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MUTAGENICITY AND BIOTRANSFORMATION STUDIES WITH NITROFURANS

by

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ABSTRACT

The mutagenicity of 12 nitrofurans was tested in the Salmonella typhimurium tester strains TA100 and TA98 developed from the standard tester strains TA1535 and TA1538, respectively, by the incorporation of an R-factor plasmid, pKM101. The standard TA1535 and TA1538 tester strains were not mutated by the nitrofurans. All the nitrofurans tested were mutagenic in both the TA100 and TA98 tester strains. Nitrofurazone and nitrofurantoin dose-response curves in TA100 and TA98 indicated that TA100 was a more sensitive indicator of nitrofuran mutagenic activity than TA98.

The mutagenicity of nitrofurazone and nitrofurantoin was further evaluated by the micronucleus test in bone marrow of male Sprague-Dawley rats. The test compounds were administered intraperitoneal, one-half the dose 30 hours and the rest 6 hours before sacrifice. Neither nitrofurazone at 15, 30, and 60 milligrams per kilogram nor nitrofurantoin at 50, 100, and 200 milligrams per kilogram caused a statistically significant increase in the percentage of reticulocytes with micronuclei. Nitrofurazone at 60 milligrams per kilogram in male Long-Evans rats also gave negative results. Furylfuramide administered at 60, 120, and 240 milligrams per kilogram as a suspension in physiological saline caused a slight but significant

(statistical significance less than 0.05) increase in the percentage of reticulocytes with micronuclei.

Nitrofurazone, further evaluated for the induction of chromosomal aberrations in in vivo cytogenetic testing of the bone marrow of male Sprague-Dawley rats, was administered intraperitoneal as a single dose at 60 milligrams per kilogram and did not induce chromosomal aberrations in bone marrow samples after 6 and 24 hours.

The enzymatic reduction of nitrofurazone to electrophilic intermediates which interact with deoxyribonucleic acid has been proposed as a mechanism for its mutagenic activity. These theoretical electrophilic intermediates may also form stable adducts with the nucleophile cysteine. The reductive metabolism of carbon-14-nitrofurazone was therefore carried out in the presence or absence of cysteine to determine 1) if cysteine altered the metabolite pattern and 2) if cysteine adducts were formed. Carbon-14-nitrofurazone was reduced by a xanthine oxidase-hypoxanthine system under anaerobic conditions in the presence or absence of cysteine. The metabolites were separated by reverse phase high pressure liquid chromatography. The radioactive elution profile of the metabolites of carbon-14-nitrofurazone was dramatically changed by the presence of cysteine. Two major peaks, a polar and a relatively non-polar metabolite, were formed in the absence of cysteine. In the presence of cysteine, there was an increase in the amount of polar metabolite at the

expense of the non-polar metabolite and two additional metabolites were formed. However, the radioactive elution profile of carbon-14-cysteine alone was not significantly different from that of carbon-14-cysteine which had been incubated with unlabelled nitrofurazone during the reaction. Therefore, under these conditions, cysteine does alter the metabolite pattern of nitrofurazone, but these changes are not due to the formation of a cysteine adduct with reactive metabolites of nitrofurazone.

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Ci	Curie
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic aicd
DPM	disintegrations per minute
e	extinction coefficient
HPLC	high pressure liquid chromatograph(y)
ID	internal diameter
ip	intraperitoneal(ly)
M	moles per liter
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFZ	nitrofurazone
nm or λ	nanometers
RNA	ribonucleic acid

I. INTRODUCTION - REVIEW OF LITERATURE ON NITROFURANS AND STATEMENT OF PROBLEM

Historical Background and Usage

In 1939, Eaton Laboratories (Norwich Pharmacal) embarked on a program of research involving synthesis of nitrofurans and investigation of their antibacterial properties (Miura and Reckendorf, 1967). This program led to the discovery of nitrofurazone, the antibacterial properties of which were reported by Dodd and Stillman in 1944 (Paul and Paul, 1964; Miura and Reckendorf, 1967; International Agency for Research on Cancer, 1974). In 1944, nitrofurazone was used successfully in the treatment of infected battle wounds during the invasion of France. Nitrofurazone was marketed for general used in 1945. Since then, hundreds of nitrofuran derivatives have been synthesized and screened for bactericidal properties. Nitrofurantoin was introduced in 1952 for clinical trials as a urinary antiseptic. It was the first nitrofuran recommended for peroral use (Miura and Reckendorf, 1967). Since the introduction of nitrofurazone as the first nitrofuran marketed for clinical use the number of nitrofurans produced and the application of their use have been greatly expanded. Certain nitrofurans exhibit antifungal, antischistosomal, and antiprotozoan properties as well as antibacterial properties. Only the more common usages of nitrofurans, with emphasis on nitrofurazone, nitrofurantoin, and furylfuramide, will be discussed in this

section. A more comprehensive summary on the use of nitrofurans in clinical and veterinary medicine is presented in Tables I-1 and I-2.

