1982

Dna Damage And Repair In Developing Embryos.

Saowalak Jirakulsomchok
University of Alabama at Birmingham

INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University Microfilms International
300 N. Zeeb Road
Ann Arbor, MI 48106

Jirakulsomchok, Saowalak

DNA DAMAGE AND REPAIR IN DEVELOPING EMBRYOS

The University of Alabama in Birmingham PH.D. 1982

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages __√__
2. Colored illustrations, paper or print _____
3. Photographs with dark background __√__
4. Illustrations are poor copy _____
5. Pages with black marks, not original copy _____
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Other _____

DNA DAMAGE AND REPAIR IN DEVELOPING EMBRYOS

by

Saowalak Jirakulsomchok

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in The Graduate School of The University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1982

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

Degree Ph.D. Major Subject Biochemistry

Name of Candidate Saowalak Jirakulsomchok

Title DNA damage and repair in developing embryos

DNA damage and repair were studied directly in developing mouse embryos. Biological and biochemical studies helped provide evidence that DNA is the principal target for many teratogens, and that DNA repair is important in determining sensitivity to teratogenesis.

The extent and types of anomalies were dependent upon the gestational day on which embryotoxic agents, including X-rays, cytoxan, and methylnitrosourea, were administered. Concurrent administration of chloroquine, an inhibitor of DNA repair, enhanced the frequency of malformations induced by these DNA-damaging agents, suggesting that DNA damage and repair mechanisms play important roles in the production of birth defects.

DNA damage and repair in the intact mouse embryos were performed using the method of nucleoid sedimentation in neutral sucrose gradients on cell preparation isolated after intrauterine damage and repair. Cells lysed with high salt and a non-ionic detergent yield nucleoids which contain nuclear DNA and RNA but are depleted of nuclear proteins, and sediment as supercoiled DNA. Changes in supercoiling of DNA represent a sensitive means of detecting the first and last step of DNA repair processes. Nucleoids were prepared from the

cells of whole embryos, liver, and limb buds at intervals following damage by X-rays, methyl methanesulfonate, and methylnitrosourea on gestation days 10 1/2, 11 1/2, 12 1/2. Preparations of nucleoids from liver and limb buds were especially useful in monitoring DNA damage and repair. At equitoxic doses used in this study, methyl methanesulfonate was not teratogenic to mouse embryos, while methylnitrosourea produced limb bud but not liver abnormalities. The liver cells repaired the damage induced by both agents completely within 24 hours. The cells of limb buds repaired the damage induced by methyl methanesulfonate completely within 24 hours but 48 hours was required for repair of methylnitrosourea.

These observations demonstrated correlation between the capacity of the cells to repair damage in DNA and the induction of malformations in the organs from which they were derived. The cells that repaired DNA lesions rapidly were not reflected in organ abnormality, whereas malformations occurred in organs from which the cells showed slow repair.

Abstract Approved by: Committee Chairman R. Lemore Yielding

Program Director John M. McLellan

Date 6/4/82 Dean of Graduate School Kenneth Bozer

ACKNOWLEDGEMENTS

I wish to express my deep appreciation to my research advisor, Dr. K. Lemone Yielding, for his guidance, encouragement, support and understanding, which were of great value to me and helped me getting through this work. I am also happy to thank Dr. Lerena W. Yielding, Dr. James C. Lacey, Dr. Gerald L. Carlson, and Dr. Charles P. Dagg, who served as members of my research committee.

I am grateful for the financial support from the Department of Anatomy, University of South Alabama.

I wish to thank Sarla Sharma for her generous help in preparing all photographs, and Wendy Hill for her invaluable and dedicated assistance in typing this manuscript.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued) Page

TABLE OF CONTENTS (Continued) Page

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

FIGURE Page

LIST OF ABBREVIATIONS

BND-cellulose	Benzoylated-naphthoylated diethylaminoethyl-cellulose
BUdR	5-bromodeoxyuridine
BU	5-bromouracil
EDTA	Ethylenediamine tetraacetic acid
ENU	Ethylnitrosourea
INH	Isonicotinic acid hydrazide
MMS	Methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	Methylnitrosourea
nm	nanometer
PBS	Phosphate buffer saline
SCE	Sister chromatid exchange
TCA	Trichloroacetic acid
UV	Ultraviolet
γ	gamma

INTRODUCTION

STATEMENT OF HYPOTHESIS

DNA damage and repair mechanisms play important roles in teratogenic processes and should be studied during embryonic development. Mammalian embryonic tissue is an especially sensitive target for attack by a variety of physical and chemical agents in the environment. During development, mammalian embryos are most susceptible to environmentally-induced malformations at the period of organogenesis. Many induction processes which lead to high rates of cell replication and cell differentiation occur during this stage of embryonic development. Because of the importance of these cell activities, agents which interfere with nucleic acid synthesis are known to be potent teratogens. However, modes of action of most teratogens are still unknown. DNA lesions and repair processes have been suggested as central to mechanisms of teratogenesis and carcinogenesis for some compounds (Nomura, 1977); but the details of the induction by teratogens and transplacental carcinogens of genetic changes in the embryo are poorly understood. Studies have been done of DNA damage and repair resulting from administration of DNA-damaging agents to embryo cells in culture (Peleg et al., 1976; Raineri et al., 1978). The response of cultured cells, however, to in vitro damage does not yield full understanding of the mechanisms of teratogenesis in vivo. Therefore, this dissertation research was pursued on the premise that DNA damage and repair could be studied directly in

developing embryos by adapting those methods applied previously to cells *in vitro* to freshly isolated embryonic organs or cells. Based on preliminary studies with embryonic liver cells, it was found possible to study and characterize DNA repair in developing embryos by using various DNA-damaging agents and specific inhibitors. Consequently, the objective of these studies was to explore the relationships between DNA damage, DNA repair and teratogenesis.

BACKGROUND

A. Mechanisms of DNA damage

It is now clear that DNA is a common target macromolecule with which a variety of insults, including ultraviolet radiation, ionizing radiation, and many alkylating agents, interact in susceptible cells or tissues. Damage to DNA has more severe implications for the functional integrity of the cell than does damage to most other cellular components. Damage refers to any modification of DNA that alters its coding properties or its normal function in replication or transcription (Hanawalt *et al.*, 1979). Damage to DNA bases threatens the integrity of genetic information. Base loss may occur spontaneously, and it is also promoted by ultraviolet (UV) radiation, ionizing radiation, and alkylation (Lindahl, 1979). When living systems are exposed to far-ultraviolet radiation (<300 nm), the formation of cyclobutane dimers between adjacent pyrimidines within the same polynucleotide strand of the DNA is apparent (Setlow, 1968). This intrastrand dimer poses a potential block to replication. Ionizing radiation damages both the phosphodiester backbone and the heterocyclic base of DNA *in situ* in the living cells. The major

reactions of bases involve the addition of radicals formed by water radiolysis, most importantly by hydroxyl radicals to the aromatic ring systems (Alexander and Lett, 1968; Bloc and Loman, 1973; Roti Roti and Cerutti, 1974). Radical addition results in ring saturation and may in secondary reactions lead to ring fragmentation and ring elimination. The presence of saturated pyrimidine nucleotides has been shown to cause local disruption of the native conformation of DNA. Saturation of the 5, 6-double bond in pyrimidine nucleosides upon exposure of DNA to γ-radiation decreases substantially the stability of the ring and the N-glycosidic bond (Dunlap and Cerutti, 1975). Apyrimidinic sites are formed by the spontaneous release of the damaged bases. Ring elimination with formation of apurinic sites also represents an important secondary reaction following the formation of alkylpurines by various alkylating agents (Strauss et al., 1975). It is well known that the most reactive base in DNA towards alkylating agents is guanine, which has the highest degree of nucleophilicity. Most of the simple alkylating agents react primarily with the N-7 of guanine, which leads to the lability of the N-glycosidic bond and results in depurination of the DNA at the alkylated site (Lawley, 1966). This can produce single-strand breaks in the DNA either by spontaneous hydrolytic removal (Strauss and Hill, 1970) or by endonuclease attack (Ljungquist and Lindahl, 1974; Ljungquist et al., 1974; Hadi and Goldthwait, 1971) at the damaged sites. On the other hand, reaction at the same N-7 position of two guanine moities involving both arms of a bifunctional alkylating agent such as mustard gas is a powerful source of inactivation of the DNA template (Lawley et al., 1969), whether by inter- or intra-strand crosslinking.

In addition to the N-7 position of guanine, other sites of reaction in DNA for simple alkylating agents are N-3 and O-6 of guanine; N-1, N-3 and N-7 of adenine; N-1, N-3 and O-2 of cytosine; and N-3 and O-4 of thymine (Roberts, 1978). Among these, alkylation at the O-6 position of guanine appears to be an effective cause of direct miscoding (Loveless, 1969). Alkylation at this position would change the tautomeric structure of the base which could induce its pairing with an anomalous base. This would result in base pairing of O^6-alkylguanine with thymine instead of cytosine, the normal partner of guanine, during DNA replication. Loveless (1969) suggested that the extent of O-6 alkylation would possibly provide a better correlation with carcinogenesis and mutagenesis than that previously proposed with alkylation at the N-7 position of guanine. This evidence has been supported by findings of many investigations that the retention of O^6-alkylguanine residues in the DNA of target tissues is associated with carcinogenesis and mutagenesis (Craddock, 1973; O'Connor et al., 1973; Goth and Rajewsky, 1974a; Lawley and Martin, 1975).

Besides DNA base alkylation, certain alkylating agents are also capable of esterifying extensively the phosphate groups in the phosphodiester linkages of the DNA backbone (Rhaese and Freese, 1969; Lawley, 1973). A phosphotriester is an alkali-labile structure which would give rise to a single-strand break in DNA under conditions of alkaline hydrolysis (Walker and Ewart, 1973a). Recent studies on phosphotriesters induced in DNA in vivo by several potent chemical carcinogens have suggested that these lesions are chemically stable and that they are not eliminated by cellular DNA-repair systems (Shooter and Slade, 1977; Shooter et al., 1977). It seems possible that in long-term

carcinogenesis studies the accumulation of phosphotriesters in tissues could be monitored in relation to time of appearance of tumors (Shooter, 1978). However, one of the most important questions, whether the accumulation of such lesions can be causally related to cancer incidence, still remains unknown.

Reaction of these physical and chemical agents with DNA leads to the distortion of the macromolecular structure of DNA which impairs its normal function in replication and transcription, and causes a breakdown of the regulation of these processes. Restoration of the structural integrity of the DNA by repair processes is, therefore, a vital function of every cell, and is the final determinant of the consequences of DNA damage.

B. Mechanisms of DNA repair

Damage to DNA by a variety of physical and chemical agents as described above can be repaired in order to maintain the structural integrity of the DNA which is necessary for normal biological function and for cell replication. Recently, various mechanisms of DNA repair have been reviewed by many investigators (Roberts, 1978; Cleaver, 1974; Cleaver, 1978; Hanawalt et al., 1979; Roberts, 1980). These repair processes will be described here briefly. The three major systems for repair originally discovered and defined in bacteria, photoreactivation, excision repair and postreplication repair, appear to have different forms and importance in eukaryotic cells (Cleaver, 1978).

Photoreactivation is the simplest repair mechanism for cyclobutane pyrimidine dimers induced by UV light. An enzyme, photoreactivating enzyme, binds to the dimer-containing region of DNA, and in the presence

of visible light (310-500 nm), monomerizes the dimer without breaking any phosphodiester bond (Cook and McGrath, 1967). Thus, the DNA is restored to its original undamaged state. This repair system functions in most prokaryotic systems and lower eukaryotes. A number of observations have indicated recently that photoreactivation, in a rather complex situation, exists in mammalian cells including human cells (Sutherland and Oliver, 1976; Mortelmans et al., 1977; Harm, 1980). However, under most experimental conditions it has been extremely difficult to demonstrate photoreactivation in mammalian cells, and whether it occurs and has functional importance in vivo is not known.

Excision repair is a complex multienzymatic process. The principle of excision repair developed from studies with UV light consists of four coordinated steps (Grossman, 1974; Grossman et al., 1975): 1) the damage which causes a distortion or an unwinding of the double helical strands of all duplex DNA is recognized by an endonuclease that introduces a strand scission into DNA; 2) the lesion, along with adjacent nucleotides, is excised from the strand containing it by an exonuclease; 3) reinsertion of new bases is catalyzed by a DNA polymerase using the intact complementary strand as a template; and 4) the gap between newly synthesized DNA and the preexisting DNA is joined by a DNA ligase. After ligation, the duplex is rewound to restore its original conformation, thus excision repair is an error-free process. This type of repair process is now described as nucleotide excision repair, which identifies distinctly that this process involves the excision of nucleotide fragments of DNA by phosphodiester chain scission (Cleaver, 1978).

More recent studies have indicated the existence of another related excision repair process, now known as base excision repair (Cleaver,

1978). In this pathway, a chemically modified base is removed from DNA by an N-glycosidase enzyme which breaks the glycosidic bond between the sugar and a damaged base, leading to the formation of an apurinic site (Kirtikar and Goldthwait, 1974; Lindahl, 1976). The latter may then be recognized by a specific endonuclease, followed by subsequent action of an exonuclease, a DNA polymerase and a DNA ligase (Lindahl, 1976; Ljungquist et al., 1975). On the other hand, the apurinic site may be repaired by the direct replacement of the missing base catalyzed by an insertase enzyme (Deutsch and Linn, 1979). A comparison between the kinds of DNA damage that are presumed to be repaired by base excision and the size of repaired regions suggests that 'small patch' or X-ray-like repair (Painter and Young, 1972; Regan and Setlow, 1974) may correspond to base excision repair, whereas the 'large patch' or ultraviolet-like repair corresponds to nucleotide excision repair (Cleaver, 1978). The excision repair processes which are now known to occur in both bacteria and mammalian cells can act upon a large spectrum of chemically distinct lesions induced by numerous diverse agents both physical and chemical (Regan and Setlow, 1974; Howard-Flanders, 1973; Cerutti, 1975).

Postreplication repair is a repair process existing in both bacteria and mammalian cells that operates after replication of DNA has occurred on a template containing the unexcised damaged region. It was first observed in DNA synthesis in UV-irradiated bacteria (Rupp and Howard-Flanders, 1968). DNA synthesized after UV-irradiation in strains of bacteria unable to carry out excision repair contained a gap in the new strands opposite each pyrimidine dimer in the template strand. Such gaps result when newly synthesized DNA strand elongation is blocked at a dimer and then resumes beyond it. These gaps, rather

than the pyrimidine dimers, are the substrates for postreplication repair; the process is therefore referred to as "daughter-strand gap repair" (Hanawalt et al., 1979). The repair occurs by sister-strand exchanges which fill each gap with undamaged DNA from the isopolar parental strand. The resultant discontinuities in the parental strand can then be filled by repair synthesis, using the undamaged regions of the complementary daughter strand as templates. This mechanism provides for the transfer of information between duplexes necessary to construct strands containing the complete base sequence of a functional chromosome even though many dimers remain in the template strands. More recent evidence has indicated that dimers are randomly transferred into newly synthesized DNA as gaps are repaired, thus becoming equally distributed among parental and daughter strands (Ganesan, 1974; Ganesan and Seawell, 1975). This observation supports the idea that sister-strand exchanges accompany repair and is in agreement with current models which include DNA strand isomerization during genetic recombination (Radding, 1978). However, the production of dimer-free DNA by this mechanism in the absence of excision repair requires several rounds of replication (Ganesan and Seawell, 1975).

In mammalian cells, a similar process of postreplication repair also occurs, but interpretation is more difficult because of the much greater complexity of the mammalian genome. In UV-irradiated mammalian cells, the resultant gaps in newly synthesized DNA are thought to be filled by de novo DNA synthesis (Lehmann, 1972; Buhl et al., 1972). This phenomenon has also been reported for cells exposed to some alkylating agents (Fujiwara, 1975a). However, not all the findings are consistent with this model. Other studies suggest that the production

of low-molecular-weight DNA following UV irradiation reflects the fact that DNA synthesis is blocked by the presence of a lesion but then synthesizes past it continuously by a process requiring strand displacement and branch migration (Higgins et al., 1976; Fujiwara and Tatsumi, 1976a). However, currently available techniques are not capable of distinguishing unequivocally between these various possible mechanisms by which cells can replicate past the unexcised lesions on their DNA.

In recent years, there have been developments in the field of inducible DNA repair process in prokaryotes, and attempts have been made to demonstrate the existence of similar systems in eukaryotes. Radman (1974, 1975) proposed that a single inducible error-prone repair replication system (SOS repair) may be responsible for both the mutagenic reactivation of UV-irradiated phage and for error-prone repair of bacterial DNA. SOS repair activity is repressed in undamaged wild-type cells, but is induced in response to UV radiation and other agents, including many mutagens and carcinogens, which damage DNA or block its replication. Such treatments generate a signal to initiate a complex group of inducible processes which appear to be co-ordinately regulated. The induction of SOS response is accompanied by the appearance of a new protein, called the rec A gene product. It is currently suggested that the rec A protein can, under inducing conditions, act proteolytically to inactivate a repressor controlling the co-ordinately expressed SOS function (Witkin, 1976). As yet the nature of the SOS induction signal is not clearly understood, nor is the mode of regulation of SOS function.

Other effects which are also apparently indicative of the induction of a DNA repair process but different from those described above have

recently been reported. The exposure of E. coli to sublethal doses of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) resulted in higher survival but in a lower mutation rate when they were exposed subsequently to lethal concentrations of the same agent (Samson and Cairns, 1977). This process has been called "adaptation," and is clearly different from SOS repair. In general, SOS functions are induced by treatments that block DNA replication, while adaptation usually occurs under conditions that do not alter detectably cell growth and DNA synthesis (Hanawalt et al., 1979).

Phenomena possibly analogous to the adaptive response in bacteria have been described recently in a mammalian system. The removal of O^6-methylguanine from DNA in rat livers is more rapid in animals that have had a prolonged exposure to low levels of dimethylnitrosamine prior to a large dose of the agent (Pegg, 1978; Montesano et al., 1979). The mechanism by which O^6-methylguanine is removed from DNA is not clear.

The descriptions presented here give a general view of various DNA repair processes. The repair systems are very important to cellular survival and normal function. The correlations between DNA repair and biological end points, such as mutagenesis, transformation, carcinogenesis and teratogenesis, though studied extensively, still require additional investigation.

Inhibitors of DNA repair. Accompanying the discovery of DNA repair processes, much effort has gone into the search for compounds that would specifically inhibit DNA repair. A number of agents have been identified (Gaudin et al., 1971, 1972a, 1972b, 1974; Cleaver, 1969), but the precise sites of action on individual enzymatic steps have not

been determined. Gaudin et al. (1971, 1972b) found that a variety of cocarcinogens act by inhibiting repair synthesis induced by UV radiation in human lymphocytes. Chloroquine and quinacrine which are antimalarial drugs were found to be effective inhibitors of UV-stimulated DNA repair synthesis in human lymphocytes (Gaudin et al., 1972a; Kulkarni and Yielding, 1978). Chloroquine was also found to decrease markedly the survival rate of X-ray-treated mice (Gaudin et al., 1972a), to increase the cytotoxic effect of cyclophosphamide, and to increase the toxicity of methyl methanesulfonate (MMS) damage to mammalian cells in culture (Michael and Williams, 1974). These findings are consistent with the suggestion that chloroquine exerts its effect by inhibiting repair of DNA.

Caffeine is a well known DNA repair inhibitor due to its ability to inhibit specifically postreplication repair in cultured mammalian cells. Caffeine has not been shown to inhibit excision repair of any type of damage in any mammalian cell (Lehmann and Kirk-Bell, 1974; Regan et al., 1968; Roberts and Ward, 1973). It is now clear that caffeine inhibits postreplication repair in rodent cells (Lehmann and Kirk-Bell, 1974; Fujiwara, 1975a). In contrast to these findings, postreplication repair in many human cell lines is apparently unaffected by caffeine (Roberts and Ward, 1973; Buhl and Regan, 1974), but caffeine does inhibit the gap filling process in xeroderma pigmentosum (XP) cells (Buhl and Regan, 1974). There is a report indicating that some xeroderma variants (patients with XP who have normal excision repair) have postreplication repair system which is inhibited by caffeine (Lehmann et al., 1975).

More recently, isonicotinic acid hydrazide (INH) which is used widely as antituberculostatic agent was reported to inhibit

postreplication repair induced by methylnitrosourea (MNU) in Chinese hamster cells (Klamerth, 1978). INH also showed an effect similar to caffeine in inhibiting repair of chromosome damage induced by mitomycin C in human lymphocytes; and it was suggested that chromosome repair is closely related to a DNA repair process sensitive to caffeine or INH (Okoyama and Kitao, 1981).

Although all these agents have additional biological effects which may complicate their effects as DNA repair inhibitors, those results described above are highly suggestive. Therefore, these findings may provide useful tools for the study of the repair mechanisms and may be of importance in helping to understand the role of DNA repair in the biological effects of DNA damaging agents.

C. Summary of methods of studying DNA repair

Repair of damage in DNA can be detected by a variety of methods, depending on the types of lesions to be studied.

1. Direct measurement of base lesions. The methods are based on the removal of abnormal or altered bases such as pyrimidine dimers and alkylation products from DNA. These techniques usually require the presence of radioactively labeled lesions in DNA, so that their loss can be detected directly as, for example, in the cases of removal of labeled pyrimidine dimers (Setlow and Carrier, 1964) or chemical adducts measured chromatographically. In order to obtain a direct measure of the individual alkylation products, it is usually necessary to degrade the nucleic acid polymer to its constituent parts with acid, alkali, or enzymes to permit chromatographic separation of the normal bases or nucleosides from the reaction products. The growing multiplicity of

reaction products has required the development of alternative procedures for analysis. These include column chromatography using Sephadex for the separation of normal and alkylated purines and Dowex ion-exchange resins for the separation of normal and alkylated nucleosides and bases (Lawley and Shah, 1972). Recently, a chromatographic procedure using Aminex A-6 cation exchange resin for the resolution of O- and N-alkylated bases and nucleosides has been described (Daoud and Irving, 1977). Other techniques such as paper chromatography (Frei and Lawley, 1975), high pressure liquid chromatography (Breter et al., 1976; Bochert and Webb, 1977), and thin-layer chromatography (Issaq et al., 1977) have also been used in these studies. However, these methods have several limitations. Biologically important lesions may be undetectable if they are rare, and measurement of radioactive chemical adducts ignores lesions produced through nonradioactive intermediates. In addition, loss of radioactivity may result not only from excision but also from chemical degradation of the adduct (Regan et al., 1976).

2. DNA strand breaks. During the excision repair process, single-strand breaks should be present transiently due to the incision of the phosphodiester bonds adjacent or close to the damage sites by an endonuclease and subsequent excision of the defective nucleotides by an exonuclease. Strand breakage in DNA may be studied by measuring the molecular weights of the DNA by means of sedimentation techniques. The most used method is that of alkaline sucrose gradient sedimentation (McGrath and Williams, 1966). In this method, alkaline lysis of cells directly onto such gradients separates the two strands of DNA, so that breaks within each single strand can be detected by the resulting decreases in size. The single-stranded DNA thus produced is centrifuged

in an alkaline sucrose gradient to identify fragments of different sizes. DNA strands with low molecular weights sediment more slowly than those with high molecular weights. Repair of the breaks results in an increase in the size of DNA strands, and thus increase in the sedimentation rate. Therefore, changes in the sedimentation rates of DNA in alkaline sucrose gradients reflect the molecular weights of DNA. The major problem of this method is that the results are difficult to quantitate since the strand breaks are randomly placed to cause heterogeneity in particle size. Strand breaks are also introduced into DNA by strong alkali during the lysis of cells before centrifugation, and these may mask the breaks produced by excision repair process (Cleaver, 1974).

Another method for the detection of DNA strand breaks is that of alkaline elution technique (Kohn and Ewig, 1973). A procedure is described whereby shortened DNA single strands can be eluted selectively under alkaline conditions from filters upon which cells have been lysed. The rate of elution of DNA from filters reflects the size of DNA. Short segments of DNA due to breaks in DNA strands elute more rapidly than long chain DNA or bulk DNA. Repair of strand breaks can convert cell DNA from a rapidly eluting form to a slowly eluting form. This alkaline elution procedure is apparently more sensitive than the alkaline sucrose gradient sedimentation for the detection of DNA strand breaks. However, the physical basis for the observed increases in elution rate is still obscure, which raises questions in the interpretation of the data (Hutchinson, 1978).

3. New DNA synthesis. One step in excision repair process involves the incorporation of segments of newly synthesized DNA into

parental strands. This synthetic process is termed nonsemiconservative DNA synthesis or repair synthesis. The most general method involves the physical resolution of repair synthesis from semiconservative synthesis of DNA. This is accomplished by combining density and radioactive labeling of the newly synthesized DNA, with separation of species according to density in isopycnic gradients (Pettijohn and Hanawalt, 1964; Cleaver, 1969). The basis of the method is that ^{3}H-bromodeoxyuridine (^{3}H-BUdR) substitutes for thymidine such that semiconservative DNA replication produces DNA that is of hybrid density with ^{3}H-bromouracil (^{3}H-BU) in the newly synthesized strands. If repair replication occurs with the insertion of ^{3}H-BUdR into DNA in the form of small patches, the DNA will be ^{3}H-labeled but of normal density. Hydroxyurea has been used frequently during labeling to inhibit semiconservative replication preferentially, thereby improving resolution of repair replication by reducing the amount of radioactivity in gradients (Cleaver, 1969). Although this method appears quantitative, additional details about the biochemistry of the process are necessary for meaningful interpretation of the results (Cleaver, 1974).

Repair synthesis has also been resolved from semiconservative DNA synthesis with the aid of a benzoylated-naphthoylated DEAE-cellulose (BND-cellulose) column (Scudiero et al., 1975). Repair can be measured in the region of DNA growing points and in the bulk of DNA. Cells are incubated with radioactive DNA precursor during treatment with a repair-inducing agent. Hydroxyurea, an inhibitor of semiconservative DNA synthesis, is added to slow the progression of DNA growing points. Sheared DNA from cell lysates is passed through the BND-cellulose column. Washing with 1 M NaCl solution elutes purely native DNA.

Subsequent washing with 50% formamide elutes single-stranded DNA and DNA containing single-stranded regions (DNA growing points). Any increase in the radioactivity in the eluates is due to repair synthesis and can be quantitated by determining its specific activity.

The photolysis of BUdR-labeled DNA has also been used for measuring repair synthesis (Regan et al., 1971). In this method, the cells whose DNA has been prelabeled with radioactive thymidine are exposed to DNA-damaging agents, and then placed in medium containing BUdR. As repair proceeds, BU is incorporated into the regions of resynthesized DNA. The cells are then irradiated with 313 nm light which produces selectively alkali-labile sites at positions where BU is incorporated. Sedimentation of the fragmented DNA in alkaline sucrose gradients permits an estimate of both the number of repaired regions and their size.

The other method used for the study of repair replication is unscheduled DNA synthesis (Djordjevic and Tolmach, 1967). In this method, the incorporation of radioactive DNA precursors into individual cells is detected by autoradiography. Since semiconservative replication is confined to the S phase of the cell cycle, the cells that are out of the S phase (those in G_1, G_2 and mitosis) and that incorporate radioactive DNA precursors are performing only repair replication. Therefore, the cells in S phase will be heavily labeled and thus can be distinguished from the lightly labeled cells undergoing repair. But this method does not prove clearly that unscheduled DNA synthesis in a cellular system represents repair of DNA. Another technique for the assay of unscheduled DNA synthesis involves the use of hydroxyurea under conditions which hydroxyurea inhibits semiconservative synthesis of DNA more than repair replication. Because of this, DNA synthesis occurring in the

presence of hydroxyurea has been taken to represent repair synthesis (Lieberman et al., 1971). However, the inhibition of de novo replication of DNA, especially under in vivo conditions, is not 100%, and in addition, certain compounds may interfere with the inhibition of normal replication by hydroxyurea (Brandt et al., 1972). Hence a genotoxic compound which causes an increased incorporation of radioactive precursor into DNA in the presence of hydroxyurea is not necessarily acting by stimulation of a repair mechanism.

