differ greatly in their capacity to induce DNA lesions and to inhibit DNA repair.

3. As a trial of the DNA repair bioassay, 64 compounds representing key groups of carcinogens of different molecular structures were examined for the capacity to evoke an unscheduled DNA synthesis in cultured human fibroblasts. This includes 29 directly active proximate or ultimate carcinogens, 15 precarcinogens that require metabolic activation, 16 non-oncogenic compounds and 4 chemicals of unknown carcinogenicity. All directly acting carcinogens triggered a DNA repair synthesis, whereas no unscheduled ^{3}HTdR incorporation was observed following the application of the 16 non-oncogenic compounds. As a rule, the precarcinogens (without metabolic activation) did not elicit DNA repair synthesis.

4. The advantages, limitations and possible adaptations of the DNA repair bioassay for chemical carcinogens were presented and discussed.

5. The second objective of this study was to investigate the possibility of variations in sensitivity within the human population towards chemical carcinogens. Cells from Xeroderma pigmentosum patients (known to be deficient in correcting UV-induced DNA damage) and "normal" persons were examined for their DNA repair capacity, frequency of

chromosome aberrations and clone forming efficiency following exposure to chemical carcinogens. The XP cells show a considerably reduced DNA repair synthesis when exposed to some but not all chemical carcinogens. With chemicals for which the XP cells exhibited a deficiency in DNA repair they also elicited a higher frequency of chromosome aberrations and lower clone forming capacity than in normal persons.

6. Cells from unrelated XP patients have been found to vary in their capacity to repair UV-induced DNA damage. With chemicals for which XP cells exhibited a deficiency in DNA repair, a similar variation in repair capacity among unrelated XP patients was observed. When cells from two XP patients with different DNA repair capacities were compared, the one with the more severe repair deficiency exhibits a higher frequency of chromosome aberrations and lower clone forming capacity than in the less repair-deficient patient.

7. Cells from several XP heterozygotes included in the present study did not exhibit any DNA repair deficiency or sensitivity towards chemical carcinogens.

8. The possible role of DNA damage and chromosome aberrations in chemical carcinogenesis was discussed.

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APPENDIX 1

Cost Anaylsis of DNA Repair Bioassay for a Chemical Carcinogen.

The following calculations are based on the assumption that 50 petri plate cultures of human skin fibroblasts are required per test compound. (The cost of initiating an explant fibroblast culture from a skin biopsy has not been included).

Materials Tissue Culture	Cost ($)
50 35 mm. Petri plates (seeding) @ $61.74/500) 50 35 mm. Petri plates (ADM transfer) @ $61.74/500)	6.17
50 22 mm. Cover glass (seeding) @ $28.60/10 oz. (1 oz. = 150 cover glasses)	.48
40 ml. 0.25% Trypsin solution @ $4.80/25 gm.	.02
40 ml. Hank's Balanced Salt Solution @ $2.44/10 litres	.10
300 ml. MEM (seeding) @ $70.00/100 litres	.21
For 1400 ml. ADM, 200 ml. (blocking cell culture) 200 ml. (chemical solutions) 200 ml. (washing, no serum) 800 ml. (cold chase, no serum, no antibiotics)	
14 ml. Vitamins (100x concentrate) @ $4/100 ml.	.56
14 ml. Non-essential amino acids (100x concentrate) @ $6/100 ml.	.84
Essential amino acids @ $2.32/10 litres ADM	.32
Hank's Balanced Salt Solution @ $2.44/10 litres ADM	.34
Antibiotics for 900 ml. MEM + ADM	
Penicillin G (Final concentration @ 204 units/ml.) $11/$10^8$ units	.02
Streptomycin (Final concentration @ 29.6 µg/ml.) $4.80/25 gm.	.005
Fungizone (Amphtericin B) (Final concentration @ 2.5 µg/ml.) $7.60/100 ml. (250 µg/ml.)	.68
Kanamycin (Final concentration @ 100 ug/ml.) $10.00/100 ml. (10,000 µg./ml.)	.90
34 ml. 7.5% Sodium bicarbonate solution for 1700 ml. MEM + ADM (@ 16 ml. per 800 ml. medium) $31/5 lb.	.03
20 ml. Fetal calf serum for 400 ml. ADM (5% FCS) 45 ml. Fetal calf serum for 300 ml. MEM (15% FCS) $43.00/500 ml.	5.59