Nitrofurazone is primarily used as a topical agent in human medicine for the treatment of burns, pyodermas, skin grafts, ulcers and wounds. It is a component of a protective paste used in the Soviet Union to protect the hands of industrial workers (Miura and Reckendorf, 1967). Nitrofurazone has been used in treatment of infections of the conjuctiva and cornea (Reckendorf and Miura, 1967), and previously it was used in treatment of vaginal infections, ear infections, and for the relief of nasal congestion. However, the Food and Drug Administration has withdrawn approval of new drug applications for these uses because of lack of proved efficacy (Federal Register, 1975).

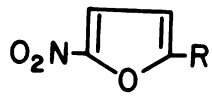
Nitrofurazone has been used extensively in veterinary medicine (See Table I-1). It was approved as the first nitrofuran for use in food-producing animals by the Food and Drug Administration in 1948. The Food and Drug Administration issued a notice for hearings on a proposal to withdraw new drug animal applications for nitrofurazone in food producing animals on August 17, 1976 (Federal Register, 1976). The reason for this action was that nitrofurazone was shown to be oncogenic in rats and that neither comprehensive oncogenic dose-response data nor comprehensive residue analysis of animals treated with nitrofurazone had been obtained or submitted for review. Three other

nitrofuran derivatives used in food-producing animals were subject to similar Food and Drug Association action. These were nihydrazone, furaltadone, and furazolidone (Federal Register, 1976). Besides treatment of animal infections, furazolidone was used for growth promotion in animals (Paul and Paul, 1964).

Nitrofurans have been used as food additives in Japan for the purpose of food preservation. Nitrofurazone was the first to be used during 1950 to 1954, but was replaced by nitrofurylacrylamide in the period of 1954 to 1965. The final nitrofuran food additive, furylfuramide, was used from 1965 until 1974. The strong mutagenic as well as carcinogenic activity of many nitrofuran derivatives led to concern over the use of furylfuramide as a food additive in Japan and resulted in a revaluation of the safety of the compound as a food additive. Because furylfuramide demonstrated mutagenicity and carcinogenicity it was removed from use as a food additive in Japan (Tazima et al., 1975).

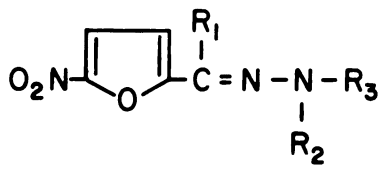
Chemical and Physical Properties

Antimicrobial effectiveness of all nitrofuran derivatives requires two essential factors: 1) the presence of a nitro group attached to C_5 of the furan ring and 2) a side chain at C_2. Thus, nitrofurans of this type can be represented as follows (Miura and Reckendorf, 1967):

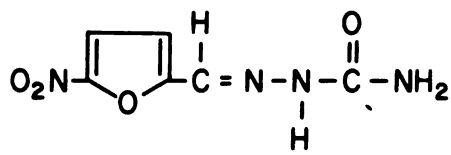


2-Substituted-5-Nitrofuran Derivative

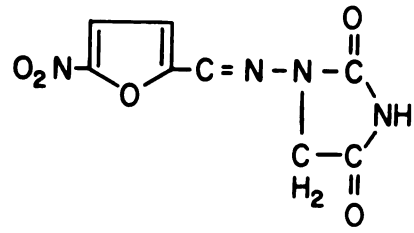
The C_2 side chain on the furan ring can be of several different classes. Azomethine type nitrofurans presently represent the most clinically important nitrofuran group including nitrofurazone and nitrofurantoin. The general structure of azomethine type nitrofurans and the structure of nitrofurazone and nitrofurantoin are as follows (Miura and Reckendorf, 1967; Tazima et al., 1975):



General Structure of Azomethine Type Nitrofurans



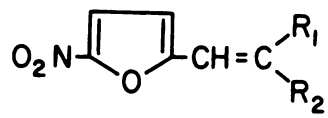
Nitrofurazone



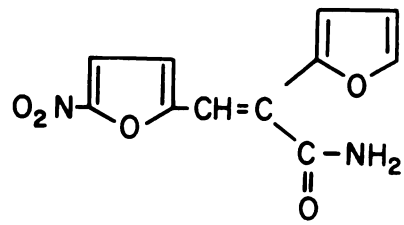
Nitrofurantoin

Other azomethine nitrofuran derivatives marketed for medical usage include guanofuracin, furazolidone, nihydrazone, furaltadone, and nifuraldazone (Miura and Reckendorf, 1967).