4. Alterations in DNA structure. Since DNA is packaged with proteins into chromatin material and organized into chromosomes, damage to DNA can be detected visibly through cytologic analysis of chromosomes. Chromosome aberrations (achromatic lesions, chromatid deletion, etc.) are induced by physical and chemical agents (Wolff, 1978). There is much circumstantial evidence to indicate that such aberrations are mediated by interference with the DNA repair mechanisms. A possible relationship between postreplication repair and production of chromosomal aberrations has been shown by posttreatment with DNA repair-antagonizing agent such as caffeine following DNA damage by chemical agents, resulting in an increase in the formation of chromosome aberrations (Roberts et al., 1974; Kirlman et al., 1974; Frei and Venitt, 1975; Okoyama and Kitao, 1981).

Currently, another cytogenetic technique which is used widely is the analysis for sister chromatid exchanges (SCE). This method has been demonstrated to afford a sensitive measure of chromosome damage (Latt, 1974; Kato, 1977; Wolff, 1977; Perry and Evans, 1975). This technique depends on exposing cells to BUdR during two rounds of replication. The metaphase (M_2) chromosomes observed subsequently consist

of substituted sister chromatids which stain differently from unsubstituted sister chromatids. A correlation between the mechanism involved in SCE and the postreplication repair of DNA damage was claimed by Kato (1973, 1974). However, other evidence indicates that the formation of SCE is not simply related to postreplication repair (Kato and Stich, 1976; de Weerd-Kastelein et al., 1977). Nevertheless, despite the lack of clear understanding of the mechanism involved in the production of SCE, it appears that their formation constitute very sensitive tests for the detection of DNA damage.

Breakage of DNA strands and rejoining of the breaks are reflected in changes in the superstructure of DNA. These conformational changes of DNA are detected by the analysis of nucleoid sedimentation in neutral sucrose gradients (Cook and Brazell, 1975). Nucleoids are structures released from cells when cells are lysed in the presence of high salt concentration and non-ionic detergent; they contain all nuclear DNA and RNA but are depleted of nuclear proteins. Nucleoids that contain intact, supercoiled DNA sediment most rapidly in neutral sucrose gradients. Any break in either of the DNA strands causes an unwinding of the supercoils to form a relaxed structure, and thus the nucleoids sediment more slowly. Repair of the breaks rewinds the extended DNA into supercoils again and reestablishes the normal sedimentation rate of nucleoids isolated subsequently. This method is very sensitive and offers the potential for detecting the low levels of DNA damage. Therefore, this nucleoid sedimentation analysis in neutral sucrose gradients should prove useful in studying DNA damage and repair in developing mouse embryos.

D. Supercoiling of DNA

The bihelical structure of DNA was first proposed by Watson and Crick (1953) and was confirmed primarily by X-ray diffraction studies of the DNA fibers. Later, Vinograd et al. (1965) proposed that closed circular duplex DNA also contains superhelical turns due to the constraints imposed by the Watson-Crick structure in a closed circular molecule. Covalently closed circular duplex DNA is widely distributed in nature. Virtually all of these duplex DNA exists naturally in a negatively supercoiled form in which the coiling of the helix axis itself is in the opposite direction to that of the double helix. This supercoiling is an important element of the process of DNA replication, transcription, and recombination (Gellert, 1981). DNA supercoiling is controlled by a class of enzymes called topoisomerases (Wang and Liu, 1979), which catalyzes the interconversion of topological isomers of DNA. In bacteria, it has been discovered that an enzyme DNA gyrase, one group of topoisomerases, catalyzes the conversion of relaxed closed circular DNA to a negative superhelical form in an ATP-dependent reaction (Gellert et al., 1976). In the absence of ATP, DNA gyrase spontaneously relaxes negatively supercoiled DNA (Gellert et al., 1977). In bacteria, there is now good evidence that gyrase is responsible for introducing at least most of the negative supercoils in vivo (Cozzarelli, 1980). Many topoisomerases with relaxing activity have been isolated from a number of eukaryotic sources (Gellert, 1981), but no eukaryotic enzyme has yet been shown to supercoil DNA. Evidence indicates that most or all supercoiling of DNA in eukaryotic cells arises from histone binding combined with the relaxing activity of a topoisomerase (Gellert et al., 1978). Binding of histone octamers

to DNA, followed by enzymatic relaxation of compensatory positive supercoils, results in a negative superhelical wrapping of DNA around the histone molecules, thus forming nucleosome units.

It is apparent that the superhelical turns are lost spontaneously when phosphodiester bonds are broken in either of the backbone strands of the duplex, hence linear derivatives of these circular DNA molecules or those bearing one single-strand scission or nick generally contain no superhelical turns. This has been proved by using the intercalating agent, ethidium bromide (Bauer and Vinograd, 1968). The binding of the intercalating agent to closed circular SV 40 DNA causes an unwinding of the duplex structure and a simultaneous and quantitatively equivalent unwinding of the superhelices. It has been suggested that superhelical turns might be a general feature of the DNA of higher organisms and that they might play a role in controlling gene expression (Cook, 1973, 1974). A model was confirmed by Cook and Brazell (1975, 1976a) showing that the DNA of higher animals including human is supercoiled. The superhelical state of DNA has been analyzed by examining the sedimentation properties of nucleoids of those higher cells through sucrose gradient containing the intercalating agent, ethidium bromide. Nucleoids are structures released when cells are lysed in the presence of non-ionic detergent and high salt concentration. The structures of nucleoids resemble nuclei, they contain nearly all nuclear DNA and RNA, but are depleted of nuclear proteins (Cook and Brazell, 1975). Since the DNA of intact nucleoids is supercoiled, they sediment in sucrose gradients more rapidly than relaxed or broken and extended forms. Therefore, the rate of sedimentation of nucleoids is a sensitive index of the integrity of their constituent DNA and its response to damage and repair. The

intercalating agent, ethidium bromide, affects the sedimentation of supercoiled DNA in a characteristic manner (Cook and Brazell, 1975; Crawford and Waring, 1967). As the concentration of ethidium bromide is increased, the distance travelled by the nucleoids falls to a minimum and then rises again. At low concentration, ethidium bromide unwinds the superhelical turns to form the relaxed molecule. The addition of more concentration of ethidium bromide leads to the formation of supercoiling of the opposite sense to that initially present and the molecules become more compact again. Thus supercoiled DNA exhibits a biphasic response to ethidium bromide. If the DNA is already nicked, the sedimentation of nucleoids should no longer show the biphasic effect in response to different concentrations of the intercalating agent. Rejoining of the breaks restores the DNA to its original conformation and reestablishes the normal sedimentation rate (Cook and Brazell, (1976b). The nucleoid sedimentation technique is the most sensitive means available for detecting single-strand breaks and repair of these breaks in nuclear DNA since any single-strand break is amplified by the resulting relaxation of supercoils. The major advantage of this method is that damage events which cause random single-strand breaks are still reflected as uniform shifts in the properties of a homogeneous population of particles. At the present time, there has been no detailed study of DNA damage and repair in developing embryos. It is of interest, therefore, to use this highly sensitive method to study directly the nature and consequences of DNA damage and repair in developing mouse embryos following treatment with teratogenic agents.

E. Development of mouse embryos

The development of the mouse embryos, from the time of fertilization, which is determined by the presence of a vaginal plug, to the time of delivery extends for a period of 18-21 days, depending on the strain of the animals used. A vaginal plug results when the mouse ejaculate mixes with the cornified epithelial cells sloughed from the vaginal lining at the time of estrus (Chahond and Kwasigroch, 1977). In many inbred lines, the duration of normal pregnancy is approximately 19 days (Rice, 1969). The gestation period is generally divided into 3 sequential intervals: 1) a period of embryogenesis, comprising the first seven days of gestation, from fertilization to the appearance of the third germ layer (the mesoderm); 2) a period of organogenesis, from the seventh day to approximately the twelfth day, during which the major organ systems are formed; and 3) a period of tissue differentiation, between the twelfth day and birth, during which differentiation of most tissue is largely completed. Most differentiation is in fact completed by the sixteenth day, except in certain organs such as the eyes and the cerebellum that do not mature until after birth. The final three days or so of gestation form a true fetal stage of development, characterized more by growth than by differentiation.

It is during the period of organogenesis that malformations of specific organs or systems are easily induced by teratogens, while during the period of tissue differentiation and true fetal stage, the susceptibility to transplacental carcinogenesis tends to be maximum. Treatment of animals during the last stage of development can produce malformations in certain organs, including the eyes, which still continue differentiating. The big advantage of studying the

maldevelopment of the eyes during the last embryonic development is that other major congenital abnormalities are produced rarely during the last trimester and fetal survival is high. Therefore, the eye abnormalities can be studied easily in the postnatal life of the offspring.

Normal development requires the integrity of the genome, appropriate and differential gene expression, and normal functioning metabolic processes. It follows, therefore, that any changes in these processes may lead to abnormal development such as death, malformation and growth retardation.

F. Some general principles of abnormal development

1. Susceptibility to teratogenic agents varies with the developmental stage at the time of exposure. It is well known that immature or developing organisms are more susceptible to changes than are mature or fully developed ones. There is a varying degree of vulnerability for induction of structural defects in organs or systems throughout the total span of development, but the greatest susceptibility is during or before the early formative stages of that organ, which are called the "critical periods" in organogenesis. After organogenesis begins, malformation of specific organs or systems is produced with relative ease, reflecting special sensitivities and needs of rapidly differentiating and growing tissues. Most organs, however, have a period of particular susceptibility to extrinsic teratogenic influences during the early developmental events in that organ, although the total period of organ-specific sensitivity may extend over several days (Wilson, 1973a). Many organs show more than one susceptible period,

indicating the fact that their embryogenesis is multiphasic, with vulnerable stages and cell types at different times (Russell and Russell, 1954). The generally elevated sensitivity of most tissues during organogenesis also makes the embryo vulnerable to death if the dosage of the agent is at high teratogenic levels. As organogenesis advances to the later period of embryonic development, teratogenic susceptibility and embryo-lethality decrease, while transplacental carcinogenesis tends to increase. Susceptibility to transplacental carcinogenesis increases rapidly after the definitive organ rudiments have formed, at approximately day 11 of gestation in the mouse, and becomes maximal a few days prior to birth (Rice, 1979a). The essential feature of transplacental carcinogenesis is that target cells are, to a variable degree, morphologically and functionally immature (Rice, 1979b). The degree of tissue differentiation at the time of exposure to a carcinogenic agent and the genetics of the subject have been shown to affect susceptibility to neoplastic transformation and induction of a tumor. Some chemical agents, such as ethylnitrosourea (ENU), can act as teratogens and transplacental carcinogens depending upon the time of administration during gestation period. However, the exact nature of the phenomena that make these variable embryonic stages more vulnerable to teratogenesis and carcinogenesis has so far not been explained satisfactorily. This differentiation may provide a capacity of tissue to repair damage in DNA. No studies of DNA repair during embryonic development have been reported. Analysis of DNA damage and repair directly in embryonic tissues would contribute to an understanding of the correlation between the role of DNA repair and the production of birth defects.

2. Transplacental passage and embryonic/fetal accumulation of teratogenic agents are important. The placenta serves as a barrier behind which the embryo or fetus is protected from foreign agents. Many molecules of small size and low ionic charge cross the placenta by simple diffusion, others by active transport or by carrier-mediated transport (Waddell and Marlowe, 1981). Chemical agents or their degradation products usually reach the embryo or fetus in some fractions of their concentration in maternal blood depending upon many factors including maternal functional capacity to store the number of molecules in the plasma, the nature of the agent itself, and the degree of placental transfer. Various chemicals which reach the embryo or fetus in considerable amounts at all gestational stages show a characteristic distribution pattern (Dencker, 1978). During the period of organogenesis, the uptake is generally found to be higher in the neuroepithelial cells than in other tissues. Particularly in small rodents, many chemicals can easily reach the fetal nervous system because of the undeveloped blood brain barrier. Furthermore, their central nervous system is still undeveloped at birth. At midgestation, around day 13 to 15, the chemicals become more evenly distributed. Later, as the organs mature, a considerable difference in organ uptake occurs. However, difficulties in the evaluation of the results remain since all accumulations do not necessarily result in tissue damage.

Besides transplacental passage, some environmental insults can reach the developing embryos directly. Ionizing radiations can pass directly through maternal tissues without modification except minimal dosage reduction. The distribution of absorbed energy is relatively uniform in embryos following irradiation to the mothers. Radiation

results in congenital malformations, growth retardation and embryolethality (Brent, 1977). However, even ionizing radiation appears to have sufficient selectivity of action to cause localized damage so that embryos or fetuses can survive in spite of the damage. It is also very significant to consider that DNA repair mechanisms in embryonic or fetal tissues provide powerful, sensitive methods to protect the embryos or fetuses from genotoxic agents. Repair of DNA lesions may be an extremely important determinant of cell survival and cell response to any DNA damaging events including mutagenic, carcinogenic and teratogenic events. Thus, apparent selectivity for any of these biologic responses may reflect different levels of repair activity rather than simple differences in dose.

3. Mechanisms of teratogenesis are complex. Many teratological experiments have been performed for determining how causative agents might act upon developing cells or tissues to initiate abnormal development. These early events are designated as "mechanisms" because they are important, not only in initiating, but also in modulating subsequent events (Wilson, 1977). The molecular events which account for many of the early changes induced in developing systems are difficult to detect by methods used at the present time. However, Wilson (1977) and others (Fraser, 1961; Kalter, 1971) have summarized some mechanisms by which developing systems might respond to causative agents in ways that would lead to abnormal development. These include germ line and somatic cell mutations, chromosomal aberrations, mitotic interference, altered nucleic acid functions other than mutation, lack of precursors or substrates needed for biosynthesis of essential substances, compromised energy source, enzyme inhibition, osmolar imbalance and altered membrane

characteristics. Among these, interference with nucleic acids and their metabolism seems to be most important and has received more attention. The embryo during organogenesis is very vulnerable to biochemical changes that interfere with nucleic acid replication, transcription and translation. Furthermore, the small numbers of cells committed to specific organ development may represent a particularly critical target for somatic cell mutations. Of all species of cellular nucleic acid, DNA is most important because it contains the only complete copy of cellular information and it is presumed therefore to be a major target of the cell for environmental insults. Thus, special attention will be focused on DNA as a principal target in teratogenesis and transplacental carcinogenesis.

Much of the previous work has been concerned with the relationship between teratogenesis and the inhibition of DNA synthesis. Short et al. (1972) have reported the inhibition of in vivo DNA synthesis in mouse embryos following administration of a teratogenic dose of cyclophosphamide. Scott et al. (1971) have observed the association of DNA synthesis inhibition and cell death with hydroxyurea teratogenesis in rat embryos. Inhibition of DNA synthesis which may lead either to inhibition of cell proliferation or cell death is thought to be one of the causes of teratogenesis (Scott et al., 1971; Ritter et al., 1971). Other studies suggest that it is the cell death which often accompanies inhibition of DNA synthesis which is the actual cause of abnormality (Scott et al., 1973; Menkes et al., 1970; Ritter et al., 1973). Recently, Sadler and Kochhar (1976) reported that the rate of DNA synthesis in whole mouse embryo culture was significantly decreased in the presence of a teratogenic dose of chlorambucil. Brookes and Lawley

(1961) and other investigators (Wheeler, 1962; Kohn et al., 1966; Jolley and Ormerod, 1973; Lawley, 1966) have reported that the DNA molecule is the site of action of those drugs. They have shown in cell culture and cell-free system that bifunctional alkylating agents, chlorambucil and cyclophosphamide can crosslink DNA by forming a covalent linkage between two opposing guanine molecules. The formation of crosslink in the DNA molecule interferes with transcription while eventually may lead to cell death. It is now generally accepted that the cell-killing action of UV- and X-irradiation and various mutagens and cytotoxic chemicals is mediated by damage to DNA (Cleaver, 1970; Griggs and Bender, 1972; Roberts et al., 1974). The role played by mutation as a fundamental mechanism in teratogenesis has also been established, but few experimental investigations have been reported. In fact, somatic cell mutations have been assigned a minor role (Wilson, 1977). Attempts to demonstrate how a drug-induced mutation could disrupt developmental processes, leading to abnormal morphogenesis, have been carried out by using the thymidine analogue, 5-bromodeoxyuridine. BUdR is a mutagen, teratogen, and carcinogen, and at proper concentration, it can inhibit or reverse differentiation without apparent cytotoxicity (Goz, 1978). In in vitro studies, BUdR has been shown to inhibit differentiation as reflected both in cellular morphology and in the synthesis of specialized cell products characteristic of differentiated cells (Stockdale et al., 1964; Rutter et al., 1975; Agnish and Kochhar, 1976). Almost all of these effects are derived from the action of BUdR through its incorporation into DNA. The mutation caused by this agent is due to the incorporation of BU in place of thymine in replicating DNA, causing mispairingof BU with guanine rather than with adenine. The more recent

in vivo study indicated that BUdR did not cause fetal malformations by inhibiting or altering differentiation of embryonic cells, but rather by its growth retarding and cytotoxic effects (Bannigan and Langman, 1978). Therefore, it seems reasonable that DNA-damaging lesions could be the initial event leading to cell death as well as mutations with resulting changes in cell behavior, and that both of these factors play important roles in causing birth defects.

Many alkylating agents are known to be potent teratogens and transplacental carcinogens. Attempts to correlate the extent of DNA alkylation with the teratogenic and transplacental carcinogenic effects of these agents have been extensive. Bochert *et al.* (1978) found no correlation between teratogenicity and the formation of N^7-methylguanine and N^3-methyladenine in mouse embryonic DNA treated with MNU and MMS. They suggested a working hypothesis that the formation of O^6-alkylguanine is responsible for the induction of malformations during pregnancy, but this still has not been proven. In a number of investigations, the major product of DNA alkylation, N^7-alkylguanine, has been shown to be nonmutagenic and is probably not responsible for tumor induction (Schoental, 1969; Swann and Magee, 1968, 1971; Kleihues and Magee, 1973). In contrast to the N^7-alkylguanine, O^6-alkylguanine which is a minor alkylation product has been suggested to be correlated with mutagenesis and carcinogenesis by a mispairing mechanism (Loveless, 1969). There is an indication that the capacity of different tissues to remove O^6-alkylguanine from DNA may correlate with the organ-specific carcinogenic effect of certain alkylating agents. In the case of ENU-induced transplacental carcinogenesis, a high incidence of nervous system tumors has been found after a single administration of ENU given to rats during the

perinatal period (Ivankovic and Druckery, 1968). This tissue specificity of the carcinogenic effect of ENU is of particular interest because the formation of the ultimate reactant, an ethyl cation, occurs non-enzymatically under physiological condition and, hence, is not tissue specific. It has been demonstrated that the initial degree of base ethylation in the DNA of different tissues does not provide an explanation for the nervous system-specificity of ENU (Goth and Rajewsky, 1972, 1974b). Instead, there is a significant difference between brain and other tissues with regard to the elimination rate of O^6-ethylguanine from DNA. O^6-ethylguanine is removed from brain DNA much more slowly than from liver DNA or from the DNA of other pooled tissue in 10-day-old rats exposed to ENU (Goth and Rajewsky, 1974a, 1974b). The observed selective persistence of O^6-ethylguanine in brain DNA, together with the high rate of DNA replication, may be an important factor in determining the probability of neoplastic transformation.

As in the case of the other related compound, it has been shown that MNU also induces neurogenic tumors in rats following transplacental treatment (Alexandrov, 1969). A low capacity for the elimination of O^6-methylguanine from DNA has been found to correlate with the organ-specific effects of MNU. After a single injection of MNU in adult rats, O^6-methylguanine is removed more slowly from DNA of the brain than from DNA of the liver (Kleihues and Margison, 1974). The brain is the principal target organ in MNU carcinogenicity, whereas the liver is not susceptible. There is no such established relationship between the persistence of O^6-methylguanine and the location of tumors produced by MNU observed in experiments with mice. Two strains of mice, A/J and C_3HeB/FJ, which differ considerably in their susceptibility to the

neuro-oncogenic effect of MNU, were chosen for a comparison of the rates of loss of O^6-methylguanine from cerebral DNA. No differences were detected in cerebral excision capacity and furthermore, both strains of mice are susceptible to hepatic tumor induction by MNU, but lost O^6-methylguanine from liver DNA at significantly different rates (Bücheler and Kleihues, 1977). It was therefore suggested that the location of tumors does not depend only on the formation and persistence of this product in DNA. However, to some extent, O^6-alkylation of guanine base does play an important role in carcinogensis.

Phosphotriesters constitute one of the principal products of the reaction of DNA with alkylating agents including MNU and ENU. Analyses of DNA alkylated in vitro by MNU (Lawley, 1973) and ENU (Sun and Singer, 1975) have shown that 20% and 70% of the total products of alkylation, respectively, are phosphotriesters. The observations on the stability of phosphotriesters in DNA in vivo suggest that these lesions are chemically stable and that they are not excised by cellular DNA-repair systems (Shooter and Slade, 1977; Shooter et al., 1977). In long-term feeding experiments with dimethylnitrosamine and diethylnitrosamine, significant concentrations of methyl and ethyl phosphotriesters can be found in DNA of liver after the course of a year of treatment (Shooter, 1978). This indicates that the accumulation of these lesions in tissues may be used as a measure of damage to DNA in long-term carcinogenesis studies. At the present time, no direct involvement of phosphotriesters in DNA in the process of carcinogenesis has been reported.

4. DNA repair may be important in both teratogenesis and transplacental carcinogenesis. A number of observations suggest that DNA repair plays an important role in determining the frequency of birth

defects. X-rays and multifunctioning alkylating agents, which produce repairable DNA damage, are the most predictable producers of birth defects (Wilson, 1973b). Studies have reported that inhibitors of DNA repair, particularly chloroquine and caffeine, can enhance the teratogenic effects of X-rays and certain alkylating agents (Yielding et al., 1976; Fujii et al., 1976; Nomura, 1977). Yielding et al. (1976) have proposed the mechanism of coteratogenesis by which some agents enhance the teratogenicity of known teratogens by inhibiting DNA repair processes. Repair of teratogenic insults during the period of organogenesis has generally been viewed in terms of tissue regeneration or of restorative proliferation of surviving cells to replace dead cells. Recently, Bochert et al. (1978) observed that the persistence of O^6-methylguanine in DNA of embryonic tissue following MNU and MMS treatment to pregnant mice on day 12 of gestation is higher than in maternal liver DNA but less than maternal brain DNA. Maternal liver and brain have been used as marker tissues with suggested high and low repair activity, respectively. Malformations of the extremities have been seen when MNU and MMS were administered to mice on this day of gestation. Although this experiment was performed by using whole embryonic tissues instead of particular organs that undergo malformation after exposure to teratogens, it at least showed a possible correlation between slow rates of excision of O^6-alkylguanine in embryonic tissues and the production of malformations.

A similar relationship between the persistence of O^6-ethylguanine and the development of tumor in animal organs exposed transplacentally to a direct-acting alkylating agent, ENU, has been reported by Goth and Rajewsky (1974b). They observed that only neuroectodermal neoplasms in

the central and peripheral nervous system developed after a single dose of ENU given to rats during the perinatal period. They showed that O^6-ethylguanine was removed from the brain DNA at a very much slower rate than from other tissues. They concluded that this slow rate of excision combined with the relatively high rate of DNA replication which takes place in the developing brain could account for the increased probability of neoplastic conversion. Evidence of a strong correlation between the accumulation (lack of repair) of O^6-alkylguanine in the DNA of certain tissues and their susceptibility to tumor production by many carcinogens has been demonstrated (Kleihues and Margison, 1974; Margison and Kleihues, 1975; Nicoll et al., 1975). Although there are some studies arguing against this correlation (Margison et al., 1977; Craddock, 1975; Rogers and Pegg, 1977; Bücheler and Kleihues, 1977), the possibility that DNA repair plays a role in carcinogenesis and probably in teratogenesis still exists and requires further investigations.

In summary, a variety of DNA-damaging agents has been shown to be potent teratogens and transplacental carcinogens. Although it has not been established with absolute certainty that the interaction of these agents with DNA is causally related to the induction of tumors or malformations, there is considerable evidence in support of this concept. The process whereby the embryos cope with teratogenic insults is fundamental to understanding the mechanisms of teratogenesis. A deleterious response may occur only after the defense mechanisms are overwhelmed. Therefore, analysis of the capacity of embryonic cells to repair lesions in DNA during development would be of great interest and might supply a sensitive approach for establishing that there is a

relationship between DNA damage, its repair and teratogenesis. The *in vivo* study of DNA damage and repair in developing embryos was conducted to test this hypothesis.

MATERIALS AND METHODS

Two groups of studies were performed in this research: 1) the more general biological studies; and 2) the specific biochemical studies. In the biological studies, the maldevelopment of tissues or organs induced transplacentally by DNA-damaging agents was observed, and the mechanisms by which these agents reacted with cellular DNA of specific tissues or organs were investigated in the biochemical studies. The experimental procedures will be described in detail in this section. Table I provides a description of the solutions used.

BIOLOGICAL STUDIES

A. Animals

Groups of SAF/ICR male and female mice (Southern Animal Farms, Prattville, AL), 8 to 10 weeks old, were used in these studies. All animals were allowed Purina Mouse Chow and fresh tap water ad libitum. Females were mated with males (2 females to 1 male) during 4 hour period from 2 a.m. to 6 a.m., the females then were separated and examined immediately for the presence of a vaginal plug. The beginning hour of the mating period was considered as 0 hour for timing developmental stages of embryos. About 80% of vaginally plugged mice actually became pregnant.

B. Teratological experiments

These experiments were designed to determine at what times after treatment with the DNA-damaging agents, X-rays and cytoxan, abnormalities of specific organs or tissues could be observed. It was then determined whether chloroquine, an inhibitor of DNA repair, could enhance the teratogenic effects of these agents. In these studies, pregnant mice were treated during the period of organogenesis from day 6 1/2 to 10 1/2. A minimum of 9 animals was used for each experimental group. For X-irradiation, pregnant females received whole body X-irradiation at a dosage of 200 rads. A Picker Cobalt 60 was used as an X-irradiation source, giving a dose rate of 98.4 rads/min at a target distance of 30 cm. Cytoxan (Mead Johnson) was dissolved in sterile water and used immediately. Cytoxan was injected intraperitoneally into animals at 10 mg/Kg body weight. Chloroquine, 25 mg/Kg, was given as a single intraperitoneal injection in 0.1 ml of normal saline 3 hours before X-irradiation or cytoxan administration. Treatment groups consisted of pregnant females, at different gestational days, given with the following: (1) saline only; (2) chloroquine only; (3) saline and X-irradiation; (4) chloroquine and X-irradiation; (5) saline and cytoxan; and (6) chloroquine and cytoxan.

All females were sacrificed by cervical dislocation 18 days after the appearance of vaginal plugs. The uterine contents from these females were removed, and the fetuses were recorded as being alive, dead or resorbed. The live fetuses were weighed and examined for gross abnormalities under a dissecting microscope.

C. Transplacental exposure to methylnitrosourea

These experiments were designed to study the effects on mouse offspring of MNU administered transplacentally late in gestation, and to determine whether some specific inhibitors of DNA repair could affect those MNU effects. Treatment late in gestation assures completion of organogenesis except for the eye and certain other parts of central nervous system. It is possible then to study specific and limited developmental defects in fetuses which otherwise are viable. Groups of SAF/ICR pregnant mice, 16 days of gestation, were used in these studies. MNU (K and K Chemicals) at different doses, 5, 20, or 50 mg/Kg, was prepared freshly and given to pregnant animals as listed below. Chloroquine (25 mg/Kg) or INH (50 mg/Kg) was given to some groups of animals immediately after MNU treatment. All chemicals were dissolved in normal saline and given as a single intraperitoneal administration not exceeding 0.1 ml in volume. Experimental animals were treated as follows: (1) 5 mg/Kg MNU; (2) 20 mg/Kg MNU; (3) 50 mg/Kg MNU; (4) 5 mg/Kg MNU and chloroquine; (5) 20 mg/Kg MNU and INH; (6) chloroquine only; and (7) INH only.

One day prior to delivery, pregnant mice were placed in separate cages and were allowed to rear their young. The offspring were weaned at 3 weeks. At the age of 3 to 6 weeks, the mouse offspring were sacrificed by cervical dislocation, the visceral organs and the eyes were removed and fixed in 10% buffered formaldehyde solution. These organs were examined for gross abnormalities. After suitable fixation, the eyes were dehydrated through series of 70%, 95%, and absolute ethanol, cleared in xylene and embedded in paraffin. Sections were cut at 8 μ and stained with hematoxylin and eosin. For the examination of

the retina, sagittal sections of the eyes through or close to optic nerves were used.