0.5 mCi ^{3}HTdR (50 ml. @ 10 μCi/ml.) $80.00/5 mCi	8.00
Sampling of Treated Cultures:	
800 ml. 1% Sodium Citrate Solution $26/5 lb.	.09
1400 ml. EtOH (100%) $5/gal	1.84
200 ml. Glacial Acetic Acid $60/50 pt.	.10
50 Gold Seal Microscope slides $4.35/gross	1.51
NTB-3 Emulsion (Coating @ 200 slides/2 oz.) $50/4oz.	6.00
Processing and Staining of Autoradiograms:	
500 ml. Kodak D-19 Developer $1.98/gal.	.26
750 ml. Fixer $11.95/5 gal.	.47
200 ml. Hypoclearing Agent $2.35/2 gal.	.06
4 gm. Orcein (for 200 ml. 2% aceto-orcein) $25/25 gm.	4.00
200 ml. Glacial acetic acid (for 200 ml. 2% aceto-orcein) $60/50 pt.	.10
200 ml. 100 % EtOH $5/gal.	.26
300 ml. Butanol $54/5 gal.	.85
500 ml. Xylene $30/5 gal.	.79
Permount (1000 slides per 4 fl. oz.) $4/4fl. oz.	.20
50 22 mm. Cover Glass $28.60/ 10 oz. (1 oz. = 150 cover glasses)	.48
Total Cost of Materials:	$41.38

Labour

	Man-Hours
Preparation of 40 ml. Trypsin (@ 2 hr./litre)	0.08
Preparation of 300 ml. MEM (@ 2 hr./20 litres)	0.03
Preparation of 1400 ml. ADM (@ 4 hr./10 litres)	0.56
Preparation of 200 ml. Aceto-orcein (@ 1 hr./1.5 litres)	0.13
Seeding and changing culture medium	2.00
Transfer of cultures into ADM	1.00
Planning experiment	4.00
Running experiment	8.00
Mounting, washing and coating of cell cultures	6.00
Developing and staining autoradiograms	4.00
Analysis of autoradiograms (@ 3-4 slides per hour) and tabulation of data (compilation)	16.00
	41.80

Labour cost (based on the service of a technician earning $850 per month, 37.5-hr. work week)	$220.34
Cost of Materials	$ 41.38
	$261.72

Additional Cost for Activation with Mouse Liver Microsomes (S9 Mix).

Microsomal Preparation (12 gm. mouse liver required) @ 1.3 gm. liver(approx.)/adult mouse - 9 mice @ $1.50	$13.50
NADP 150 mg. @ $89.75/gm.	13.50
NADPH (For DMN control) 12 mg. @ $120.00/500 mg.	4.80
Clucose-6-Phosphate 225 mg. @ $30.00/5 gm.	1.35
$MgCl_2.6H_2$) 203 mg. @ $4.35/ lb.	(.002)
Labour (2 man-hours)	10.50
	$43.65

APPENDIX 2

Human Skin Fibroblasts

The great advantage of diploid human skin fibroblastoid cell cultures is that they permit controlled studies of individual strain variation. Except for a variable but significant degree of tetraploidy and occasional nondisjunctional progeny, the genotype of the cells established in culture is that of the donor. Without exception, human skin fibroblasts have a limited replicative life span which places limits on the types of experiments which can be performed.