A second large group of antimicrobial nitrofurans are those which contain a vinyl residue at the C_2 position of the furan ring. Furylfuramide is an example of a vinyl type nitrofuran. The general structure of vinyl type nitrofurans and the structure of furylfuramide are as follows:

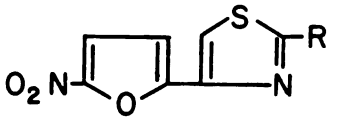


General Structure of Vinyl Type of Nitrofurans



Furylfuramide

A third group of antimicrobial nitrofurans consist of 5-thiazole derivatives attached to the C_2 position on the furan ring. The general structure of 5-thiazolylnitrofurans is as follows (Tazima et al., 1975):



General Structure of 5-thiazole Type Nitrofurans

The 5-thiazolylnitrofurans, a group of antimicrobial nitrofurans that have seen only limited clinical use, have been widely investigated among researchers for mutagenicity and carcinogenicity.

Finally, there is a large number of miscellaneous C_2 substituted nitrofurans which do not fall into the three previously mentioned categories. Some are commercially available and possess antimicrobial properties. (Miura and Reckendorf, 1967; Tazima et al., 1975).

In general, the antimicrobial effective nitrofurans are brightly colored reddish-orange or yellow compounds. They can exist as crystalline or amorphous powders. Most exist in solid form, either decomposing or melting above 150 °C (Miura and Reckendorf, 1967; International Agency for Research on Cancer, 1974).

In general, nitrofurans are only slightly soluble in

water. Most nitrofurans are soluble in dimethyl formamide or dimethyl sulfoxide. If there is no interference in biological or chemical procedures, an aqueous solution of a nitrofuran of desired concentration may be prepared by dissolving a weighed amount of the nitrofuran in a small volume of suitable organic solvent and introducing this to the aqueous system (Paul and Paul, 1964; Miura and Reckendorf, 1967).

The nitrofurans have characteristic ultraviolet absorption spectra. There are usually two absorption maxima in the ultraviolet region of nitrofuran solutions. One absorption maximum occurs in the 300 nm to 400 nm region and the other absorption maximum occurs in the 200 nm to 300 nm region. These absorption maxima in the ultraviolet region, particularly at the higher wavelength, have been valuable for the monitoring of nitrofurans, since changes in the spectral characteristics of these compounds appear as they are degraded or metabolized (Paul and Paul, 1964; Miura and Reckendorf, 1967; International Agency for Research on Cancer, 1974).

Nitrofurans are stable when shielded from light, however, nitrofurans in dilute solution are highly photosensitive and must be shielded from daylight or fluorescent light. Water or saline solutions of most commercially available nitrofurans are stable upon autoclaving. The nitrofurans are not stable to autoclaving when dissolved in bacterial media or organic solvents.

Aqueous solutions of nitrofurazone are stable in the physiological pH range but most are quite unstable at pH greater than 10. The azomethine linkage of a number of nitrofurans will undergo acid hydrolysis at a very low pH (Paul and Paul, 1964; Miura and Reckendorf, 1967).

Since nitrofurazone is the subject of prime investigation for this dissertation its physical and chemical properties are summarized in Appendix I-1.

Mutagenicity and Mechanism of Action

It is difficult to discuss the mutagenicity of nitrofurans, particularly in bacteria, without also discussing the mechanism of bactericidal action, since the two seem to be related. Therefore, both mutagenicity and mechanism of bactericidal action will be discussed together in this section.

In the 1950's it was thought that nitrofurazone inhibited in some manner metabolic activity necessary for bacterial survival. Early work showed that nitrofurans could inhibit respiration of intact bacteria as well as pyruvic oxidase in cell free extracts. Other enzymes have been reported to be inhibited by nitrofurans. However, there has been insufficient evidence to pinpoint an inhibition of enzyme as the critical step in the antimicrobial activity of nitrofurans (Paul and Paul, 1964; McCalla and Voutsinos, 1974).

