

DAMAGE TO MAMMALIAN CELL DNA BY NITROFURANS

DAMAGE TO MAMMALIAN CELL DNA BY NITROFURANS

By

PEGGY LOUISE OLIVE, M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

February 1976

© Peggy Louise Olive 1976

DOCTOR OF PHILOSOPHY (1976)
(Biochemistry)

MCMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Damage to Mammalian Cell DNA by Nitrofurans

AUTHOR: Peggy Louise Olive, B.Sc. (Bishop's University)
M.Sc. (University of Western
Ontario)

SUPERVISOR: Professor D. R. McCalla

NUMBER OF PAGES: xv, 278

ABSTRACT

Nitrofurans, initially developed in the 1940's as antibacterial agents, have recently been shown to be mutagens and carcinogens. Present studies indicate that DNA may be an important target and that reductive 'activation' of the nitrofurans is likely before DNA damage occurs.

Mammalian cells contain the enzymes necessary for reduction of nitrofurans, a process which occurs at maximum rates under anaerobic conditions. Toxicity of nitrofurans, as measured by cell survival, is maximum under nitrogen, and is also dependent on temperature, medium of incubation and position of the cell in the growth cycle. ATP and DNA synthesis are inhibited in air as well as in nitrogen, while RNA and protein synthesis are often elevated in air but not in nitrogen. Phytohemagglutinin stimulation of human lymphocytes is inhibited when these cells are incubated with nitrofurans under nitrogen prior to stimulation.

DNA single-strand breaks can be found in several cultured mammalian cell lines after incubation with nitrofurans. The extent of damage is dependent upon the oxygen concentration, nitrofuran used, drug concentra-

tion (including drug/cell ratio), and the duration of exposure. Rejoining of breaks is influenced by the extent of damage with proportionately less repair after extensive breakage. Ascites cells incubated with nitrofurans in situ also show single-strand breaks which can be rejoined following elimination of the drug.

Cells irradiated with UV or gamma rays, then incubated with nitrofurans, show enhanced damage (single strand breaks, or cell death) when incubation occurs in air or nitrogen. The position of the cell in the growth cycle influences the extent of DNA damage with cells being most sensitive at the G1/S interface. Plateau phase cells are more sensitive to such damage than exponentially growing cells.

Some organs of mice labelled with radioactive thymidine show a loss of radioactivity following nitro-furan feeding. It is not possible to conclude whether this loss is due exclusively to cell death and replacement, or to repair of damaged DNA. This decrease occurs in a biphasic manner, with a rapid initial loss that suggests DNA repair as well as cell replacement.

ACKNOWLEDGEMENTS

The author wishes to express her gratitude to Dr. D. R. McCalla of the Biochemistry Department under whose direction this research was carried out.

Acknowledgements must also be made to Dr. K. B. Freeman, Biochemistry Department and Dr. S. Mak, Biology Department, who acted as advisors for this project.

The author is indebted to Dr. M. B. Yatvin and Dr. K. Clifton, Radiobiology Department, University of Wisconsin, for the use of space and equipment during the latter stages of this research and for helpful suggestions. Finally, I wish to thank my husband, Ralph Durand, Radiobiology Department, for constant encouragement, advice and very competent help.

This work was supported by the National Cancer Institute of Canada.

TABLE OF CONTENTS

	Page
Descriptive Note.....	ii
Abstract.....	iii
Table of Contents.....	vi
List of Figures.....	ix
List of Tables.....	xiii
Abbreviations.....	xiv
1. INTRODUCTION	
1.1 Nitrofurans: Current Use and Controversy.....	1
1.2 Mode of Action.....	5
1.3 Mutagenic and Carcinogenic Properties.....	15
1.4 Effect of Nitrofurans on DNA	
1.4.1 DNA as a Target.....	18
1.4.2 Quantification of Damage.....	19
1.5 Rationale and Experimental Design.....	26
2. MATERIALS AND METHODS	
2.1 Nitrofurans.....	28
2.2 Mammalian Cells: Source and Maintenance.....	28
2.3 Measurement of Toxicity: Cell Survival.....	31
2.4 Incorporation of Radioactive Precursors.....	34
2.5 Metabolic Reduction of Nitrofurans.....	35
2.6 ATP Measurements.....	37

2.7	Stimulation of Lymphocytes by PHA.....	38
2.8	Alkaline Sucrose Gradients.....	39
2.9	Histology and Autoradiography.....	41
2.10	Incorporation of ³ H-TdR into Mouse Tissue....	42
2.11	Irradiation Procedures.....	43
3. RESULTS		
3.1	Reduction of Nitrofurazone.....	44
3.2	Toxicity of Nitrofurans	
3.2.1	Cell Survival.....	49
3.2.2	Inhibition of the Stimulation of Lymphocytes by PHA.....	68
3.2.3	Inhibition of ATP Synthesis.....	72
3.2.4	Inhibition of Macromolecule Synthesis.	82
3.3	Production and Repair of Single-Strand Breaks	
3.3.1	Cultured Cells.....	88
3.3.2	Ascites Cells <u>In Vivo</u>	115
3.4	Factors Influencing DNA Damage	
3.4.1	Properties of Cells.....	119
3.4.2	Incubation Conditions.....	139
3.4.3	Enhancement of Nitrofuran Damage By Radiation.....	147
3.5	Turnover of DNA in Nitrofuran-Fed Mice	
3.5.1	Effects of Nitrofuran Diets.....	165
3.5.2	Loss of Prelabelled DNA.....	171
3.5.3	Autoradiography and Histology.....	181

4.	DISCUSSION	
4.1	Activation as a Requirement for DNA Damage...	188
4.2	Relation of Cell Toxicity to DNA Damage.....	198
4.3	Single-Strand Breaks: Direct or Indirect Damage?.....	207
4.4	DNA Turnover: Replacement or Repair?.....	212
5.	SUMMARY.....	216
6.	BIBLIOGRAPHY.....	220
7.	APPENDIX 1	251
	Calculation of Average Molecular Weights of DNA on Alkaline Sucrose Gradients	
8.	APPENDIX 2	260
	Nitrofurans Effects on Bacteria: Relation to Mammalian Cells	
9.	APPENDIX 3	270
	Analysis of Autoradiography Distribution in Turnover Experiments	
10.	APPENDIX 4	274
	Nitrofurans: Chemical Structure and Properties	
	VITA.....	277

LIST OF FIGURES

	Page
1. Pathways of Nitrofurazone Reduction.....	14
2. Turnover of DNA: Outline of Experimental Procedure, Distribution of Mice Among Groups.....	33
3. Reduction of Nitrofurazone by Mouse Liver Microsomes.....	48
4. Toxicity of Nitrofurazone under Anaerobic Conditions.....	53
5. Toxicity of Nitrofurans under Anaerobic Conditions.....	55
6. Effect of Oxygen Concentration on Toxicity.....	57
7. Effect of Incubation Medium on Toxicity.....	59
8. Effect of Glucose on Nitrofurantoin Toxicity.....	61
9. Effect of Temperature of Incubation on Toxicity...	63
10. Effect of Nitrofurantoin on Survival of Plateau Phase and Exponentially Growing Cells.....	65
11. Variation in the Shape of the Survival Curve with Duration of Exposure to Nitrofurantoin.....	67
12. Effect of Nitrofurazone on Incorporation of ³ H-TdR	76
13. Inhibition of DNA Synthesis by Nitrofurans.....	78
14. Effect of Nitrofurazone on RNA Synthesis.....	80
15. Inhibition of ATP Synthesis by Nitrofurazone.....	85
16. Time Course of ATP Inhibition by Nitrofurazone....	87
17. Production of DNA Single-Strand Breaks in Three Cultured Cell Lines.....	92
18. Production of Single-Strand Breaks by Several Nitrofurans.....	94
19. Effect of Nitrofurazone and NF-167 on Production of Breaks after 2 and 18 Hour Lysis.....	96

	Page
20. Production of Breaks at a Low Concentration.....	98
21. Effect of Time of Incubation on Production of Single-Strand Breaks.....	100
22. Calculation of Molecular Weight as a Function of Duration of Nitrofurantoin Treatment.....	102
23. Effect of Concentration of Nitrofurazone on Production of Single-Strand Breaks.....	104
24. Effect of Oxygen Concentration on Production of Breaks.....	106
25. Relation between DNA Breaks, Oxygen Concentration Nitrofurazone Concentration and Duration of Incubation.....	108
26. Rejoining of Single-Strand Breaks.....	110
27. Analysis of Molecular Weights During Repair Incubation.....	112
28. Production of DNA Breaks in XP Cells by Nitrofurans.....	114
29. Production of DNA Breaks in Ascites Cells <u>In Vivo</u>	118
30. Effects of Nitrofurans on Production of Breaks in Plateau Phase and Exponentially Growing Cells..	124
31. Rejoining of Breaks by Exponential and Plateau Phase Cells.....	126
32. Production of DNA Breaks in Synchronized Cells by Nitrofurans.....	128
33. Molecular Weight Analysis of Data from Synchrony Experiments.....	130
34. Comparison of Data from Two Synchronizing Methods.....	132
35. Toxicity of ³ H-TdR Incorporated into DNA.....	134
36. Effect of Incorporated ³ H-TdR on Survival of L cells.....	136

	Page
37. Effect of Amount of Incorporated ^3H -TdR on Production of DNA Breaks by Nitrofurazone.....	138
38. Effect of pH on Damage to L Cells by Furaltadone..	142
39. Effect of pH on Production of Breaks by Nitrofurantoin.....	144
40. Effect of Medium of Incubation on Production of Single-strand Breaks by Nitrofurantoin.....	146
41. Effect of Nitrofurazone on Uptake of ^3H -TdR by Irradiated Lymphocytes.....	152
42. Effect of UV Radiation on Production of Single- Strand Breaks by Nitrofurantoin.....	154
43. Effect of Gamma Radiation on Production of Single-Strand Breaks by Nitrofurazone.....	156
44. Effect of Treatment Time Following Irradiation on Production of Breaks by Nitrofurazone.....	158
45. Effect of Irradiation on Metabolic Reduction by L Cells.....	160
46. Pattern of ^3H -TdR Incorporation after Irradiation and Incubation with Nitrofurazone.....	162
47. Survival of Irradiated Cells Treated with Nitrofurazone.....	164
48. Loss of Prelabelled DNA from Mouse Tissues.....	180
49. Histology of Tissues of Mice Fed Nitrofurazone....	184
50. Autoradiography of Sections from Mouse Tissue....	186
51. Correlation Between Number of Breaks, Rate of Nitrofurazone Reduction and Toxicity.....	195
52. Interaction of Nitrofurans and DNA.....	197
53. Interpretation of Single-Strand Break Production by Nitrofurans.....	206
54. Analysis of Variation of Molecular Weight with Peak Position.....	257

	Page
55. Effect of Cell Numbers on Sedimentation Behaviour.....	259
56. Effect of Nitrofurazone on Liquid Holding Recovery.....	264
57. Effect of Duration of Incubation on Inhibition of Liquid Holding Recovery.....	266
58. Alkaline Elution of <u>E. coli</u> DNA Treated with Nitrofurans.....	269
59. Autoradiographic Analysis of DNA Turnover.....	273

LIST OF TABLES

	Page
1. Metabolic Reduction of Nitrofurazone by Mammalian Cells.....	46
2. Inhibition of the Response of Lymphocytes to PHA Stimulation by Nitrofurazone.....	70
3. Inhibition of the Response of Lymphocytes to PHA Stimulation by FANFT.....	71
4. Effect of Nitrofurans on Incorporation of ³ H-Leucine.....	81
5. Inhibition of Mouse Growth by Nitrofurans.....	167
6. Change in Total Body and Liver Weights Following Nitrofurazone Feeding.....	168
7. Tissue Weights after Nitrofurazone Feeding.....	169
8. Effect of Nitrofurazone on DNA and RNA Content of Mouse Tissues.....	170
9. Effect of Nitrofurans on Loss of Prelabelled DNA after Partial Hepatectomy.....	175
10. Effect of Nitrofurazone on Tissue Specific Activity.....	176
11. Statistical Significance: Analysis of Variance....	177
12. Effect of Nitrofurazone on Total Organ Activity...	178
13. Factors Influencing Extent of DNA Damage by Nitrofurans.....	204
14. Biochemical and Pharmacological Properties of Nitrofurans.....	274

ABBREVIATIONS

Nitrofurans*

AF-2 (Furylfuramide)	2-(2-furyl)-3-(5-nitro-2-furyl) acrylic acid
FANFT	N-[4-(5-nitro-2-furyl)-2-thiazole] formamide
FNT	Formic acid-2-4-(5-nitro-2-furyl)-2-thiazolyl hydrazide
NFT	3-amino-6-[2-(5-nitro-2-furyl) vinyl]-1,2,4-triazone
NFTA	N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide
NF-167	1-(5-nitro-2-furfurylidine)-3 N,N-diethylpropylaminourea HCl

Incubation Solutions

MEM	Minimal Essential Medium
FCS	Fetal Calf Serum
PBS	Phosphate Buffered Saline (Dulbecco formulation)
PBS ⁻	PBS without divalent cations
PSG	PBS with glucose (0.1%)

Chemicals

BUdR	Bromodeoxyuridine
DMSO	Dimethylsulphoxide

* Common chemical names for nitrofurans are used in the text. Chemical names are given in Appendix 4.

Abbreviations, continued

EDTA	Ethylenediaminetetraacetate
HU	Hydroxyurea
POPOP	1,4-Bis [2-(5-phenyloxazolyl)] Benzene
PPO	2,5-diphenyl oxazole
PHA	Phytohemagglutinin
TCA	Trichloroacetic acid

Terminology

LHR	Liquid Holding Recovery
PLD	Potentially Lethal Damage
SEM	Standard Error of the Mean
SSB	Single Strand Break
S phase	DNA synthetic period in cell cycle
UDS	Unscheduled DNA Synthesis

1. INTRODUCTION

1.1 Nitrofurans: Current Use and Controversy

There has been a continued interest in the recognition and development of new antibacterial agents. In 1944, Dodd and Stillman discovered that the introduction of a nitro group into the 5 position of a 2-substituted furan ring resulted in significant antibacterial activity. Innumerable reports have since appeared, dealing with aspects of synthesis, antibacterial activity, metabolism and toxicity of a growing list of derivatives. Several nitrofurans have found widespread uses in human and veterinary medicine and as food preservatives.

Many derivatives are effective against a wide spectrum of microorganisms with no immediate harmful complications to the host. The distribution of the drug achieved within different body fluids was related to the antibacterial action of each derivative (Buzard 1962). Nitrofurantoin, with a half-life in animals of 30 minutes, reached an appreciable antibacterial level only within the bladder and most of the drug was rapidly eliminated without degradation (Paul et al. 1960). Thus, its current use as an antibacterial agent has been confined principally to the treatment of urinary tract infections. Other nitro-

furans with different solubilities, rates of degradation or binding to plasma components have found uses in enteric infections or as topical agents (Paul et al. 1960, Buzard 1962). Nitrofurazone was used as a food preservative in a variety of meats and fish for several years in Japan. Peripheral neuritis and joint pain led to its replacement in 1965 with the less toxic furylfuramide (AF-2) (De Serres 1974). In 1974, the use of AF-2 was discontinued.

In the early 70's, nitrofurans were found to sensitize anoxic mammalian cells to ionizing radiation (Reuvers, Chapman and Borsa 1972). Due to their high electron affinity, nitrofurans were thought to mimic the sensitizing action of oxygen by reacting with target radicals and 'fixing' radiation damage (Adams and Cooke 1969, Adams 1970). Radiosensitization occurred if the nitrofuran was present only milliseconds before and during irradiation, but no enhancement was observed after irradiation (Adams, personal communication). Several nitrofurans had enhancement ratios that suggested they might be useful in the radiotherapy of solid tumours thought to contain radioresistant hypoxic cells (Chapman et al. 1973, Appendix 4). However, nitrofurans caused severe physiologic effects in mice injected with doses required for tumor sensitization (Olive 1972), and sensitization was not observed in dense cell suspensions (Agnew and Skarsgard 1974). Rapid metabolism of nitrofurans would also limit

their usefulness as hypoxic cell radiosensitizers in humans, and recently, evidence of nitrofurans interference with electron transport was described (Biaglow, Nygaard and Greenstock 1975).

A controversy over the use of nitrofurans arose from studies indicating nitrofurazone to be mutagenic in Escherichia coli (Szybalski 1958, Zamperi and Greenberg 1964). Later work revealed many nitrofurans were highly mutagenic in different procaryote and eukaryote test systems (McCalla and Voutsinos 1974, Tazima, Kada and Murakami 1975, Ujjie 1974, Ong and Shanin 1974, Tazima and Onimura 1974). In 1966, Stein et al. reported that some nitrofurans might be carcinogenic. A year later, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) was shown to be a potent carcinogen, producing bladder carcinomas in all rats fed 0.2% FANFT in the diet (Erturk et al. 1967,1969). Standard rodent assays indicated that many other nitrofurans induced tumours, and that the principal tumour site was dependent upon the nitrofuran tested (Erturk et al. 1969,1970a,1970b,1971, Cohen et al. 1970, 1973a,1973b).

Studies by Tonomura and Sasaki (1973) revealed that some nitrofurans, including the food preservative AF-2, induced exchange-type chromosome abberations in human lymphocytes and stimulated DNA repair synthesis in cultured fibroblasts. However, no measurable effect was observed

with several clinically used derivatives. Chromosome breaks were caused by AF-2 feeding or intraperitoneal injection in rats (Sugiyama et al. 1975, Tazima et al. 1975); chromatid breakage in bone marrow cells was used as the criterion, and young animals appeared to be more sensitive than older ones. However, AF-2 and nitrofurazone did not show teratogenic effects when administered to mice as 0.0125% of each nitrofurans in the diet (Miyaji 1971).

The past use of nitrofurans as food preservatives, their present medical use, and their proposed use as hypoxic cell radiosensitizers requires a re-evaluation of the potential hazards. The mode of action of nitrofurans on mammalian cells should be studied with the aim of determining and quantifying damage to target molecules.

1.2 Mode of Action

The present understanding of the mode of action of nitrofurans is as yet fragmentary. Earlier studies attempted to differentiate between nitrofuran actions on mammalian and bacterial cells, with the aim of understanding the selective toxicity to bacteria. These studies led to attempts to determine the metabolic pathway(s) during nitrofuran degradation in both bacteria and animals. Metabolic conversion seemed to be a prerequisite for many of the effects of nitrofurans but the steps involved in nitrofuran 'activation' and the critical target remain elusive.

Nitrofurans were selected for clinical use according to their toxicity, solubility, effective lifetime in vivo and spectrum of antibacterial activity. The side group on the furan ring was found to be important in determining solubility, toxicity and perhaps specificity of action, but not antibacterial activity (Buzard 1962). Nitrofurans were found to inhibit respiration of intact bacteria (Green 1948), ATP synthesis (Tu, personal communication), and the activity of pyruvic oxidase in cell-free extracts (Green 1948, Buzard 1962). Treatment of E. coli B with nitrofurazone resulted in an almost equal inhibition of growth, DNA, RNA and protein synthesis (Terawaki and Greenberg 1965), while 3-amino-6-[2-(5-nitro-2-furyl)vinyl]-1,2,4-triazine (NFT) inhibited only DNA synthesis (Endo et al. 1963), and

FANFT inhibited DNA synthesis to a greater extent than protein or RNA synthesis (McCalla, Reuvers and Kaiser 1971).

Nitrofurans were found to be radiomimetic (Woody-Karrer and Greenberg 1963, McCalla 1965), and were able to induce development of lambda phage from the prophage state in a lysogenic strain of E. coli (Endo et al. 1963, McCalla et al. 1970). The DNA template was altered after NFT treatment of E. coli and after high doses, DNA broke down (Kato, Sugino and Endo 1966).

Although the reductive pathway of nitrofuran metabolism is not completely known, it has been established that the nitro group of nitrofurazone is reduced by pyridine nucleotide-dependent enzymes in animal tissue and bacteria (Asnis 1957, McCalla et al. 1970). E. coli has been shown to contain at least 3 nitrofuran-reducing enzymes (McCalla et al. 1970,1975). Reductase I was active under aerobic conditions in the presence of NADPH or NADH. Two higher molecular weight enzymes required NADH and had maximum activity under anaerobic conditions. Reductase I appeared to be lacking in nitrofuran-resistant mutants suggesting that the intermediates in the reduction of nitrofurazone are more toxic than the parent drug (McCalla et al. 1970). Such mutants were nonetheless susceptible under anaerobic conditions, probably as a result of activation of nitrofurans by the higher molecular weight enzymes which

are inactive except at low oxygen tensions (McCalla et al. 1975).

Reduction of ^{14}C -nitrofurazone resulted in unstable intermediates which formed stable (probably covalent) linkages to macromolecules (McCalla et al. 1970). In wild-type strains of E. coli, nitrofurans caused alkali-labile damage to DNA, and the number of breaks caused by three quite different derivatives was roughly correlated with the carcinogenicity of the compound (McCalla, Reuvers and Kaiser 1971). Bacterial mutants lacking reductase I did not show DNA damage when incubated with nitrofurans under aerobic conditions; however, anoxic incubation resulted in single-strand breaks (McCalla et al. 1975). Nitrofurazone inhibited transformation of B. subtilis when transforming factor DNA was extracted from cells treated in vivo but not in vitro (Terawaki and Greenberg 1965) suggesting that metabolic activation of nitrofurazone was required for this effect.

Several enzymes may reduce nitrofurans in animal tissues, including xanthine oxidase (Taylor, Paul and Paul 1951, McCalla et al. 1971b), aldehyde oxidase (Wolpert et al. 1973) and the microsomal-associated NADPH:cytochrome c reductase (McCalla et al. 1971b, Wang et al. 1975). Maximum rates of reduction generally occur in the presence of NADPH, a NADPH regenerating system, and under anaerobic conditions. However, the sensitivity to oxygen concentration

appears to be a function of the particular enzyme (Wolpert et al. 1973).

Allopurinol, an inhibitor of xanthine oxidase, greatly inhibited the degradation of nitrofurazone and AF-2 in rat small intestine indicating that xanthine oxidase was of major importance in this tissue; however, in liver, other enzymes were important since allopurinol inhibited only 19% of the degradation (Tatsumi, Yamaguchi and Yoshimura 1973). The combined nitrofurazone reductase activity attributable to xanthine oxidase plus aldehyde oxidase was only one third of the total nitrofurazone reductase activity in rat, mouse and rabbit liver (Wolpert et al. 1973). Also, tissues like muscle with low levels of xanthine oxidase were still able to reduce nitrofurans (Bender and Paul 1951). It has been suggested that the microsomal enzyme, NADPH: cytochrome c reductase, may be involved in metabolism of nitrofurans under anaerobic conditions (McCalla et al. 1971b, Wang et al. 1975). Tissue slices were found to be much less active in reducing nitrofurans than was a similar weight of anaerobic extracts plus NADPH. This was probably due to diffusion limitations and auto-oxidation of intermediates (McCalla et al. 1971b).

Many drugs are absorbed from the gut by passive diffusion dependent on the oil/water partition ratio. However, most nitrofuran reduction in animals occurs in the lumen of the small intestine, and degradation and insolubil-

ity in the digestive tube apparently acted as more important factors than the partition ratio (Tatsumi et al. 1973). In most cases, metabolism was rapid. Half-lives of the parent compound were as low as 15 minutes with as much as 75% of the drug being excreted unaltered (Paul et al. 1960). Non-ionized nitrofurans such as nitrofurazone were bound to plasma proteins to a slight extent, while 50 to 70% of anionic compounds such as nitrofurantoin were bound. This binding appeared to be reversible and could not be correlated with systemic effects or antibacterial activity (Paul et al. 1960). Changes in pH affected the antibacterial activity of nitrofurans with anionic or cationic activity; the greatest activity was achieved with nonionized molecules which are able to cross membranes more readily (Paul et al. 1960).

During incubation with animal tissues, the 375 nm absorption maximum of nitrofurazone and related semicarbazones was replaced by 335 nm absorbing material. Beckett (1959) concluded that reduction of nitrofurazone resulted in the production of 5-amino-2-furaldehyde semicarbazone. However, in studies with purified xanthine oxidase, 5-hydroxylamino-2-furaldehyde semicarbazone seemed a more likely candidate (Paul et al. 1960) (Figure 1). The metabolism of nitrofurazone postulated by Paul et al. (1960) and the reduction of 2-methyl-4-(5-nitro-2-furyl)thiazole suggested by Wang et al. (1975) indicate that the hydroxyl-

amine may be further reduced to inactive products (Figure 1). However, the structure of the 'activated' intermediate remains elusive due to its reactivity and correspondingly short half-life.

At least two mammalian enzymes are inhibited by nitrofurans under physiologic conditions. These are pyruvate oxidase and glutathione reductase, both with flavin cofactors (Paul, Bryson and Harrington 1956, Buzard and Kopko 1963). Paul et al. (1956) found that nitrofurazone interfered with oxygen uptake by rat tissues when glucose was used as a substrate but had no effect on anaerobic glycolysis. The formation of acetyl CoA from pyruvate was markedly inhibited. This effect may be a function of the nitro group as some other nitro compounds have the same effect (Buzard 1962). The inhibition of glutathione reductase does not involve sulphhydryl groups since neither cysteine nor oxidized or reduced glutathione could prevent or reverse the effect (Buzard et al. 1963). However, these compounds reduced the binding of NFTA to protein while not affecting the rate of reduction by microsomes (Wang et al. 1975), and both cysteine and lysine inhibited the binding of ^{14}C -nitrofurazone to protein in vitro (McCalla et al. 1970). There was a similarity between the inhibitory pattern of the two enzymes since both responded to the same nitrofurans and to about the same degree (Appendix 4). In contrast to the inhibition of pyruvic

oxidase and glutathione reductase, RNA polymerase appears to be stimulated in liver nuclei (Akao et al. 1971, Miyaki et al. 1969). As was predicted, simultaneous feeding of nitrofurans with 3'-methyl-4-dimethylaminoazobenzene (3'Me-DAB), which inhibits RNA polymerase, retarded the induction of hepatomas normally produced after feeding 3'Me-DAB to rats (Miyaki et al. 1971).

It was suggested that nitrofurans may have 'anti-cancer' activity since the life span of mice implanted with Ehrlich ascites tumours was lengthened by treatment with nitrofurans (Ujje 1966). It is not unusual for a carcinogenic compound to be used in cancer therapy; many agents which are selectively lethal to growing cells are themselves carcinogenic, the best-known examples being alkylating agents. Use of nitrofurans in testicular cancer therapy (Marshall, Johnson and Price 1964) followed pharmacologic studies in which high doses were found to be selectively cytotoxic to the testicular germinal epithelium in rats (Friedgood 1952), presumably because of the high activity of nitrofuran reductase in such cells. Adrenal cortical atrophy has been observed following nitrofurazone feeding in mice. This effect suggested a possible mode of action of nitrofurazone as an inhibitor of tumour growth since it was ineffective as an antitumour agent in adrenalectomized mice (Friedgood and Ripstein 1951).

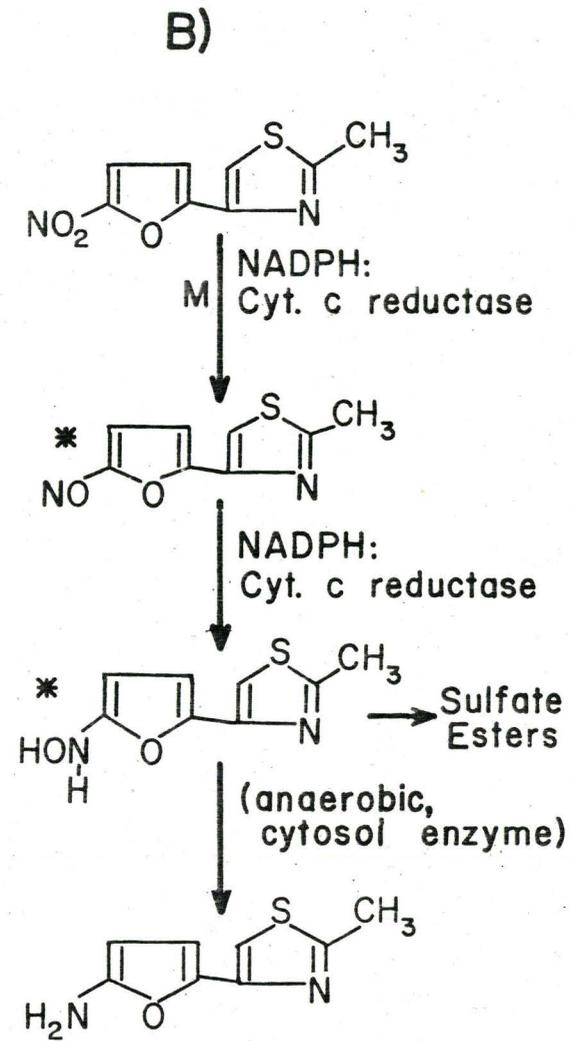
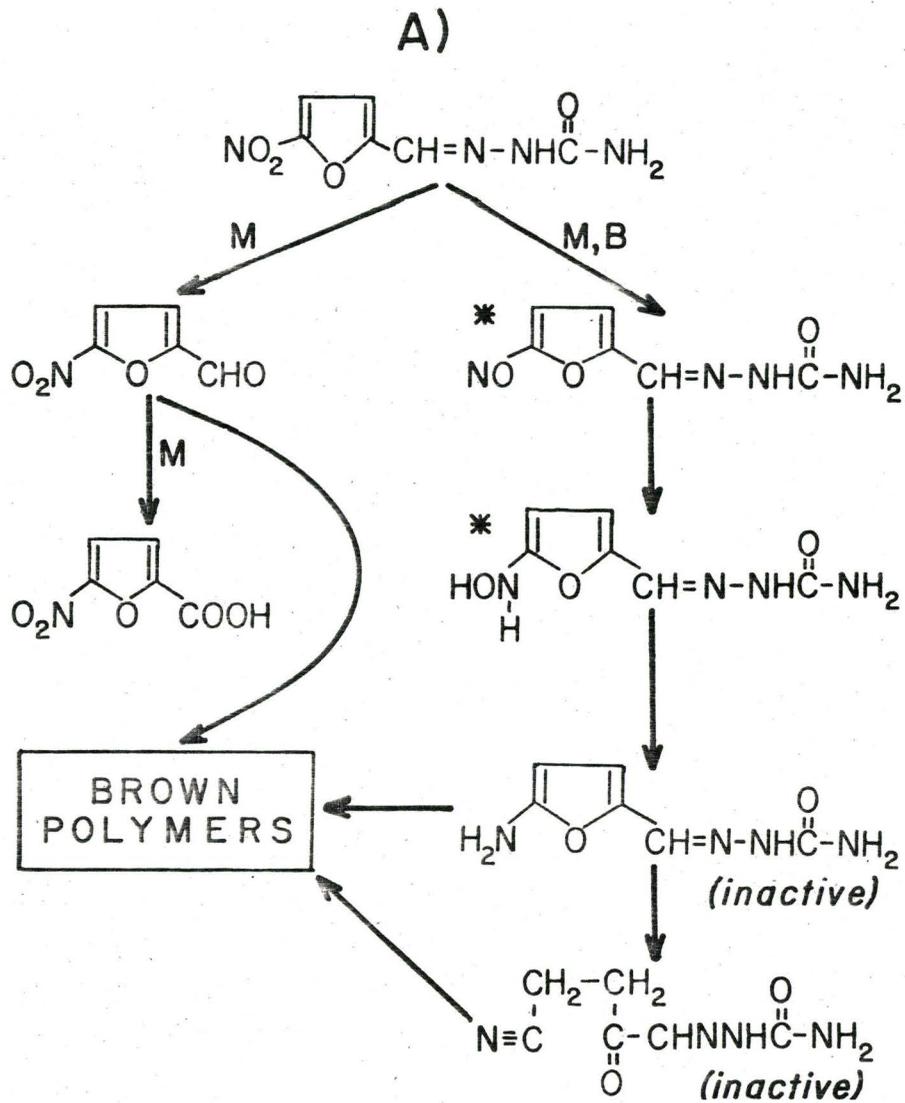
The present understanding of nitrofuran actions

appears to be a collection of seemingly unrelated observations. One common factor is the requirement for metabolic reduction. As will many other carcinogens, conversion of nitrofurans to more electron-affinic compounds may be required. It has been suggested that the hydroxylamine intermediate (Erturk et al. 1971) or the nitrofuryl radical anion (Mason and Holtzman 1974) may be the reactive species. These electrophillic reactants may induce malignant transformation by reacting with nucleophiles in a critical target tissue. Covalent binding of residues of chemical carcinogens has been noted in all cases which have been adequately examined, and long-term binding has been associated mainly with DNA. However, the relatively non-specific nature of these interactions could result in numerous secondary effects on cellular metabolism.

Figure 1: Pathways of Nitrofuran Reduction

A) Possible pathways in the degradation of nitrofurazone by mammalian (M) and bacterial (B) enzymes. Intermediates indicated by stars have not been isolated and are only postulated. From Paul et al. (1960).

B) Metabolic reduction of NFTA by microsomal cytochrome c reductase may also result in the hydroxylamine intermediate with final reduction to the amine. From Wang et al. (1975).



1.3 Mutagenic and Carcinogenic Properties

Tumour initiation is considered by many to be associated with genetic changes in somatic cells. Although the nature of these changes is not yet understood, malignancy probably occurs either as a result of a mutation or as a result of a functional change in genetic makeup (Pogosianz 1973). Attempts to correlate mutagenicity and carcinogenicity have not yet firmly established the parallelism.

Enzyme activation of nitrofurans appears to be a requirement for DNA damage and mutation in bacteria. In cells lacking reductase I, ^{14}C -nitrofurazone did not bind to macromolecules. The other nitrofuran-reducing enzymes were not active in air, however, under anaerobic conditions, nitrofurazone was activated with subsequent damage to DNA and mutation in bacteria (McCalla et al. 1975). The final products of reductases II and III have not been identified, but they appear to be different from the cyano-derivative (Figure 1) formed by reduction of nitrofurazone by reductase I (Asnis 1958, McCalla et al. 1975).

The lack of induced mutation in exr^- or rec^- bacterial strains suggested that the 'error-prone' repair system was necessary for the mutagenic effect of the nitrofurans (McCalla and Voutsinos 1974). Reduction of the nitro group was probably required for mutation under aerobic

conditions; the absence of reduction in nitrofurans resistant mutants correlated with the absence of induced mutations in these strains (McCalla and Voutsinos 1974).

A wide spectrum of tumours has been produced in rats after feeding with nitrofurans (Erturk et al. 1967, 1969, 1970a, 1970b, 1971; Morris et al. 1969; Cohen et al. 1970, 1973a, 1973b). FNT was non-specific in production of neoplastic lesions (Erturk et al. 1971), while nitrofurazone and NFT caused predominantly mammary tumours and FANFT was a bladder carcinogen (Erturk et al. 1969). In addition, some nitrofurans were suggested to act in a bifunctional manner, such as FNT, which contains a hydrazine side group shown to be weakly carcinogenic itself (Erturk et al. 1971).

The molecular basis of the carcinogenicity of nitrofurans is not known. However, metabolic reduction of the nitro group is required for mutagenesis in bacteria, and the 5-nitro group probably also plays a major role in the carcinogenic activity (Erturk et al. 1971). All nitrofurans tested, with the exception of 5-nitrofuroic acid, are mutagenic in bacteria. Some nitrofurans, like AF-2, have been tested in eukaryotes and are also mutagenic to higher forms. Since all carcinogens are mutagens, an understanding of the relationship between these processes is of critical importance. Some nitrofurans, such as nitrofurantoin, do not appear to cause tumours; this may

indicate that these nitrofurans are truly non-carcinogenic, or that they are only weakly tumorigenic and cannot be recognized as such using small numbers of animals, short exposure times or small doses.

1.4 Effect of Nitrofurans on DNA

1.4.1 DNA as a Target

The heritable nature of neoplastic transformation has led to attempts to measure the interaction between chemical carcinogens and DNA (Sneider 1974). Miller (1970) found that azo dyes and polycyclic hydrocarbons displayed long-term binding only to DNA. Of course, reactions with other important cellular macromolecules occur in vivo. For example, the association of DNA with the nuclear membrane and the finding that 2-acetylaminofluorene and β -propiolactone react with membranes in such a way that their ability to bind DNA was altered, suggested that other targets may be at least of initial importance in transformation (Kubinski and Kasper 1971).

Much evidence implicates DNA as the critical target for the mutagenic activity of nitrofurans in bacteria. Nitrofurans bind to DNA, induce prophage development, are radiomimetic, inhibit DNA synthesis, prevent transformation and cause single-strand DNA breaks in bacteria. Determining whether DNA is also a primary target for carcinogenic activity in mammalian cells is crucial to both the understanding of the specific action of nitrofurans and the mechanism(s) of human carcinogenesis.

1.4.2 Quantification of Damage

At least 4 approaches are currently used to examine DNA damage and repair in eukaryotes. These include (1) induction or removal of chemical alterations or bound chemicals, (2) rupture of a DNA strand and subsequent availability of new groups to enzymic attack, (3) incorporation of deoxynucleotides into DNA which is not actively involved in replication, and (4) measurement of a change in the average size of a DNA strand.

The first of these has been useful for studying the removal of thymine dimers following UV irradiation, or loss of methyl groups after treatment of cells with methylating agents (Carrier and Setlow 1971; Roberts 1971). However, this approach would be of little value when the chemistry of the DNA-carcinogen interaction is not understood.

Damage to DNA resulting in a break will also produce new 3-OH and 5' termini. Polynucleotide kinase and DNA polymerase can be used to label both these termini; the extent of DNA damage and its repair can be monitored by measuring the decrease in enzyme activity with time (Aposhian and Kornberg 1962; Weiss and Richardson 1967; Dalrymple et al. 1970). DNA polymerase I has been used to measure single-strand breaks in rat liver DNA following injection of methylating agents in vivo (Saffhill, Cooper

and Itzhazi 1974). Nondenaturing conditions can be used for such assays, however, only breaks formed in regions of DNA accessible to DNA polymerase I would be observed thus limiting the technique.

A third approach has been most successful in quantifying damage by a number of chemicals and radiation. 'Repair replication' and 'unscheduled DNA synthesis' (UDS) are two different assay systems for studying the same event: the replacement of precursors in excised regions of damaged parental DNA strands (Schaeffer and Merz 1971). The relevance of repair synthesis was recognized when it was discovered that defects in DNA repair can be correlated with several hereditary diseases causing increased sensitivity of the skin to sunlight, abnormal growth, increased sensitivity to carcinogens, increased incidence of cancer and premature aging (Howard-Flanders 1973).

Correlations have been found between the carcinogenicity of a compound and the level of DNA repair synthesis as for example with analogues of 4-nitroquinoline-N-oxide (Stich et al. 1971,1973). The measurement of repair replication using equilibrium density centrifugation is time-consuming and generally requires rebanding of DNA for accurate measurement of repair; autoradiography is more easily applied to such studies. Since both techniques assume that damage can be readily repaired and monitored by incorporation of precursors, little data is available on

agents which might have low levels of repair for long periods of time. Most studies using autoradiography to determine repair have required highly toxic drug or radiation doses. Slightly soluble compounds, such as nitrofurans, might be difficult to apply at sufficiently high concentrations to cause measurable repair synthesis.

Repair can also be followed if damaged cells are allowed to incorporate BUdR during the repair interval. Cells are then irradiated with 313 nm light and placed in alkali; breaks appear at the sites of repair (Wilkins 1973). Since breaks are seen as a cumulative incidence rather than an instantaneous rate, the assay can be more sensitive than the alkaline sucrose gradient method. However, denaturation of DNA is required, a measurable amount of damage results from UV irradiation, and the assay is again one step removed from direct measurement of DNA damage.

Analysis of bacterial DNA using alkaline sucrose gradients was first described by McGrath and Williams (1966). When whole protoplasts were layered on top of alkaline gradients, the bacterial membrane was disrupted, proteins denatured, RNA degraded and duplex DNA separated into single strands with minimal shear damage. DNA was then sedimented in a linear sucrose gradient at several thousand g. The average size of the DNA strand could then be determined, and changes in size as a result of DNA damage were calculated. Although it is not possible to determine

the size of eukaryote DNA directly using the alkaline sucrose gradient technique (Lett et al. 1967; McBurney and Whitmore 1972), a representative and reproducible fragment can usually be obtained when denaturing and sedimenting conditions are carefully standardized. The observed number of single-strand breaks, or alkali-labile lesions, also included any double-strand breaks present since these cannot be distinguished from two single breaks. McGrath and Williams (1966) demonstrated a relationship between cell killing and single-strand breaks in E. coli. The method was subsequently adopted and modified to study the effects of radiation on mammalian cells (Lett et al. 1967; Humphrey, Steward and Sedita 1968; Moroson and Furlan 1969; Elkind and Kamper 1970). Several chemical carcinogens have since been shown to produce single-strand breaks in DNA (Andoh and Ide 1972; Cox et al. 1973; Peterson et al. 1974; Regan and Setlow 1974).

There are currently many valid criticisms concerning the use of alkaline sucrose gradients (McBurney and Whitmore 1972; Simpson et al. 1973; Ehmann and Lett 1973; Cleaver 1974). Care must be taken in standardizing the procedure, interpreting results, and confirming them using other techniques. Cells lysed in alkali are subject to numerous artifacts (for a critical review, see Cleaver 1974). Since apurinic sites will become breaks in alkali,

single-strand breaks cannot be equated with alkali-labile lesions, nor do observed breaks directly represent biologically meaningful damage. The use of formamide gradients to replace alkali has been suggested as an alternative to indicate direct damage to DNA rather than breaks due to alkali-labile lesions (Gaudin 1972). However, recent evidence indicates that only a portion of nitrofurazone damage to bacterial DNA results from alkali-labile lesions; the remainder is a result of direct breaks, or breaks caused by the actions of endonucleases on nucleotide derivatives formed in DNA (Tu and McCalla 1975). Rejoining of breaks may prove more important since faulty repair may lead to permanent damage to DNA.

The question of the significance of single-strand breaks is inevitably raised. Double-strand breaks are thought to be lethal events (Chapman et al. 1975) although rejoining of such breaks has been observed (Corry and Cole 1968; Stewart and Farber 1973). The number of single-strand breaks cannot always be correlated with toxicity (Dalrymple et al. 1970; Fox and Fox 1973; Peterson et al. 1974a), and breaks appear to be readily rejoined under proper incubation conditions. Despite these criticisms, relative damage on alkaline sucrose gradients has shown correlations with radiation survival (Palcic and Skarsgard 1972a), radiosensitizing ability (Moroson and Furlan 1969), and the degree of carcinogenicity in structurally related

compounds (McCalla et al. 1971). The gradient method can provide a rapid, reproducible analysis of DNA damage using physiologically relevant drug concentrations.

DNA turnover is a steady-state, continuous breakdown and resynthesis of molecules. Attempts have been made to determine this by measuring the loss of pre-labelled DNA (Goodman and Potter 1972), uptake of radioactive precursors (Yager and Potter 1975), or the appearance of DNA breakdown products in the urine of animals (Chu and Lawley 1975). In most mature tissues, metabolic turnover of DNA does not occur in the absence of toxic influences. Fresco and Bendich (1960) observed a half-time of turnover of the order of 680 days for ^{14}C -thymidine incorporated into rat liver. Livers of weanling rats doubled in size after the labelling period but the percentage of labelled cells remained relatively constant.

Loss of radioactivity in prelabelled tissue may represent excision repair (Goodman and Potter 1972). The relative stability of incorporated ^3H -thymidine in tissues of mice fed nitrofurans may indicate whether DNA turnover occurs, suggesting either cell replacement or repair. Measurement of the cumulative damage by nitrofurans feeding through loss of radioactive label is a more sensitive technique than following incorporation after a single injection of DNA precursor. However, it is essential to

consider reutilization of labelled breakdown products since DNA-synthesizing cells can incorporate labelled products which have been released from dying cells (Clifton and Cooper 1971).

1.5 Rationale and Experimental Design

DNA may be the critical cellular target for carcinogenic action. It also appears to be a target in many of the effects of nitrofurans on bacteria. Although work with bacteria has provided insight into pathways of nitrofuran reduction and resulting mutagenic effects, mammalian cells are a more relevant test system to study the mechanism of carcinogenicity of these drugs.

The experiments outlined below have determined several effects on intact mammalian cells, both in vitro and in vivo, with emphasis on damage to DNA, its repair, and the relation of DNA damage to metabolic 'activation' of nitrofurans. A prerequisite to this study was the determination of whether nitrofurans are metabolized by mammalian cells.

Additional types of cellular damage were investigated including macromolecule synthesis, effects on ATP production and cell survival under various conditions of incubation. 'Direct' interaction between nitrofurans and mammalian DNA was estimated using the alkaline sucrose gradient technique for cells growing in culture or in the peritoneal cavity of mice. Some of the conditions which affected this damage were also determined by altering incubation conditions or cell growth state. Repair of damage was measured by rejoining of breaks.

The action of nitrofurans on tissues in vivo was studied using mice having DNA pre-labelled with ^3H -thymidine. Loss of DNA in tissues could then be demonstrated following nitrofuran feeding. An attempt was made to correlate DNA damage with a requirement for metabolic reduction of nitrofurans. Speculations resulting from single-strand break data, and factors influencing the amount of damage were also discussed.

Many of these experiments survey the effects of nitrofurans on processes which may or may not be directly related to damage to critical cellular targets resulting in transformation. Several findings are ambiguous and thus provide questions rather than answers. However, this work was a pioneering study and has suggested profitable areas of further research. Because of the diversity of experiments and their implications, a specific introduction and limited discussion accompany each section of the results.

2. MATERIALS AND METHODS

2.1 Nitrofurans

FANFT (N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide) was supplied by Abbott Laboratories, Chicago, Ill. Nitrofurazone was in part synthesized in our laboratory and in part supplied by Norwich Pharmacal Co., Norwich N.Y. NFT (3-amino-6-[2-(5-nitro-2-furyl)-vinyl]-1,2,4-triazine) was synthesized by the method of Miura et al. (1961). Niridazole, a nitroheterocycle, was obtained from Ciba, Basel. Nitrofurantoin, furaltadone, furazolidone and nifuroxime (for chemical names, see Appendix 4) were obtained from Norwich Pharmacal Co.

Stock solutions of nitrofurans in DMSO were stored at 4° in the dark for no longer than one month. Prior to use, they were diluted in medium or buffer to the appropriate nitrofuran concentration. The final concentration of DMSO was less than 1%.

2.2 Mammalian Cells: Source and Maintenance

Mouse L-929, hamster BHK-21 and human KB cells were obtained from Dr. K.B. Freeman, McMaster University.

Hela cells were obtained from Dr. C. Heidelberger, McArdle Laboratories, Madison, Wisconsin. Human Xeroderma Pigmentosum XP-2 (XP1B) cells were supplied by Dr. S. Goldstein, McMaster University. For later experiments, L cells were purchased from Gibco, Grand Island, N.Y. Primary cultures of rat liver cells were obtained from Dr. M.B. Yatvin, Department of Radiobiology, University of Wisconsin, and were isolated using a method developed by Bonney et al. (1973).

Cell lines were maintained in suspension culture in glass spinner flasks (Bellco) at 37°. Gibco modified Eagles medium (MEM-Joklik modified) containing 10% fetal calf serum (FCS) and antibiotics (penicillin, 100 I.U./ml, and streptomycin, 0.1 mg/ml) was added daily to maintain cell numbers at approximately 2×10^5 cells/ml. For labelling, cells were grown as monolayers attached to 100 mm Falcon plastic tissue culture dishes at 37° in a humidified atmosphere of 3% CO₂ in air. Cells were typically removed from dishes by aspiration of the medium, rinsing with PBS⁻ (phosphate-buffered saline without calcium or magnesium), and incubating in one ml of 0.15% trypsin (Gibco) until the cells lifted from the plates (about 4 minutes). Trypsinization was terminated by adding several ml complete medium.

L cells were synchronized by two methods. Cells labelled for 20 hours with 0.2 μ C/ml ³H-TdR were washed and

reincubated for 20 hours in 2.0 mM hydroxyurea (HU). HU is known to selectively kill rodent cells in S phase, while blocking non-S cells at the G1/S interface (Sinclair 1967, 1969).

In the second method, cells were partially synchronized using 2.0 mM HU for 12 hours. They were then labelled with $0.2 \mu\text{Ci/ml}$ $^3\text{H-TdR}$ in the presence of colcemid ($0.6 \mu\text{g/ml}$) (Gibco) for 15 hours. It was assumed that cells killed in S phase by the action of HU were not able to incorporate $^3\text{H-TdR}$. Cells were released from the metaphase block produced by colcemid by washing and reincubating in fresh medium. The degree of synchrony was checked at specific times after release by incubating unlabelled cells (otherwise treated as above) with $^3\text{H-TdR}$ for 15 minutes. These cells were fixed in acetic acid and alcohol (1:4), dispersed on a clean microscope slide, coated with photographic emulsion, exposed, developed and the percent of labelled cells determined.

Ehrlich ascites cells, obtained from Dr. S. Bhandari, McMaster University, were transferred weekly by intraperitoneal (i.p.) injection of 0.4 ml ascitic fluid into young Swiss Webster mice.

Partial hepatectomies (Higgins and Anderson 1931) were performed on 12 to 15 week old female Swiss Webster mice. Newborn mice were used for total body DNA labelling. Mice were housed in metal cages, 4 to 8 per cage, and fed

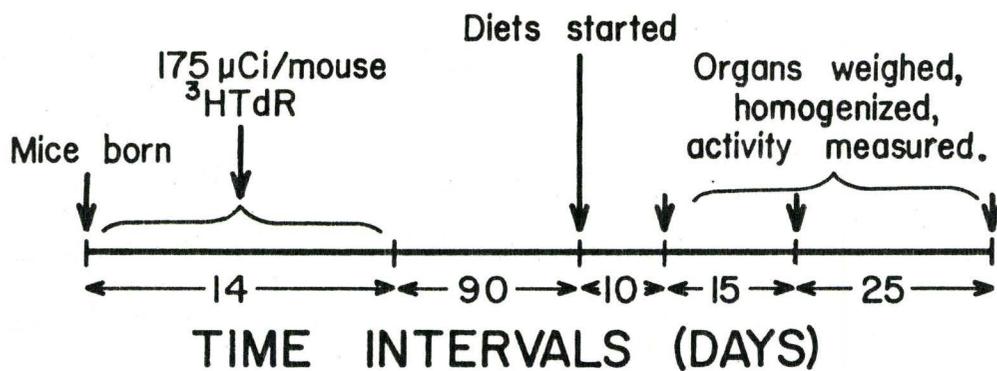
Purina Lab Chow ad libitum for 3 months after DNA labelling. Mice were then assigned to specific diets (Figure 2). They were weighed at the start of the diet and before killing by cervical dislocation. At this time, organs were removed, weighed and samples of tissue were fixed in buffered neutral formalin for histological examination. DNA content of tissues was determined by the method of Burton (1956). The orcinol test was used to determine RNA content (Ceriotti 1955) and the Lowry (1951) method for protein. In preparation for these determinations, tissues were homogenized in ice-cold buffer. Acid insoluble material was precipitated with 0.6 N perchloric acid, washed with 0.2 N perchloric acid and subjected to 0.3 N potassium hydroxide for 4 hours before precipitation with 1.2 N perchloric acid. Duplicate determinations were performed to determine RNA content using the KOH hydrolyzate, and DNA content using the final precipitate. The original tissue homogenate was used for determination of protein content.

2.3 Cell Survival: Toxicity of Nitrofurans

Approximately 2×10^5 cells/ml were suspended in 20 ml of medium or buffer containing the nitrofurans to be tested. Glass water-jacketed Bellico suspension culture vessels were gassed with a humidified mixture of 97% nitrogen and 3% CO₂ at 37° for the duration of exposure.

Figure 2: Turnover of DNA: Outline of Experimental
Procedure and Distribution of Mice Among
Groups

Seven litters of new-born mice received 7 injections of ^3H -TdR during a 14 day period to a total dose of 0.175 mC/mouse. Three months were allowed for mice to reach stable weight and for replacement of transient labelled cells. They were then assigned to groups as shown. 'T' represents the total number of mice per group at 10, 25 and 50 days at the start and end of the diet when mice were killed and tissue specific radioactivity determined. 'C 10' refers to the group of mice that were fed control (C) diets for 10 days. Similarly, C 25 and C 50 refer to groups of control mice that were killed 25 and 50 days after the start of the diet. 'F' refers to mice that were fed 0.1% nitrofurazone for the same periods as above.



DISTRIBUTION OF MICE AMONG GROUPS

Litter	1	2	3	4	5	6	7	T
C 10					♂			6
C 25					♀			6
C 50		♂		♂		♀		11

F 10	♂						♂	12
F 25		♀				♂		9
F 50	♀		♀	♀			♀	11

In some experiments, 2 to 20% oxygen was added, and for cells incubated in buffer without bicarbonate, CO₂ was eliminated. At specified times, an aliquot of cells was removed, a sample counted using an electronic particle counter (Coulter Electronics, Hialeah, Fla.), and the remainder washed in MEM and resuspended at a suitable concentration. A final count was made to determine the number of cells plated. Normally two dilutions were plated in duplicate, allowed 12 days for colony growth, and stained with methylene blue for colony counting (Puck and Marcus 1956). Plating efficiency was determined as the number of colonies counted divided by the number of cells plated. Percent survival represented the plating efficiency of the drug-treated samples divided by the plating efficiency of the corresponding control multiplied by 100. 'Surviving fraction' was generally used to refer to either of the above descriptions, and a precise explanation is given in the figure captions.

2.4 Incorporation of Radioactive Precursors

Cells used for alkaline sucrose gradient experiments were labelled on 100 mm plastic petri dishes (Falcon # 3003) in medium containing 0.1 to 2.0 μ C/ml ³H-TdR (New England Nuclear or Amersham, methyl group labelled, in aqueous solution, specific activity about 18 C/mmol). Twenty hours

later, the medium was removed and replaced with fresh medium for one hour to deplete DNA precursor pools.

Ascites cells were labelled by injecting 0.15 mC ^3H -TdR per mouse i.p. six days after transfer of the tumour inoculum.

To follow the uptake and incorporation of radioactive precursors in the presence of nitrofurans, cells were maintained in conditions normal for growth with the exception of using 97% nitrogen and 3% CO_2 . Control cells were incubated both aerobically and anaerobically. Approximately 2×10^5 cells/ml were incubated with various concentrations of nitrofurans and either 2 $\mu\text{C}/\text{ml}$ ^3H -TdR, 2 $\mu\text{C}/\text{ml}$ ^3H -L-leucine (58 C/mmmole, Amersham) or 1 $\mu\text{C}/\text{ml}$ ^{14}C -uridine (110 mC/mmmole, Amersham). At specified times, aliquots were removed, washed twice in medium precipitated with 5% TCA on 0.45 μ membrane filters, washed twice with ethanol and counted in fluor (Anderson and McClure 1973) containing 25% Triton X-114 (Rohm and Haas, Philadelphia), 75% xylene (Fisher), 3 gm/l PPO and 0.2 gm/l POPOP (Fisher).

2.5 Metabolic Reduction of Nitrofurans

To measure rates of nitrofurazone metabolism, one ml of packed cells or one ml of 9000 g supernatant from mouse liver homogenate, supplemented with 75 μ M NADPH (Sigma), 0.2 ml glucose-6-phosphate dehydrogenase (2 units,

Worthington), and two ml of glucose-5-phosphate (0.04 mM) was incubated at 37° with one ml PBS⁻ (2.68 mM KCl, 1.47 mM KH₂PO₄, 0.14 M NaCl and 6.5 mM Na₂HPO₄·H₂O) plus 0.1% glucose and a final concentration of 80 μM nitrofurazone. Mixtures were gassed by bubbling prior to addition of cells, and during incubation, by blowing the gas over the surface of stirred solutions. A flowmeter controlled the rate of bubbling. Gas mixtures used were pure nitrogen (Canada Liquid Air, Hamilton; certified oxygen content less than 3 ppm) or with gas mixtures containing 2 or 5% oxygen in nitrogen (Matheson of Canada, Toronto, certified standard). Samples (0.5 ml) of incubation fluid were withdrawn at specified times, added to 3 ml cold 90% ethanol, and the mixture centrifuged. The absorption of the clear supernatant was determined at 375 nm in a Hitachi-Perkin-Elmer Model 139 spectrophotometer. Rates of metabolism were calculated during an interval when the rate of change of absorbance was linear.