Biopsy Technique

Vigorous cultures of predominantly diploid fibroblastoid cultures can often be established from a single dermalepidermal punch biopsy about 2-3 mm. in diameter. The minimum depth of biopsy varies with the thickness of the epidermis. At the usual site of biopsy, the mesial aspect of the upper-forearm, a depth of 1 mm. will usually suffice. The site was chosen because of cosmetic considerations, the relative ease of access, the minimal keratinization, and the sparseness of hair follicles. However, successful cultures can be established with dermal explants from other regions of skin. For well-controlled experiments, consistency in the site of biopsy is advised.

Sterile skin preps are obtained using 70% ethanol and local anesthetics.

Transport and Storage of Tissue

The biopsy tissues are best stored and transported in a standard sterile tissue culture medium (MEM) with 15-20% fetal calf serum. The main precaution in shipment is the avoidance of freezing temperatures.

Initiating Explant Cultures

While enzymatic digestion has been successfully employed for both mass cultures and primary cloning, explant cultures are much more convenient and are probably more reliable in the case of small specimens of skin.

Given a limited amount of material, we prefer a "sandwich" explant technique. It is the surest method of obtaining growth since it minimizes the possibility of loss of explants because of detachment, dessication, pH fluctuation or contamination.

The specimen is washed in 3 changes of WASH MEM (MEM

without serum) with antibiotics (penicillin, streptomycin, fungizone, kanamycin, anti-PPLO agent). The tissue is then transferred to a 15 cm. sterile glass petri dish previously loaded with a small amount of medium. The tissue is then diced, using 2 sharp scapel blades, into approximately pin-head sized pieces (about 50-60 pieces for a 3 mm punch). With a sterile pasteur pipette, transfer the pieces to 35 mm petri dishes. Transfer 3-4 pieces per tetri dish. Place sterile 22 mm. square glass coverslips on top of the pieces in each dish. The tissue fragments are therefore sandwiched between the glass coverslips and the bottom surfaces of the petri dishes. Two mls. of medium (* MEM supplemented with 15-20% FCS and antibiotics) are added to each dish. These are then incubated at 37°C in a CO_2 incubator.

In the early stages of cell migration from the explants one often observes epitheloid cells, probably derived from the epidermis. However, these are invariably and rapidly outgrown by the more actively migrating spindle-shaped fibroblastoid cells which eventually cover the surfaces of the petri dishes and coverslips. The time for confluency varies greatly depending upon the strain and the number of viable explants, but it is generally of the order of several weeks when the medium is changed twice weekly after an initial 7-day interval in the original explant medium. When the petri dishes reach approximately 40-60% confluency, the cells are transferred to 10 cm. petri dishes (Falcon plastic) using trypsin. The skin fragments may be used again with the "sandwich" technique.

* With bicarbonate-buffered media, our experience has been that open petri dishes (in a humidified atmosphere with the appropriate concentration of CO_2) are superior to closed systems; this is probably because of better pH control.

APPENDIX 3

Arginine Deficient Medium (ADM)

To prepare 10 litres of ADM:-

1. Hank's Balanced Salt Solution (BSS)

   To prepare 1 litre of 10x stock solution:-

   1.1. Sodium Chloride (NaCl) 80 gm.
   Potassium Chloride (KCl) 4 gm.
   Magnesium Sulphate ($MgSO_4.7H_2O$) 1 gm.
   Sodium Phosphate, dibasic (Na_2HPO_4) 0.48 gm.
   Potassium Phosphate, monobasic (KH_2PO_4) 0.6 gm.
   Glucose 10.0 gm.
   (Dissolved in 800 ml. of distilled water)

   1.2. Calcium Chloride ($CaCl_2$) 1.4 gm.
   (Dissolved in 100 ml. of distilled water)

   1.3. Phenol Red 0.1 gm.
   (Dissolved in distilled water. Before making up to a final volume of 100 ml., the pH has to be adjusted to 7.0 with 0.05 N NaOH)