BIOCHEMICAL STUDIES

A. Animals

Groups of CD-1 male and female mice (Charles-River Breeding Laboratories, Wilmington, MA), 8 to 10 weeks old, were used in these experiments. Females were caged with males (2 females to 1 male) for 15 hours from 5:00 p.m. to 8:00 a.m., then the females were separated and examined for the presence of a vaginal plug. The 24 hour period beginning approximately at the middle of the mating period was considered as day 0 of gestation. Pregnant mice at 10 1/2, 11 1/2, and 12 1/2 days of gestation were used because these were days of high sensitivity to teratogenesis by DNA-damaging agents. All animals were maintained on Purina Mouse Chow and water.

B. Treatment of animals with DNA-damaging agents

Pregnant mice were injected intraperitoneally on the selected days of gestation with 500 μCi/Kg ^{3}H-thymidine. One and a half hour after ^{3}H-thymidine administration, during which extensive labeling of cells was achieved, the mice were treated with DNA-damaging agents, including X-rays, MMS, and MNU. The X-irradiation was given as a single whole-body exposure at 600 rads or 235 rads. Both MMS and MNU were dissolved in normal saline immediately before administration. In most cases, MMS was given as a single intraperitoneal injection at 25 mg/Kg, but in some cases 50 or 100 mg/Kg MMS was used. MNU at a dose of 20 mg/Kg was

given as a single intraperitoneal administration. Control animals received only ^{3}H-thymidine without DNA-damaging agents.

C. Administration of inhibitors of DNA repair for biochemical experiments

Chloroquine. In studies with the effect of chloroquine (a DNA excision repair inhibitor) on MMS-induced DNA damage and repair, chloroquine (Sigma) was given as a single intraperitoneal injection at 25 mg/Kg in 0.1 ml normal saline immediately or 30 minutes after MMS treatment.

Caffeine. For studies of the effect of caffeine (reported to inhibit postreplication repair (Roberts et al., 1974) on MNU-induced DNA damage and repair), caffeine (Eastman Organic Chemicals) at 50 mg/Kg in 0.1 ml normal saline was given intraperitoneally as a single dose or as 2 doses at different times after MNU treatment. A single dose of caffeine was given immediately, 1 hour or 2 hours after MNU treatment. Two doses of caffeine were given 2 hours and 5 hours after MNU administration, respectively.

Isonicotinic acid hydrazide. In studies with the effect of INH (reported to inhibit postreplication repair on MNU damage (Klamerth, 1978)) on MNU-induced DNA damage and repair), INH (Sigma) at 50 mg/Kg in 0.1 ml normal saline was given intraperitoneally as a single dose at 30 min after MNU treatment or as 2 doses at 2 hours and 5 hours after MNU injection, respectively.

D. Isolation of embryonic tissues

After varying experimental periods of time, the mothers were sacrificed by cervical dislocation. The embryos were removed from the

uterine horns and placed in a petri dish containing cold PBS for tissue homogenization, or placed in a petri dish containing PBS at room temperature for tissue trypsinization. Under a dissecting microscope, the embryos were dissected free from their membranes by using 2 pairs of forceps. Care was taken not to damage the embryos. To remove the whole embryonic liver, 2 pairs of forceps were used to open the abdomimal wall and to clear other tissues attached to the liver. The liver then was carefully removed and placed in a test tube containing PBS. The limb buds of the embryos were pinched off by a pair of forceps and placed in a test tube containing PBS. Cold PBS or PBS at room temperature was used depending upon the methods of cell disaggregation to be used.

E. Tissue homogenization

The whole embryonic tissue of 10 1/2-day-old mouse embryos and the liver tissue of 12 1/2-day-old embryos were used for tissue homogenization. The tissue to be studied was washed twice in cold PBS. Three embryos or the liver tissue from 6 embryos were homogenized in 1 ml cold PBS using a Ten Broeck homogenizer, clearance 0.005-0.007 inches. The tissue was homogenized with 5 strokes. The homogenate was filtered through nylon mesh in order to remove clumps of unbroken tissue and connective tissue. The filtrate was kept in an ice bath for the analysis of nucleoid sedimentation.

F. Tissue trypsinization

The whole embryonic tissue of 10 1/2-day-old embryos, the liver tissue of 12 1/2-day-old embryos and the tissue of limb buds of 11 1/2- or 12 1/2-day-old embryos were used for tissue trypsinization. The

tissue to be studied was washed twice in PBS at room temperature, and placed in a test tube containing trypsin-EDTA solution (Flow Laboratories). Five embryos, 6 to 8 embryonic livers, or all limb buds of the entire litter were incubated in 3 ml trypsin-EDTA solution at 37°C for 30 min with occasional agitation. After incubation, the mixture was pipetted up and down to dissociate cells, then filtered through nylon mesh. The filtrate was centrifuged for 5 min at 4000 rpm, the supernatant was discarded and the pellet was washed twice with 2 ml cold PBS, followed by centrifugation for 5 min at 4000 rpm. The final pellet of cells was suspended in 0.25-0.5 ml cold PBS in order to get 4×10^6 to 1×10^7 cells per ml, and gently pipetted up and down to break up clumps. Cell counts were performed using trypan blue stain (0.4 g in 100 ml of 0.9% NaCL) and a hemocytometer. The use of trypan blue was based on the ability of viable cells to exclude the dye. Therefore, both total and viable cell counts were obtained by this method. The cell suspension was then kept in an ice bath for nucleoid sedimentation analysis.

G. Neutral sucrose gradient sedimentation analysis

Changes in density of nuclear DNA particles were measured by sedimentation velocity in neutral sucrose gradients. The procedure was based upon that of Cook and Brazell (1975) with some modifications. Gradients of 15 to 30% sucrose (Schwarz/Mann), 4.2 ml, were prepared over a 0.5 ml shelf of 60% sucrose in 1/2 x 2" cellulose nitrate tubes by using a Buchler density gradient maker. When studying the sedimentation response of nucleoids to ethidium bromide, the drug was added to sucrose gradients at concentrations varying from 4 to 20 µg/ml. A

layer of 150 µl lysing solution was added carefully to the surface of the gradients immediately prior to addition of the cell suspension, 50 µl containing 2 to 5 x 10^5 cells. Another aliquot of 50 µl lysing solution was layered gently on the top of the cell suspension to ensure complete lysis. The cells were allowed to lyse for 15 min at room temperature and another 15 min at 20°C. The tubes were centrifuged at 20°C using an SW 50.1 rotor in a Beckman L2-65b ultracentrifuge at 30,000 rpm for 30 min. After centrifugation, fractions of 8 drops each were collected on Whatman #1 chromatography paper strips. The papers were allowed to dry, then submerged successively in jars of 5% cold trichloroacetic acid (TCA) and cold 95% ethanol. After drying, the paper strips were cut, then each piece was placed in a glass vial containing 3 ml of counting cocktail 302a (Research Products International Corp.), and the radioactivity was counted with a Beckman scintillation counter. Data were expressed as percent of total radioactivity.

TABLE I

SOLUTIONS

15 or 30% sucrose solution (pH 8.0)

For 100 ml

1.95 M NaCl (11.31 g)

0.001 M EDTA (0.034 g)

0.01 M Tris-base (0.121 g)

15 or 30% sucrose (15 or 30 g)

Lysing solution (pH 8.0)

For 100 ml

2.6 M NaCl (15.08 g)

0.133 M EDTA (4.482 g)

0.003 M Tris-base (0.032 g)

0.67% Triton X-100 (0.67 ml)

Phosphate buffered saline (pH 7.2)

For one liter

0.14 M NaCl (8.0 g)

0.003 M KCl (0.2 g)

0.008 M Na_2HPO_4 (1.15 g)

0.001 M KH_2PO_4 (0.2 g)

RESULTS AND DISCUSSION

BIOLOGICAL EXPERIMENTS

A. Effects of X-rays on developing mouse embryos

A single exposure of X-rays, 200 rads, to groups of pregnant SAF/ICR mice on gestational days 6 1/2 through 10 1/2 resulted in increased numbers of resorptions and a variety of teratogenic effects. As illustrated in Table II, days of treatment may be selected to produce abnormalities of specific tissues or organs in characteristic high frequency. Treatment on days 7 1/2 and 8 resulted in a marked increase in numbers of resorptions. Resorptions refer to early post-implantation deaths. Dead implants are represented as "deciduomata" which actually are endometrial tissue stimulated into growth by the eggs which were about to implant but have died without trace (Bateman, 1973). These deciduomata are visible as small, brown masses containing necrotic tissue and fibrin clots interspersed between live fetuses. Exencephaly was observed in a high level when X-irradiation was administered on days 7 1/2 through 8 1/2. Supernumerary digits were frequent anomalies of the limbs. They were identified as an excess number of digits with an extra tiny digit usually protruding from the lateral side of the paw. A high incidence of this defect occurred when the pregnant mice were exposed to X-rays on days 8 1/2 and 9. Other limb abnormalities such as ectrodactyly, syndactyly, clubbed feet, also occurred but at lower

TABLE II

DEFECTS WHICH OCCUR IN TYPICAL HIGH FREQUENCY FROM X-IRRADIATION (200 RADS) OF MOUSE EMBRYOS ON SELECTED DAYS OF GESTATION.

Day of Treatment	Frequency of Abnormalities
7 1/2 and 8	Resorptions (56.2%)
7 1/2 through 8 1/2	Exencephaly (13.7%)
8 1/2 and 9	Supernumerary digits (15.6%)
10 and 10 1/2	Cleft palate (21.0%)
10 and 10 1/2	Tail abnormality (23.6%)

Groups of pregnant mice were treated with 200 rads X-rays on days 6 1/2 through 10 1/2 of gestation. Pregnancies were terminated on the 18th day, and the fetuses were examined for gross abnormalities. The numbers in parentheses represent the average percentages of resorptions and other malformations observed in the minimum of 9 litters. The calculations of all abnormalities were based on fetuses affected per total number of live fetuses, except for resorptions, for which the calculation was based on the number of resorptions per total number of fetal implantations. In untreated control mice, all abnormalities except resorptions were apparent less than 1.0%. The occurrence of resorptions was about 20.0%.

frequency. Cleft palate and tail abnormalities were observed at high levels when the mothers were X-irradiated on days 10 and 10 1/2. Abnormal tails were noted as kinky tails or unusually short tails. In all embryos exposed to X-irradiation from days 6 1/2 through 10 1/2, body weights were reduced from normal. This is in agreement with previous

observations (Brent, 1977) that at sufficiently high doses, intrauterine growth retardation can result from radiation exposure delivered any time during gestation after the time of implantation. Growth retardation is closely associated with teratogenesis. In groups of irradiated embryos that exhibit major congenital anomalies, there also is a concomitant reduction in weight and various physical measurements at term. The results obtained in these SAF/ICR mice support those reported previously (Russell and Russell, 1952) that abnormalities are produced in great abundance by irradiating during the period of major organogenesis, and that there are very clearly defined critical periods for almost all the abnormalities obtained.

B. Effects of cytoxan on developing mouse embryos

A single intraperitoneal injection of cytoxan, a radiomimetic alkylating agent, to groups of pregnant SAF/ICR mice on gestational days 6 1/2 through 10 1/2 in a dosage of 10 mg/Kg also produced a great variety of abnormalities. Characteristic high frequency of abnormalities of specific tissues or organs on selected days of treatment was illustrated in Table III. A high increase in number of resorptions occurred when the drug was administered on days 8 1/2 and 9. Treatment on day 8 resulted in a high incidence of exencephaly. Tail abnormalities were noted in animals treated on day 9 1/2. Cleft palate and open eyes were apparent with high frequencies from treatment on days 9 1/2 and 10. As in the case of X-ray teratogenesis, cytoxan resulted in decreased growth rates of all fetuses treated on days 6 1/2 through 10 1/2. At the same dosage of cytoxan, 10 mg/Kg, Gibson and Becker (1968) observed an increase in resorptions and a decrease in growth

TABLE III

DEFECTS WHICH OCCUR IN TYPICAL HIGH FREQUENCY FROM CYTOXAN TREATMENT (10 mg/Kg) OF MOUSE EMBRYOS ON SELECTED DAYS OF GESTATION.

Day of Treatment	Frequency of Abnormalities
8	Exencephaly (7.1%)
8 1/2 and 9	Resorptions (32.0%)
9 1/2	Tail abnormality (8.9%)
9 1/2 and 10	Cleft palate (13.8%)
9 1/2 and 10	Open eyes (14.0%)

Groups of pregnant mice were treated with 10 mg/Kg cytoxan on days 6 1/2 through 10 1/2 of gestation. Pregnancies were terminated on the 18th day, and the fetuses were examined for gross abnormalities. The number in parentheses represent the average percentages of resorptions and other malformations observed in the minimum of 9 litters. The calculations of all abnormalities were based on fetuses affected per total number of live fetuses, except for resorptions, for which the calculation was based on the number of resorptions per total number of fetal implantation. In untreated control mice, all abnormalities except resorptions were apparent less than 1.0%. The occurrence of resorptions was about 20.0%.

rates but they found no discernible anomalies of other tissues or organs in Swiss Webster mice. The dosage required to induce the fetal anomalies in Swiss Webster mice was 20 mg/Kg which was higher than the dosage required in SAF/ICR strain. Thus, the SAF/ICR mice appear to be more sensitive to the teratogenic effects of cytoxan.

As illustrated here, the results of X-ray and cytoxan teratogenesis have confirmed other observations that the extent and types of fetal anomalies are dependent upon the gestational day on which these agents are administered. Both X-rays and cytoxan are DNA-damaging agents, X-rays induce single-strand breaks in DNA whereas cytoxan is known to produce alkylated bases and cross-linkages in DNA either between adjacent areas of one strand or by joining together opposite strands of the helix (Brookes and Lawley, 1961). DNA is the genetic material for all higher organisms, and intracellular alterations of this information system can result in severe damage and abnormality or death of cells. Since both of these agents are potent teratogens, it is possible that these agents cause gene damage that is phenotypically expressed as gross embryonic abnormalities. Such damage is normally subject to repair mechanisms, but if repair inhibitors are also present in the system, the efficiency of maintaining the gene integrity is decreased so that more damage persists.

C. Effects of chloroquine on X-ray and cytoxan teratogenesis

Chloroquine, an antimalarial drug which has been reported to inhibit DNA excision repair (Gaudin et al., 1972a; Michael and Williams, 1974), was used in conjunction with X-rays or cytoxan. Cytoxan and X-rays produced high frequencies of many abnormalities when the animals were treated on days 10 and 10 1/2, respectively. Chloroquine at the dosage of 25 mg/Kg was given 3 hours before cytoxan and X-ray administrations to pregnant mice on gestation days 10 and 10 1/2, respectively. The influence of pretreatment with chloroquine on the frequencies of gross abnormalities induced by X-rays and cytoxan is shown in Table IV.

TABLE IV

EFFECT OF CHLOROQUINE ON X-RAY AND CYTOXAN TERATOGENESIS

Abnormality	Saline	Chloroquine	X-ray + Saline	X-ray + Chloroquine	Cytoxan + Saline	Cytoxan + Chloroquine
Cleft palate	0/90	0/70	7/70 (10.0%)	18/90 (20.0%)[A]	18/157 (11.5%)	31/98 (31.6%)[B]
Tail abnormality	0/90	0/70	9/70 (12.9%)	41/90 (45.6%)[B]	4/157 (2.5%)	5/98 (5.1%)
Open eyes	0/90	0/70	0/70	0/90	22/157 (14.0%)	22/98 (22.4%)[A]

A = Significance at $p \leq 0.05$
B = Significance at $p \leq 0.01$

Groups of pregnant mice were treated with 200 rads X-rays or 10 mg/Kg cytoxan on days 10 1/2 and 10 of gestation, respectively, with and without added treatment with chloroquine. Pregnancies were terminated on the 18th day, and the fetuses were examined for gross abnormalities. The numbers in the categories of abnormalities represent the numbers of malformed fetuses per total number of live fetuses.

Chloroquine enhanced significantly the teratogenic effects of X-rays expressed as cleft palate and tail abnormalities, and the frequencies of cleft palate and open eyes caused by cytoxan. The significance of levels of enhancement was evaluated by using the binomial expansion method of Goldstein (1964). All calculations were based on the numbers of fetuses affected per total number of live fetuses. Control experiments showed that saline and chloroquine alone produced no gross abnormalities.

The present data showed that concurrent administration of chloroquine with X-rays or cytoxan increased significantly the incidence of abnormalities. Chloroquine was also found to decrease the survival rate in X-ray-treated adult mice (Gaudin et al., 1972a), to increase the cytotoxic effect of cyclophosphamide (Kovacs and Steinberg, 1972), and to increase the toxicity of MMS damage to mammalian cells in culture (Michael and Williams, 1974). Preliminary observations on the effect of chloroquine on neutron irradiation teratogenesis (Yielding, L. W., unpublished data) showed that there was no synergistic effect of the drug on teratogenesis. The damage from neutron irradiation is largely non-repairable due to the extensive DNA double-strand breaks (Ritter and Cleaver, 1977). From these previous observations, it was concluded that the enhancing effect of chloroquine on the effectiveness of those DNA-damaging agents could be a consequence of the inhibition of repair. The results observed here, therefore, suggest that DNA repair may be important to the teratogenic effects of X-rays and cytoxan. At the present time, the roles of DNA repair processes in birth defects have not been determined. Repair of teratogenic insults has generally been regarded in terms of tissue regeneration or of restorative hyperplasia of surviving cells to replace dead cells. Thus, the analysis of the

capacity of embryonic cells to repair lesions in DNA during development may contribute to an understanding of the mechanisms of teratogenesis.

It is well known that the fetal and neonatal periods of development are states of generally high susceptibility to carcinogens. Many chemical carcinogens, including cytoxan, require enzyme-mediated metabolism for conversion to a chemically reactive derivative, or ultimate carcinogen, in order to induce neoplastic transformation. Since the enzyme systems required for these transformations are present at low levels or not at all in fetal rodent tissues until shortly before birth (Klinger and Muller, 1976), the ultimate metabolite must be found in maternal or placental tissues and must be sufficiently stable to reach the fetus. Therefore, in studying the transplacental effect of carcinogens, cytoxan seems not to be satisfactory, whereas enzyme-independent agents such as direct-acting alkylating agents are more effective. Methylnitrosourea, a direct-acting alkylating agent, is a potent mutagen, teratogen, and carcinogen. This compound has been found to be interesting because its action is independent of metabolic activation. MNU is a very short-acting agent (lifetime *in vivo* about 20 min) (Bochert *et al.*, 1978), it is uniformly distributed throughout the fetal organism shortly after transplacental administration. This substance decomposes spontaneously *in vivo* and yields an electrophilic residue which reacts readily with biological macromolecules, particularly DNA. It is likely that MNU produces its mutagenic, teratogenic and carcinogenic effects through its alkylation products. MNU methylates many sites of DNA *in vivo*. Of these alkylation products, O^6-methylguanine has been suggested to play an important role in carcinogenesis; since the ability of target tissues to remove this product is less than that of non-target tissues (Kleihues

and Margison, 1974). In addition to O^6-methylguanine, phosphotriesters now have received more attention. Since these lesions are chemically stable and they are not excised by cellular DNA-repair systems (Shooter and Slade, 1977; Shooter et al., 1977), it has been suggested that the accumulation of these lesions in tissues may be used as a measure of the extent of damage to DNA in long-term carcinogenesis study (Shooter, 1978).

D. Transplacental effects of methylnitrosourea

The effects on mouse offspring of administering MNU in different doses to pregnant SAF/ICR mice on day 16 of gestation are presented in Table V with data referring to incidence of abnormalities. The abnormalities found in these offspring observed at 3 to 6 weeks of age included lens opacity, lung nodules, cystic kidneys, and retinal dysplasia. The frequency of induction of abnormalities was clearly dependent on the dose of MNU except for the lens lesion which showed no difference between the 2 higher dose levels of MNU, 20 mg/Kg and 50 mg/Kg. The striking finding was the occurrence of retinal dysplasia since the nervous system is not a primary target in MNU carcinogenicity in mice. Retinal dysplasia is a maldevelopment of retina characterized by the formation of rosettes which consist of complicated infoldings of the outer part of the retina so that their lumina are bounded by the external limiting membrane through which project the rods and cones (Lahav et al., 1973). At a lower dose, 5 mg/Kg, MNU resulted in mild or moderate degree of retinal defects. Few rosettes were found in the photoreceptor layer, particularly in the anterior retina, while the cytoarchitecture of the remaining retina appeared normal (Fig. 2).

TABLE V

TRANSPLACENTAL EFFECTS OF METHYLNITROSOUREA ON MOUSE OFFSPRING

Dose	Retinal Dysplasia	Lens Opacity	Lung Nodules	Cystic Kidneys
5 mg/Kg	7/36 (19.4%)	0/36	2/36 (5.6%)	0/36
20 mg/Kg	72/77 (93.5%)	4/77 (5.2%)	41/77 (53.2%)	43/77 (55.8%)
50 mg/Kg	13/13 (100.0%)	4/83 (4.8%)	52/83 (62.7%)	78/83 (94.0%)

Groups of pregnant mice were treated with methylnitrosourea in different doses on day 16 of gestation. The offspring were killed at the age of 3 to 6 weeks, and examined for macroscopic and microscopic lesions. The numbers in the categories of lesions represent the numbers of affected offspring per total number of offspring observed. In untreated control mice, no abnormalities had been found.

At higher doses, 20 mg/Kg and 50 mg/Kg, the cytoarchitectural lesions of MNU-treated retina were most pronounced. The retina was severely atrophic with many rosettes over the entire retina (Fig. 3). The normal appearance of mouse retina is seen in Fig. 1. Retinotoxic effects induced by MNU have also been observed in Syrian hamsters (Herrold, 1967) and in rats (Murthy et al., 1972), but the retinal changes are not similar to those observed in these mouse studies. No retinal

Figure 1: Photomicrograph of the normal retina of 5-week-old mouse (320X). Note the clearly visible cell layers of the retina.

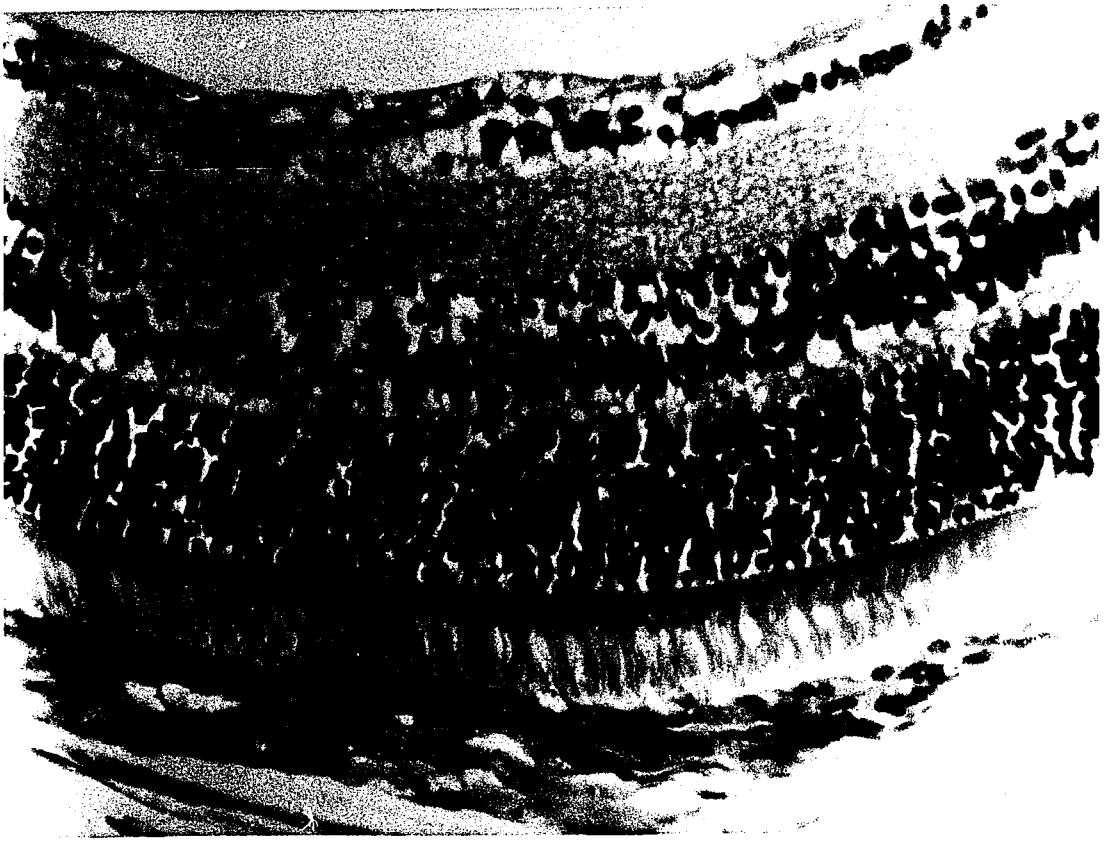
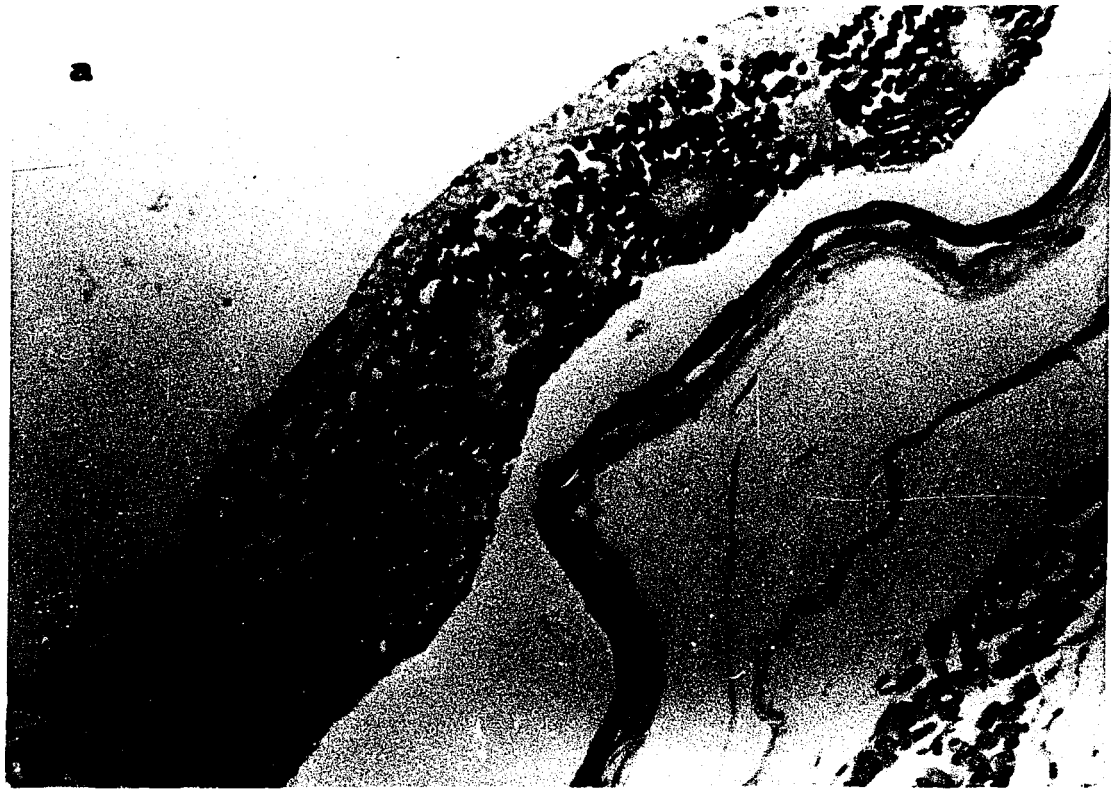
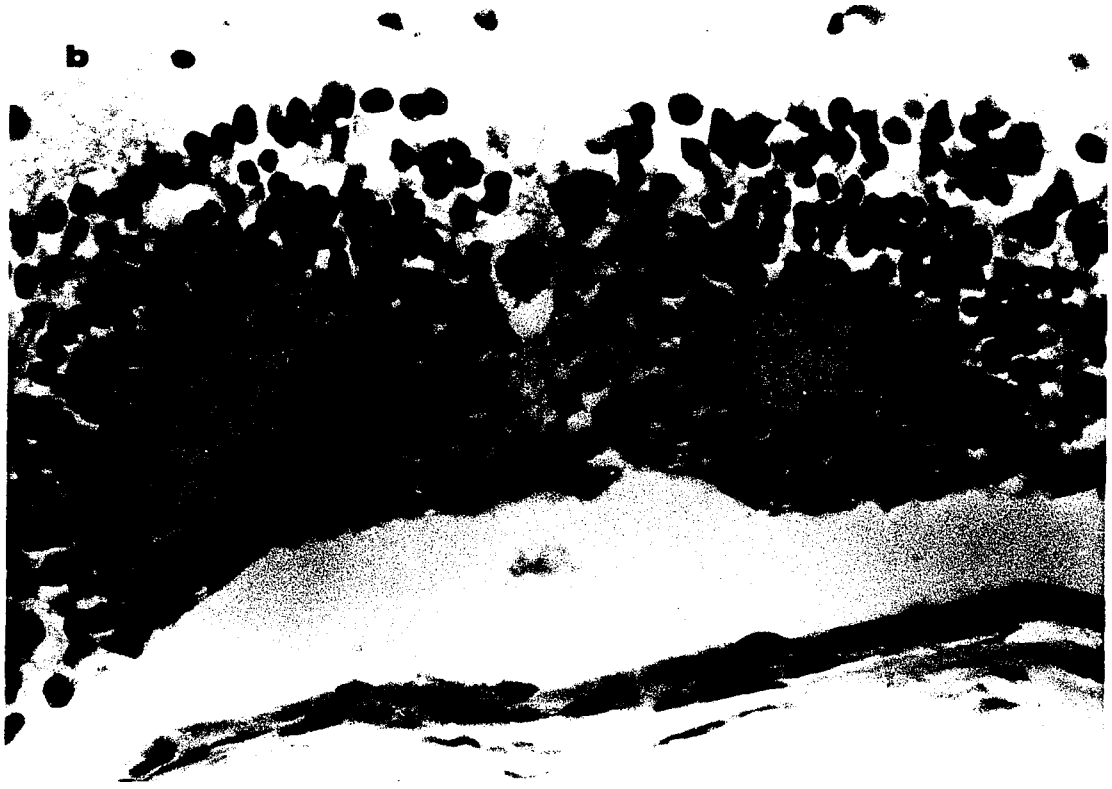


Figure 2: Photomicrograph of the retina from the mouse treated with 5 mg/Kg methylnitrosourea on day 16 of intrauterine life, and sacrificed at 5 weeks of age (320X). Note a few rosettes formed in the outer nuclear layer, while the cytoarchitecture of the remaining retina appears normal.