During the 1960's nitrofurans were demonstrated to be radiomimetic and mutagenic. Mutants of E. coli B selected for resistance to the bactericidal action of nitrofurazone were also resistant to ultraviolet light; mutants resistant to ultraviolet light were also resistant to the bactericidal action of nitrofurazone. E. coli strain B/r (resistant to the bactericidal action of radiation and radiomimetic chemicals), a strain already in existance, demonstrated the same resistance pattern as those mutants of E. coli strain B initially selected for resistance to ultra violet light or nitrofurazone. It is believed that the resistant strains of bacteria contain a more effective repair mechanism for damaged DNA than does the susceptible strain. Mutants of E. coli B were also resistant to nitrofurantoin, nihydrazone, and furazolidone. These data suggest that nitrofurans may exert their bactericidal action on E. coli through damage to DNA (McCalla, 1965).

E. coli B/r has been thoroughly investigated with respect to its interactions with nitrofurans. In E. coli B/r two different classes of nitrofurazone reductase have been discovered. The first, nitroreductase I, uses either NADH or NADPH as a hydrogen donor and is active in the presence of oxygen. In contrast, nitroreductase II has a higher molecular weight, does not reduce nitrofurans in the presence of oxygen and uses only NADH. E. coli B/r mutants selected for resistance to nitrofurazone lack nitroreductase I activity. Cells which contain nitroreductase I bind ^{14}C

from radiolabelled nitrofurazone to trichloroacetic acid insoluable material while resistant mutants lacking this reductase do not. Serum albumin will also bind covalently ^{14}C from radiolabelled nitrofurazone upon reduction of the nitrofurazone (McCalla et al., 1970). In addition, exposure of E. coli B/r, which contain nitroreductase I, to nitrofuran derivatives results in single-strand breaks in DNA whereas exposure of E. coli strain nfr-207, a mutant which lacks nitroreductase I, to nitrofurans results in no single strand breaks in DNA (McCalla et al., 1971b). These data support the theory that nitrofurans must be metabolically activated to become bactericidal and to interact with DNA and cellular proteins.

The ability of antibacterial nitrofurans to inhibit bacterial synthesis of certian macromolecules has also been investigated. Nitrofurans inhibit DNA, RNA and protein synthesis in intact cells and in protoplast lysate systems of E. coli strain K-12 (Nakamura and Shimizu, 1973). Exposure of E. coli B/r to nitrofurazone strongly inhibits the synthesis of all classes of RNA, ribosomal sub-units, and formation of polysomes. However, nitroreductase I deficient E. coli nfr-207, upon exposure to nitrofurazone, does not exhibit inhibition in synthesis of RNA or ribosomal sub-units and formation of polysomes are not significantly effected. The ribosomes isolated from nitrofurazone treated E. coli B/r have a lower ability to carry out poly-U directed polyphenylalanine synthesis than untreated cells

(Tu and McCalla, 1976). These data indicate that nitrofurans may cause bactericidal action through inhibition of molecular synthesis essential for cell survival.

The discovery that nitrofurazone was radiomimetic led to concern that it would also be mutagenic since radiomimetic chemicals generally are mutagenic as well. In the early 1960's it was shown that nitrofurazone reverted lactose non-fermenting E. coli to lactose fermenting E. coli. This early work led to suspicions that nitrofurazone may also be a potential carcinogen (Zampieri and Greenberg, 1964). Development of interest in genetic toxicology lead to interest in studying the mutagenicity of nitrofurans in the late 1960's and 1970's. These tests have also lead to further confirmation that bactericidal action is related to DNA interaction.

Bacterial mutagenicity testing has been the most exhaustive area of mutagenicity testing for the nitrofurans. A variety of E. coli auxotroph tester strains are reverted to prototrophy by nitrofurans (Kada, 1973; Kondo and Ichikawa-Ryo, 1973; McCalla and Voutsinos, 1974; Yahagi et al., 1974; McCalla et al., 1975; Tazima et al., 1975). E. coli WP2 tryp- (tryptophan dependent) is reverted (tryp- tryp+) by furylfuramide (Kada, 1973). E. coli WP2 tryp- strains defective in the excisional repair system (UvrA-) for DNA are more sensitive to reversion and killing (McCalla and Voutsinos, 1974). Forty of 41 nitrofuran derivatives tested induced reversions in E. coli WP2 tryp- (Tazima et al.,

1975). Only 5-nitro-2-furoic acid did not, but it induces reversions in Salmonella typhimurium TA100 (Yahagi et al., 1976). Mutant strains of E. coli WP2 tryp- isolated for resistance to nitrofurazone were not mutated by nitrofurazone or other nitrofurans. These bacteria lacked nitroreductase I, suggesting that a reduction product may be the ultimate mutagen (McCalla and Voutsinos, 1974). A furylfuramide resistant derivative (C801) was also isolated from E. coli B/r WP2 tryp-. This new strain was not reverted by furylfuramide. However, addition of 9,000 x g rat liver homogenate during treatment of the C801 with furylfuramide bacteria markedly increased the frequency of reversion (Kada, 1975). These results show that nitrofurans can be activated to mutagens by mammalian enzymes.