Microsomes were isolated by a procedure developed by Kamath, Kummerow and Narayan (1971) which takes advantage of the fact that microsomes bind calcium. Differential centrifugation was also employed using the method described by Masters (1971). A microsomal suspension (105,000 g pellet) was then substituted for the 9000 g supernatant in the above assay. In some experiments, β-diethylaminoethylidiphenyl-n-propylacetate-HCl (SKF-525 A), a gift from

Smith, Kline and French, Montreal, was added to incubation solutions at concentrations of 5×10^{-5} to 2×10^{-2} M. SKF-525 A is an inhibitor of microsomal drug metabolism (Jenner and Netter 1972).

2.6 ATP Measurements

The luciferin-luciferase system was used for ATP determinations (Addanki, Sotos and Rearich 1966). Approximately 10^9 HeLa or L cells were harvested by centrifugation. They were then incubated in PBS⁻ or PSG (phosphate-buffered saline plus glucose) in an air or nitrogen atmosphere in water-jacketed (37°) spinner flasks at a concentration of about 10^6 cells/ml. Nitrofurans were added from stock solutions, and at specified times, samples were removed, the cells counted, sonicated (Branson Sonifier S-125) and kept on ice, or resuspended in 0.2 ml PBS⁻ and rapidly pipetted into one ml boiling distilled water, kept at 100° for 10 minutes, and then stored on ice. The assay mixture consisted of 1.7 ml ice-cold distilled water, 0.1 ml firefly extract (Sigma Firefly Lantern Extract) and 0.2 ml cell suspension. A Packard refrigerated Tri-Carb liquid scintillation counter was set to the tritium window with a gain setting of 52% and discriminator at 0.5 to 10.5. The sample was added and a series of fluorescence readings taken starting 15

seconds later. A decay curve was plotted and compared to a standard curve prepared from Sigma ATP just before use.

2.7 Stimulation of Lymphocytes by Phytohemagglutinin

Human lymphocytes were collected from heparinized blood of one individual using the Ficol/Isopaque technique (Perper, Zee and Mickelson 1968; Mendelsohn, Skinner and Kornfeld 1971). Ficol (9.12 gm, Sigma) was mixed with 130 ml distilled water and 20 ml Ispopaque (Worthington Chemical Corporation). Blood was diluted 1:2 with Hanks buffer (Gibco), and 30 ml was layered, using sterile technique, on top of 15 ml Ficol/Isopaque, spun at 1500 g for 10 minutes, and the lymphocytes withdrawn by pasteur pipette from the middle layer (approximately 10^6 lymphocytes/ml whole blood). Cells were then collected by centrifugation and resuspended in MEM plus 20% FCS for two hours. They were treated with nitrofurans under nitrogen in complete medium, washed, and at specified times, 0.1 ml PHA (Phytohemagglutinin-m, Gibco) was added to approximately 10^6 cells. At the same time, $^3\text{H-TdR}$ (1 $\mu\text{C/ml}$) was added, and 24, 48 or 72 hours later, cells were spun down, precipitated with 5% TCA on membrane filters and washed with 95% ethanol. Samples were counted in omnifluor in a Beckman Liquid Scintillation Counter. In some

cases, TCA precipitates were solubilized in NCS tissue solubilizer (Nuclear Chicago) before addition of fluor. In order to prevent replicative synthesis, 4 mM hydroxy-urea was added to inhibit the enzyme ribonucleotide diphosphate reductase (Cleaver 1969).

2.8 Alkaline Sucrose Gradients

Cells were incubated with nitrofurans in 20 ml glass tubes sealed with rubber serum stoppers and containing 3 to 5 ml medium or buffer. Solutions were equilibrated with different humidified gas mixtures by bubbling through filling needles for approximately one hour before adding cells (10^5 cells/ml). After injecting the cells through the stopper, the gas was circulated above the liquid. Incubation was terminated by centrifuging the tube contents and collecting cells attached to the glass walls using trypsin (see Section 2.2). Cells were kept on ice at a concentration of about 5×10^5 cells/ml before lysing on gradients.

Approximately 10^4 intact cells in 0.025 ml cold PBS⁻ were lysed directly on top of an alkaline sucrose gradient using a method modified from that of McGrath and Williams (1966). Lysing solution (0.25 ml) containing 0.5 M NaOH, 0.4 M NaCl and 0.01 M Na₂EDTA, pH 12.9, was layered onto a 4.6 ml alkaline sucrose gradient (5 to 20%)

containing 0.9 M NaOH, 3 mM Na₂EDTA and 0.1 M NaCl (pH 12.8). All solutions were filtered and stored in glass bottles. Cells were lysed for 2 to 6 hours at room temperature (20°) in the dark, then spun at 18,000 rpm for 4 hours or 30,000 rpm for 90 minutes at 20° using a Beckman SW 50.1 rotor in a Model L, L-2 or L-265B ultracentrifuge. Approximately 20 fractions were collected from the top of the Beckman cellulose nitrate tube (# 305050) using an Isco Model 183 or 260 density gradient fractionator. Samples were dissolved in Triton X-114 Fluor to which 10% water and 10 ml/l acetic acid had been added to maximize counting efficiency, aid solubilization and prevent fluorescence due to the presence of alkali. Results were expressed as the percent of total radioactivity found in each fraction. Molecular weights of DNA were calculated as indicated in Appendix 1.

The gradient forming apparatus was constructed from two resevoirs drilled into a teflon block and connected by a stopcock and thin tygon tubing into the upper edge of the 5 ml cellulose nitrate tube. Both sucrose containers were filled from syringe resevoirs through holes which pierced the teflon at a level equivalent to 2.3 ml. This insured that every gradient was identical. Gradients were formed one hour before use and stored at 20°. Linearity of gradients was tested using a refractometer.

The loading apparatus advanced a 0.1 ml Hamilton

syringe to gently layer cells onto the surface of the lysing solution. The entire unit was mounted on a Lab Jack so that the syringe needle could be brought into contact with the lysing solution. About 45 seconds was taken to deposit 0.02 ml cells onto the gradient.

2.9 Histology and Autoradiography

Histology was performed on representative samples of tissue from mice which had been fed control diets or diets containing 0.1% nitrofurazone for 50 days. Sections were fixed in buffered neutral formalin, embedded in paraffin and sectioned about $6\ \mu$ thick. They were cleared with xylol and stained using hematoxlin and eosin.

For autoradiography, sections were cleared of paraffin by immersing slides in three washes of xylene for 5 minutes, two washes in 100% alcohol for one minute, and one minute each of 70%, 50% and 30% alcohol in distilled water. This was followed by several washes in distilled water. Slides remained immersed in water until coated with emulsion.

Eastman Kodak nuclear emulsion, NTB-3, was melted at 43° and thinned with an equal volume of warmed distilled water. Slides were coated in absolute darkness, allowed to dry while resting flat on cold metal shelves, then stacked in light tight boxes. Slides were developed in

Kodak D-19 developer at 16° for 5 minutes with a quick wash in distilled water followed by a 5 minute period of fixation and another wash. Cells were stained with aceto-orcein (75% distilled water, 25% acetic acid, 10 gm/l orcein) for one minute then washed in distilled water.

2.10 Incorporation of ^3H -TdR into Mouse Tissue

Approximately 60% of the liver was removed from Swiss Webster mice. The total dose of ^3H -TdR received by each mouse during liver regeneration was 0.5 mC, given as i.p. injections 20, 24, 40 and 48 hours following liver excision.

Newborn mice were injected with 0.025 ml ^3H -TdR (1 mC/ml) subcutaneously in the neck region by sliding a 27 gauge Hamilton syringe needle from just above the base of the tail under the skin of the back to the neck (Stich, personal communication). The total dose received over a 14 day period was 0.175 mC/mouse with injections every other day. Mice were assigned to experiments within three months at which time the ^3H -label would be associated mainly with tissue DNA (Craddock and Magee 1967).

2.11 Irradiation Procedures

Cells were irradiated in air with UV light (16.6 ergs/mm²/sec) calibrated with a UV dosimeter using a Mineralight source (Ultraviolet Products Inc., San Gabriel, Calif.). Cells were attached to plastic petri dishes and medium was removed during irradiation at room temperature. Gamma irradiation was performed using a Cesium¹³⁷ unit (2010 rads/min, U.S. Nuclear Corporation) with cells suspended in buffer in 15 ml plastic tubes. Irradiation was performed in air at room temperature.

3. RESULTS

3.1 Metabolic Reduction of Nitrofurazone

Reduction of nitrofurans by bacterial cells was found to be essential for both mutagenic activity and production of single-strand DNA breaks on alkaline sucrose gradients. Since mammalian cells may also 'activate' nitrofurans, some of the conditions affecting the rate of nitrofurazone reduction were determined.

Intact cells of each of the cell lines tested reduced nitrofurazone under appropriate conditions, as did Ehrlich ascites cells and rat liver cells incubated in vitro (Table 1). The most important factor found to affect the rate of nitrofurazone reduction was the concentration of oxygen during incubation. Intact L cells exposed to 5% oxygen in nitrogen reduced nitrofurazone at only 20% of the rate of reduction in pure nitrogen. Mouse liver homogenate (9000 g supernatant) was also most active in reducing nitrofurazone when assay solutions were anaerobic. However, some reduction did occur when solutions were gassed with mixtures containing 5% or less oxygen in nitrogen. The rate of nitrofurazone reduction was highest

in rat liver cells from a primary culture; these cells might be expected to contain high numbers of microsomal-associated enzymes responsible for much drug metabolism.

Reduction by a liver microsomal preparation obtained from Swiss mice indicated that most of the nitroreductase activity was associated with a microsomal enzyme (Figure 3). Reduction appeared to be enhanced by addition of a factor or enzyme in the 105,000 g supernatant (Figure 3a), possibly NADPH, although this supernatant by itself had no activity (Figure 3b). Addition of an inhibitor of microsomal drug metabolism, SKF-525A (Jenner and Netter 1972), did not alter the rate of reduction even at high inhibitory concentrations (Figure 3b), suggesting that cytochrome P-450 was not directly involved with metabolism of nitrofurans, but probably associated with NADPH: cytochrome c reductase as previously suggested (McCalla et al. 1971; Wang et al. 1974).

TABLE 1

METABOLIC REDUCTION OF NITROFURAZONE BY MAMMALIAN CELLS

Source of Reductase ^a	Rate of Reduction ^b
Mouse Liver Homogenate	
0% O ₂	8.2 μ moles/hr/gm liver
2%	4.5
5%	1.7
21%	0
Intact Cells (0% O ₂)	
L-929	2.1 μ moles/hr/10 ⁸ cells
KB	1.3
Hela	1.0
EA	0.5
BHK	0.4
Rat liver	18.7

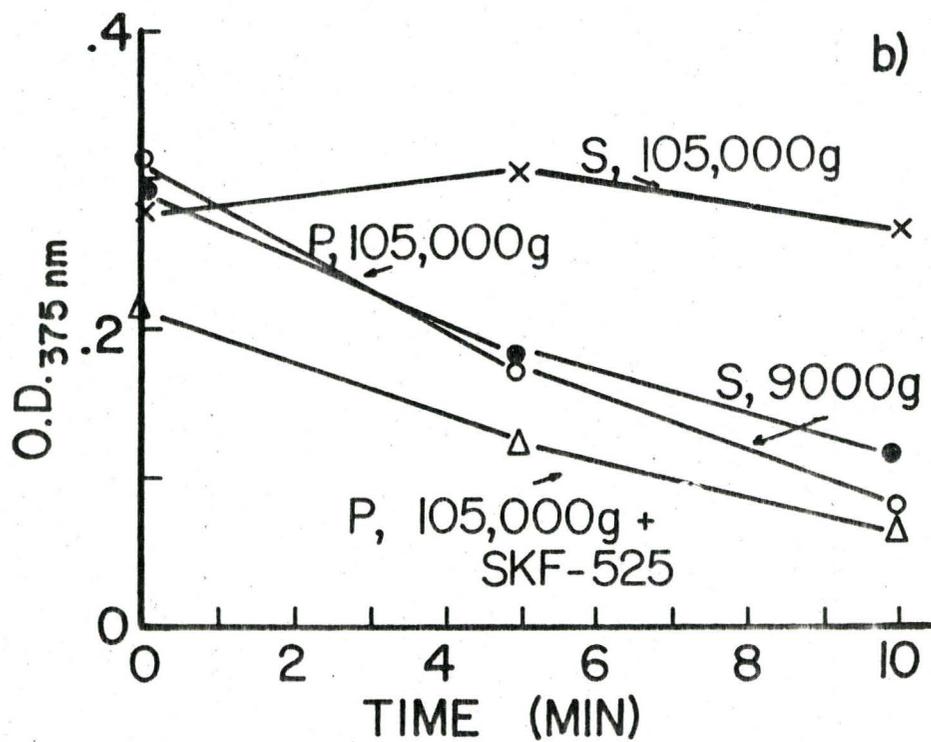
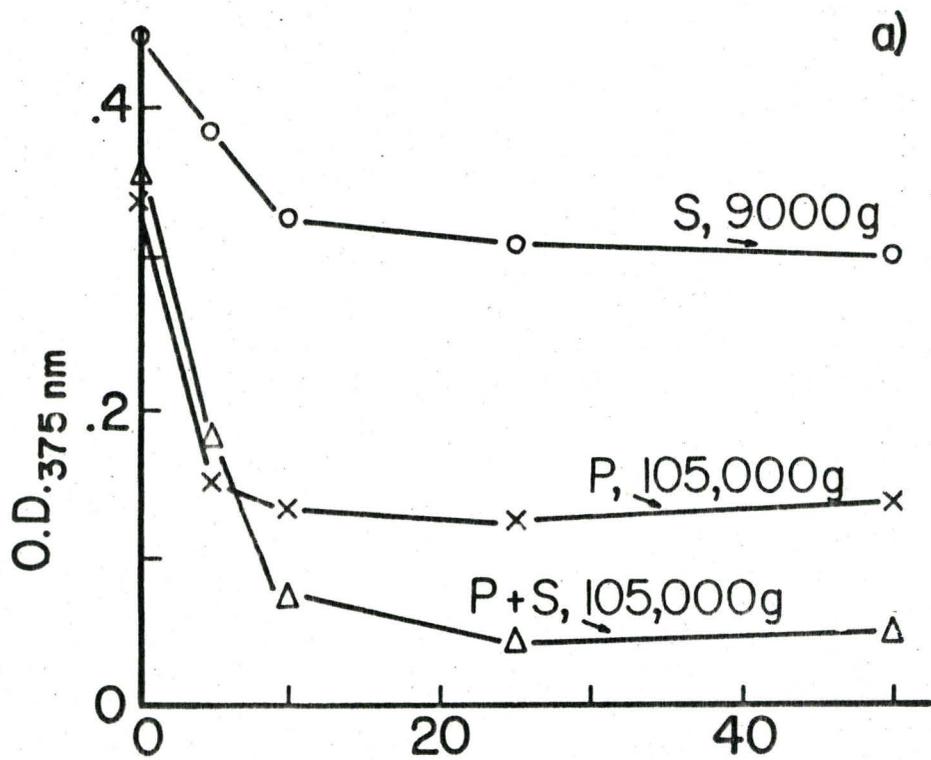
^a Mouse liver homogenate (9000 g supernatant) and intact cells (see Section 2.2) were incubated with nitrofurazone at 37°.

^b Rate of reduction was calculated from a change in absorbance at 375 nm during the first 20 minutes of incubation (see Section 2.5).

Figure 3: Reduction of Nitrofurazone by Mouse
Liver Microsomes

Microsomes were obtained from the livers of 6 mice as outlined in Section 2.5. One ml of 9000 g supernatant (S), 105,000 g pellet (P) representing the microsomes, and 105,000 g supernatant (S) which was the soluble portion of the cytosol were incubated with 1 ml nitrofurazone (final concentration, $80 \mu\text{M}$) under anaerobic conditions. An NADPH generating system was also included.

- a) Optical density at 375 nm plotted against time of incubation. A decrease in optical density at this wavelength represents reduction of nitrofurazone.
- b) Optical density is plotted against time (note the shorter interval). SKF-525A (10^{-3}M) was added to the microsomal preparation as indicated.



3.2 Toxicity of Nitrofurans

3.2.1 Cell Survival

Cytotoxicity is often associated with DNA-damaging agents. One of the most common measures of toxicity is reduction of colony formation (Puck and Marcus 1956) which equates reproductive ability with cell integrity. Using this endpoint, an attempt was made to determine whether nitrofurans were cytotoxic per se, or whether metabolic reduction resulted in toxic products. Since reduction could be greatly reduced (using loss of nitrofurazone as a measure of reduction) by incubating cells in air, this provided the means for comparing the effects of nitrofurans themselves or their metabolic intermediates and end-products on cellular processes.

Under anaerobic conditions, Hela cells showed a reduced survival two hours after incubation in complete medium with 750 μM nitrofurazone and after three hours with 250 μM of the drug (Figure 4). However, under aerobic conditions, where no significant reduction of the drug occurred, nitrofurazone was non-toxic at concentrations up to 750 μM for 6 hours. Anoxic cells incubated in MEM without FCS were more sensitive to nitrofurazone toxicity (Figure 4), presumably due to decreased binding of nitrofurazone to serum components in the medium.

Nitrofurantoin, FANFT and furazolidone were also toxic to Hela cells under anaerobic conditions in complete medium (Figure 5). However, 430 μ M nitrofurantoin in buffer containing glucose equally damaged L cells gassed with 2% oxygen in nitrogen, or pure nitrogen (Figure 6a). Nitrofurazone was toxic to L cells incubated in PSG gassed with 2%, 5% or 21% oxygen in nitrogen, however, anoxic incubation with the drug was most damaging (Figure 6b).

The nature of the incubation solution influenced the extent of toxicity by nitrofurantoin under anaerobic conditions. Survival of L cells was greatly decreased when cells were incubated in PSG at 37°, although the plating efficiency of control cells incubated in PSG was also decreased (Figure 7). Toxicity was greatest in PSG, followed by PBS⁻, MEM, PBS⁻ plus FCS, and MEM plus FCS (Figure 8). Since PBS⁻ differs from PSG by lacking Ca⁺⁺, Mg⁺⁺ and glucose, an experiment was required to determine whether PBS containing divalent cations was as effective in decreasing the survival of L cells as PSG, or whether glucose was the 'missing' factor. Survival of cells incubated with nitrofurantoin was similar in the presence or absence of divalent cations in the buffer; however, glucose added to PBS⁻ significantly increased the toxicity of nitrofurantoin when compared to PBS⁻ alone (Figure 8).

When L cells were incubated with 430 μ M nitro-

furantoin at 0° under anaerobic conditions, no toxicity was observed (Figure 9).

Plateau phase cells were less sensitive to the toxic effects of nitrofurans than exponentially growing cells (Figure 10). Other assay techniques indicated greater sensitivity of plateau phase cultures to nitrofurans damage to DNA. However, lethality is a 'late' endpoint and cell survival can be affected by numerous events, including 'repair', which intervene between the initial damage and lethality. It should also be emphasized that the colony formation technique does not indicate functional integrity of a given cell; only the proliferative ability of the cell can be assessed. This was reflected in the wide distribution of colony sizes and densities of cells which survived drug treatment.

A further limitation of the cytotoxicity data can be demonstrated when surviving fraction is plotted as a function of the drug dose (Figure 11). Note that the curves cannot be simply described in terms of 'sublethal' (shoulder) and 'lethal' (exponential region) damage. The shape of the curve is a much more complex function of dose and treatment time. A comparison of Figures 6b and 47b suggests that cells may be conditioned by incubation in nitrogen before addition of nitrofurazone; the shoulder width is greatly reduced when cells are incubated in nitrogen for one hour before addition of nitrofurazone.

Figure 4: Toxicity of Nitrofurazone under
Anaerobic Conditions

MEM containing FCS and nitrofurazone (250 or 750 μM) was equilibrated with nitrogen plus 3% CO_2 for one hour before addition of Hela cells. Anaerobic conditions were maintained throughout the experiment. At the times specified, a sample of cells was removed, counted using a Coulter counter, and plated. Colonies formed from survivors were counted 12 days later and divided by the number of cells plated to obtain surviving fraction. In one case, FCS was omitted from the medium. Since each point represents 600 or more surviving cells counted, and initial cell counts are accurate to within 1%, the error of survival data is considered to be smaller than the plotting symbols.

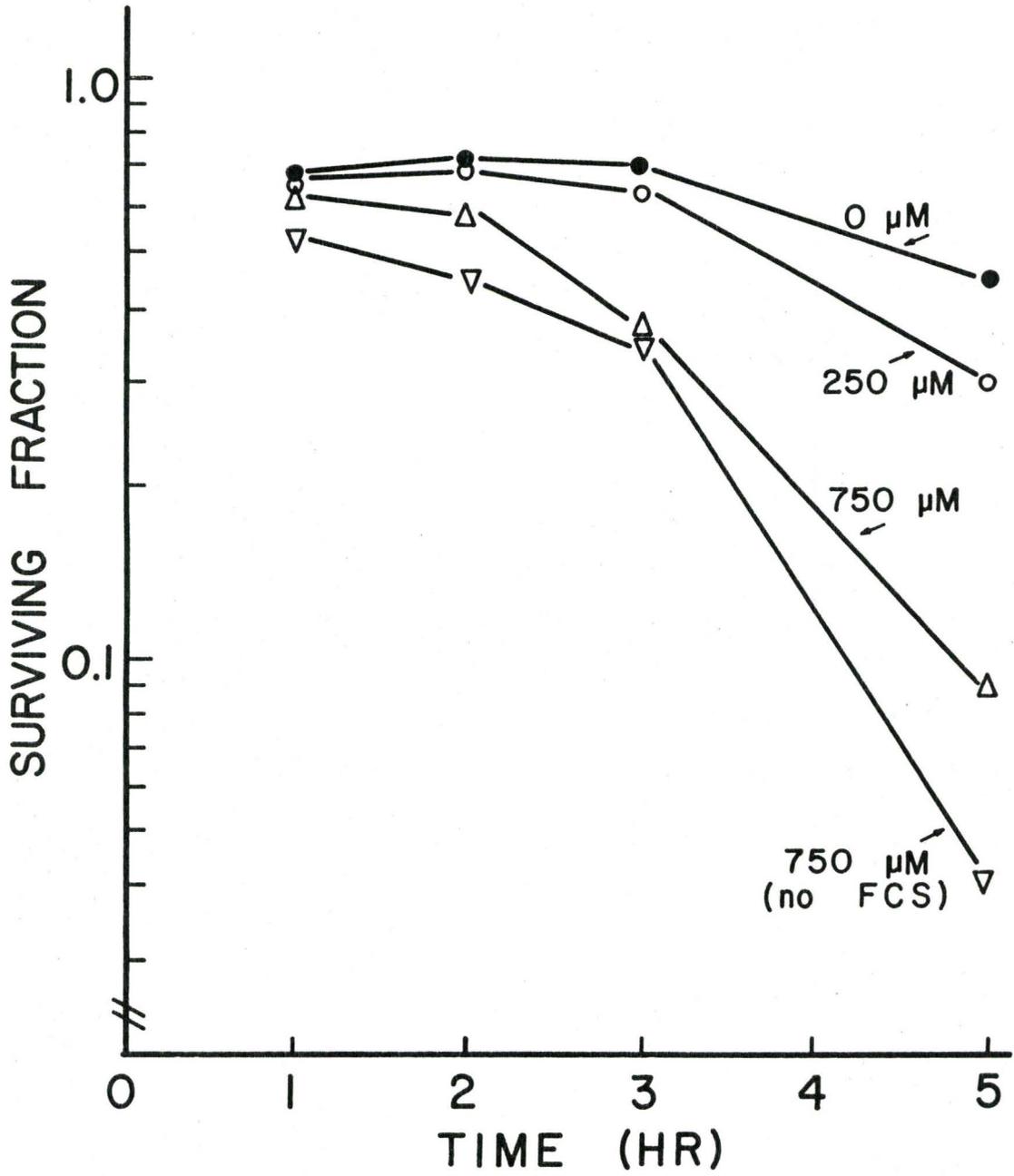


Figure 5: Toxicity of Nitrofurans under Anaerobic
Conditions

Hela cells were incubated under anaerobic conditions in MEM plus FCS equilibrated with nitrogen for one hour before addition of cells. Surviving fraction was calculated by dividing the plating efficiency of the nitrofuran-treated cells by the plating efficiency of the control cells ($0.86 \pm .02$) during the six hour incubation period. The numbers following the nitrofuran represent the concentration of the drug in μM . Data from Figure 4 is included.

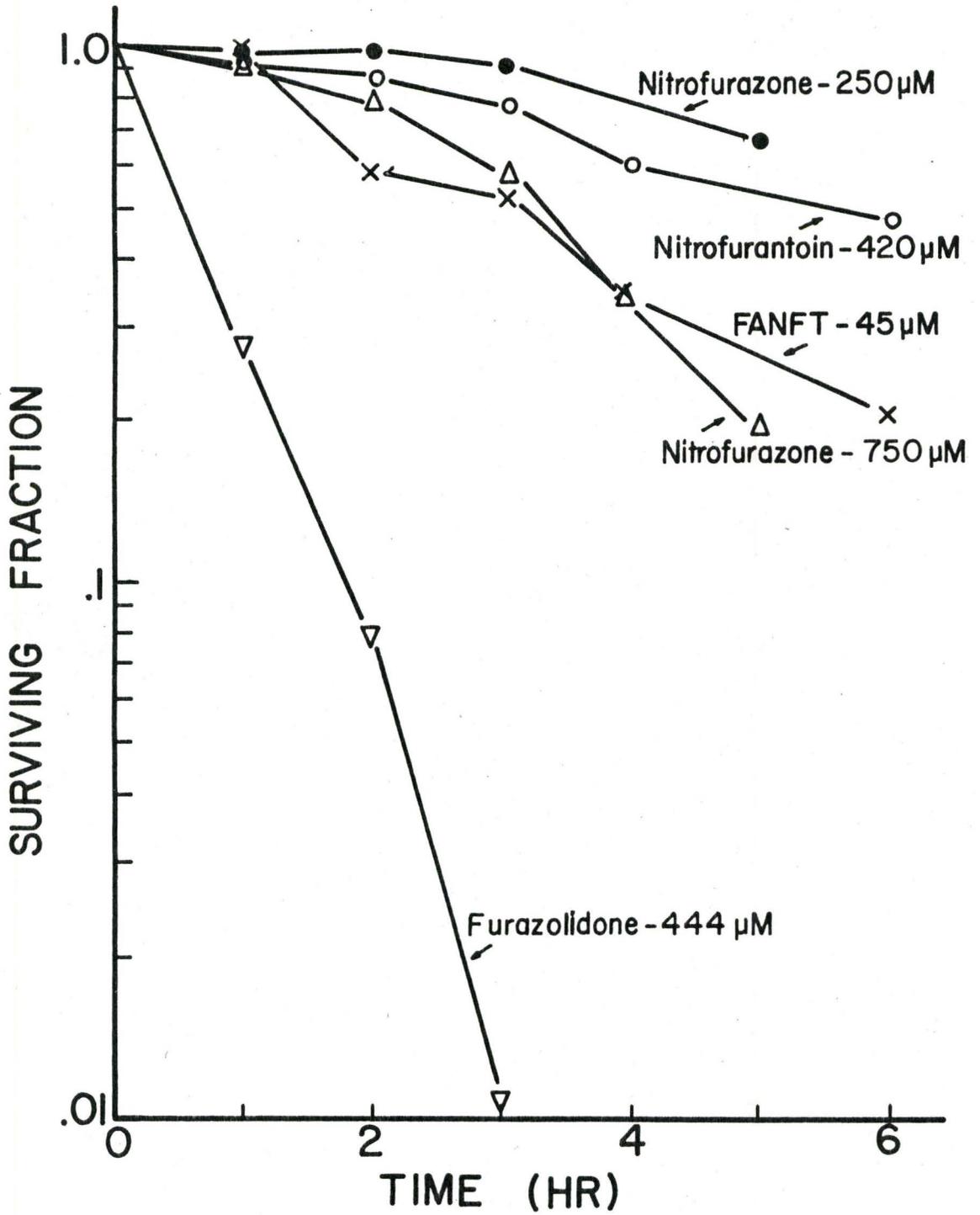


Figure 6: Effect of Oxygen Concentration on Toxicity
of Nitrofurans

PSG containing 10^5 L cells/ml was equilibrated with 0%, 2%, 5% or 21% oxygen in nitrogen for one hour before addition of 430 μ M nitrofurantoin (a) or 250 μ M nitrofurazone (b). Control cells were equilibrated with nitrogen (C,0) or with air (C,21). Surviving fraction was determined as the number of colonies counted divided by the number of cells plated.

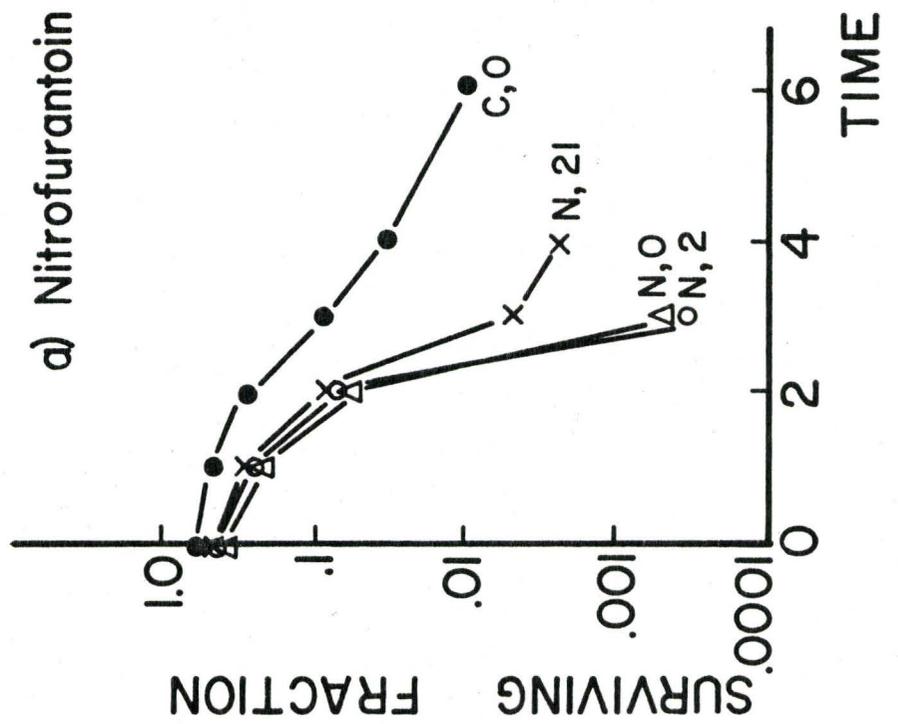
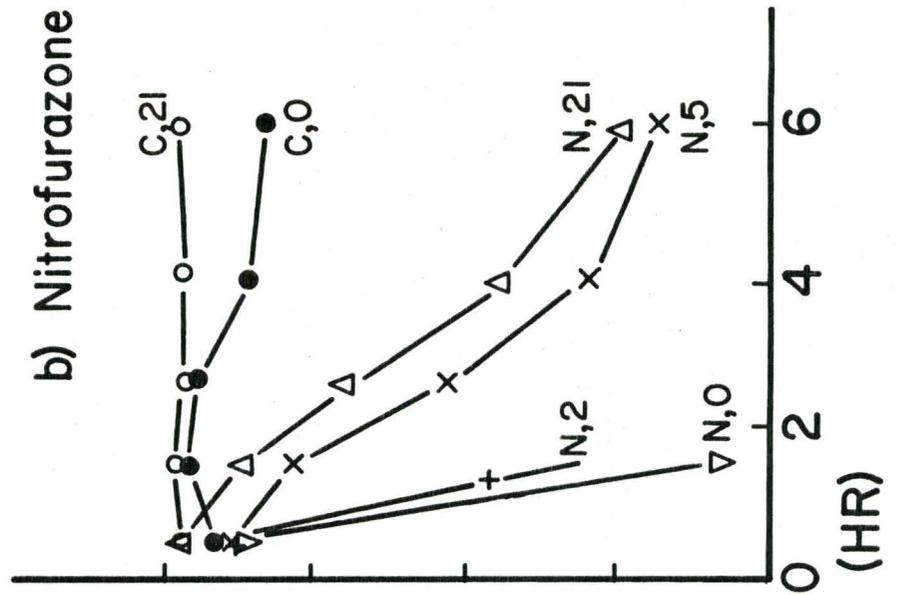


Figure 7: Effect of Incubation Medium on Toxicity

L cells were incubated in the dark under anaerobic conditions with 430 μ M nitrofurantoin in the solutions indicated, equilibrated for one hour with nitrogen before addition of cells. Numbers in brackets indicate the plating efficiencies of the corresponding controls after six hours. Surviving fraction represents the ratio of the plating efficiencies of treated cells divided by the plating efficiency of the corresponding control.

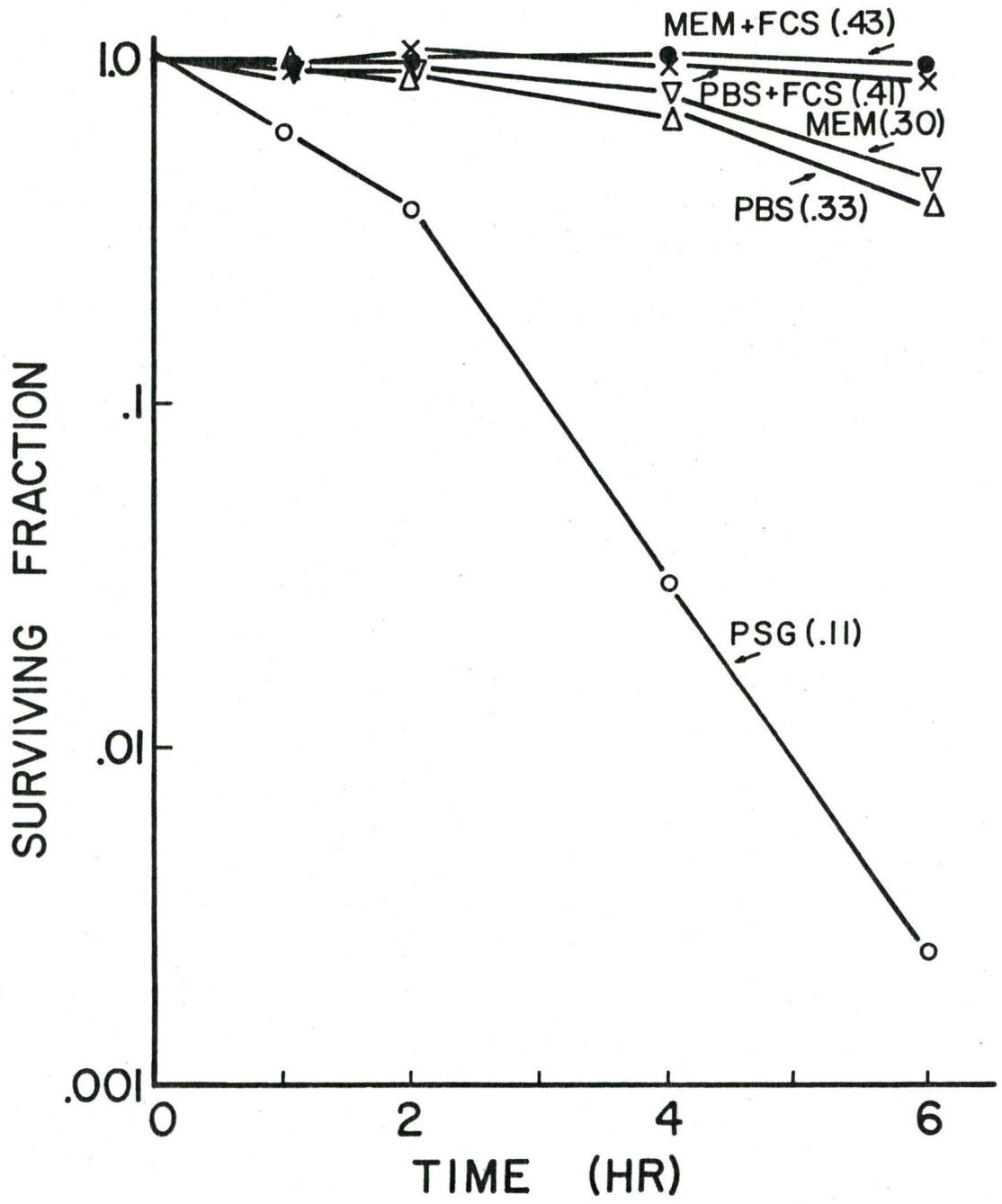


Figure 8: Effect of Glucose on Nitrofurantoin
Toxicity

L cells were incubated in PBS, PBS⁻, PSG or PBS plus 0.1% glucose under nitrogen. Solutions were equilibrated with nitrogen for one hour before addition of cells. 'D' indicates that 430 μ M nitrofurantoin was included. 'C' indicates control samples. Surviving fraction was calculated by dividing the number of colonies counted 12 days later by the number of cells plated.

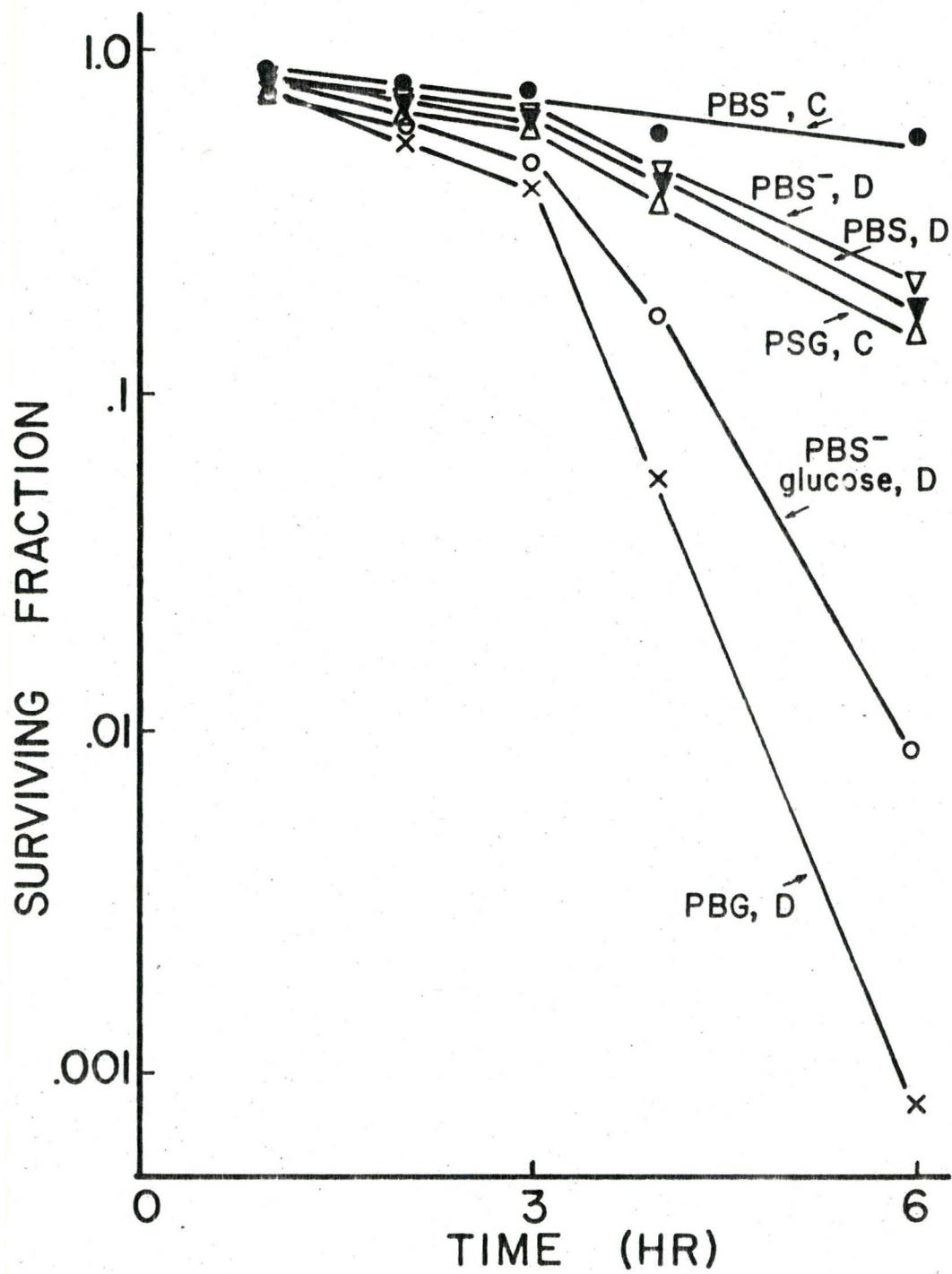


Figure 9: Effect of Temperature of Incubation on
Toxicity of Nitrofurantoin

Surviving fraction is plotted versus time of incubation calculated by dividing plating efficiencies of HeLa cells treated with 430 μM nitrofurantoin by those of the corresponding anoxic controls.

Solutions were equilibrated with nitrogen for one hour before addition of cells.

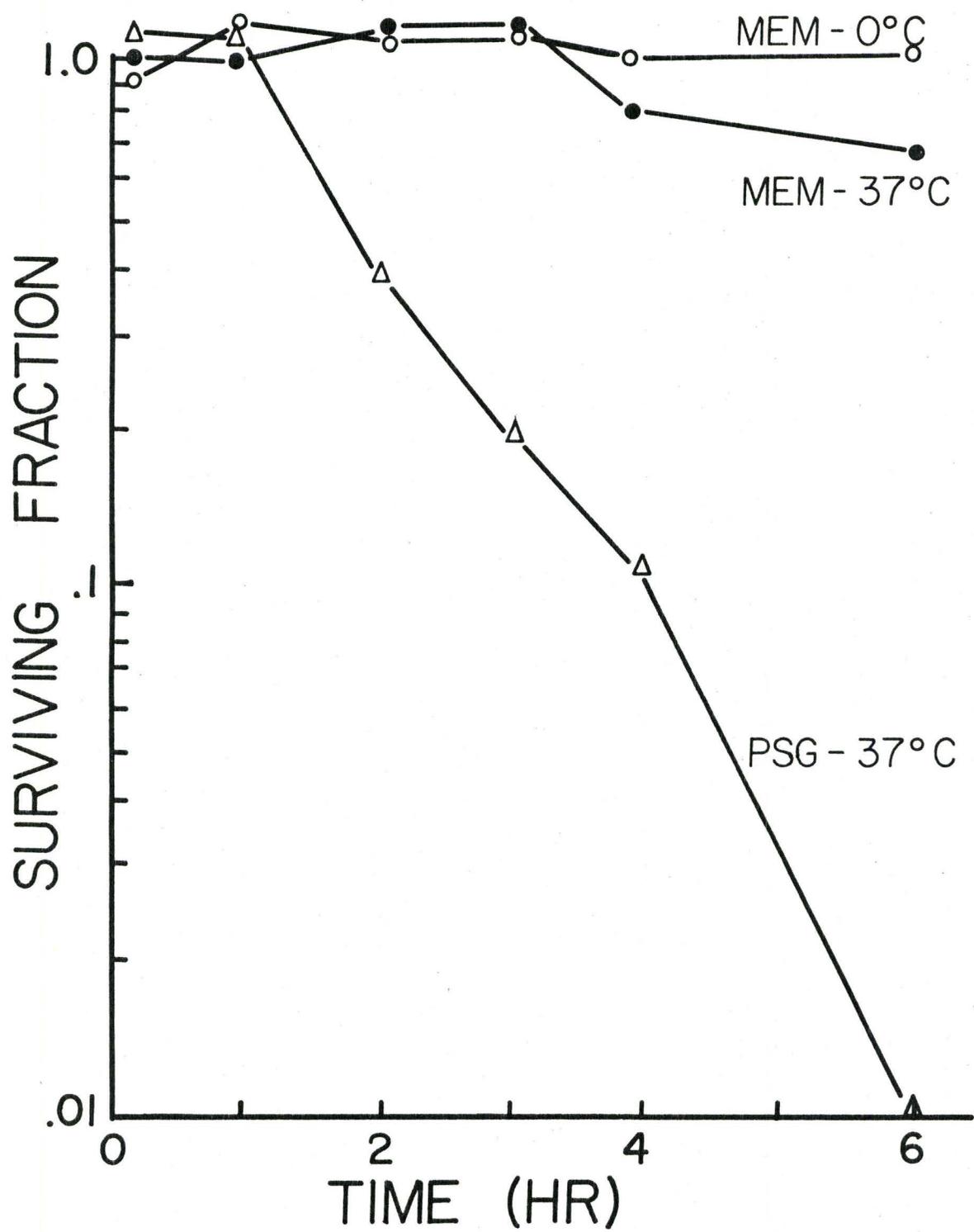


Figure 10: Effect of Nitrofurantoin on Survival
of Plateau Phase and Exponentially-
Growing Cells.

L cells were suspended in MEM and equilibrated with 97% nitrogen and 3% carbon dioxide for one hour before addition of nitrofurantoin (concentration in μ M is indicated in figure). Plateau phase cells were grown on petri dishes for 3 days in depleted medium. Zero time controls indicated that plating efficiencies were comparable to cells growing exponentially in suspension. Surviving fraction was determined by the number of colonies counted divided by the number of cells plated.

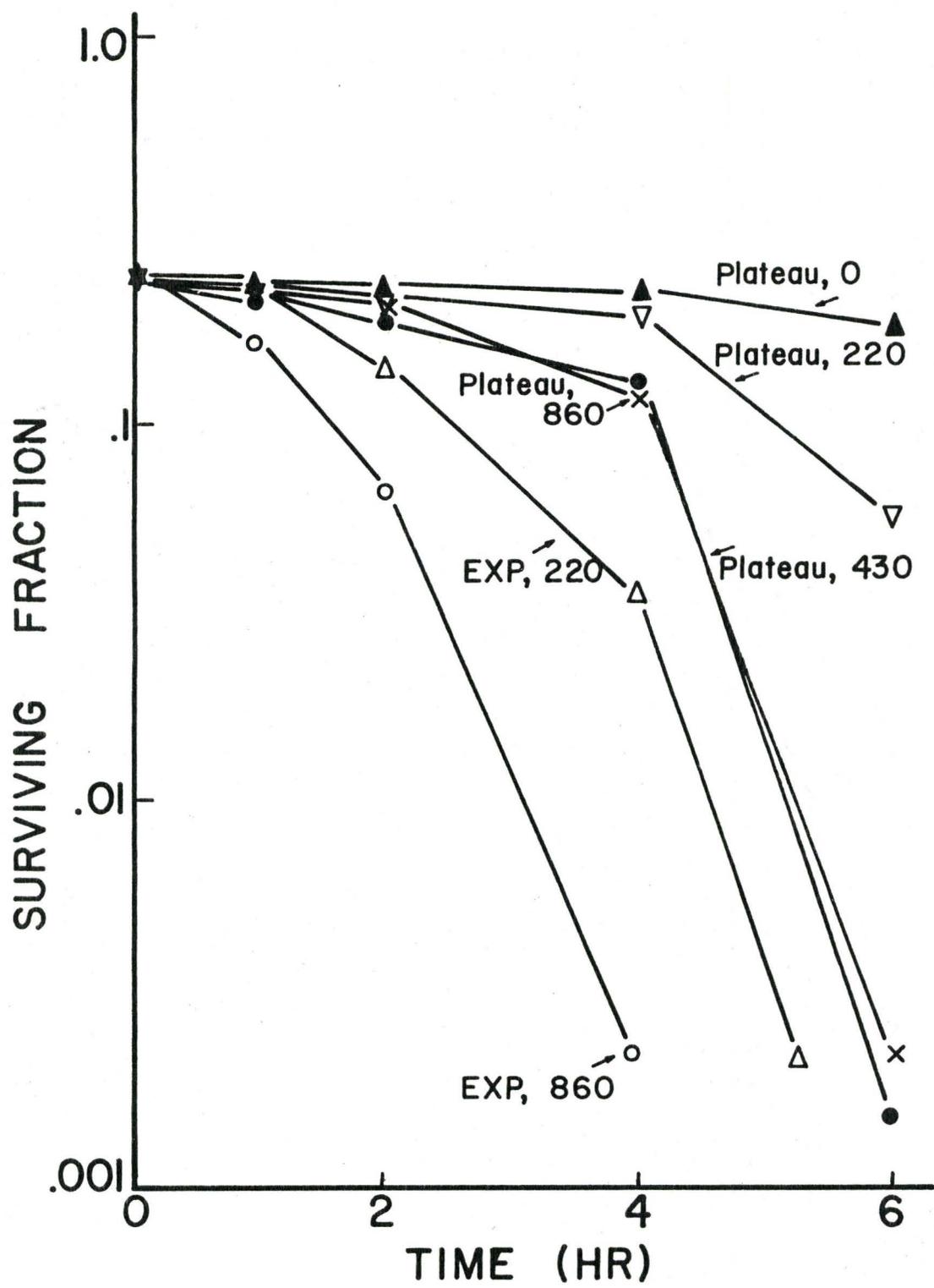
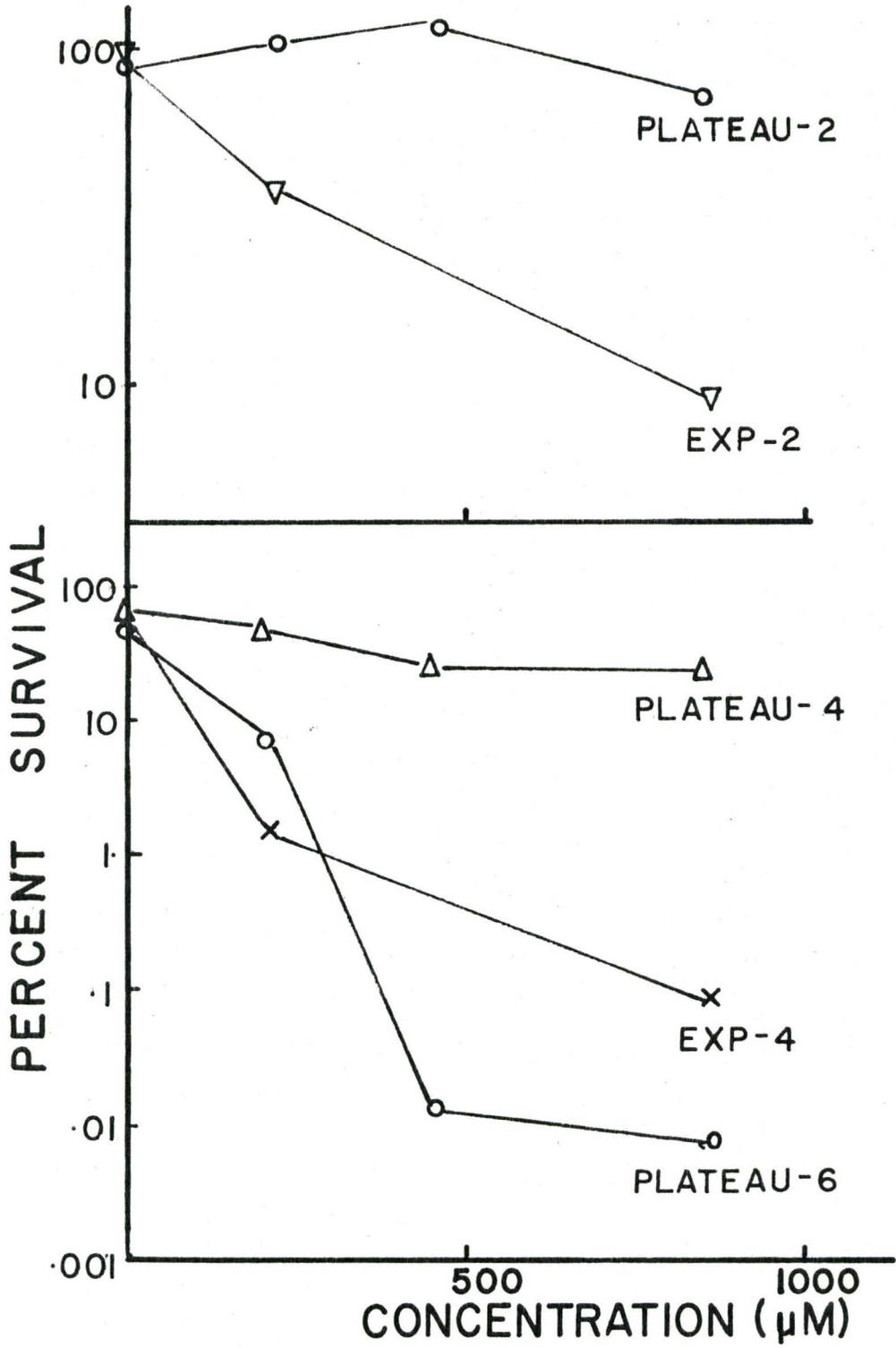


Figure 11: Variation in Shape of Survival Curve
with Duration of Exposure to Nitro-
Furantoin

Data from Figure 10 is plotted as surviving fraction versus concentration of nitrofurantoin. Numbers following the description of cell growth state (Exponential or Plateau-phase) indicate the duration of treatment, i.e. the time of plating, under anoxic conditions in MEM.



3.2.2 Inhibition of the Stimulation of Lymphocytes by Phytohemagglutinin

Human peripheral lymphocytes normally do not divide in vivo, or when maintained in culture. However, in the presence of a mitogenic stimulant, such as phytohemagglutinin (PHA), a series of events is initiated that lead to DNA synthesis and cell division. After 20 hours in the presence of PHA, there is a marked stimulation of DNA polymerase with a parallel rise in thymidine incorporation (Elves 1972). The ability of a cell to transform after PHA stimulation is a measure of cell integrity; the initial requirement of binding of PHA involves functional membrane receptors, and later events require membrane transport, RNA synthesis and DNA synthesis.

Human lymphocytes incubated with nitrofurazone under anoxic conditions for 4 hours, then washed and resuspended in MEM plus ^3H -thymidine with PHA showed a decreased incorporation of labelled thymidine compared to controls (Table 2). Hydroxyurea was added to prevent replicative DNA synthesis in the small number of lymphocytes that might be cycling (Leiberman et al. 1971).

Incorporation into DNA was decreased when lymphocytes were incubated with low doses of FANFT for two hours in PBS⁻ equilibrated with nitrogen, then washed

and resuspended either immediately or two days later in medium containing PHA and $^3\text{H-TdR}$ (Table 3). The ability to stimulate nitrofurazone-treated lymphocytes returned to some extent after two days 'repair' incubation although this was true only in a relative sense since control incorporation was depressed after two days.

A very small percent of viable cycling lymphocytes might account for uptake after 18 hours in $^3\text{H-TdR}$ and would suggest that the low activity in lymphocytes treated with 22 μM FANFT was due to toxicity, although trypan blue exclusion studies did not indicate this. Similar incubation times of L cells with 44 μM FANFT resulted in only 20% cell death, and 20 to 50% after treatment with 500 μM nitrofurazone. However, these results are not directly comparable due to possible differences in cell sensitivity.

Thirty minutes anoxic incubation with either 500 μM nitrofurazone or 22 μM FANFT did not inhibit the PHA response to a measurable degree (data not shown).

TABLE 2
 INHIBITION OF THE RESPONSE OF LYMPHOCYTES TO PHA
 STIMULATION BY NITROFURAZONE

PHA	HU	Concentration of Nitrofurazone (μ M) (cpm/ 10^5 cells)		
		0	50	500
-	-	1562	1435	1490
-	+	1938	1611	1421
+	-	23800	8877	3885

Lymphocytes were collected from 60 ml heparinized blood and incubated for 4 hours in the above concentrations of nitrofurazone dissolved in MEM in nitrogen. After washing, 2 ml aliquots were made to the above tubes in triplicate and ^3H -TdR added until TCA precipitation 72 hours later. Toxicity was not measured.

