CLASTOGENIC EFFECTS OF THE CARCINOGENS, METHYLNITROSOUREA AND ETHYLNITROSOUREA, ON CHROMOSOMES FROM HUMAN FIBROBLAST CELL LINES

by

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TABLE OF CONTENTS

Page

INTRODUCTION 1

Carcinogenesis by the Nitroso Compounds, Ethylnitrosourea (ENU) and Methylnitrosourea (MNU) 1

Transplacental Effects of ENU and MNU 3

Anti-tumor Activity of Nitrosoureas 4

Mutagenicity of ENU and MNU 4

In Vitro Transformation by MNU 5

Sensitivity of Man to Nitroso Compounds 5

Environment and Synthesis of Nitroso Compounds 6

RATIONALE 8

MATERIAL AND METHODS 9

Cell Sources 9

Cell Culture Technique 9

Chemicals and Treatment 11

Synchronization Attempts 13

Time and Long Term Series 17

Combination Short and Long Term Series 17

Short Term For Banding 18

Staining, Observation and Photography 19

Criteria for Metaphase Scoring 19

RESULTS 22

Lymphocyte Series - Pilot Study 22

Synchronization Attempts 23

Fibroblast MNU Time Series 23

Fibroblast ENU Time Series 24

Long Term MNU and ENU 25

Combination Short and Long Term Cultures 26

Short Term 26

Long Term 27

Distribution of Chromatid Aberrations 29

Distribution of Chromatid Gaps and Breaks in Giemsa Banded (G-banded) Chromosomes 29

DISCUSSION 31

Pilot Studies with Lymphocytes 31

Synchronization Attempts 31

Fibroblast MNU Time Series 33

Fibroblast ENU Time Series 34

Long Term MNU and ENU Fibroblast Studies 35

Combination Short and Long Term Cultures 35

Distribution of Chromatid Abnormalities Induced by MNU and ENU 38

Distribution of Chromatid Gaps and Breaks in G-banded Chromosomes 39

Mechanism of Action 40

SUMMARY. 43

TABLES AND FIGURES 45

LITERATURE CITED 141

LIST OF TABLES

NUMBER OF TABLE		PAGE
1	Results obtained after lymphocyte cultures were treated with MNU or ENU four hours prior to harvest.	46
2	Results obtained after lymphocyte cultures were treated with MNU or ENU 14 hours prior to harvest.	48
3	Results obtained after lymphocyte cultures were treated with MNU or ENU 26 hours prior to harvest.	50
4	Results obtained after lymphocyte cultures were treated with DMN 14 hours and 26 hours prior to harvest. . . .	52
5	Results obtained by harvesting the 46,XY-1 cell line five hours following the initiation of a one-hour MNU pulse. 62	
6	Results obtained by harvesting the 46,XY-1 cell line 13 hours following the initiation of a one-hour MNU pulse.	64
7	Results obtained by harvesting the 46,XY-1 cell line 23 hours following the initiation of a one-hour MNU pulse.	66
8	Results obtained by harvesting the 46,XY-1 cell line 48 hours following the initiation of a one-hour MNU pulse.	68
9	Results obtained by harvesting the 46,XY-1 cell line five hours following the initiation of a one-hour ENU pulse.	84
10	Results obtained by harvesting the 46,XY-1 cell line 13 hours following the initiation of a one-hour ENU pulse.	86
11	Results obtained by harvesting the 46,XY-1 cell line 23 hours following the initiation of a one-hour ENU pulse.	88
12	Results obtained by harvesting the 46,XY-1 cell line 48 hours following the initiation of a one-hour ENU pulse.	90

NUMBER OF TABLE | PAGE

13 Results obtained when 46,XY-1 cell line was harvested one week following one-hour MNU pulse. 104

14 Results obtained when 46,XY-1 cell line was harvested one week following one-hour ENU pulse. 106

15 Results obtained when approximately equal numbers of cells from four cell lines were combined, allowed to approach a logarithmic phase of proliferation and harvested five hours following initiation of a one-hour MNU pulse. 110

16 Results obtained when approximately equal numbers of cells from four cell lines were combined, allowed to approach a logarithmic phase of proliferation and harvested five hours following initiation of a one-hour ENU pulse. 112