   1.4. Mixing of the above three solutions gives 1 litre of 10x stock Hank's BSS.

2. Essential Amino Acids

L-Histidine	310 mg.	
L-Leucine	520 mg.	
L-Lysine	580 mg.	
L-Isoleucine	520 mg.	
L-Methionine	150 mg.	
L-Phenylalanine	320 mg.	
L-Threonine	480 mg.	
L-Tryptophan	100 mg.	
L-Valine	460 mg.	
(Dissolved in 100 ml. 1 x Hank's BSS)		
L-Tyrosine	360 mg.	(Dissolved in 100 ml. 0.1 N HCl)
L-Cystine	240 mg.	(Dissolved in 100 ml. 0.1 N HCl)
L-Glutamine	2.92 gm.	(Dissolved in 100 ml. 1 x Hank's BSS)

3. Non-Essential Amino Acids

L-Alanine	89 mg.
L-Asparagine	150 mg.
L-Aspartic Acid	133 mg.
L-Glutamic Acid	147 mg.
L-Proline	115 mg.
L-Serine	105 mg.
Glycine	75 mg.
(Dissolved in 100 ml. 1 x Hank's BSS)	

4. Vitamins

Choline Chloride	100 mg.
Nicotinamide	100 mg.
i-Inositol	200 mg.
Pyridoxal	100 mg.
Riboflavin	10 mg.
D-Ca-Pantothenate	100 mg.
Thiamine HCl	100 mg.
(Dissolved in 100 ml. 1x BSS)	
Folic Acid	10 mg.
(Dissolved in 100 ml. 1x BSS)	

The solutions from (2), (3), (4) and the amount left from (1) are thoroughly mixed. Distilled water is added to bring the final volume to 10 litres.

The culture medium can be sterilised by passage through a millipore filter (pore size: 0.22 microns; Millipore Filter Corporation, Mass., U.S.A).

Antibiotics, fetal calf serum and sodium bicarbonate are added to the culture just prior to use.

In lieu of weighing out the individual items, the vitamins and non-essential amino acids are obtainable in the form of 100x concentrated mixture from Flow Laboratories, Inc. (Inglewood, California).

APPENDIX 4

Statistical Analysis of Autoradiograms

In the analysis of autoradiograms, at least 30 nuclei, at random locations throughout the entire coverslip culture, were scored for grain number. Background count was taken into consideration by reckoning the number of grains over an area equal in size to that of the nucleus. Routinely, grain counts were made on small interphase nuclei. The data from a typical experiment are shown in Table A. As the mean value of grains per nucleus becomes smaller, the coefficient of variation increases for a given number of nuclei analysed (50 in this example, except for the highest concentration).

Based on statistical calculations, Rogers and England (1973) have demonstrated that the accuracy of estimating the radioactivity per nucleus will depend, not on the number of nuclei counted, nor on the total area of emulsion scanned, but on the total number of silver grains counted in one sampling of the population. England and Miller have prepared charts from which the optimal allocation of effort in grain counting can be obtained once a rough estimate of the ratio of counts over the labelled sources to counts over the background is known [J. Microscropy, 92; 167 (1970)]. For example, the optimal number of grains to be counted for three stated values of the coefficient of variation is given in Table B. When the

background count is low (e.g. 1), only 7 nuclei need to be counted (for a source to background ratio of 20) to obtain a coefficient of variation not exceeding 10%. Referring to the first set of grain counts in Table A and using England and Miller's calculations, only 12 nuclei need to be counted to obtain a coefficient of variation of 5%. The actual number (35) of nuclei scored is therefore adequate. For the lowest concentration ($6x10^{-6}M$), a total of 1500 grains would have to be counted. From these considerations, it is apparent that a relatively small number of nuclei need to be counted when the average grains per nucleus and the source to background ratio are high. Many nuclei have to be scored if the counts are low. However, since the main objective of an experiment is to obtain a dose response profile rather than the statistical significance of mean value for grains per nucleus (e.g. 2), the analysis of 30 to 50 nuclei per sample (irrespective of mean grains per nucleus) may be justified.