The Salmonella typhimurium tester strains TA1535, TA1536, TA1537, and TA1538 were not reverted by nitrofurans (see Section III on Mutagenicity Testing of Nitrofurans for details on Salmonella typhimurium tester strains). It was observed that TA1538, a strain which possesses normal enzymatic excisional repair for DNA, was not as sensitive to the killing properties of nitrofurans as TA1938, a similar strain only enzymatic excisional repair deficient. The lack of induction of his- → his+ reversions in the TA1535 and TA1538 tester strains was puzzling. Then introduction of an R-factor for ampicillin resistance, pKM101, into the above strains (designated TA100 and TA98 respectively) rendered them both sensitive to his+ → his- reversions by

furylfuramide (McCann et al., 1975; Wang et al., 1975b). A wide variety of nitrofurans were subsequently found to be mutagenic in TA100 (Goodman and Vore, 1975; Yahagi et al., 1976) and in TA98 (Goodman and Vore, 1975). A nitrofurazone resistant mutant (TA100-FRI) which is nitroreductase deficient has been selected and isolated from the TA100 tester strain (Rosenkranz and Speck, 1975). The TA100-FRI strain was nitroreductase deficient (Rosenkranz and Speck, 1975). TA100-FRI was resistant to the reversion effects of nitrofurantoin under aerobic incubation conditions, while TA100 was reverted under the same conditions. This is consistent with the theory that it is the oxygen insensitive nitroreductase I previously characterized in E. coli that is deficient in TA100-FRI. However, under anaerobic conditions TA100-FRI is reverted by nitrofurantoin, consistent with the theory that oxygen sensitive nitroreductase II is present in resistant bacteria. Finally, the post-mitochondrial fraction from rat livers containing nitroreductases will activate nitrofurantoin to revert TA100-FRI under aerobic incubation conditions. Under anaerobic incubation conditions in the presence of post-mitochondrial liver fraction the number of revertants observed with TA100-FRI is markedly increased over the number of revertants observed under anaerobic incubation conditions in absence of liver postmitochondrial fraction or in aerobic conditions with the post-mitochondrial liver fraction. This reinforces the evidence that mammalian enzymes can activate nitrofurans to

mutagens (Rosenkranz and Speck, 1976). Similar studies with TA100-FRI showed that metronidazole, a nitroimidazole, had similar mutagenic properties to nitrofurantoin. Also, TA-100-FRI was resistant to the lethal effects of other nitro compounds, such as picrolonic acid, 2 nitrofluorene and 2-nitronaphthalene indicating that the strain is deficient in a non-specific nitroreductase (Rosenkranz and Speck, 1975). Indeed, if a nitroreductase must be present to activate the nitrofurans to mutagens it would appear that the nitro group in the 5-position of the furan ring is essential for mutagenicity.

It has been discovered that a wide variety of nitroheterocyclic compounds are mutagenic in Salmonella typhimurium and E. coli and that aminoheterocyclic derivatives of these compounds are inactive mutagens in these same strains (Wang et al., 1975b; McMahon et al., 1976). Furan derivatives of mutagenic nitrofurans lacking the 5-nitro group are not mutagenic in E. coli (McCalla and Voutsinos, 1974).

Metabolites from the urine of rats fed nitrofuran compounds have been shown to be mutagenic in bacteria. The urine of rats fed N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT) were analyzed for metabolites of the nitrofuran. Of the three metabolites identified, one (2-amino-4-(5-nitro-2-furyl) -thiazole or ANFT) was mutagenic in non-activated TA100 (Hayashida et al., 1976).

Many mutagenic agents cause prophage induction in bacteria. Nitrofurans have been shown to induce mass lysis through prophage induction in E. coli T44(λ). Corresponding furan derivatives are less lysogenic in E. coli T44 (λ) (McCalla and Voutsinos, 1974).