17 Results obtained when approximately equal numbers of cells from four cell lines were combined, pulse treated with MNU and harvested seven days later. . . 114

18 Results obtained when approximately equal numbers of cells from four cell lines were combined, pulse treated with ENU and harvested seven days later. . . 120

19 Distribution of chromatid aberrations induced by a one-hour MNU pulse beginning five hours before harvest. 126

20 Distribution of chromatid aberrations induced by a one-hour ENU pulse beginning five hours before harvest. 128

21 Data obtained after Giemsa-banding (G-banding) of chromosomes from the 46,XY-1 cell line (MNU). 130

22 Data obtained after Giemsa-banding (G-banding) of chromosomes from the 46,XY-1 cell line (ENU). 132

LIST OF FIGURES

NUMBER OF FIGURE		PAGE
1	Experiment No. 1 with 46,XY-1 cell line showing mitotic index at hourly intervals following initiation of a single 2.5 x 10^{-3} M thymidine block.	54
2	Experiment No. 2 with 46,XY-1 cell line showing mitotic index at hourly intervals following initiation of a single 2.5 x 10^{-3} M thymidine block.	54
3	Average of Experiments 1 and 2.	56
4	Experiment No. 1 with the 46,XY-1 cell line showing mitotic index at hourly intervals following the release of the second thymidine block.	58
5	Experiment No. 2 with the 46,XY-1 cell line showing mitotic index at hourly intervals following the release of the second thymidine block.	58
6	Average of Experiments 1 and 2.	58
7	Graph showing the mitotic index at hourly intervals following removal of the second 2.5 x 10^{-3} M thymidine block in the 46,XY-2 cell line.	60
8	Graph showing the mitotic index at hourly intervals following removal of the second 2.5 x 10^{-3} M thymidine block in the 46,XY-3 cell line.	60
9	A typical severely fragmented metaphase plate as was observed when cells were harvested 23 hours following initiation of a 10^{-3} M or 5 x 10^{-4} M one-hour MNU pulse.	70
10	Experiment No. 1 with the 46,XY-1 cell line showing the per cent of cells in mitosis at 5, 13, 23 and 48 hours after initiation of a one-hour pulse with MNU.	72
11	Experiment No. 2 with the 46,XY-1 cell line showing the per cent of cells in mitosis at 5, 13, 23 and 48 hours after initiation of a one-hour pulse with MNU.	72
12	Average of Experiments 1 and 2.	72

NUMBER OF FIGURE

PAGE

13 Graph showing the per cent of abnormal metaphases plotted against concentrations of MNU when cells were harvested 5 hours subsequent to initiation of a one-hour MNU pulse. 74

14 Graph showing the per cent of abnormal metaphases plotted against concentrations of MNU when cells were harvested 13 hours subsequent to initiation of a one-hour MNU pulse. 74

15 Graph showing the per cent of abnormal metaphases plotted against concentrations of MNU when cells were harvested 23 hours subsequent to initiation of a one-hour MNU pulse. 76

16 Graph showing the per cent of abnormal metaphases plotted against concentrations of MNU when cells were harvested 48 hours subsequent to initiation of a one-hour MNU pulse. 76

17 Graph showing average percent (from experiments 1 and 2) plotted against concentrations of MNU when cells were harvested 5 hours subsequent to initiation of a one-hour MNU pulse. 78

18 Graph showing average per cent (from experiments 1 and 2) plotted against concentrations of MNU when cells were harvested 13 hours subsequent to initiation of a one-hour MNU pulse. 78

19 Graph showing average per cent (from experiments 1 and 2) plotted against concentrations of MNU when cells were harvested 23 hours subsequent to initiation of a one-hour MNU pulse. 80

20 Graph showing average per cent (from experiments 1 and 2) plotted against concentrations of MNU when cells were harvested 48 hours subsequent to initiation of a one-hour MNU pulse. 80

21 Graph showing average per cent (from experiments 1 and 2) of abnormal metaphase cells, including gaps, plotted against time of harvest following initiation of a one-hour MNU pulse. 82