Figure 3: a) Photomicrograph of the retina from the mouse treated with 20 mg/Kg methylnitrosourea on day 16 of intrauterine life, and sacrificed at 5 weeks of age (320X). Note the marked atrophy and extreme dysgenesis of the retina with many rosettes scattered over the entire retina.

b) The higher power (800X) shows that the cells of the outer nuclear layer are arranged in a radiating formation about a central lumen, through which project the rods and cones, to form a rosette.


a


b

rosettes were found in those animals which were treated after weaning. It appears therefore that MNU, when administered prenatally to mice, can produce selective damage to the retinal elements.

The development of the eyes in mice is later than other organs. At the last stage of development (day 16 to birth), mouse fetuses are nearly mature. The differentiation of most organs is complete, but not the lens and especially not the retina. During this period and in early postnatal life, active cellular proliferation is taking place in the neural retina producing neurons at the outer and inner nuclear layers. It has been shown that the developing retina is very vulnerable to X-rays (Lahav et al., 1973) and cytosine arabinoside (Shimida et al., 1973). The appearance of rosettes in the retina exposed to these agents was the striking feature. These authors suggested that the maldevelopment of the retina might be a sequel to the inhibition of cellular proliferation caused by these agents. Both X-rays and cytosine arabinoside interfere with DNA structure or function, as does MNU. Therefore, it seems likely that these agents exert their effects upon the DNA of the developing retina. Damage to cellular DNA leads to an interference with replication or transcription, or both. Thus, DNA synthesis is usually affected first. Inhibition of DNA synthesis may lead to cellular killing or to inhibition of cellular proliferation and thus cause maldevelopment of the retina. Alternatively, mutated cells may persist as abnormal clones.

Since many MNU-induced lesions in DNA are known to be repaired, evaluations of the consequences of damage and repair must take into account the fact that the cells must not only be protected from damage but also from errors which might arise from error-prone repair processes.

It has been suggested that such error-prone postreplication repair accounts for the major biological effects of MNU (Roberts et al., 1974). The earlier studies of both X-ray and cytoxan teratogenesis were consistent with the conclusion that chloroquine exerted its enhancing effect on teratogenesis by inhibiting DNA repair, presumably excision repair. Isonicotinic acid hydrazide, a very effective tuberculostatic agent, has been reported to inhibit the postreplication repair process of MNU-induced damage (Klamerth, 1978), and to have the same effect as caffeine as inhibitors of postreplication repair of mitomycin C-induced chromosome damage (Okoyama and Kitao, 1981). It was of interest, therefore, to study the effects of these two types of repair inhibitors on the production of retinal damage induced by MNU. Chloroquine was studied at low teratogen dose for its possible enhancing effect while INH was tested at high level of insult for its protective effect.

E. Effects of chloroquine and isonicotinic acid hydrazide on MNU-induced retinal dysplasia

The effects of chloroquine and INH, when given to pregnant mice on day 16 of gestation immediately after MNU administration, on the production of retinal dysplasia in mouse offspring are shown in Table VI. The frequency of retinal dysplasia obtained, when chloroquine was given in conjunction with the low dose of MNU (5 mg/Kg), was higher than that induced by MNU alone, but the difference was not statistically significant. Concurrent administration of INH with the high dose of MNU (20 mg/Kg) did not reduce the incidence of retinal damage. Chloroquine or INH alone had no effects on the production of retinal dysplasia. Thus, it appeared that the concentrations of chloroquine and INH used in these experiments did not affect the retina.

TABLE VI

EFFECT OF CHLOROQUINE AND ISONICOTINIC ACID HYDRAZIDE ON MNU-INDUCED RETINAL DYSPLASIA

Treatment	Retinal Dysplasia
5 mg/Kg MNU	7/36 (19.4%)
5 mg/Kg MNU + 25 mg/Kg chloroquine	21/64 (32.8%)
20 mg/Kg MNU	72/77 (93.5%)
20 mg/Kg MNU + 50 mg/Kg INH	34/36 (94.4%)
25 mg/Kg chloroquine	0/107
50 mg/Kg INH	0/35

Groups of pregnant mice, 16 days of gestation, were treated with different doses of methylnitrosourea with and without added treatment of inhibitors. The offspring were sacrificed at 3 to 6 weeks of age, and their eyes were examined for the appearance of retinal dysplasia. The data were expressed as the number of mice with retinal maldevelopment per total number of mice observed.

The interesting finding was the enhancing effect of chloroquine on retinal dysplasia induced by the low dose of MNU, although the difference could not be proved to be statistically significant ($p \leq 0.08$). This trend is consistent with enhancing effects of chloroquine observed on birth defects induced by X-rays and cytoxan. A low dose of MNU may produce mild damage to the DNA of retinal cells so that the cellular repair process can operate on it. Since chloroquine is thought to inhibit excision repair, and the mammalian excision repair is mainly an error-free process (Maher and McCormick, 1979), it is possible that the repair of this kind of damage in retinal cells is an

error-free process, and thus the inhibitor serves an enhancing role for retinal damage.

Another possibility that the induction of retinal dysplasia may be closely connected with misrepair of DNA lesions was also investigated. Since the frequency of the occurrence of retinal dysplasia was closely dependent on the dose of MNU, a high dose of MNU was used to cause severe damage to the retinal cells. It is possible that after such a high dose of MNU, the extent of DNA damage was so high that the error-free repair process was overloaded. Therefore, an alternative repair pathway may have been induced and this process may have been error-prone, leading ultimately to the net increase of retinal lesions. There are suggestions that tolerance repair or postreplication repair mechanisms can increase the frequency of tumors induced in animals by chemical carcinogens (Maher and McCormick, 1979), and that the incidence of these tumors is reduced in the presence of caffeine which is commonly known to inhibit postreplication repair. It has been suggested that the reducing effect of caffeine on tumor production may be related to the possibility that caffeine suppresses the repairing action of error-prone repair system (Kondo, 1977). Since INH has been reported to inhibit postreplication repair of MNU damage (Klamerth, 1978) and to have the same effect as caffeine in decreasing the frequency of interchromatid exchanges induced by mitomycin C (Okoyama and Kitao, 1981), it should have a protective effect on retinal dysplasia induced by MNU if this incidence is closely connected with misrepair of DNA lesions. The finding that INH had no influence on the frequency of retinal dysplasia following MNU damage did not support a role for the error-prone repair process in the induction of retinal maldevelopment. However, it has not

been established that INH, under these *in vivo* conditions, has an effect on such repair process.

The results obtained here may help provide evidence that the final expression of malformations after teratogenic insults is related to effects on DNA repair, but which pathways for repair are operating is still unclear. Based on these teratological studies, it is reasonable to hypothesize that DNA damage and repair mechanisms play important roles in teratogenic processes and should be studied during embryonic development. These studies must be direct, however, since such biological experiments as reported here can only provide suggestions of their importance.

BIOCHEMICAL STUDIES

A. DNA damage and repair in whole embryonic cells

From previous biological studies, it has been found that mouse embryos at 10 to 10 1/2 days of intrauterine life are very susceptible to many teratogens which damage DNA. Therefore, embryos at 10 1/2 days of age were used to study DNA damage and repair *in vivo*. Low levels of damage and the small amount of tissue available have presented a formidable challenge to provide a method of sufficient sensitivity. The method of nucleoid sedimentation has proved to be the most sensitive method available for detecting the production and closure of single-strand breaks in nuclear DNA during damage and repair processes (Cook and Brazell, 1976b). The rapid sedimentation of nucleoids in neutral sucrose gradients is consistent with a highly supercoiled structure of their DNA. Introduction of single-strand breaks either directly by damage or during the course of excision repair results in a release of

such supercoils and a decrease in sedimentation rate. Repair of the breaks restores the DNA to its original state and reestablishes the high sedimentation rate. Therefore, treatment of embryonic cells with agents which are known to induce single-strand breaks should be expected to change the sedimentation properties of nucleoids isolated subsequently. Since X-rays have been shown to produce many malformations during this period of embryonic development in earlier studies, and they have also been known to produce single-strand breaks in DNA of cultured mammalian cells, X-rays are suitable for this purpose. Another agent, methyl methanesulfonate, a direct-acting alkylating agent, is known to produce adducts in DNA which result secondarily in single-strand breaks due to spontaneous chemical events and DNA excision repair processes. Because of their properties, both X-rays and MMS were used as DNA-damaging agents and also agents for testing whether the nucleoid sedimentation method could be used for studying strand breaks and repair of DNA in developing mouse embryos. In each instance the insult was administered in utero followed by removal of embryos at selected time intervals to follow the time course of DNA damage by comparison with untreated controls. Fig. 4 shows the effect of X-rays, 600 rads, delivered in utero, on the sedimentation of nucleoids from whole embryonic cells of 10 1/2-day-old mouse embryos isolated subsequently. The cells were obtained from the whole embryonic homogenate as described in Materials and Methods. There was no difference in the sedimentation rates between control and X-irradiated nucleoids. Both of them sedimented slowly, as seen by the presence of sharp peaks near the top of the gradients. A similar result was obtained with MMS treatment (Fig. 5). MMS was administered intraperitoneally to the pregnant mouse at the dose

Figure 4: Effect of X-rays on the sedimentation of nucleoids from whole embryonic cells in neutral sucrose gradients. Whole body X-irradiation was administered to pregnant mice on day 10 1/2 of gestation at a dose of 600 rads. Whole embryonic cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation.
●: untreated control; o: X-rays, 10 minutes.

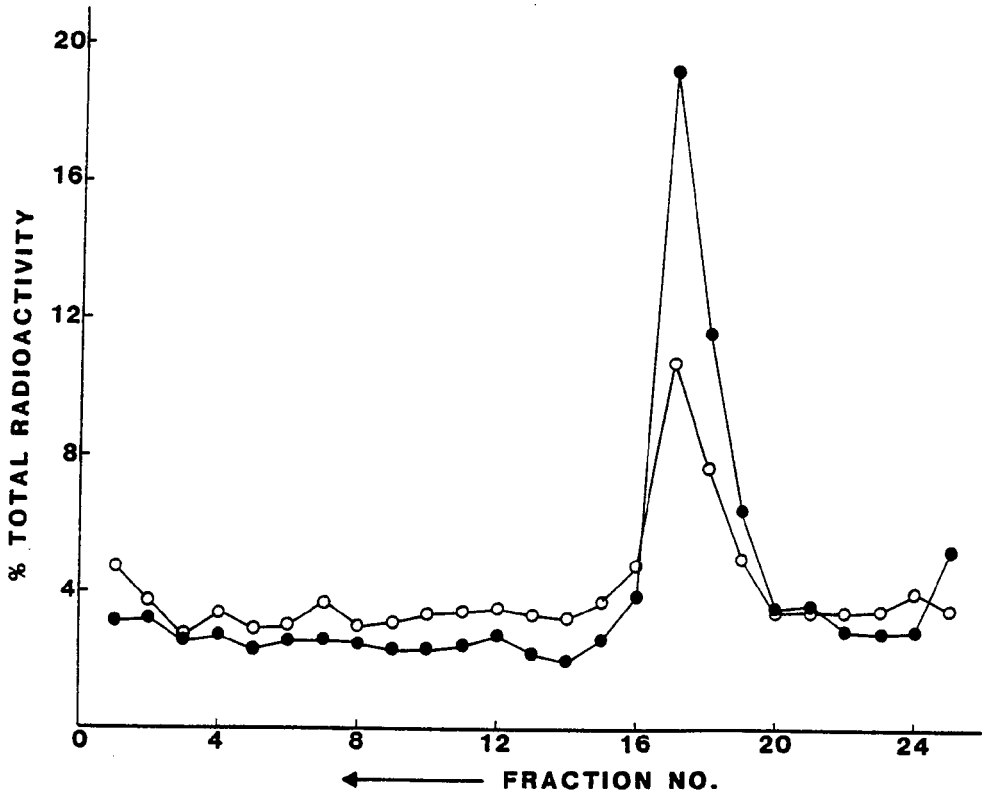
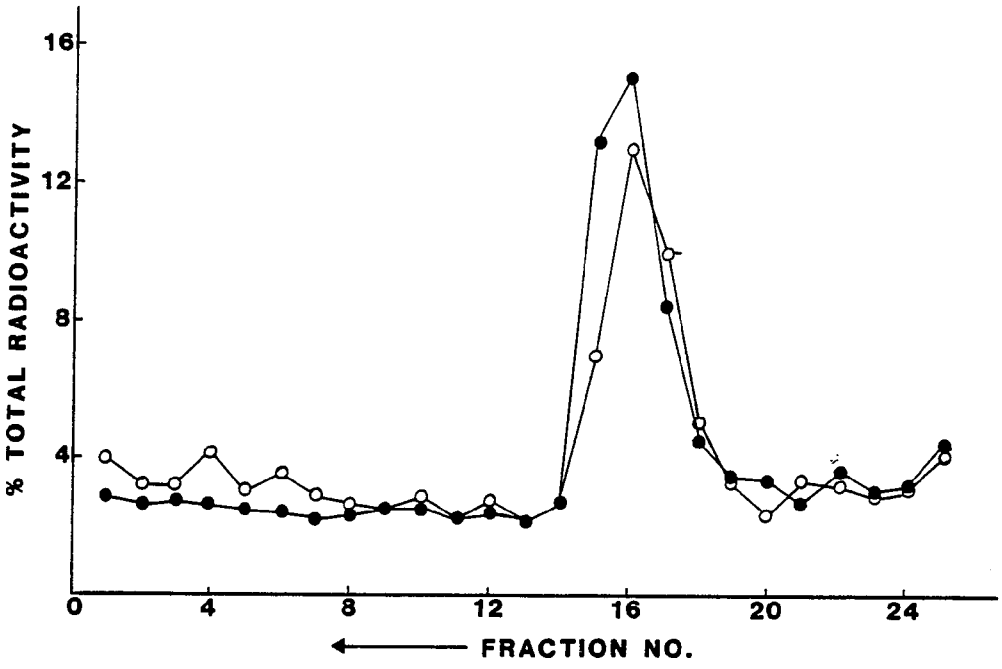
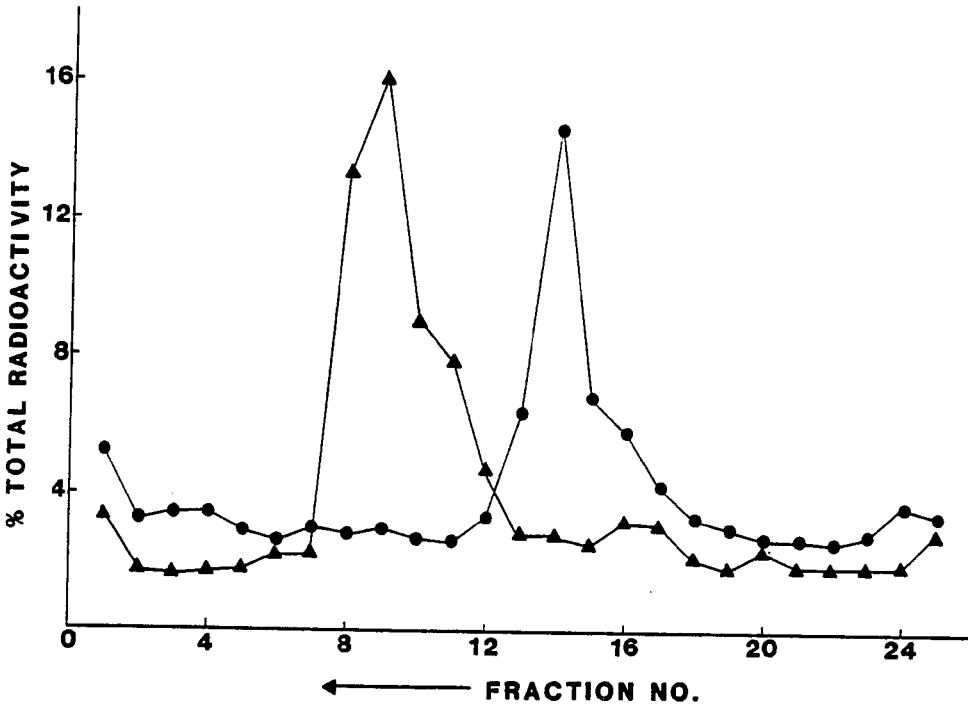

20
16
12
8
4
% TOTAL RADIOACTIVITY
0
4
8
12
16
20
24
FRACTION NO.

Figure 5: Effect of methyl methanesulfonate on the sedimentation of nucleoids from whole embryonic cells in neutral sucrose gradients. Methyl methanesulfonate was given as a single intraperitoneal injection to pregnant mice on day 10 1/2 of gestation at a dose of 100 mg/Kg body weight. Whole embryonic cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; o: methyl methanesulfonate, 30 minutes.


% TOTAL RADIOACTIVITY
16
12
8
4
0
4
8
12
16
20
24
FRACTION NO.

of 100 mg/Kg body weight. High doses of X-rays and MMS were used to insure that the damage by these agents could be detected by this experimental approach. The DNA of higher animals usually is in the superhelical state. Therefore, the nucleoids which contain supercoiled DNA should sediment rapidly toward the bottom of the sucrose gradients. Since control nucleoids sedimented at the same slow rate as treated nucleoids, three possibilities were considered: 1) the DNA of mouse embryonic cells is not intact supercoiled, it may already be nicked or in the relaxed form; 2) the high dose of ^{3}H-thymidine, 500 μCi/Kg, may produce damage to the DNA of embryonic cells and thus cause the loss of its superhelical structure; 3) the phosphodiester bonds may be nicked by the action of nucleases released after homogenization of the whole embryos, causing the loss of supercoils in both control and experimental specimens. It was of interest, therefore, to investigate the properties of DNA of more differentiated and homogeneous cells such as embryonic liver cells, for comparison with DNA from the whole embryos. Mouse embryos at 12 1/2 days of intrauterine life were used because their livers could be removed and enough material could be obtained to perform the experiment. Whole embryonic cells and liver cells from untreated embryos were prepared by homogenizing the tissues in cold PBS, and used for neutral sucrose gradient analysis. Fig. 6 shows the sedimentation profiles of nucleoids from whole embryonic cells and liver cells of 12 1/2-day-old mouse embryos. There was a marked difference in the sedimentation rates between whole embryonic and liver nucleoids. The nucleoids of liver cells sedimented more rapidly than those of whole embryonic cells. The peak of liver DNA was near the bottom of the gradient whereas the DNA peak of whole embryonic cells

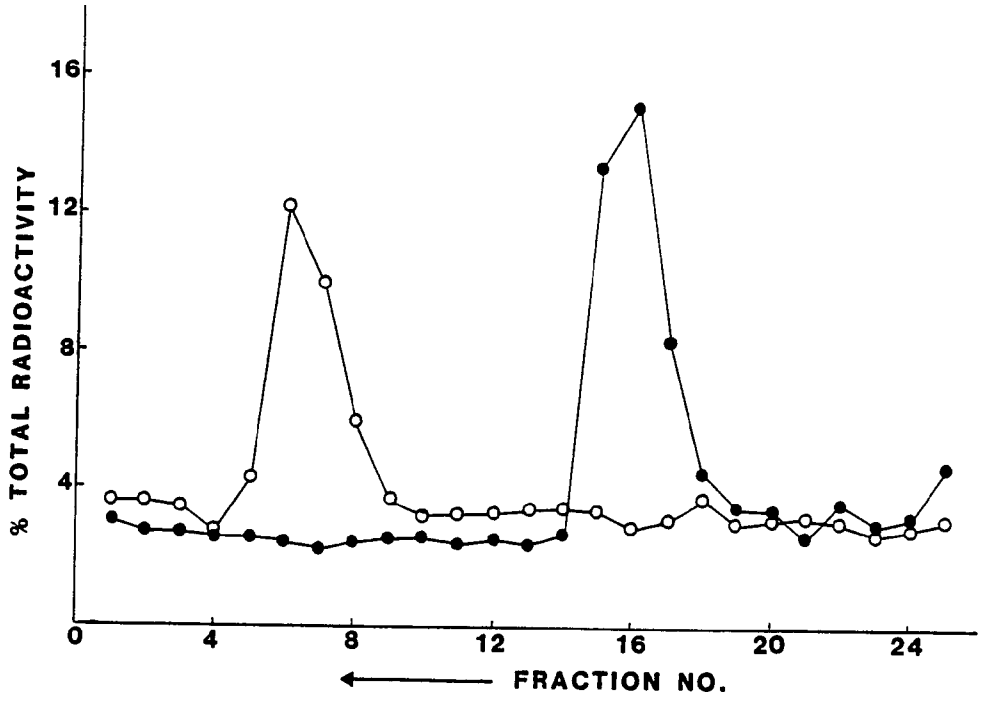
Figure 6: Sedimentation profiles of nucleoids from whole embryonic cells and liver cells of 12 1/2-day untreated mouse embryos in neutral sucrose gradients. Both types of cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation. ●: nucleoids of whole embryonic cells; ▲: nucleoids of liver cells.


% TOTAL RADIOACTIVITY
16
12
8
4
0
4
8
12
16
20
24
FRACTION NO.

was near the top of the gradient. This indicated that the isolated nucleoids of embryonic liver cells either were larger in size or were more supercoiled than those of whole embryonic cells. If the latter were true, the embryonic liver cells could be used conveniently for studying DNA damage and repair in developing embryos. These results also indicated that the use of ^{3}H-thymidine at the dose of 500 μCi/Kg did not cause detectable strand breaks in DNA of embryonic liver cells. ^{3}H-thymidine passes the placenta, but the labeling of the embryos is up to 8 times lower than that of the mother (Herken, 1977). Thus, the high dose of ^{3}H-thymidine was used in order to obtain satisfactory labeling, and at a dose of 500 μCi/Kg, ^{3}H-thymidine proved effective in labeling DNA of embryonic tissues.

The slow sedimentation of nucleoids from whole embryonic cells suggested that their DNA was not in the intact superhelical state. The question was asked whether the homogenization of the whole embryos may have caused disruption of the cells prior to nucleoid preparation, releasing nucleases and allowing extensive enzymatic breakdown of endogenous DNA. In order to answer this question, another disaggregation procedure, trypsinization of the tissue, which causes little or no damage to the cells, was used to dissociate the whole embryos into its constituent cells. A comparison of the sedimentation of nucleoids prepared by homogenization and by trypsinization was carried out in neutral sucrose gradients. Whole embryonic cells of 10 1/2-day-old mouse embryos were prepared by incubating the tissue of whole embryos in trypsin-EDTA solution as described in Materials and Methods, and this preparation was used for the analysis of nucleoid sedimentation in neutral sucrose gradients. Fig. 7 shows the sedimentation of

Figure 7: Neutral sucrose gradient profiles of nucleoids from whole embryonic cells of 10 1/2-day-old mouse embryos prepared by 2 different methods. The arrow indicates the direction of nucleoid sedimentation. ●: cells obtained by homogenization; o: cells obtained by trypsinization.


% TOTAL RADIOACTIVITY
16
12
8
4
0
4
8
12
16
20
24
FRACTION NO.

nucleoids from whole embryonic cells obtained by the two different methods, homogenization and trypsinization. There was a difference in the sedimentation rates of nucleoids prepared by these two methods. The nucleoids obtained by trypsinization sedimented more rapidly than those obtained by homogenization. The DNA peak of the former banded near the bottom of the gradient, indicating that the DNA of whole embryonic cells actually was also in a superhelical state prior to homogenization. The striking finding was the difference in the viability of cells prepared by these two methods. Most or all of the cells obtained by trypsinization were alive whereas many cells obtained by mechanical homogenization were dead. The viability of cells was detected by the use of trypan blue staining, based on the ability of viable cells to exclude the dye. Therefore, mechanical homogenization of the tissue of whole embryos caused damage to the cells, leading to cell death. Upon the death of cells, the membranes of the lysosomes disintegrated, releasing lytic enzymes, probably nucleases which induced breaks in DNA strands, with consequent loss of supercoils. Alternatively, the use of trypsin-EDTA solution to disaggregate embryonic cells did not cause cell damage. The chelating agent, ethylenediamine tetraacetic acid (EDTA), also inactivated nucleases by removing divalent cations required for nuclease activity (Shack and Byrum, 1964), preventing rapid fragmentation of DNA. Thus, the cells obtained by trypsinization were alive. In contrast, most of the liver cells prepared by homogenization were still alive, and their nucleoids sedimented rapidly toward the bottom of the gradient. Consequently, it can be predicted that liver nucleoids retain their supercoiling.

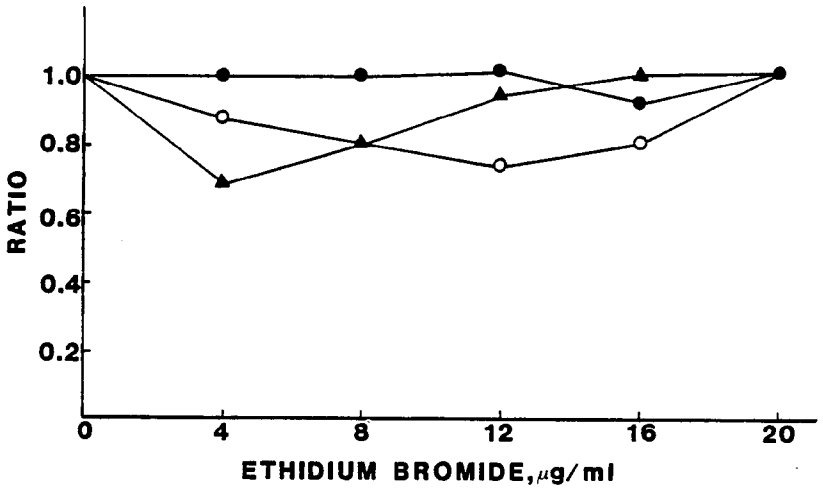
In order to confirm the superhelical state of DNA in nucleoids from whole embryos and embryonic liver cells, sedimentation experiments were performed with ethidium bromide added to the sucrose gradients. The intercalating agent affects the sedimentation of supercoiled DNA in a characteristic manner (Cook and Brazell, 1975). Fig. 8 illustrates the characteristic response of nucleoids from cells of untreated mouse embryos to varying concentrations of ethidium bromide. Whole embryonic cells were obtained from 10 1/2-day-old mouse embryos, where liver cells were obtained from 12 1/2-day-old mouse embryos. The distance sedimented by nucleoids in sucrose gradients containing different concentrations of ethidium bromide is expressed as a ratio relative to that of nucleoids sedimenting in the absence of ethidium bromide. As expected, the sedimentation of nucleoids from whole embryonic cells prepared by trypsinization and from liver cells prepared by homogenization showed biphasic effects in response to increasing concentrations of ethidium bromide, confirming that the DNA of mouse embryonic cells actually was supercoiled. In contrast, there was no change in the sedimentation rates of whole embryonic nucleoids prepared by homogenization in response to varying concentrations of ethidium bromide. The presence of strand breaks in DNA has been shown to abolish the biphasic response to ethidium bromide (Cook and Brazell, 1976b). Thus, the results obtained here indicated that there were indeed strand breaks in DNA prepared from cells disrupted by homogenization, and also explained why there were no differences in the sedimentation rates between the control and X-ray-treated or MMS-treated nucleoids in previous experiments (Fig. 4 and Fig. 5). Introduction of strand breaks results in an unwinding of supercoils and a decrease in sedimentation. If the

Figure 8: Effect of ethidium bromide on the sedimentation of nucleoids from whole embryonic cells of 10 1/2-day-old mouse embryos and liver cells of 12 1/2-day-old mouse embryos. The cells were prepared either by homogenization or by trypsinization. The distance sedimented by nucleoids in neutral sucrose gradients containing different concentrations of ethidium bromide is expressed as a ratio relative to that of nucleoids sedimenting in the absence of ethidium bromide.