Another problem arises when data from different experiments are to be compared, e.g. the level of unscheduled DNA synthesis induced by two different chemical carcinogens. To obtain a meaningful comparison, a control slide has been included in each experiment to ensure that any differences between two experiments are not due to technical variations alone. Table C shows the grain counts from control slides of 5 experiments. Before a comparison is made between two sets of data from different experiments,

the control slides are first analysed for any statistically significant difference. Adjustments can then be made when the two sets of data are compared.

Table A. Unscheduled ^{3}HTdR Incorporation in Cultured Human Fibroblasts Following Exposure to N-Hydroxy-2-AAF (5 hr.) and ^{3}HTdR (1.5 hr.)

CONCENTRATION (M)																			
10^{-4}				5×10^{-5}				2.5×10^{-5}				1.2×10^{-5}				6×10^{-6}			
GRAIN COUNT*																			
S+B	B	S+B	B	S+B	B	S+B	B	S+B	B	S+B	B	S+B	B	S+B	B	S+B	B	S+B	B
45	2	40	1	34	2	26	2	12	2	16	2	7	0	6	1	6	8	7	5
42	0	31	0	29	0	31	0	12	1	14	4	10	3	9	5	10	8	6	3
44	1	29	0	17	0	17	2	13	1	16	4	6	2	10	4	8	5	9	4
37	2	34	0	18	0	18	1	12	0	17	2	8	2	10	4	9	4	2	2
35	2	40	1	19	1	27	1	11	1	18	3	10	2	7	1	4	3	3	4
47	0	29	0	21	1	30	1	11	2	17	4	10	2	10	4	7	4	4	4
50	1	42	0	28	0	25	0	15	1	19	0	11	1	13	3	7	3	8	5
47	1	43	1	24	1	15	0	8	2	22	3	5	0	10	4	6	4	5	4
37	0	36	0	22	1	21	0	16	1	22	3	14	4	5	2	5	2	5	5
40	1	38	1	14	0	24	4	10	1	10	2	12	5	10	5	7	7	7	4
48	0			12	0	26	3	19	3	15	2	14	5	8	3	4	2	4	3
50	1			34	2	17	1	9	4	11	1	7	1	4	2	5	4	5	3
45	1			32	1	24	1	16	1	12	5	13	4	8	2	5	4	3	3
46	0			22	2	25	4	13	1	23	5	7	4	12	3	6	4	4	2
40	0			15	0	24	3	19	4	10	4	8	3	8	2	7	4	4	4
35	1			24	1	23	1	11	0	11	0	5	0	8	3	7	4	9	5
41	2			17	0	28	1	12	0	10	1	2	4	10	5	5	6	7	4
26	2			19	0	24	2	13	0	12	2	8	3	5	3	9	6	6	3
45	1			18	1	22	1	14	1	17	2	11	3	7	0	5	3	6	4
38	2			27	0	14	2	10	1	11	2	8	2	7	0	8	4	4	2
37	0			22	0	21	1	8	1	7	2	8	2	8	3	4	3	5	2
37	0			18	0	19	2	15	2	11	3	12	4	12	4	6	3	3	3
38	0			15	2	20	1	10	0	9	0	8	4	7	1	7	4	4	2
36	1			21	1	24	1	9	0	9	1	7	1	12	4	9	6	5	2
28	1			16	1	18	0	16	3	8	4	11	4	5	1	7	4	5	3

	10^{-4}M		5×10^{-5}M		2.5×10^{-5}M		1.2×10^{-5}M		6×10^{-6}M	
	S+B	B	S+B	B	S+B	B	S+B	B	S+B	B
Σx_i	1376	26	1101	52	661	94	433	134	293	194
$\bar{x}$	39.3	0.7	22.0	1.0	13.2	1.9	8.7	2.7	5.9	3.9
S.D.(S)	6.3		5.5		4.2		3.1		2.4	
S.E.(S)	1.06		0.77		0.60		0.44		0.33	
C.V.(S)	16%		26%		37%		52%		120%	

* S+B = grains over nucleus due to source including background.
B = background count.
S.D.(S) = Standard deviation of grain count due to source alone = $\sqrt{S.D.(S+B)^2 + S.D.(B)^2}$
S.E.(S) = standard error of the mean of counts due to source alone.
C.V.(S) = coefficient of variation of counts due to source alone.