TABLE 3
 INHIBITION OF THE RESPONSE OF LYMPHOCYTES TO PHA
 STIMULATION BY FANFT

Time(hr) of PHA Stimulation	Harvest Time (hr)	Concentration of FANFT (μ M) (cpm/ 10^6 cells)		
		0	10	22
0	48	650,000	292,000	508
	72	750,000	250,000	2,000
48	48	175,000	110,000	3,000
	72	400,000	203,000	2,500
Background (no PHA)	18	2,270	2,225	800

Lymphocytes were incubated for 2 hours in the above concentrations of FANFT in MEM under nitrogen. Time of PHA stimulation is the length of time after treatment with nitrofurantoin that PHA was added. Harvest time is the length of time that cells were stimulated with PHA in the presence of ^3H -TdR. After this time, duplicate samples of cells were collected, washed in medium and precipitated with 5% TCA for determination of incorporated radioactivity.

3.2.3 Inhibition of Macromolecule Synthesis

Nitrofurans are known to affect macromolecule synthesis in bacteria. Since DNA, RNA and protein synthesis are required for cell growth and maintenance, alterations in synthetic rate can be a measure of toxicity and perhaps an indication of cellular targets for nitrofuran action. For example, a decrease in the rate of DNA synthesis might reflect death of a fraction of cells in the population rather than inhibition of synthesis in all cells in S phase. It has also been suggested that errors in DNA synthesis might result in malignant alterations (Loeb, Springgate and Battula 1974).

Nitrofurazone inhibited the incorporation of ^3H -TdR into Hela cell DNA (Figures 12,13); incorporation was inhibited both in air and nitrogen, although to a greater extent in nitrogen. The degree of inhibition was dependent upon the concentration of nitrofurazone (Figure 12) and the duration of exposure. Nitrofurantoin and furazolidone inhibited DNA synthesis in air but not in nitrogen, while FANFT inhibited incorporation to about the same extent in air and nitrogen (Figure 13).

Incorporation of ^{14}C -uridine was enhanced after incubation of L cells with 500 μM nitrofurazone in air and to a slight extent in nitrogen (Figure 14). Alkaline

extractions were performed on cell contents but data were not corrected for incorporation of ^{14}C -uridine into DNA. RNA synthesis was also increased by incubation with $430\ \mu\text{M}$ nitrofurantoin in air and in nitrogen, but incubation of L cells with FANFT for 2 hours resulted in a 55% inhibition of incorporation both in air and nitrogen.

^3H -leucine incorporation was stimulated in air in the presence of nifuroxime and furazolidone and inhibited during incubation with FANFT. In nitrogen, FANFT inhibited incorporation of ^3H -leucine and nifuroxime stimulated incorporation at early times (Table 4). Nitrofurazone and nitrofurantoin had little effect on incorporation in air or nitrogen.

Macromolecule synthesis in cultured cells was affected by nitrofuran treatment but no obvious pattern of action was found. Also, no correlation was observed between oxygen concentration during incubation (i.e., level of activated intermediate) and effects on precursor incorporation, probably due to the large effects of oxygen itself on macromolecule synthesis. In a study of the effects of nitrofurans on incorporation of radioactive precursors into the DNA, RNA and protein of Ehrlich ascites cells, inhibition of incorporation was generally observed (Fuska, Fuskova and Jurasek 1973), however, nitrofurantoin inhibited thymidine but not uridine incorporation. No relationship could be found between antiprotozoal, antibacterial or

bleaching effects in *Euglena* and precursor incorporation
(Fuska et al. 1973).

Figure 12: Effect of Nitrofurazone on Incorporation
of ^3H -TdR by Hela Cells

a) Hela cells were incubated in complete medium in air or nitrogen containing $1 \mu\text{C/ml}$ ^3H -TdR with or without $500 \mu\text{M}$ nitrofurazone. At specified times, a sample of cells was removed, DNA precipitated with 5% TCA and specific activity measured and expressed as cpm/cell.

b) The percent of inhibition of DNA synthesis is plotted as a function of the concentration of nitrofurazone during incubation under nitrogen in complete medium after four hours.

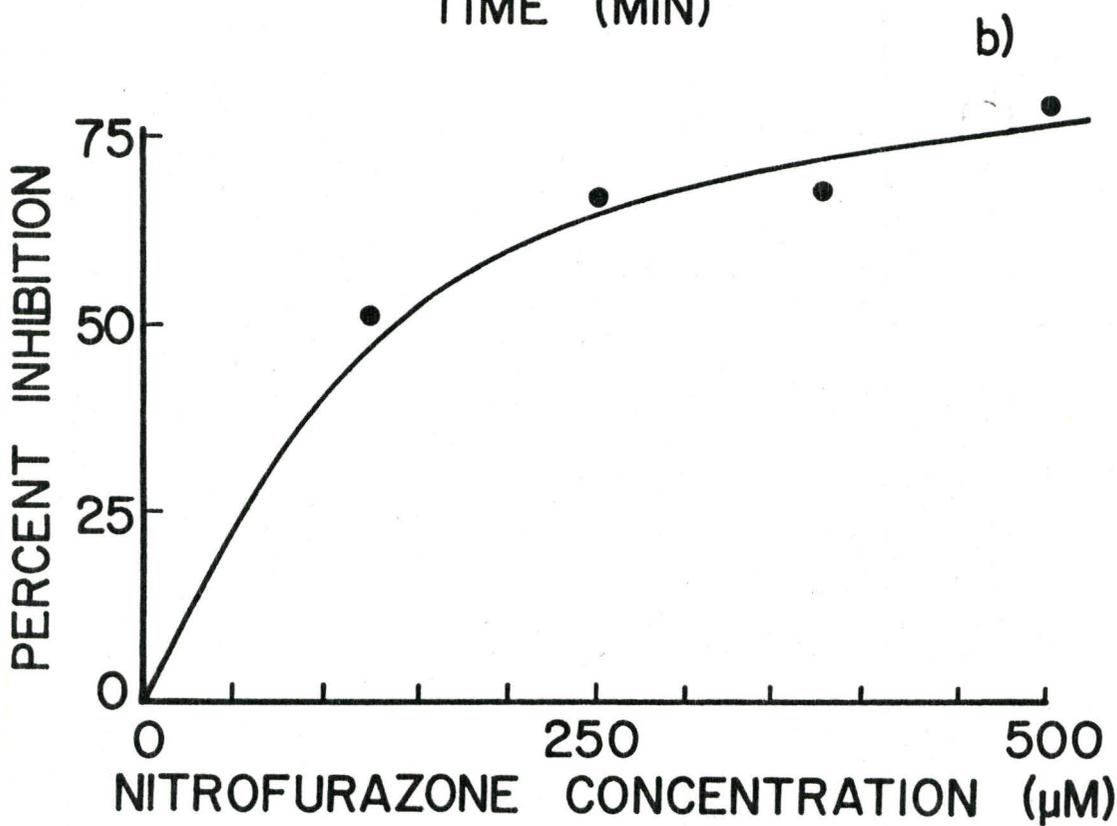
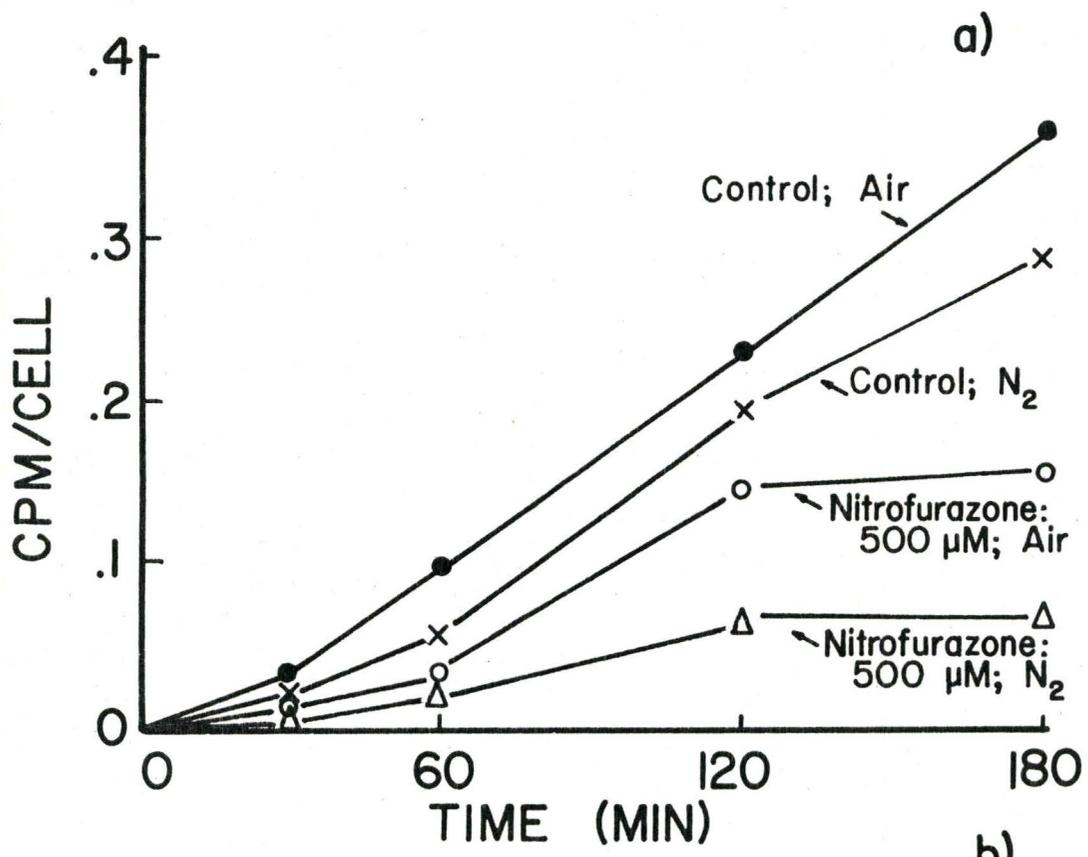


Figure 13: Inhibition of DNA Synthesis by Nitrofurans

Hela cells were incubated in complete medium in either air or nitrogen with 430 μ M nitrofurantoin, 444 μ M furazolidone or 43 μ M FANFT. Continuous uptake of ^3H -TdR (2 μ C/ml) was expressed as cpm/cell over a period of four hours, after TCA precipitation. The mean of duplicate determinations is shown.

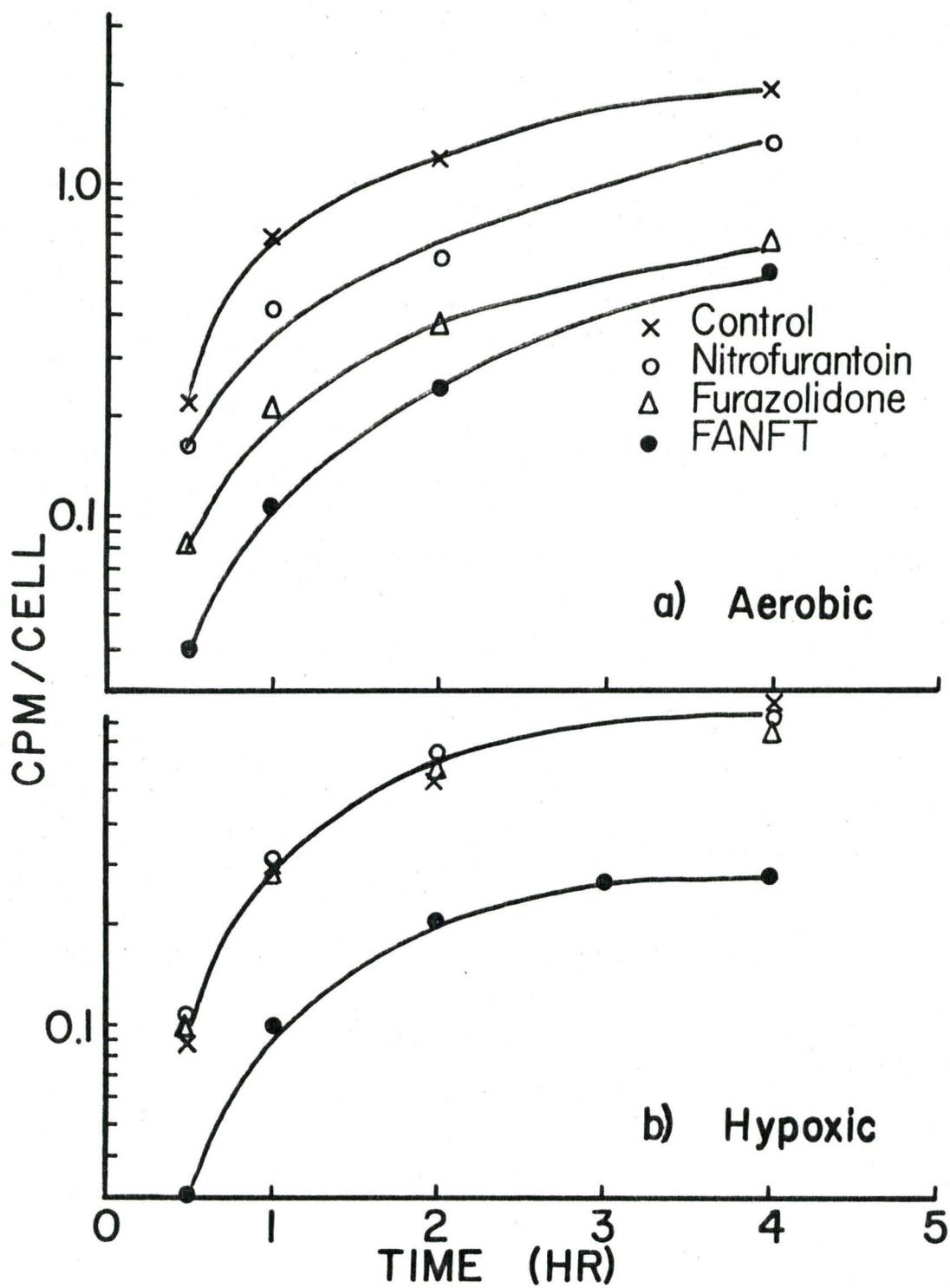


Figure 14: Effect of Nitrofurans on RNA Synthesis

Incorporation of ^{14}C -uridine into HeLa cell RNA is plotted versus incubation time in 500 μM nitrofurazone. Perchloric acid extractions were followed by incubation of samples in alkali and measurement of radioactivity in the supernatant after centrifugation.

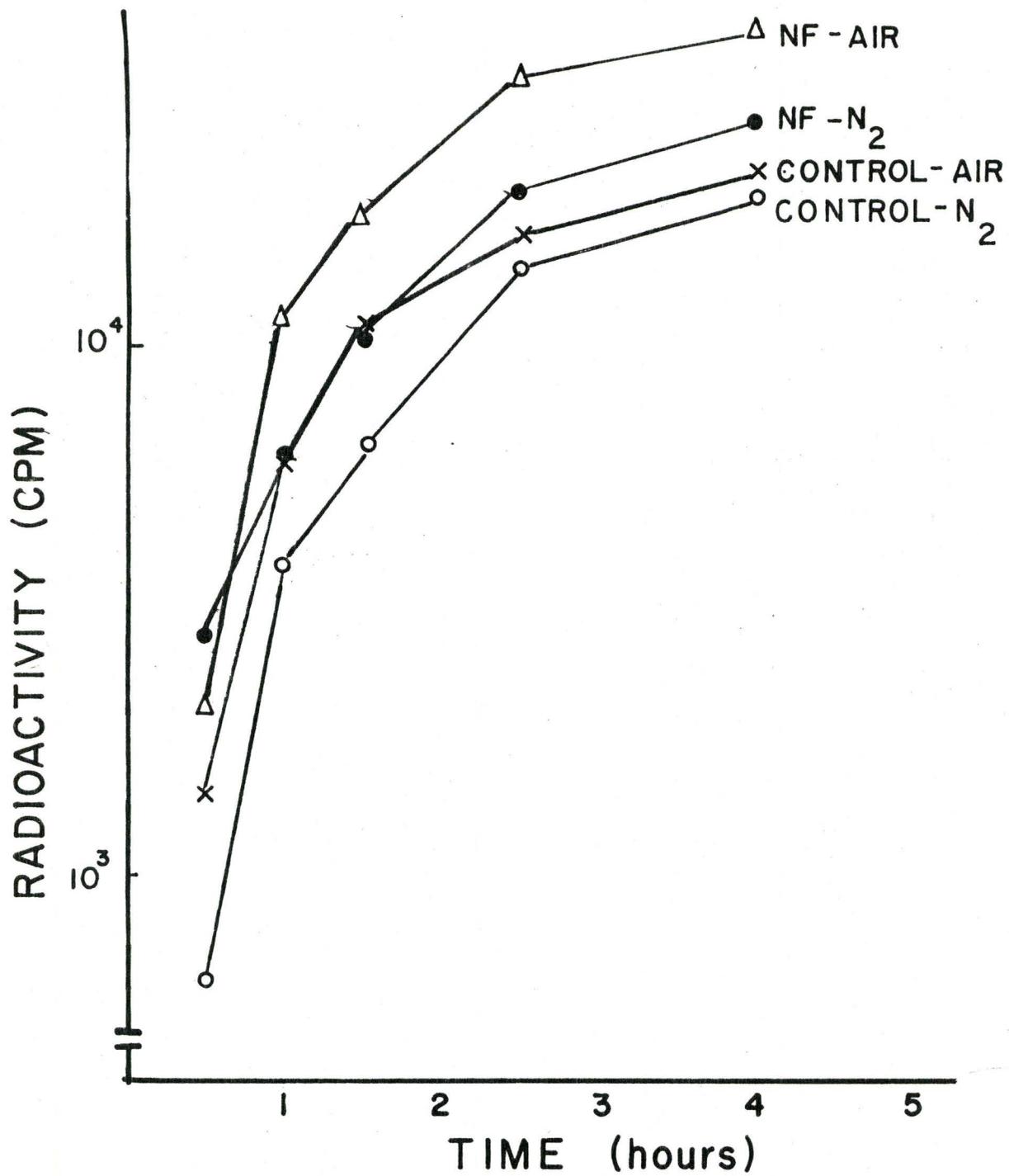


TABLE 4
EFFECT OF NITROFURANS ON INCORPORATION OF ^3H -LEUCINE

Nitrofurans	Concentration (μM)	Time of Incubation (min)			
		30	60	90	120
A. Incubation in Air					
Nitrofurazone	500	98	88	98	105
Furazolidone	444	160	138	130	133
Nitrofurantoin	430	105	110	110	115
FANFT	44	99	81	61	41
Nifuroxime	630	238	210	180	162
B. Incubation in Nitrogen					
Nitrofurazone	500	94	98	84	94
Nitrofurantoin	430	82	84	81	76
FANFT	44	89	79	74	64
Nifuroxime	630	138	120	85	91

L cells were incubated in complete medium. At the times specified, a sample of cells was removed and incubated with $2 \mu\text{C/ml}$ ^3H -leucine under aerobic conditions for 30 minutes. Cells were precipitated on membrane filters with TCA. Data are expressed as a percent of the control incorporation. Single determinations were performed at the time specified.

3.2.4 Inhibition of ATP Synthesis

ATP is required for all synthetic pathways within cells, including the repair of DNA by ligase. Inhibition of ATP production for long periods of time could well result in cell death and contribute to the toxicity of nitrofurans. The intracellular concentration of ATP is known to be a good indicator of cell injury or cell death caused by several mechanisms (Trump, Croker and Mergner 1971).

Incubation of Hela cells with nitrofurazone decreased the level of ATP in both air and nitrogen (Figures 15,16). The extent of reduction was dependent upon the concentration of nitrofurazone, time and medium of incubation. After 45 minutes, there was no inhibition of ATP production in cells incubated in air-equilibrated PSG, and in fact, there appeared to be some stimulation. After 120 minutes, there was strong inhibition (Figure 15). After two hours, the decrease in ATP was apparently independent of dose with concentrations of nitrofurazone greater than $150\ \mu\text{M}$ (Figure 15). Cells incubated with nitrofurazone in PSG showed a smaller decline in ATP with time than those incubated in PBS^- (Figure 16). Although it appears that ATP inhibition is greatest under aerobic conditions (when calculated as a percent of the corresponding control), the rapid loss of ATP in control cells

incubated in buffer and/or nitrogen prevents a meaningful comparison.

There is some indication from work with bacteria that the concentration as well as the particular nitrofurantoin determine whether ATP production is stimulated or inhibited (McCalla, personal communication). Thus at 45 minutes, the slight stimulation in ATP production, particularly in air, may indicate an early inhibitory effect on other synthetic reactions so that the ATP level increases, while at later times (or higher concentrations), synthetic reactions required for ATP production are also inhibited.

Figure 15: Inhibition of ATP Formation by Nitrofur-
azone

The amount of ATP per cell is plotted as a function of the concentration of nitrofurazone in PSG using Hela cells incubated under 0%, 5% or 21% oxygen in nitrogen for 45 or 120 minutes. Samples of cells were pipetted into boiling water for assay of ATP (see Section 2.6). Zero values were obtained by incubating cells without nitrofurazone for the same period of time. Each point represents the mean of 5 determinations of ATP content using the same sample.

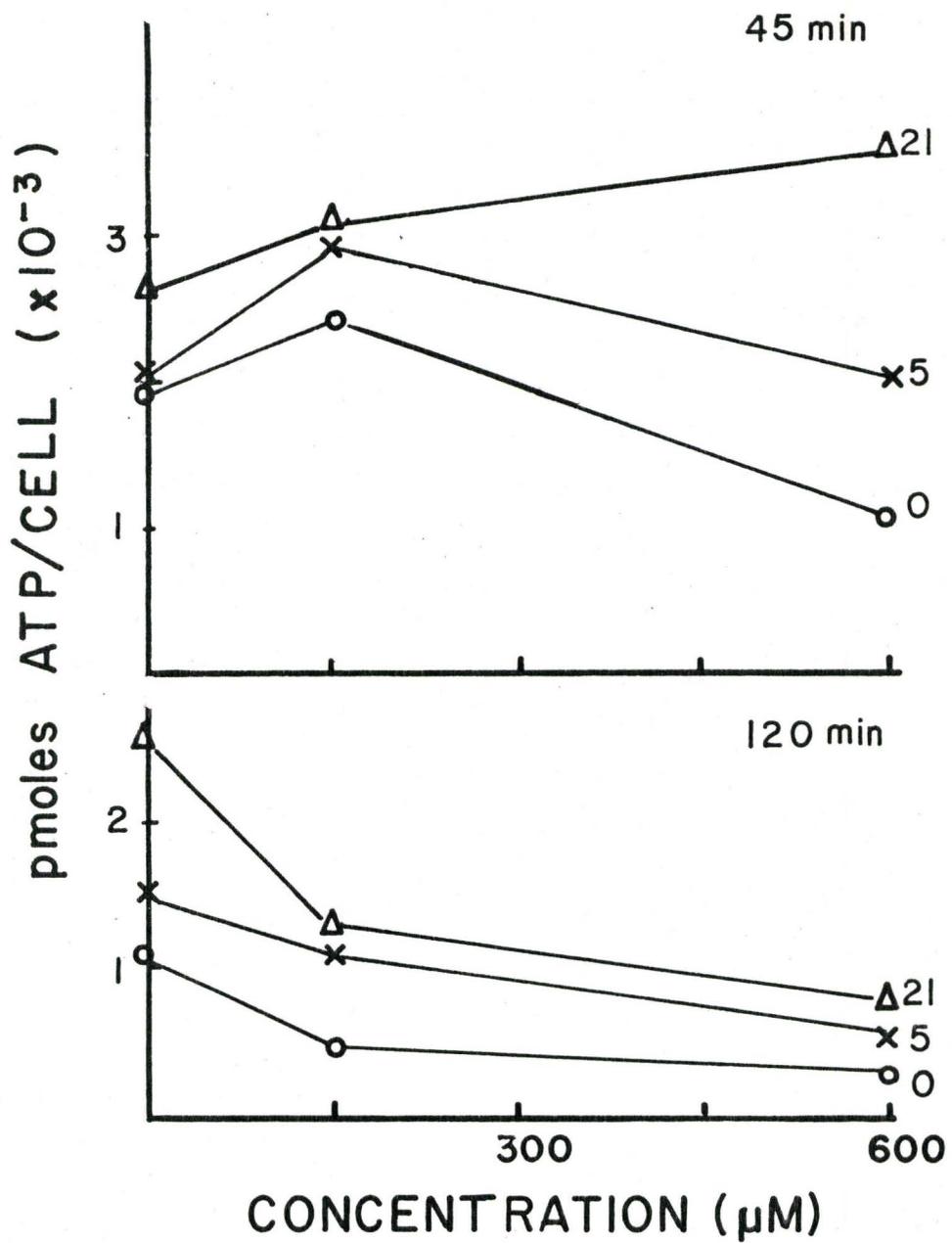
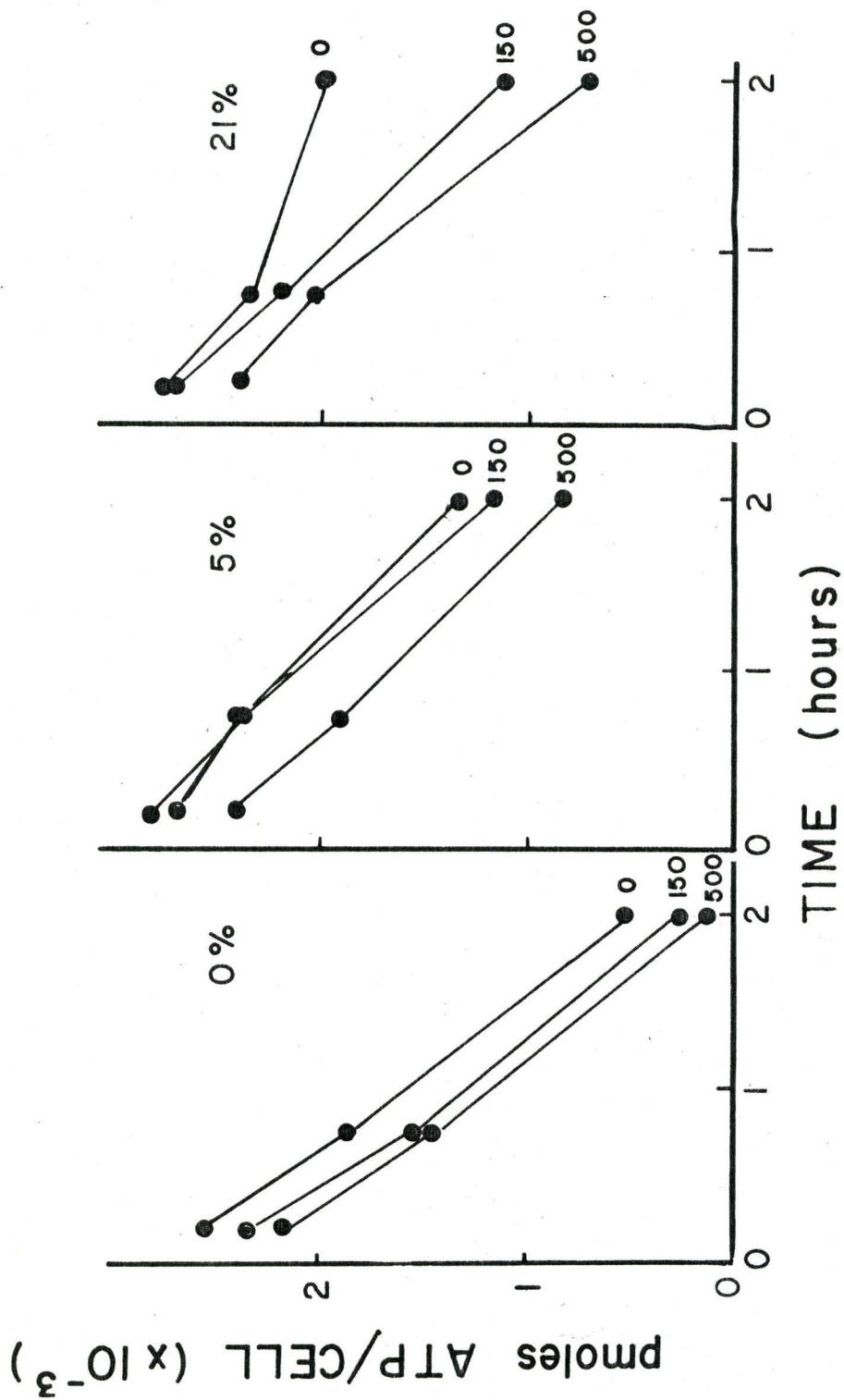


Figure 16: Time Course of ATP Inhibition

Hela cells were incubated in PBS containing 0, 150 or 500 μ M nitrofurazone, gassed with 0, 5% or 21% oxygen in nitrogen. At specified times, samples of cells were removed for ATP determination. Each point represents the mean of 4 determinations on a single sample of treated cells.



3.3 Production and Repair of Single-Strand DNA Breaks

3.3.1 Cells in Culture

A decrease in the molecular weight of single-strands of DNA on alkaline sucrose gradients demonstrates certain types of DNA damage. Rejoining of 'breaks' may be equated with repair although the fidelity of such repair cannot be determined from sedimentation data. The breaks that are rejoined incorrectly may result in mutation; breaks that are not rejoined most likely result in cell death.

Nitrofurantoin treatment of mouse L, hamster CHO and human KB cells resulted in single-strand breaks in DNA (Figure 17). The amount of damage depended upon the nitrofurantoin tested (Figures 18,19,20). Although no relationship between toxicity (see Section 3.2.1) and the number of single-strand breaks was observed at high concentrations, when doses of the order of 10 μ g/ml were tested, FANFT was the only nitrofurantoin to cause single-strand breaks, and was also toxic to cells at this concentration (Figure 20).

The amount of damage increased with the duration of exposure (Figures 21,22,25). Although there was little or no damage after 15 minutes incubation with 430 μ M

nitrofurantoin, after 120 minutes, there was extensive damage. However, the resolution of material at the top of the gradient is poor and a normal distribution is not present leading to inaccuracies in the calculations of average molecular weight at longer incubation times. Data can be corrected by calculating molecular weights using only those fractions surrounding the peak of the profile (see Appendix 1), and the peak position itself can be a good indication of changes in molecular weight (Figure 22).

The concentration of nitrofurazone also determined the amount of damage on alkaline sucrose gradients (Figures 23,25). There appeared to be a linear relationship between the concentration of nitrofurazone and the number of single-strand breaks (Figure 25).

The amount of DNA damage decreased with greater concentrations of dissolved oxygen in the medium (Figures 19, 24,25). This may indicate interference of nitrofurans with repair phenomena, or a requirement for nitrofuran 'activation' to elicit DNA damage.

Repair of strand-breaks occurred within four hours when damage resulted from anoxic incubation of L cells with 630 μ M nifuroxime but not within 16 hours (using peak position as the criterion) when 430 μ M nitrofurantoin was the damaging agent (Figure 26). However, damage was far more extensive after exposure of cells to nitrofurantoin.

Some indication of strand rejoining was suggested from calculations of the molecular weight during repair after nitrofurantoin damage (Figure 27). Often, attempts to measure repair gave results which indicated further degradation in DNA, both in control and treated cells. This may represent cell death and degradation after incubation in buffer gassed with nitrogen.

Human Xeroderma Pigmentosum (XP) cells are not able to repair damage caused by UV and many chemicals as efficiently as normal cells, presumably because of the lack of the endonuclease required for incision around the damaged region (Cleaver 1968). It seems likely that the same endonuclease recognizes nitrofurans damaged regions in DNA. However, breaks resulted from incubation of XP cells with nitrofurans (Figure 28) implying 'direct' action of the nitrofurans or their metabolic intermediates. It is however possible that sufficient repair enzyme was present in XP cells to allow excision of damaged DNA.

Figure 17: Production of DNA Single-Strand Breaks
in Three Cultured Cell Lines

Alkaline sucrose gradient analysis of DNA from cells of three cultured cell lines incubated for 90 minutes with 250 μ M nitrofurazone (NFZ) in PBS⁻, or in PBS⁻ alone (C) under anaerobic conditions. Cells were lysed for 3.5 hours before sedimenting at 18 Krpm for 4 hours. Sedimentation is from left to right.

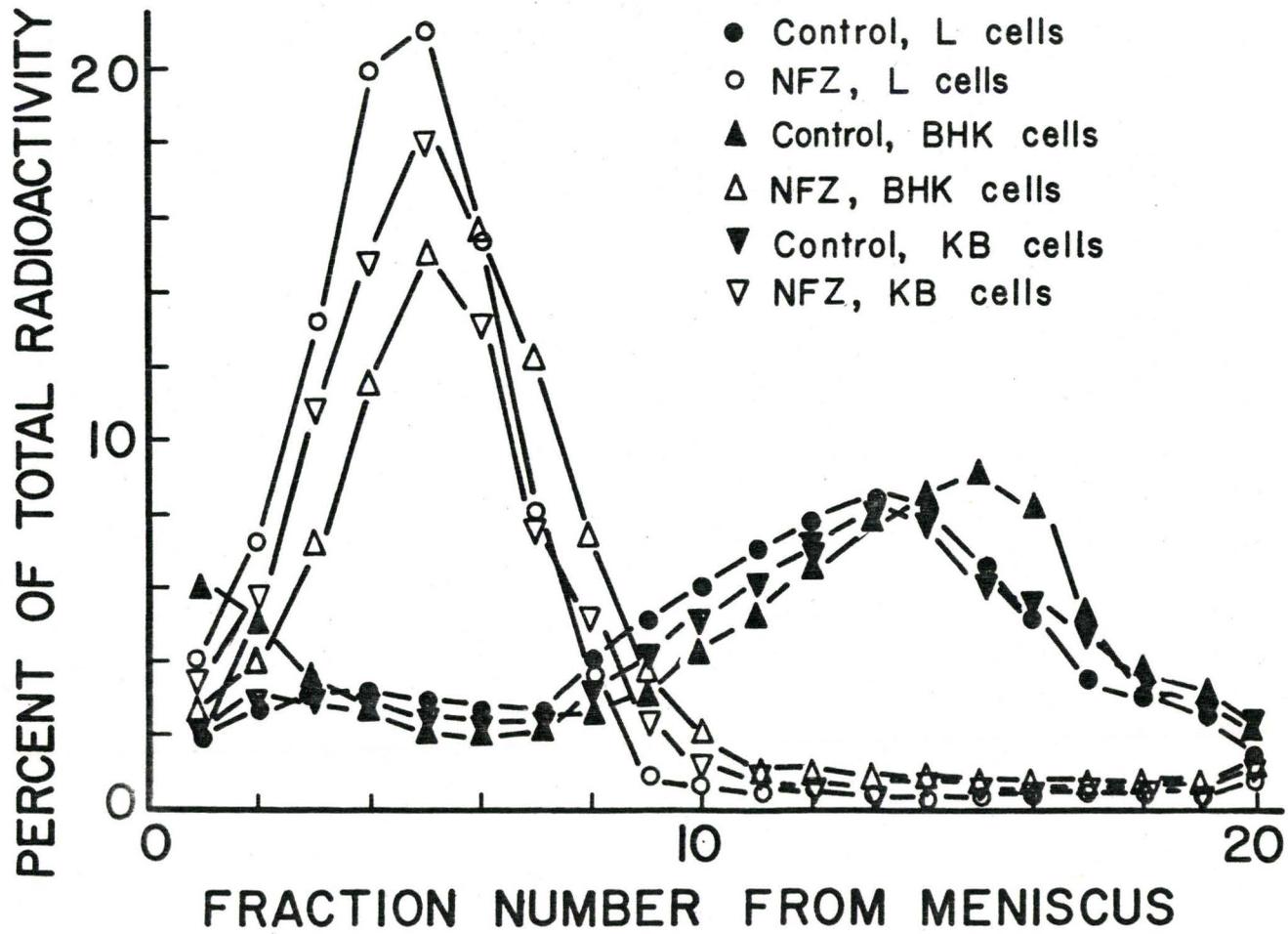


Figure 18: Production of DNA Single-Strand Breaks
by Several Nitrofurans

Alkaline sucrose gradient analysis of DNA from L cells incubated in PSG under nitrogen with 450 μ M nitrofurantoin, 630 μ M nifuroxime, 444 μ M furazolidone or 310 μ M furaltadone for 2.5 hours at 37^o. Cells were lysed 3 hours before sedimentation at 30 Krpm for 90 minutes.

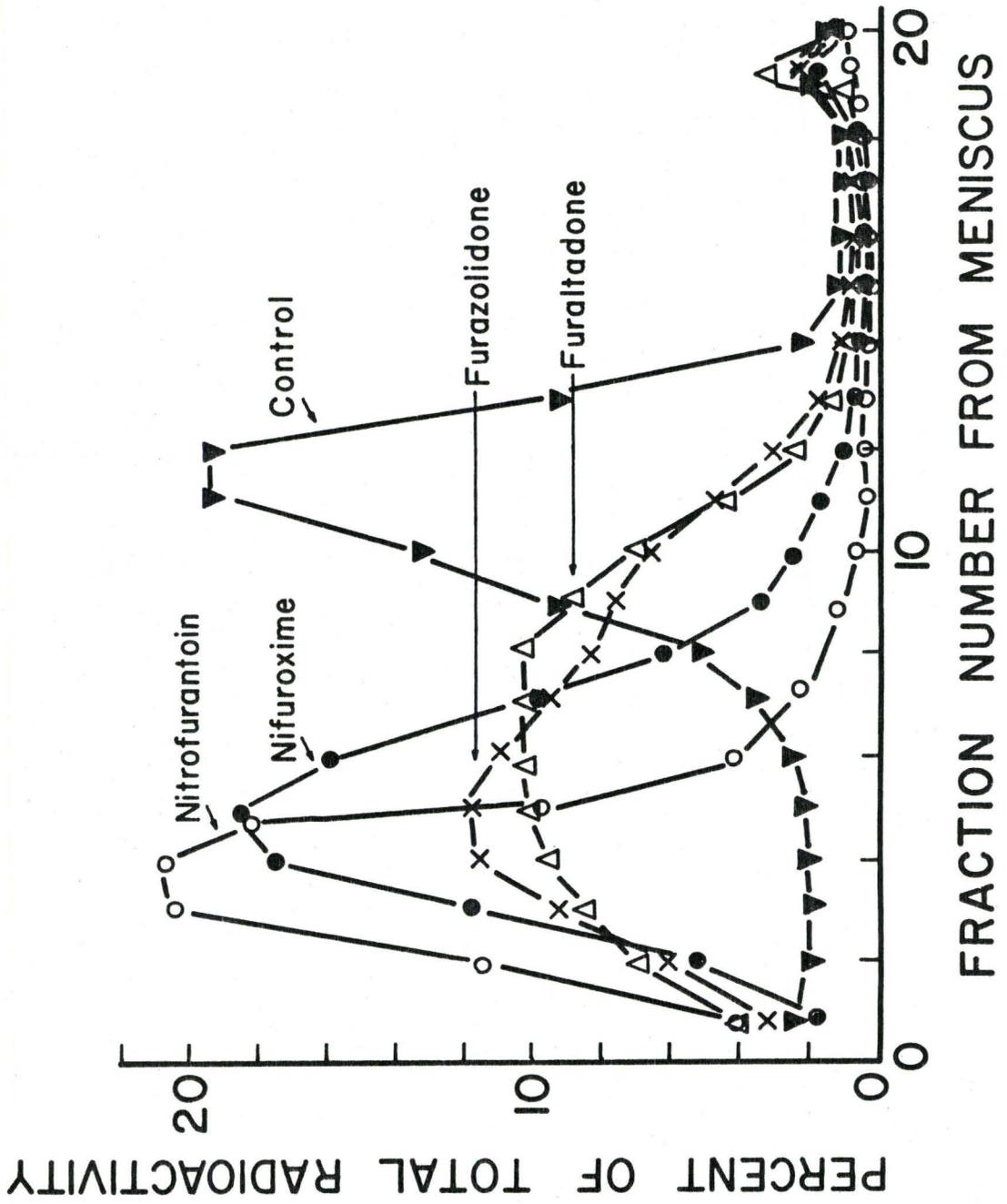


Figure 19: Effect of Oxygen Concentration on Damage
by Nitrofurazone and NF-167

L cells were incubated for 60 minutes with 250 μ M nitrofurazone dissolved in PBS⁻ equilibrated with air (NF,21) or nitrogen (NF,0), or with 250 μ M NF-167 in air (167,21) or nitrogen (167,0). Cells were lysed for two hours or for 18 hours before sedimenting at 16 Krpm for 4 hours.

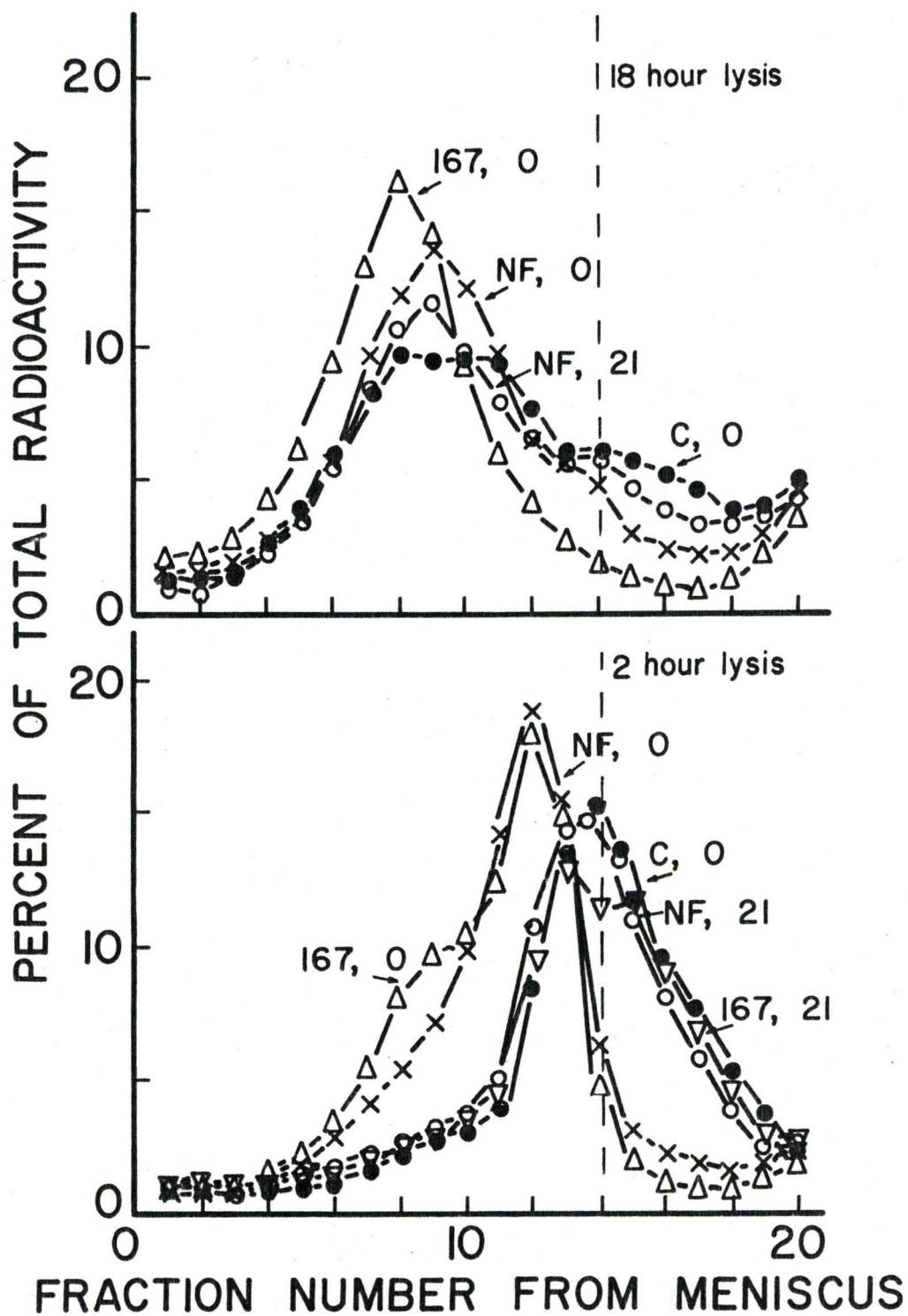


Figure 20: Production of Single-Strand Breaks
at a Low Concentration (10 μ g/ml)

3 H-TdR labelled L cells were incubated for 90 minutes under anoxic conditions with 10 μ g/ml (about 50 μ M) nitrofurazone, FANFT, NFT or nitrofurantoin in PBS⁻. Approximately 10^4 cells were layered on alkaline sucrose gradients, lysed four hours and sedimented at 18 Krpm for four hours.

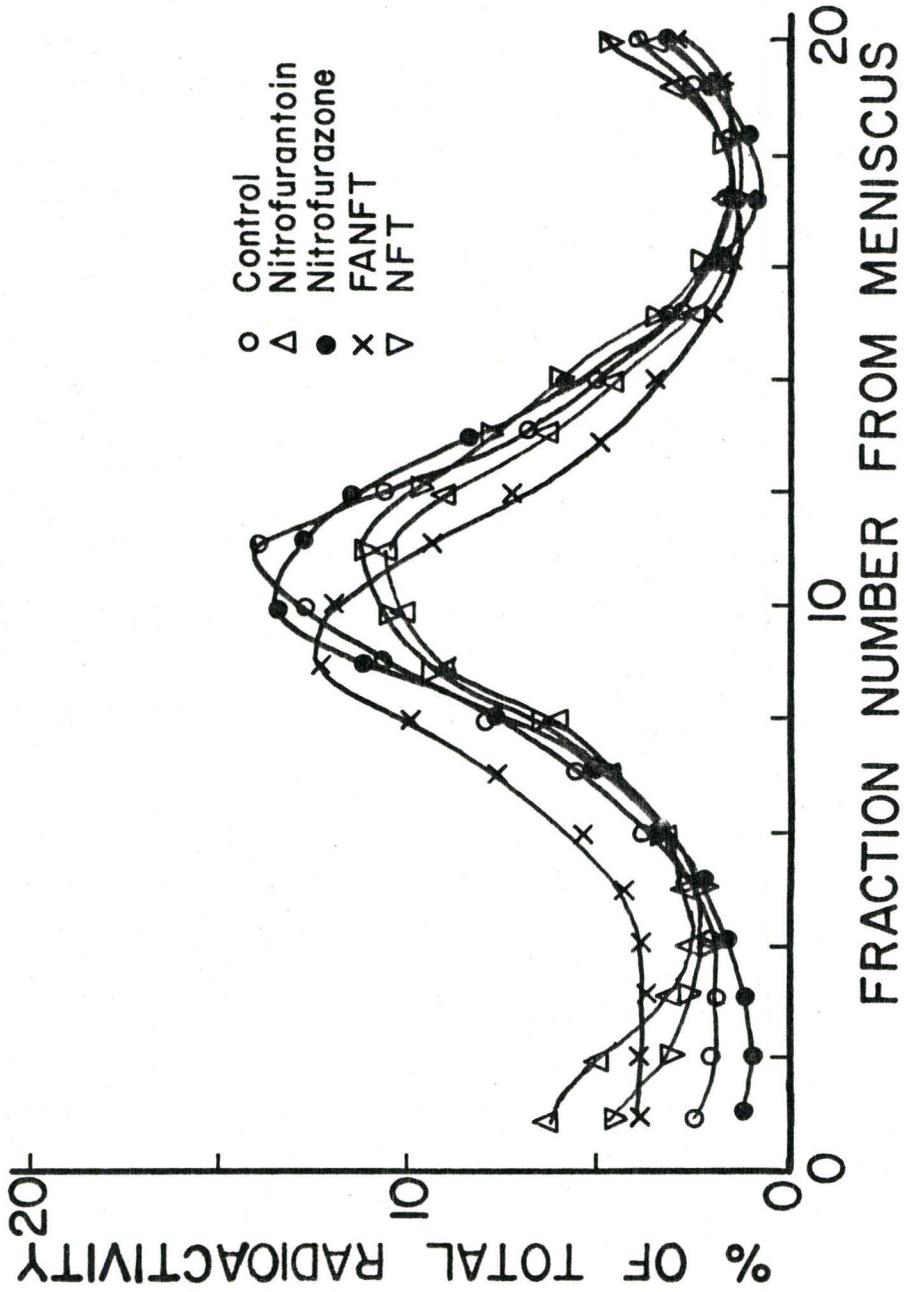


Figure 21: Effect of Duration of Incubation on
Production of Single-Strand Breaks

L cells labelled with ^3H -TdR were incubated under anoxic conditions with $430\ \mu\text{M}$ nitrofurantoin in PSG for the times indicated. They were then lysed for 3.5 hours on alkaline sucrose gradients and spun at 18 Krpm for 4 hours.

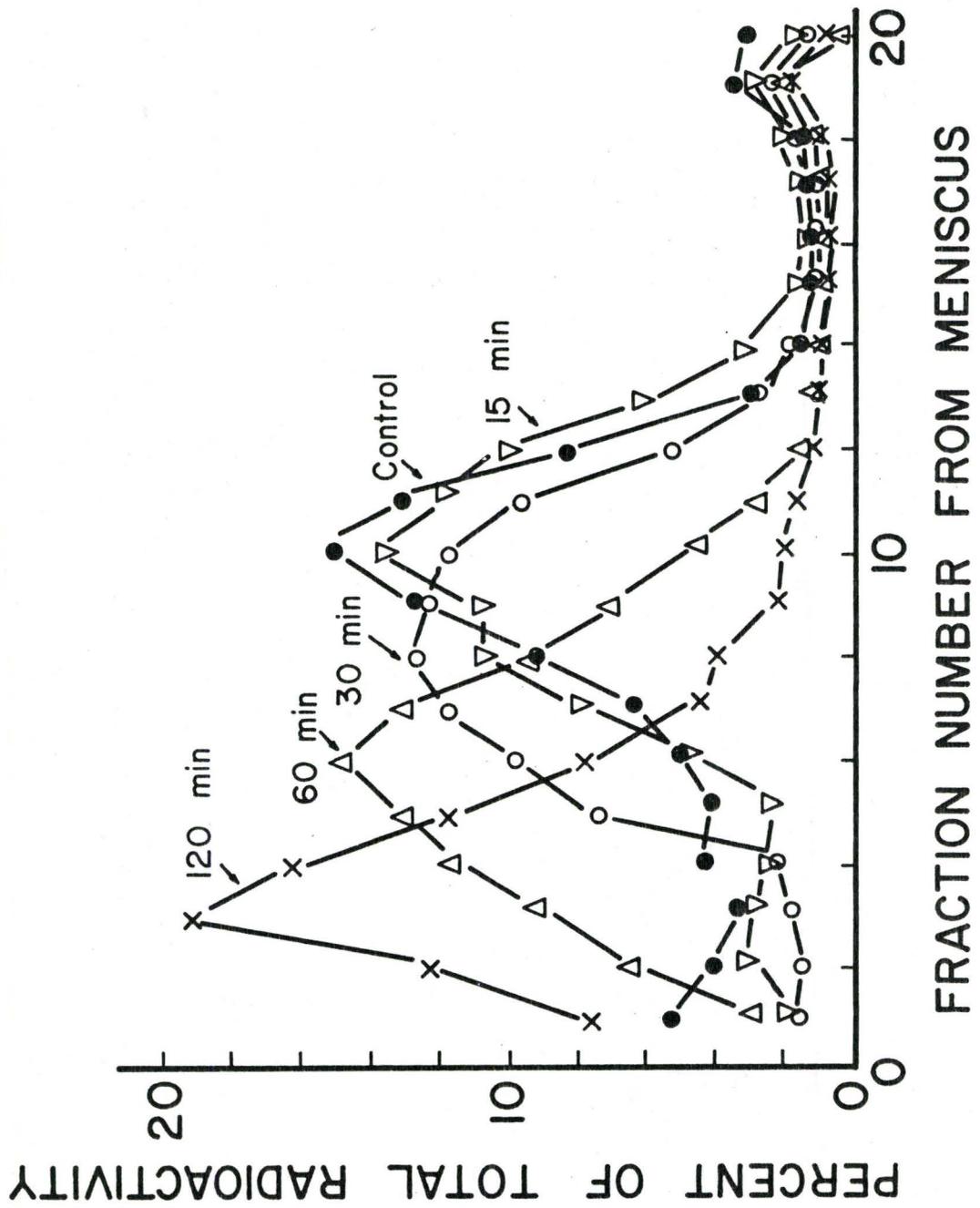


Figure 22: Calculations of Average Molecular Weight,
Corrected Molecular Weight and Peak
Position as a Function of Duration of
Nitrofurantoin Treatment

Time of incubation of L cells in 430 μ M nitrofurantoin under anoxic conditions (Figure 21) is plotted versus peak position, average molecular weight and corrected molecular weight as calculated from formulae given in Appendix 1.

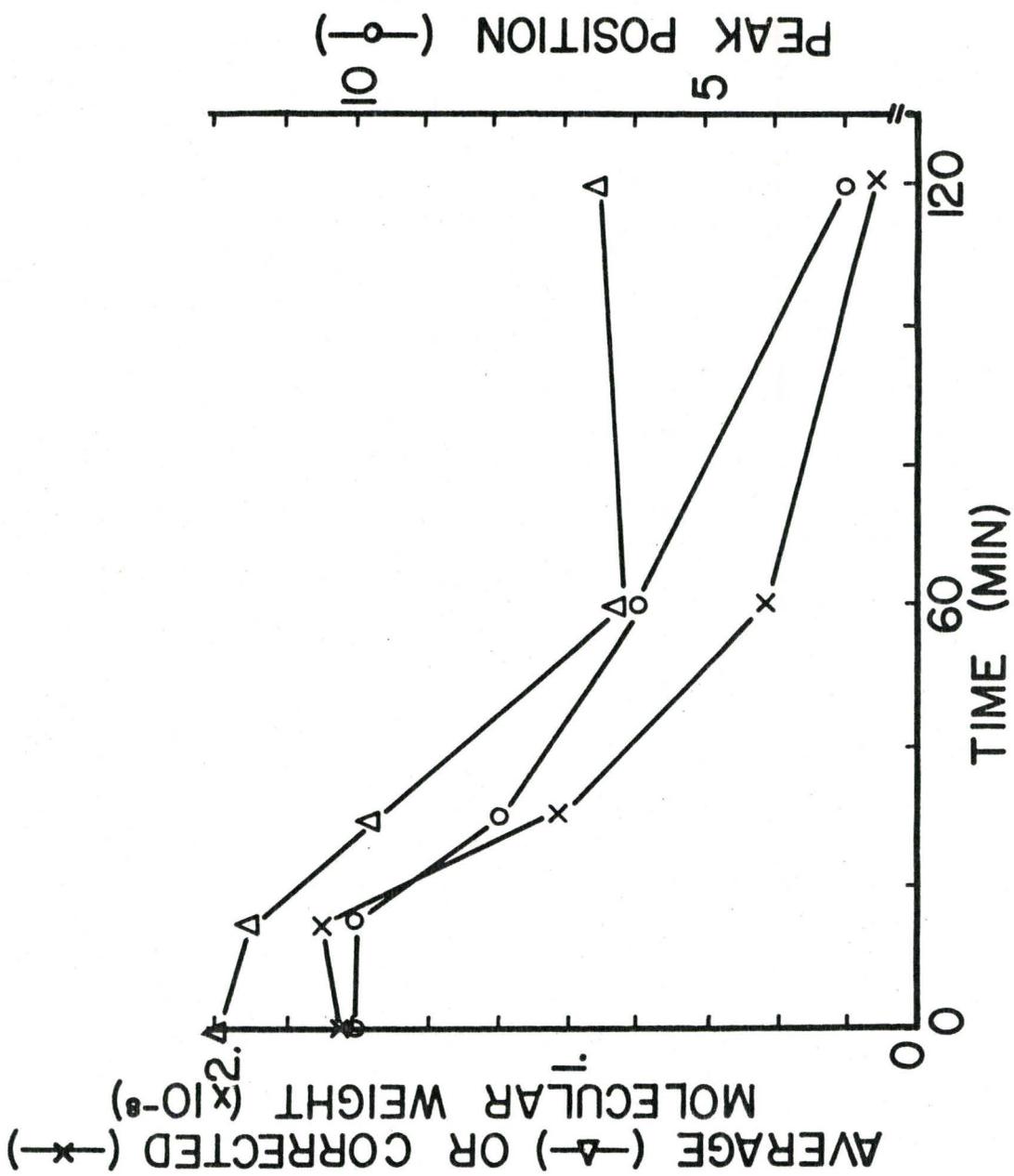


Figure 23: Effect of Concentration of Nitrofurazone
on Production of Single-Strand Breaks

Alkaline sucrose gradient analysis of DNA from L cells that were incubated for 90 minutes with different concentrations of nitrofurazone under anaerobic conditions. Cells were lysed for 3.5 hours before sedimenting at 18 Krpm for 4 hours.