●: whole embryonic cells (homogenization);

o: whole embryonic cells (trypsinization);

▲: embryonic liver cells (homogenization).


1.0
0.8
0.6
0.4
0.2
RATIO
0
4
8
12
16
20
ETHIDIUM BROMIDE,μg/ml

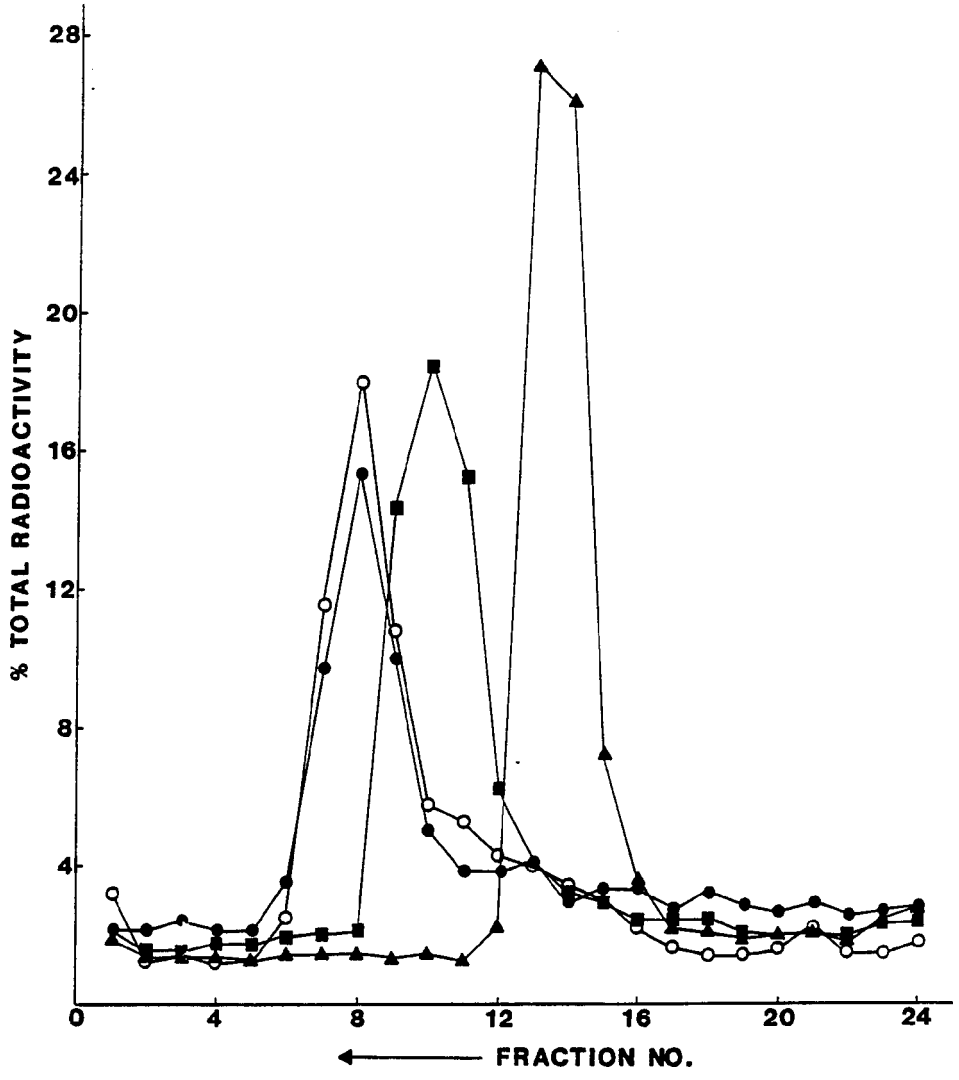
supercoils are completely unwound, then a minimum sedimentation rate should occur. In case of X-ray- and MMS-treated nucleoids, high doses of these agents may cause many breaks in DNA strands which lead to a complete unwinding of supercoils. In the same way, it is possible that in dying or dead cells, there might be a rapid fragmentation of DNA, and thus complete loss of supercoils. Therefore, both control and treated nucleoids sedimented at the same minimum rates.

B. DNA damage and repair in embryonic liver cells

From the earlier experiments, liver cells proved useful for studying DNA damage and repair by the nucleoid sedimentation method. Therefore, the embryonic liver cells of 12 1/2-day-old mouse embryos prepared by homogenization were used for this purpose. Agents capable of producing damage to DNA, including X-rays, MMS, and MNU, were used in these studies.

1. The effect of X-irradiation on the sedimentation of nucleoids. A single exposure of pregnant mice to X-rays on day 12 1/2 of gestation resulted in damage to DNA of embryonic liver cells isolated subsequently, which was reflected by a decrease in sedimentation rate of nucleoids in neutral sucrose gradients. Fig. 9 shows the effect of X-rays to pregnant mice at a dose of 235 rads on the sedimentation of nucleoids isolated from embryonic liver cells. The embryos were removed from mothers at different times after X-irradiation. Ten minutes after X-irradiation, the nucleoids sedimented most slowly indicating a decrease in superhelical density of liver DNA. Such a decrease in sedimentation rate can be attributed to the presence of single-strand breaks induced by X-rays resulting in loss of supercoils. At 30 min,

Figure 9: Effect of X-rays on the sedimentation of embryonic liver nucleoids in neutral sucrose gradients. Whole body X-irradiation was administered to pregnant mice on day 12 1/2 of gestation at a dose of 235 rads. The embryos were removed from the mothers at 10 minutes, 30 minutes, and 5 hours after X-irradiation. The embryonic liver cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: X-rays, 10 minutes; ■: X-rays, 30 minutes; o: X-rays, 5 hours.

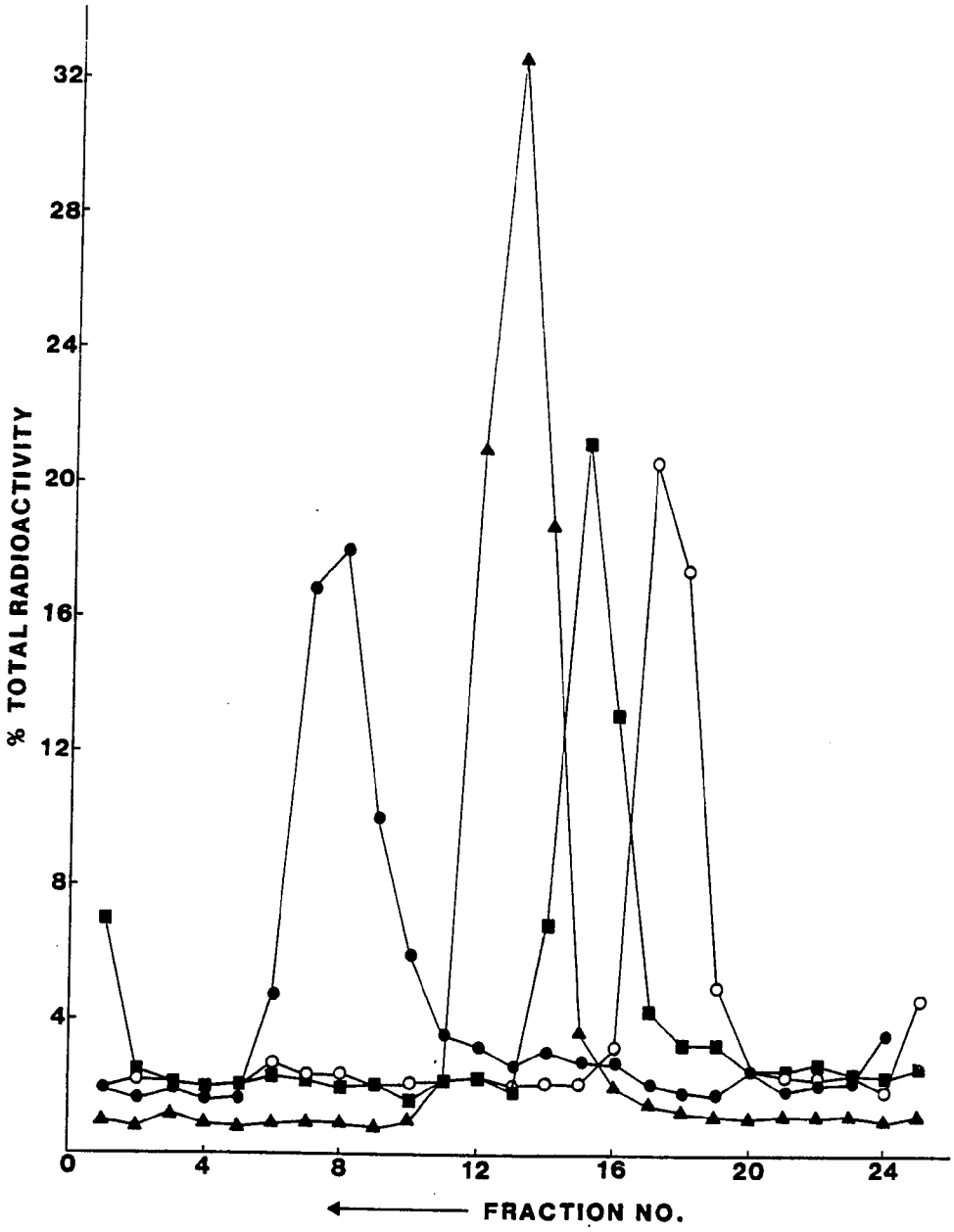

28
24
20
16
12
8
4
0
4
8
12
16
20
24
% TOTAL RADIOACTIVITY
FRACTION NO.

the sedimentation profile began to shift toward the bottom of the gradient, indicating a partial repair of DNA damage. By 5 hours, the repair was complete as shown by the restoration of the sedimentation rate to that of control nucleoids. These results suggested that X-ray damage to the DNA in embryonic cells could be detected almost immediately after transplacental exposure. The rapid repair of X-ray-induced breaks is in agreement with other studies. This rapid rejoining of strand breaks is widely presumed to result from excision repair (Hutchinson, 1978). The finding that the embryonic liver cells can repair X-ray-induced strand breaks rapidly may explain why the liver is not a target organ of X-ray teratogenicity. This study also demonstrated that the method of nucleoid sedimentation is sensitive and useful for the study of DNA damage and repair of embryonic cells during development. The nucleoids sediment as a single peak, and strand breaks are expressed as a change in density of the whole structures rather than heterogeneous fractions. The change in sedimentation (Fig. 9) resulted from rejoining of strand breaks rather than new DNA synthesis because the DNA of the embryonic liver cells had been prelabeled with ^{3}H-thymidine and any new DNA would not be detected as labeled. ^{3}H-thymidine has been used experimentally to label DNA in proliferating cells, the major reason being its unique specificity in labeling, thymidine is incorporated only into DNA. Cells can be labeled with ^{3}H-thymidine only during the S-phase. In mouse embryos, about 6-8 times more cells are in the S-phase of the cell cycle than in mitosis (Herken, 1977). The percentage of cells incorporating ^{3}H-thymidine in control mouse embryos is high at 90 minutes. Injected thymidine is available only a short time due to breakdown of the

thymidine by hepatic enzymes. Therefore, it is reasonable to assume that the normal high sedimentation rate of liver nucleoids after X-ray damage is due to repair of DNA.

2. The effect of methyl methanesulfonate on the sedimentation of nucleoids. Methyl methanesulfonate, a well known methylating agent, has been used widely in studying DNA damage and repair both in vivo and in vitro due to its property of producing adducts in DNA. It was of interest, therefore, to use this agent for studying the appearance and elimination of DNA strand breaks during embryonic development. MMS was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation, and the embryos were removed at varying periods of time thereafter. Embryonic liver cells were prepared by homogenization and the nucleoids were analyzed in neutral sucrose gradients as described in Materials and Methods. Fig. 10 shows the effect of MMS, at a dose of 100 mg/Kg, on the sedimentation of nucleoids from liver of treated embryos. The nucleoids after MMS treatment of the embryos sedimented more slowly than did those from untreated controls. Furthermore, the decrease in sedimentation was dependent on the interval of time after treatment. As time increased, the distance sedimented decreased progressively. At 24 hours after MMS treatment, all embryos removed from the uterine horns were already dead, and the mother looked sick. Therefore, the high dose of MMS, 100 mg/Kg, was very toxic to the cells of both mothers and embryos. It is possible that such a high dose of MMS caused such severe damage to DNA, that the cells could not repair successfully. Another possibility is that MMS had some inhibitory action on the repair enzyme system so that an increase in dose, in addition to causing more damage to DNA, would also inhibit repair.

Figure 10: Effect of methyl methanesulfonate, 100 mg/Kg, on the sedimentation of nucleoids from embryonic liver cells in neutral sucrose gradients. Methyl methanesulfonate was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation. The embryos were removed from the mothers at 30 minutes, 5 hours, and 10 hours after methyl methanesulfonate administration. The embryonic liver cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation.
●: untreated control; ▲: methyl methanesulfonate, 30 minutes; ■: methyl methanesulfonate, 5 hours; o: methyl methanesulfonate, 10 hours.


% TOTAL RADIOACTIVITY
32
28
24
20
16
12
8
4
0
4
8
12
16
20
24
FRACTION NO.

Such a mechanism has been suggested to account for the lack of a dose response in repair replication following MMS induced damage in other systems (Thielman et al., 1975). The inability of cells to repair DNA lesions would probably result in cell lethality.

Since the high dose was very toxic, lower doses of MMS, 50 mg/Kg and 25 mg/Kg, were used instead. Fig. 11 shows the effect of MMS, 50 mg/Kg, on the sedimentation of nucleoids from liver cells of 12 1/2-day-old mouse embryos. DNA damage was detected at 30 min after MMS treatment, the nucleoids from MMS-treated embryos sedimented more slowly than those from untreated controls. Partial repair of DNA damage was apparent by 5 hours following treatment as shown by the shift of the DNA peak toward the bottom of the gradient. At 24 hours, there was a complete return to the control sedimentation rate, indicating a repair of the damage induced by MMS. Similar results were obtained when MMS was given at a dose of 25 mg/Kg, as shown in Fig. 12. Therefore, the low dose of MMS, 25 mg/Kg, was used as the reference dose for all MMS experiments.

In order to contrast the degree of supercoiling in DNA of MMS-treated nucleoids, sedimentation experiments were performed with different concentrations of ethidium bromide added to the sucrose gradients. Fig. 13 shows the effect of ethidium bromide on the sedimentation of nucleoids of embryonic liver cells from embryos treated with MMS after varying periods of time. At 30 min, the sedimentation rate of MMS-treated nucleoids was not affected by increasing concentrations of ethidium bromide (Cook and Brazell, 1976b). After 16 and 24 hours of MMS treatment, the nucleoids had regained their biphasic responses to

Figure 11: Effect of methyl methanesulfonate, 50 mg/Kg, on the sedimentation of nucleoids from embryonic liver cells in neutral sucrose gradients. Methyl methanesulfonate was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation. The embryos were removed from the mothers at 30 minutes, 5 hours, and 24 hours after methyl methanesulfonate administration. The embryonic liver cells were prepared by the homogenization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methyl methanesulfonate, 30 minutes; ■: methyl methanesulfonate, 5 hours; o: methyl methanesulfonate, 24 hours.

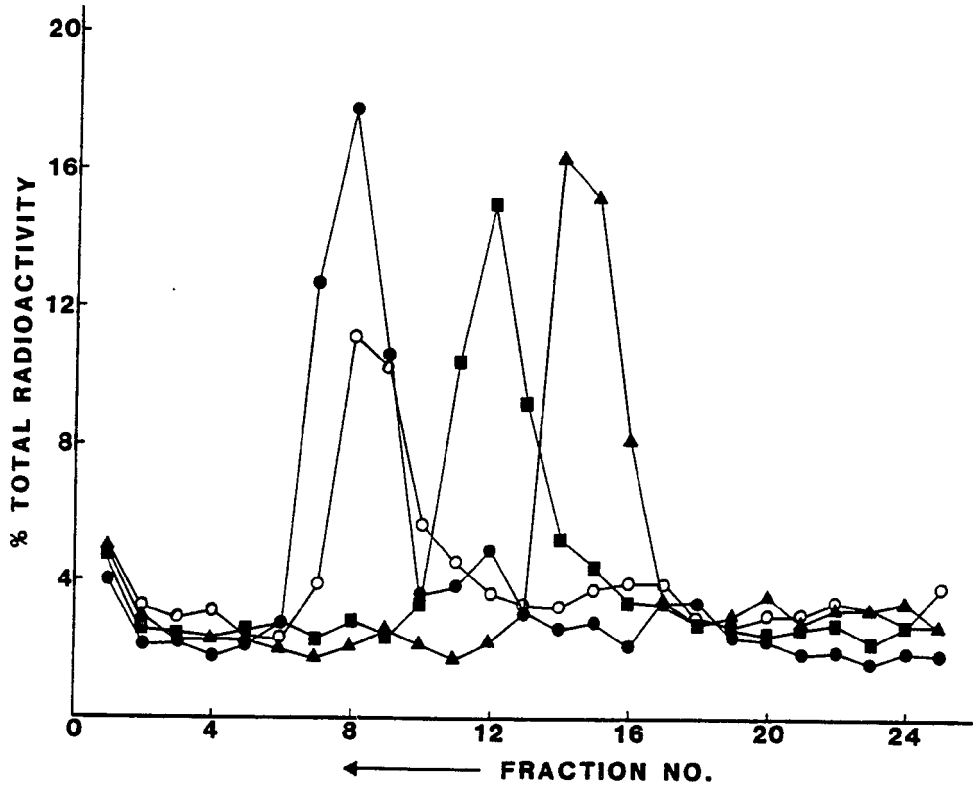

% TOTAL RADIOACTIVITY
20
16
12
8
4
0
4
8
12
16
20
24
FRACTION NO.

Figure 12: Effect of methyl methanesulfonate, 25 mg/Kg, on the sedimentation of nucleoids from embryonic liver cells in neutral sucrose gradients. Methyl methanesulfonate was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation. The embryos were removed from the mothers at 30 minutes, 7 hours and 24 hours after methyl methanesulfonate administration. The embryonic liver cells were prepared by the homogenization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methyl methanesulfonate, 30 minutes; ■: methyl methanesulfonate, 7 hours; o: methyl methanesulfonate, 24 hours.

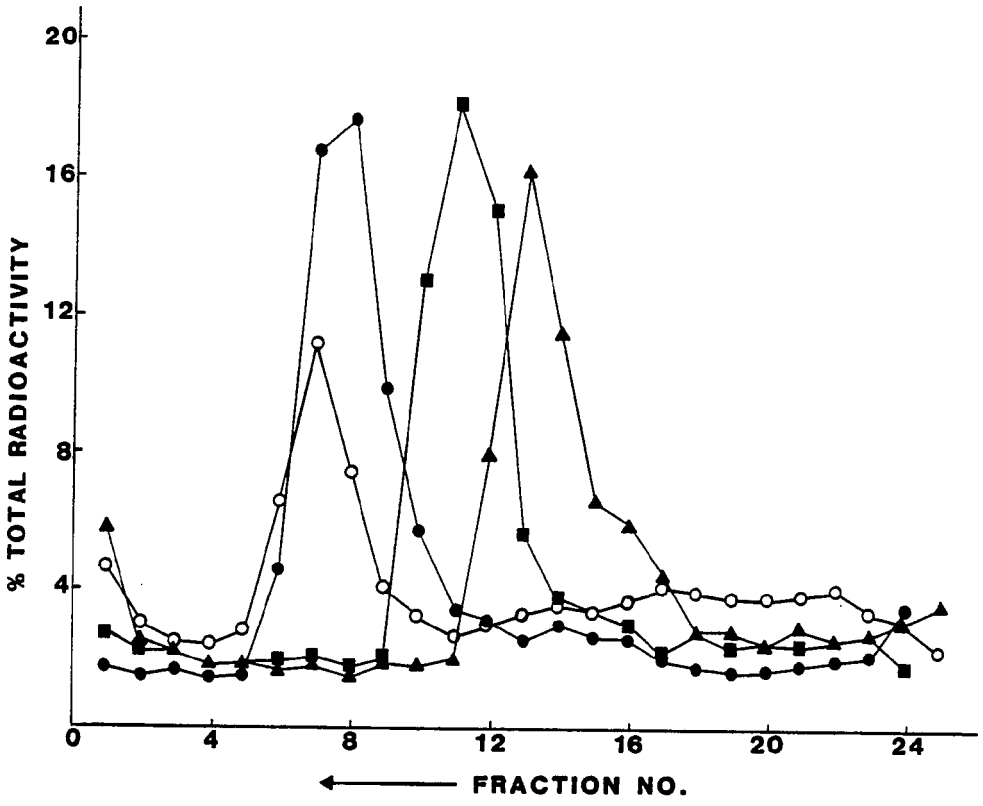
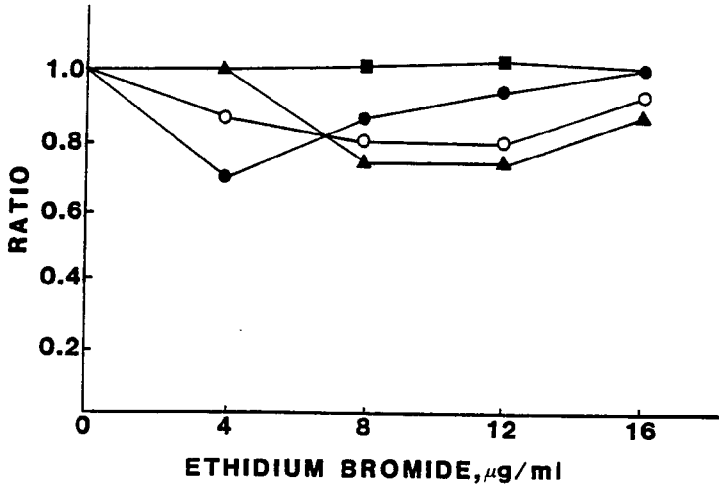

20
16
12
8
4
% TOTAL RADIOACTIVITY
0
4
8
12
16
20
24
FRACTION NO.

Figure 13: Effect of ethidium bromide on the sedimentation of nucleoids from liver cells of mouse embryos treated with 25 mg/Kg methyl methanesulfonate. The embryos were removed from the mothers at 30 minutes, 16 hours, and 24 hours after methyl methanesulfonate treatment. The distance sedimented by nucleoids in neutral sucrose gradients containing different concentrations of ethidium bromide is expressed as a ratio relative to that of nucleoids sedimenting in the absence of ethidium bromide. ●: untreated control; ■: methyl methanesulfonate, 30 minutes; ▲: methyl methanesulfonate, 16 hours; o: methyl methanesulfonate, 24 hours.


1.0
0.8
0.6
0.4
0.2
RATIO
0
4
8
12
16
ETHIDIUM BROMIDE,μg/ml

ethidium bromide, indicating that the breaks were rejoined and the DNA had returned to its normal superhelical state.

These observations indicated that transplacental administration of MMS resulted in single-strand breaks in DNA of embryonic cells which was reflected in a loss of supercoils in nucleoid DNA. Completed repair of these breaks restored the supercoiling.

3. The effect of chloroquine on the sedimentation of MMS-treated nucleoids. In biological studies, chloroquine was found to enhance the teratogenic effects of X-rays and cytoxan, and it has been concluded that chloroquine may have exerted its enhancing effect by inhibiting DNA repair. Chloroquine also has been demonstrated to inhibit the repair of DNA damage induced by MMS in cultured mammalian cells (Michael and Williams, 1974). It was of interest, therefore, to investigate the role of chloroquine in DNA damage and repair induced by MMS in developing mouse embryos. Chloroquine was given to pregnant mice at a dose of 25 mg/Kg body weight 30 min after MMS administration, when the maximum breakage in DNA was obtained. The effect of chloroquine on the sedimentation of MMS-treated nucleoids from embryonic liver cells observed at 7 hours and 24 hours after MMS injection are presented in Fig. 14 and Fig. 15, respectively. There were no differences in the sedimentation rates between nucleoids treated with MMS alone and those treated with MMS plus chloroquine. Partial repair was apparent at 7 hours, and complete repair was observed at 24 hours after MMS plus chloroquine treatment. Treatment with chloroquine alone caused no change in the sedimentation of liver nucleoids from that of control (Fig. 15), indicating that the dose of chloroquine used here did not produce any strand break in the DNA of embryonic liver cells. These

Figure 14: Neutral sucrose gradient profiles of nucleoids from liver cells of mouse embryos treated with methyl methanesulfonate with and without chloroquine. Chloroquine, 25 mg/Kg, was given as a single intraperitoneal injection to 12 1/2 day pregnant mice 30 minutes after methyl methanesulfonate administration. The embryos were removed from the mothers at 7 hours after methyl methanesulfonate treatment. The embryonic liver cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methyl methanesulfonate, 7 hours; ■: methyl methanesulfonate plus chloroquine, 7 hours.