22 Graph showing average per cent (from experiments 1 and 2) of abnormal metaphase cells, excluding gaps, plotted against time of harvest following initiation of a one-hour MNU pulse. 82

NUMBER OF FIGURE — PAGE

23 Experiment No. 1 with the 46,XY-1 cell line showing the per cent of cells in mitosis at 5, 13, 23 and 48 hours following initiation of a one-hour pulse with ENU. 92

24 Experiment No. 2 with the 46,XY-1 cell line showing the per cent of cells in mitosis at 5, 13, 23 and 48 hours following initiation of a one-hour pulse with ENU. 92

25 Average of Experiments 1 and 2. 92

26 Graph showing the per cent of abnormal metaphases plotted against concentrations of ENU when cells were harvested 5 hours subsequent to initiation of a one-hour ENU pulse. 94

27 Graph showing the per cent of abnormal metaphases plotted against concentrations of ENU when cells were harvested 13 hours subsequent to initiation of a one-hour ENU pulse. 94

28 Graph showing the per cent of abnormal metaphases plotted against concentrations of ENU when cells were harvested 23 hours subsequent to initiation of a one-hour ENU pulse. 96

29 Graph showing the per cent of abnormal metaphases plotted against concentrations of ENU when cells were harvested 48 hours subsequent to initiation of a one-hour ENU pulse. 96

30 Graph showing average percent of abnormal metaphases (from experiments 1 and 2) plotted against concentrations of ENU when cells were harvested 5 hours subsequent to initiation of a one-hour ENU pulse. . . 98

31 Graph showing average per cent of abnormal metaphases (from experiments 1 and 2) plotted against concentrations of ENU when cells were harvested 13 hours subsequent to initiation of a one-hour ENU pulse. . . 98

32 Graph showing average per cent of abnormal metaphases (from experiments 1 and 2) plotted against concentrations of ENU when cells were harvested 23 hours subsequent to initiation of a one-hour ENU pulse. . .100

33 Graph showing average per cent of abnormal metaphases (from experiments 1 and 2) plotted against concentrations of ENU when cells were harvested 48 hours subsequent to initiation of a one-hour ENU pulse. . 100

NUMBER OF FIGURE — PAGE

34 Graph showing average per cent of abnormal metaphase cells (from experiments 1 and 2), including gaps, plotted against time of harvest following initiation of a one-hour ENU pulse. 102

35 Graph showing average per cent of abnormal metaphase cells (from experiments 1 and 2),excluding gaps, plotted against time of harvest following initiation of a one-hour ENU pulse. 102

36 Photograph of a typical metaphase plate with "distorted fuzzy" chromosomes from all MNU-treated 46,XY-1 cultures harvested one week subsequent to one-hour MNU pulse. 108

37 A typical example of an endoreduplicating cell observed one week following a 10^{-3} M one-hour MNU pulse. . . 116

38 Photograph of a typical metaphase plate with "distorted fuzzy" chromosomes from all MNU-treated combination cultures harvested one week after one-hour MNU pulse.118

39 Photograph of a typical example of a probable "hypertetraploid" metaphase plate observed in combination cultures treated with 5×10^{-4} M or 10^{-3} M one-hour MNU or ENU pulses one week prior to harvest. 122

40 Photograph of one of the few "hypertetraploids" from the MNU and ENU treated combination long term series which showed no apparent chromosome abnormalities. . 124

41 Photograph of ENU-treated chromosomes following the Giemsa-banding (G-band) procedure. 134

42 Graphic representation of an idealized cell cycle. . . 136

43 Photograph of a diploid metaphase plate from the 46,XY-1 cell line showing a possible pericentric inversion of an A_2 chromosome after one-hour MNU pulse treatment one week prior to harvest. 138

44 Photographic representation of some of the more common types of chromatid aberrations observed after treatment of human fibroblasts with MNU and ENU 140