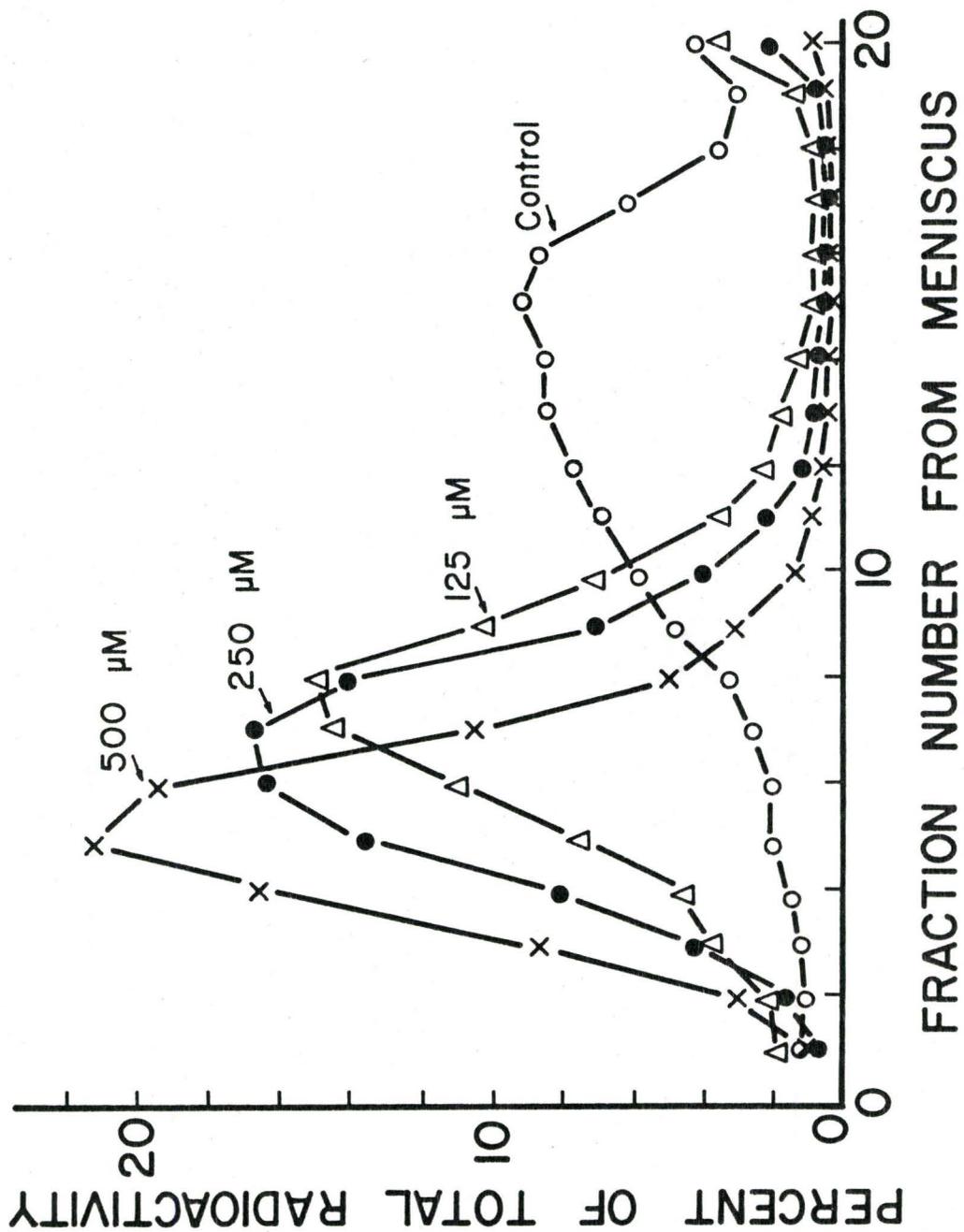


Figure 24: Effect of Oxygen Concentration on
Production of Single-Strand Breaks

³H-TdR labelled L cells were incubated with a) 500 μ M nitrofurazone or b) 430 μ M nitrofurantoin in PSG equilibrated with air, or 0%, 2%, 5% oxygen in nitrogen. After two hours, cells were lysed on top of alkaline sucrose gradients and spun at 18Krpm for 4 hours.

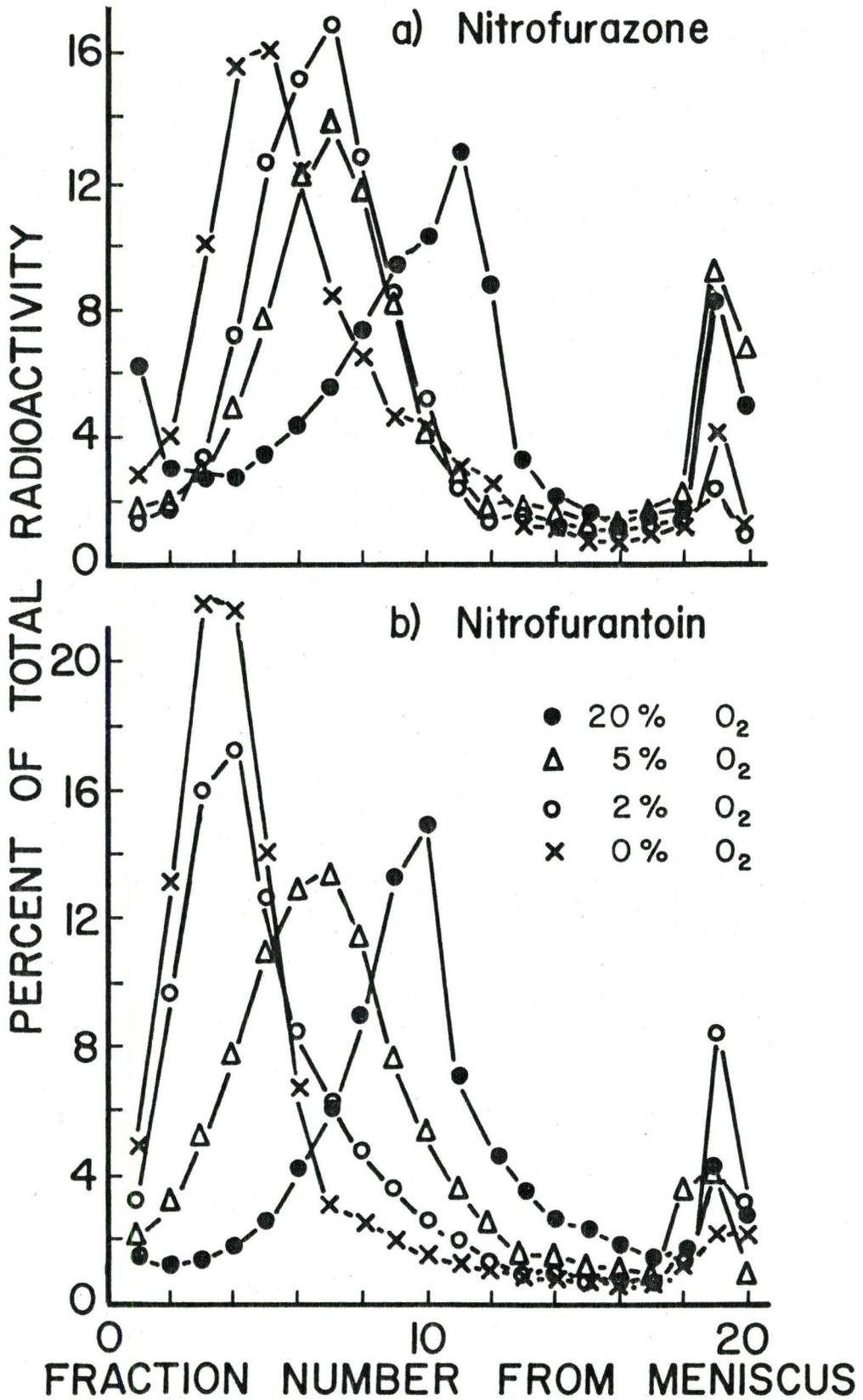


Figure 25: Relation of DNA Breaks to Oxygen Concentration, Nitrofurazone Concentration and Duration of Incubation

Data from Figures 21, 23 and 24 were used to calculate molecular weights (see Appendix 1). Results are plotted as $1/\text{Molecular weight}$, which is proportional to the number of breaks.

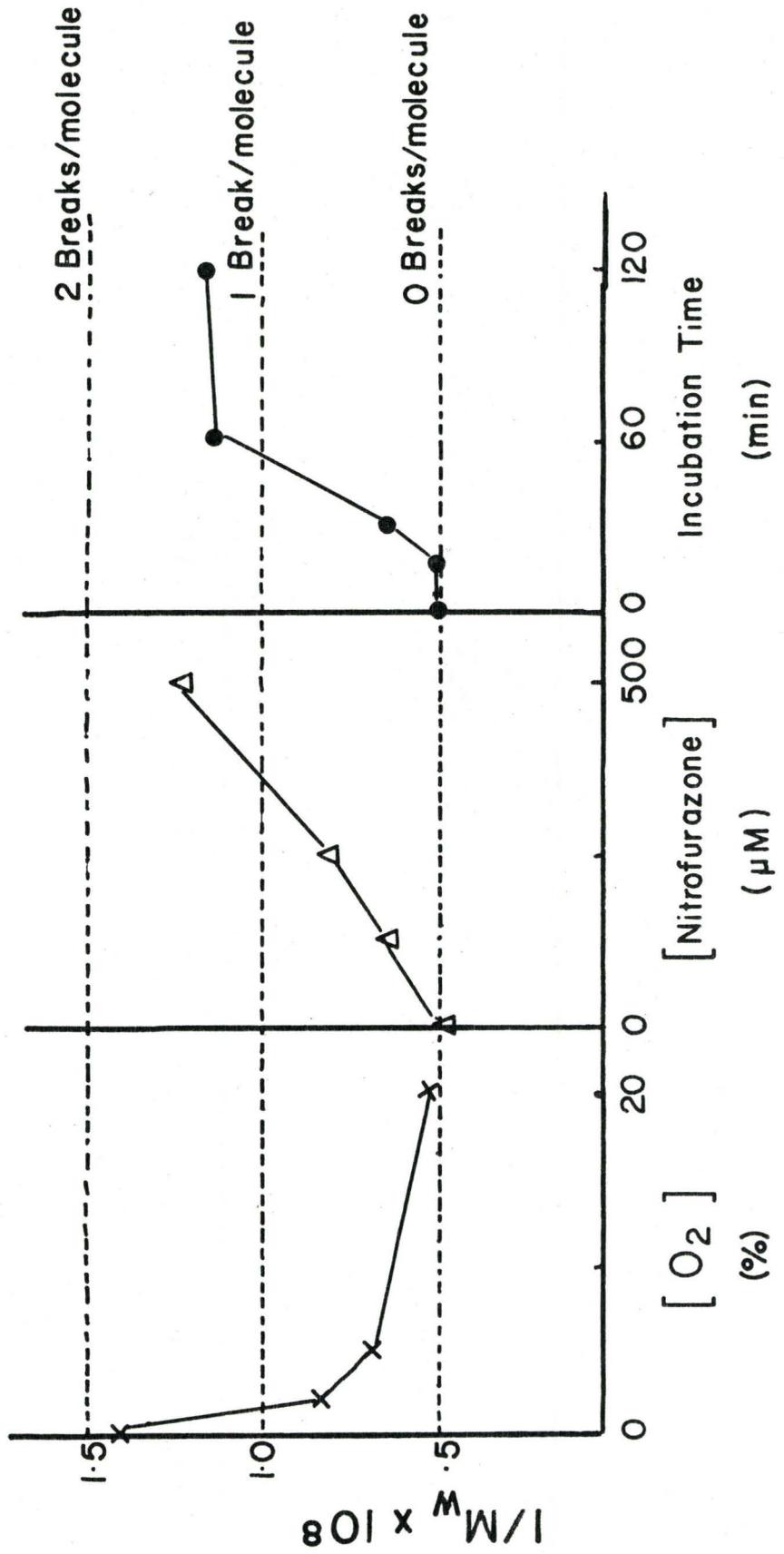


Figure 26: Rejoining of Single-Strand Breaks

³H-TdR labelled L cells were incubated with 430 μ M nitrofurantoin or 500 μ M nifuroxime in PSG under anoxic conditions for 2 hours. After incubation, cells were either lysed immediately (0 time) or washed and reincubated in complete medium for 4, 8, 12 or 16 hours before lysis on alkaline sucrose gradients for 4 hours. Gradients were spun at 30 Krpm for 90 minutes.

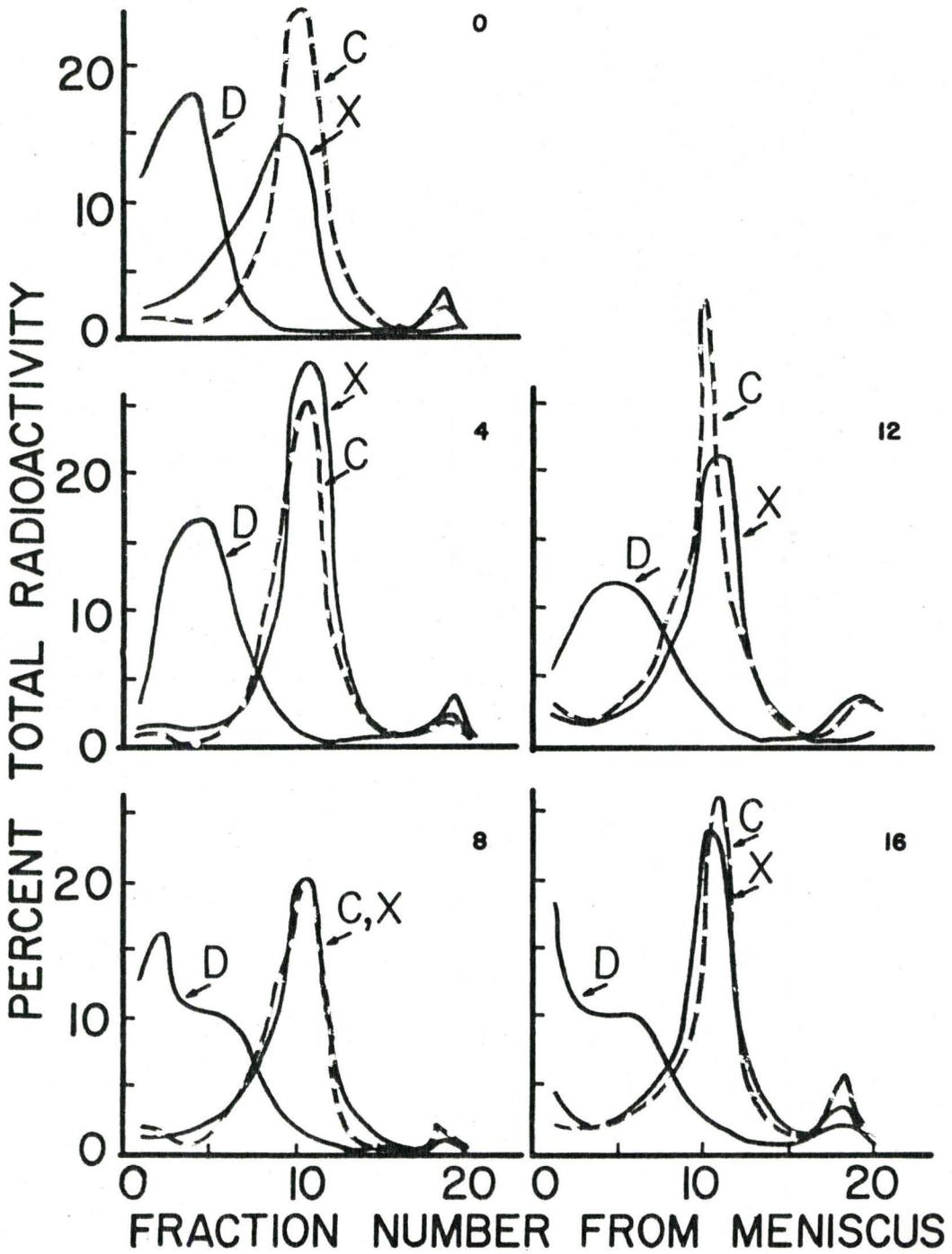


Figure 27: Analysis of Molecular Weights During
Repair Incubation

Time of repair incubation is plotted versus the reciprocal of weight average molecular weight from data in Figure 26.

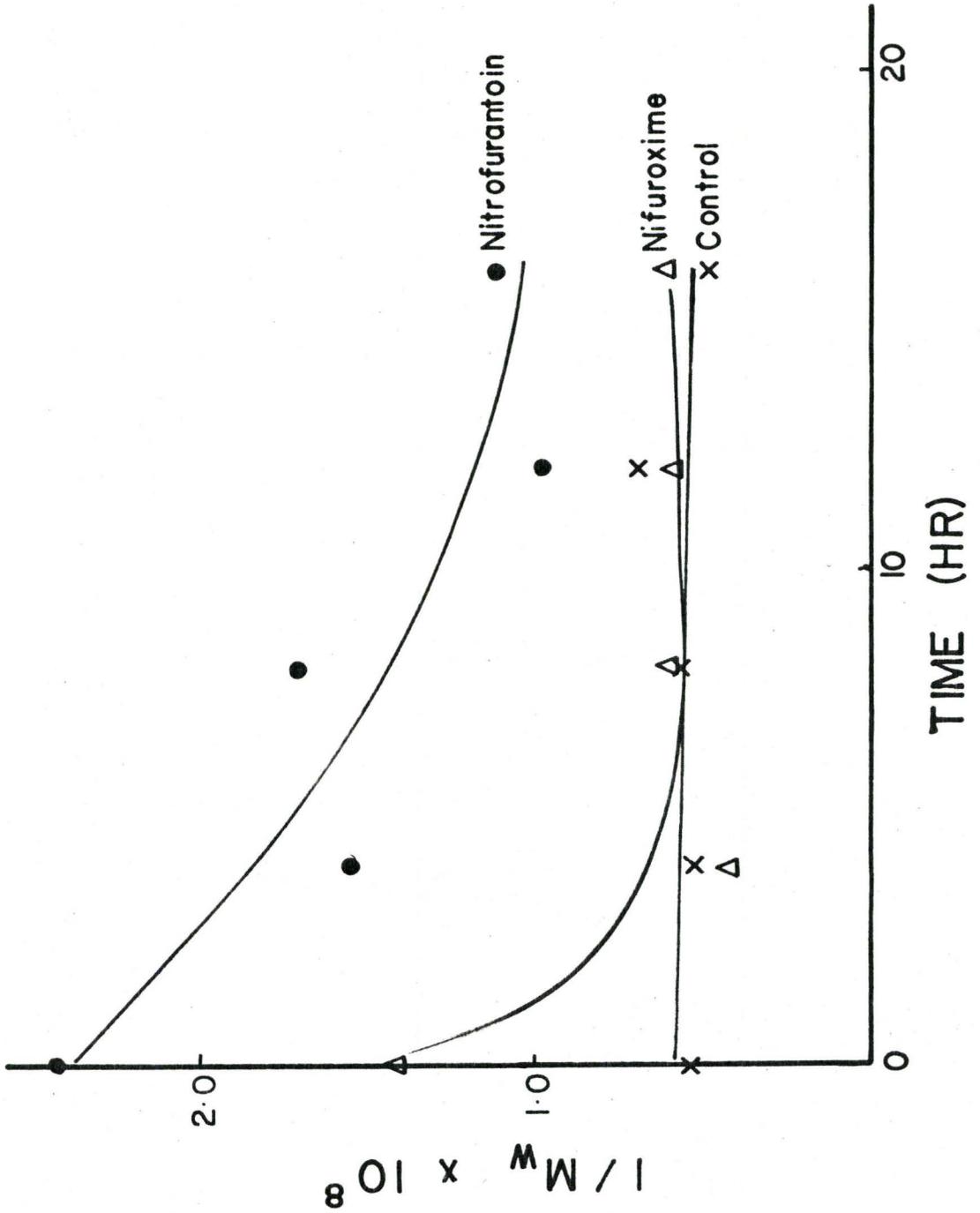
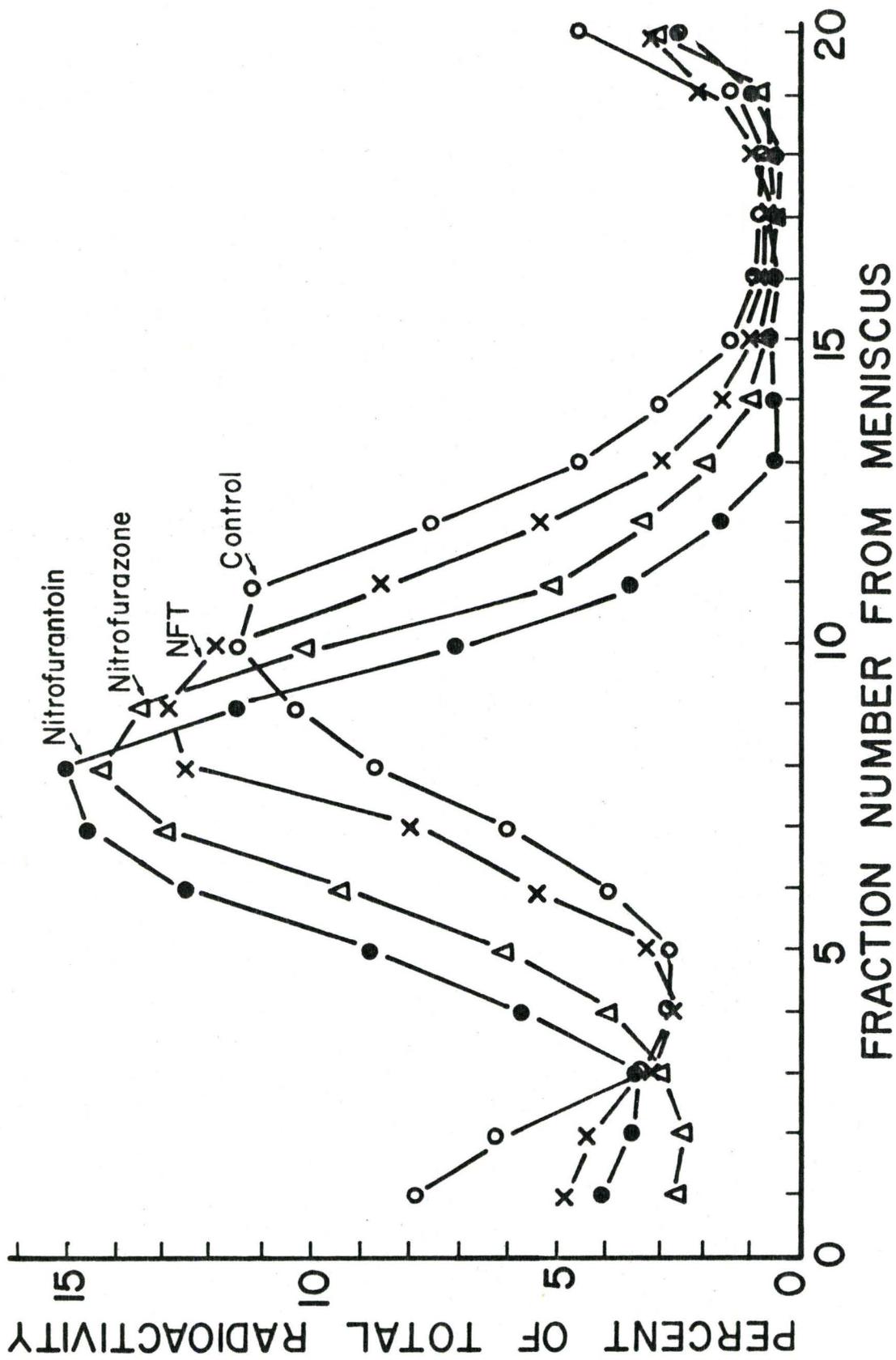


Figure 28: Production of DNA Breaks in Xeroderma
Pigmentosum Cells by Nitrofurans

XP cells were incubated for 90 minutes under anoxic conditions in PBS with 42 μ M NFT, 250 μ M nitrofurazone and 250 μ M nitrofurantoin. Cells were lysed for four hours and DNA sedimented at 18 Krpm for four hours.



3.3.2 Ascites Cells In Vivo

Ascites cells provide a convenient system for assessing nitrofuran damage in vivo, although they are not equivalent to tissue since they grow as individual cells rather than organized structures. The fact that they divide rapidly in the peritoneal fluid suggests that nutrient supplies are comparable to those available to cells in vascularized tissue. Oxygen delivery is, however, less adequate (Del Monte 1969), allowing for greater nitrofuran reduction.

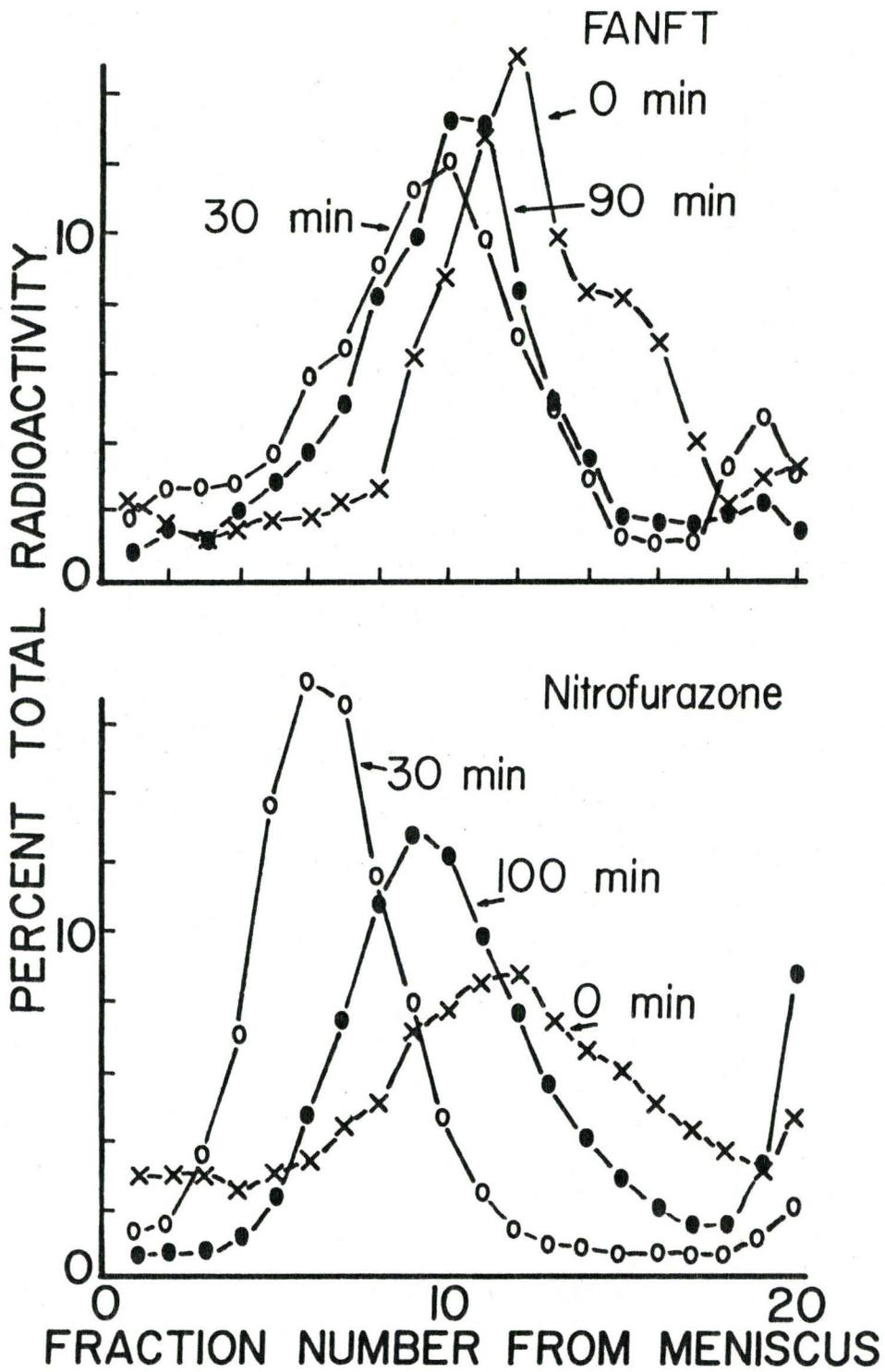
When ascites cells were exposed to nitrofurazone or FANFT in vivo, little or no breakage was observed if the cell mass was large, presumably due to the relatively rapid reduction and elimination. However, when cell numbers were reduced by withdrawing a 5 to 10 ml portion of the ascitic fluid, and replacing it with oxygen-free PBS⁻, a large shift in the molecular weight of the DNA was observed 30 minutes after injection of either one mg FANFT (Figure 29a) or 2 mg nitrofurazone (Figure 29b) dissolved in 30% DMSO. The peak position of a sample withdrawn 100 minutes after nitrofurazone injection had shifted back toward the control DNA peak; a smaller shift was observed with FANFT-treated DNA. Mice survived these injections of nitrofurans, but ascites growth was subsequently inhibited

for several days, probably reflecting the toxicity of nitrofurazone under anaerobic conditions. Injection of DMSO by itself was not found to cause single-strand breaks.

Figure 29: Production of Breaks in Ascites Cell

DNA In Vivo

Ehrlich ascites cells were labelled in the peritoneal cavity of mice with 0.15 mC ^3H -TdR and 24 hours later, samples of cells were removed and used as controls (0 min). One mg FANFT or 2 mg nitrofurazone dissolved in 30% DMSO were injected into mice. At the times specified, samples were withdrawn and cells lysed on alkaline sucrose gradients. DNA was sedimented at 18 Krpm for 4 hours.



3.4 Factors Influencing the Extent of DNA Damage

3.4.1 Properties of Cells

Small differences in alkaline sucrose gradient profiles were observed for different nitrofurantoin-treated mammalian cells. L cells in exponential growth appeared less sensitive to nitrofurantoin treatment than did plateau phase cells (Figure 30). These findings appear to conflict with those found when cell survival was the endpoint (Figure 10). Using cells treated as above, breaks appeared to be rejoined with roughly equal efficiency (Figure 31).

Synchronized cells showed a pattern of damage that was similar for two different synchronizing techniques (Figures 32,33,34). Cells in G1/S were most sensitive to nitrofurantoin and nifuroxime while more resistant periods were observed immediately after release from the metaphase block and again in late S.

The sedimentation pattern for nifuroxime shows two peaks, one beneath the control DNA peak and another at a lower molecular weight around fraction 7, perhaps representing cells that escaped synchrony. With time after release from synchrony, the relative amount of DNA in each peak changed. After 8 hours, the least amount of overlap occurred between profiles of control and nifuroxime-treated DNA indicating the greatest amount of DNA damage to the

treated cells. This corresponds with late G1 and the beginning of S (Figure 33, top panel). The profile for nitrofurantoin gave little indication of the effects on synchronized cells since the single peak remained at fraction 4 regardless of the time of treatment. However, analysis of molecular weights using these data revealed a large change in the sedimentation pattern with nitrofurantoin-treated cells, and a comparatively small change with nifuroxime (Figure 34). As discussed in Appendix 1, molecular weight determinations have little meaning if either 1) DNA sediments in more than 1 peak, or 2) peak position is near the top of the gradient. It is, however, interesting to note that the molecular weight analyses of the nitrofurantoin profiles also revealed that the time of greatest sensitivity was 8 hours after release from synchrony.

The radioactive disintegrations in ^3H -labelled cells were of concern since radiation and nitrofurans are synergistic in their actions under hypoxic conditions (Reuvers *et al.* 1972). Labelling cells with 0.01 to 2 $\mu\text{C}/\text{ml}$ ^3H -TdR resulted in significant cell death which was dependent on the extent of labelling (cpm/cell) in both HeLa and L cells (Figures 35,36). However, the survival of cells attached to plates was considerably higher than of cells suspended in medium above the plates (Figure 36b). Selection of attached cells was routinely done in these experiments.

Although toxicity was dependent on the extent of incorporation, cells labelled for 8 or 20 hours with $2.0 \mu\text{C/ml}$ $^3\text{H-TdR}$ were equally damaged and only marginally different from those labelled with $0.2 \mu\text{C/ml}$ when production of single strand breaks was measured (Figure 37). However, in highly labelled cells, there was degradation of some of the DNA indicated by the amount of radioactive material at the top of the gradient.

Occasionally, pleural-pneumonia-like organisms (PPLO) are found to contaminate cultures of mammalian cells to such an extent that they interfere with cell growth, and uptake of radioactive precursors (Paul, J. 1970). All cell lines were periodically tested for PPLO. Several experiments were performed before contamination was recognized, and two major peaks of label were noted in sedimentation profiles, apparently due to $^3\text{H-TdR}$ incorporation into both mammalian cell DNA and PPLO DNA. Interestingly, this may suggest a relatively easy method of testing for gross PPLO contamination. Damage by nitrofurans appeared to occur to a greater extent in mycoplasma DNA than in mammalian DNA, although both peaks were shifted to lower molecular weights. These impressions were not pursued as cell lines free from PPLO were obtained and used for all experiments presented in this thesis.

Incubation of L cells with $430 \mu\text{M}$ nitrofurantoin under anaerobic conditions at 0° did not result in a shift

from control molecular weight, suggesting that metabolically active cells were required for DNA damage, or simply that the chemical reaction rate was lowered, although this seems a less likely alternative.

Figure 30: Production of Breaks in Plateau Phase and
Exponentially Growing Cells by Nitrofurans

Plateau phase cells (grown at confluence for 4 days) and exponentially growing L cells labelled with $^3\text{H-TdR}$ were incubated with $430 \mu\text{M}$ nitrofurantoin or $630 \mu\text{M}$ nifuroxime in nitrogen equilibrated PBS. After two hours, cells were lysed for 4 hours and spun at 18 Krpm for 4 hours.

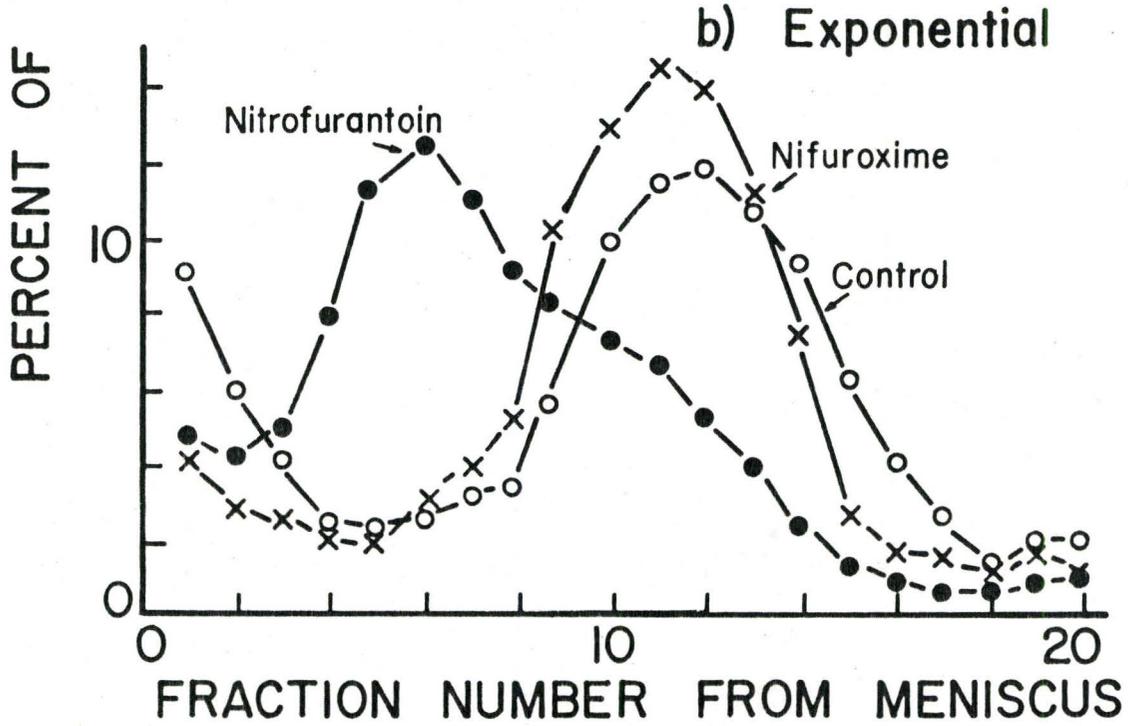
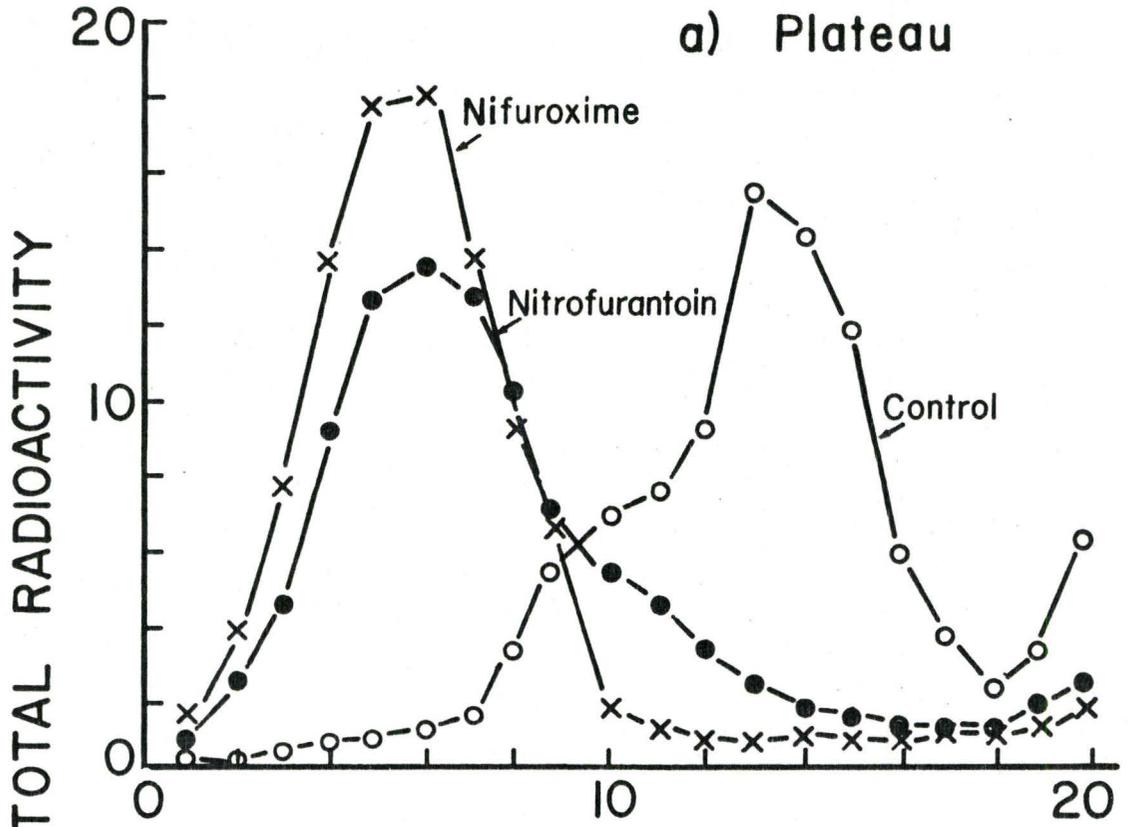


Figure 31: Rejoining of Breaks by Exponential and
Plateau Phase Cells

Cells treated as in Figure 30 were allowed 4 hours repair incubation in complete medium before lysing for four hours on alkaline sucrose gradients and sedimenting at 18 Krpm for 4 hours.

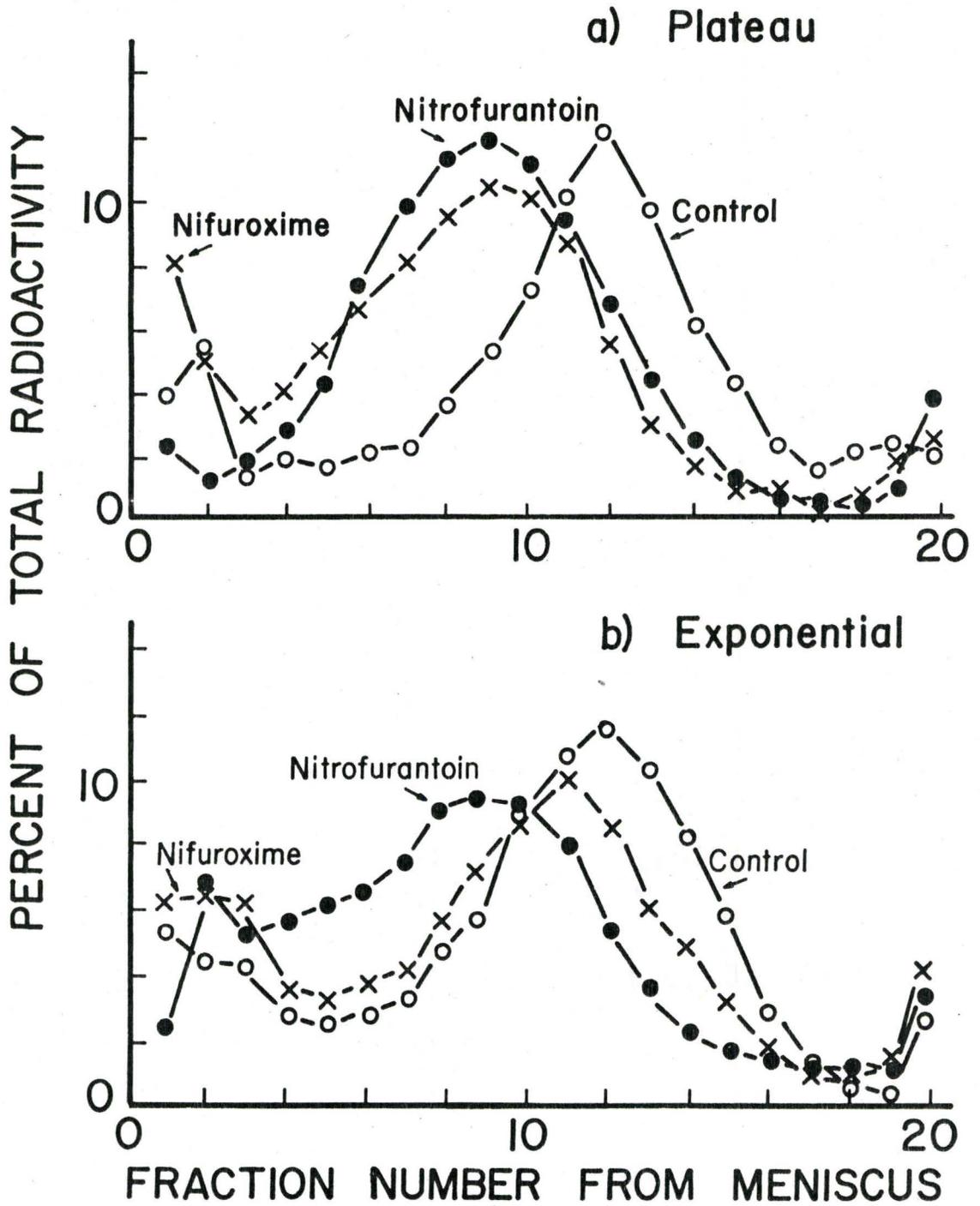


Figure 32: Production of Breaks in Synchronized
Cells by Nitrofurans

L cells were synchronized (see Section 2.2) using hydroxyurea followed by colcemid. After washing and plating in fresh medium, cells were incubated immediately or at 4 hour intervals under nitrogen with 430 μ M nitrofurantoin (D), or 630 μ M nifuroxime (X) in PSG for 90 minutes. Control cells are indicated by (C). Numbers above and to the right of each set of profiles indicate time after release from the blocking agent that cells were incubated with nitrofurans. Cells were lysed for 4 hours before sedimenting at 30 Krpm for 90 minutes.

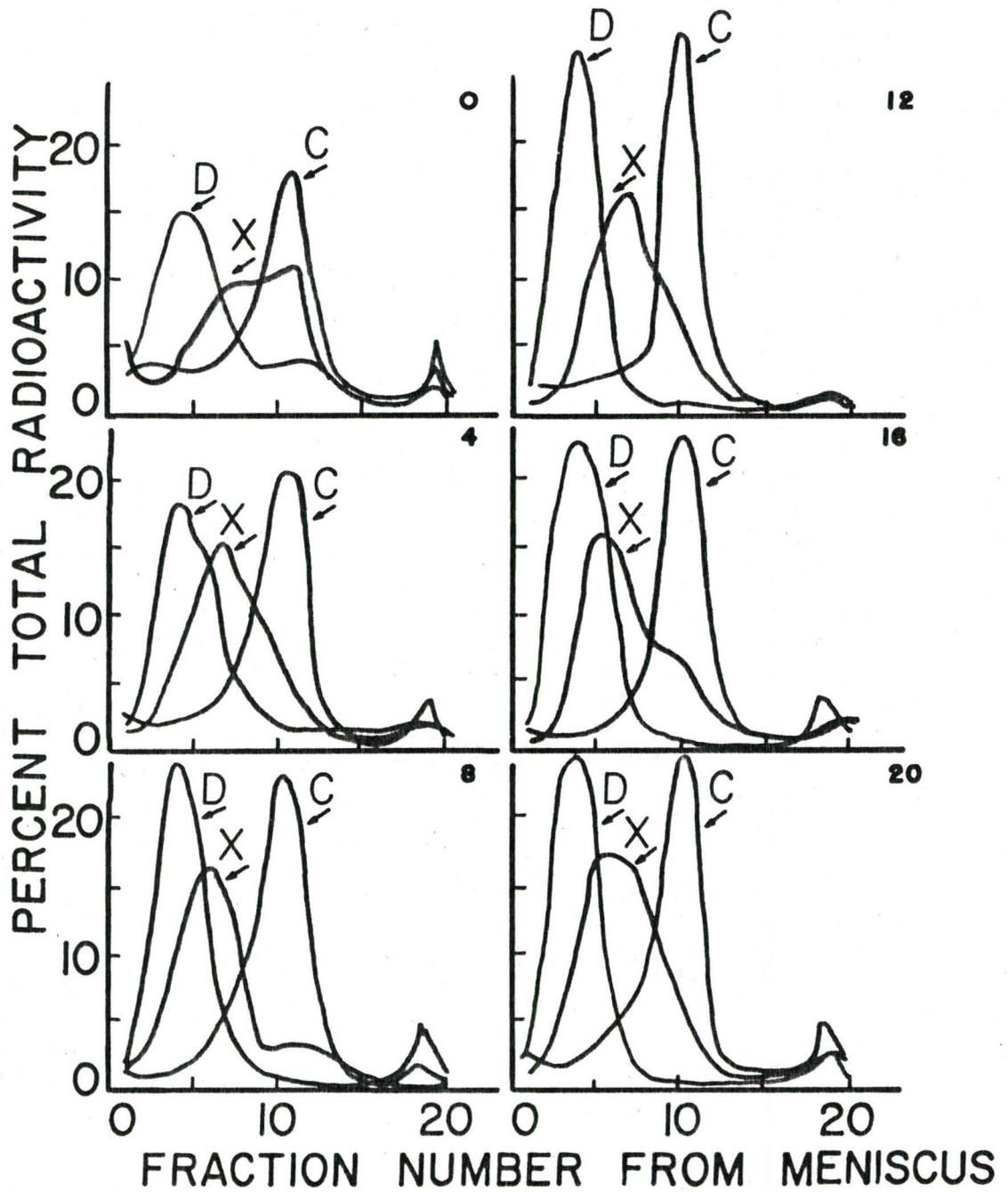


Figure 33: Molecular Weight Analysis of Synchrony
Data

Results from Figure 32 were used to calculate molecular weights of DNA from control and nitro-furan treated cells. Labelling index was determined using cells synchronized by the same method, however, $^3\text{H-TdR}$ was not included with colcemid and was later added to cells for a 15 minute pulse before fixing and coating with emulsion (see Section 2.5).

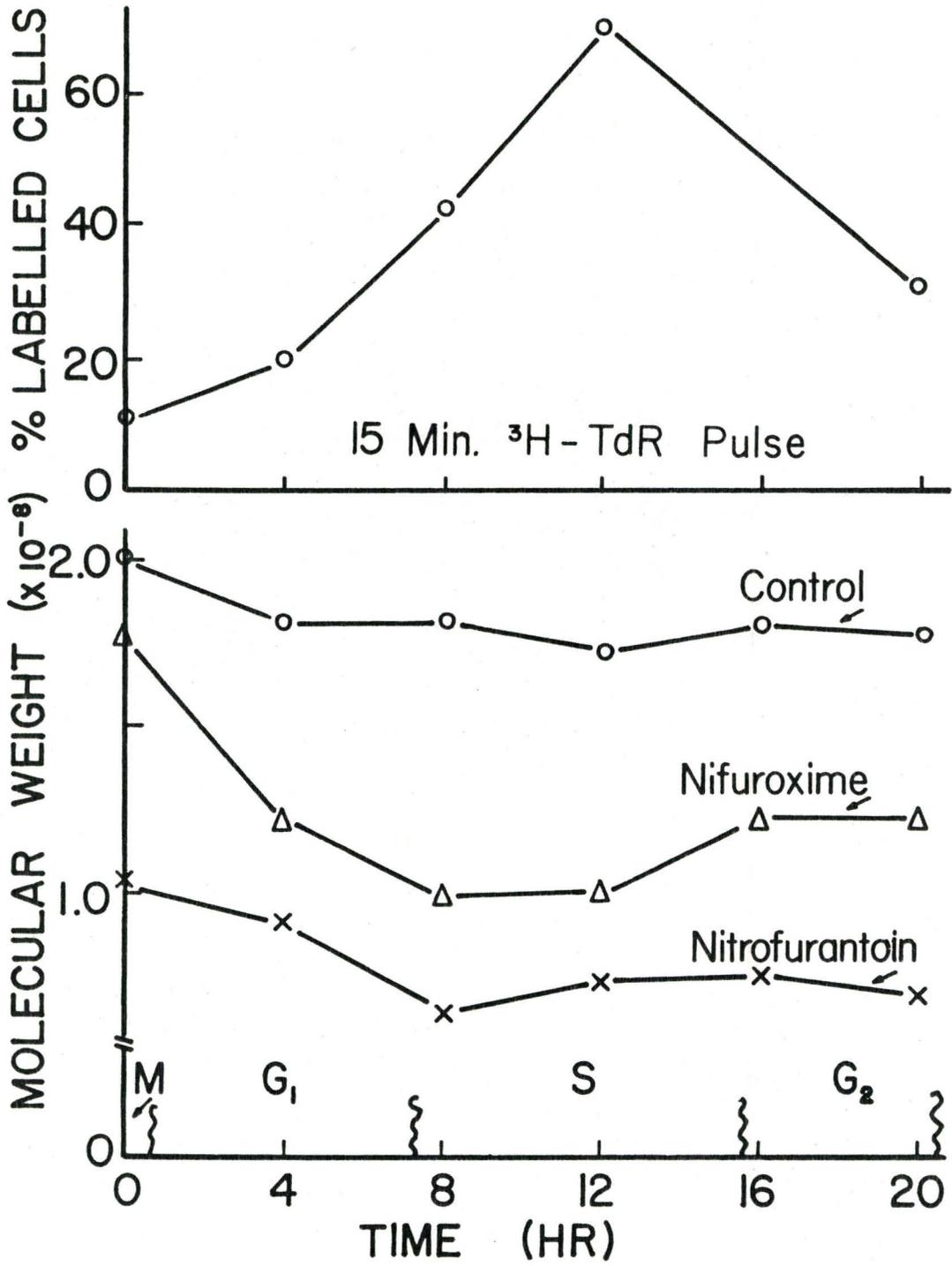


Figure 34: Comparison of Data from Two Synchronizing
Methods

a) Cells were partially synchronized by incubating L cells labelled with $^3\text{H-TdR}$ for 20 hours in 4 mM hydroxyurea. After this time, they were washed and incubated at 4 hour intervals in 250 μM nitrofurantoin dissolved in PBS for 90 minutes.

b) L cells were synchronized as indicated in Figure 32. Hydroxyurea arrests cells at the G1/S border (a) while colcemid leaves cells in M (b). The data are plotted to facilitate direct comparison.

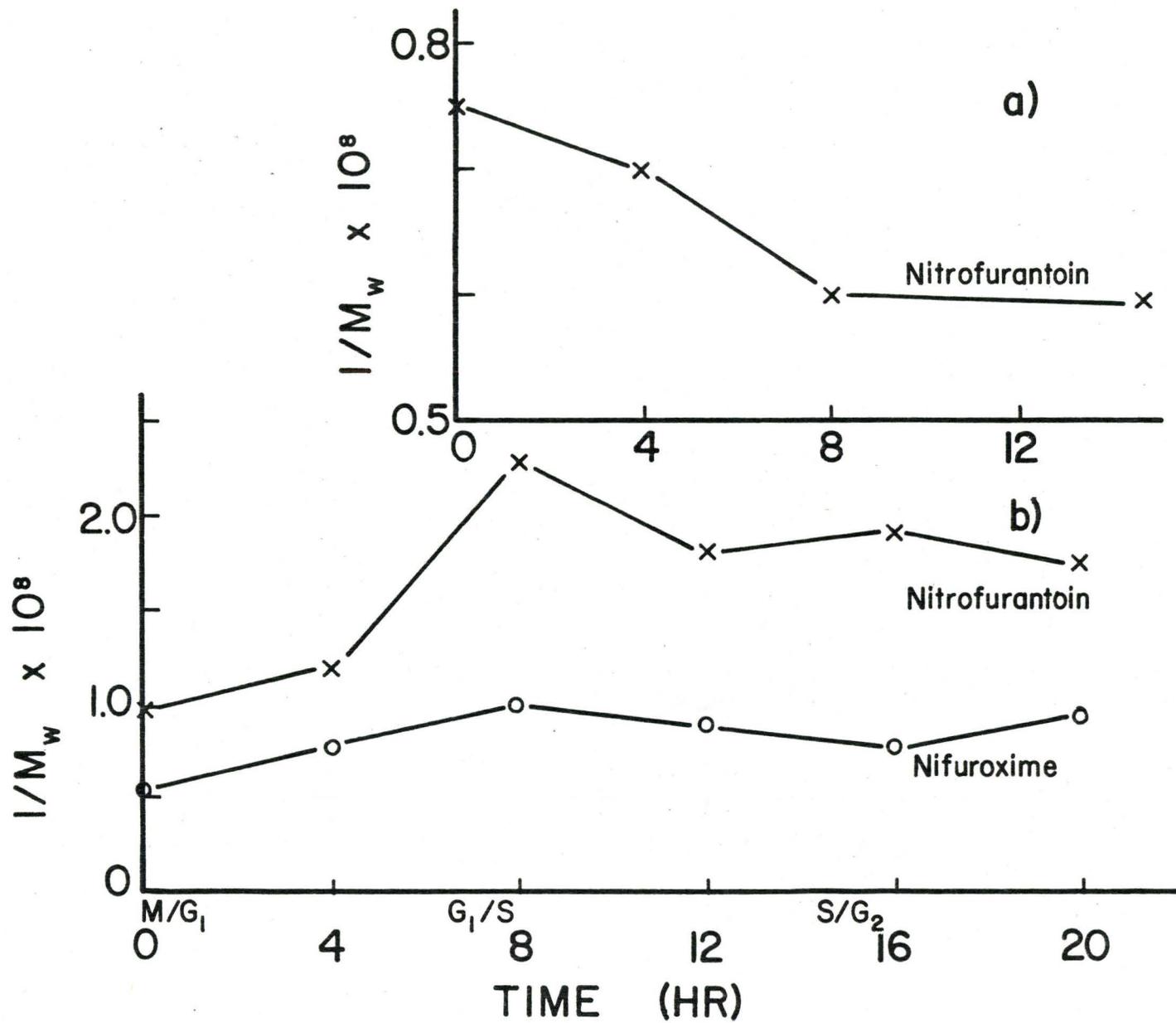


Figure 35: Toxicity due to Incorporation of ^3H -TdR

Growth of HeLa cells, expressed as an increase in cell number with time, was measured after incubation in complete medium containing ^3H -TdR (0.2 to 2 $\mu\text{C}/\text{ml}$). The specific activity is expressed as cpm/cell. Survival of cells treated with 0.2 to 2.0 $\mu\text{C}/\text{ml}$ for 24 hours is also shown.