Table B. Optimal Allocation of Effort in Grain Counting Between the Labelled Sources and Background

Rough Estimate of Grain Count S+B[1]	B	(S+B)/B Ratio	Number of Background Grains to Count[2]			Number of Grains Over Sources to Count			Number of Nuclei to Count		
			CV=.10	CV=.05	CV=.025	CV=.10	CV=.05	CV=.025	CV=.10	CV=.05	CV=.025
2	1	2	70	300	1200	370	1500	6000	185	750	3000
5	1	5	18	70	300	210	840	3400	42	168	680
10	1	10	5	20	80	160	650	2600	16	65	260
20	1	20	1.5	6	25	135	540	2150	7	27	107
40	1	40	0.5	2	8	120	490	1980	3	12	49
6	3	2	70	300	1200	370	1500	6000	62	250	1000
15	3	5	18	70	300	210	840	3400	14	53	226
30	3	10	5	20	80	160	650	2600	5	22	87
60	3	20	1.5	6	25	135	540	2150	2	9	36
120	3	40	0.5	2	8	120	490	1980	1	4	17

1. S+B = grain count over source, including background.
   B = background grain count.
2. CV = three stated values of the coefficient of variation.

Table C. UV Control Slide from 5 Different Experiments (100 ergs/mm^2 Followed by 1.5 hr. ^{3}HTdR)

EXPERIMENT									
1		2		3		4		5	
GRAIN COUNT*									
S+B	B	S+B	B	S+B	B	S+B	B	S+B	B
70	3	70	2	80	0	70	0	61	0
54	2	68	3	62	0	62	0	55	4
46	2	60	1	53	0	73	1	47	4
52	1	52	0	62	0	54	0	78	6
59	1	62	1	68	1	80	0	65	4
46	3	57	0	77	0	72	0	86	5
70	1	52	0	67	0	48	1	51	3
59	2	26	0	63	0	66	1	54	0
62	3	35	2	50	0	58	0	76	5
63	2	70	1	68	0	46	0	60	3
54	2	57	2	66	1	56	0	50	5
58	2	52	1	56	1	64	0	60	3
57	2	78	1	64	0	80	0	63	5
60	1	61	0	44	0	48	0	76	6
66	2	82	0	68	1	68	0	72	4
56	1	82	2	56	0	60	0	58	4
57	2	48	0	58	0	78	1	74	6
45	0	52	2	64	0	60	0	68	4
43	2	60	1	62	1	54	0	57	2
65	1	58	1	58	0	50	0	65	6
				63	0	69	1		
				72	1	80	0		
				70	0	64	1		
				61	0	61	0		
				62	0	83	0		
Σx_i	1142	33	1182	20	1574	6	1604	6	1276	79
$\bar{x}$	57.1	1.7	59.1	1.0	63.0	0.2	64.2	0.2	63.8	4.0
S.D.(S)	8.0		14.1		7.9		11.0		10.7	
S.E.(S)	1.8		3.2		1.6		2.2		2.4	
C.V.(S)	14.4%		24.2%		12.5%		17.1%		17.8%	

* S+B = grains over nucleus due to source including background.
B = background count.
S.D.(S) = standard deviation of grain count due to source alone = $\sqrt{S.D.(S+B)^2 + S.D.(B)^2}$
S.E.(S) = standard error of the mean of counts due to source alone.
C.V.(S) = coefficient of variation of counts due to source alone.

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