INTRODUCTION

Research on nitroso compound toxicity and carcinogenesis was initially performed because of the indication of human toxicity caused by the industrial use of dimethylnitosamine (DMN) as a solvent (1); however, until 1960, the presence of these compounds other than in the industrial environment was not suspected. In 1960, an outbreak of serious liver disease in sheep occurred in Norway which was linked to a diet of fish meal preserved with sodium nitrite. Since fish contain relatively large amounts of amines, nitrosamines were suspected as the etiological agent, because nitrites and secondary amines are precursors to nitrosamine formation. Subsequent treatment of healthy sheep with DMN induced liver lesions similar to those observed in sheep which had received the fish meal diet (2). This raised the possibility that smaller amounts of this nitroso compounds, or others, might be present in other foods including those for human consumption, perhaps not at high enough concentrations to cause obvious toxic effects, but high enough to be carcinogenic if the consumption of such foods were continued for a sufficiently long period of time.

Carcinogenesis by the Nitroso Compounds, Ethylnitrosourea (ENU) and Methylnitrosourea (MNU)

Nitrosoureas are considered to be the most effective of all carcinogens known in inducing nervous system tumors (3,4). Ethylnitrosourea (ENU) was for the first time shown to cause teratogenic and transplacental carcinogenic effects in the offspring of rats by Druckery in 1966 (5). Since that time, Ivankovic and Druckery

(6), Koestner et al. (7), Wechsler et al. (8) and Swenberg et al. (9) showed that single or repeated intravenous inoculations of ENU into pregnant rats resulted in a high incidence of neural tumors in their offspring. Newborn rats given ENU either subcutaneously or intracerebrally developed a high incidence of tumors of the nervous system (10). In rats, strain differences in the carcinogenic response to ENU were demonstrated (11). In mice, strain differences in response to neural tumor induction by ENU were shown by Searle and Jones (12). This revealed younger animals to be more susceptible to the induction of neural tumors than were older individuals.

Methylnitrosourea (MNU) is an extremely active carcinogen (13) and has been studied quite extensively. Single oral doses of 90 mg. MNU/kg. to rats yielded tumors of the kidney, stomach, small and large intestine and skin (14). Tumors of the brain and spinal cord developed within eight months in a group of rats given monthly intravenous injections of 25 mg. MNU/kg. of body weight (15). Guinea pigs given 2.5 mg. MNU/kg. per day developed tumors of the stomach, pancreas, ear and the nervous system and some developed leukemia (13). Carcinomas of the small and large intestine, as well as upper respiratory tract tumors, in the Syrian hamster were induced by intravenous, intraperitoneal and oral doses of MNU (16). Carcinomas were produced in rats after in vitro exposure of epithelial cells to MNU and subsequent transplantation of these cells to newborn or X-irradiated syngenic rats (17).

Transplacental Effects of ENU and MNU

In 1969, Rice (18) intraperitoneally introduced .25-1.0 mM ENU/kg. to mice on days 12-19 of gestation. This single injection yielded offspring with pulmonary adenomas. Offspring exposed to the above treatment five days before birth developed lymphocytic leukemia and hepatomas. Intravenous injection of 50 mg. ENU/kg. of body weight into Sprague Dawley and Fisher rats at day 20 of gestation yielded 69% and 27%, respectively, of offspring with brain tumors (9). These authors reported that all dosages (as low as 1 mg. ENU/kg.) produced some type of neoplasm following transplacental exposure. Furthermore, strain and age differences in response to transplacental carcinogenic effects of ENU were shown in mice (18, 19). Tumors of the nervous system were produced in fetal rats treated through the mother (7). On the other hand, Givelber and DiPaolo (20) treated 22 pregnant Syrian hamsters with .1-.5 mM ENU/kg. and found that there was a high incidence of teratogenic effects, but no evidence was found that ENU acted as a transplacental carcinogen in hamsters.

MNU also acts as a transplacental carcinogen and teratogen in rats. Offspring of pregnant rats given intraperitoneal doses of MNU subsequently developed tumors of the uterus, vagina, mammary gland, cerebrum, the femoral nerve and the kidney (21). Koyama et al. (15) treated rats with 20-40 mg. MNU/kg. between days 8.5 and 11.5 of gestation and found a high incidence of fetal resorptions. Rats treated on days 11.5 and 12.5 with 10 mg. MNU/kg. revealed 70% and 100%, respectively, of the progeny to be malformed and to exhibit CNS defects: hydrocephaly in those treated on day 11.5 and microcephaly

in those treated on day 12.5. Various other CNS anomalies were prominent findings in embryos from rats treated on day 9, while from day 12.5 onwards, marked inhibition of the brain was reported.