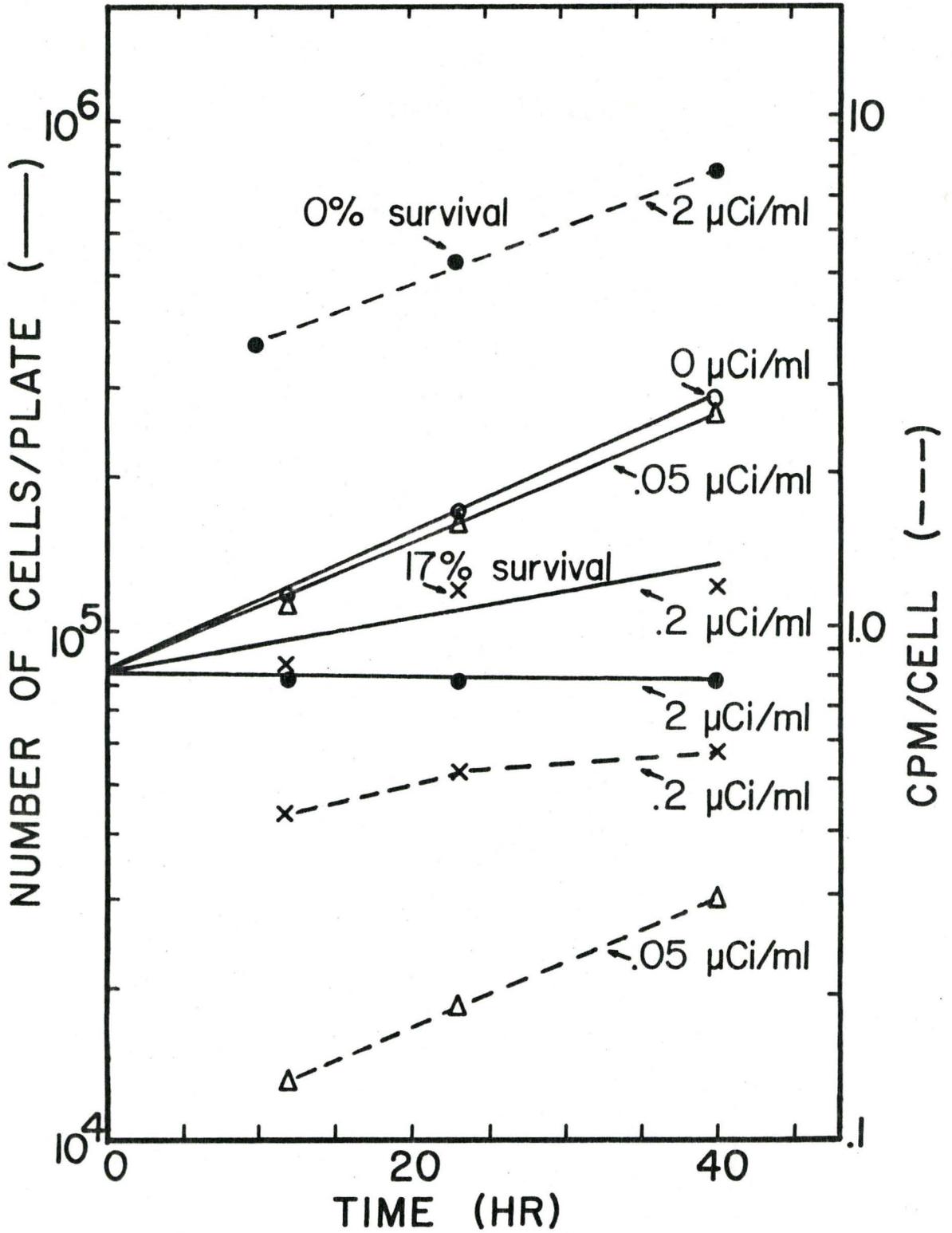


Figure 36: Effect of Incorporated $^3\text{H-TdR}$ on Survival
of L Cells

L cells were incubated with $^3\text{H-TdR}$ and a) net survival of the total population (both attached and floating cells) was measured as a function of time of incubation in the presence of $^3\text{H-TdR}$ (0 to 26 hours) at various times after its removal. Note the surviving fraction can be increased by both division of viable cells and disintegration of dead cells (dead cells would not be included in the initial count when cells are plated, thus increasing the plating efficiency).

b) Viability of attached cells only, measured as a function of incorporated $^3\text{H-TdR}$. Cells attached to plates were removed with trypsin and plated, while unattached cells were discarded. Note that, compared to (a), the surviving fraction is enhanced by selection against non-viable (unattached) cells.

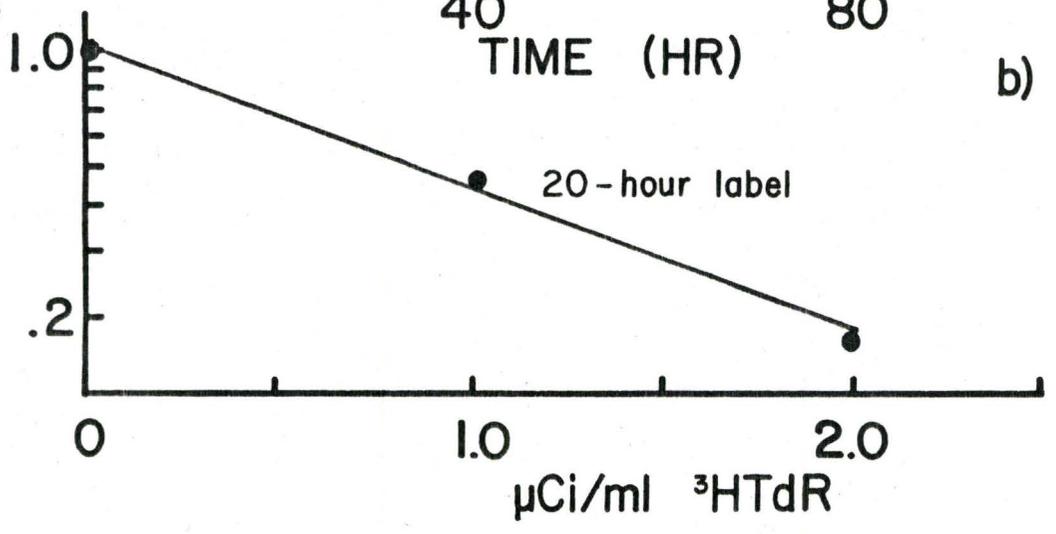
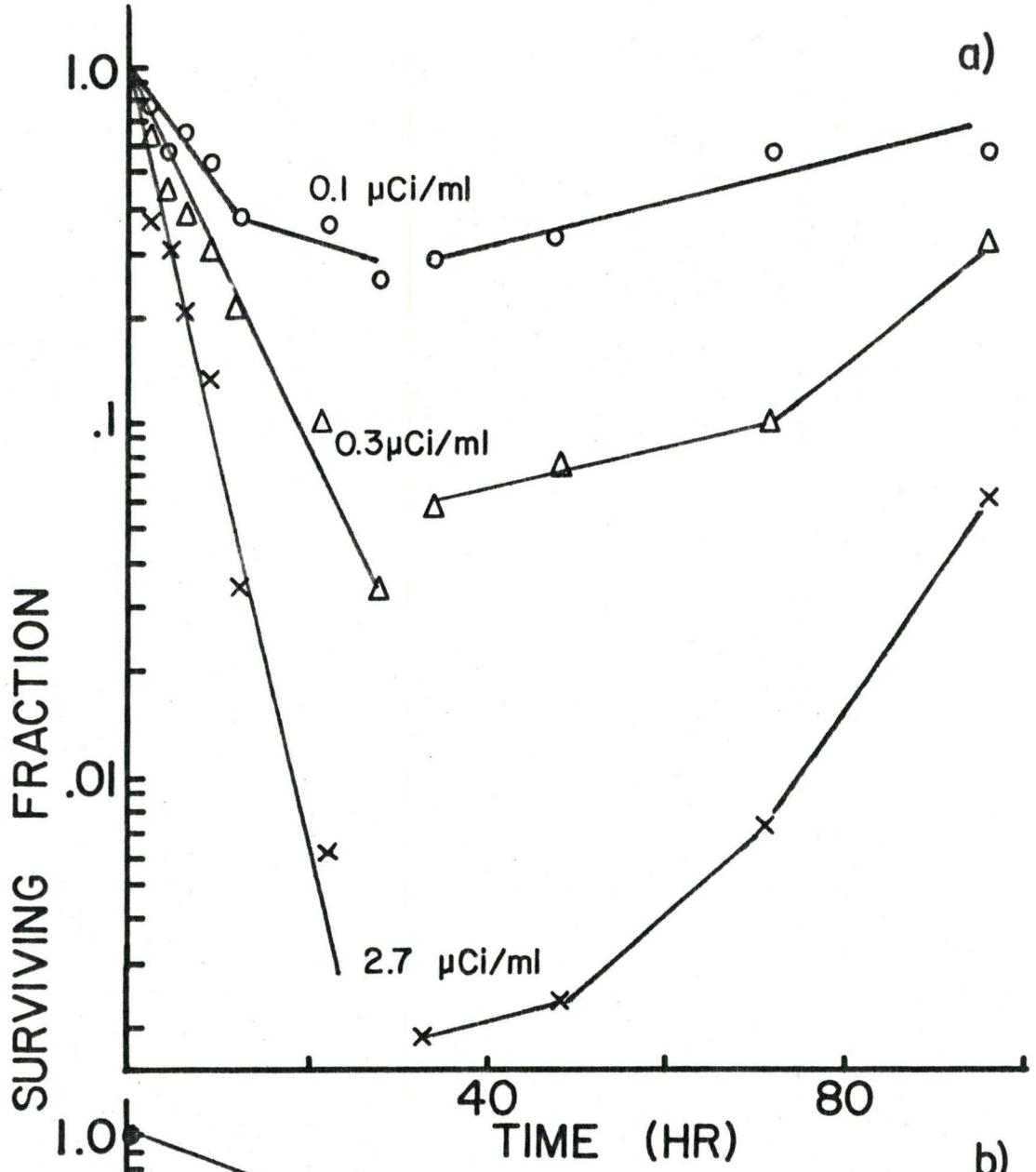
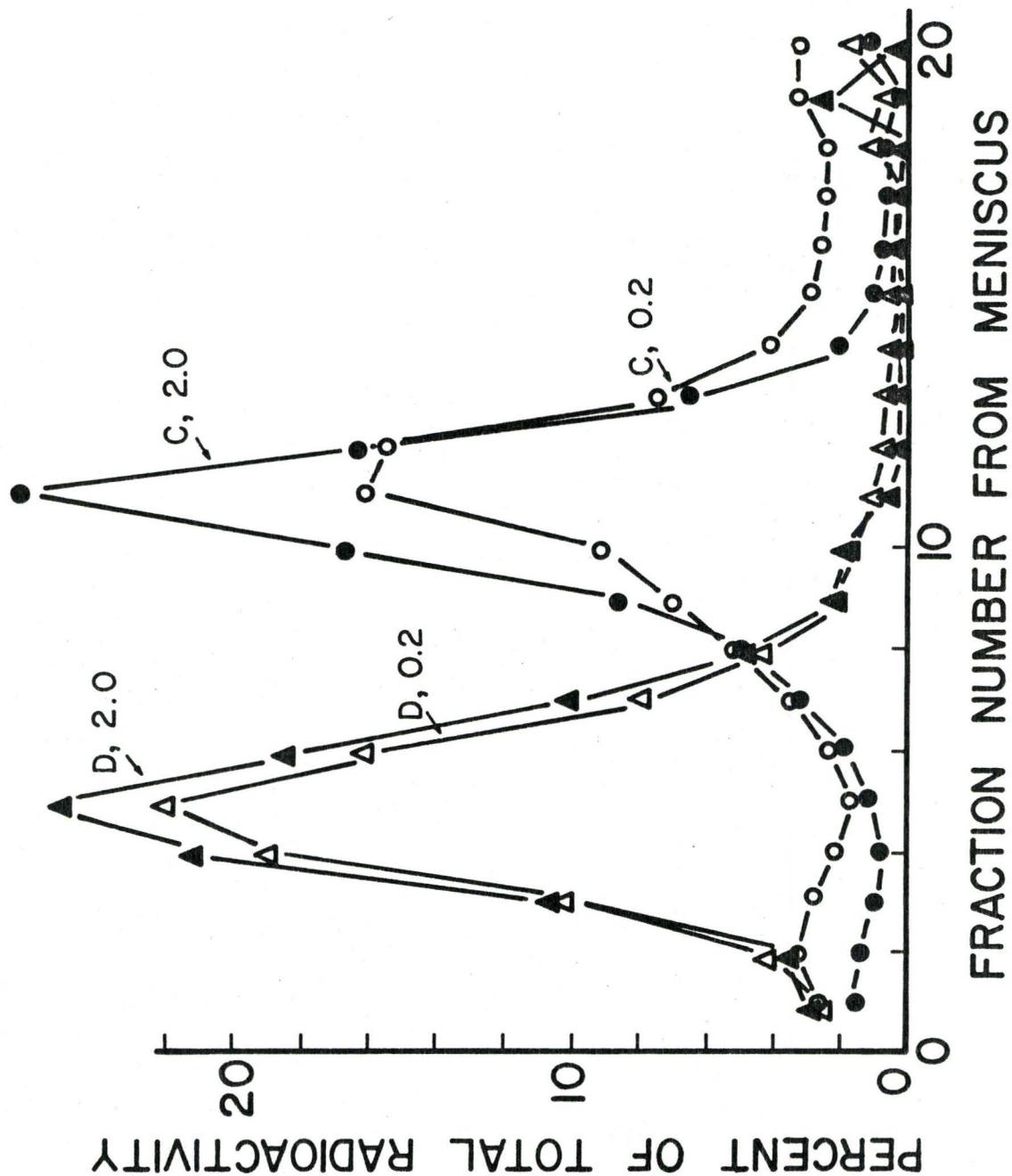


Figure 37: Effect of Amount of Incorporated ^3H -TdR on
Production of Single-Strand Breaks by
Nitrofurazone

L cells were labelled with 0.2 to 2.0 $\mu\text{C}/\text{ml}$ ^3H -TdR for 20 hours in complete medium. They were then incubated with 500 μM nitrofurazone for 2 hours under anoxic conditions (D) or in PSG (C). Cells were lysed on alkaline sucrose gradients for four hours before spinning at 30 Krpm for 90 minutes.



3.4.2 Incubation Conditions

Single-strand breaks in mammalian cell DNA may be a result of the direct interaction of the agent with the sugar-phosphate backbone, repair of damage by endonuclease, or an indirect effect due to interaction with damage by another agent. Incubation conditions detrimental to the cell may enhance any of the above possibilities.

Due to the low water solubility of many of the nitrofurans (Appendix 4), sufficiently high concentrations could be attained only by solubilizing them in DMSO and then diluting in the appropriate incubation solution. This procedure led to some concern about cell membrane integrity in the presence of high concentrations of DMSO. While addition of 10% DMSO was occasionally found to enhance damage by 500 μ M nitrofurazone to L cells slightly, this effect was not consistently observed. In all other experiments, the concentration of DMSO was never greater than 2%.

Cells treated with the non-ionic drug nitrofurazone showed no significant change in peak position when the pH of the buffer was varied by addition of 0.5N HCl from 5.9 to 7.4. However, varying pH with the anionic nitrofurantoin or cationic furaltadone was found to produce marked changes in the extent of DNA damage (Figures 38,39), perhaps due to

changes in ionization of these compounds and the efficiency with which they penetrated membranes.

There was some indication that the incubation medium could determine the amount of DNA damage. Cells incubated for 8 hours in PSG under anoxic conditions showed a significant decrease in molecular weight (2 fractions) while those in complete medium appeared to be undamaged when compared to zero time controls. Plating efficiency after this incubation time in PSG was correspondingly lower. However, L cells incubated for 60 minutes with 430 μ M nitrofurantoin were equally damaged in PBS⁻, MEM or PBS⁻ plus 0.5% glucose (Figure 40).

Further interpretation is complicated by recognition that DNA single-strand break measurements represent an averaging procedure so that death and degradation of a small part of the population cannot be distinguished from net damage to the entire population. Presumably, if damage to a small proportion of cells was extensive, two separate peaks could be resolved on gradients. The presence of two peaks might also indicate a specificity of nitro-furan interaction with DNA.

Figure 38: Effect of pH on Damage to L Cells by
Furaltadone

L cells labelled overnight with ^3H -TdR were incubated for 90 minutes with $360\ \mu\text{M}$ furaltadone in PBS at pH 5.2 or 7.0 under nitrogen. Control cells were incubated at pH 5.2 under nitrogen. After two hours, cells were lysed for four hours on top of alkaline sucrose gradients before sedimenting at 18 Krpm for 4 hours.

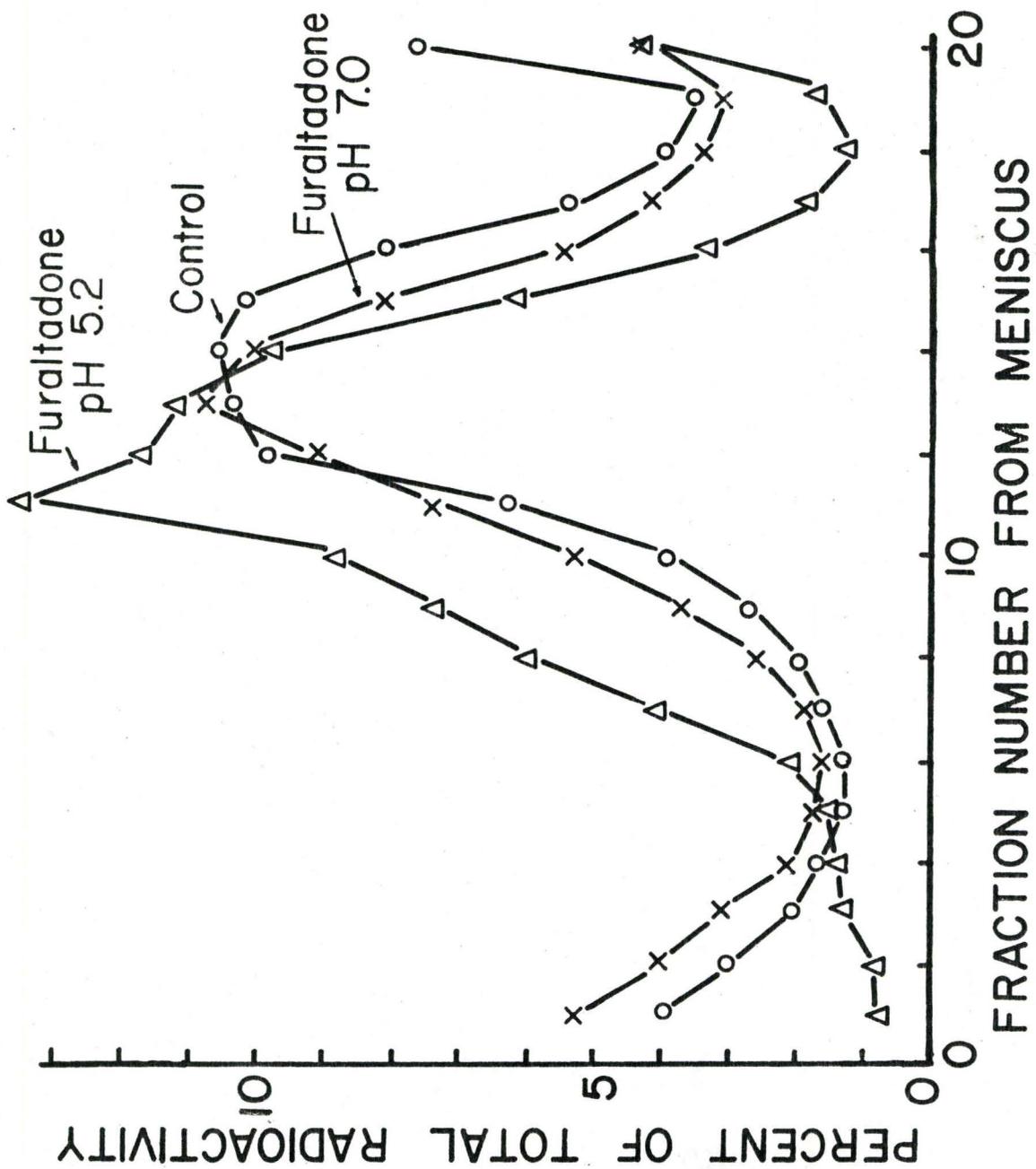


Figure 39: Effect of pH on Production of Breaks
by Nitrofurantoin

L cells were labelled overnight with ^3H -TdR and incubated under anoxic conditions with $110\ \mu\text{M}$ nitrofurantoin at pH 5.2 or 7.0 in PBS. Control cells were incubated at pH 7.0 under nitrogen. After two hours, cells were lysed for 4 hours before sedimenting at 18 Krpm for 4 hours.

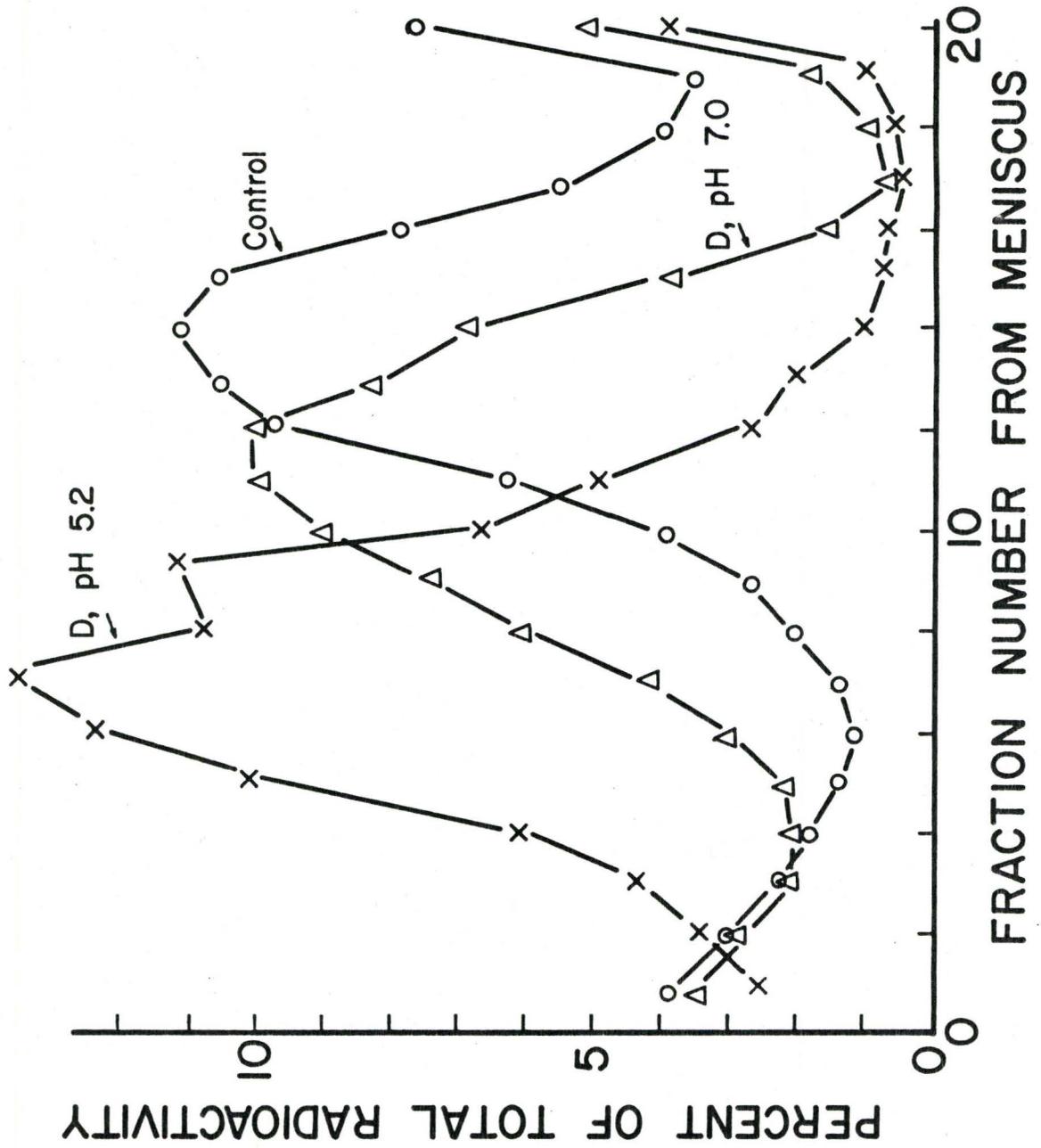
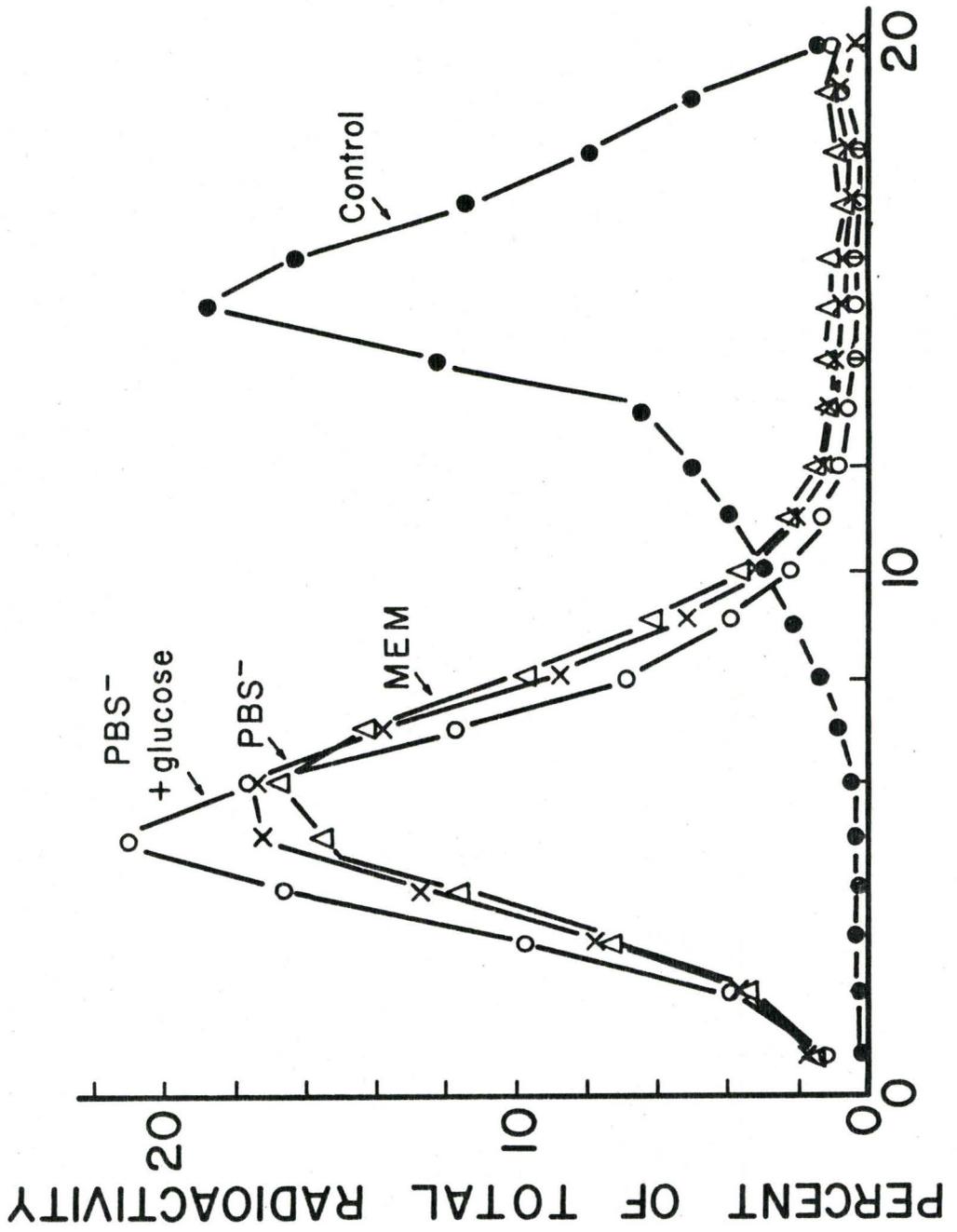


Figure 40: Effect of Medium of Incubation on
Production of Single-Strand Breaks
by Nitrofurantoin

L cells were incubated with $430 \mu\text{M}$ nitrofurantoin for 60 minutes in MEM, PBS^- or PBS^- plus 0.5% glucose. Cells were lysed for 3.5 hours and gradients spun at 18 Krpm for four hours.



3.4.3 Enhancement of Nitrofurans Damage by Radiation

There is no clear understanding of the nature of the interaction between nitrofurans and DNA. When a non-cycling population of human lymphocytes was incubated with nitrofurans under nitrogen, and the level of unscheduled DNA synthesis measured, there was no significant uptake of ^3H -TdR. However, nitrofurans may interact with DNA by inhibiting repair and perhaps incorporation of precursors. This has been suggested as a mode of action for other radiosensitizers (Moroson and Furlan 1971). To test this possibility, cells were irradiated, and then repair of this damage followed in the presence of nitrofurans using several assay systems.

Human lymphocytes were irradiated with 350 ergs/mm² UV under air, then incubated with 500 μM nitrofurazone plus ^3H -TdR under anaerobic conditions. Incorporation of the radioactive label was greatly enhanced, both over control cells receiving only radiation, or those incubated in nitrofurazone without radiation (Figure 41). Since 4 mM HU was present during incorporation, the increase in radioactivity in DNA could only be a result of repair. The presence of nitrofurazone apparently increased either the size of the newly incorporated patch, or the number of repaired regions. Although the incorporation of precursors

was increased, it was possible that the 'joining' enzyme, ligase, was not active and that repair was inhibited in this manner.

In fact, the number of single-strand breaks appeared to be increased after incubation of irradiated cells with nitrofurans. L cells irradiated with 350 ergs/mm² UV also showed enhanced damage, measured as single-strand breaks, when cells were incubated in air for 4 hours with nitrofurantoin following irradiation. Only a small increase in damage was observed in cells incubated in nitrogen (Figure 42), possibly due to the large amount of damage to DNA by nitrofurantoin alone, and poor resolution at the top of the gradient. This response was also observed following doses of gamma radiation from 1000 to 3000 rads. Cells incubated in nitrofurazone for 4 hours showed increased damage following irradiation if incubated in air or in nitrogen (Figure 43), but a greater increase was seen in air. A large shift in molecular weight occurred if incubation with nitrofurazone in air lasted only two hours, but began two hours following irradiation. Conversely, incubation for two hours beginning immediately after irradiation produced only slight differences (Figure 44).

Irradiation of cells did not result in an enhanced rate of nitrofurazone reduction (Figure 45). Destruction of nitrofurazone was affected only by doses in excess of

30 krads.

The pattern of incorporation of ^3H -TdR into human KB cell DNA after UV radiation was observed during subsequent anoxic incubation with nitrofurazone and ^3H -TdR. If nitrofurans damaged DNA by inhibiting repair, the DNA pieces would remain small following incorporation into replicating DNA (i.e., the presence of dimers should prevent base pairing at those regions, or inhibition of ligase would prevent strand rejoining). However, it was not possible to distinguish between inhibition of repair of UV damage and inhibition of DNA synthesis by nitrofurazone (Figure 46). The main difference between irradiated cells and those which were irradiated and then treated with nitrofurazone was the decrease in total incorporation into high molecular weight DNA (perhaps due to inactive ligase, or inhibition of DNA synthesis), and the presence of two peaks with different relative amounts of radioactivity (Figure 46). The two peaks were analyzed further in cells partially synchronized by overnight incubation with 4 mM HU, then allowed 6 hours to reach the beginning of S phase before following incorporation in the presence of nitrofurans after irradiation. The higher molecular weight peak increased markedly in relative size in the control gradients but was unchanged in the nitrofurazone treated sample, suggesting that this peak represented newly synthesized DNA while the lighter peak might

be DNA undergoing repair or in the midst of synthesis.

In view of the anomolous effect of nitrofurans on irradiated cells, survival of irradiated cells incubated with nitrofurans was examined. Two findings emerged. First, nitrofurazone and radiation acted in an additive manner to enhance damage (toxicity) both in air and nitrogen (Figure 47a). Secondly, nitrofurazone appeared to inhibit potentially lethal damage repair in air but not in nitrogen (Figure 47b). This effect was more marked in plateau phase cells. One hour following irradiation, control cells incubated in buffer containing glucose showed a 100% increase in surviving fraction. However, cells incubated with nitrofurazone in buffer under aerobic conditions showed only a small increase in survival, but no significant inhibition was observed in nitrogen.

The combined effects of irradiation and post-incubation with nitrofurans raise many interesting questions. It appears that radiation either alters the response of cell to nitrofurans, perhaps obviating the requirement for 'activation', or nitrofurans react with cellular repair mechanisms after irradiation, perhaps preventing the action of ligase. Whether these findings are important in the mechanism of action of nitrofurans, both as DNA damaging agents, or as radiosensitizers, is not clear at this time.

Figure 41: Effect of Nitrofurazone on Uptake of $^3\text{H-TdR}$
by Irradiated Lymphocytes

Human lymphocytes were irradiated with 350 ergs/mm^2 UV and incubated in complete medium plus $500 \mu\text{M}$ nitrofurazone and $1 \mu\text{C/ml}$ $^3\text{H-TdR}$ under anoxic conditions. After specified times, a sample of cells was homogenized, DNA precipitated with 5% TCA and dissolved in NCS plus omnifluor for liquid scintillation counting. Data is represented as a percent of control incorporation and corrected for cell number (approximately $500 \text{ cpm}/10^6$ cells).

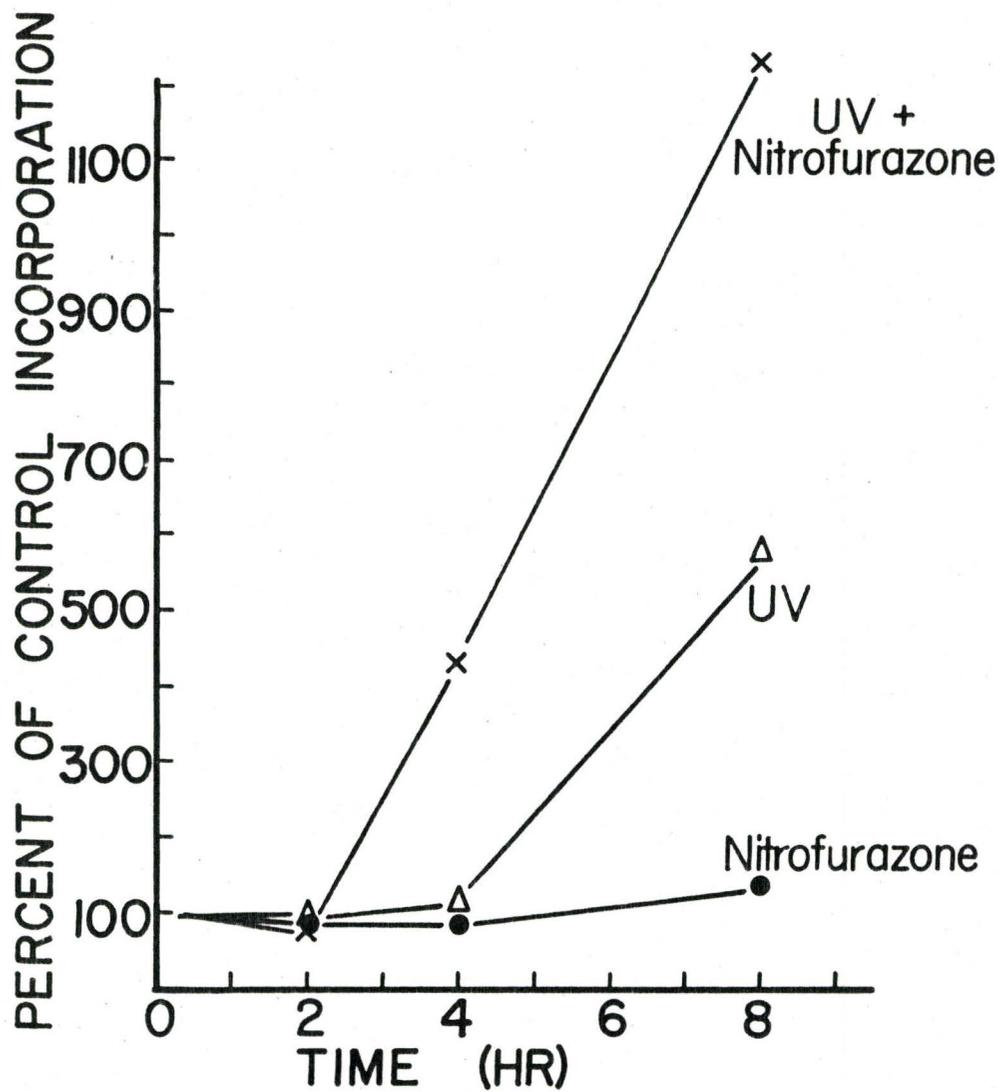


Figure 42: Effect of UV Radiation on Production of
Single-Strand Breaks by Nitrofurantoin

³H-TdR labelled L cells were incubated with 210 μ M nitrofurantoin in air (A) or nitrogen (N) for 4 hours following a dose of 0 ergs/mm² UV or 350 ergs/mm² UV. Control cells are indicated by (C). Cells were then lysed on alkaline sucrose gradients for 3.5 hours before sedimenting at 18 Krpm for 4 hours.

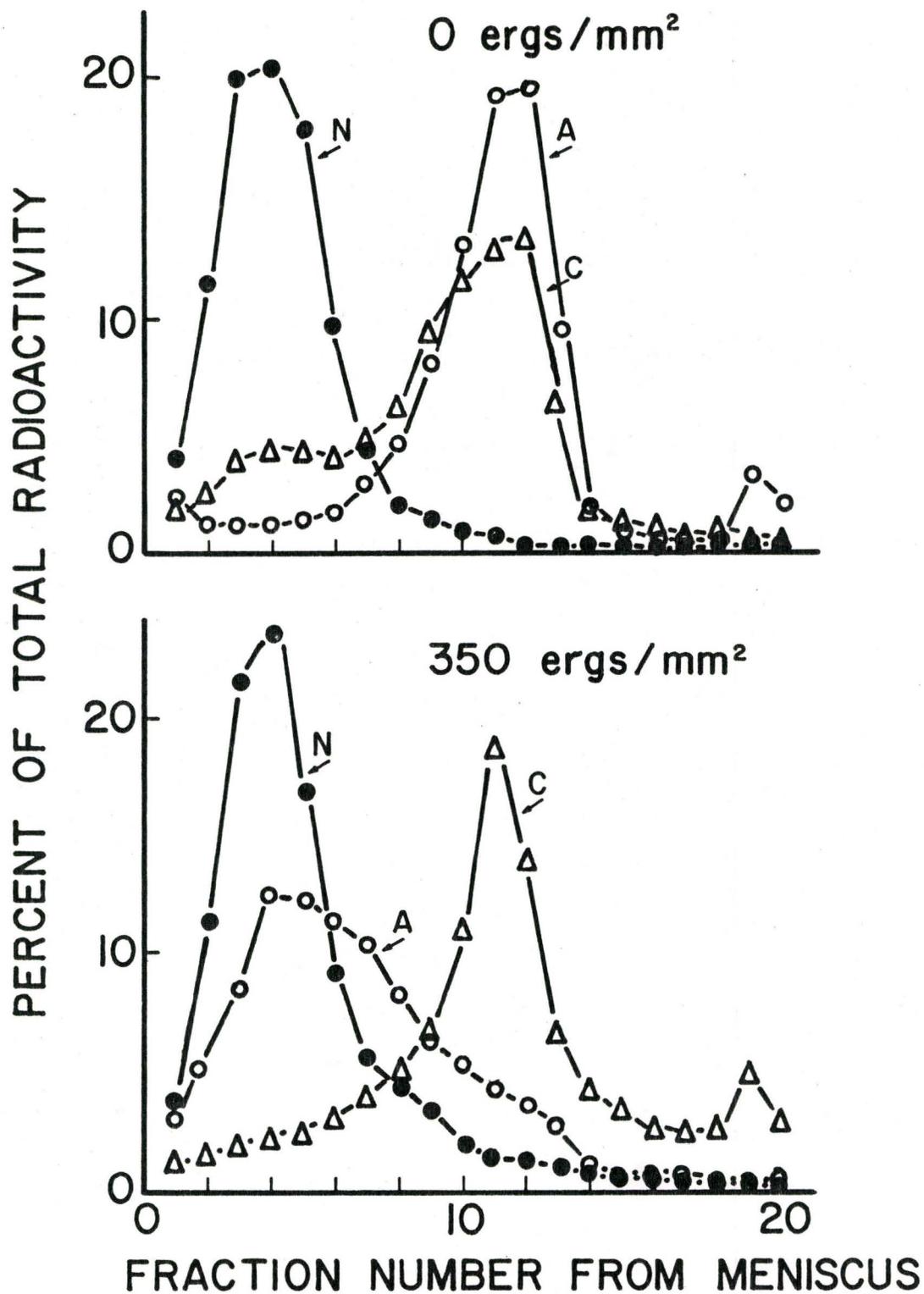


Figure 43: Effect of Gamma Radiation on Production
of Single-Strand Breaks by Nitrofurazone

Following irradiation with 0 or 100 rads in air, L cells were incubated for four hours with 125 μ M nitrofurazone under air or nitrogen in PBS⁻. Cells were lysed for four hours and gradients spun at 18 Krpm for 4 hours.

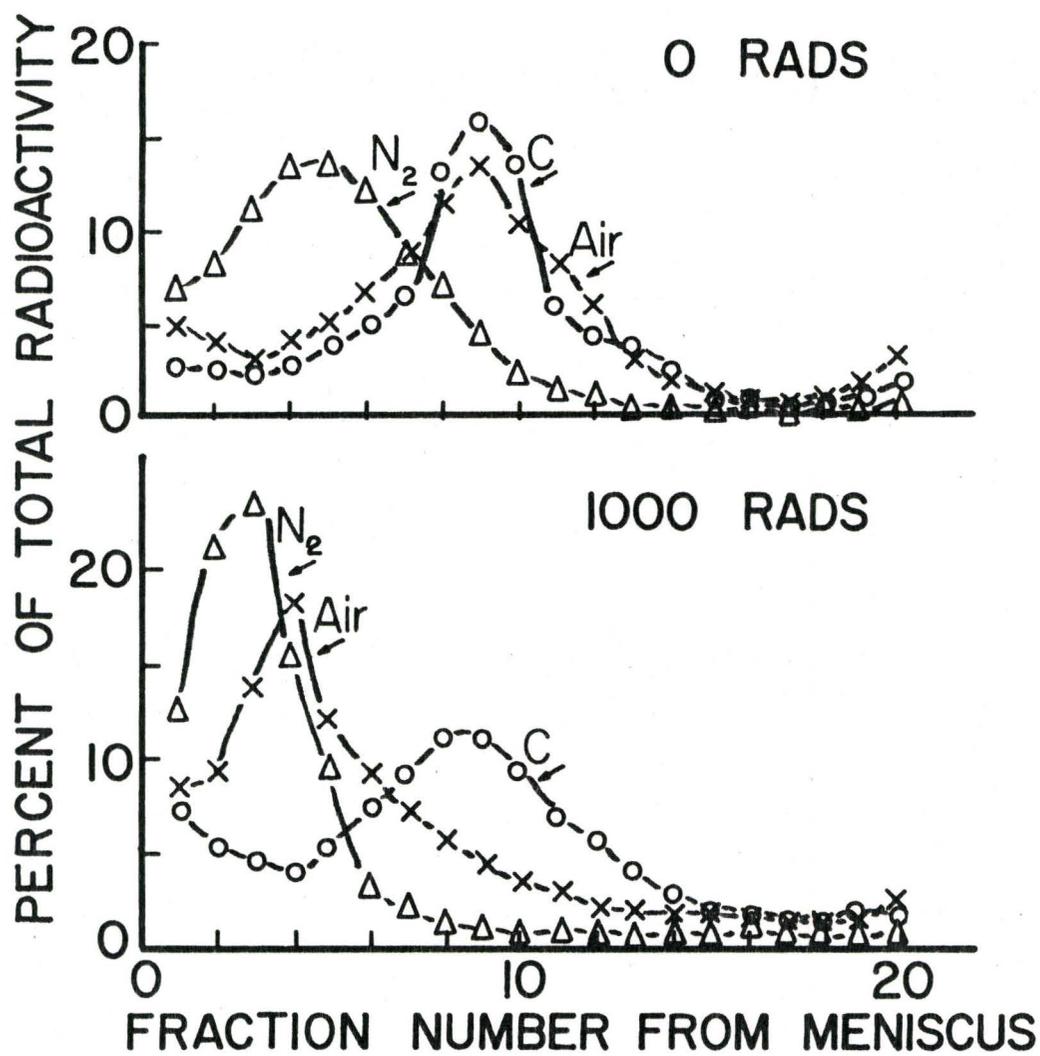


Figure 44: Effect of Treatment Time Following
Irradiation on Production of Breaks by
Nitrofurazone

L cells irradiated with 0 or 500 rads were incubated for various times after irradiation in 125 μ M nitrofurazone in PBS⁻ under air or nitrogen. Cells were lysed for 4 hours and DNA sedimented at 18 Krpm for 4 hours. Numbers in the upper right hand corners indicate the time and duration of incubation following irradiation.

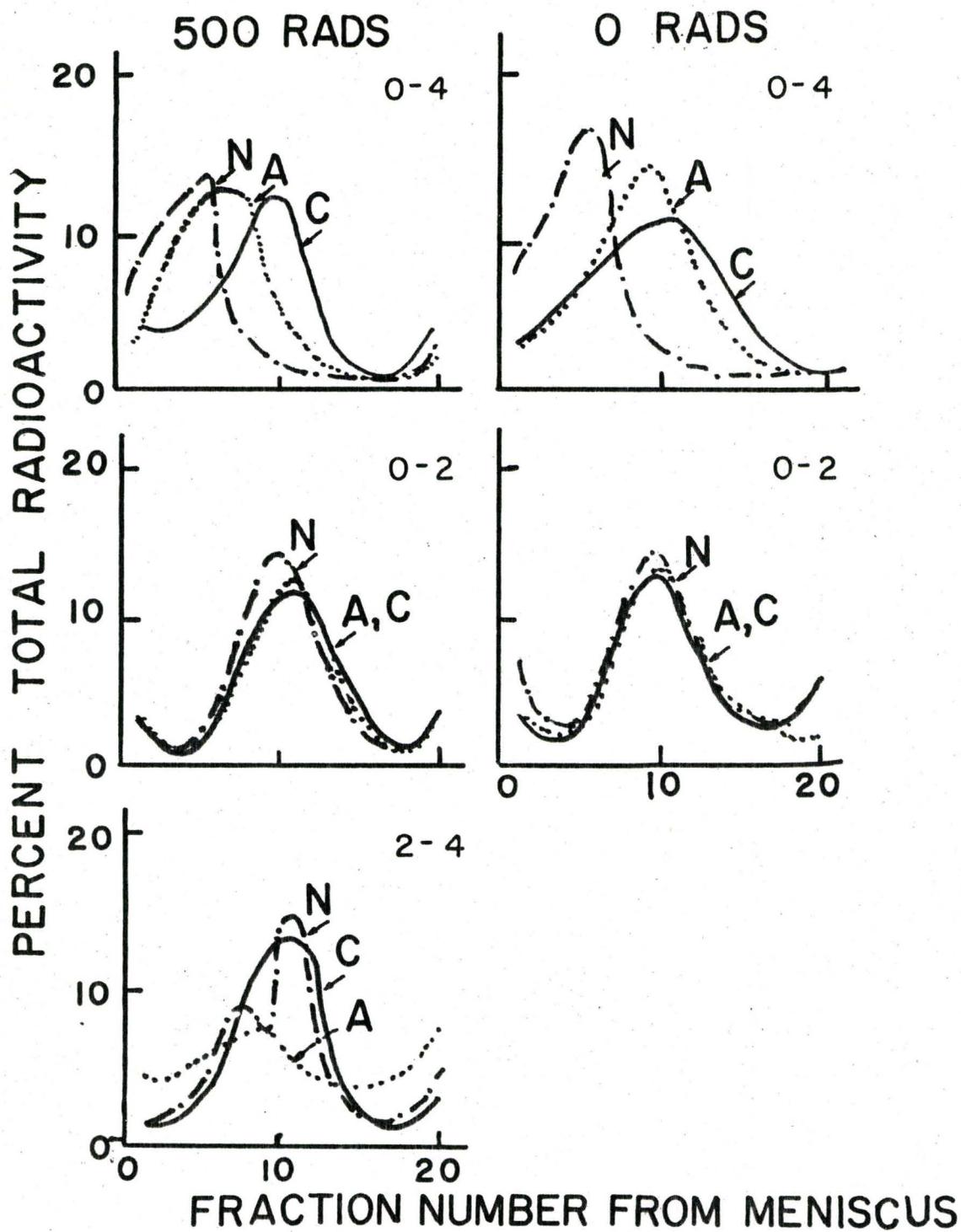


Figure 45: Effect of Irradiation on Metabolic
Reduction by L cells

L cells (2×10^7) were incubated with nitrofurazone under air or nitrogen, and the reduction of nitrofurazone was compared to that in cells irradiated with 500 rads (γ).

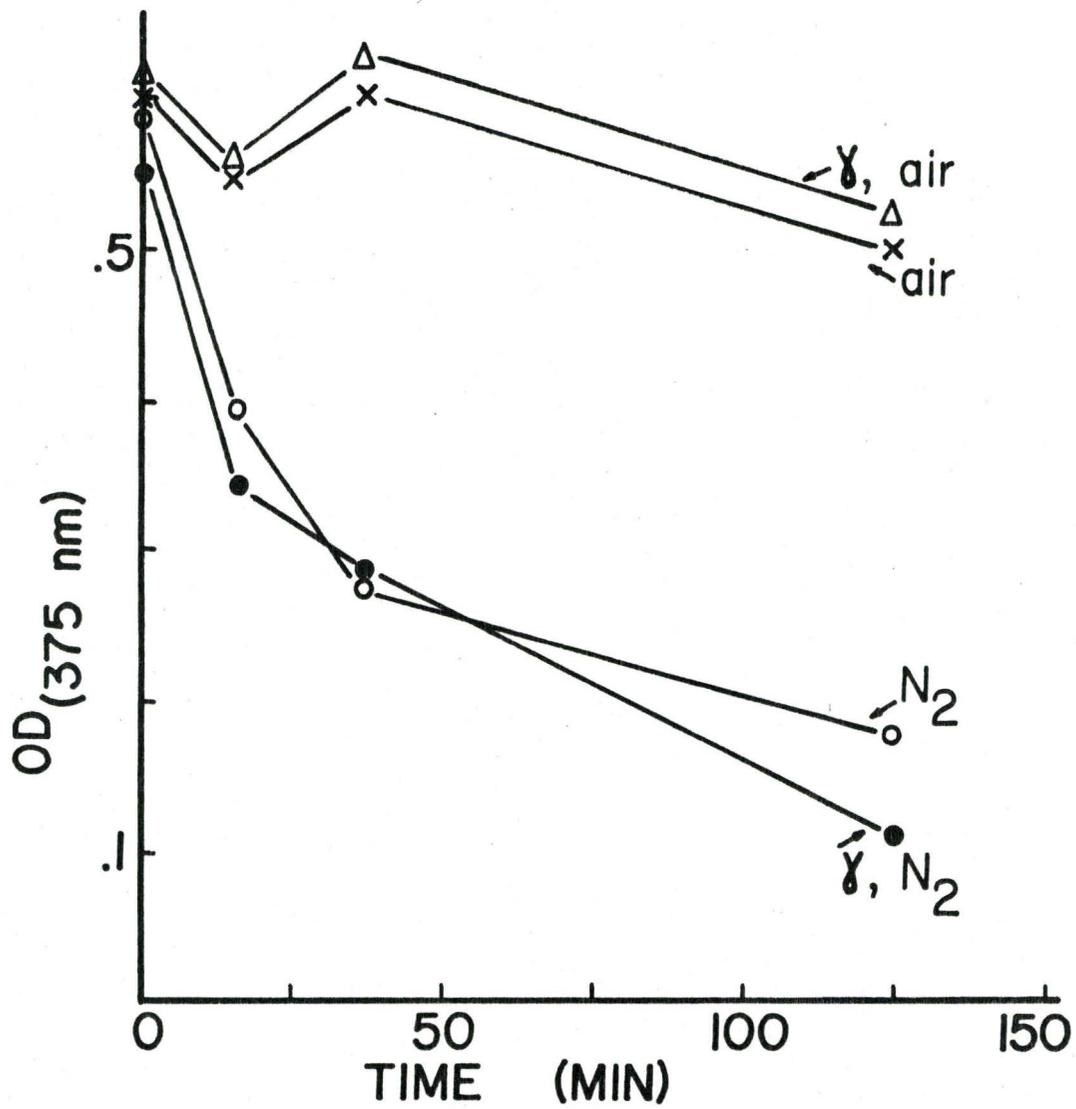


Figure 46: Pattern of ^3H -TdR Incorporation after
Irradiation and Incubation with Nitro-
Furazone

Human KB cells were irradiated with 100 ergs/mm^2 UV. They were then incubated in complete medium with or without $100 \mu\text{M}$ nitrofurazone under anoxic conditions. ^3H -TdR ($1.0 \mu\text{C/ml}$) was added and after 4 hours, cells were washed and lysed for 3.5 hours on alkaline sucrose gradients. a) asynchronous cells and b) synchronized (partially) using 4 mM HU for 20 hours then allowing six hours to reach S phase. The first few fractions, containing large amounts of unincorporated ^3H -TdR (or very small pieces of DNA) were omitted. Gradients were spun at 28 Krpm for 2 hours.