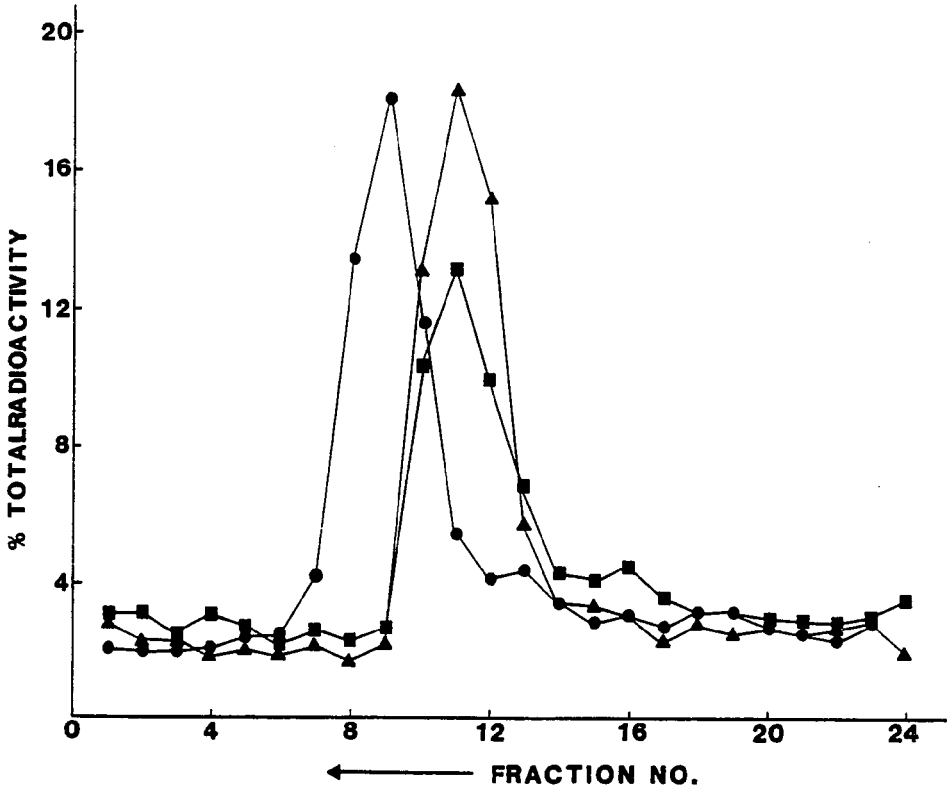
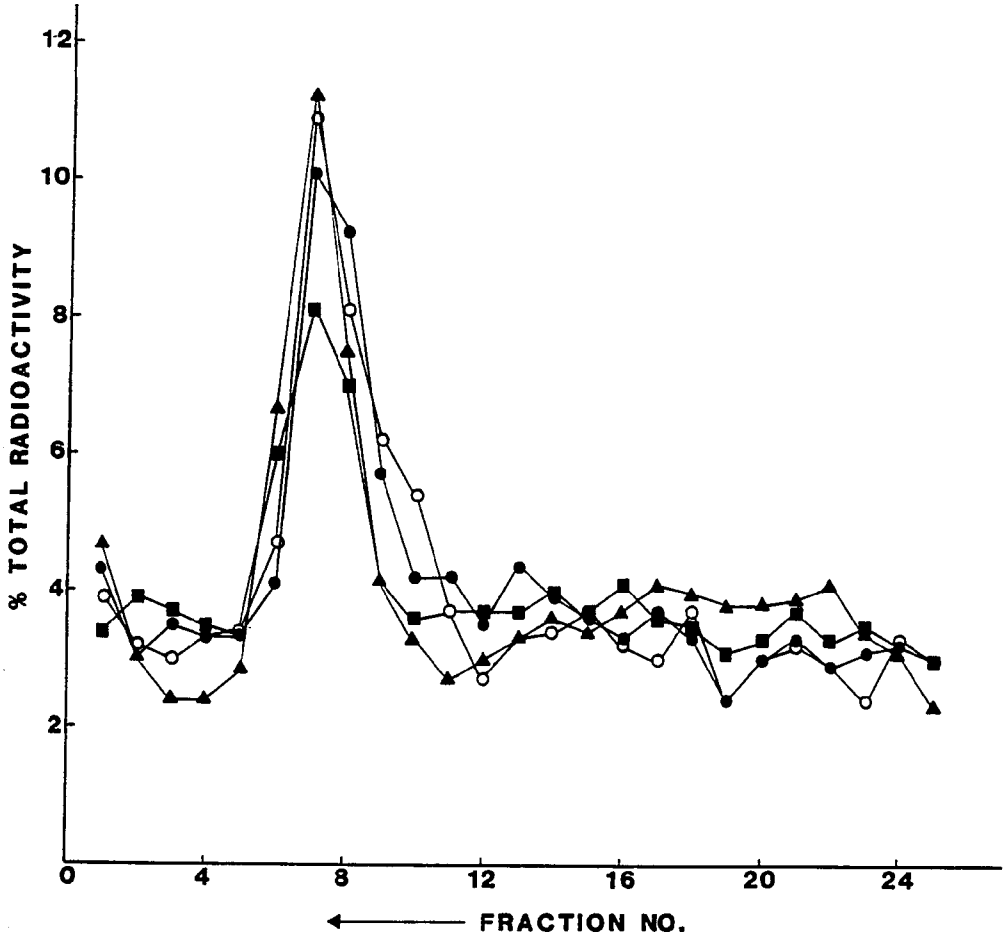

20
16
12
8
4
% TOTALRADIOACTIVITY
0
4
8
12
16
20
24
FRACTION NO.

Figure 15: Neutral sucrose gradient profiles of nucleoids from liver cells of mouse embryos treated with methyl methanesulfonate with and without chloroquine and chloroquine alone. Chloroquine, 25 mg/Kg, was given as a single intraperitoneal injection to 12 1/2 day pregnant mice either alone or with methyl methanesulfonate (30 minutes after methyl methanesulfonate administration). The embryos were removed from the mothers at 24 hours after methyl methanesulfonate or chloroquine treatment. The embryonic liver cells were prepared by the homogenization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methyl methanesulfonate, 24 hours; ■: methyl methanesulfonate plus chloroquine, 24 hours; o: chloroquine, 24 hours.


% TOTAL RADIOACTIVITY
12
10
8
6
4
2
0
4
8
12
16
20
24
FRACTION NO.

results also did not show any inhibitory effect of chloroquine on the repair of MMS-induced DNA damage in embryonic liver cells. The effect of chloroquine observed here, therefore, contrasted sharply from that observed in cultured mammalian cells (Michael and Williams, 1974), and did not support the earlier conclusion that chloroquine exerted its enhancing effect on birth defects by inhibiting DNA repair. Based on previous teratogenic studies, chloroquine enhanced the frequencies of cleft palate, abnormal tails, etc., which are common targets for X-ray teratogenesis. The liver is not a target for teratogenesis, and one possible explanation for this might be due to its high capacity to repair DNA that has been damaged sublethally by the agents. Therefore, the possible argument is that the wrong systems (wrong target and wrong agent) have been used to demonstrate the inhibitory effect of chloroquine, and thus it could not be simply proved that way.

Earlier studies demonstrated that transplacental exposure of methylnitrosourea produced abnormalities in many organs in mouse offspring. MNU, like MMS, is a direct-acting alkylating agent, but its chemical reaction mechanism is different from that of MMS. MNU reacts through an S_N1 mechanism which follows first-order kinetics, the rate depending only on the concentration of the alkylating agent but not on the nucleophiles, whereas MMS reacts through an S_N2 mechanism, in which the reaction is selectively directed by the nucleophilicity of the site, as well as the concentration of the alkylating agent. As a result of this difference in reaction mechanism, alkylation of DNA is affected differentially by these two agents. Although both MMS and MNU react with DNA primarily at the principal nucleophilic center, N-7 of

guanine, MMS is less likely to attack the less nucleophilic O-6 atoms in DNA, while MNU reacts more extensively at the less nucleophilic O-6 position of guanine in DNA and the phosphate groups of the sugar-phosphate backbone (Lawley, 1974). The alkylated bases can be removed from DNA either by spontaneous chemical hydrolysis or by enzymatic excision, resulting in the formation of apurinic sites which are subsequently converted to single-strand breaks by specific endonucleases (Ljungquist and Lindahl, 1974; Ljungquist et al., 1974). Available evidences suggest that the apurinic sites are probably subjects to repair reactions in vivo, and thus DNA strand breaks may result from the action of endonucleases in the initiation step in repairing the damage (Verly and Paquette, 1972; Lindahl and Anderson, 1972). Studies with phosphotriesters, another kind of O-alkylation products, have suggested that these lesions are chemically stable and are not excised by the enzymatic repair system of cells (Shooter and Slade, 1977). However, these lesions could lead to DNA strand breaks under alkaline hydrolysis (Walker and Ewart, 1973a).

Several investigations have demonstrated that MNU is able to induce DNA damage and subsequent repair both in vitro (Walker and Ewart, 1973b) and in vivo (Zubroff and Sarma, 1976). In the present research, experiments with MMS have shown that repair of DNA strand breaks induced transplacentally by this agent can be observed in mouse embryonic liver cells. Since MNU is a potent teratogen, it was of interest to study the effect of MNU on the DNA of developing mouse embryos by using nucleoid sedimentation technique.

4. The effect of methylnitrosourea on the sedimentation of embryonic liver nucleoids. The experiments were carried out in the

same way as MMS experiments but MNU at a dose of 20 mg/Kg body weight was used instead. The results are shown in Fig. 16. At 30 min after MNU treatment, the nucleoids of embryonic liver cells sedimented most slowly, reflecting the presence of DNA strand breaks. An increase in the sedimentation rate was apparent at 7 hours, and by 24 hours the sedimentation rate had been restored to that of control nucleoids, indicating partial and complete repair of MNU damage, respectively. When nucleoids from MNU-treated embryonic liver cells were sedimented in sucrose gradients containing different concentrations of ethidium bromide, the results obtained were consistent with the above finding that at 30 min, the presence of strand breaks in DNA caused the loss of supercoiling and abolished the biphasic response to ethidium bromide. At 16 and 24 hours, repair of the damage restored normal supercoiling, and regained the biphasic response to ethidium bromide (Fig. 17). These observations showed that MNU, after reaching the embryos, reacted directly with DNA of embryonic cells to cause damage and measurable repair. This is consistent with the hypothesis that MNU exerts its teratogenic effect through reaction with DNA of embryonic cells.

5. Effects of caffeine and isonicotinic acid hydrazide on the sedimentation of MNU-treated nucleoids. Both caffeine and INH have been reported to inhibit postreplication repair of MNU-induced DNA damage in cultured mammalian cells (Fujiwara, 1975b; Klamerth, 1978). Teratogenic studies have shown that caffeine altered the frequency of malformations induced by some teratogens, and suggested that these changes were mediated by caffeine-induced inhibition of DNA repair (Nomura, 1977; Yielding et al., 1976). Although these studies

Figure 16: Effect of methylnitrosourea on the sedimentation of embryonic liver nucleoids in neutral sucrose gradients. Methylnitrosourea was given as a single intraperitoneal injection to pregnant mice on 12 1/2 day of gestation at a dose of 20 mg/Kg body weight. The embryos were removed from the mothers at 30 minutes, 7 hours, and 24 hours after methylnitrosourea treatment. The embryonic liver cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methylnitrosourea, 30 minutes; ■: methylnitrosourea, 7 hours; o: methylnitrosourea, 24 hours.

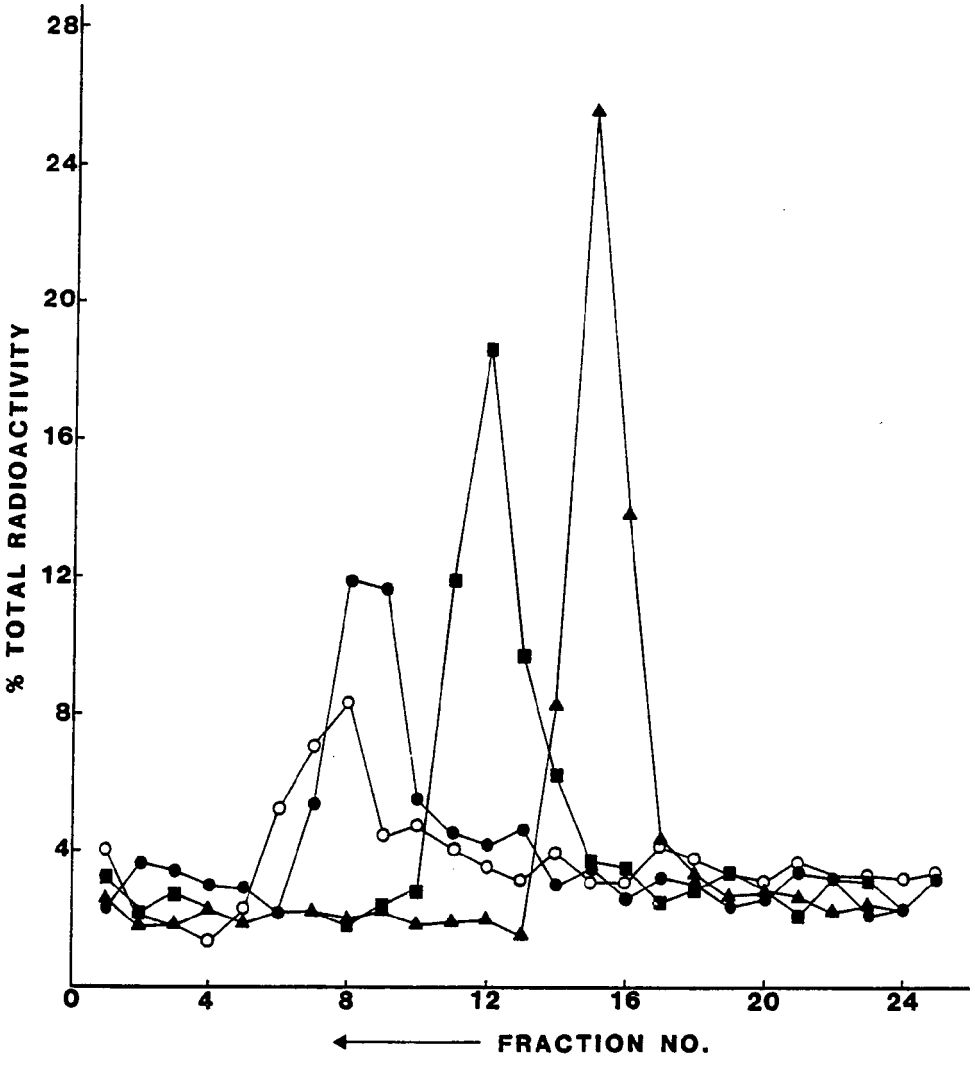
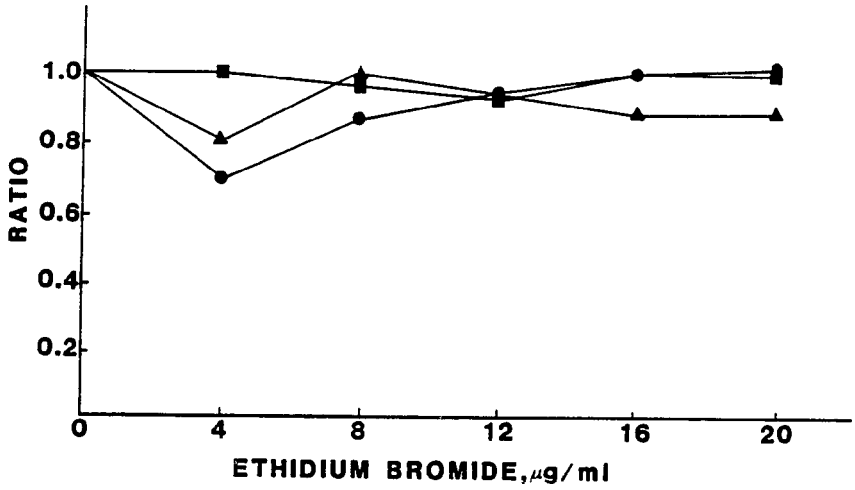

28
24
20
16
12
8
4
% TOTAL RADIOACTIVITY
0
4
8
12
16
20
24
FRACTION NO.

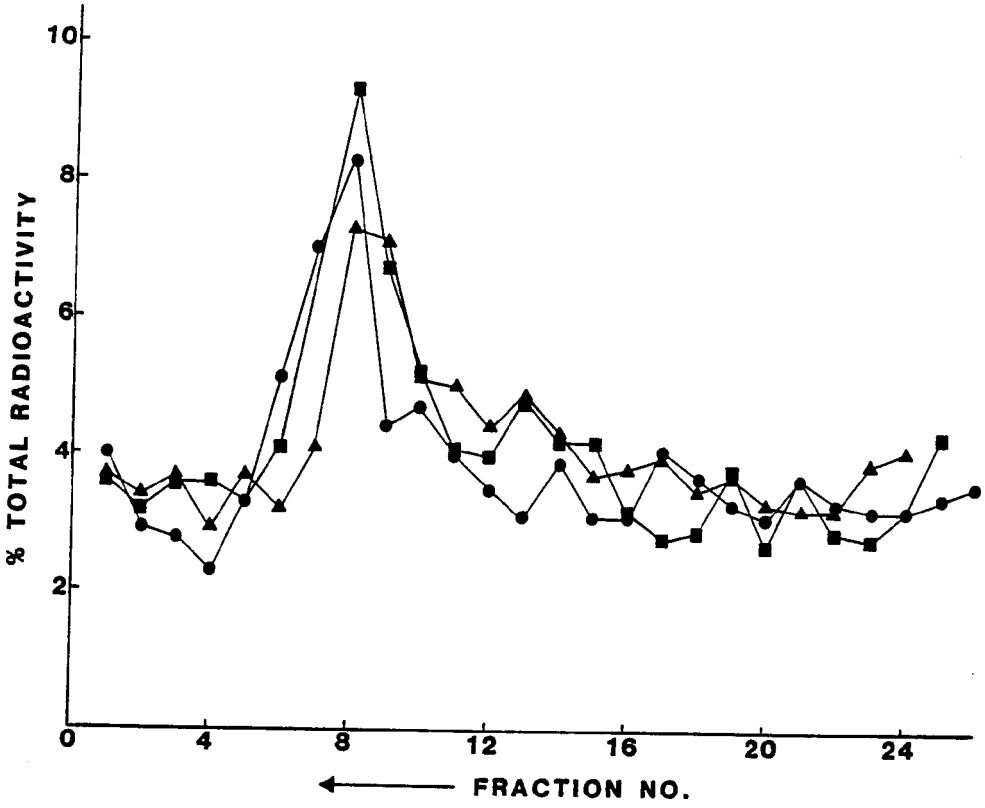
Figure 17: Effect of ethidium bromide on the sedimentation of nucleoids from liver cells of mouse embryos treated with 20 mg/Kg methylnitrosourea. The embryos were removed from the mothers at 30 minutes and 16 hours after methylnitrosourea administration. The distance sedimented by nucleoids in neutral sucrose gradients containing different concentrations of ethidium bromide is expressed as a ratio relative to that of nucleoids sedimenting in the absence of ethidium bromide. ●: untreated control; ■: methylnitrosourea, 30 minutes; ▲: methylnitrosourea, 16 hours.


1.0
0.8
0.6
0.4
0.2
RATIO
0
4
8
12
16
20
ETHIDIUM BROMIDE,μg/ml

have not demonstrated that DNA repair is related directly to birth defects, they provided evidence in support of this concept. Since the damage and repair of DNA induced by MNU can be demonstrated in embryonic liver cells by using the method of nucleoid sedimentation, it was of interest to examine whether caffeine and INH would inhibit the repair of DNA damage caused by MNU in these liver cells.

Caffeine and INH, each at a dose of 50 mg/Kg, were given intraperitoneally to pregnant mice as a single dose or two doses at different times after MNU treatment as described in Materials and Methods. At 7 and 24 hours after MNU administration, the embryos were removed from the uterine horns, and the embryonic liver cells prepared by homogenization method were used for nucleoid sedimentation analysis. The results of these experiments were similar and will not all be presented, but Fig. 18 was selected to show that posttreatment of both caffeine and INH had no measurable effect on the sedimentation of MNU-treated nucleoids. This figure represents the sedimentation profiles of nucleoids from animals treated with MNU alone and those treated with MNU plus 2 doses of caffeine or INH administered at 2 and 5 hours after MNU treatment. The effect of caffeine or INH on the sedimentation of MNU-treated nucleoids was observed at 24 hours. It can be seen that there was no difference in the sedimentation rates between nucleoids treated with MNU alone and those treated with MNU plus inhibitors. All nucleoids sedimented rapidly toward the bottom of the gradients, indicating the repair of MNU-induced DNA damage. Therefore, the effects of caffeine and INH observed here contrasted from their inhibitory effects on the repair of MNU damage observed in cultured mammalian cells. It is possible that caffeine-sensitive or

Figure 18: Neutral sucrose gradient profiles of nucleoids from liver cells of mouse embryos treated with methylnitrosourea with and without caffeine or isonicotinic acid hydrazide. Methylnitrosourea, 20 mg/Kg, was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation. Two doses of caffeine or isonicotinic acid hydrazide, each at 50 mg/Kg, were given intraperitoneally to methylnitrosourea-treated mice at 2 hours and 5 hours after methylnitrosourea administration. The embryos were removed from the mothers at 24 hours after methylnitrosourea treatment. The embryonic liver cells were prepared by homogenization. The arrow indicates the direction of nucleoid sedimentation. ●: methylnitrosourea, 24 hours; ▲: methylnitrosourea plus 2 doses of caffeine, 24 hours; ■: methylnitrosourea plus 2 doses of isonicotinic acid hydrazide, 24 hours.


% TOTAL RADIOACTIVITY
10
8
6
4
2
0
4
8
12
16
20
24
FRACTION NO.

INH-sensitive DNA repair mechanisms are not present in embryonic liver cells. Not all DNA repair mechanisms are caffeine-sensitive and the difference in caffeine-response found between different cell cultures may reflect the relative importance and efficiency of the different repair mechanisms in the cell lines (Timson, 1977). Caffeine does not inhibit repair replication in Hela cells or in normal human fibroblasts, and it has been suggested that these cells may perform an alternative replication repair which is not caffeine-sensitive (Fujiwara and Tatsumi, 1976b). Another possibility is that caffeine inhibits an S-phase specific postreplication repair by inhibiting the filling of gaps in newly synthesized DNA strands. In the present experiment with MNU, all embryonic DNA was prelabeled before the damage was introduced, so that it was the repair of those lesions in prelabeled DNA strands, not newly synthesized DNA strands, which was demonstrated by the nucleoid sedimentation assay. Therefore, the inhibitory effect of caffeine could not be seen in the present experiment due to the different assay used. There are only a few reports in the literature of the inhibitory effect of INH on postreplication repair in cultured mammalian cells, and the mechanism by which INH exerts its effect has not been established. However, the effects of caffeine and INH on DNA repair mechanisms may depend on many factors, and thus require further investigations.

Experiments with MNU and MMS demonstrated that these two agents administered transplacentally produced damage in the DNA of mouse embryonic liver cells which provoked subsequent DNA repair processes as measured by the sedimentation rate of liver nucleoids in neutral sucrose gradients. Liver nucleoids prepared by trypsinization responded

to the effects of both MMS and MNU in a manner similar to those prepared by homogenization. The DNA strand breaks induced by these agents were observed in 30 min, and complete repair of the damage was apparent within 24 hours (Fig. 12 and Fig. 16). In mice, both MNU and MMS are distributed rather uniformly in the body (Bochert et al., 1978). In pregnant animals, transplacental passage into embryos occurs shortly after an intraperitoneal injection. The approximate life time of these substances in vivo have been shown to be 120 min for MMS and about 20 min for MNU. Thus, the initial reactions of these chemicals with DNA in vivo are quite rapid; methylation of DNA is maximal within 3 hours after MMS administration and within 1 hour after MNU treatment. This is in agreement with the present finding that DNA strand breaks induced by both MNU and MMS could be detected in embryonic liver cells within 30 min after administration. It was also shown in the present experiments that the embryonic liver cells had the ability to repair the damage induced by either MNU or MMS, and the repair occurred rather rapidly. There is evidence that cells that can repair lesions in DNA are less sensitive to the effects of alkylating agent. An alternate, but not exclusive, explanation for the organotropic carcinogenicity of alkylating agents involves the capacity of various animal tissues to repair DNA damage induced by those agents. Michael and Sarma (1973) demonstrated different rates of DNA repair in animal livers exposed to various carcinogens. Preliminary data suggest that DNA damage induced by MNU which is not a hepatocarcinogen is rapidly repaired, whereas damage induced by hepatocarcinogens, such as acetoxyacetyl-aminofluorene and dimethylnitrosamine, is slowly restored.

Thus, the ability of embryonic liver cells to repair lesions in their DNA rapidly might be correlated with the observation that the liver is not the target organ for teratogenesis caused by many teratogens, including MNU, MMS and X-rays. Because of this, therefore, it is difficult to evaluate the significance of DNA repair in the process of teratogenesis by using embryonic liver cells.

C. Effects of methylnitrosourea and methyl methanesulfonate on the sedimentation of nucleoids from limb bud cells

MNU is a potent teratogen, while MMS is a weak teratogen. Both MNU and MMS have been reported to cause malformations of the extremities when given to pregnant NMRI mice on days 10 1/2, 11 or 12 of gestation (Bochert et al., 1978). The minimal dose that causes a teratogenic effect for MNU is 2.4-2.8 mg/Kg, and for MMS is 85-95 mg/Kg. No differences in the type of malformations were seen after MNU or MMS treatment. Thus, the limb buds seem to be the principal targets in MNU and MMS teratogenesis in mice. In order to examine whether MNU and MMS, at the doses studied earlier in embryonic liver cells, produced any malformation in mouse embryos of CD-1 strain, it was first necessary to study the teratogenic effects of these substances in CD-1 mice.

MNU or MMS was given as a single intraperitoneal injection to pregnant CD-1 mice on days 11 1/2 or 12 1/2 of gestation, and the occurrence of malformations was studied on day 18. Two pregnant mice were used for each experiment. Treatment with 20 mg/Kg MNU resulted in decreased fetal body weights, and produced 100% malformations of extremities, but the degree of severity observed in animals treated on day 11 1/2 was higher than in those treated on day 12 1/2. The limb

abnormalities included oligodactyly and adactyly of the fore and the hind limbs. In contrast, treatment with 25 mg/Kg MMS either on day 11 1/2 or on day 12 1/2 produced no malformation. At a higher dose of MMS, 50 mg/Kg, almost all embryos died, and a few embryos that were still alive showed no malformation. MMS at 100 mg/Kg caused 100% fetal death. Therefore, the dose of MMS at 25 mg/Kg was non-toxic and non-teratogenic to the mouse embryos. As a rough measure of the toxicity to the animals of the doses of MNU and MMS used, the doses of MNU (20 mg/Kg) and MMS (25 mg/Kg) were about 1/6 of the LD_{50} of MNU (123.7 mg/Kg) and 1/7 of the LD_{50} of MMS (176.2 mg/Kg), respectively. Thus, the selection of the doses of MNU and MMS seemed to be appropriate for a comparison of the effects of these two agents. From the teratogenic studies of MNU and MMS, it was shown clearly that at doses of similar toxicity to animals, MNU produced limb abnormalities, whereas MMS did not. It is important to contrast the nature of the lesions and their fate following treatment with these two agents. There are differences in reaction mechanisms between MNU and MMS as described earlier. Outstanding among these differences is the production of significant levels of O^6-methylguanine and phosphotriesters by MNU, contrasting with the barely detectable levels of these products following reaction of MMS with DNA. It is possible, therefore, that the potent teratogenic effect of MNU is largely attributable to these additional DNA modifications. Since the limb buds responded to the treatment of MNU and MMS differently, it was important to study DNA damage and repair in the cells of limb buds, and to determine whether there were any differences in the capacity of limb bud cells to repair DNA lesions induced by MNU and MMS.

MNU (20 mg/Kg) or MMS (25 mg/Kg) was given intraperitoneally to pregnant mice on day 11 1/2 or 12 1/2 of gestation 90 minutes after ^{3}H-thymidine administration as in previous experiments. At varying times after treatment, the embryos were removed from the uterine horns. The limb buds of embryos were removed and incubated in trypsin-EDTA solution in order to dissociate the cells as described in Materials and Methods. By using the trypsinization method, most of the cells were still alive, whereas those obtained from mechanical homogenization were dead. The limb bud cells were then used for nucleoid sedimentation analysis in neutral sucrose gradients. Fig. 19 shows the sedimentation profiles of nucleoids from limb bud cells of 12 1/2-day-old mouse embryos after MNU treatment. At 30 min and 7 hours, the nucleoids sedimented most slowly as seen by the sharp peaks near the top of the gradients. At 24 hours, there was a shift of the peak toward the bottom of the gradient, and at 48 hours, the peak was in the same position as the control peak, indicating the partial and complete repair of DNA strand breaks induced by MNU, respectively. Similar sedimentation patterns were obtained when the embryos were treated with MNU on day 11 1/2 of intrauterine life (Fig. 20).

In contrast, MMS affected the sedimentation of limb bud nucleoids differently as shown in Fig. 21. At 30 min, the MMS-treated nucleoids from limb bud cells of 12 1/2-day-old mouse embryos sedimented slowly, and at 24 hours they sedimented at the same rate as the untreated control nucleoids, indicating complete repair had already occurred by 24 hours after MMS treatment. Similar results were obtained with MMS treatment on day 11 1/2 of gestation (Fig. 22). These observations demonstrated that the cells of limb buds responded differently to the

Figure 19: Effect of methylnitrosourea on the sedimentation of nucleoids from the cells of limb buds in neutral sucrose gradients. Methylnitrosourea, 20 mg/Kg, was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation. The embryos were removed from the mothers at 30 minutes, 7 hours, 24 hours, and 48 hours after methylnitrosourea administration. The cells of limb buds were prepared by the trypsinization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methylnitrosourea, 30 minutes; ■: methylnitrosourea, 7 hours; o: methylnitrosourea, 24 hours; x: methylnitrosourea, 48 hours.