Nitrofurans have been tested in many eukaryotic systems. In non-mammalian eukaryotes the mutagenicity of nitrofurans has been confirmed in Saccharomyces cerevisiae (yeast) (Ong and Shahin, 1974), Neurospora crassa (Ong and Shahin, 1974), Bombyx mori (silkworm) (Tazima et al., 1975), and Euglena gracilis (Ikushima, T., 1975).

A number of mammalian test systems have been used to analyze the potential mutagenicity of nitrofurans. In a host-mediated study, a suspension of wild (Rec^+) and recombinational repair deficient (Rec^-) strains of Bacillus subtilis was injected into the peritoneal cavity of mice one hour after oral administration of furylfuramide. More Rec^- cells than Rec^+ cells were killed in the peritoneum of mice after doses of furylfuramide greater than 9 mg/kg (Tutikawa and Kada, 1975). A host-mediated reversion assay with E. coli WP2 $tryp^-$ cells injected into peritoneum of mice orally administered with furylfuramide was positive (Tazima et al., 1975).

In cultured embryonic human lung cells positive mutagenicity of furylfuramide has also been demonstrated. This system detects mutation through a drug resistance marker: the reversion from azoguanine sensitive to

azoguanine resistant cells ($\underline{AG}^{S} \rightarrow \underline{AG}^{r}$). Furylfuramide at 3 μg/ml induced a mutational frequency 2.62 times as high as the spontaneous mutational frequency (Kuroda, 1974).

Analysis of metaphase cell spreads for chromosomal aberrations *in vitro* has been conducted using cultured human lymphocytes exposed to a number of nitrofurans. Dose ranging from 0.5 μM to 100 μM were tested. The chromosomes were evaluated for gaps, breaks, and exchanges. In cultures tested with furamizole, nitrofurylacrylamide, furpyrinol, and furylfuramide a significant number of all these chromosomal structural aberrations were observed in every case. The levels of chromosomal aberrations produced by equimolar concentrations of these compounds decreased in the following order: furamizole > nitrofurylacrylamide > furpyrinol > furylfuramide. However, nitrofurazone, nitrofurantoin, and furazolidone produced no significant increase in chromosomal aberrations (Tonomura and Sasaski, 1973). DNA repair synthesis (unscheduled DNA synthesis) was examined using cultured fibroblasts exposed to nitrofurans obtained from the skin biopsies from a normal person and from a patient with xeroderma pigmentosum. None of the nitrofurans tested induced the unscheduled DNA synthesis in the xeroderma pigmentosum cells. However, in normal cells, furamizole, nitrofurylacrylamide, furpyrinol, and furylfuramide were effective in inducing unscheduled DNA synthesis in the dose range 5.0 μM to 100 μM. The level of unscheduled DNA synthesis stimulated by an equimolar

concentration of these compounds decreased in the following order: furamizole > nitrofurylacrylamide > furypyrinol > furylfuramide. Within the dose range tested, nitrofurazone, nitrofurantoin, and furazolidone did not significantly increase unscheduled DNA repair synthesis (Tonomura and Saski, 1973).

The mutagenicity of furylfuramide was investigated by cytogenetic analysis on rat bone marrow cells in vivo. Male Long-Evans rats treated with furylfuramide demonstrated chromosomal aberrations following both oral and ip administration. Upon ip administration of 240 mg/kg furylfuramide in physiological saline the peak of observed chromosomal aberrations occurred at 6 hours after administration with a return to baseline frequency within 24 hours. The lowest doses causing a statistically significant increase in chromosomal aberrations from negative control were 15 mg/kg following i p administration and 30 mg/kg following oral administration. The mean value of aberrant cells in untreated animals was 1.1 percent; in animals treated with 240 mg/kg i p the mean value of aberrant cells was 8.9 percent 6 hours post-treatment. Breaks constituted 76 percent of the aberrations and gaps constituted 24 percent of the aberrations. No exchanges were observed between chromosomes (Sugiyama et al., 1975). In mice administered as much as 200 mg/kg furylfuramide orally the number of chromosomal aberrations was not

statistically different from control (Miyaji, T., 1976; Tazima et al., 1975).

Several dominant lethal tests have been conducted on furylfuramide. Male mice were orally treated with up to 513 mg/kg then mated with female mice and the number of early fetal deaths and resorptions were recorded. All dominant lethal tests with furylfuramide have been negative (Soares and Sheridan, 1975; Tazima et al., 1975; Tutikawa and Kada, 1975).

Carcinogenicity

The mutagenic properties of nitrofurazone lead to speculation that it might induce mutations leading to neoplasms (Zampieri and Greenberg, 1964). The subsequent evaluation of the carcinogenicity of nitrofurazone is a good example of the subjective analyses and controversial criteria used in carcinogenicity testing of chemicals.