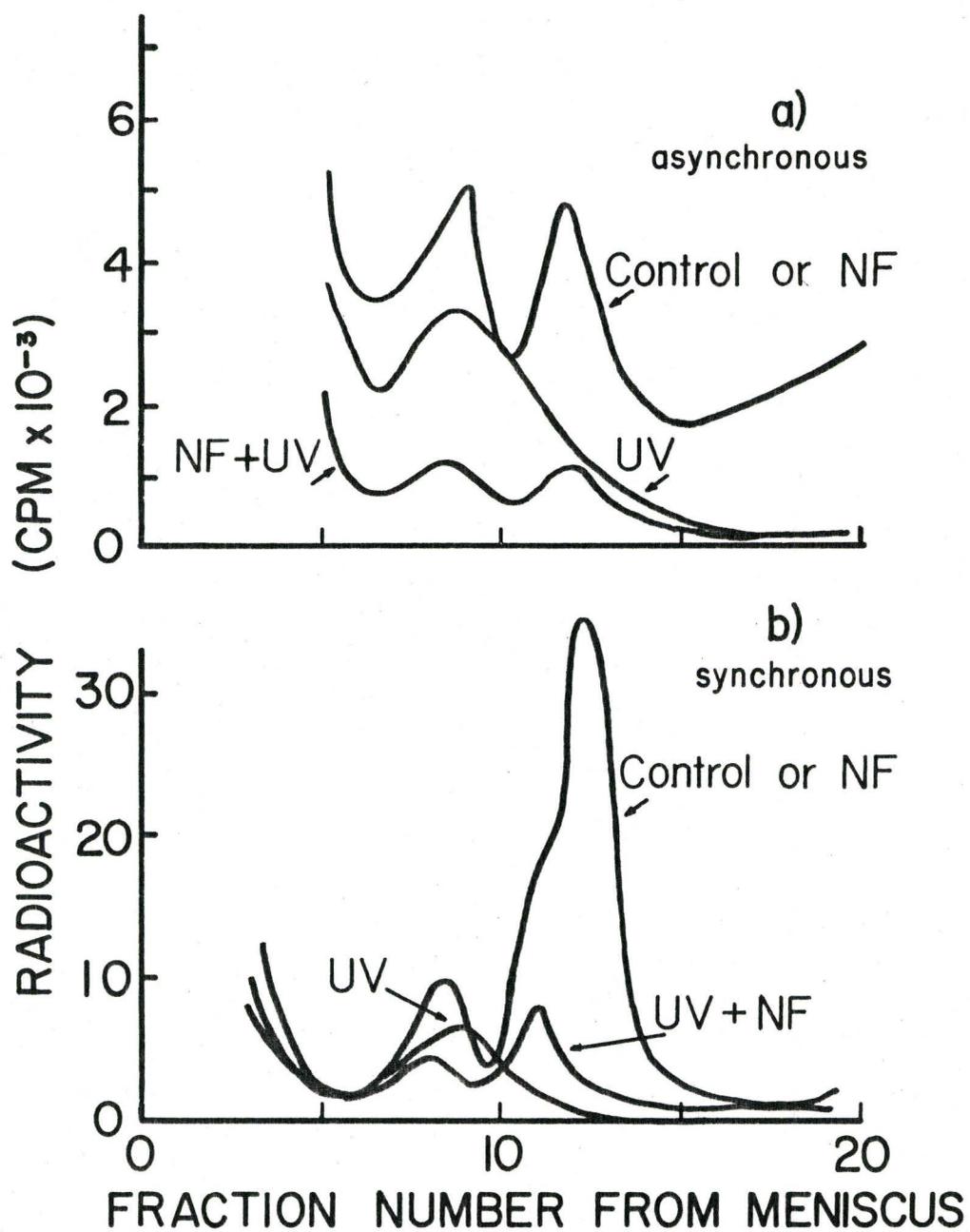
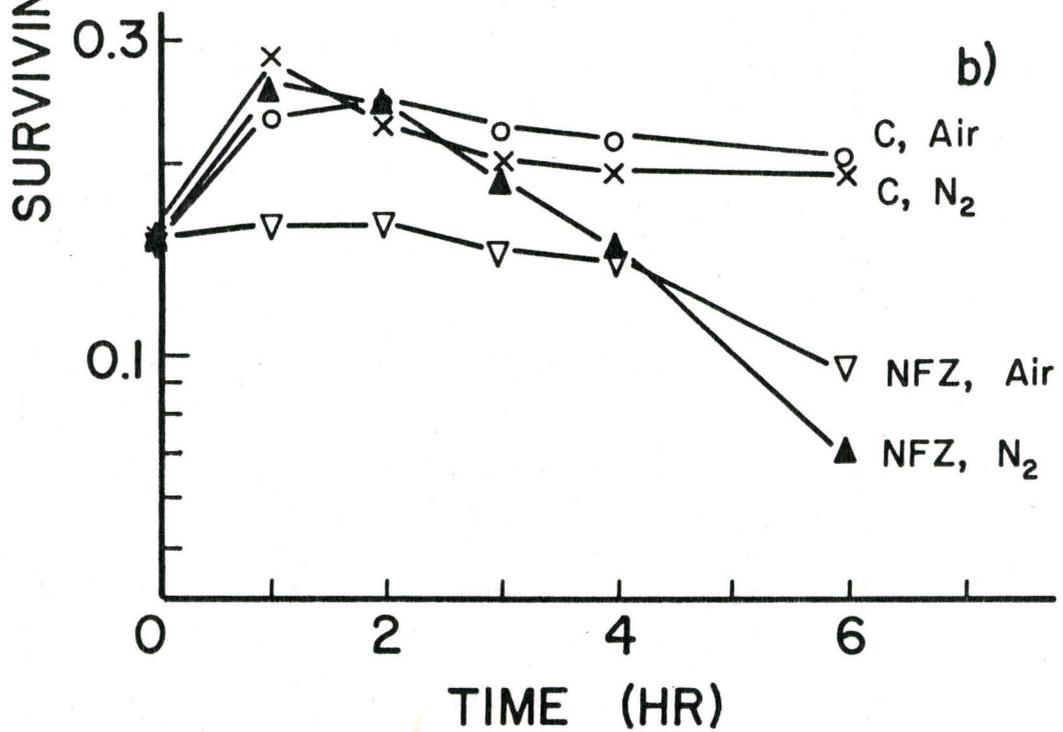
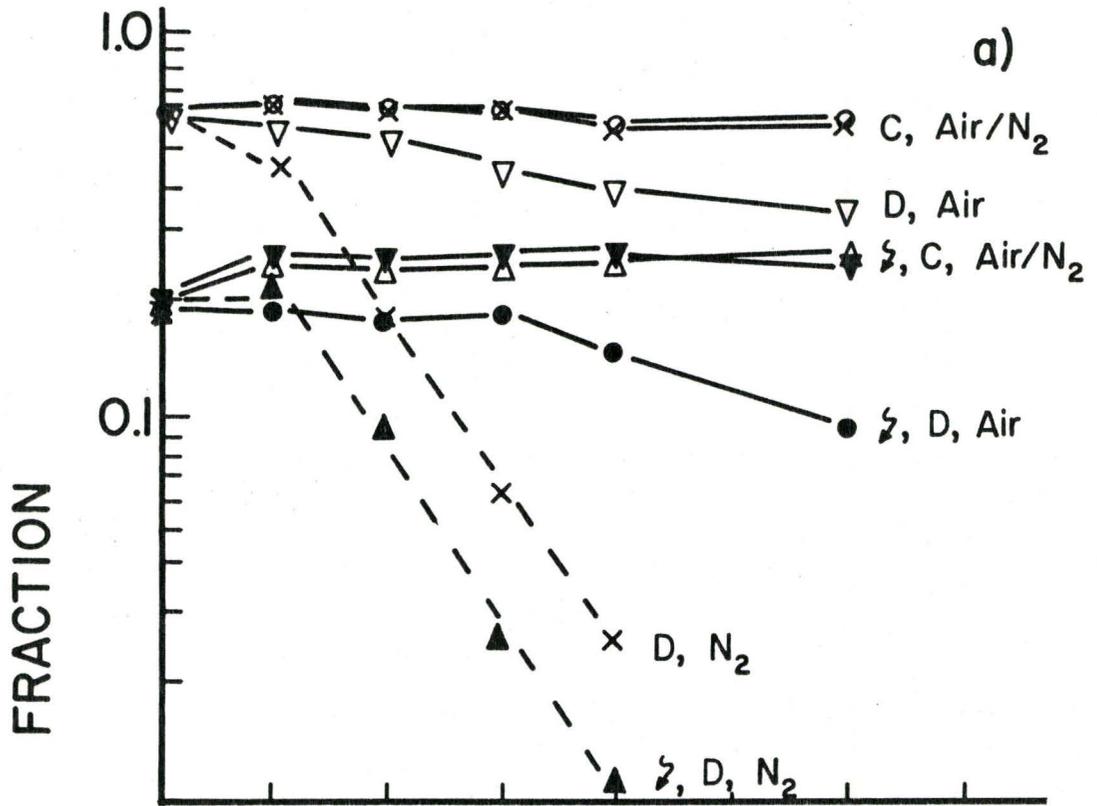


Figure 47: Survival of Irradiated Cells Treated with
Nitrofurazone or Nitrofurantoin

L cells were irradiated with 500 rads (~~5~~) followed by incubation in 250 μ M nitrofurazone (NFZ) or 430 μ M nitrofurantoin (D) in PBS⁻ plus 0.5% glucose under air or nitrogen. At specified times, aliquots of cells were removed, counted and plated. Surviving fraction is determined by the ratio of the number of colonies counted after 12 days to the number of cells initially plated. a) exponentially growing cells, b) irradiated plateau phase cells. Note survival does not increase after irradiation when plateau phase cells are incubated with nitrofurazone in air.



3.5 DNA Turnover in Nitrofurantoin-Fed Mice

3.5.1 Effect of Nitrofurantoin Diets on Mice

Results with mammalian cells in vitro or in vivo cannot be directly extrapolated to humans. The pharmacokinetics of absorption, distribution and excretion of nitrofurantoin, and the nature and amount of the metabolites may all affect the extent and character of DNA damage. Measurement of the repair of damage to tissues of mice fed nitrofurantoin would offer more relevant results. Loss of radioactivity from prelabelled tissue represents another method of assessing the effects of nitrofurantoin on mammalian DNA. Since 'turnover' can result from either a loss of cells or from DNA repair, it is necessary to distinguish between these possibilities.

Preliminary experiments indicated that nitrofurantoin resulted in slight toxicity to mice as measured by animal weight and liver size. Young Swiss Webster mice fed 0.1% furazolidone or nitrofurantoin in the diet grew less rapidly, whereas a slight increase in growth rate was noted in mice fed 0.2% nitrofurantoin (Table 5). The relative size of the liver increased after nitrofurantoin feeding and decreased after furazolidone ingestion (Table 5).

Mice fed 0.1% nitrofurantoin for 10, 25 or 50 days

showed a decrease in body weight in part dependent upon the sex of the animal (Table 6). Male mice appeared less tolerant to the drug than females as indicated by percent weight loss and observations of irritability. The effect on organ enlargement appeared to be specific for liver as other tissues showed changes in weight in accordance with body size (Table 7). Spleen weight is known to be highly variable (Green 1966).

Mice fed 0.1% nitrofurazone for 50 days showed a decrease in the RNA concentration in liver (Table 8), and since DNA concentration was not significantly decreased, organ enlargement was probably the result of hypertrophy (cell enlargement) rather than hyperplasia (increase in cell numbers).

TABLE 5

INHIBITION OF MOUSE GROWTH BY NITROFURANS

Diet	Number of Mice	Weight Before Diet(gm)	Weight After Diet(gm)	% Body Weight Gain	% Body Weight of Liver
Control	10	19.4	22.7	17.0	4.9±0.2
0.1% Nitrofurazone	15	20.0	21.6	8.2	5.6±0.2*
0.2% Nitrofurantoin	10	18.4	22.9	24.4	5.3±0.2
0.1% Furazolidone	10	18.3	18.5	1.0	4.3±0.2*
0.05% FANFT	5	18.0	21.4	19.0	5.3±0.2

* Value significantly different from control at 5% level
using the t test.

TABLE 6

CHANGE IN TOTAL BODY AND LIVER WEIGHTS FOLLOWING NITROFURAZONE FEEDING

Group	Days on Diet	Number/ Sex	Weight Before Diet (gm)	Weight After Diet (gm)	%Weight Loss	Liver Weight	%Body Weight
Control	10 to 50	6m, 16f	32.6±1.0*	32.6±1.0	0	1.67±0.1	5.1
NFZ	10	12m	39.4±1.0	36.5±1.0	7.4	2.12±0.1	5.8
NFZ	25	9m	35.2±1.1	27.1±1.0	23.0	1.38±0.1	5.1
NFZ	50	11f	34.0±1.0	30.7±1.0	9.7	1.74±0.1	5.7

* Mean ±standard error of the mean

TABLE 7
TISSUE WEIGHTS AFTER NITROFURAZONE FEEDING

Group	Time on Diet	Liver	Lung	Spleen	Kidney	Brain
Control	10 to 50	1.67±.08*	0.24±.01	0.23±.02	0.13±.01	0.43±.01
NFZ	10	2.12±.13	0.28±.01	0.30±.01	0.23±.004	0.47±.01
NFZ	25	1.38±.01	0.24±.01	0.20±.01	0.13±.002	0.44±.02
NFZ	50	1.74±.08	0.24±.01	0.22±.01	0.14±.002	0.46±.01

* Mean weight in gm ± standard error of the mean

TABLE 8

EFFECT OF NITROFURAZONE FEEDING ON DNA AND RNA CONTENT OF MOUSE TISSUES

Group	Days on Diet	Liver	Lung	Spleen	Kidney	Brain
A. DNA Concentration ^a						
Control	50	3.3±.15	5.3±.67	25.1±3.1	5.4±.67	2.0±.13
NFZ	50	2.9±.21	5.9±.46	28.2±3.7	5.2±.65	1.7±.15
B. RNA Concentration ^b						
Control	50	9.6±0.4	11.2±1.1	21.5±3.2	15.7±.88	8.6±0.8
NFZ	50	7.1±0.3*	9.7±0.6	18.3±3.0	13.3±.77	7.8±0.5

^aMean DNA concentration (mg/gm tissue) ± standard error of the mean

^bMean RNA concentration (mg/gm tissue) ± standard error of the mean

*Value significantly different from control, using the t test

3.5.2 Loss of Prelabelled DNA

DNA in tissues of mice can be labelled with radioactive thymidine by any one of at least three methods. The tissue can be labelled directly if cells are dividing, and some tissues can be partially excised and then labelled during the subsequent period of regeneration. The entire animal can be labelled by injecting ^3H -TdR into neonatal mice. The last two methods allow measurement of nitrofurantoin-induced turnover in DNA after tissues have reached a stable state. Incorporation of ^3H -TdR after nitrofurantoin injection was not measured since no unscheduled DNA synthesis was observed in vitro at much higher concentrations of nitrofurantoin than could be achieved in vivo. The loss of labelled DNA could be correlated with time on the diet as a measure of DNA replacement, and thus cumulative repair of damage was measured. As nitrofurantoin itself inhibits DNA synthesis in vitro, proliferation would be unlikely to occur without cell death.

Mouse livers labelled with ^3H -TdR after partial hepatectomy showed a loss of radioactivity following 2 to 4 weeks feeding with 0.1% nitrofurantoin (Table 9), however, no significant decrease followed feeding of 0.05% FANFT at the times studied. Using partial hepatectomy to label liver, reproducibility of specific activity in livers

of different mice was poor, partly due to the difficulty in removing the same proportion of liver each time. An attempt was made to control this by performing biopsies on mice with labelled livers one month before the start of the diets. A few mg liver tissue was removed and the specific activity measured so that each animal served as his own control. However, as only a portion of the liver was removed during the original hepatectomy, the specific activity may vary throughout the tissue, and the radioactivity of the biopsy sample may not be representative of the entire liver. Measurement of levels of radioactivity in the blood by sampling from the tail vein (cpm/white blood cell) was also attempted, however, reutilization of label may make such determinations questionable, and nitrofurazone is known to stimulate leukocyte production.

Labelling neonatal mice should result in tissue levels of radioactivity that are comparable among mice and litters. Results plotted as cpm/mg tissue in Table 10 indicated a loss of radioactivity in DNA of tissues of mice fed nitrofurazone for 10, 25 and 50 days. A significant loss occurred in the liver, lung, brain at 25 and 50 days (Table 11). Since the test for significance assumes that the sample variances were identical, that is, that the individual groups represent the same population, it is essential to investigate whether they are indeed the

same. The variance ratio test (F-test) was used to test the significance of the difference between sample variances (Moroney 1951). Analysis of the variance between groups indicated that there was no significant difference; the mice were randomly distributed in groups (Table 11). Some ambiguity in presentation of data is inherent, as cpm/mg tissue is dependent on organ weight. Alternatively, data presented as cpm/organ (Table 12) assumes a density of counts dependent on size. A larger mouse would have fewer cpm/mg tissue than a small mouse, although the same total cpm/organ. Since all mice received identical injections of ^3H -TdR immediately after birth, when body weight and growth rate were comparable, the latter assumption should be valid. However, analysis of statistical significance in different tissues indicated that calculations using both methods showed a similar pattern of loss of radioactivity with time on diet. The indication that radioactivity was also lost from control mice suggested that the rate of loss may be more important than the actual net decrease.

Analysis of radioactivity in two separate control groups that were originally litter mates (Control at 10 days, and Control at 20 days, Table 10) indicate that tissue specific activities were comparable in liver, lung and brain, but not in spleen or kidney. This may

indicate a sex difference in the pattern of incorporation, or actual variation due to thymidine reutilization.

When data were plotted as time on diet versus radioactivity (cpm/mg tissue) as a percent of control, the loss of counts appeared to be biphasic (lung, brain) or exponential (liver) with a rapid initial loss followed by a slower decrease, or in some cases a slight increase (spleen, kidney) (Figure 48). The exponential loss in the liver suggests random loss of prelabelled DNA.

TABLE 9

EFFECT OF NITROFURANS ON LOSS OF PRELABELLED DNA AFTER
PARTIAL HEPATECTOMY

Diet	Weeks on Diet	Number of Mice	CPM/mg DNA ± SEM
Control	2	6	1089±300
	4	7	957±181
0.1% NFZ	2	7	534±18*
	4	7	276±88*
0.05% FANFT	2	4	1435±181
	4	4	775±173

* Values significantly different from corresponding controls
at 5% level using t test.

TABLE 10

EFFECT OF NITROFURAZONE ON TISSUE SPECIFIC ACTIVITY

Group	Days on Diet	Liver	Lung	Kidney	Spleen	Brain
Control	10	382±55*	2272±375	1933±98	837±101	1101±159
Control	25	388±67	1903±222	2617±239	499±144	913±35
Control	50	299±53	1618±178	1965±156	601±147	888±86
NFZ	10	265±43	1522±182	1218±160	400±39	826±60
NFZ	25	172±19	1103±151	1449±90	262±63	584±64
NFZ	50	106±21	1042±70	1505±198	471±60	534±44

* cpm/mg tissue (mean ± standard error of the mean)

TABLE 11
 STATISTICAL SIGNIFICANCE: ANALYSIS OF VARIANCE

Days on Diet	Liver	Lung	Kidney	Spleen	Brain
10	.871 ^a (.591) ^b	<u>.999</u> (.153)	<u>.983</u> (.279)	<u>.998</u> (.841)	.916(.462)
25	<u>.997</u> (.790)	<u>.989</u> (.751)	<u>.998</u> (.853)	.886(.533)	<u>.998</u> (.801)
50	<u>.989</u> (.494)	<u>.991</u> (.279)	.887(.283)	.565(.210)	<u>.997</u> (.779)

^a Probability that the difference between two means (control and nitrofurazone treated) is significant, calculated using the t test and data from Table 10.

^b Probability that variances between control and nitrofurazone treated groups are significantly different, calculated using the F ratio test.

TABLE 12

EFFECT OF NITROFURAZONE ON TOTAL ORGAN ACTIVITY

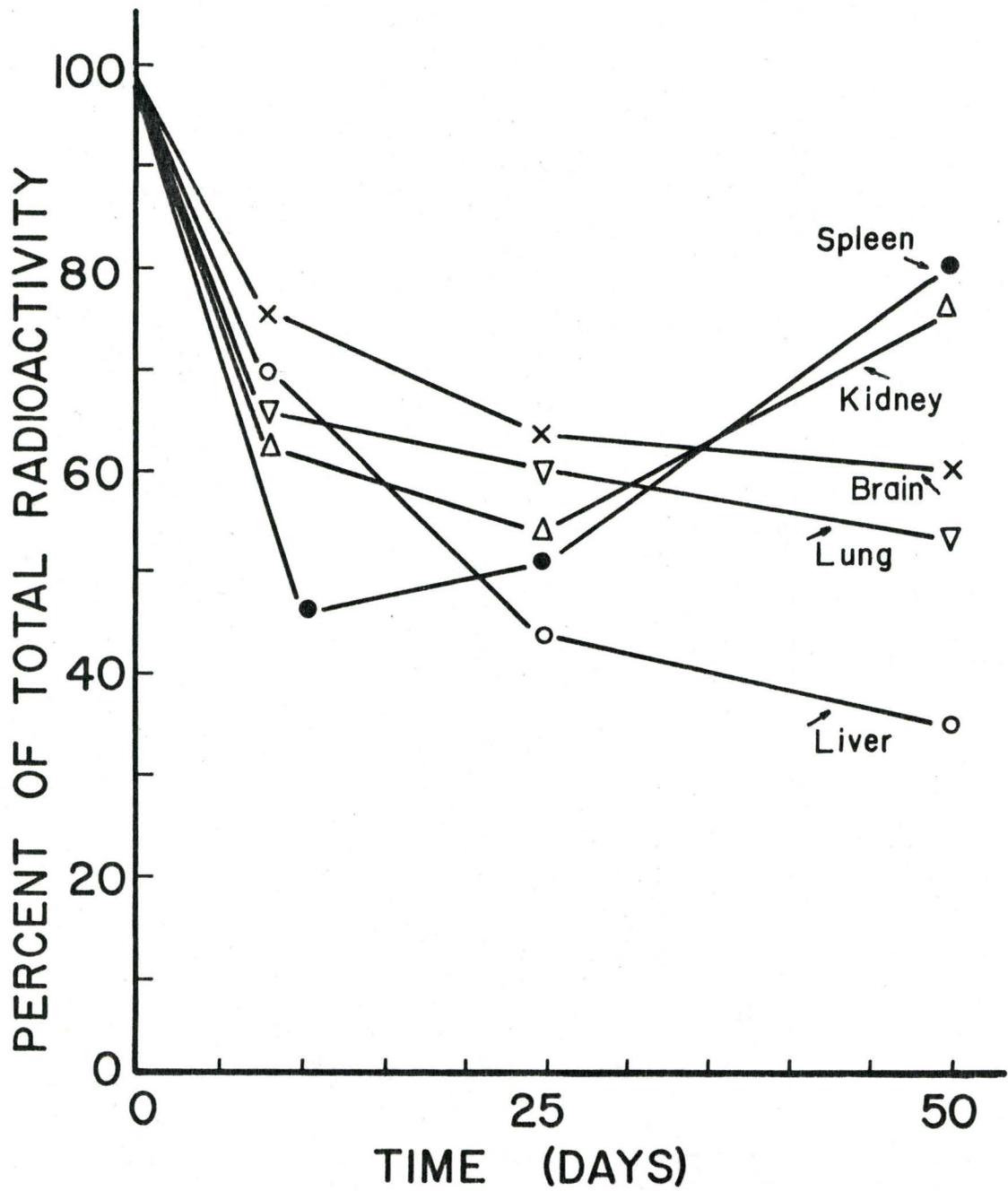
Group	Days on Diet	Liver	Lung	Kidney	Spleen	Brain
Control	10	68.3±6.7 ^a	48.9±10.6	40.4±2.8	11.7±1.2	50.6±0.8
Control	25	37.8±4.2	42.0±3.3	42.6±1.4	4.5±0.8	41.3±1.1
Control	50	45.0±3.5	38.5±3.2	42.3±1.9	6.7±0.7	38.4±4.2
NFZ	10	51.1±4.7	43.4±3.1	36.3±4.0	8.9±0.5	34.3±3.0
NFZ	25	22.6±1.0*	15.3±1.8*	29.0±1.5*	2.9±0.5	25.2±3.5*
NFZ	50	17.4±2.7*	25.5±1.9*	32.7±3.3	5.4±0.5	25.2±2.5*

^acpm/organ x 10⁻⁴ (mean ± standard error of the mean)

* Values significantly different from corresponding controls using t test .

Figure 48: Loss of Prelabelled DNA from Mouse
Tissues

Mice with ^3H -TdR labelled DNA (see Figure 2) were fed 0.1% nitrofurazone in the diet. 10, 25 and 50 days later, the specific activity of 5 organs (cpm/mg tissue) was compared to tissues of mice on control diets by calculating the percent of control activity using data from Table 10.



3.5.3 Histology and Autoradiography

A microscopic examination of sections of tissues from control or nitrofurazone-fed mice after 50 days revealed no gross changes in the five tissues studied. The relative ratio and size of non-parenchymal (oblong nuclei) to parenchymal (round nuclei) was unchanged in liver (Figure 49a,b,d,e), and no atrophy of the adrenal cortex was noted (Figure 49c).

Autoradiography of tissues at 50 days indicated that in kidney and spleen, cells comprising or in close proximity to blood vessels showed a larger number of grains than surrounding areas (Figure 50a,h). In spleen, both the white pulp (containing forming lymphocytes) and the red pulp (containing maturing lymphocytes and phagocytosed leukocytes) were labelled. In kidney, the proximal convoluted tubules and collecting ducts were also densely labelled (Figure 50c,f). In brain, only a small percent of nuclei were labelled in control or nitrofurazone-fed mice. These however, were generally heavily labelled (Figure 50j). Scoring the grain number of cells indicated that the distribution of counts was not significantly different between control and treated cells in liver and brain at 50 days. The large variety of cell types in kidney, spleen and brain made grain counting difficult as only segregated areas were highly

labelled. The labelling itself also tended to obscure the underlying cells, thus preventing identification.

Autoradiography can theoretically distinguish between turnover due to repair of DNA, or turnover due to cell death and replacement (see Appendix 3). As no obvious change in the distribution of radioactive label was observed, and no significant cell enlargement occurred, the loss of counts may be due, at least in part, to DNA repair.

Figure 49: Histology of Tissues of Mice Fed
Nitrofurazone

After 50 days on control diets or diets containing 0.1% nitrofurazone, sections of tissue were prepared from various organs. (a) and (b) represent sections of liver from control animals. Some binucleate cells are evident. (c) is a section of adrenal from an animal fed nitrofurazone. (d) and (e) are liver sections from nitrofurazone-fed mice. (f) is a section of bladder from a mouse fed nitrofurazone.

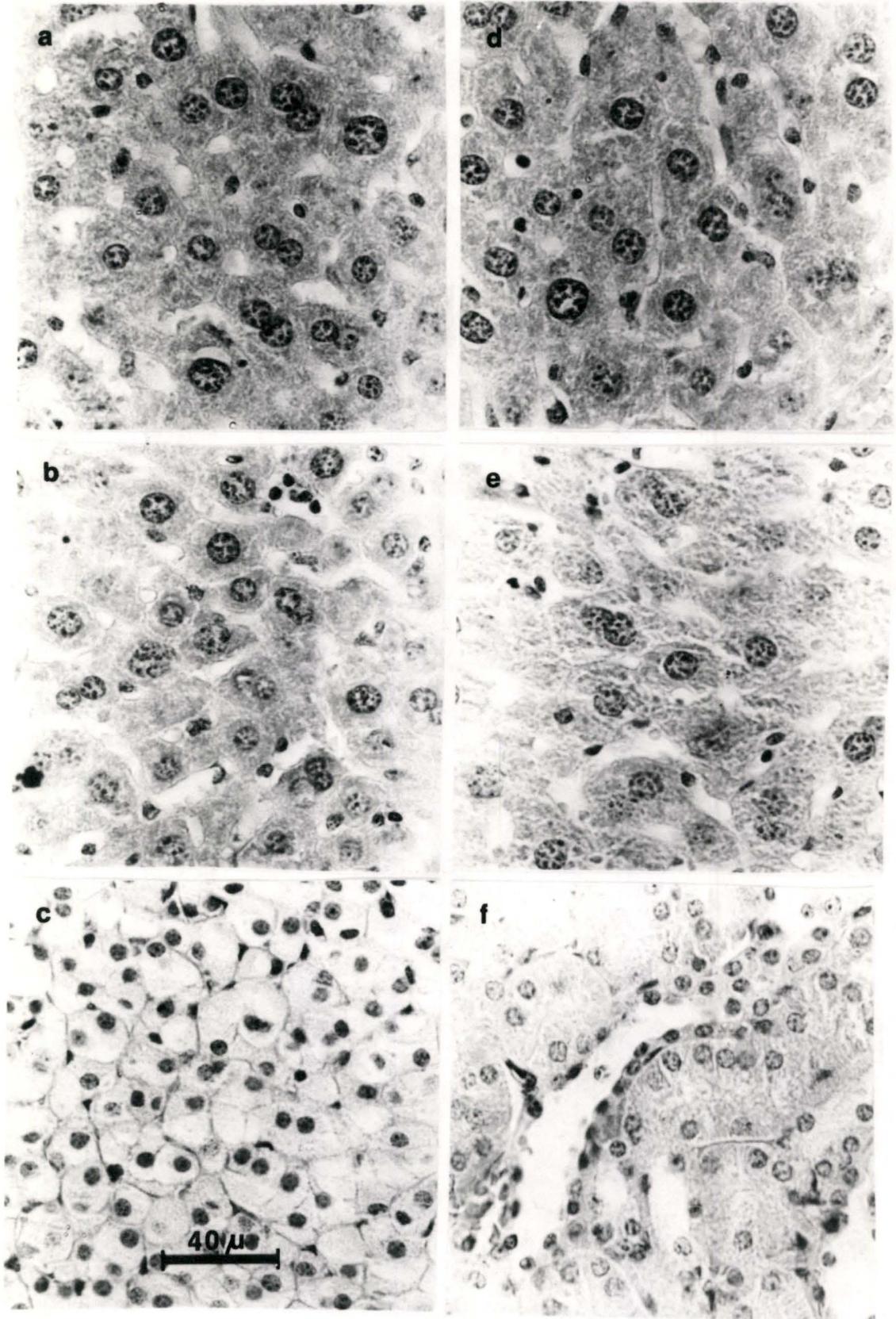
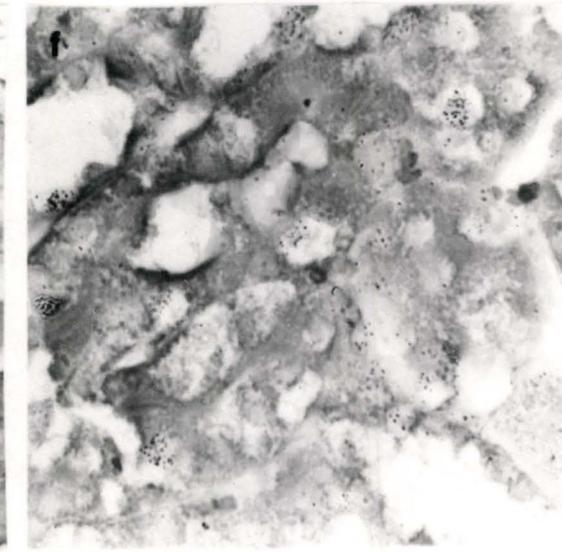
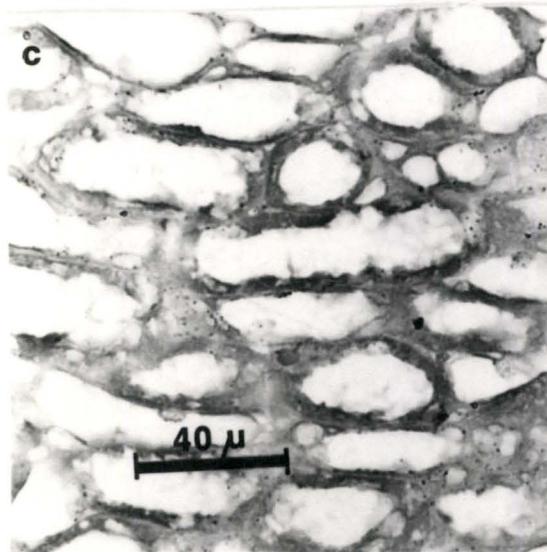
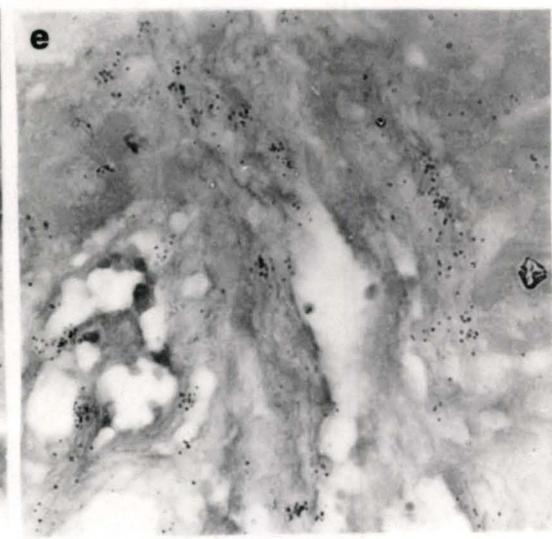
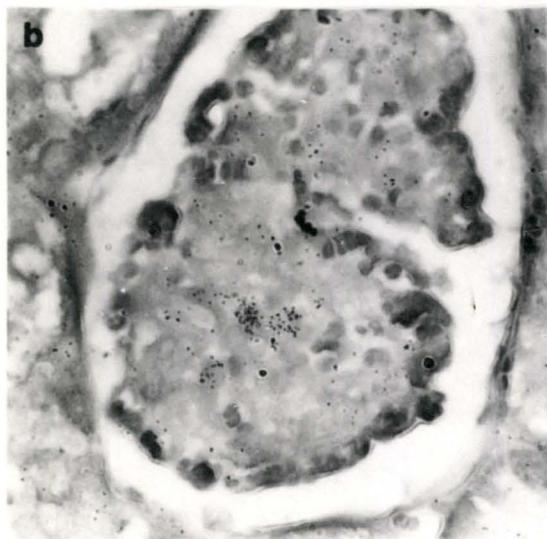
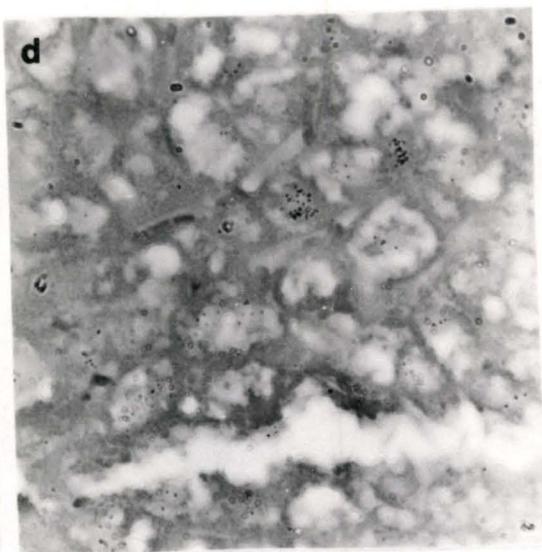
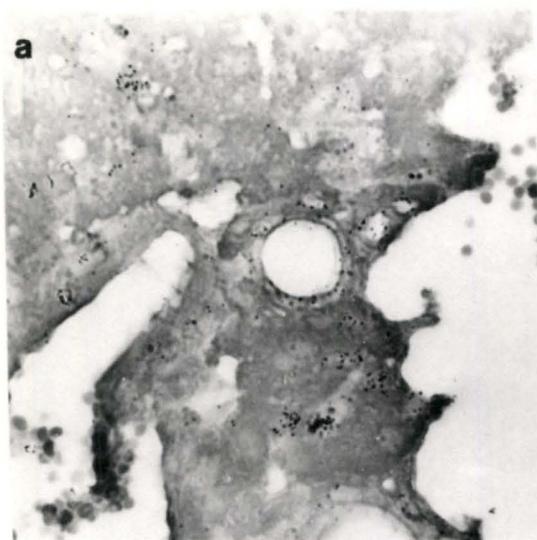
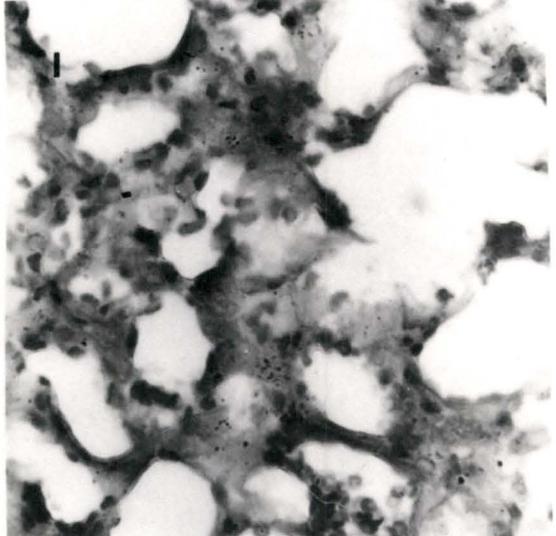
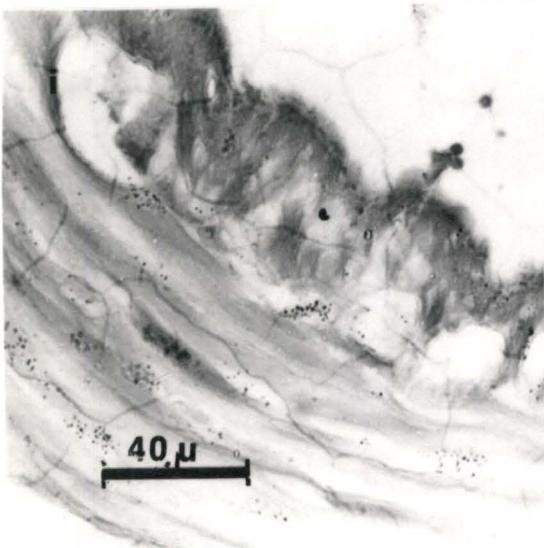
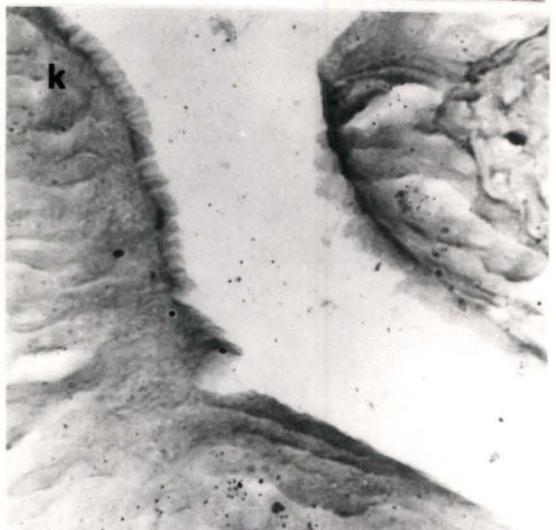
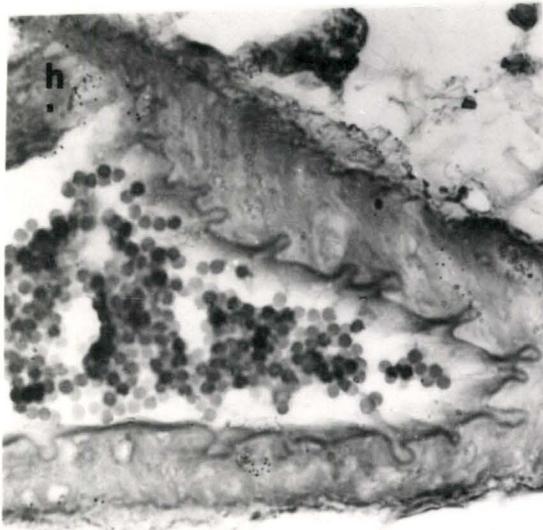
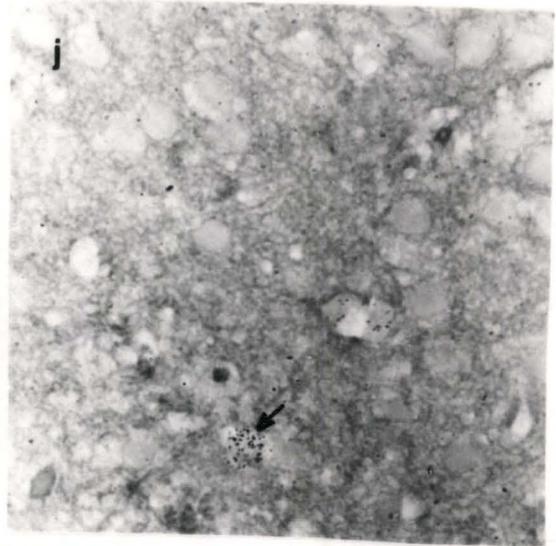
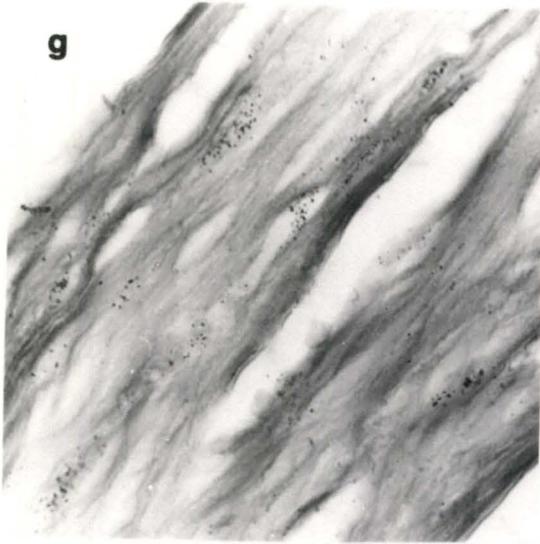


Figure 50: Autoradiography of Sections from Mouse
Tissue

Autoradiography of various tissues in mice fed 0.1% nitrofurazone or control diet indicated a similarity in grain distribution. Sections of liver from control (a) and treated mice (d, e) indicate a relation between grain density and proximity to blood vessels. (b) kidney glomerulus from control animal; note extensive labelling within glomerulus. (c) collecting ducts within kidney; most epithelial cells are labelled in this control animal. (f) section of kidney cortex from treated mouse. (g) muscle cells of stomach, control animal. (h) section of artery in the kidney of a control mouse. Note the labelled cells within the muscle layers and intima of the artery. (i) section of stomach showing epithelium and muscle layers in a mouse fed nitrofurazone. (j) Brain tissue of treated mouse; note sparcity of labelled cells. (k) section of bladder of treated animal (l) lung tissue of control mouse.





4. DISCUSSION

4.1 'Activation' as a Requirement for Damage

In mammalian cells as in bacteria, the reduction of nitrofurazone appears to be a prerequisite for production of single-strand breaks in DNA. While maximum rates of reduction are measured under anaerobic conditions, a significant amount of reduction does take place in mouse liver homogenate and in cultured cells equilibrated with gas mixtures that have oxygen concentrations as high as 5% (Table 1). This figure is within the range encountered by tissue in vivo (partial pressure of oxygen equivalent to 40 mm Hg). There appears to be a direct relation between the number of single-strand breaks and the rate of reduction of nitrofurazone (Figure 51). However, this data is only inferential as it was obtained from two separate experiments in which the oxygen concentration was varied. Mammalian reductase systems are relatively nonspecific and would thus be expected to reduce a wide variety of these compounds, not only nitrofurazone. Since aminofurans and other end products of reduction are known to be biologically inactive, it seems likely that reactive intermediates formed during the reduction of nitrofurans are the ultimate carcinogens.

The spectrophotometric assay for reduction is a measure of the loss of nitrofurazone. This is apparently irreversible in the absence of oxygen, however, unstable auto-oxidizable intermediates could be produced. Such compounds would be re-oxidized in the presence of oxygen and thus the amount of unreduced nitrofurans would remain relatively constant. This might explain the small amount of breakage measured when cells were incubated with nitrofurans in air at high concentrations (Figure 43). A similar suggestion was made by Morita et al. (1971) concerning the reduction of the nitroheterocycle, niridazole. Incubation with hypoxanthine and purified rat liver xanthine oxidase resulted in significant oxidation of hypoxanthine to uric acid with no apparent decrease in the niridazole concentration. Incubation of NFTA with dialyzed liver cytosol also resulted in formation of uric acid under aerobic conditions, while the rate of reduction of NFTA was minimal (Wang et al. 1974). Other studies with EA cells have shown that oxygen uptake is stimulated by nitrofurans in the presence of glucose (Biaglow, Nygaard and Greenstock 1975).

The decrease in molecular weight of DNA resulting from incubation with nitrofurazone under hypoxic conditions was similar for three cell lines, despite the observation that reduction was five times greater in L cells than in BHK cells. However, the rate of reduction is a mis-

leading value since it is not a measure of the concentrations of active intermediates. Analysis of molecular weights of nitrofurantoin-treated DNA and comparison with rates of reduction indicated that in excess of 10^4 molecules of nitrofurantoin were reduced for every break.

There is a continued interest in the mechanism of radiosensitization by nitrofurans and related compounds. Other cell radiosensitizers also cause single-strand breaks on alkaline sucrose gradients (Moroson and Furlan 1971; Voiculetz, Smith and Kaplan 1974). It was suggested that the radiosensitizing effect could be due to inhibition of repair of radiation damage (Moroson and Furlan 1971) or to depression of macromolecule synthesis (Voiculetz *et al.* 1974). The importance of metabolic effects of nitrofurans has been stressed (Biaglow *et al.* 1975), however, the relative amounts of radiosensitization which are due to 'fixation' of radiation damage and to interference with normal cellular processes is not known.

An enhancement of damage by radiation before incubation of cells with nitrofurans was suggested from data obtained using bacteria to test for the inhibition of 'liquid-holding recovery' (LHR) by nitrofurans (see Appendix 2). This type of repair is analogous to the repair of potentially lethal damage (PLD) in mammalian cells. However, the finding that LHR was inhibited only

if bacteria were held before plating in medium containing nitrofurans but not in buffer with nitrofurans suggested that metabolically active bacteria were required. This was also suggested from data obtained with mammalian cells. PLD was only inhibited when incubation took place in air, but not in nitrogen.

The enhancement of single-strand breaks by irradiation of mammalian cells followed by incubation with nitrofurans in air suggests that radiation may inhibit normal repair of damage, or sensitize the target so that much less 'activated' nitrofurans (or none at all) is required for DNA damage (Figure 52). Analysis of single-strand breaks produced after irradiation indicated that extensive damage occurred after two hours. Two to four hours following a dose of about 1000 rads, cells initiate DNA synthesis with a decrease in the oxygen consumption rate (Okada 1971). Prior to this, biochemical changes related to an impairment of ATP production, alterations of membrane permeability and nuclear disorganization occur. In order to correlate this damage and metabolic reduction, further studies into the mechanisms and intermediates of nitrofurans reduced by mammalian cells are required. Recent experiments on the effect of split doses of UV or chemical mutagens indicate that mammalian cells are less able to repair DNA damage which occurs within two to four hours

after the first insult (Thilly and Heidelberger 1973; Warren and Stich 1975). Incubation of irradiated cells with nitrofurans may be a similar situation; if so, cells in this 'refractive period' following irradiation may be much more susceptible to DNA damage by nitrofurans.

It has been found that high cell densities decrease the ability of nitrofurans to sensitize hypoxic cells to ionizing radiation (Agnew and Skarsgard 1974). If radiosensitization depends upon the continued presence of unreduced nitrofuran within the cells, the reductive metabolism of the drug by dense cell cultures may lead to much lower intracellular drug concentrations than those that can be obtained when less dense cultures (in which the ratio of drug to cells is much higher) are used. Nifuroxime sensitized the inner hypoxic core of an in vitro tumour model composed of V79 cells to radiation damage (Sutherland and Durand 1972). Apparently the drug was able to diffuse through the outer layers of oxygenated cells of the 'spheroids' without being metabolized. Furthermore, the inner hypoxic cells would have access to a large amount of nitrofuran surrounding the entire structure, a situation that is considerably different from the one in which cells are spun down to form a pellet (Agnew and Skarsgard 1974). In the pellet experiments, both diffusion and metabolism of nitrofurans become

important considerations in determining the extent of radiosensitization.

These considerations also apply to the data obtained using ascites cells (Figure 29). Reduction of the drug coupled with rapid absorption from the peritoneal cavity rapidly reduced the effective drug level. The drug/cell ratio could only be increased by reducing the number of cells, and replacing them with buffer. In contrast to ascites cells, experiments using rat liver cells lysed on gradients (Cox et al. 1973) suggested that although nitrofurans might be expected to be reduced more rapidly by liver cells, cells in situ are insufficiently hypoxic for damage to be observed at the systemic drug levels which were tolerated by mice during a single injection.

The 5-nitro group has been shown to play a major role in the carcinogenic activity of nitrofurans (Erturk et al. 1971). It is also of interest that the presence of the nitro group is required for high antibacterial activity (Dodd and Stillman 1944), inhibition of pyruvate oxidase (Buzard 1962), radiosensitizing ability (Chapman et al. 1972) and electron withdrawing properties (Raleigh et al. 1973). The implications of these observations have yet to be fully resolved. An attempt to correlate the Hammett constants (a measure of electron withdrawing power) with the carcinogenicity of different nitrofurans should be undertaken.

Figure 51: Correlation Between Number of Breaks,
Rate of Nitrofurazone Reduction and
Toxicity

Data from Table 1 (mouse liver homogenate), Figure 6 (nitrofurazone) and Figure 24 (nitrofurazone) were used to determine the correlation between the rate of reduction of nitrofurazone and the number of single-strand breaks by equating values obtained when the oxygen concentration was the independent variable.

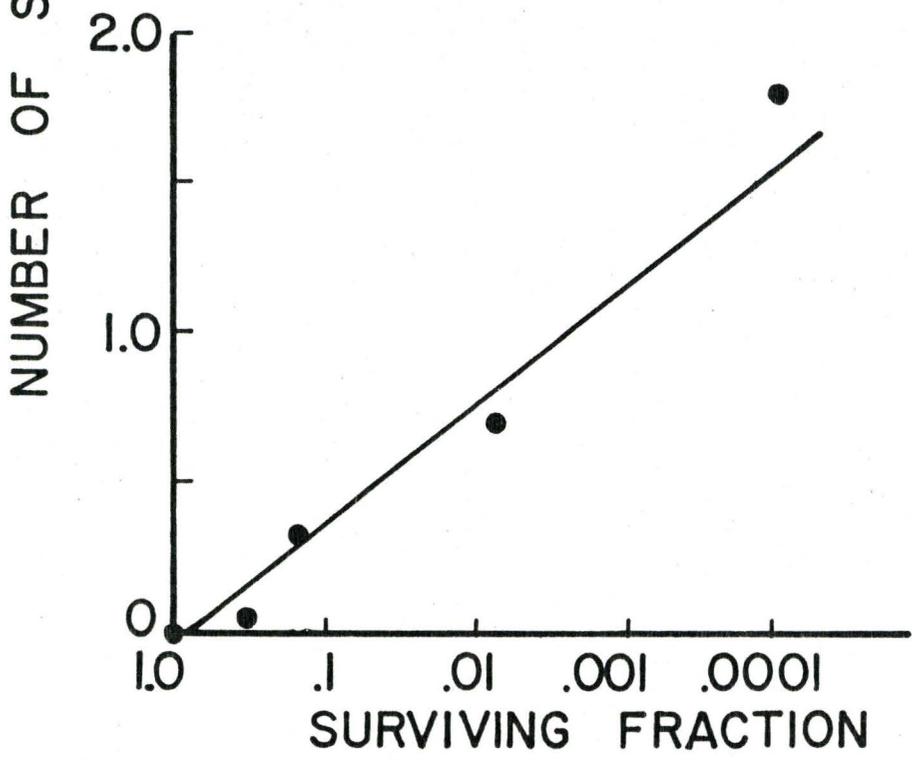
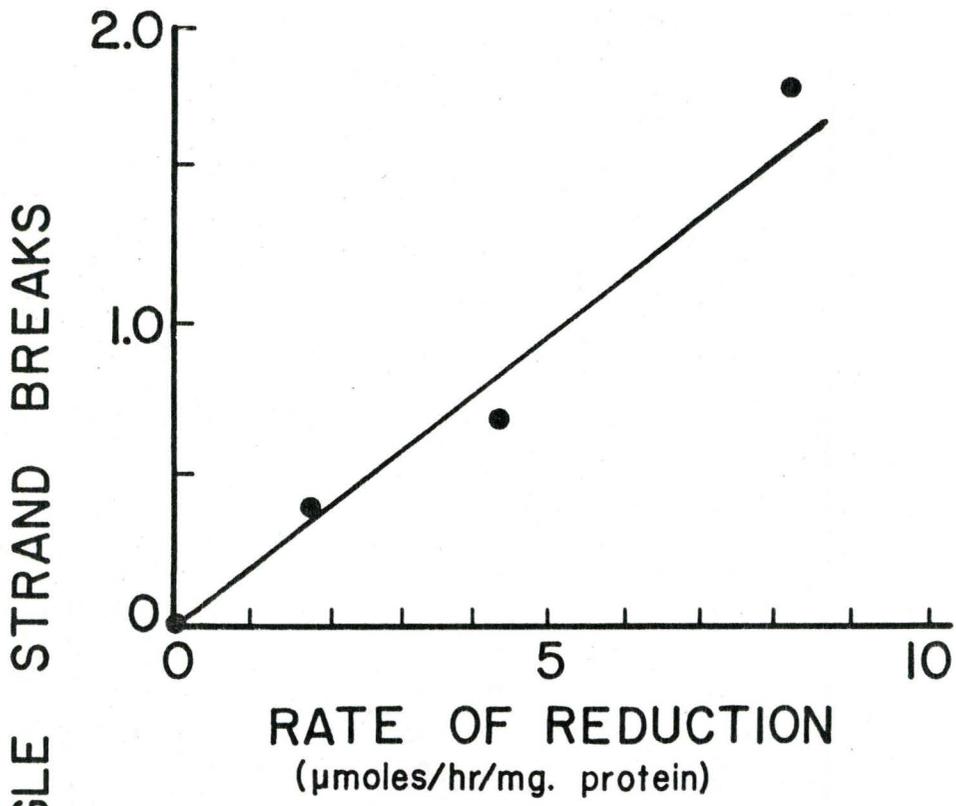
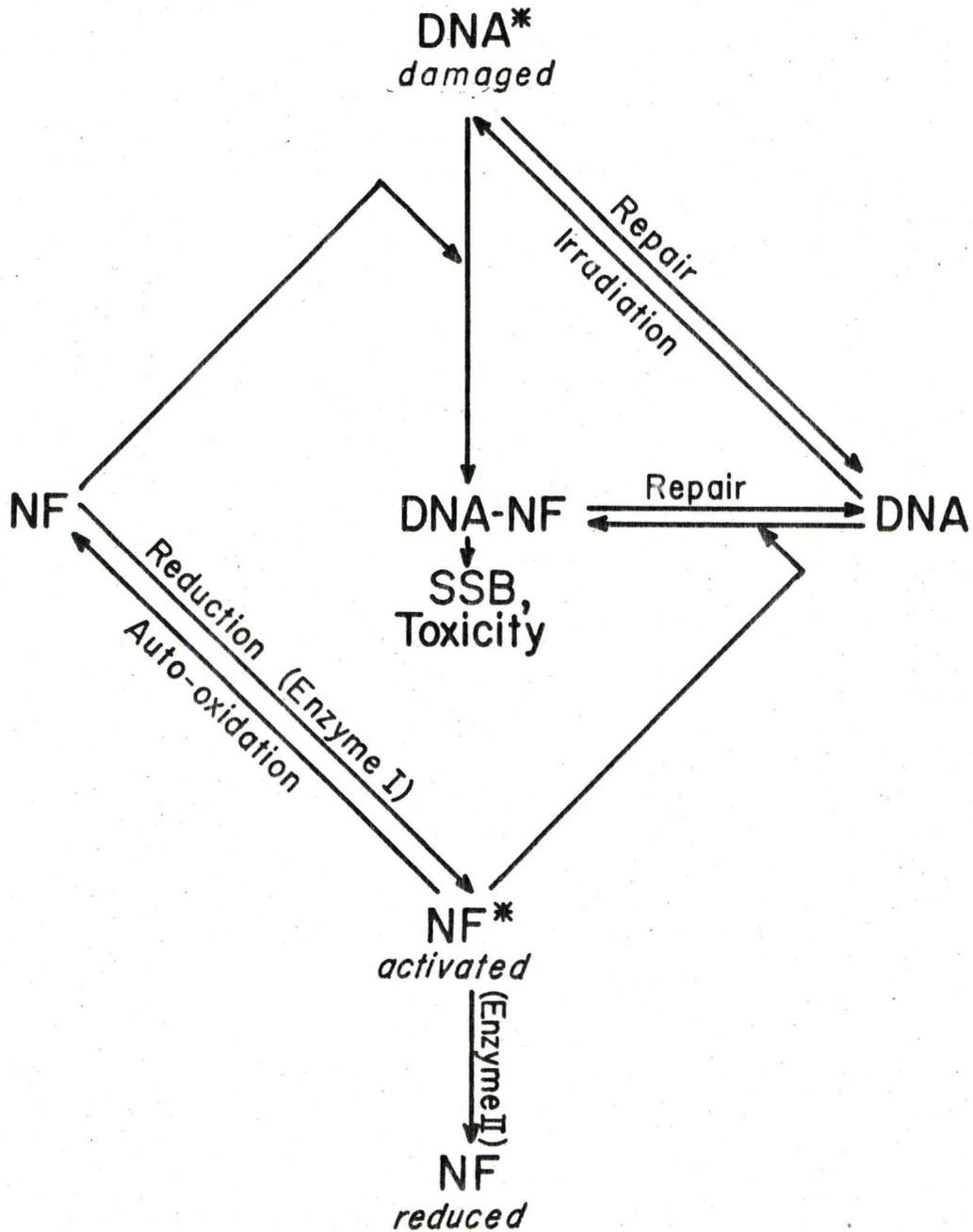


Figure 52: Interaction of Nitrofurans with DNA

DNA and nitrofurans will not normally react in solution. However, 'activation' of nitrofurans by enzymes present in mammalian cells allows them to interact with DNA causing single-strand breaks in DNA and cell death. DNA may also be made to interact with nitrofurans by radiation damage. Nitrofurans could react with damaged sites in irradiated DNA, or affect repair capabilities. These effects may not require metabolic conversion of nitrofurans. Enzymes which repair radiation damage may also repair damage by 'activated' nitrofurans.