Anti-tumor Activity of Nitrosoureas

In contrast to the carcinogenic and teratogenic effects of the nitrosoureas, certain members of this group show promise of becoming clinically useful compounds in cancer chemotherapy (22). As of 1973, Oliverio (22) reported three nitrosoureas which had reached the stage of clinical trial and which had shown marked cytostatic activity in a wide spectrum of advanced human malignancies: 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). In 1973, Schabel (23) observed that MNU showed limited but reproducible therapeutic activity in leukemic mice.

Mutagenicity of ENU and MNU

In 1963, Rapoport (24) reported a mutation yield comprising sex-linked lethals, "semi-lethals" and visibles of 92% after treatment of Drosophila with ENU which he calculated to correspond to an overall mutation incidence of six per haploid cell. Rapoport also found MNU to be mutagenic but not as extensively as ENU. Mutations and chromosome breaks were reported after treatment of moss (*Pogonatum aloides*) spores with ENU and MNU (25). MNU was shown to be mutagenic in the bacterium, *Escherichia coli* (26) and in *Aspergillus nidulans* (27). Parkin et al. (28), employing the dominant lethal test in mice, showed

MNU to induce dominant lethal mutations. MNU was classified as mutagenic to Djungarian hamster (Phodopus sungorus) cells grown in vitro on the criterion that it produced chromosome aberrations (29). Mutations and chromosome aberrations were induced in Chinese hamster ovary cells treated with MNU (30, 31). ENU and MNU, when applied separately to primary roots of Vicia faba, produced chromatid aberrations (32). When MNU was applied first, followed by ENU, an additive effect was noted; however, when ENU was first applied and MNU second, an interaction of induced lesions was observed in the production of interchanges. Selezneva and Korman (33) reported no increase in lymphocyte chromosome aberrations after intravenous treatment of cancer patients with MNU.

In Vitro Transformation by MNU

MNU was shown to be effective in producing in vitro transformations (based upon loss of cellular contact inhibition) in Chinese hamster lung cells (34, 35) and in Syrian hamster kidney cells (36) in cell culture. MNU has transformed mouse embryo cells in vitro based upon the same criterion (37). Williams et al. (17) reported transformation of epithelial cells from rat liver following in vitro exposure to MNU. These cells were injected into newborn or X-irradiated syngenic rats which developed carcinomas after latent periods of two to eight months.

Sensitivity of Man to Nitroso Compounds

The wide range of species susceptible to carcinogenesis by the nitroso compounds indicates that man is probably also susceptible; however, definite proof of such is lacking. Some indirect evidence

was provided by Montesano and Magee (38). They conducted *in vitro* experiments to compare the metabolism of ^{14}C DMN in liver slices from man and in liver and kidney slices from the rat. The liver and kidney of rats are especially susceptible to carcinogens by DMN (39). There was a similarity between the rate of CO_2 production and between the levels of nucleic acid methylation in the liver slices from man and from the rat on incubation with DMN. This led Montesano and Magee to conclude that man is probably about as sensitive as the rat to the carcinogenic action of DMN and by extrapolation, perhaps to other nitroso compounds.

Environment and Synthesis of Nitroso Compounds

Fairly large quantities of nitrates are found in certain vegetables, in some kinds of cheese, in cured meats and other foods which man includes in his diet (40). Nitrate concentrations in drinking water has been reported to exceed 100 mg./liter in certain areas (41).

Hawksworth and Hill (42) reported that certain common bacteria convert nitrates to nitrites *in vitro* and *in vivo*. They also reported bacterial reduction of nitrate to nitrite in the human stomach and postulated that such reduction could also occur in the urinary tract, especially during urinary tract infections. Nitrites also are found in foods such as cured meats, fish, some medications and certain vegetables (40).

Nitrites can react with amines (43) or urea derivatives (44) to form nitroso compounds under proper physiological conditions. Asahina et al. (45) demonstrated that secondary amines and nitrites

can form nitrosoureas either in the stomach or in the urinary tract of mice.