In 1966 and 1967 Norwich Pharmacal submitted data on two studies to the Food and Drug Administration on the cumulative effect of feeding diets containing nitrofurazone to female Sprague-Dawley (Holtzmann strain) rats. The first study comprised 3 groups, each containing 35 female rats: 1) one group served as an untreated negative control, 2) one group received 28 mg nitrofurazone/kg in the diet daily, and 3) one group received 55 mg nitrofurazone/kg in the diet daily. The rats received their respective diets for 45

weeks, followed by maintenance on an unmedicated diet for 8 weeks before sacrifice. Upon histological examination by Norwich a total of 19 mammary tumors were observed in 13 of the 30 rats examined that were treated with 55 mg nitrofurazone/kg daily. Norwich reported 18 mammary tumors in 12 of the 33 rats fed 28 mg nitrofurazone/kg daily and no mammary tumors of any type in the 33 control rats examined. Norwich reported that most of the tumors were fibroadenomas; no adenocarcinomas were reported. However, an ad hoc committee was established to review the histopathology in the nitrofurazone feeding study. This committee concluded that 14 of the 30 rats treated with 55 mg nitrofurazone/kg daily had tumors that were primarily fibroadenomas and that 4 of these rats had mammary adenocarcinomas. In the rats fed 28 mg nitrofurazone/kg daily the ad hoc committee concluded that 12 of the 33 rats examined had mammary tumors. One rat had a mammary adenocarcinoma; the remainder of the tumors were fibroadenomas. A pathologist from the Food and Drug Administration confirmed the ad hoc committee findings. However, the ad hoc committee did not review all the negative control slides, thus leaving a somewhat incomplete comparison to negative control (Federal Register, 1976).

In the second study, nitrofurazone was fed to 20 male and 20 female Carworth Farms rats at 50 mg/kg daily for 45 weeks followed by an unmedicated diet for an additional 7 weeks before sacrifice. A comparable control group of rats

was maintained on a nitrofurazone-free diet for 52 weeks. Again, as in the previous study, nitrofurazone induced tumors of the mammary glands in female rats. Norwich reported that no tumors were observed in the 15 female control and 18 male control rats examined. Norwich reported from histopathological examination that 11 out of 19 nitrofurazone treated female rats examined had a total of 28 mammary tumors. No mammary tumors were observed in the male rats treated with nitrofurazone. Norwich reported 3 mammary adenocarcinomas among the 28 mammary tumors reported; it was not indicated by Norwich if these adenocarcinomas were seen in 3 different rats or in the same rat. The ad hoc committee reviewed the slides of the mammary tissues from the 19 nitrofurazone treated female rats provided by Norwich. They also concluded that 11 of the nitrofurazone treated rats had mammary tumors. However, from the slides reviewed they found only one rat with mammary adenocarcinomas. It is not known if the ad hoc committee had all the slides that Norwich had diagnosed as mammary adenocarcinomas. The pathologist from the Food and Drug Administration reviewed slides of the mammary tumors reported by the ad hoc committee. He confirmed mammary tumors in each of the 11 rats with the following diagnostic distribution: 9 slides of mammary adenoma, 1 slide of mammary fibroma, 19 slides of mammary fibroadenoma, and 1 slide of mammary adenocarcinoma. However, neither the ad hoc committee nor the Food and Drug Administration reviewed

the histopathology from control rats (Federal Register, 1976).

Even with the relative confusion of the analysis of the histopathology data of the two studies mentioned two points have emerged: 1) nitrofurazone causes an induction of mammary tumors in female rats upon chronic oral administration for a year and 2) there is a strong indication that nitrofurazone induces mammary adenocarcinomas upon chronic oral administration to female rats (Federal Register, 1976).

In similar chronic feeding studies, induction of mammary fibroademomas by nitrofurazone was confirmed in female Sprague-Dawley rats (Erturk et al., 1970). However, furaldehyde semicarbazone, the anitro analog of nitrofurazone, did not cause an induction of mammary fibroadenomas in female Sprague-Dawley rats upon chronic oral administration (Erturk et al., 1970). From these data the nitro group in the 5-position of the furan ring appears to be essential for the induction of tumors in female rats and may be essential for the carcinogenicity of nitrofurans.