Interaction of Nitrofurans with DNA



4.2 Relation of Toxicity to DNA Damage

All nitrofurans tested were toxic under anoxic conditions suggesting that reduction products or intermediates caused cell death. Rauth (personal communication) found that plating efficiency was significantly decreased when Hela cells were incubated with nitrofurazone in complete medium equilibrated with gas containing less than 0.1% oxygen; incubation of cells under air with nitrofurazone resulted in cell death at much longer incubation times. However, nitrofurantoin and nitrofurazone were toxic to L cells incubated in PSG both under air and nitrogen (Figures 7,8). These data could be correlated with reduction of nitrofurazone as well as the production of breaks on alkaline sucrose gradients (Figure 51). Cells are severely depleted of ATP and nutrients required for biosynthesis after incubation in buffer ; toxicity in air may represent a decreased repair capacity or an increased sensitivity to damage by another agent.

Serum decreased the toxicity of nitrofurazone and nitrofurantoin probably due to binding to plasma proteins (Paul et al. 1960, Zinneman et al. 1962). It has been found that plasma proteins bind up to 70% of nitrofurantoin in a reversible manner so that the effective drug concentration is reduced by this amount. Since

toxicity is almost eliminated in the presence of serum (Figure 7), other factors must also be involved in the deactivation of nitrofurans, such as the requirement for intracellular levels of specific cofactors, amino acids or thiols. There appeared to be a slight enhancement of damage when cells were incubated in buffer under nitrogen for four hours prior to incubation with nitrofurazone in air. This may indicate that the metabolic state of the cell (level of glucose, ATP, NADPH etc.) is an important part of the effect of nitrofurans.

Nitrofurantoin was more toxic to cells incubated in buffer containing glucose than in buffer alone (Figure 8), perhaps reflecting the enhanced reduction of nitrofurans in the presence of glucose. Using ascites cells in vitro, Biaglow et al. (1974) found that the rate of reduction of nifuroxime was dependent upon the concentration of glucose. The half-time for reduction by 3×10^8 cells was four minutes if 0.01 M glucose was added, compared to 23 minutes without glucose. Glucose could be required to maintain or accelerate the pentose phosphate shunt which in turn supplies NADPH required for reduction of nitrofurans by NADPH: cytochrome c reductase. Glucose might also alter membrane properties such as permeability to nitrofurans.

Nitrofurantoin is degraded at low pH to a toxic compound which is subsequently inactivated by light (McCalla

and Reuvers 1970). However, cell suspensions monitored in the present experiments indicated that the pH at the end of incubation was about 6.8, whereas chemical degradation requires a pH below 4.0 (Paul et al. 1960). Light has also been shown to degrade nitrofurantoin to nitro-furaldehyde which is more toxic (McCalla and Reuvers 1970). Experiments performed in darkened conditions indicated that degradation of nitrofurantoin by light would not represent a serious problem, at least in terms of toxicity (Figure 7).

Dugle et al. (1973) reported that nitrofurans were able to diffuse to the target in V79 cells within four minutes for complete radiosensitization. In the present studies, the shoulder on the drug survival curves, which was influenced by dose and incubation time, might indicate that a certain period of time was required for reduction of toxic quantities of drug or for interaction with targets, rather than repair.

The production of DNA single-strand breaks at low oxygen tensions corresponded with reduction of drug. Toxicity (cell survival) was also correlated with oxygen concentration when cells were incubated in buffer with glucose. However, effects on macromolecule synthesis did not appear to be related to oxygen tension. Experiments which examined incorporation of radioactive precursors required maintaining cells in conditions optimal for growth,

that is, in medium containing serum. Since toxicity is dependent on the medium of incubation, and most of the measurements of DNA breaks were performed after incubation of cells in buffer, a direct comparison of toxicity with effects on macromolecule synthesis may not be valid. Inhibition of ATP production was independent of the oxygen concentration implying that metabolic reduction was not a prerequisite for this effect. Other workers have also found an enhancement or inhibition of damage which is not necessarily associated with changes in macromolecule and ATP synthesis, but may be involved with inhibition of repair (Moroson and Furlan 1971; Palcic and Skarsgard 1972; Moss and Dalrymple 1970). The decrease in ATP as a result of nitrofurazone is evident after 15 minutes of incubation, but incorporation experiments indicated that macromolecule synthesis was also affected soon after drug exposure. Further studies on the effects of nitrofurans at early times may be able to establish whether the decrease in ATP preceded effects on DNA, RNA and protein synthesis.

Inhibition of the response of lymphocytes to PHA could indicate cell death, alteration of a critical target, inhibition of DNA synthesis, or a change in membrane receptors for PHA. Recent experiments (Nir, personal communication) indicate that nitrofurans (AF-2, FANFT and nitrofurazone) are almost as effective in inhibiting stimulation

of lymphocytes before and after addition of PHA suggesting that either membrane receptors for PHA were not altered by nitrofurans, or that nitrofurans exert their effects at several levels.

Other results suggest that membranes may play an important role in mammalian cell damage by nitrofurans (Table 13). Maintaining cells under hypoxia has been shown to alter membrane permeability (Pentilla and Trump 1974) suggesting that toxic intracellular concentrations of nitrofurans might be achieved only under hypoxic conditions. Membrane damage by radiation could explain a part of the enhanced post-irradiation damage by nitrofurans in air. While no consistent pattern developed, it was also noted that recently trypsinized cells, stripped of their surface glycoproteins, often appeared somewhat more sensitive to nitrofuran treatment than non-trypsinized cells.

Toxicity (cell survival) and single-strand breaks after nitrofuran treatment were dependent on the oxygen concentration. Thus the relative value of each can be compared for the various oxygen concentrations (Figure 51). By inference, the rate of reduction is also proportional to toxicity of nitrofurans. This was true for data obtained when cells were incubated in PSG, however, when cells were incubated in complete medium, there was no toxicity in air. Also, nitrofurantoin appeared less toxic than FANFT or

nitrofurazone, and yet produced more single-strand breaks. These observations may reflect the error in comparing data with several inherent variables. Nitrofurantoin damage may be more readily repaired than FANFT or nitrofurazone damage and hence less toxic. The nature of the lesion and not the extent of damage may be the determining factor in this case. Since alkaline sucrose gradient analysis gives no indication of the type of DNA damage, or the nature of repair, the inability to find correlations between breaks and toxicity with different drugs is not surprising. Similarly, production of breaks in complete medium may require nitrogen because toxicity and interaction with DNA have been reduced by drug binding to serum components; lengthy incubation in air might have more toxic effects.

TABLE 13

FACTORS INFLUENCING EXTENT OF DNA DAMAGE BY NITROFURANS

VARIABLE	NITROFURAN REDUCTION	MEMBRANE PERMEABILITY	DECREASE IN ATP	CELL DEATH	OTHER EFFECTS
TEMPERATURE	X	X	X		
PH		X			NITROFURAN DEGRADATION
GLUCOSE	X	X	X	X	
TRYPSIN		X			
MEDIUM	X	X	X	X	
SERUM		X		X	NITROFURAN BINDING
NITROGEN	X	X	X	X	
PPLO	X		?	?	
CO ₂		X			
CELL LINE	X			X	
DIVALENT CATIONS		X			LIGASE REQUIREMENT
POSITION IN CELL CYCLE	<u>?</u>	X		X	
ISOTOPE INCORPORATION		?		X	RADIATION-INDUCED BINDING

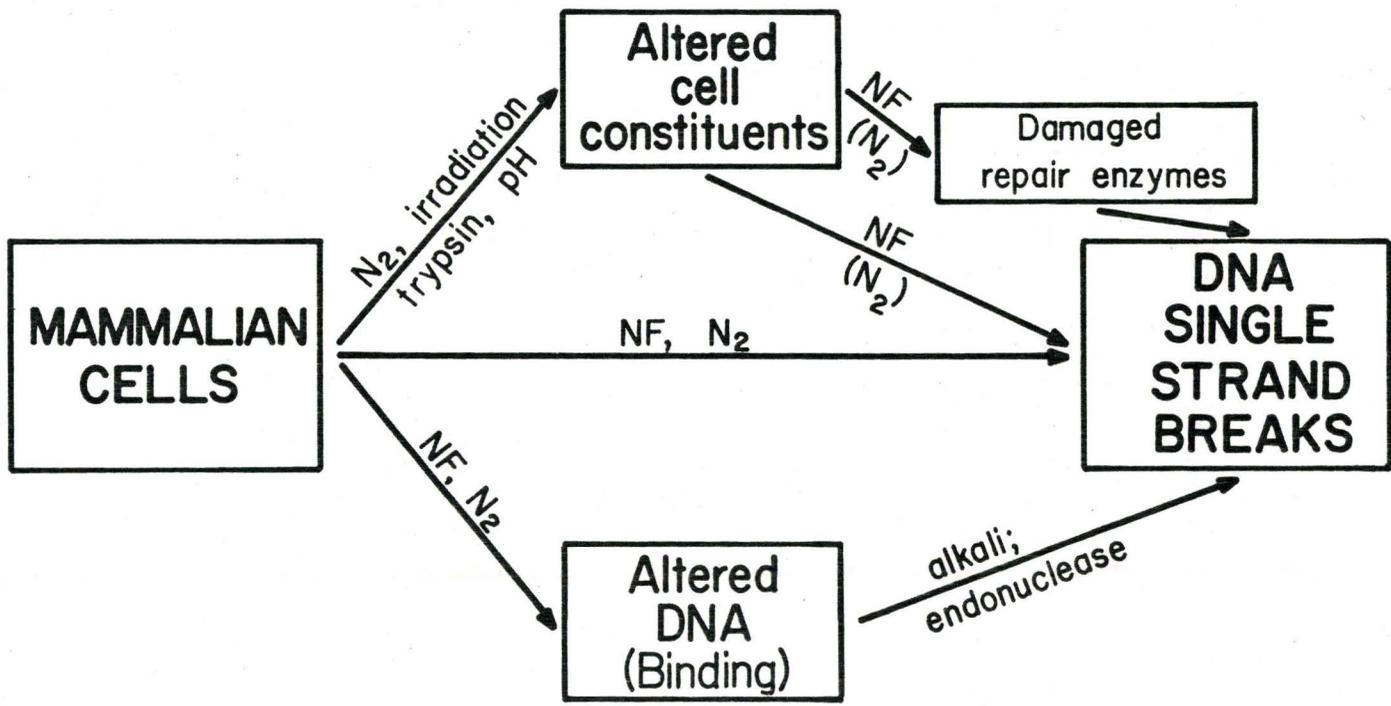
4.3 DNA Breaks: Direct or Indirect Damage?

The biological relevance of DNA single-strand breaks in mammalian cells is not known. Equally uncertain is their cause: DNA breaks may be a result of a direct or indirect effect by the nitrofurans on DNA, inhibition of repair of damage, or the first step in excision repair (Figure 53). Repair of nitrofuran damage does occur when cells are allowed a post-incubation period in oxygen-equilibrated drug-free medium. The oxygen concentration has been shown to have little effect on rejoining of radiation induced single-strand breaks (Koch 1974). The experiments reported here with ascites cells in situ also suggest that repair occurs when at least some of the cells are anoxic. Thus the mechanisms involved can be related directly to the action of nitrofurans, rather than the hypoxic conditions maintained during treatment.

The indication that cell cycle position influenced the extent of damage by nifuroxime and nitrofurantoin (Figures 30 to 34) suggests that changes during the cycle affect nitrofuran reduction, target susceptibility, or repair. During G₁, cell permeability to small molecules increases, and synthesis of some proteins occurs. G₁ appears to be the time when some carcinogens are bound more readily and when UV irradiation produces more dimers (Watanabe and Horikawa 1975). It is also interesting to

Figure 53: Interpretation of Single-Strand Break
Production by Nitrofurans

Mammalian cells may be damaged by nitrofurans by more than one mechanism. Single-strand breaks may result by direct interaction of activated nitrofurans with DNA. However, damage by nitrofurans may be indirect, requiring recognition by endonuclease or the action of alkali before breaks are produced. Also, incubation conditions before nitrofuran treatment may alter essential enzymes so that subsequent incubation results in nitrofuran damage that may not require 'activated' intermediate.



note that nitrofurazone was found to be slightly more effective in sensitizing G1 than S phase cells to radiation damage (Chapman et al 1972).

Rejoining of breaks appeared to be complete four hours after nifuroxime exposure, yet only partial repair had occurred 16 hours after treatment with nitrofurantoin (Figure 27). The extent of damage may limit the amount of repair, whereas the rate of repair may remain constant. Repair experiments included all cells that were plated after treatment, not just those attached to the dish. Thus, dying cells were included in the analysis and their degraded DNA would have contributed to the calculation of molecular weight. It should also be remembered that the measurement of DNA breaks on alkaline sucrose gradients is an instantaneous determination of the number of breaks present. An increase in the rate of their production would thus suggest more damage even when the total number of breaks remained constant.

The finding that non-dividing cells (in a G1-like state) are less sensitive to the toxic effects of nitrofurans than are exponentially growing cells (Figure 10) was somewhat surprising; cells in the process of rapid division are usually less sensitive to the action of nitrofurans (Paul et al. 1964). Although nitrofurans arrest spermatogenesis, they do so at the developmental stage just before

certain cell divisions take place. However, repair of damage may be an important consideration as more DNA breaks were measured in plateau phase cells (Figure 30). Exponentially growing cells would have less time to complete repair before division so that damage becomes 'fixed', whereas arrested cells may be able to repair damage before proliferation occurs after plating. Thus the presence of more breaks measured at any one time may represent a balance between endonuclease 'cutting' and subsequent 'patching', rather than unrepaired damage.

Nitrofurans have been shown to be reduced and to produce single-strand breaks detectable in neutral gradients using supercoiled DNA from minicells of the strain E. coli X1256. By layering these minicells containing supercoiled DNA on either neutral or alkaline sucrose gradients, the percent of damage attributed to alkali was determined (Tu and McCalla 1975). Nitrofurans caused breaks attributable to alkali as well as to the direct action of nitrofurans, but apparently direct breaks were readily repaired while repair of alkali-labile damage was not. Treatment of supercoiled DNA from drug-treated minicells with Micrococcal endonuclease also caused breaks suggesting that the first step in excision repair may be partially responsible for the damage seen on neutral sucrose gradients. Purified nitrofuran reductase, free from endonuclease contamination, is required for in vitro

experiments to determine conclusively if breaks are due to direct damage or a result of repair.

The finding that Xeroderma Pigmentosum cells do not exhibit unscheduled DNA synthesis after treatment with nitrofurans (Yamamoto et al. 1974) agrees with the indication that some of the breaks are the result of endonuclease action. However, breaks were produced in XP cells after incubation with nitrofurans (Figure 28) suggesting that these breaks may be the result of incubation of damaged DNA in alkali. Supporting this idea, extensive incubation of control and nitrofuran-treated cells in alkali caused a relatively greater reduction in molecular weight of nitrofuran-damaged DNA suggesting that some nitrofuran lesions are alkali-labile (Figure 19). However, the XP cell line used (XP-2) also exhibited 9 to 25% of the repair capabilities of normal cells which may be adequate for partial if not complete excision of damaged areas. Cleaver (1974) recently reported measuring production of breaks with XP cells irradiated with UV and analyzed on alkaline sucrose gradients, suggesting that poor resolution using conventional gradient techniques prevented their earlier detection. If lesions are the result of incubation of DNA in alkali, then at various times after treatment, the number of breaks should remain constant, indicating the continued presence of nitrofuran-induced damage.

The carcinogenicity of a nitrofurantoin did not appear to be related to the extent of DNA damage as measured on alkaline sucrose gradients. Nitrofurantoin has not been shown to be carcinogenic, yet it actively produced breaks in DNA. However, FANFT, a highly carcinogenic nitrofurantoin, was the only compound tested that caused a measurable decrease in the molecular weight of DNA at a concentration of 10 $\mu\text{g/ml}$ (Figure 20). As indicated in Figure 52, single-strand breaks may be due to a combination of events, the proportion of which is dependent upon the particular nitrofurantoin. Another problem in interpretation was the inability to obtain consistent quantitative results from one experiment to the next. Thus, some speculation remains as to the exact nature of the DNA breaks resulting from nitrofurantoin treatment.

4.4 DNA Turnover: Replacement or Repair?

DNA was lost from mouse liver labelled with ^3H -TdR after partial hepatectomy when nitrofurans were included in the diet. The loss was considerable (71%) after four weeks feeding of 0.1% nitrofurazone, but no significant loss occurred after feeding of 0.05% FANFT, perhaps due to the low solubility of this nitrofuran, or to its specificity for bladder tissue. Some decrease in growth rate occurred in mice fed nitrofurazone but no effect was seen after feeding of FANFT. Liver enlargement occurred to a slight extent after nitrofurazone treatment only (Table 6). Other studies have found that rats failed to gain weight while on a diet containing 2% nitrofurazone and after four weeks, enlarged liver cells appeared (Miyaji et al. 1971), while Dodd (1946) observed no histologic damage to tissues of mice fed 5 mg nitrofurazone/day for 6 days. Although some cell replacement probably occurs after nitrofurazone feeding, extensive repopulation would not be likely to occur without visual histological damage.

Loss of DNA after nitrofuran feeding also occurred in other mouse tissues. Significant loss of radioactivity was measured after 25 and 50 days in liver, lung and brain of animals fed diets containing 0.1% nitrofurazone. Measurement of radioactivity in the urine of nitrofuran-fed

mice indicated that much of the loss of ^3H could not be accounted for by excretion (10^3 to 10^4 cpm/day in urine, loss of 10^7 cpm/mouse in turnover) suggesting that there must have been substantial reutilization. Kidney and spleen are tissues which would be expected to reutilize ^3H -TdR originating from DNA breakdown. Areas of high grain counts were associated with blood vessels, proximal convoluted tubules in kidneys, and white and red pulp in spleen.

If no cell death occurred in the tissues of treated animals, then loss of radioactivity might result from an excision-repair system, assuming DNA turnover is minimal in normal tissues. Liver is an organ in which damage might be easily detectable since it is responsible for most drug detoxification and contains the highest density of microsomal enzymes involved in nitrofurans 'activation'. The target organ for the carcinogenic activity of nitrofurazone is the mammary gland in rats but insufficient quantities of this tissue could be obtained for assay of DNA turnover. Nitrofurazone is known to cross the blood-brain barrier (Buzard 1962) so that turnover might be expected to occur there as well. In rats, nitrofurans are metabolized in order of activity by liver and kidney, small intestine, spleen and lung, stomach and skin, and finally muscle and brain (Westerfield and Richart 1949; Paul et al. 1960). An examination of the

maximum loss of ^3H -TdR reveals that liver is most active and brain least responsive to damage by nitrofurazone.

A mouse ingesting 5 gms food a day is also eating 5 mg nitrofurazone, corresponding to about 1 mM if stable and equally distributed. However, reduction, metabolism and elimination would lower this figure dramatically so that plasma levels would be closer to 100 μM (see Appendix 4). Focal necrosis in the liver and coagulated albumatous material in kidney tubules were found after feeding rats 0.4% nitrofurazone in the diet (Krantz and Evans 1962). Nitrofurazone-feeding of monkeys caused swelling of liver cells with no focal concentration. Obviously, the concentration of nitrofuran determines the amount of damage, and in chronic feeding to animals, may be toxic without a requirement for 'activation' of the drug.

Autoradiography could be the strongest tool for such studies since it indicates changes in the distribution of radioactivity as well as loss of total counts. Appendix 3 indicates the rationale behind scoring of sections and the ability of this technique to distinguish between loss of radioactivity due to repair or due to cell death and replacement. It might be possible that the rapid initial loss is due to turnover in areas close to blood vessels. However, at longer times on the diets, reutilization would also occur preferentially in these areas. Thus complications of reutilization prevent

meaningful measurements at later times unless areas which would be favored for accumulation of recycled thymidine (i.e., blood vessels) are avoided in scoring. This requires a three-dimensional knowledge of tissue which can only be achieved using serial sections and painstaking grain counts. Data would also be more easily interpreted if turnover were followed as a function of time and special attention paid to earlier times using autoradiography.

Damage might occur in control animals simply because of the relatively high level of radioactivity in DNA. The tissue level (500 dpm/mg tissue) would be equivalent to about 0.5 rad/day/cell assuming equal distribution. However, when the more realistic assumption of the label being confined to the DNA is made, the dose is in excess of 50 rad/day to DNA.

The DNA turnover technique has been used with some success by Goodman and Potter (1972), Sneider et al. (1970) and Yager and Potter (1975), however, the crucial question of whether cell death or DNA repair or both are responsible for the observed effects has not been answered. It seems reasonable to assume that if cell death occurs, it is preceded by an attempt to repair damage; the rapid initial decrease in radioactivity may be explained by DNA as well as cell turnover.

5. CONCLUDING REMARKS

5.1 Summary

Although a number of nitrofurans are carcinogenic, many are also beneficial against a wide variety of infectious diseases. It is important to weigh these benefits against the harmful effects which requires a thorough understanding of the interactions of nitrofurans with mammalian cells. The work reported in this thesis has contributed to our understanding of the mode of action of nitrofurans on mammalian cells, both in animals and grown as established cell lines in culture. Several new findings have emerged:

- 1) Nitrofurans are reduced by enzymes present in mammalian cells grown in culture. Nitrofuran reduction or 'activation' is dependent on the intracellular oxygen concentration; maximum reduction occurs under nitrogen, and the presence of 2% oxygen allows about 50% reduction by mouse liver homogenates.
- 2) Nitrofurans kill mammalian cells in culture, when loss of reproductive ability is the criterion for cell death. Toxicity is greatly increased under anaerobic conditions, in the absence of serum or proteins, and the presence of glucose. Metabolic activation of nitrofurans is implicated

in their toxicity.

- 3) Nitrofurans inhibit DNA and ATP synthesis in cultured mammalian cells, incubated both under air and nitrogen. Interference with RNA and protein synthesis also occurs when nitrofurans are incubated with cultured mammalian cells. Nitrofurans inhibit the response of lymphocytes to stimulation by PHA.
- 4) Nitrofurans cause single-strand breaks in DNA of ascites tumour cells or mammalian cells grown in culture, as measured by a decrease in molecular weight of DNA sedimented on alkaline sucrose gradients.
- 5) DNA strand breakage by nitrofurans requires low oxygen concentrations during incubation. The presence of 2% oxygen results in about half the number of DNA strand breaks as observed with pure nitrogen, correlating with reduction and thereby implicating metabolic intermediates in the toxic effects of nitrofurans.
- 6) The amount of strand breakage is dependent on nitro-furan concentration, time of exposure, pH of incubation solution and position of the cell in the growth cycle.
- 7) Repair of DNA strand breaks resulting from nitrofuran treatment occurs both in vitro and in vivo. The time required for rejoining of most of the breaks is dependent upon the amount of initial damage.
- 8) UV or gamma irradiated cells are damaged more by nitro-furan treatment after irradiation than unirradiated cells,

as measured by cell death, DNA strand breakage, incorporation of ^3H -TdR as a measure of UDS, and inhibition of the repair of potentially lethal damage.

9) Mice whose tissue DNA had been prelabelled with ^3H -TdR during growth lost a significant amount of radioactivity when fed a diet containing 0.1% nitrofurazone for several weeks. DNA turnover might be associated with cell death and replacement or DNA repair.

5.2 Future Work

Nitrofurans have been shown to interact with mammalian DNA in situ. Further studies may uncover important interactions of nitrofurans with other macromolecules or cellular constituents. Membrane integrity may influence damage to DNA by nitrofurans, and the importance of nitrofurans effects on intracellular ATP concentration cannot be overlooked. Nitrofuran derivatives differing slightly in chemical structure have proved useful in determining the requirements for mutagenesis and carcinogenesis. Further analyses may indicate which biochemical changes are associated with carcinogenic properties. Analyses of DNA turnover in different tissues as a result of nitrofuran feeding may prove useful in the recognition of target organs and the biochemical or molecular requirements for tissue damage by nitrofurans.

In vitro experiments are required to determine whether DNA breaks are due to the action of an endonuclease or alkali, or represent direct breakage of DNA by nitrofurans. Slight DNA damage measured in air may be the result of intermediates of nitrofurane reduction which are rapidly reoxidized in air, but may accumulate over a sufficient period to cause cell death. Analysis of mammalian chromatin treated with mammalian nitrofurane reductase(s) in vitro should be undertaken.

As another link between nitrofurane damage and mammalian cells, mammalian cells could be treated with nitrofurans and the mutation frequency scored (Kakunaga 1973; Dipaulo, Nelson and Donovan 1971). Ultimately the DNA lesions responsible for nitrofurane mutagenesis could be determined, probably by characterizing bacterial mutant cell lines resistant to nitrofurans.

As with other carcinogens, nitrofurans represent a potential tool for studying DNA. However, until such time as the major molecular interactions are elucidated, the sequence of events beginning with potentially damaging lesions and ending as neoplasms must be studied at the cellular level with the aim of defining conditions for damage and maintaining relevance to the in vivo effects. Studies with mammalian cells thus are crucial to the rational clinical use of nitrofurans.

10. BIBLIOGRAPHY

- ADAMS, G.E. and COOKE, M.S. Electron-affinic sensitization. I. A structural basis for chemical radiosensitization. *Int. J. Radiat. Biol.* 15: 457-463, 1969.
- ADAMS, G.E. Molecular mechanisms of cellular radiosensitization and protection. In *Radiation Protection and Sensitization*, edited by H. Moroson, and M.L. Quintiliani. London: Taylor and Francis, 1970.
- ADDANKI, A.S., SOTOS, J.F. AND REARICK, P.D. Rapid determination of picomole quantities of ATP with a scintillation counter. *Anal. Biochem.* 14: 261-266, 1966.
- AGNEW, D.A. and SKARSGARD, L.D. Chemical radiosensitization of anoxic mammalian cells: effects of cell concentration. *Radiat. Res.* 57: 246-259, 1974.
- AKAO, M., KURODA, K., KANESAWA, M. and MIYAKI, K. Influence of some nitrofurans on carcinogenesis in rats fed DAB. *Gann* 62: 479-484, 1971.
- AKAO, M., KURODA, K. and MIYAKI, K. Selective inhibition of RNA polymerase activity in rat liver nuclei by DAB and effect of nitrofurans on liver RNA metabolism. *Gann* 63: 1-10, 1972.
- AKAO, M., KURODA, K., TSUTSU, Y., KANISAWA, M. and MIYAKI, K. Effect of nitrofurans antagonistic to 3'-methyl-

- 4-dimethyl aminoazobenzene in hepatocarcinogenesis and RNA polymerase activity in liver cell nuclei in rats. *Cancer Res.* 34: 1844-1850, 1974.
- ALEXANDER, P., DEAN, C.J., LEHMANN, A.R., ORMEROD, M.G., FELDSCHREIBER, P. and SERIANI, R.W. The repair of DNA and the mode of action of sensitizers and protectors in biological systems of different complexities. In Radiation protection and sensitization, edited by H.L. Moroson and M. Quintiliani. London: Taylor and Francis, 1970.
- ANDERS, M.W. and MANNERING, G.J. Kinetics of the inhibition of the N-demethylation of ethylmorphine by SKF-525A and related compounds. *Mol. Pharmac.* 2: 319-327, 1966.
- ANDERSON, E.H. Growth requirements of virus-resistant mutants of E. coli strain B. *P.N.A.S.* 32: 120-128, 1946.
- ANDERSON, L. and McCLURE, W. An improved scintillation cocktail of high solubilizing power. *Anal. Biochem.* 51: 173-179, 1973.
- ANDOH, T. and IDE, T. Strand scission and rejoining of DNA in cultured mammalian cells induced by 4-nitroquinoline-1-oxide. *Cancer Res.* 32: 1230-1235, 1972.
- APOSHIAN, H.V. Enzymic synthesis of DNA IX. The polymerase formed after T2 bacteriophage infection of E. coli: a new enzyme. *J. Biol. Chem.* 237: 519-525, 1962.

- ASNIS, R.E., COHEN, B. and GOTS, J.S. Studies on bacterial resistance to Furacin. *Antibio. Chem.* 2: 123-130, 1952.
- ASNIS, R.E. The reduction of Furacin by cell-free extracts of Furacin-resistant and parent-susceptible strains of *E. coli*. *Arch. Biochem. Biophys.* 66: 208-216, 1957.
- ASNIS, R.E., GLICK, M.C. and FRITZ, M. Effect of Furacin on the dissimilation of pyruvate and formate by cell-free extracts of bacteria. *J. Biol. Chem.* 227: 863-869, 1957.
- BARRENCO, S.C., NOVAK, J.K. and HUMPHREY, P.M. Studies on recovery from chemically induced damage in mammalian cells. *Cancer Res.* 35: 1194-1204, 1975.
- BECKETT, A.H. and ROBINSON, A.E. The reaction of nitrofurans with bacteria. II. *J. Med. Pharmac. Chem.* 1: 135-164, 1959.
- BENDER, R.C. and PAUL, H.E. Metabolism of the nitrofurans. II. Incubation of Furacin with mammalian tissues. *J. Biol. Chem.* 191: 217-222, 1952.
- BELLI, J.A., COOPER, S. and BROWN, J.A. Sedimentation properties of mammalian cell DNA. *Int. J. Radiat. Biol.* 21: 603-606, 1972.
- BIAGLOW, J.E., NYGAARD, O.F. and GREENSTOCK, C.L. Electron transfer in Ehrlich ascites tumor cells in the presence of nitrofurans. *Biochem. Pharmac.* (in press).

- BONNEY, R.J., BECKER, J.E., WALDER, P.R. And POTTER, V.R.
Primary monolayer cultures of adult rat liver paren-
chymal cells suitable for study of the regulation
of enzyme synthesis. *In Vitro* 9: 399-413, 1974.
- BRANT, N., FLAMM, W. and BERNHEIM, N. The value of hydrox-
yurea in assessing repair synthesis of DNA in Hela
cells. *Chem-Biol. Inter.* 10: 327-339, 1972.
- BRODIE, B.B. Pathways of drug metabolism. *J. Pharm.
Pharmac.* 8: 1-17, 1956.
- BROWN, D. and PHILLIPS, J. Mechanism of the mutagenic
action of hydroxylamine. *J. M. B.* 11: 663-671, 1965.
- BUZARD, J.A., VRABLIC, D.M. and PAUL, M.R. Colorimetric
determination of nitrofurazone, nitrofurantoin and
furazolidone in plasma. *Antibio. Chemo.* 6: 702-707,
1956.
- BUZARD, J.A., KOPKO, F. and PAUL, M.F. Inhibition of
glutathione reductase by nitrofurantoin. *J. Lab
and Clin. Med.* 56: 884-890, 1960.
- BUZARD, J.A. Studies on the distribution, metabolism and
mode of action of the nitrofurans. *Giorn. Ital.
Chemo.* 59: 1-12, 1962.
- BUZARD, J.A. and KOPKO, F. The flavin requirement and
some inhibition characteristics of rat tissue gluta-
thione reductase. *J. Biol. Chem.* 238: 464-468, 1963.
- CAPPS, M.J., O'CONNOR, P.J. and CRAIG, A.W. The influence
of liver regeneration on the stability of 7-methyl

- guanine in rat liver DNA after treatment with N,N-dimethylnitrosamine. *Biochem. Biophys. Acta* 331: 33-40, 1973.
- CARRIER, W.L. and SETLOW, R.B. The excision of Pyrimidine dimers. *Methods Enzymol.* 21: 230-237, 1971.
- CERIOTTI, G. Determination of nucleic acids in animal tissues. *J. Biol. Chem.* 214: 59-70, 1955.
- CHAPMAN, J.D., WEBB, R.G., and BORSA, J. ATP pool levels in synchronously growing Chinese hamster cells. *J. Biol. Cell* 49: 229-233, 1971.
- CHAPMAN, J.D., REUVERS, A.P., BORSA, J., PETKAU, A. and McCALLA, D.R. Nitrofurans as radiosensitizers of hypoxic mammalian cells. *Can. Res.* 32: 2616-2632, 1972.
- CHAPMAN, J.D., GREENSTOCK, C.L., REUVERS, A.P., McDONALD E. and SUNLOP, I. Radiation chemical studies with nitrofurazone as related to its mechanism of radiosensitization. *Radiat. Res.* 53: 190-203, 1973.
- CHAPMAN, J.D., REUVER, A.P. and BORSA, J. Effectiveness of nitrofurane derivatives in sensitizing hypoxic mammalian cells to X-rays. *Brit. J. Radiol.* 46: 623-630, 1973.
- CHAPMAN, J.D., REUVERS, A.P. and BORSA, J. Nitroheterocyclic drugs as selective radiosensitizers of hypoxic mammalian cells. *Cancer Chemo.* 58: 559-570, 1974.

- CHAPMAN, J.D., GILLESPIE, C.J., REUVERS, A.P. and DUGLE, D.L. The inactivation of Chinese hamster cells by X-rays: The effects of chemical modification on single and double events. *Radiat. Res.* (in press)
- CHIA, D. and SCHUMAKER, V.N. A rotor speed dependent crossover in sedimentation velocities of DNA of different sizes. *B.B.R.C.* 56: 241-245, 1974.
- CHU, B. and LAWLEY P. Increased urinary excretion of nucleic acid and nicotinamide derivatives by rats after treatment with alkylating agents. *Chem-Biol. Inter.* 10:333-338, 1975.
- CLEAVER, J.E. Defective repair replication in Xeroderma Pigmentosum. *Nature* 218: 652-656, 1968.
- CLEAVER, J.E. Repair replication of mammalian cell DNA: effects of compounds that inhibit DNA synthesis or dark repair. *Radiat. Res.* 37: 334-348, 1969.
- CLEAVER, J.E. Sedimentation of DNA from human fibroblasts irradiated with UV: possible detection of excision breaks in normal and repair deficient Xeroderma Pigmentosum Cells. *Radiat. Res.* 57: 207-227, 1974.
- COHEN, S.M., ERTURK, E. and BRYAN, G. Carcinogenicity of formic acid 2-(4-(5-nitro-2-furyl)-2-thiazolyl)Hydrazide in Swiss mice. *Can. Res.* 30: 2320-2325, 1970.
- COHEN, S.M., LOWER, G.M., ERTURK, E. and BRYAN, G.T. Comparative carcinogenicity in Swiss Mice of N-(4-(5-nitro-2-furyl)-2-thiazolyl)acetamide and structur-

- ally related 5-nitrofurans and nitrobenzenes.
Cancer Res. 33: 1593-1597, 1973.
- CLIFTON, K.H. and COOPER, J. Reutilization of thymidine and iododeoxyuridine by mouse mammary carcinoma strain MTG-B'. Proc. Soc. Exp. Biol. Med. 142: 1145-1151, 1973.
- COHEN, S.M., ALTER, A. and BRYAN G.T. Distribution of radioactivity and metabolism of formic acid 2-(4-(5-nitro-2-furyl)-2-¹⁴C-2-thiazolyl)hydrazide following oral administration to rats and mice. Cancer Res. 33: 2802-2809, 1973.
- COHEN, S.M., ERTURK, E., VON ESCH, A., CROVETTI, A. and BRYAN, G.T. Carcinogenicity of 5 nitrofurans, 5 nitroimidazoles, 4-nitrobenzenes and related compounds. Cancer Res. 33: 1593-1597, 1973.
- CONE, R. and ANDERSON, B. Inhibition of lymphocyte blast formation by streptolysin O. Fed. Biol. Soc. Abst. 31313, 1974.
- CONKLIN, J.D. Biopharmaceutics of nitrofurantoin. Pharmacol. 8: 178-181, 1972.
- CORY, P.M. and COLE, A. Radiation induced double-strand scission of the DNA of mammalian metaphase chromosomes. Radiat. Res. 36: 528-535, 1968.
- COX, R., DAMJANOV, I., ABANOBI, S.E. and SARMA, D.S. A method for measuring DNA damage and repair in the liver in vivo. Can Res. 33: 2114-2121, 1973.

- CRADDOCK, V.M., MAGEE, P.N. The conservation of isotopically labelled formate and orotate after administration to neonatal animals. *Biochem. Biophys. Acta* 134: 182-184, 1967.
- CRATHORN, A.R. and ROBERTS, J.J. Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for removal of alkylated groups from DNA. *Nature* 211: 150-153, 1966.
- DALRYMPLE, G.V., SANDERS, J.J., MOSS, A.J., and WILKINSON K.P. Radiation induced breaks increase the priming activity of rat sarcoma DNA in the DNA polymerase reaction. *Biochem. Biophys. Res. Commun.* 39: 538-542, 1970.
- DALRYMPLE, G.V., MOSS, A.J., BAKER, M., NASH, J., SANDERS J. and WILKINSON, K. The repair of sublethal radiation injury and the rejoining of radiation-induced DNA breaks by cultured mammalian cells. In *Molecular and cellular repair processes*, edited by Beers, Herriot and Tilghman. Baltimore: Johns Hopkins University Press, 1972.
- DEL MONTE, U. Consideration of factors influencing the oxygenation of ascites tumors. *Eur. J. Cancer* 5: 639-640, 1969.
- DE SERRES, F.J. AF-2: Food preservative or genetic hazard. *Mutat. Res.* 26: 1-2, 1974.
- DODD, M.E. The chemotherapeutic properties of 5-nitro-2-

- furaldehyde semicarbazone (Furacin). *J. Pharmacol. Exp. Ther.* 86: 311-323, 1946.
- DIPAOLLO, J.A., NELSON, R.L. and DONOVAN, J. Morphological, oncogenic and karyological characteristics of Syrian hamster cells transformed in vitro by carcinogenic polycyclic hydrocarbons. *Cancer Res.* 31: 1118-1127, 1971.
- DODD, M.E. and STILLMAN, W.B. The in vitro bacteriostatic action of some simple furan derivatives. *J. Pharmacol. Exp. Ther.* 82: 11-18, 1944.
- DUGLE, D.L., CHAPMAN, J.D., GILLESPIE, C.J., BORSA, J., WEBB, R.G., MEEKER, B.E. and REUVERS, A.P. Radiation-induced strand breakage in mammalian cell DNA. I. Enhancement of single-strand breaks by chemical radiosensitizers. *Int. J. Radiat. Biol.* 22: 545-555, 1972.
- EDWARDS, D.I., DYE, M. and CARNE, H. The selective toxicity of antimicrobial nitroheterocyclic drugs. *J. Gen. Microbiol.* 76: 135-145, 1973.
- EHMANN, U.K. and LETT, J.T. Review and evaluation of molecular weight calculations from the sedimentation profiles of irradiated DNA. *Radiat. Res.* 54: 152-162, 1973.
- ELKIND, M.M. and KAMPER, C. Two forms of Repair of DNA in mammalian cells following irradiation. *Biophys. J.* 10: 237-245, 1970.

- ELVES, M.W. (ed) The Lymphocytes. 2nd edition, Chicago: Year Book Medical Publishers, 1972, p 393-410.
- ENDO, H., ISHIZAWA, M., KAMIYA, T. and KUWANO M. A nitrofurantoin derivative, a new inducing agent for phage development in lysogenic E. coli. Biochem. Biophys. Acta 68: 502-505, 1963.
- ERTURK, E., COHEN, W.M., PRICE, J.M. and BRYAN G.T. Pathogenesis, histology and transplantability of urinary bladder carcinomas induced in albino rats by oral administration of FANFT. Cancer Res. 29: 2219-2228, 1969.
- ERTURK, E., COHEN, W.M. and BRYAN, G.T. Urinary bladder carcinogenicity of N-4-(5-nitro-2-furyl)-2-thiazolyl)formamide in female Swiss mice. Cancer Res. 30: 1309-1311, 1970a.
- ERTURK, E., MORRIS, J.E., COHEN, W.M., PRICE, J.M. and BRYAN, G.T. Transplantable rat mammary tumors induced by 5-nitro-2-furaldehyde semicarbazone and by formic acid 2-(4-(5-nitro-2-furyl)-2-thiazolyl)hydrazide. Cancer Res. 30: 1409-1412, 1970b.
- ERTURK, E., MORRIS, J.E., COHEN, S.M., VONESCH, A.M. CROVETTI, A.J., PRICE, J.M. and BRYAN, G.T. Comparative carcinogenicity of formic acid 2-(4-(5-nitro-2-furyl)-2-thiazolyl)-hydrazide and related chemicals in the rat. J. National Cancer Inst. 47: 437-445, 1971.

- EVANS, R. and NORMAN, A. Radiation stimulated incorporation of thymidine into the DNA of human lymphocytes. *Nature* 217: 455-456, 1968.
- FARRANT, J. Pharmacologic actions and toxicity of DMSO. *J. Pharmac. Pharmacol.* 16: 472-483, 1964.
- FELLER, D.R., MORITA, M. and GILLETTE, J.R. Enzymatic reduction of niridazole by rat liver microsomes. *Biochem. Pharmac.* 20: 203-215, 1971.
- FOX, B.W. and FOX, M. DNA single strand rejoining in two pairs of cell lines showing the same and different sensitivities to X-rays. *Intern. J. Radiat. Biol.* 24: 127-135, 1973.
- FOX, M. and AYAD, S.R. Characteristics of repair synthesis in P388 cells treated with methyl methanesulphonate. *Chem.-Biol. Inter.* 3: 193-211, 1971.
- FOX, M., GILBERT, C.W., LAJTHA, L. and NIAS, A. The interpretation of split dose experiments in mammalian cells after treatment with alkylating agents. *Chem.-Biol. Inter.* 1: 241-246, 1970.
- FRESCO, J.R. and BENDICH, A. The metabolic stability of rat liver deoxyribonucleic acid; a turnover study. *J. Biol. Chem.* 235: 1124-1128, 1960.
- FRIEDGOOD, C.E. and RIPSTEIN, C.B. The relationship of nitrofurazone to the adrenal cortex and its effect on tumor growth. *Cancer Res.* 11: 248-249, 1951.

- FRIEDGOOD, C.E., DANZA, A.H. and BOCCABELLA, A. The effect of nitrofurans on the normal testis and on testicular tumours. *Cancer Res.* 12: 262-263, 1952.
- FUSKA, J., FUSKOVA, A., JURASEK, A. Effect of 5-nitro-furan derivatives on uptake of labelled nucleic acid and protein in the acid-insoluble portion of Ehrlich ascites. *Neoplasma* 20: 171-179, 1973.
- GAUDIN, D. and YEILDING, K.L. The use of formamide gradients to distinguish between alkali-labile regions and single strand breaks in DNA. *Biochem. Biophys. Res. Commun.* 47: 1396-1401, 1972.
- GILES, K.W. and MYERS, A. An improved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* 206: 93-95, 1965.
- GILLETTE, J.R., KAMM, J.J. and SASAME, H.A. Mechanism of p-nitrobenzoate reduction in liver; the possible role of cytochrome P-450 in liver microsomes. *Mol. Pharmac.* 4: 541-548, 1968.
- GOODMAN, J.I. and POTTER, V.R. Evidence for DNA Repair synthesis and turnover in rat liver following ingestion of 3'-Me-DAB. *Cancer Res.* 32: 766-775, 1972.
- GOODMAN, J.I. Hepatic DNA repair synthesis in rats fed 3'-Me-DAB. *Biochem. Biophys. Res. Commun.* 56: 52-59, 1974.
- GREEN, E.L. (ed.) *Biology of the Laboratory Mouse*. New York: McGraw Hill, 1966.

- GREEN, M.H. DONCH, J. and GREENBERG, J. An immediate effect of inhibition of DNA synthesis on cell division in a strain of E. coli. Mut. Res. 8: 409-411, 1969.
- GREEN, M.N. The effect of furacin on the metabolism of bacteria. Arch. Biochem. 19: 397-406, 1948.
- GRUNBERG, E. and TITSWORTH, E.H. Chemotherapeutic properties of heterocyclic compounds: monocyclic compounds with 5-membered rings. Ann. Rev. Microbiol. 27: 317-346, 1973.
- HAHN, G.M. and LITTLE, J.B. Plateau-phase cultures of mammalian cells. Current Topics in Radiat. Res. 8: 39-83, 1972.
- HARIHARAN, P. and CERUTTI, P. Formation and repair of gamma ray induced thymine damage in M. radiodurans. J. Mol. Biol. 66: 65-81, 1972.
- HARM, W. The role of host-cell repair in liquid-holding recovery of UV-irradiated E. coli. Photochem. Photobiol. 5: 747-760, 1966.
- HIGGINS, M. and ANDERSON, R. Experimental pathology of the liver. Arch. Path. 31: 186-202, 1931.
- HOLLAENDER, A. and CLAUS, W.D. Liquid Holding Recovery. Bull. Nat. Res. Counc. U.S. 100, 78-88, 1937.
- HOWARD-FLANDERS, P. DNA repair and recombination. Brit. Med. Bull 29: 226-235, 1973.
- HRISHI, N.J. A comparison of environmental influences on the resistance of strains B and B/r of E. coli to

- radiation and to Furacin. *Biochem. Biophys. Acta* 74: 490-494, 1963.
- HUMPHREY, R.M., STEWARD, D.L. and SEDITA, B.A. DNA strand breaks and rejoining following exposure of synchronized Chinese hamster cells to ionizing radiation. *Mut. Res.* 6: 459-465, 1968.
- JENNER, S. and NETTER, K.J. On the inhibition of microsomal drug metabolism by SKF-525A. *Biochem. Pharmac.* 21: 1921-1927, 1972.
- KAKUNAGA, T. A quantitative system for assay of malignant transformation by chemical carcinogens using a clone derived from Balb/3T3. *Intern. J. Cancer* 12: 463-473, 1973.
- KAMATH, S.A., KUMMEROW, F.A. and NARAYAN, K. A simple procedure for the isolation of rat liver microsomes. *FEBS Letters* 17: 90-95, 1971.
- KANISAWA, M. KATCH, H. and AISO, K. Carcinogenicity of potassium 1-methyl-7-(2,5)-vinyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate in ICR mice. *Gann* 65: 1-11, 1974.
- KATO, K., SUGINO, Y. and ENDO, H. Effects of a prophage inducer, a nitrofurantoin derivative, on the DNA metabolism of *E. coli*. *Biochem. Biophys. Acta* 119: 309-321. 1966.
- KLEIJER, W.J., HOWKSEMA, J.L., SLUYTER, M.L. and BOOTSMA D. Effects of inhibitors on repair of DNA in

- normal human and Xeroderma Pigmentosum cells after exposure to X-rays and ultraviolet irradiation. Mut. Res. 17: 385-394, 1973.
- KOCH, C. Oxygen dependence of single strand break repair. Proceedings of the 3rd Annual Hypoxic Cell Sensitizer Meeting, Seattle, Washington, 1974.
- KOHN, K.W. and GRIMEK-EWIG, R.A. Alkaline elution analysis, a new approach to the study of DNA single-strand interruptions in cells. Cancer Res. 33: 1849-1853, 1973.
- KRANTZ, J.C. and EVANS, W.E. A contribution to the pharmacology of 5-nitro-2-furaldehyde semicarbazone. J. Pharmac. Exp. Ther. 85: 324-335, 1962.
- KUBINSKI, H. and KASPER, C.B. Carcinogen and microsomal membrane interactions; change in membrane density and ability to bind nucleic acids. Science 171: 201-203, 1971.
- LEE, Y.C., BENNETT, L.R. and BYFIELD, J. Inhibition of repair of DNA single-strand breaks in mouse leukemia cells by Actinomycin D. Biochem. Biophys. Res. Commun. 49: 758-765, 1972.
- LEGATOR, M.S. and FLAMM, W.G. Environmental mutagenesis and repair. Ann. Rev. Biochem. 42: 683-708, 1973.
- LEIBERMAN, M.W., SELL, S. and FARBER, S. Deoxyribonucleotide incorporation and the role of hydroxyurea in a model lymphocyte system for studying DNA repair in

- carcinogenesis. *Cancer Res.* 31: 1307-1312, 1971.
- LEIBERMAN, M.W. and DIPPLE, A. Removal of bound carcinogen during DNA repair in non-dividing human lymphocytes. *Cancer Res.* 32:1855-1860, 1972.
- LETT, J.T., CALDWELL, I., DEAN, C. and ALEXANDER, P. Rejoining of X-ray induced breaks in the DNA of leukaemia cells. *Nature* 214: 790-792, 1967.
- LETT, J.T. and SUN, C. The production of strand breaks in mammalian DNA by X-rays at different stages in the cell cycle. *Radiat. Res.* 44: 771-787, 1970.
- LEWIS, A.J. (ed.) *Modern Drug Encyclopedia*. New York: Dunn-Donnelley Publishing Corp. 1975.
- LITTLE, J. Factors influencing the repair of potentially lethal radiation damage in growth-inhibited human cells. *Radiat. Res.* 56: 320-333, 1973.
- LOEB, L.A., SPRINGGATE, C.F. and BATTULA, N. Errors in DNA replication as a basis for malignant change. *Cancer Res.* 34: 2311-2331, 1974.
- LOHMAN, P.H.M. Induction and rejoining of breaks in the DNA of human cells irradiated at various phases of the cell cycle. *Mut. Res.* 6: 449-458, 1968.
- LOWRY, O.H., ROSEBROUGH, N.F., FAR, A.L. and RANDALL, R.J. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275, 1951.
- MAJUMDAR, C. and FRANKEL, F. Role of ATP in DNA replication. *Nature* 243: 33-36, 1973.

- MARSHALL, M., JOHNSON, H. and PRICE, S. Metastatic seminoma of testes treated by oral nitrofurazone and radiotherapy. *J. Urol.* 91: 392-395, 1964.
- MASON, R.R and HOLTZMAN, J.L. The mechanism of microsomal and mitochondrial nitroreductase. Electron spin resonance evidence for nitroaromatic free radical intermediates. *Biochem.* 14: 1626-1633, 1974.
- McBURNEY, M.W. and WHITMORE, G.F. Molecular weight analysis of mammalian DNA. *Biochem. Biophys. Res. Commun.* 46: 898-904, 1972.
- McCALLA, D.R. Effect of nitrofurans on the chloroplast system of *E. gracilis*. *J. Protozool.* 12: 34-41, 1965.
- McCALLA, D.R. Nitrofuran derivatives as radiomimetic agents: cross resistance studies with *E. coli*. *Can. J. Microbiol.* 11: 185-191, 1965.
- McCALLA, D.R., REUVERS, A. and KAISER, C. Mode of action of nitrofurazone. *J. Bact.* 104: 1126-1134, 1970a.
- McCALLA, D.R. and REUVERS, A. Activation of nitrofuran derivatives on the chloroplast system of *Euglena gracilis*: Effect of light. *J. Protozool.* 17: 129-134, 1970b.
- McCALLA, D.R., REUVERS, A. and KAISER, C. "Activation" of nitrofurazone in animal tissues. *Biochem. Pharmac.* 20: 3532-3537, 1971.

- McCALLA, D.R., REUVERS, A. and KAISER, C. Breakage of bacterial DNA by nitrofurans derivatives. *Cancer Res.* 31: 2184-2188, 1971a.
- McCALLA, D.R. and VOUTSINOS, D. On the mutagenicity of nitrofurans. *Mutat. Res.* 26: 3-16, 1974.
- McCALLA, D.R., OLIVE, P.L., TU, Y. and FAN, M. Nitrofurazone reducing enzymes in *E. coli* and their role in drug activation in vivo. *Can. J. Microbiol.* (in press) 1975.
- McGRATH, R.A. and WILLIAMS, R.W. Reconstruction in vivo of irradiated *E. coli* deoxyribonucleic acid: the rejoining of broken pieces. *Nature* 212: 534-535, 1966.
- MENDELSON, J., SKINNER, A. and KORNFELD, S. The rapid induction by Phytohemagglutinin of increased alpha-aminoisobutyric acid uptake by lymphocytes. *J. Clin. Invest.* 50: 818-826, 1971.
- MENEGHINI, R. Repair replication of opossum lymphocyte DNA: effect of compounds that bind to DNA. *Chem.-Biol. Interact.* 8: 113-126, 1974.
- MILLER, J.A. Carcinogenesis by chemicals. *Cancer Res.* 30: 559-576, 1970.
- MILLER, E.C. and MILLER, J.A. Biochemical mechanisms of chemical carcinogenesis. In *The molecular biology of cancer*. New York: Academic Press, 1974, p 377-402.

- MITCHISON, J.M. The Biology of the Cell Cycle. Cambridge: Cambridge University Press, 1971.
- MIURA, K., IKEDA, M., OHASHI, T., IGARASHI, Y. and ICHIMURA, K. Chemical and chemotherapeutic studies on nitrofurans derivatives. XX. Yakkugaku Zasshi 81: 1372-1374, 1961.
- MIURA, K. and RECKENDORF, H.K. The nitrofurans. J. Med. Chem. 5: 320-381, 1967.
- MIYAKI, K., AKAO, M., TERAOKA, K. and KURODA, K. Modes of action of 3 bacteriostatic agents and their inhibitory effect on induction of hepatoma in rats fed DAB. Gann 60: 167-179, 1969.
- MIYAJI, T. Acute and chronic toxicity of furylfuramide in rats and mice. Tohoku J. Med. 103: 331-369, 1971.
- MIYAJI, T. Effect of furylfuramide on reproduction and malformation. Tohoku J. Med. 103: 381-388, 1971.
- MORITA, M., FELLER, D.R. and GILLETTE, J.R. Reduction of niridazole by rat liver xanthine oxidase. Biochem. Pharmac. 20: 217-226, 1971.
- MORONEY, M.J. Facts from Figures. Baltimore: Penguin Books Inc., 1951.
- MOROSON, H. and FURLAN, M. Single strand DNA breaks in DNA of Ehrlich ascites tumor cells produced by methylhydrazine. Radiat. Res. 40: 351-365, 1969.
- MORRIS, J.E., PRICE, J.M., LALICH, J.J. and STEIN, R.J. The carcinogenic activity of some 5-nitrofurans der-

- ivatives in the rat. *Cancer Res.* 29: 2145-2156, 1969.
- OKADA, S. *Radiation Biochemistry*. Vol. I. Cells, edited by K. Altman, G. Gerber and S. Okada. New York: Academic Press, 1971.
- OLIVE, P.L. *Radiosensitization of the C3HBA Mouse Mammary Tumour*. M.Sc. Thesis, University of Western Ontario, 1972.
- ONG, T. and SHANIN, M.M. Mutagenic and recombinogenic activities of the food additive furylfuramide in eukaryotes. *Science* 184: 1086-1087, 1974.
- ORMEROD, M.G. and STEVENS, U. The rejoining of X-ray induced single strand breaks in the DNA of a murine lymphoma cell L5178Y. *Biochem. Biophys. Acta* 232: 72-82, 1971.
- ORMEROD, M.G. and LEHMANN, A.R. Artifacts arising from the sedimentation of high molecular weight DNA on sucrose gradients. *Biochem. Biophys. Acta* 247: 369-372, 1971.
- PAINTER, R.B. and CLEAVER, J.E. Repair replication, unscheduled DNA synthesis, and the repair of mammalian DNA. *Radiat. Res.* 37: 451-466, 1969.
- PAINTER, R.B. Repair of mammalian DNA damaged by UV. *Photophysiol.* 5: 182-185, 1969.
- PALCIC, B. and SKARSGARD, L.D. DNA single strand breaks produced in mammalian cells by ionizing radiation after treatment with 2,4-dinitrophenol. *Int.J.*

- Radiat. Biol. 21: 535-544, 1972.
- PALCIC, B. and SKARSGARD, L.D. The effect of oxygen on DNA single strand breaks produced by ionizing radiation in mammalian cells. Int. J. Radiat. Biol. 21: 417-433, 1972.
- PARDEE, A.B. The surface membrane as a regulator of animal cell division. In Vitro 7: 95-104, 1971.
- PATTERSON, M.C., LOHMAN, P. and SLUYTER, M.L. Use of a UV endonuclease from M. Luteus to monitor the progress of DNA repair in UV-irradiated human cells. Mut. Res. 19: 245-256, 1973.
- PAUL, M.F., PAUL, H.E., KOPKO, F., BRYSON, M.J. and HARRINGTON, C. Inhibition by Furacin of citrate formation in testis preparations. J. Biol. Chem. 206: 491-497, 1954.
- PAUL, F., BRYSON, M.J. and HARRINGTON, C. Effect of Furacin on pyruvate metabolism. J. Biol. Chem. 219: 463-470, 1956.
- PAUL, H.E., ELLS, V.R., KOPKO, F. and BENDER, R.C. Metabolic degradation of the nitrofurans. J. Med. Pharmac. Chem. 2: 563-583, 1960.
- PAUL, H.E. and PAUL, M.F. The nitrofurans - chemotherapeutic properties. In Experimental Chemotherapy, edited by R.J. Schnitzer and F. Hawking. New York: Academic Press, 1966, p 521-536.