Methylation of stomach, liver and small intestine nucleic acids was demonstrated in rats after simultaneous ingestion of ^{14}C methylurea and sodium nitrite (38). These results led to the conclusion that MNU was formed in the stomach and immediately absorbed into the circulation of the rat.

Of particular significance is the fact that the nitroso compounds can be formed in vivo by the reaction of nitrite ion and amines or urea derivatives in the gastrointestinal tract, in amounts sufficient in rodents for carcinogenesis in distant organs (43, 44, 46) or for transplacental passage to the fetus with subsequent development of abnormalities before birth or tumors after birth (47, 48).

Until recently, it was thought that efficient nitroso compound synthesis was limited to a low pH environment; however, Keefer and Roller (49) demonstrated that nitroso compound synthesis occurred quite easily in the presence of formaldehyde or chloral at neutral and even alkaline conditions. Formaldehyde is widely distributed in our environment and is used in germicides and fungicides for house plants and vegetables (50), is used along with nitrite as a preservative for fish (40), and is especially abundant in smoked ham and fish (51). Chloral is used as a sedative and anesthetic for farm animals (45).

RATIONALE

The nitroso compounds, if not the actual cause of cancer in man under certain circumstances, are, at the very least, a potential hazard to man either as a mutagen, teratogen, or carcinogen. It would seem that it would behoove man to discern just how severe this hazard might be.

This study proposes to determine, on human cells grown in vitro, if the nitrosamides, ENU and MNU, both of which have been shown to be mutagenic in experimental animals, cause chromosome damage [clastogenesis (52)] and, therefore, are possibly mutagenic in man.

MATERIALS AND METHODS

Cell Sources

Whole blood, from which the lymphocytes for the pilot study were cultured, was obtained via venipuncture from a chromosomally normal 41 year-old female. Fibroblast cell lines employed were derived from three chromosomally normal males (46,XY-1; 46,XY-2; 46,XY-3), from a chromosomally normal female (46,XX), from a female with "Cri du Chat" Syndrome (46,XX,$5p^-$) and from a balanced translocation carrier male with exchange points in the distal portion of the long arms of an A_1 chromosome and in the proximal portion of the long arms of a D_{15} chromosome (46,XY,t(1:15)(q44:q15)). The latter three cell lines were purchased from the Human Genetic Mutant Cell Repository in Camden, New Jersey. The 46,XY-1 cell line was initiated in 1965, and the 46,XY-2 and 46,XY-3 cell lines were initiated in 1973. All had been maintained in liquid nitrogen at the Human Genetics Laboratory of the University of Nebraska Medical Center. Each fibroblast cell line was brought to passage 12, frozen in liquid nitrogen and was at passage 12-16 when used for experimental purposes.

Cell Culture Technique

Whole blood was transferred immediately after venipuncture to a heparinized test tube and .2 ml. was dispensed to two-ounce prescription bottles, each of which contained 8 ml. of Minimum Essential Medium (MEM)-Eagles with Hank's salts (GIBCO). Added to the MEM were 50 USPU/ml. sodium potassium penicillin and 51 µg/ml. of streptomycin sulfate plus 10 units/ml of sodium heparin. Two-tenths ml.

of Phytohemagglutinin-M (PHA) was added to each bottle and the cultures were incubated at 37° C. for a total of 74 hours before harvesting.

All fibroblasts were cultured in medium containing the same ingredients as described for lymphocytes, minus the sodium heparin and PHA. Fibroblasts were grown at 37° C. in 250 ml. disposable Falcon cell culture flasks initially planted with approximately 10^6 cells. The medium was changed every two or three days as needed. When cells attained confluency, they were passed to three or four Falcon flasks of the same size. Fibroblasts were released from the flask surface for passage by removing the media, rinsing with a trypsin-versene solution (4 gm. NaCl, 2 gm. KCL, .29 gm. sodium bicarbonate, .5 gm. dextrose, .25 gm. trypsin, .19 gm. EDTA in 500 ml. H_2O), incubating with the solution for 5-10 minutes, rinsing the flask with medium, followed by centrifugation at approximately 900 RPM and subsequent replanting or harvesting. The pH of the final MEM mixture and trypsin-versene was always adjusted before use to 7.2 and 7.8 respectively by adding sterile HCL or sodium bicarbonate.