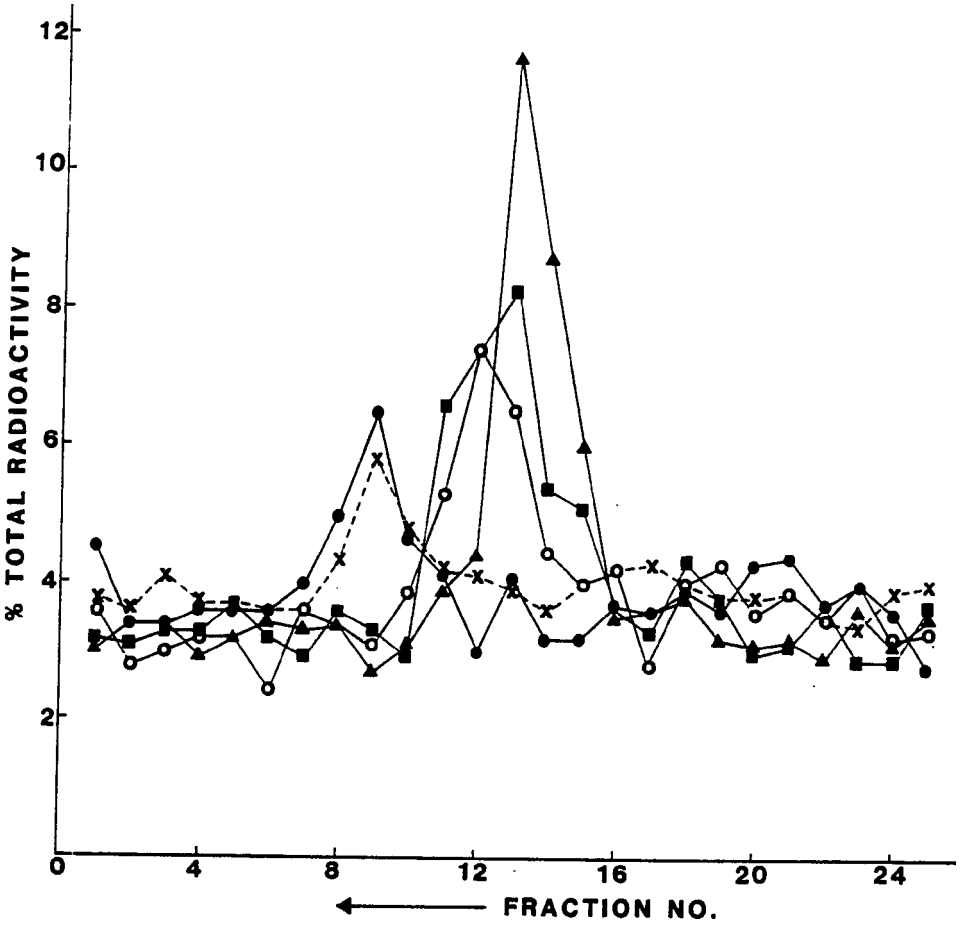

% TOTAL RADIOACTIVITY
12
10
8
6
4
2
0
4
8
12
16
20
24
FRACTION NO.

Figure 20: Effect of methylnitrosourea on the sedimentation of nucleoids from the cells of limb buds in neutral sucrose gradients. Methylnitrosourea, 20 mg/Kg, was given as a single intraperitoneal injection to pregnant mice on day 11 1/2 of gestation. The embryos were removed from the mothers at 30 minutes, 24 hours, and 48 hours after methylnitrosourea administration. The cells of limb buds were prepared by the trypsinization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methylnitrosourea, 30 minutes; o: methylnitrosourea, 24 hours; x: methylnitrosourea, 48 hours.

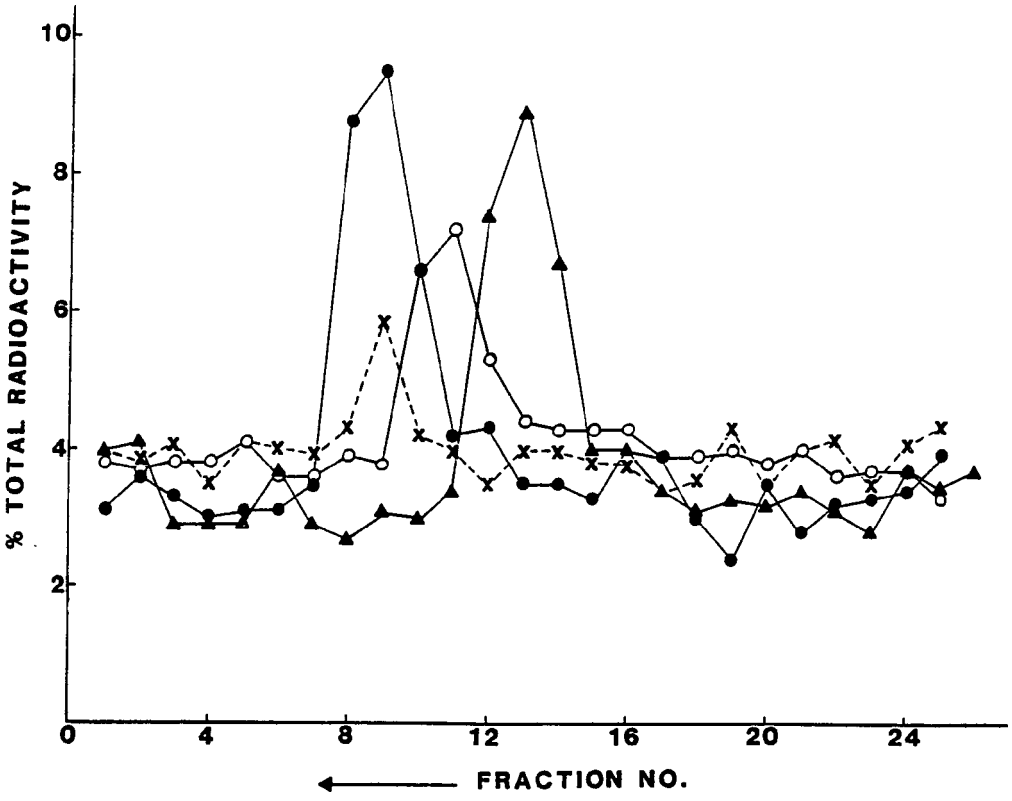

10
8
6
4
2
% TOTAL RADIOACTIVITY
0
4
8
12
16
20
24
FRACTION NO.

Figure 21: Effect of methyl methanesulfonate on the sedimentation of nucleoids from the cells of limb buds in neutral sucrose gradients. Methyl methanesulfonate, 25 mg/Kg, was given as a single intraperitoneal injection to pregnant mice on day 12 1/2 of gestation. The embryos were removed from the mothers at 30 minutes and 24 hours after methyl methanesulfonate administration. The cells of limb buds were prepared by the trypsinization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methyl methanesulfonate, 30 minutes; o: methyl methanesulfonate, 24 hours.

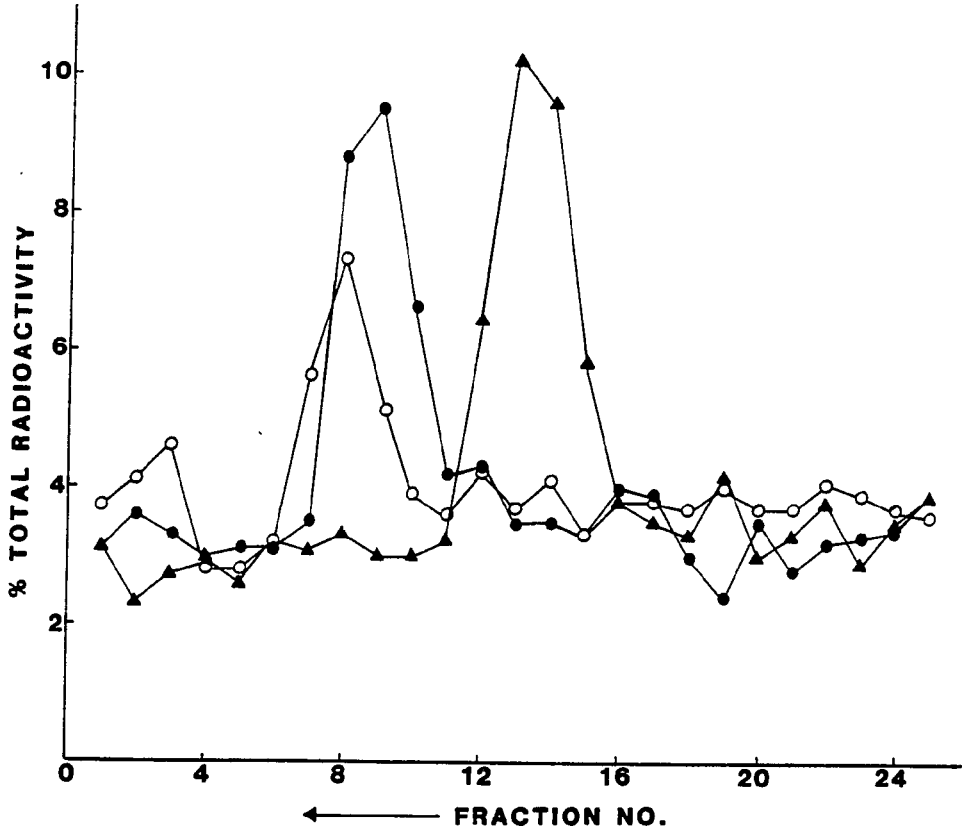
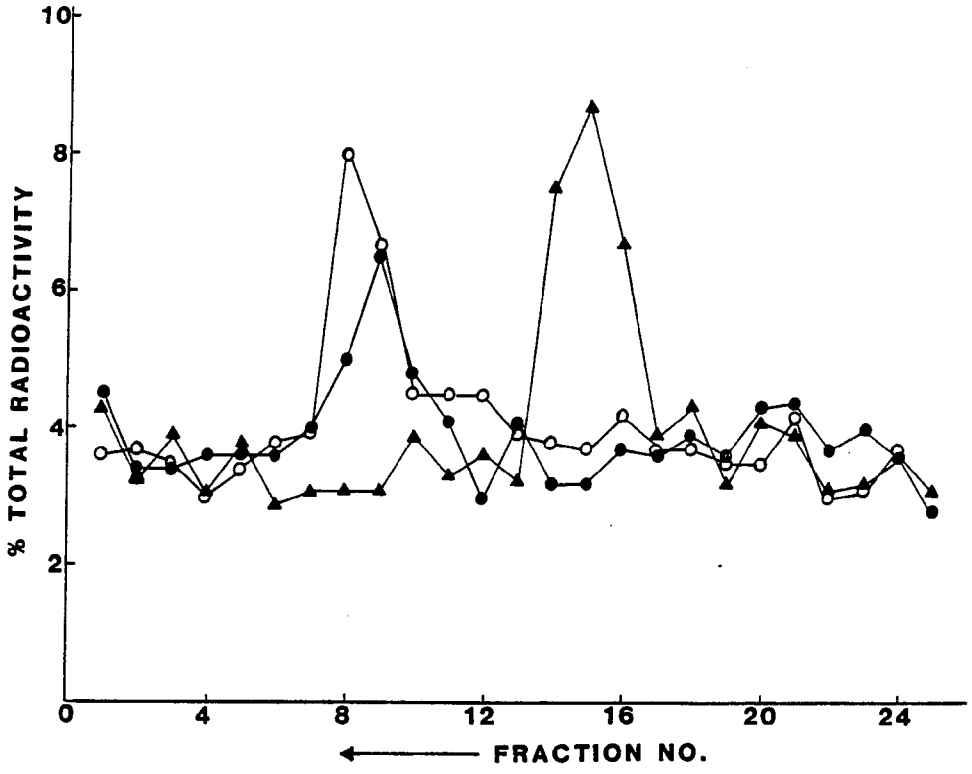

% TOTAL RADIOACTIVITY
10
8
6
4
2
0
4
8
12
16
20
24
FRACTION NO.

Figure 22: Effect of methyl methanesulfonate on the sedimentation of nucleoids from the cells of limb buds in neutral sucrose gradients. Methyl methanesulfonate, 25 mg/Kg, was given as a single intraperitoneal injection to pregnant mice on day 11 1/2 of gestation. The embryos were removed from the mothers at 30 minutes and 24 hours after methyl methanesulfonate administration. The cells of limb buds were prepared by the trypsinization method. The arrow indicates the direction of nucleoid sedimentation. ●: untreated control; ▲: methyl methanesulfonate, 30 minutes; o: methyl methanesulfonate, 24 hours.


10
8
6
4
2
% TOTAL RADIOACTIVITY
0
4
8
12
16
20
24
FRACTION NO.

damage of DNA induced by MNU and MMS. They could repair the damage induced by MMS completely within 24 hours, but that by MNU required 48 hours.

The fact that MNU produces more alkylation of the DNA in limb bud cells than does MMS might have been predicted by their different reaction mechanism. MNU, an S_N1 reagent, not only can react with strongly nucleophilic centers in DNA such as the N-7 of guanine, but also can react relatively more extensively with less nucleophilic centers, such as the O-6 of guanine and the phosphate groups in DNA, than can MMS which reacts through an S_N2 mechanism. An attempt has been made by Bochert et al. (1978) to correlate the teratogenic effects of MNU and MMS with the extent of methylation of a given base in embryonic DNA. They showed that a correlation between teratogenicity and the formation of N^7-methylguanine or N^3-methyladenine in embryonic DNA did not exist, and they suggested a hypothesis that the formation of O^6-alkylguanine is responsible for the induction of malformations. Since MNU produced limb abnormalities, whereas MMS did not, it therefore seemed reasonable to consider that the formation of O^6-methylguanine and phosphotriesters might also correlate with the production of limb malformations in the present study. Nucleoid sedimentation analysis experiments demonstrated that the limb bud cells in contrast to liver cells repaired MNU-induced DNA damage more slowly than MMS-induced DNA damage, suggesting that the slow rate of repair of limb bud cells might also be related to their susceptibility to the teratogenic effect of MNU. In contrast, the MMS-induced lesions were repaired as rapidly in limb buds as they were in embryonic liver cells. Since the liver showed no susceptibility to teratogenic effects of either MNU or

MMS, it is possible that the non-target nature of the liver might be due to its ability to repair lesions in DNA rapidly. The similar conclusion may be suggested for the non-target response of the limb buds to MMS administration. In the present study, no attempt has been made to determine the extent of DNA methylation caused by MNU and MMS in the embryonic tissues, because not enough DNA could be obtained from the very small amount of these embryonic tissues. Based on the studies with chemically-induced carcinogenesis, it has been shown that O^6-alkylguanine is the most likely lesion responsible for the production of tumors, and the persistence of this product in the DNA of certain tissues correlates with their susceptibility to tumor induction (Goth and Rajewsky, 1974a; Margison and Kleihues, 1975). In addition to O^6-alkylguanine, phosphotriesters have recently been considered to be involved in other biological effects caused by some alkylating agents including MNU (Shooter, 1978; Frei and Venitt, 1975). An indication that these chemically stable products are slowly lost from the DNA of animal tissues led to the conclusion that phosphotriesters are not excised by cellular enzymatic repair system. Because of this, the accumulation of phosphotriesters might play a more significant role in biological effects including carcinogenic and teratogenic effects of those alkylating agents. Studies with the RNA-containing bacteriophage R17 showed that a phosphotriester group in a single-stranded RNA does not itself constitute a lethal lesion but becomes lethal when hydrolysis of the group leads to chain scission (Shooter et al., 1974). It seems probable, therefore, that a phosphotriester might not be itself a lethal lesion, a carcinogenic lesion, or a teratogenic lesion in DNA. It has been shown that phosphotriesters are resistant to nuclease

activity (Jensen and Reed, 1978). Exhaustive enzymatic hydrolysis of MNU-treated poly [dA-dT] with nucleases and alkaline phosphatase generated nuclease-resistant methyl phosphotriesters, as well as unmodified nucleosides, base methylated nucleosides or free-methylated bases (Jensen, 1978). Due to its resistance to enzymic repair system, it is possible that the presence of phosphotriesters might interfere with excision repair of other adjacent DNA lesions which are carcinogenic or teratogenic lesions. Thus, the persistence of phosphotriesters may contribute to the biological effects of those damaging lesions. At the present time, other than indications that phosphotriesters are only slowly removed from cellular DNA, the biological consequences of these lesions remain obscure.

SUMMARY

The experiments reported here helped provide evidence that DNA is the principal target for many teratogens including physical and chemical agents. The aim of this dissertation research was to examine the capacity of embryonic cells to repair lesions in DNA during embryonic development so that this may provide an approach for understanding the basic mechanisms of teratogenesis.

In conventional experiments involving the induction of malformations in mouse fetuses by X-rays and alkylating agents, it was apparent that the extent and types of fetal anomalies were dependent upon the gestational day on which the teratogens were administered. Concurrent administration of some specific DNA repair inhibitors altered the frequency of malformations induced by those teratogens, suggesting that DNA repair may play an important role in modifying the teratogenic consequences of DNA damage. These observations led to the consideration that DNA damage and repair could be studied directly in developing embryos.

Analysis of the sedimentation of nucleoids derived from undifferentiated and differentiated cells of mouse embryos in neutral sucrose gradients containing different concentrations of ethidium bromide indicated that their DNA is supercoiled. Nucleoids are structures resembling nuclei, containing nuclear DNA and RNA, but depleted of nuclear proteins. These structures are released from cells when

cells are lysed in the presence of high salt concentrations and non-ionic detergents (Cook and Brazell, 1975; 1976). As their DNA is intact supercoiled, nucleoids sediment in neutral sucrose gradients rapidly. Introduction of single-strand breaks into DNA causes an unwinding of supercoils and a decrease in sedimentation rate. Repair of the breaks restores the DNA to its original superhelical structure and reestablishes the high normal sedimentation rate. Therefore, this method proved useful and highly sensitive for studying DNA damage and its repair through detection of single-strand breaks. This approach is possible because each strand break results in a measurable change in supercoiling. The experiments were performed by treating the pregnant mice, during day 11 1/2 or 12 1/2 of gestation, with various DNA-damaging agents, including methyl methanesulfonate and methylnitrosourea. At different times after treatment, the embryos were removed, and the cells from whole embryos, livers, or limb buds were isolated and used for nucleoid sedimentation analysis. At the dosages of MMS and MNU used in these experiments, it was shown that MMS was not teratogenic to mouse embryos, while MNU produced malformations of the extremities. The embryonic liver cells obtained from MMS-treated and MNU-treated embryos showed the same ability to repair the damage induced by these two agents. Complete repair was apparent within 24 hours. In contrast, the cells of limb buds responded differently to the damage of DNA induced by comparable doses of MMS and MNU. They repaired the damage induced by MMS completely within 24 hours, but that induced by MNU within 48 hours. These observations suggested a correlation between the capacity of the cells to repair DNA damage and the induction of malformations. The cells that can repair DNA lesions rapidly showed

no abnormality, whereas malformations occurred in organs from which the cells showed slow repair. Therefore, these experiments have established the importance of DNA repair processes in the production of birth defects.

BIBLIOGRAPHY

Agnish, N. D. and Kochhar, D. M. 1976 Direct exposure of postimplantation mouse embryos to 5-bromodeoxyuridine *in vitro* and its effect on subsequent chondrogenesis in the limbs. J. Embryol. Exp. Morphol. 36:623-638.

Alexander, P. and Lett, J. 1968 Effects of ionizing radiation on biological macromolecules. In M. Florkin and E. Stotz, eds., Comprehensive biochemistry, pp. 267-356. Elsevier, Amsterdam.

Alexandrov, V. A. 1969 Transplacental blastomogenic effect of N-nitrosomethylurea on the offspring of rats. Vop. Onkol. 15:55-61.

Bannigan, J. and Langman, J. 1978 The cellular effect of 5-bromodeoxyuridine on the mammalian embryo. In D. Neubert *et al.*, eds., Role of pharmacokinetics in prenatal and perinatal toxicology, pp. 545-557. Georg Thieme, Stuttgart.

Bateman, A. J. 1973 The dominant lethal assay in the mouse. Agents and Actions 3:73-76.

Bauer, W. and Vinograd, J. 1968 The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV40 DNA in the presence and absence of dye. J. Mol. Biol. 33:141-171.

Bloc, J. and Loman, H. 1973 The effects of γ-radiation in DNA. Curr. Top. in Rad. Res. 9:165-245.

Bochert, G. and Webb, J. 1977 The use of liquid ion-exchange chromatography for the determination of alkylated nucleic acid bases in maternal and fetal tissues. In D. Neubert *et al.*, eds., Methods in prenatal toxicology, pp. 456-464. Georg Thieme, Stuttgart.

Bochert, G., Rahm, U., and Schnieders, B. 1978 Pharmacokinetics of embryotoxic direct-acting alkylating agents: Comparison of DNA alkylation of various maternal tissues and the embryo during organogenesis. In D. Neubert *et al.*, eds., Role of pharmacokinetics in prenatal and perinatal toxicology, pp. 235-251. Georg Thieme, Stuttgart.

Brandt, W. N., Flamm, W. G., and Bernheim, N. J. 1972 The value of hydroxyurea in assessing repair synthesis of DNA in Hela cells. Chem. Biol. Interact. 5:327-339.

Brent, R. L. 1977 Radiations and other physical agents. In J. G. Wilson and F. C. Fraser, eds., Handbook of Teratology, vol. I, pp. 153-223. Plenum Press, New York.

Breter, H. J., Siebert, G., and Zahn, R. K. 1976 The use of high pressure liquid cation exchange chromatography for determination of the 5-methylcytosine content of DNA. J. Chromatogr. 118:242-249.

Brookes, P. and Lawley, P. D. 1961 The reaction of mono- and di-functional alkylating agents with nucleic acids. Biochem. J. 80:496-503.

Bücheler, J. and Kleihues, P. 1977 Excision of O^6-methylguanine from DNA of various mouse tissues following a single injection of N-methyl-N-nitrosourea. Chem. Biol. Interact. 16:325-333.

Buhl, S. N., Setlow, R. B., and Regan, J. D. 1972 Steps in DNA chain elongation and rejoining after ultraviolet irradiation of human cells. Int. J. Radiat. Biol. 22:417-424.

Buhl, S. N. and Regan, J. D. 1974 Effect of caffeine on postreplication repair in human cells. Biophys. J. 14:519-527.

Cerutti, P. A. 1975 Repairable damage in DNA: Overview. In P. C. Hanawalt and R. B. Setlow, eds., Molecular mechanisms for repair of DNA, part A, pp. 3-12. Plenum Press, New York.

Chahond, I. and Kwasigroch, T. E. 1977 Controlled breeding of laboratory animals. In D. Neubert et al., eds., Methods in prenatal toxicology, pp. 78-91. Georg Thieme, Stuttgart.

Cleaver, J. E. 1969 Repair replication of mammalian cell DNA: Effects of compounds that inhibit DNA synthesis of dark repair. Radiat. Res. 37:334-348.

Cleaver, J. E. 1970 DNA repair and radiation sensitivity in human (xeroderma pigmentosum) cells. Int. J. Radiat. Biol. 18:557-565.

Cleaver, J. E. 1974 Repair processes for photochemical damage in mammalian cells. Adv. Radiat. Biol. 4:1-75.

Cleaver, J. E. 1978 DNA repair and its coupling to DNA replication in eukaryotic cells. Biochim. Biophys. Acta 516:489-516.

Cook, P. R. 1973 Hypothesis on differentiation and the inheritance of gene superstructure. Nature 245:23-25.

Cook, P. R. 1974 On the inheritance of differentiated traits. Biol. Rev. 49:51-84.

Cook, P. R. and Brazell, I. A. 1975 Supercoils in human DNA. J. Cell Sci. 19:261-279.

Cook, P. R. and Brazell, I. A. (1976a) Conformational constraints in nuclear DNA. J. Cell Sci. 22:287-302.

Cook, P. R. and Brazell, I. A. (1976b) Detection and repair of single-strand breaks in nuclear DNA. Nature, Lond. 263:679-682.

Cook, J. S. and McGrath, J. R. 1967 Photoreactivating-enzyme activity in metazoa. Proc. Natl. Acad. Sci. U.S.A. 58:1359-1365.

Cozzarelli, N. R. 1980 DNA gyrase and the supercoiling of DNA. Science. 207:953-960.

Craddock, V. M. 1973 The pattern of methylated purines formed in DNA of intact and regenerating liver of rats treated with carcinogen dimethylnitrosamine. Biochim. Biophys. Acta 312:202-210.

Craddock, V. M. 1975 Effect of a single treatment with the alkylating carcinogens dimethylnitrosamine, diethylnitrosamine and methyl methanesulfonate on liver regeneration after partial hepatectomy. II. Alkylation of DNA and inhibition of DNA replication. Chem. Biol. Interact. 10:323-332.

Crawford, L. V. and Waring, M. J. 1967 Supercoiling of polyoma virus DNA measured by its interaction with ethidium bromide. J. Mol. Biol. 25:23-30.

Daoud, A. H. and Irving, C. C. 1977 Methylation of DNA in rat liver and intestine by dimethylnitrosamine and N-methylnitrosourea. Chem. Biol. Interact. 16:135-143.

Dencker, L. 1978 Localization and diffusible substances in the embryo. In D. Neubert et al., eds., Role of pharmacokinetics in prenatal and perinatal toxicology, pp. 535-544. Georg Thieme, Stuttgart.

Deutsch, W. A. and Linn, S. 1979 DNA binding capacity from cultured human fibroblasts that is specific for partially depurinated DNA and that inserts purines into apurinic sites. Proc. Natl. Acad. Sci. U.S.A. 76:141-144.

de Weerd-Kastelein, E. A., Keijzer, W., Rainaldi, G., and Bootsma, D. 1977 Induction of sister chromatid exchanges in xeroderma pigmentosum cells after exposure to ultraviolet light. Mutat. Res. 45:253-270.

Djordjevic, B. and Tolmach, L. J. 1967 Response to synchronized populations of Hela cells to ultraviolet irradiation at selected stages of the generation cycle. Radiat. Res. 32:327-346.

Dunlap, B. and Cerutti, P. 1975 Apyrimidinic sites in gamma irradiated DNA. FEBS Lett. 51:188-190.

Fraser, F. C. 1961 The use of teratogen in the analysis of abnormal development mechanisms. In M. Fishbein, ed., First international conference on congenital malformation genetics, pp. 179-186. Lippincott, Philadelphia.

Frei, J. V. and Lawley, P. D. 1975 Methylation of DNA in various organs of C57Bl mice by a carcinogenic dose of N-methyl-N-nitrosourea and stability of some methylation products up to 18 hours. Chem. Biol. Interact. 10:413-427.

Frei, J. V. and Venitt, S. 1975 Chromosome damage in the bone marrow of mice treated with the methylating agents methyl methanesulfonate and N-methyl-N-nitrosourea in the presence or absence of caffeine, and its relationship with thymoma induction. Mutat. Res. 29:89-96.

Fujii, T., Nakatsuka, T., Inouye, M., and Shoji, R. 1976 Caffeine enhancement of the teratogenic action of chlorambucil in the mouse. (Abs. in Teratology 16:103).

Fujiwara, Y. (1975a) Postreplication repair of alkylation damage to DNA of mammalian cells in culture. Cancer Res. 35:2780-2789.

Fujiwara, Y. (1975b) Caffeine-sensitive postreplication repair of N-methyl-N-nitrosourea damage in mouse L cells. Mutat. Res. 31:260-261.

Fujiwara, Y. and Tatsumi, M. (1976a) Replicative by-pass repair of ultraviolet damage to DNA of mammalian cells: Caffeine sensitive and caffeine resistant mechanisms. Mutat. Res. 37:91-110.

Fujiwara, Y. and Tatsumi, M. (1976b) Replicative repair of UV damage to normal human cells. Mutat. Res. 38:335-336.

Ganesan, A. K. 1974 Persistence of pyrimidine dimers during post-replication repair in ultraviolet light-irradiated Escherichia coli K-12. J. Mol. Biol. 87:103-119.

Ganesan, A. K. and Seawell, P. C. 1975 The effect of lex A and rec F mutations on postreplication repair and DNA synthesis in Escherichia coli K-12. Mol. Gen. Genet. 141:189-205.

Gaudin, D., Gregg, R. S., and Yielding, K. L. 1971 DNA repair inhibition: A possible mechanism of action of cocarcinogens. Biochem. Biophys. Res. Commun. 45:630-636.

Gaudin, D., Gregg, R. S., and Yielding, K. L. (1972a) Inhibition of DNA repair replication by DNA binding drugs which sensitize cells to alkylating agents and X-rays. Proc. Soc. Expl. Biol. Med. 141:543-547.

Gaudin, D., Gregg, R. S., and Yielding, K. L. (1972b) Inhibition of DNA repair by cocarcinogens. Biochem. Biophys. Res. Commun. 48:945-949.

Gaudin, D., Gutherie, L., and Yielding, K. L. 1974 DNA repair inhibition: A new mechanism of action of steroids with possible implications for tumor therapy. Proc. Soc. Exp. Biol. Med. 146:401-405.