- PAUL, J. Cell and Tissue Culture, 4th ed. Baltimore: Williams and Wilkins, 1970.
- PAUL, M.F., PAUL, H.E., BENDER, R.C., KOPKO, F., HARRINGTON C.M., ELLS, V.R. and BUZARD, J.A. Studies on the distribution and excretion of certain nitrofurans. Antibio. Chemo. 10: 287-302, 1960.
- PENTILA, A. and TRUMP, B. Extracellular acidosis protects Ehrlich ascites tumor cells and rat renal cortex against anoxic injury. Science 185: 277-278, 1974.
- PERPER, R.J., ZEE, T.W. and MICKELSON, M.M. Purification of lymphocytes and platelets by gradient centrifugation. J. Lab. Clin. Med. 72: 842-849, 1968.
- PETERSON, A.R., BERTRAM, J.S. and HEIDELBERGER, C. DNA damage and its repair in transformable mouse fibroblasts treated with MNNG. Cancer Res. 34: 1593-1599, 1974a.
- PETERSON, A.R., BERTRAM, J.S. and HEIDELBERGER, C. Cell cycle dependency of DNA damage and repair in transformable mouse fibroblasts treated with MNNG. Cancer Res. 34: 1600-1607, 1974b.
- PHILLIPS, R.A. and TOLMACH, L.J. Repair of potentially lethal damage in X-irradiated Hela cells. Radiat. Res. 29: 413-432, 1966.
- POGOSIANZ, A. Comparative study of mutagenic and carcinogenic action of certain chemicals. Neoplasma

- 20: 527-530, 1973.
- PUCK, T.T. and MARCUS, P. Action of X-rays on mammalian cells. *J. Exp. Med.* 103: 653-666, 1956.
- RALEIGH, J.A., CHAPMAN, J.D., BORSA, J., KREMERS, W. and REUVERS, A.P. Radiosensitization of mammalian cells by p-nitroacetophenone. III. Effectiveness of nitrobenzene analogues. *Int. J. Radiat. Biol.* 23: 377-387, 1973.
- RAJALAKSHMI, S. and SARMA, D.S. Rapid repair of hepatic DNA damage induced by camptothecin in the intact rat. *Biochem. Biophys. Res. Commun.* 53: 1268-1272, 1973.
- REGAN, J.D., SETLOW, R.B. and LEY, R.D. Normal and defective repair of damaged DNA in human cells: a sensitive assay utilizing the photolysis of bromodeoxyuridine. *Proc. Nat. Acad. Sci.* 68: 703-712, 1971.
- REGAN, J.D. and SETLOW, R.B. Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens. *Cancer Res.* 34: 3318-3325, 1974.
- REUVERS, A.P., CHAPMAN, J.D. and BORSA, J. Potential use of nitrofurans in radiotherapy. *Nature* 237: 402-403, 1972.
- ROBBINS, E. and MORRILL, G.E. Oxygen uptake during the HeLa cell life cycle and its correlation with macromolecule synthesis. *J. Cell Biol.* 43: 629-633, 1969.

- ROBERTS, A.B. and ALDOUS, E. Recovery from ultraviolet irradiation in *E. coli*. *J. Bacteriol.* 57: 363-375, 1949.
- ROBERTS, J.J. Repair of alkylated DNA in mammalian cells. In *Molecular and Cellular Repair Processes*, edited by Beers, Herriott, and Tilghman. Johns Hopkins University Press, 1972, p 226-247.
- ROSSI, E.C., LEVIN, N. and LOUIS, G. Inhibition of primary ADP-induced platelet aggregation in normal subjects after administration of Furadantin. *J. Clin. Invest.* 52: 2457-2467, 1973.
- RUGH, R. *The Mouse: Its Reproduction and Development*. Minneapolis: Burgess Publishing Co, 1968.
- SAFFHILL, R., COOPER, H. and ITZHAZI, R. Detection by DNA polymerase I of breaks produced in rat liver chromatin in vivo by alkylating agents. *Nature* 248: 153-156, 1974.
- SANDER, G. and PARDEE, A.B. Transport changes in synchronously growing CHO and L cells. *J. Cell Physiol.* 80: 267-272, 1972.
- SETLOW, R.B. and REGAN, J.D. Repair in human Progeroid cells. *Ann. Biophys. Soc. Meeting*, ABST. 1871, 1974.
- SHARGEL, L. and MAZEL, P. Influence of DPEA and SKF-525A on hepatic microsomal azoreductase activity from phenobarbitol or methylcholanthrene induced rats. *Biochem. Pharmac.* 21: 69-75, 1972.

- SIMPSON, J.R., NAGLE, W.A., BRICK, M.D. and BELLI, J.A.
Molecular nature of mammalian cell DNA in alkaline
sucrose gradients. Proc. Nat. Acad. Sci. 70:
3360-3364, 1973.
- SINCLAIR, W.K. Hydroxyurea: differential lethal effects
on cultured mammalian cells during the cell cycle.
Cancer Res. 27: 297-308, 1967.
- SINCLAIR, W.K. Hydroxyurea: effects on Chinese hamster
cells grown in culture. Science 150: 1729-1731,
1969.
- SNEIDER, T.W., BUSNELL, D.E. and POTTER, V.R. The
distribution and synthesis of DNA in 2 classes of
rat liver nuclei during azo dye-induced carcino-
genesis. Cancer Res. 30: 1867-1873, 1970.
- SOLIMAN, M., JOHNSON, H.D. and WADE, W.E. The inter-
actions of inducers, inhibitors and substrates of
drug metabolizing enzymes with rat liver. Drug
Metab. Disp. 2: 87-96, 1974.
- SPEIGLER, P.A. and NORMAN, A. Unscheduled DNA synthesis
at low X-ray doses. Mut. Res. 10: 379-387, 1970.
- STEIN, R.J., YOST, D. and PETROLIUMAS, F. Carcinogenic
activity of nitrofurans. A histological evaluation.
Fed. Proc. 25: 291-295, 1966.
- STEWART, B.W. and FARBER, E. Strand breakage in rat
liver DNA and its repair following administration
of cyclic nitrosamines. Cancer Res. 33: 3209-3215,
1973.

- STICH, H.F. and SAN, H.C. DNA repair and chromatid anomalies in mammalian cells exposed to 4-nitroquinoline-1-oxide. *Mut. Res.* 10: 389-404, 1970.
- STICH, H.F., SAN, H.C. and KAWAZOE, Y. DNA repair synthesis in mammalian cells exposed to a series of oncogenic and non-oncogenic derivatives of 4-nitroquinoline-1-oxide. *Nature* 229: 416-419, 1971.
- STICH, H.F., SAN, H.C., MILLER, J.A. and MILLER, E.C. Various levels of DNA repair synthesis in XP cells exposed to the carcinogens N-hydroxy and N-acetoxy-2-acethylaminofluorene. *Nature* 238: 9-10, 1972.
- STICH, H.F. The use of DNA repair in the identification of carcinogens, precarcinogens and target tissue. *Proc. 10th Ann. Canad. Cancer Res. Conf.*, 1973.
- STUDIER, F.W. Sedimentation studies on the size and shape of DNA. *J. Mol. Biol.* 11: 373-390, 1965.
- SUGIYAMA, T., GOTO, K., UENAKA, H. and NISHIO, K. Acute cytogenic effect of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide on rat bone marrow cells *in vivo*. *Mut. Res.* 31: (in press) 1975.
- SUTHERLAND, R.M. and DURAND, R.E. Radiosensitization by nifuroxime of the hypoxic cells in an *in vitro* tumour model. *Int. J. Radiat. Biol.* 22: 613-618, 1972.
- SWENSON, P.A. and SETLOW, R.B. Effects of ultraviolet

- radiation on macromolecule synthesis in E. coli.
J. Mol. Biol. 15: 201-206, 1966,
- SZYBALSKI, W. Special microbiological systems II.
Observations on chemical mutagenesis in micro-organisms. Ann. N.Y. Acad. Sci. 76: 475-489, 1958.
- TATSUMI, K., OU, T., YAMAGUCHI, T. and YOSHIMURA, H.
The metabolic rate of nitrofurans derivatives (2).
Degradation by small intestinal mucosa and absorption from G.I. Tract. Chem. Pharm. Bull. 21: 191-201, 1973.
- TATSUMI, K., YAMAGUCHI, T., and YOSHIMURA, H. The metabolic fate of nitrofurans derivatives (3).
Studies on enzymes in small intestinal mucosa of rat catalyzing degradation of nitrofurans derivatives. Chem. Pharm. Bull. 21: 622-628, 1973.
- TAYLOR, J.D., PAUL, H.E. and PAUL, M.F. Metabolism of the nitrofurans. III. Studies with xanthine oxidase in vitro. J. Biol. Chem. 191: 223-231, 1951.
- TAZIMA, Y. and ONIMURU, K. Results of mutagenicity testing for some nitrofurans derivatives in a sensitive test system with silkworm oocytes. Mut. Res. 26: 440, 1974.
- TAZIMA, Y., KADA, T. and MURAKAMI, A. Mutagenicity of nitrofurans derivatives including furylfuramide, a food preservative. Mut. Res. 32: 35-80, 1975.

- TERAWAKI, A. and GREENBERG, J. Effect of some radio-mimetic agents on DNA synthesis in E. coli and transformation in B. subtilis. Biochem. Biophys. Acta 95: 170-173, 1965.
- THILLY, W.G. and HEIDELBERGER, C. Cytotoxicity and mutagenicity of UV-irradiation as a function of the interval between split doses in cultured Chinese hamster cells. Mut. Res. 17: 287-290, 1973.
- TONOMURA, A. and SASAKI, M.S. Chromosome aberrations and DNA repair synthesis in cultured human cells exposed to nitrofurans. Jap. J. Genet. 48: 291-294, 1973.
- TROSHIN, A.S. Problems of Cell Permeability. London: Pergamon Press, 1966, p 236-237.
- TRUMP, B., CROKER, B. and MERGNER, W. The role of energy metabolism ion and water shifts in the pathogenesis of cell injury. In Cell Membranes, Biological and Pathological Aspects, edited by G. Richter and D. Scarpelli. Baltimore: Williams and Wilkins Co., 1971, p 84-128.
- TU, Y. and McCALLA, D.R. Effect of activated nitrofurans on DNA. Can. J. Microbiol. (in press) 1975.
- UJJIE, T. Experimental anticancer activity of some nitrofurans derivatives. Chem. Pharm. Bull. 14: 461-466, 1966.

- UMAR, M.T. and MITCHARD, M. The competitive inhibition of nitroreductase by some analogues of nitrofurantoin. *Biochem. Pharm.* 17: 2057-2060, 1968.
- VEATCH, W. and OKADA, S. Radiation induced breaks of DNA in cultured mammalian cells. *Biophys. J.* 9: 330-346, 1969.
- VOICULETZ, N., SMITH, K. and KAPLAN, H. Effect of quinocrine on survival and DNA repair in X-irradiated Chinese hamster cells. *Cancer Res.* 34: 1038-1044, 1974.
- WANG, C.Y., BEHRENS, B.C., ICHIKAWA, M. and BRYAN, G.T. Nitroreduction of 5-nitrofuranyl derivatives by rat liver xanthine oxidase and reduced NADPH-cytochrome c reductase. *Biochem. Pharmacol.* 23: 3395-3404, 1974.
- WANG, C.Y., CHIU, C.W. and BRYAN, G.T. Metabolism and disposition of N-(4-(5-nitro-2-furyl)-2-¹⁴C-thiazolyl)acetamide in the rat. *Drug. Met. Disp.* 3: 89-95, 1975.
- WANG, C.Y., CHIU, C.W., KARMAN, B. and BRYAN, G.T. Identification of 2-methyl-4-(5-amino-2-furyl)thiazole as the reduced metabolite of 2-methyl-4-(5-nitro-2-furyl)thiazole. *Biochem. Pharmacol.* 24: 29-31, 1975.
- WARREN, P.M. and STICH, H.F. Reduced DNA repair capacity and increased cytotoxicity following split doses of the mutagen 4-nitroquinoline-1-oxide in cultured

- human cells. *Mut. Res.* 28: 285-293, 1975.
- WATANABE, M. and HORIKAWA, M. Analyses of differential sensitivities of synchronized HeLa S3 cells to radiations and chemical carcinogens during the cell cycle. *Mut. Res.* 28: 295-304, 1975.
- WATERBURY, W.E. and FREEDMAN, R. Induction of Phage formation by nitrofurans. *Can. J. Microbiol.* 10: 932-934, 1964.
- WEISS, J.J. and RICHARDSON, C.C. Enzymic breakage and joining of DNA. I. Repair of single-strand breaks in DNA by an enzyme system of E. coli infected with T4 bacteriophage. *Proc. Nat. Acad. Sci.* 57: 1021-1022, 1967.
- WESTERFIELD, W.W. and RICHART, D.D. The santhine oxidase activity in rat tissues. *Proc. Soc. Exp. Biol. Med.* 71: 181-185, 1949.
- WILKINS, R.J. DNA repair: a simple enzymic assay for human cells. *Int. J. Radiat. Biol.* 24: 609-613, 1973.
- WOLPERT, M.K., ALTHAUS, J. and JOHNS, D. Nitroreductase activity of mammalian liver aldehyde oxidase. *J. Pharm. Exp. Ther.* 185: 202-213, 1973.
- WOODY KARRER, P. and GREENBERG, J. Resistance and cross resistance of E. coli S mutants to the radio-mimetic agent nitrofurazone. *J. Bacteriol.* 85: 1208-1216, 1963.

- YAGER, J.D. and POTTER, V.R. A comparison of the effects of 3'-Me-DAB, 2-Me-DAB and 2-AAF on rat liver DNA stability and new synthesis. *Cancer Res.* 35: 1225-1234, 1975.
- YAHAGI, T., NAGAO, M., HARA, K., MATSUSHIMA, T., SUGIMURA, T. and BRYAN, G. Relationships between the carcinogenic and mutagenic of DNA-modifying effects of nitrofurans derivatives, including AF-2, a food additive. *Cancer Res.* 34: 2266-2273, 1974.
- ZAMPIERI, A. and GREENBERG, J. Nitrofurazone as a mutagen in E. coli. *Biochem. Biophys. Res. Commun.* 14: 172-176, 1964.
- ZINNEMAN, H.H., HALL, W.H., HONG, L. and SEAL, U.S. Binding of erythromycin, novobiocin, chloramphenicol, chlorotetracycline and nitrofurantoin by serum proteins. In *Antimicrobial Agents and Chemotherapy*. Ann Arbor, Michigan: Braun and Brumfield, 1962, p 637-643.

7. APPENDIX 1

Determination of the Molecular Weight of DNA From Alkaline Sucrose Gradients

Molecular weights of DNA were calculated according to the formulae developed by McGrath and Williams (1966) and applied to mammalian cells by Lett et al. (1967).

Sedimentation measurements were normalized using ³H-TdR labelled adenovirus 2 (AD-2) provided by Dr. A. Rainbow, McMaster University, as a marker. This DNA has a sedimentation value of 35.2 S, and in these gradients sedimented to fraction 5 (1.2 cm) when run in a SW 50.1 rotor at 28 Krpm for 2 hours. The constant, β , for these gradient solutions and lysing conditions could then be calculated using the formula:

$$\beta = \frac{\omega^2 t S_i}{d_i} = 5.7 \times 10^{10}$$

where ω is the angular velocity of centrifugation in rpm, t is the time of centrifugation in hours, d_i is the distance sedimented, and S_i is the sedimentation constant of the molecule in water at 20°. The above value is in agreement with the values of 5.3 and 6.2 x 10¹⁰ obtained by Palcic and Skarsgard (1972) using different lysing and

gradient solutions.

The weight average molecular weight is defined as that molecular weight which is exceeded by one-half the mass of molecules in the distribution.

$$M_w = \frac{\sum n_i M_i^2}{\sum n_i M_i}$$

where n_i is the number of molecules in the i^{th} fraction, and M_i is the molecular weight of a molecule which sediments to the middle of that fraction.

Bergi and Hershey (1963) found:

$$M_i = \frac{s_i^{2.5}}{.0528^{2.5}}$$

The constants are those given by Studier (1965).

It has been shown that the molecular weight of DNA sedimenting in alkaline sucrose gradients can be expressed by the following equation:

$$M_w = \frac{\beta^{2.5}}{.0528\omega^{2t}} \frac{\sum c_i (d_i)^{2.5}}{\sum c_i}$$

where c_i is the number of counts in the i^{th} fraction and d_i is the distance they have sedimented (cm). Refinements of this equation by Palcic and Skarsgard (1972)

give:

$$M_w = A \frac{\sum c_i (i - 1/2)^{2.5}}{\sum c_i}$$

A represents the constant in the previous formula which is dependent upon speed and time of centrifugation.

In the experiments reported in this thesis, A = 52159 when $\omega = 30$ Krpm, t = 90 min; A = 57756 when $\omega = 18$ Krpm, t = 4 hours.

If the distribution of molecules occurs from random degradation, then using Poisson statistics it can be shown that the number average molecular weight, M_n is equal to $0.5 M_w$, where M_n is defined as the total mass of DNA per cell divided by the number of pieces of DNA per cell. Then the number of breaks per cell which is equal to the mass of DNA $\times 1/M_n$ (treated) - $1/M_n$ (control) is also proportional to $1/M_w$. If the size distribution is not random then the above calculations will be inaccurate to an extent which will depend upon the actual distribution (Ehmann and Lett 1973). Calculations of M_n directly from experimental data are complicated by slowly sedimenting components in the tailing edge.

Molecular weights were calculated using a Hewlett Packard 35 or programmable 55 calculator. To determine peak molecular weight, the value of A was multiplied by

the distance the peak sedimented $(i - 1/2)^{2.5}$. 'Corrected' molecular weights arbitrarily assumed that counts less than 25% of the peak height were below background, and weight average molecular weight was computed as above. This allowed molecular weights of samples with a profile near one end of the tube to be calculated assuming a more normal distribution so that contributions of counts at the opposite end would not prejudice the weight calculation. Molecular weight increases as a power function with sedimentation distance so that at the top of the tube, resolution between peaks becomes very poor (Figure 54). Maximum resolution of a single fraction peak would occur at the bottom of the gradient, however, because the distribution of mammalian DNA covers several fractions, sedimentation to the bottom of the tube would distort the peak position. Therefore, the control molecular weight should sediment about one-half to two thirds down the gradient. For example, assuming a normal distribution, and 20 fractions, a change in peak molecular weight from 0.4 to 0.12×10^8 , representing about 2 breaks per molecule, corresponds to a change in peak position from fraction 6 to fraction 4. However, for a peak at position 12, a sample with two breaks per molecule would sediment to fraction 8 (Figure 54). Resolution is also limited in a different way; at a level of incorporation of 1 cpm/cell of $^3\text{H-TdR}$, approximately 1 in 100 molecules

of thymidine in duplex DNA would be labelled with tritium.

Some dependency of molecular weight was found with cell concentrations greater than 2×10^5 cells/gradient (Figure 55). Peak molecular weights were comparable when gradients were sedimented at 30 K rpm for 90 minutes or 18 K rpm for 4 hours, however, because profiles were slightly skewed, molecular weights were falsely low.

It is becoming more evident that determinations of molecular weight using the above formula have limited application (Cleaver 1974). Speed dependent artifacts (McBurney and Whitmore 1972; Lett et al. 1969), anomalous sedimentation behaviour of DNA associated with other cell constituents (Elkind and Kamper 1970; Simpson et al. 1973), presence of double strand pieces and irreproducibility of molecular weights in different laboratories indicate that assigning a number to a profile is merely a convenience and does not describe the sedimentation characteristics as well as the original data. Average molecular weight analysis assumes a homogenous population with equal susceptibility to an agent, whereas two or more populations may actually exist. Under such conditions strand break calculations based on weight average molecular weights may well be meaningless (Ehmann and Lett 1973).

Figure 54: Analysis of Variation of Molecular Weight
with Peak Position

Calculations of peak molecular weight as described in Appendix 1 indicate that maximum resolution is achieved at the bottom of the gradient (one fraction difference gives the smallest change in molecular weight). However, this assumes that DNA sediments in one peak (top panel). When fraction number from meniscus is plotted as a function of the difference in fraction number, a series of lines would emerge indicating the number of strand breaks per molecule. Assuming control DNA sediments to position 10, an example of the number of breaks per molecule for a sample sedimenting to lower fraction numbers is shown (bottom panel).

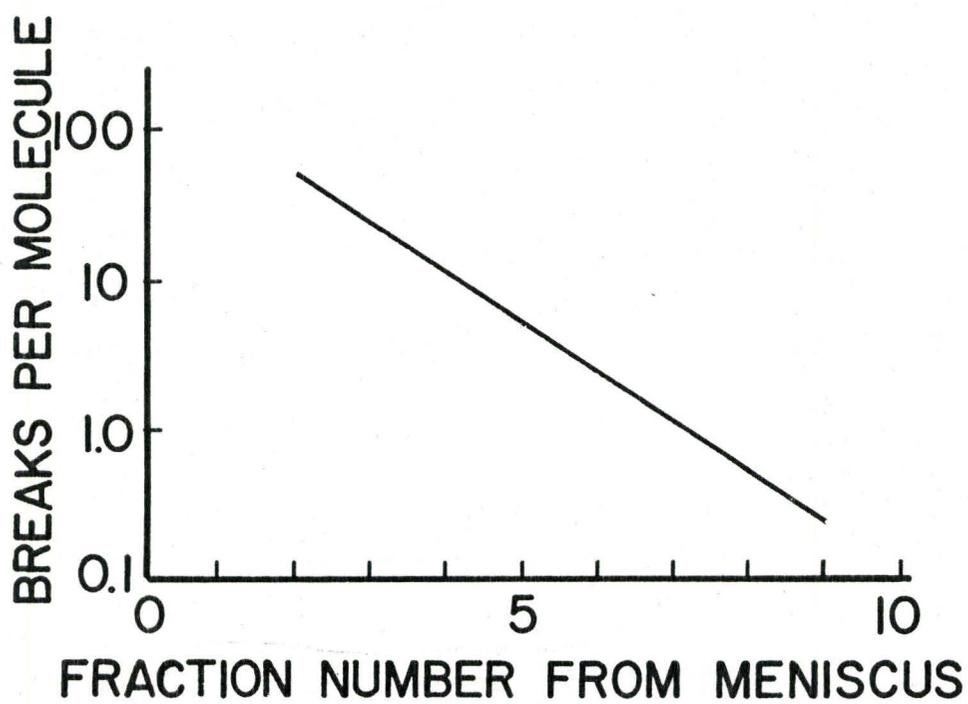
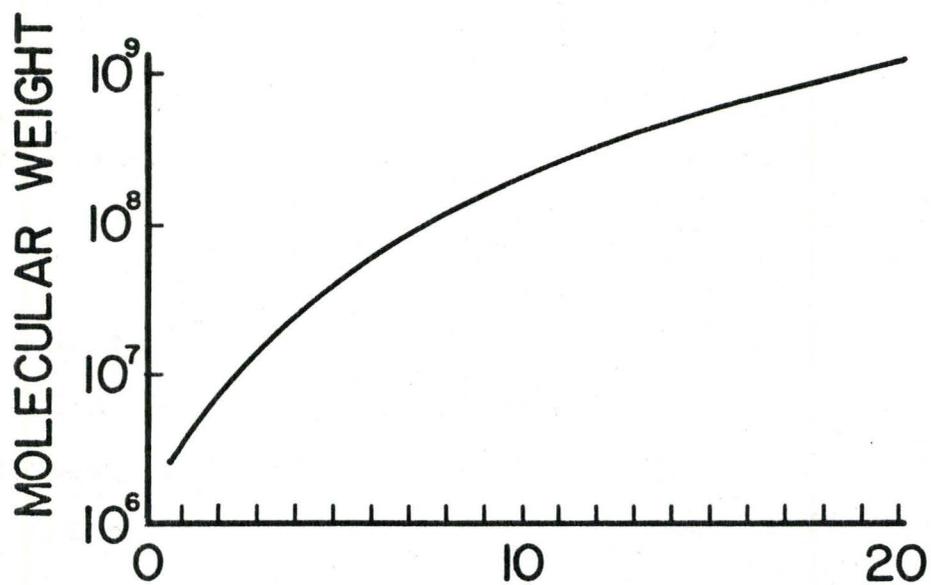
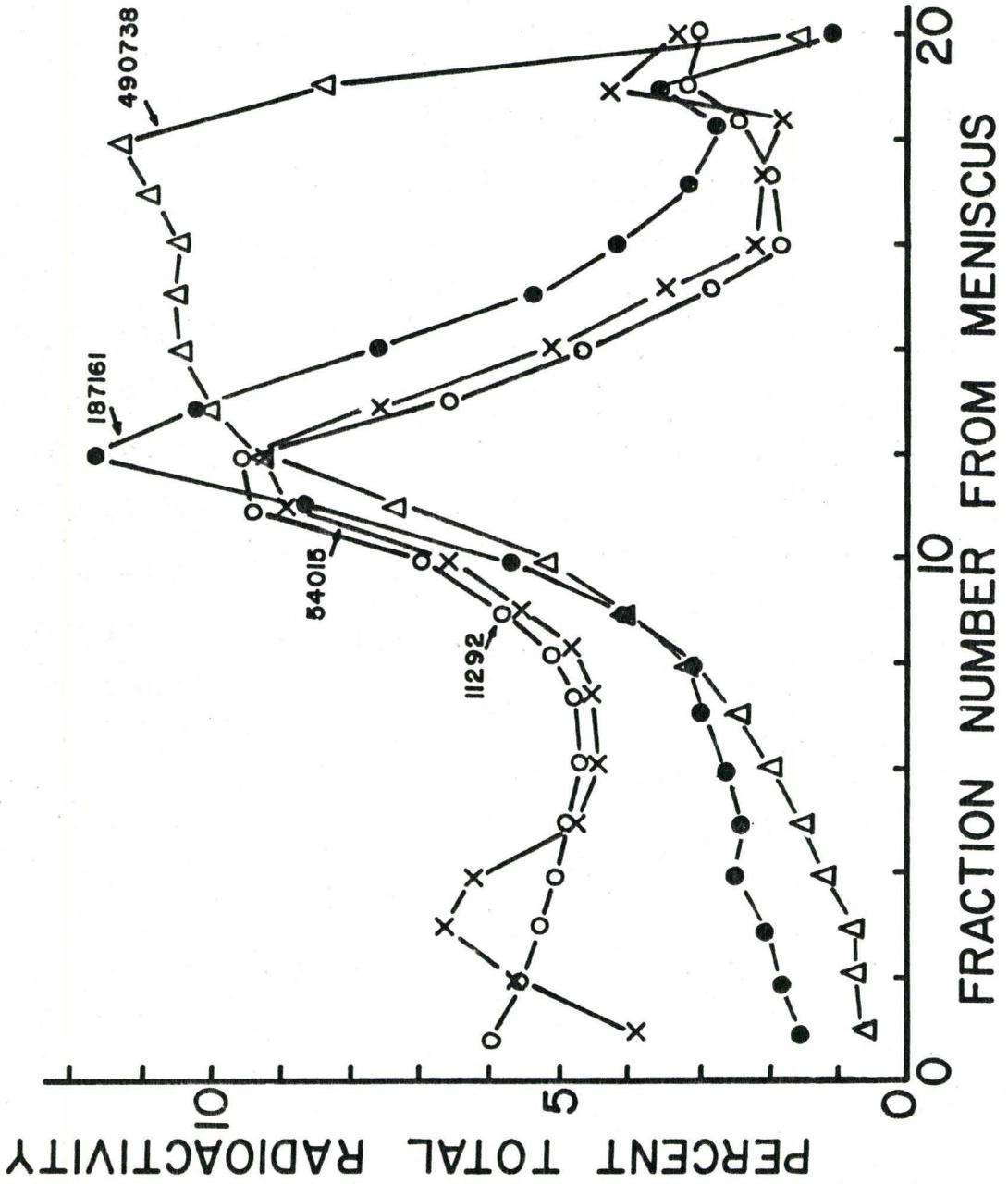


Figure 55: Effect of Cell Numbers on Sedimentation
Behaviour

L cells labelled with ^3H -TdR (1 cpm/cell) were lysed for four hours on alkaline sucrose gradients before sedimenting at 18 Krpm for 4 hours. Numbers beside profiles indicate the total number of counts layered on gradients. Material at the top of the gradients represents fluorescence due to the lysing solution. Since the contribution remains constant, it becomes less significant as higher numbers of counts are layered onto the gradients.



8. APPENDIX 2

Nitrofurantoin Effects on Bacteria: Relation to the Mammalian System

Several experiments were performed using bacteria as a simpler system for testing techniques such as alkaline sucrose gradients and alkaline elution, before application to mammalian cells. Other advantages include the ease of testing survival of bacteria compared to the mammalian colony forming assay, better understanding of nitrofurantoin metabolism in bacteria, and availability of reductase-less mutants and bacteria lacking complete DNA repair capabilities. The following experiments added some new information concerning techniques and nitrofurantoin effects on bacteria which may be related to damage to mammalian cells.

Inhibition of Liquid-Holding Recovery

Liquid-holding recovery was first observed when bacteria irradiated with UV showed an increased survival if allowed to rest in liquid for a few hours before plating on solid medium (Hollander and Claus 1937, Roberts and Aldous 1949). This enhancement of survival was thought to be related to an ability to repair damage. If bacteria were not required to undergo replication immediately

following irradiation, they had more time to repair damage. An analogous effect was found in mammalian cells and called repair of potentially lethal damage (Phillips and Tolmach 1965). Cells irradiated with UV or gamma rays will repair some of this damage if, after irradiation, they are incubated in buffer rather than complete medium. Experiments to determine if nitrofurans inhibited repair of potentially lethal damage (PLD) were preceded by the simpler experiments looking at LHR in bacteria.

The ability of nitrofurans to inhibit LHR is dependent on the choice of incubation medium (Figures 56,57). Cells irradiated and incubated in buffer showed no inhibition of LHR after four hours in 50 μ M nitrofurazone. There was some toxicity at this concentration and greater toxicity when bacteria were incubated in nutrient broth rather than buffer. The extent of recovery was smaller when cells were incubated in medium rather than buffer. LHR appeared to be completely inhibited in medium but was not affected when bacteria were incubated in buffer. This suggests that inhibition of repair of LHR requires metabolic activation of nitrofurazone, or metabolically active cells. Experiments using bacteria lacking nitrofuran reductase I, such as nfr 207, would clarify this finding. Inhibition of excision of UV dimers or rejoining of breaks and improper repair would decrease survival.

While bacteria exhibit LHR in both buffer and medium, potentially lethal damage repair is apparent in mammalian cells only after incubation in buffer. For this reason, experiments using mammalian cells were limited to conditions in which the level of metabolic activity was low. Although inhibition of PLD is not necessarily related to production of single-strand breaks, data obtained using irradiated cells incubated with nitrofurans in air suggests that there might be some correlation between the enhanced damage and inhibition of PLD.

Alkaline Elution

This technique was developed by Kohn and Grimek-Ewig (1973) for use with mammalian cells as an alternative to the alkaline sucrose sedimentation method for estimating decreases in molecular weight of single-strand DNA. It involves depositing a sample of cells with ^3H -TdR labelled DNA on a stack of membrane filters, lysing them with SDS and EDTA, then washing through pieces of denatured DNA of increasing size as a function of time of elution with alkaline medium. Fractions can be collected and counted.

This procedure was applied to bacterial DNA. Results were fairly reproducible indicating that DNA breaks resulted from incubation of E. coli B with nitrofurazone

Figure 56: Effect of Nitrofurazone on Liquid Holding
Recovery

Exponentially growing E. coli B cells were irradiated in M9 medium (Anderson 1946) and either diluted and plated immediately on agar, or plated 4 hours later after holding in phosphate buffer (.67M) or nutrient broth (Difco) with or without 50 μ M nitrofurazone at 37^o in the dark. Surviving cells formed colonies which were counted 30 hours later.

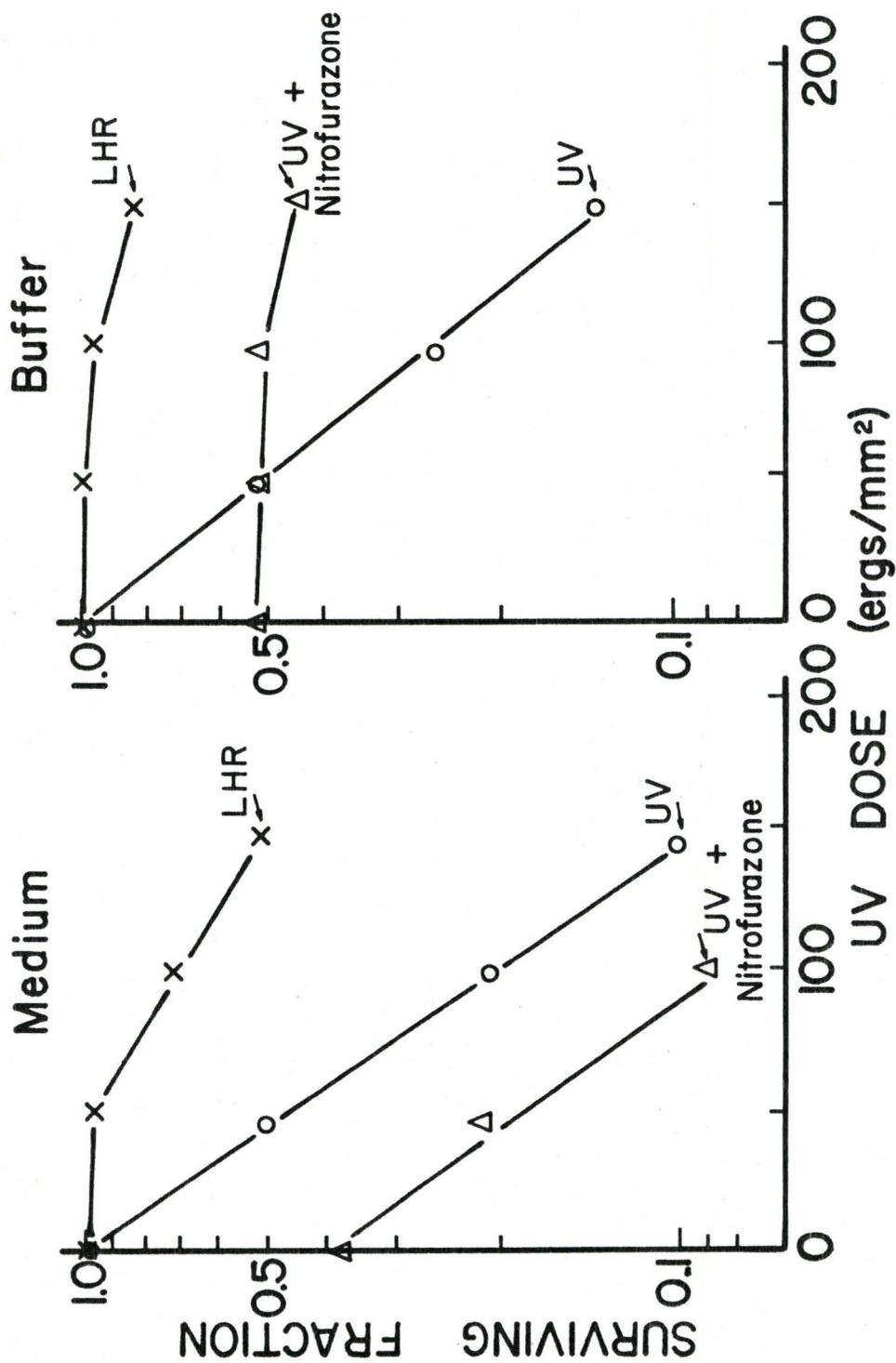
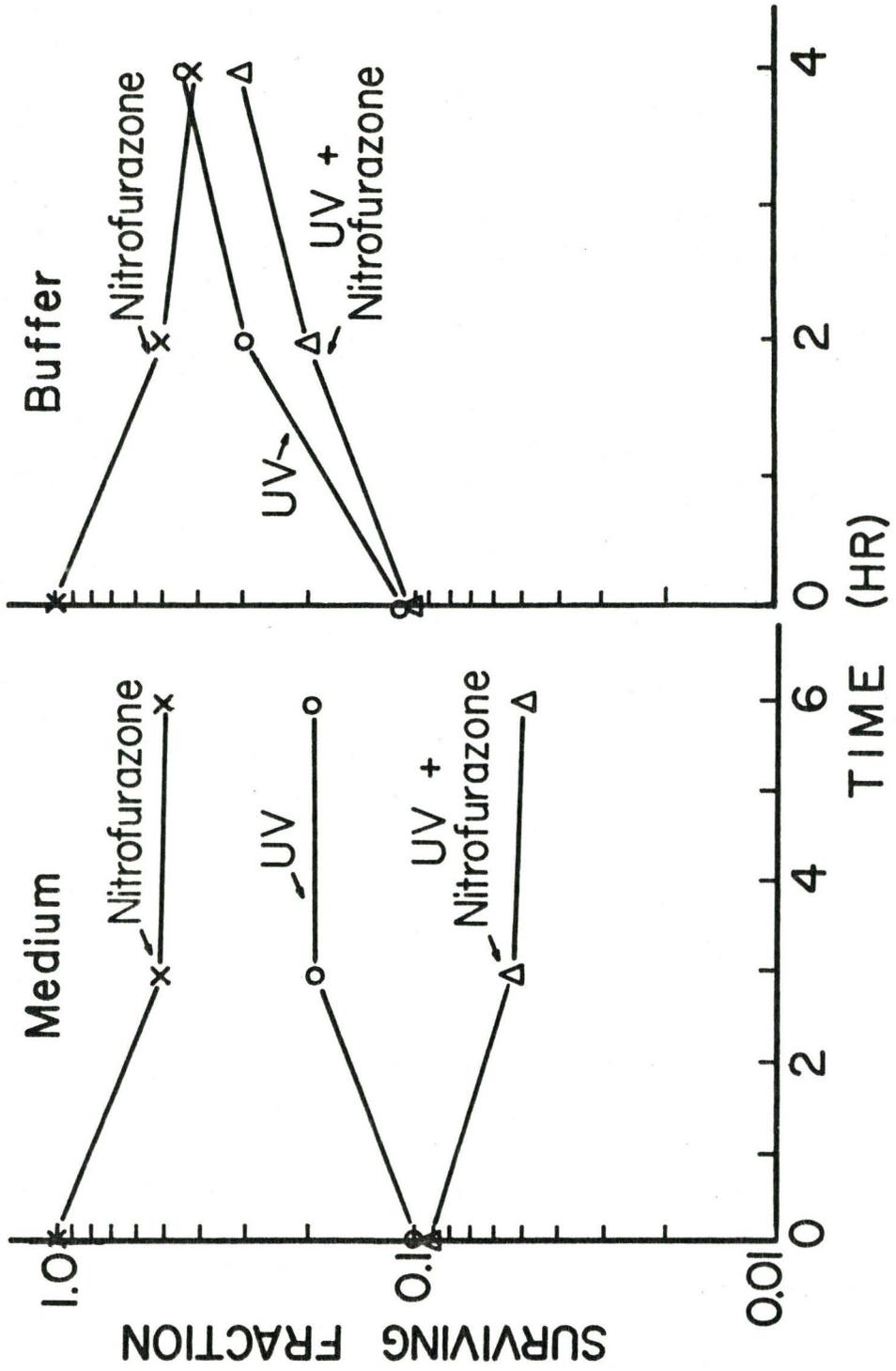


Figure 57: Effect of Duration of Incubation on
Inhibition of Liquid Holding Recovery

Exponentially growing E. coli B cells were irradiated with 150 ergs/mm² UV and held in buffer or nutrient broth with or without 50 μM nitrofurazone for 3 or 6 hours after irradiation.

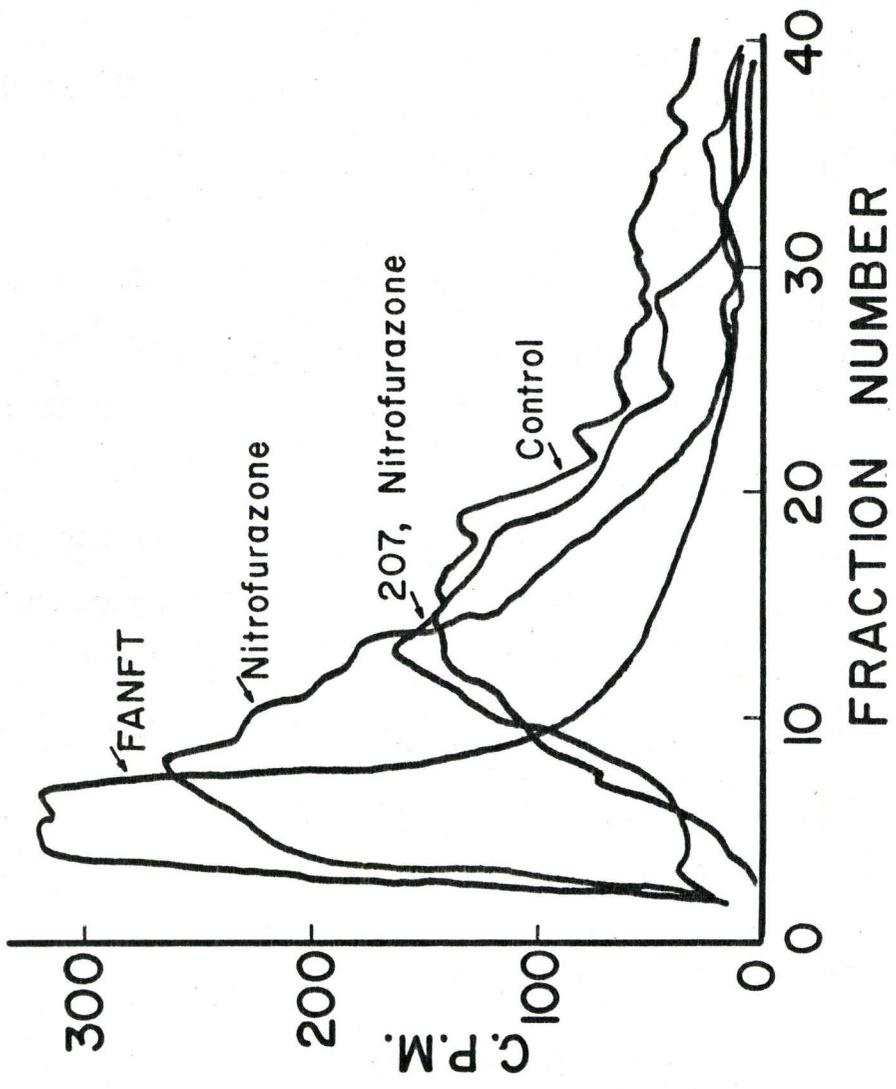


or FANFT, but not from incubation of E. coli nfr 207 lacking nitrofurane reductase I with nitrofurazone (Figure 58).

As this technique provides a convenient way of confirming results obtained from gradient analysis, and since it is based on a different physical principal, new information may be added. However, when this system was applied to mammalian cells, no reproducible profiles were obtained. The complexity of chromatin, membrane attachments and the size of mammalian DNA may account for this failure. Modifications of filter number and pore size, alkalinity of the elution solution and rate of elution did not improve resolution or reproducibility.

Figure 58: Alkaline Elution of E. coli DNA Treated
with Nitrofurans

E. coli B and nfr 207 were incubated with 100 μ M nitrofurazone or 10 μ M FANFT for 60 minutes. They were then deposited on a stack of 4 membrane filters (Gelman, 0.22 μ) and lysed using SDS and EDTA. Selective elution of small pieces of DNA was accomplished by forcing an alkaline solution through the filters. Fraction number is proportional to the time after start of elution (one fraction is approximately 2 minutes).



9. APPENDIX 3

Autoradiographic Analysis in DNA Turnover Studies

The specific radioactivity of labelled DNA may decrease in tissue as a result of cell loss, organ enlargement, DNA turnover without cell loss, or some combination of the above.

If cell loss occurs (Figure 59A), the total counts per organ would decrease, the organ size decrease, but the density of label (cpm/mg tissue) remain constant. If the organ enlarges due to hyperplasia(B) or hypertrophy (C), then the total counts per organ would remain constant but the organ size would increase and the density of label decrease. Finally, if DNA turnover occurs as a result of DNA loss (D), the total counts per organ would decrease, the density of label drop, but the organ size would remain constant. However, this could also result from cell turnover, that is, cell loss and replacement (E).

Autoradiography should be able to distinguish between these last two possibilities. In (D), the distribution of grain counts should indicate a rather narrow peak, suggesting that most cells are labelled to the same extent. The 'nitrofurantoin-treated' distribution should be displaced from the control only if some cells are more vulnerable to nitrofurans than others. If this

is not the case, only the peak height should decrease.

In (E), cell loss and replacement will result in a wider distribution in grain counts from tissue of nitrofuran-fed mice. If a large amount of cell division has occurred, it might be possible to observe two populations, indicating some cells have half the grain counts of others. Alternatively, if only a small population of 'stem' cells are dividing, areas with low grain counts might result. The entire tissue should then be searched for such fields.

Figure 59: Autoradiographic Analysis of DNA Turnover

Diagrammatic analysis of DNA turnover. Dots indicate grains resulting from exposure of emulsion to incorporated ^3H -TdR and boxes represent cells. See text for details.

DNA TURNOVER

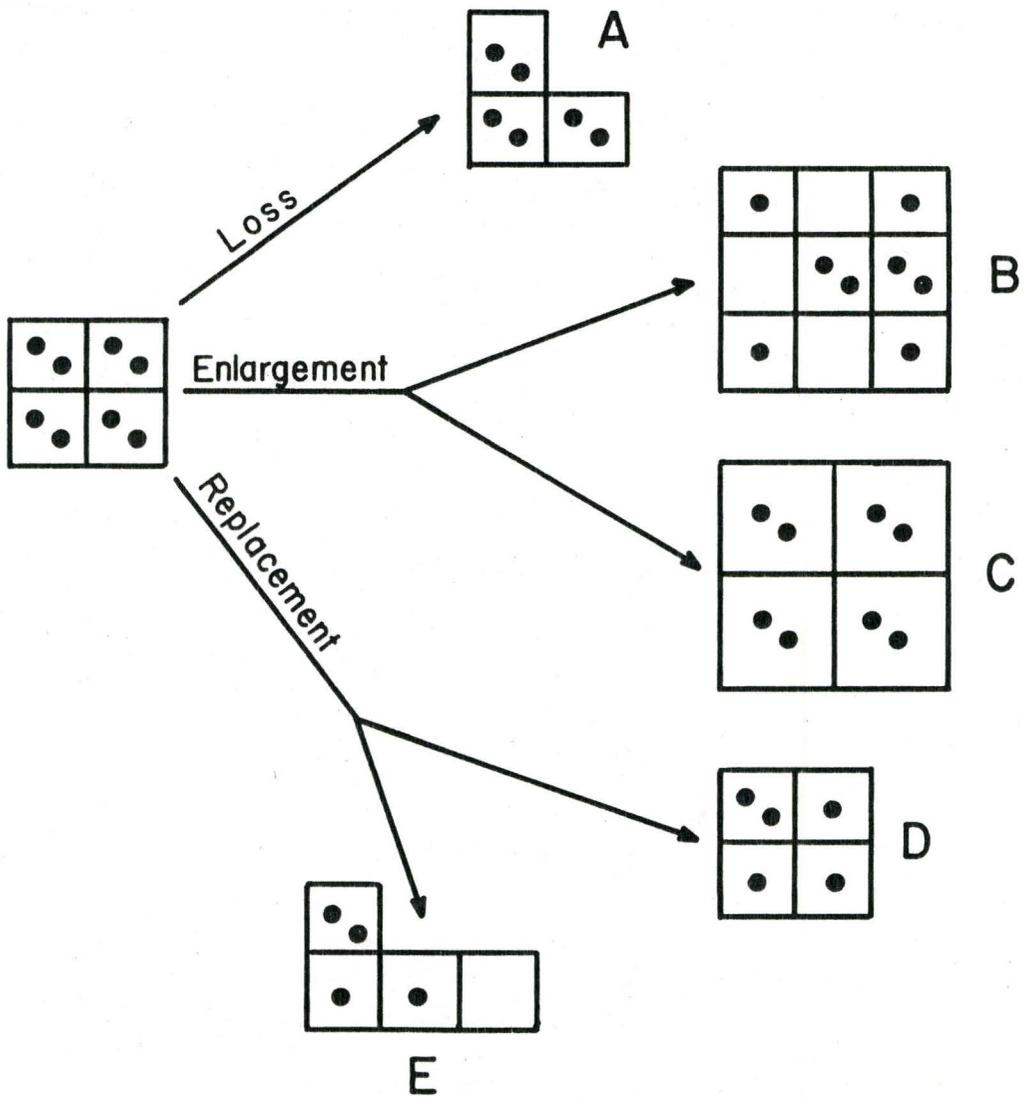


TABLE 14

BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF NITROFURANS

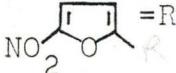
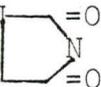
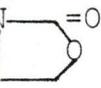
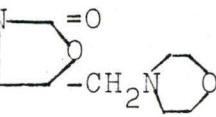
Chemical Name	Common Name (Trade Name)	Structure
1. 5-nitro-2-furaldehyde- semicarbazone	nitrofurazone (Furacin) NF-7	
2. N-(5-nitro-2-furfurylidine) -]-amino-hydantoin	nitrofurantoin (Furadantin) NF-153	$R-CH=NN$ 
3. N-(5-nitro-2-furfurylidine) -3-amino-β-oxazolidone	furazolidone (Furoxone) NF-180	$R-CH=NN$ 
4. 5-nitro-2-furyl-oxyzyl-N- ethylene-3-azomethyloxazol	furaltadone (Altafur) NF-260	$R-CH=NN$ 
5. 1-(5-nitro-2-furfurylidine) -3-N,N-diethylpropyl-amino urea HCl	NF-167	$R-CH=NNHCOCONH(CH_2)_3 \cdot$ $NEt_2 \cdot HCl$
6. 5-nitro-2-furaldehyde oxime	nifuroxime (Microfur)	$R-CH=NOH$

TABLE 14 CONTINUED

Clinical Use ^a	Solubility mg/l at pH 7.0 ^a	LD ₅₀ , Oral mg/kg mouse ^b	pK _a ^c	Absorption Maximum (nm)	Minimum Inhibitory Concentration (μM) (Radiosensitizing Enhancement Ratio) ^d
1. Surface Bacterial Infections	210	746	n.i.	375	44 (1.4)
2. Urinary Tract Infections	190	426	7.2	367	72 (1.2)
3. Enteric Infections	100	2,765	n.i.	367	39 (1.4)
4. Systemic Staphylococcal Infections	753		5.0	366	68 (1.4)
5.	13,400				11 (1.4)
6. Vaginitis	1,038				44 (1.5)

^a From Lewis (1975)

^b From Buzard et al (1956)

^c From Paul et al (1960) n.i = nonionized

^d From Chapman et al (1973) using V79 cells, measuring inhibition of growth

TABLE 14 CONTINUED

	Plasma Level ^e (mg/l) Rats after Oral Feeding, 500 mg/kg Time (hr)				Percent Plasma Binding ^f	Concentration For Inhibition Of GSSG Reductase ^f (μM)	Concentration For Inhibition Of Pyruvic Oxidase (μM) ^f	Mutagenicity (Carcinogenic) ^g
	4	8	16	24				
1.	5.7	10	14.5	16.0	34	20	0.04	+ (+)
2.	2.6	2.4	4.0	8.3	53	20	0.33	+ (-)
3.					30	20	0.27	++
4.	3.2	3.6	1.3	1.6	10	200	0.50	
5.								+
6.								++

^e From Buzard et al (1956)

^f From Buzard (1962)

^g From Tazima et al (1975)

VITA

NAME: Peggy Louise OLIVE

PLACE OF BIRTH: Montreal, Quebec, Canada

DATE OF BIRTH: May 30, 1948

MARITAL STATUS: Married (R.E. Durand), no children

POST-SECONDARY EDUCATION AND DEGREES:

Bishop's University
Quebec, Canada
1965-1969 B.Sc. (Honors) - Physics

The University of Western Ontario
Ontario, Canada
1969-1972 M.Sc. - Biophysics

McMaster University
Ontario, Canada
1972-1975 Ph.D. - Biochemistry

HONORS AND AWARDS:

Montreal School Board Scholarships, 1960-1965
Bishop's University Founder Scholarship, 1965
Research Student of the National Cancer
Institute of Canada, 1973

RELATED WORK EXPERIENCE:

Teaching Assistant
Department of Physics
Bishop's University
1967-1968

Student Research Assistant
Ontario Cancer Institute
Toronto, Ontario
Summer, 1968

Graduate Assistantship
Ontario Cancer Foundation
London, Ontario
1969-1972

Teaching Assistant
Department of Biology
McMaster University
1972-1973

Teaching Assistant
Department of Biochemistry
McMaster University
1973-1974

PUBLICATIONS:

- P.L. Olive, W.R. Inch and R.M. Sutherland. The effect of triacetoneamine-N-oxyl on oxygenation and radio-curability of a mouse mammary carcinoma. *Radiat. Res.* 52: 618-626, 1972.
- P.L. Olive and W.R. Inch. Effect of inhaling oxygen/carbon dioxide mixtures on oxygenation and cure by X-rays of the C3HBA mouse mammary carcinoma. *Radiology* 106: 673-678, 1973.
- P.L. Olive and D.R. McCalla. Damage to mammalian cell DNA by nitrofurans. *Cancer Res.* 35: 781-784, 1975.
- D.R. McCalla, D. Voutsinos and P.L. Olive. Mutagen screening with bacteria: niridazole and nitrofurans. *Mut. Res.* 31: 31-37, 1975.
- D.R. McCalla, P.L. Olive, Y. Tu and M.L. Fan. Nitrofurazone-reducing enzymes in *E. coli* and their role in drug activation in vivo. *Can. J. Microbiol.* (in press) 1975.
- D.R. McCalla, P.L. Olive and Y. Tu. Damage of DNA by activated nitrofurans. Procedures of the 5th International Meeting of the Princess Takamatsu Cancer Research Fund. Nov. 1975.
- P. L. Olive. DNA turnover in mouse tissue as a result of nitrofurans feeding. (in preparation)
- P. L. Olive, and D.R. McCalla. Toxicity and DNA-modifying effects of nitrofurans. (in preparation)