When sufficient cells were available at the desired passage, cells were planted for experimental series in 30 ml. (25 cm^2) disposable Falcon cell culture flasks at an inoculation density of 10^4-10^5 cells/ml.. Cell counts were made via the use of a Fisher Scientific hemacytometer and appropriate dilutions were made for a final cell inoculum of 10^4 - 10^5 cells/ml.. After this was done, cells were mixed in the media, using a magnetic stirrer for 15 minutes to insure equal distribution and separation of cells to each experimental Falcon flask. The cells were dispensed to the flasks with an

automatic Corning Syringe which uniformly dispensed 4 ml. of the cell suspension into each Falcon flask.

Chemicals and Treatment

Chemicals were obtained from the Eppley Cancer Institute: dimethylnitrosamine (DMN) from Dr. Bella Toth; ethylnitrosourea (ENU) and methylnitrosourea (MNU) from Dr. Robert Langenbach. DMN, a liquid, was stored in an air-tight bottle at 4° C. ENU and MNU are both crystalline in the pure form and will degrade upon contact with moisture, heat or high pH. These chemicals were consequently stored in sealed vials, wrapped in aluminum foil and kept surrounded by $CaCl_2$ in a Revco freezer at approximately -70° C.

For the lymphocyte studies, DMN was added to the cultures by volume; ENU and MNU were weighed on a Mettler analytical balance and dissolved in anhydrous Dimethylsulfoxide (DMSO) - Pierce Chemical Company. The appropriate amounts were dispensed into the cultures using a microliter pipette (Microtol Bores, Drummond Scientific Company). To determine cytotoxicity in this pilot study, the following final concentrations of DMN, ENU and MNU were used:

DMN - 1 M, .1 M, 1×10^{-2} M, 1×10^{-3} M, and 1×10^{-4} M.

ENU and MNU - 8×10^{-3} M, 4×10^{-3} M, 2×10^{-3} M, 1×10^{-3} M, and 1×10^{-5} M.

Two controls were run in the ENU and MNU lymphocyte studies, one with nothing added and another with only DMSO, the amount added being equal to the maximum amount used as solvent in the particular series. All cultures were pulse-treated with ENU and MNU (or solvent) for one

hour, after which time the cells were rinsed twice with fresh complete media. At the third change, the MEM mixture was left on the cells. Lymphocyte cultures were treated at hours 48, 60 and 70 of cell culture time. At hour 72, 2 mcg. of Colcemid (GIBCO) was added and chromosomes were harvested following a variation of the technique of Hungerford (53). The flame-dry technique was utilized (54).

After fibroblasts were planted, they attached to the flask surface and were allowed to approach logarithmic phase (24-48 hours). When it was apparent from observation of the cultures, using a Zeiss inverted microscope, that the cells had begun a logarithmic phase of growth, they were treated with ENU or MNU. ENU and MNU concentrations utilized throughout this study were 1×10^{-3} M, 5×10^{-4} M and 1×10^{-4} M. Hereafter, for the sake of brevity, 1×10^{-3} M and 1×10^{-4} M concentrations will be referred to as 10^{-3} and 10^{-4}, respectively. These chemicals were weighed on a Mettler analytical balance and immediately dissolved, using a magnetic stirrer, in MEM previously adjusted to a pH of 7.0. The half life of MNU is 125 hours at pH 4, 24 hours at pH 6.5, 1-2 hours at pH 7 and six minutes at pH 8 (55). Within five minutes from the time the chemicals were placed in the complete medium (pH 7.0), 4 ml. of the latter was dispensed into the culture flasks containing the living cells. MEM adjusted to a pH of 7.0 was used for the controls in each treatment group. All cultures were pulse-treated for 60 minutes, after which time the MEM with or without ENU or MNU was removed. The cells were then washed twice with the MEM mixture. Fresh media was added to all cultures, which were then allowed to incubate until transfer or harvest. Colcemid was added at various intervals four hours before