Gellert, M. 1981 DNA topoisomerases. Ann. Rev. Biochem. 50:879-910.

Gellert, M., Mizuuchi, K., O'Dea, M. H., and Nash, H. A. 1976 DNA gyrase: An enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. U.S.A. 73:3872-3876.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., and Tomizawa, J. 1977 Nalidixic acid resistance: A second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. U.S.A. 47:4772-4776.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Ohmori, H., and Tomizawa, J. 1978 DNA gyrase and DNA supercoiling. Cold Spring Harbor Symp. Quant. Biol. 43:35-40.

Gibson, J. E. and Becker, B. A. 1968 The teratogenicity of cyclophosphamide in mice. Cancer Res. 28:475-480.

Glücksmann, A. and Tansley, K. 1936 Some effects of gamma radiation on the developing rat retina. Br. J. Ophthal. 20:497-509.

Goldstein, A. 1964 Biostatistics: An introductory text. McMillan, New York.

Goth, R. and Rajewsky, M. F. 1972 Ethylation of nucleic acids by ethylnitrosourea-1-^{14}C in the fetal and adult rat. Cancer Res. 32:1501-1505.

Goth, R. and Rajewsky, M. F. (1974a) Persistence of O^6-ethylguanine in rat brain DNA: Correlation with nervous system-specific carcinogenesis by ethylnitrosourea. Proc. Natl. Acad. Sci. U.S.A. 71:639-643.

Goth, R. and Rajewsky, M. F. (1974b) Molecular and cellular mechanisms associated with pulse-carcinogenesis in the rat nervous system by ethylnitrosourea: Ethylation of nucleic acids and elimination rates of ethylated bases from the DNA of different tissues. Z. Krebsforsch. 82:37-64.

Goz, B. 1978 Halogenated deoxyuridines. Pharmacol. Rev. 29:249-272.

Griggs, H. G. and Bender, M. A. 1972 Ultraviolet and gamma-ray induced reproductive death and photoreactivation in a Xenopus tissue culture cell line. Photochem. Photobiol. 15:517-526.

Grossman, L. 1974 Enzymes involved in the repair of DNA. Adv. Radiat. Biol. 4:77-129.

Grossman, L., Braun, A., Feldberg, R., and Mahler, I. 1975 Enzymatic repair of DNA. Ann. Rev. Biochem. 44:19-43.

Hadi, S. and Goldthwait, D. A. 1971 Endonuclease II of Escherichia coli. Degradation of partially depurinated deoxyribonucleic acid. Biochemistry 10:4986-4994.

Hanawalt, P. C., Cooper, P. K., Ganesan, A. K., and Smith, C. A. 1979 DNA repair in bacteria and mammalian cells. Ann. Rev. Biochem. 48:783-836.

Harm, H. 1980 Damage and repair in mammalian cells after exposure to non-ionizing radiations. III. Ultraviolet and visible light irradiation of cells of placental mammals, including humans, and determination of photo-repairable damage in vitro. Mutat. Res. 69:167-176.

Herken, R. 1977 Application of autoradiography in teratology. In D. Neubert et al., eds., Methods in prenatal toxicology, pp. 322-327. Georg Thieme, Stuttgart.

Herrold, K. McD. 1967 Pigmentary degeneration of the retina induced by N-methyl-N-nitrosourea: An experimental studies in Syrian hamsters. Arch. Ophthal. 78:650-653.

Higgins, N. P., Kato, K., and Strauss, B. 1976 A model for replication repair in mammalian cells. J. Mol. Biol. 101:417-425.

Howard-Flanders, P. 1973 DNA repair and recombination. Br. Med. Bull. 29:226-235.

Hutchinson, F. 1978 DNA strand break repair in eukaryotes. In P. C. Hanawalt et al., eds., DNA repair mechanisms, pp. 457-463. Academic Press, New York.

Issaq, H. J., Barr, E. W., and Zielinski, W. L., Jr. 1977 Separation of alkylated guanines, adenines, uracils, and cytosines by thin-layer chromatography. J. Chromatogr. 131:265-273.

Ivankovic, S. and Druckery, H. 1968 Transplacentare Erzeugung maligner tumoren des nervensystems. I. Athylnitrosoharnstoff (ÄNH) an BD-IX Ratten. Z. Krebsforsch. 71:320-360.

Jensen, D. E. 1978 Reaction of DNA with alkylating agents. Differential alkylation of poly [dA-dT] by methylnitrosourea and ethylnitrosourea. Biochemistry 17:5108-5113.

Jensen, D. E. and Reed, D. J. 1978 Reaction of DNA with alkylating agents. Quantitation of alkylation by ethylnitrosourea of oxygen and nitrogen sites on poly [dA-dT] including phosphotriester formation. Biochemistry 17:5098-5107.

Jolley, G. M. and Ormerod, M. G. 1973 An improved method for measuring cross links in the DNA of mammalian cells: The effect of nitrogen mustard. Biochim. Biophys. Acta 308:242-251.

Kalter, H. 1971 Correlations between teratogenic and mutagenic effects of chemicals in mammals. In A. Hollaender, ed., Chemical Mutagens, pp. 57-82. Plenum Press, New York.

Kato, H. 1973 Induction of sister chromatid exchanges by UV light and its inhibition by caffeine. Exp. Cell. Res. 82:383-390.

Kato, H. 1974 Induction of sister chromatid exchanges by chemical mutagens and its possible relevance to DNA repair. Exp. Cell Res. 85:239-247.

Kato, H. 1977 Spontaneous and induced sister chromatid exchanges as revealed by the BUdR-labeling method. Int. Rev. Cytol. 49:55-97.

Kato, H. and Stich, H. F. 1976 Sister chromatid exchanges in aging and repair-deficient human fibroblasts. Nature, Lond. 260: 447-448.

Kirlman, B. A., Sturelid, S., Hartly-Asp, B., and Nilsson, K. 1974 The enhancement by caffeine of the frequencies of chromosomal aberrations induced in plant and animal cells. Mutat. Res. 26:105-122.

Kirtikar, D. M. and Goldthwait, D. A. 1974 The enzymatic release of O^6-methylguanine and 3-methyladenine from DNA reacted with the carcinogen N-methyl-N-nitrosourea. Proc. Natl. Acad. Sci. U.S.A. 71:2022-2026.

Klamerth, O. L. 1978 Inhibition of postreplication repair by isonicotinic acid hydrazide. Mutat. Res. 50:251-256.

Kleihues, P. and Magee, P. N. 1973 Alkylation of rat brain nucleic acids by N-methyl-N-nitrosourea and methyl methanesulfonate. J. Neurochem. 20:595-606.

Kleihues, P. and Margison, G. P. 1974 Carcinogenicity of N-methyl-N-nitrosourea: Possible role of excision repair of O^6-methylguanine from DNA. J. Natl. Cancer Inst. 53:1839-1841.

Klinger, W. and Muller, D. 1976 Developmental aspects of xenobiotic transformation. Environ. Health Perspect. 18:13-23.

Kohn, K. W., Spears, L. S., and Doty, P. 1966 Interstrand crosslinking of DNA by nitrogen mustard. J. Mol. Biol. 19:266-268.

Kohn K. W. and Ewig, R. A. G. 1973 Alkaline elution analysis, a new approach to the study of DNA single-strand interruptions in cells. Cancer Res. 33:1849-1853.

Kondo, S. 1977 A test for mutation theory of cancer: Carcinogenesis by misrepair of DNA damaged by 4-nitroquinoline-1-oxide. Br. J. Cancer 35:595-601.

Kovacs, K. and Steinberg, A. D. 1972 Cyclophosphamide: Drug interactions and bone marrow transplantation. Transplantation 13:316-321.

Kulkarni, M. S. and Yielding, K. L. 1978 Inhibition of UV-induced DNA repair at different steps by quinacrine and anthralin. Biochem. Biophys. Res. Comm. 83:1531-1537.

Lahav, M., Albert, D. M., and Wyand, S. 1973 Clinical and histopathologic classification of retinal dysplasia. Amer. J. Ophthal. 75:648-667.

Latt, S. A. 1974 Sister chromatid exchanges, indices of human chromosome damage and repair: Detection by fluorescence and induction by mitomycin C. Proc. Natl. Acad. Sci. U.S.A. 71:3162-3166.

Lawley, P. D. 1966 Effects of some chemical mutagens and carcinogens on nucleic acids. Prog. Nucleic Acid Res. Mol. Biol. 5:89-131.

Lawley, P. D. 1973 Reaction of N-methyl-N-nitrosourea (MNU) with ^{32}P-labeled DNA: Evidence for formation of phosphotriesters. Chem. Biol. Interact. 7:127-130.

Lawley, P. D. 1974 Some chemical aspects of dose-response relationships in alkylation mutagenesis. Mutat. Res. 23:283-295.

Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V. 1969 Inactivation of bacteriophage T_7 by mono- and di-functional sulfur mustards in relation to crosslinking and depurination of bacteriophage DNA. J. Mol. Biol. 39:181-198.

Lawley, P. D. and Martin, C. N. 1975 Molecular mechanisms in alkylation mutagenesis: Induced reversion of bacteriophage T_4r 11AP72 by ethyl methanesulfonate in relation to extent and mode of ethylation of purines in bacteriophage deoxyribonucleic acid. Biochem. J. 145:85-91.

Lawley, P. D. and Shah, S. A. 1972 Methylation of RNA by the carcinogens dimethylsulfate, N-methyl-N-nitrosourea and N-methyl-N'-nitro-N-nitrosoguanidine: Comparison of chemical analyses at the nucleoside and base levels. Biochem. J. 128:117-132.

Lehmann, A. R. 1972 Postreplication repair of DNA in ultraviolet-irradiated mammalian cells. J. Mol. Biol. 66:319-337.

Lehmann, A. R. and Kirk-Bell, S. 1974 Effects of caffeine and theophylline on DNA synthesis in unirradiated and UV-irradiated mammalian cells. Mutat. Res. 26:73-82.

Lehmann, A. R., Kirk-Bell, S., Arlett, C. F., Paterson, M. C., Lohman, P. H. M., de Weerd-Kastelein, E. A., and Bootsma, D. 1975 Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. Proc. Natl. Acad. Sci. U.S.A. 72:219-223.

Lieberman, M. W., Baney, R. N., Lee, R. E., Sell, S., and Farber, E. 1971 Studies on DNA repair in human lymphocytes treated with proximate carcinogens and alkylating agents. Cancer Res. 31:1297-1306.

Lindahl, T. 1976 New class of enzymes acting on damaged DNA. Nature, Lond. 259:64-66.

Lindahl, T. 1979 DNA glycosylases, endonucleases for apurinic/apyrimidinic sites, and base excision-repair. Prog. Nucleic Acid Res. Mol. Biol. 22:135-192.

Lindahl, T. and Andersson, A. 1972 Rate of chain breakage at apurinic sites in double stranded deoxyribonucleic acid. Biochemistry 11:3618-3623.

Ljungquist, S. and Lindahl, T. 1974 A mammalian endonuclease specific for apurinic site in double-stranded deoxyribonucleic acid. I. Purification and properties. J. Biol. Chem. 249:1530-1535.

Ljungquist, S., Andersson, A. and Lindahl, T. 1974 A mammalian endonuclease specific for apurinic sites in double-stranded deoxyribonucleic acid. II. Further studies on substrate specificity. J. Biol. Chem. 249:1536-1540.

Ljungquist, S., Nyberg, B., and Lindahl, T. 1975 Mammalian DNA endonuclease acting at apurinic site: Absence of associated exonuclease activity. FEBS Lett. 57:169-171.

Loveless, A. 1969 Possible relevance of O-6 alkylation of deoxyguanine to mutagenicity and carcinogenicity of nitrosamine and nitrosamides. Nature, Lond. 223:206-207.

Maher, V. M. and McCormick, J. J. 1979 DNA repair and carcinogenesis. In P. L. Grover, Chemical carcinogen and DNA, vol. II, pp. 133-158, CRC Press, Boca Raton.

Margison, G. P. and Kleihues, P. 1975 Chemical carcinogenesis in the nervous system: Preferential accumulation of O^6-methylguanine in rat brain deoxyribonucleic acid during repetitive administration of N-methyl-N-nitrosourea. Biochem. J. 148:521-529.

Margison, G. P., Margison, J. M., and Montesano, R. 1977 Accumulation of O^6-methylguanine in non-target-tissue deoxyribonucleic acid during chronic administration of dimethylnitrosamine. Biochem. J. 165:463-468.

McGrath, R. A. and Williams, R. W. 1966 Reconstruction in vivo of irradiated E. coli DNA: The rejoining of broken pieces. Nature, Lond. 212:534-535.

Menkes, B., Sandar, S., and Ilies, A. 1970 Cell death in teratogenesis. Advan. Terat. 4:170-206.

Michael, R. O. and Sarma, D. S. 1973 Measurement of in vivo damage and repair of rat liver DNA induced by chemical carcinogens. Proc. Amer. Assoc. Cancer Res. 14:5.

Michael, R. O. and Williams, G. M. 1974 Chloroquine inhibition of repair of DNA damage induced in mammalian cells by methyl methanesulfonate. Mutat. Res. 25:391-396.

Montesano, R., Brésel, B., and Margison, G. P. 1979 Increase excision of O6-methylguanine from rat liver DNA after chronic administration of dimethylnitrosamine. Cancer Res. 39:1798-1802.

Mortelmans, K., Cleaver, J. E., Friedberg, E. C., Paterson, M. C., Smith, B. P., and Thomas, G. H. 1977 Photoreactivation of thyine dimers in UV-irradiated human cells: Unique dependence on culture conditions. Mutat. Res. 44:433-446.

Murthy, A. S. K., Vawter, G. F., Kopito, L., and Rossen, E. 1972 Retinal atrophy and cataract in rats following administration of N-methyl-N-nitrosourea. Proc. Soc. Exp. Biol. Med. 139:84-87.

Nicoll, J. W., Swann, P. F., and Pegg, A. E. 1975 Effect of dimethylnitrosamine on persistence of methylated guanines in rat liver and kidney DNA. Nature, Lond. 254:261-262.

Nomura, T. 1977 Similarity of the mechanism of chemical carcinogen-initiated teratogenesis and carcinogenesis in mice. Cancer Res. 37:969-973.

O'Connor, P. J., Capps, M. J., and Craig, A. W. 1973 Comparative studies of the hepatocarcinogen N-N-dimethylnitrosamine in vivo: Reaction sites in rat liver DNA and the significance of their relative stabilities. Br. J. Cancer 27:153-166.

Okoyama, S. and Kitao, Y. 1981 Inhibition of chromosome repair by caffeine or isonicotinic acid hydrazide on chromosome damage induced by mitomycin C in human lymphocytes. Mutat. Res. 81:75-80.

Painter, R. and Young, B. 1972 Repair replication in mammalian cells after X-irradiation. Mutat. Res. 14:225-235.

Pegg, A. E. 1978 Enzymatic removal of O^6-methylguanine from DNA by mammalian cell extracts. Biochem. Biophys. Res. Commun. 84:166-173.

Peleg, L., Raz, E., and Ben-Ishai, R. 1976 Changing capacity for DNA excision repair in mouse embryonic cells in vitro. Exp. Cell Res. 104:301-307.

Perry, P. and Evans, H. J. 1975 Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature, Lond. 258:121-124.

Pettijohn, D. E. and Hanawalt, P. C. 1964 Evidence for repair replication of ultraviolet damaged DNA in bacteria. J. Mol. Biol. 9:395-410.

Radding, C. M. 1978 Genetic recombination strand transfer and mismatch repair. Ann. Rev. Biochem. 47:847-880.

Radman, M. 1974 Phenomenology of an inducible mutagenic DNA repair pathway in Escherichia coli: SOS repair hypothesis. In L. Prokash et al., eds., Molecular and environmental aspects of mutagenesis, pp. 128-142. Thomas, Springfield, Ill.

Radman, M. 1975 SOS repair hypothesis: Phonomenology of an inducible DNA repair which is accompanied by mutagenesis. In P. C. Hanawalt and R. B. Setlow, eds., Molecular mechanisms for repair of DNA, part A, pp. 355-367. Plenum Press, New York.

Raineri, R., Poiley, J. A., Hillesund, T., and Pienta, R. J. 1978 Factors affecting the binding of polycyclic aromatic hydrocarbons to human embryo cells, and transformable and non-transformable hamster embryo cells. Chem. Biol. Interact. 22:257-270.

Regan, J. D. and Setlow, R. B. 1974 Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. Cancer Res. 34:3318-3325.

Regan, J. D., Setlow, R. B., and Ley, R. D. 1971 Normal and defective repair of damaged DNA in human cells: A sensitive assay utilizing the photolysis of bromodeoxyuridine. Proc. Natl. Acad. Sci. U.S.A. 68:708-712.

Regan, J. D., Trosko, J. E., and Carrier, W. L. 1968 Evidence for excision of ultraviolet-induced pyrimidine dimers from the DNA of human cells in vitro. Biophys. J. 8:319-325.

Regan, J. D., Setlow, R. B., Francis, A. A., and Lijinsky, W. 1976 Nitrosocarbaryl: Its effect on human DNA. Mutat. Res. 38:293-302.

Rhaese, H. and Freese, E. 1969 Chemical analysis of DNA alterations. IV. Reactions of oligodeoxynucleotides with monofunctional alkylating agents leading to backbone breakage. Biochim. Biophys. Acta 190:418-433.

Rice, J. M. 1969 Transplacental carcinogenesis in mice by 1-ethyl-1-nitrosourea. Ann. N.Y. Acad. Sci. 163:813-827.

Rice, J. M. (1979a) Perinatal period and pregnancy: Intervals of high risk for chemical carcinogens. Environ. Health. Perspect. 29:23-27.

Rice, J. M. (1979b) Problems and perspectives in perinatal carcinogenesis: A summary of the conference. Natl. Cancer Inst. Monogr. 51:271-278.

Ritter, E. J., Scott, W. J., and Wilson, J. G. 1971 Teratogenesis and inhibition of DNA synthesis induced in rat embryos by cytosine arabinoside. Teratology 4:7-14.

Ritter, E. J., Scott, W. J., and Wilson, J. G. 1973 Relationship of temporal patterns of cell death and development to malformations in the rat limb: Possible mechanisms of teratogenesis with inhibitors of DNA synthesis. Teratology 7:219-226.

Ritter, M. A. and Cleaver, J. E. 1977 High-LET radiation induce a large proportion of non-rejoining DNA breaks. Nature, Lond. 266:653-655.

Roberts, J. J. 1978 The repair of DNA modified by cytotoxic, mutagenic, and carcinogenic chemicals. Adv. Radiat. Biol. 7:211-436.

Roberts, J. J. 1980 Cellular response to carcinogen-induced DNA damage and the role of DNA repair. Br. Med. Bull. 36:25-31.

Roberts, J. J. and Ward, K. N. 1973 Inhibition of postreplication repair of alkylated DNA by caffeine in Chinese hamster cells but not in Hela cells. Chem. Biol. Interact. 7:241-262.

Roberts, J. J., Sturrock, J. E., and Ward, K. N. 1974 The enhancement by caffeine of alkylation-induced cell death, mutations and chromosomal aberrations in Chinese hamster cells as a result of inhibition of postreplication DNA repair. Mutat. Res. 26:129-143.

Rogers, K. J. and Pegg, A. E. 1977 Formation of O^6-methylguanine by alkylation of rat liver, colon and kidney DNA following administration of 1,2-dimethylhydrazide. Cancer Res. 37:4082-4087.

Roti Roti, J. and Cerutti, P. 1974 Gamma-ray induced thymine damage in mammalian cells. Int. J. Radiat. Biol. 25:413-417.

Rupp, W. D. and Howard-Flanders, P. 1968 Discontinuities in the DNA synthesized in an excision-defective strain of Escherichia coli following ultraviolet irradiation. J. Mol. Biol. 31:291-304.

Russell, L. B. and Russell, W. L. 1952 Radiation hazards to the embryo and fetus. Radiology 58:369-376.

Russell, L. B. and Russell, W. L. 1954 An analysis of the changing radiation response of the developing mouse embryo. J. Cell Comp. Physiol. Suppl. 1. 43:103-150.

Rutter, W. J., Picket, R. L., Githens, S., III, and Gordon, J. S. 1975 The mode of action of the thymidine analogue, 5-bromodeoxyuridine, a model teratogenic agent. In D. Neubert and H. J. Merker, eds., New approaches to the evolution of abnormal embryonic development, pp. 804-821. Georg Thieme, Stuttgart.

Sadler, T. W. and Kochhar, D. M. 1976 Biosynthesis of DNA, RNA and proteins of mouse embryos cultured in the presence of a teratogenic dose of chlorambucil. J. Embryol. Exp. Morph. 36:273-281.

Samson, L. and Cairns, J. 1977 A new pathway for DNA repair in Escherichia coli. Nature, Lond. 267-281-283.

Schoental, R. 1969 Lack of correlation between the pressure of 7-methylguanine in DNA and RNA of organs and localization of tumors after a single carcinogenic dose of N-methyl-N-nitrosourethane. Biochem. J. 114:55P-56P.

Scott, W. J., Ritter, E. J., and Wilson, J. G. 1971 DNA synthesis inhibitors and cell death associated with hydroxyurea teratogenesis in rat embryos. Dev. Biol. 26:306-315.

Scott, W. J., Ritter, E. J., and Wilson, J. G. 1973 DNA synthesis inhibition, cytotoxicity and their relationship to teratogenesis following administration of a nicotinamide antagonist, aminothiadiazole, to pregnant rats. J. Embryol. Exp. Morph. 30:257-266.

Scudiero, D., Henderson, E., Norin, A., and Strauss, B. 1975 The measurement of chemically-induced DNA repair synthesis in human cells by BND-cellulose chromatography. Mutat. Res. 29:473-488.

Setlow, R. B. 1968 The photochemistry, photobiology, and repair of polynucleotides. Proc. Nucleic Acid. Res. Mol. Biol. 8:257-295.

Setlow, R. B. and Carrier, W. L. 1964 The disappearance of thymine dimers from DNA: An error-correcting mechanism. Prog. Natl. Acad. Sci. U.S.A. 51:226-231.

Shack, J. and Byrum, B. S. 1964 Interdependence of variables in the activation of deoxyribonuclease. J. Biol. Chem. 239:3843-3848.

Shimida, M., Wakaizumi, S., Kasubuchi, Y., Kusunoki, T., and Nakamura, T. 1973 Cytosine arabinoside and rosette formation in mouse retina. Nature, Lond. 236:151-152.

Shooter, K. V. 1978 DNA phosphotriesters as indicators of cumulative carcinogen-induced damage. Nature, Lond. 274-612-614.

Shooter, K. V., Howse, R., and Merrifield, R. K. 1974 The reaction of alkylating agents with bacteriophage R17: Biological effects of phosphotriester formation. Biochem. J. 137:313-317.

Shooter, K. V. and Slade, T. A. 1977 The stability of methyl and ethyl phosphotriesters in DNA in vivo. Chem. Biol. Interact. 19:353-361.

Shooter, K. V., Slade, T. A., and O'Connor, P. J. 1977 The formation and stability of methyl phosphotriesters in the DNA of rat tissues after treatment with the carcinogen N,N-dimethylnitrosamine. Chem. Biol. Interact. 19:363-367.

Short, R. D., Rao, K. S., and Gibson, J. E. 1972 The in vivo biosynthesis of DNA, RNA and proteins in mouse embryos after a teratogenic dose of cyclophosphamide. Teratology 6:129-138.

Stockdale, F., Okazaki, K., Nameroff, M., and Holtzer, H. 1964 5-bromodeoxyuridine: Effect on myogenesis in vitro. Science 146:533-535.

Strauss, B. and Hill, T. 1970 The intermediate in the degradation of DNA alkylated with a monofunctional alkylating agent. Biochim. Biophys. Acta 213:14-25.

Strauss, B., Scudiero, D., and Henderson, E. 1975 The nature of the alkylation lesions in mammalian cells. In P. C. Hanawalt and R. B. Setlow, eds., Molecular mechanisms for repair of DNA, pp. 13-24. Plenum Press, New York.

Sugino, A., Peebles, C. L., Kreuzer, K. N., and Cozzarelli, N. R. 1977 Mechanism of action of nalidixic acid: Purification of Escherichia coli nal A gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. U.S.A. 47:4767-4771.

Sun, L. and Singer, B. 1975 The specificity of different classes of ethylating agents towards various sites of Hela cell DNA in vitro and in vivo. Biochemistry 8:1795-1802.

Sutherland, B. M. and Oliver, R. 1976 Culture conditions affect photoreactivating enzyme levels in human fibroblasts. Biochim. Biophys. Acta 442:358-367.

Swann, P. F. and Magee, P. N. 1968 Nitrosamine-induced carcinogenesis. The alkylation of nucleic acids of the rat by N-methyl-N-nitrosourea, dimethylnitrosamine, dimethylsulfate and methyl methanesulfonate. Biochem. J. 110:39-47.

Swann, P. F. and Magee, P. N. 1971 Nitrosamine-induced carcinogenesis. The alkylation of N-7 of guanine of nucleic acids of the rat by diethylnitrosamine, N-ethyl-N-nitrosourea and ethyl methanesulfonate. Biochem. J. 125:841-847.

Timson, J. 1977 Caffeine. Mutat. Res. 47:1-52.

Thielmann, H. W., Vosberg, H. P., and Reygers, V. 1975 Carcinogen-induced DNA repair in nucleotide-permeable Escherichia coli cells. Induction of DNA repair by the carcinogens methyl and ethyl nitrosourea and methyl methanesulfonate. Eur. J. Biochem. 56:433-447.

Verly, W. J. and Paquette, Y. 1972 An endonuclease for depurinated DNA in Escherichia coli. Can. J. Biochem. 50:217-224.

Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P. 1965 The twisted circular form of polyoma viral DNA. Proc. Natl. Acad. Sci. U.S.A. 53:1104-1111.

Waddell, W. J. and Marlowe, C. 1981 Biochemical regulation of the accessibility of teratogens to the developing embryos. In M. R. Juchau, ed., The biochemical basis of chemical teratogenesis, pp. 1-62. Elsevier/North-Holland, New York.

Walker, I. G. and Ewart, D. F. (1973a) The nature of single-strand breaks in DNA following treatment of L-cells with methylating agents. Mutat. Res. 19:331-341.

Walker, I. G. and Ewart, D. F. (1973b) Repair synthesis of DNA in Hela and L-cells following treatment with methylnitrosourea or ultraviolet light. Can. J. Biochem. 51:148-157.

Wang, J. C. and Liu, L. F. 1979 DNA topoisomerases: Enzymes that catalyze the concerted breaking and rejoining of DNA backbone bonds. In J. H. Taylor, ed., Molecular genetics, part III, pp. 65-88. Academic Press, New York.

Watson, J. D. and Crick, F. H. 1953 Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. Nature, Lond. 171:737-738.

Wheeler, G. P. 1962 Studies related to the mechanisms of action of cytotoxic alkylating agents: A review. Cancer Res. 22:651-688.

Wilson, J. G. (1973a) Environment and birth defects, pp. 11-32. Academic Press, New York.

Wilson, J. G. (1973b) Environment and birth defects, pp. 35-82. Academic Press, New York.

Wilson, J. G. 1977 Current status of teratology: General principles and mechanisms derived from animal studies. In J. G. Wilson and F. C. Fraser, eds., Handbook of Teratology, vol. I, pp. 47-74. Plenum Press, New York.

Witkin, E. M. 1976 Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bact. Rev. 40:869-907.

Wolff, S. 1977 Sister chromatid exchange. Ann. Rev. Genet. 11:183-201.

Wolff, S. 1978 Relation between DNA repair, chromosome aberrations, and sister chromatid exchanges. In P. C. Hanawalt et al., eds., DNA repair mechanisms, pp. 751-760. Academic Press, New York.

Yielding, L. W., Riley, T. L., and Yielding, K. L. 1976 Preliminary study of caffeine and chloroquine enhancement of X-ray induced birth defects. Biochem. Biophys. Res. Comm. 68:1356-1361.

Zubroff, J. and Sarma, D. S. R. 1976 A nonradioactive method for measuring DNA damage and its repair in nonproliferating tissues. Anal. Biochem. 70:387-396.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Saowalak Jirakulsomchok

Major Subject Biochemistry

Title of Dissertation DNA Damage and Repair in Developing Embryos

Dissertation Committee:

________________, **Chairman**

Director of Graduate Program ________________

Dean, UAB Graduate School ________________

Date 3/5/82

PS-1428