Furylfuramide, a nitrofuran formerly used as a food additive in Japan, was initially deduced to be a non-carcinogen based on chronic feeding studies in male and female ICR/Jcl mice (Miyaji, 1971). However, the reported mutagenicity of furylfuramide lead to renewed concern that it may be a potential carcinogen and that new studies should be undertaken (deSerres, 1974). Tumors in the forestomach

were induced in ddy/Slc mice after feeding on a diet containing furylfuramide for 18 months (Nomura, 1975). In another study, pregnant female ICR/Jcl mice received subcutaneous injections of 50 mg furylfuramide/kg in propylene glycol on days 13, 15, and 17 of pregnancy. The offspring were killed 32 weeks after birth and examined for tumors. The offspring whose mothers were treated with furylfuramide had a higher incidence of lung tumors (papillary adenomas) than did the offspring from mothers who received solvent alone (Nomura, 1975). In a separate test, 21 day old mice receiving 3 subcutaneous injections of 50 mg furylfuramide/kg in propylene glycol on day 21, 22, and 23 after birth developed an increase in lung tumors at week 27 when compared to solvent treated controls. A second group of mice received only one subcutaneous injection of furylfuramide at 100 mg/kg in propylene glycol on day 21 after birth. This group did not exhibit a significant increase in tumor induction over the solvent treated negative control group (Nomura, 1975). These data support three major conclusions: 1) furylfuramide can induce tumors in mice; 2) the ability of furylfuramide to induce tumors in mice is favored by multiple dosing over single dosing, and 3) furylfuramide possesses the ability to act as a transplacental carcinogen in mice.

Female Sprague-Dawley rats fed chronically up to 0.3% of their diet with nitrofurantoin over nearly a year do not

exhibit an increased frequency of tumor formation (Morris et al., 1969; Cohen et al., 1973a).

Nitrofurazone, furylfuramide, and nitrofurantoin carcinogenicity were presented as specific examples of the problems of testing the nitrofurans for carcinogenicity. Nine 5-nitrofuran compounds having the thiazole ring at the 2-position of the furan have been shown to be potent carcinogens. Carcinogenicity tests with the nitrofurans have been executed mainly in rats and mice, but dogs and hamsters have also been tested and shown to be susceptible to the carcinogenic properties of nitrofurans. Lung, breast, stomach, kidney, bladder, gall bladder, and ovary are some of the organs where nitrofurans have induced tumor formation. Nitrofurans have also induced leukemia in mice (Tazima et al, 1975).

The data on nitrofurazone and furylfuramide demonstrate that a given chemical, even though previously tested and indicated to be a non-carcinogen, should be tested or retested for carcinogenicity in animals if found to be mutagenic (Nomura, 1975). Of 25 nitrofurans tested for carcinogenicity, 22 have been found to be positive carcinogens (Tazima et al, 1975). Perhaps the 3 nitrofurans which are negative carcinogens (e.g. nitrofurantoin) should be further scrutinized for carcinogenic properties.

Metabolism, Absorption, Distribution, and Excretion

The most striking feature in biotransformation studies of nitrofurans is the ability of a wide range of biological systems to reduce the nitro group attached to the C_5 carbon in the furan ring. This is the most thoroughly investigated area of nitrofuran metabolism. The reduction of the nitro group on the furan ring has been shown to result in two major identifiable metabolites: the aminofuran analog and the open chain nitrile derivative (Figure I-1, Pathways 1A and 1B; Figure I-2) (H. Paul et al., 1960, Tazima et al., 1975). The open chain nitrile is believed to be formed from spontaneous ring cleavage of the aminofuran analog (Beckett and Robinson, 1959). Upon anaerobic incubation of nitrofurazone with xanthine oxidase or rat liver microsomes nitroreduction occurs with the formation of the aminofuran analog (5-amino-2-furaldehyde semicarbazone) (Tatsumi et al., 1976); further incubation with xanthine oxidase results in formation of the open chain nitrile derivative (4-cyano-2-oxobutyraldehyde semicarbazone) (Tatsumi et al., 1976). The aminofuran analogs of 5-thiazolylnitrofuran derivatives have been isolated upon anaerobic incubation with various mouse tissue homogenates (Chatfield, 1977). Mice administered 5-thiazolylnitrofuran derivatives ip exhibited evidence of nitroreduction in vivo as shown by isolation of the open chain nitrile derivatives in the urine (Chatfield, 1977). In rabbits administered 5-nitro-2-furaldehyde

acetylhydrazone orally or ip, 5-acetamido-2-furaldehyde acetylhydrazone (or a N-acetylamino metabolite) was isolated from the urine as well as traces of 5-diacetylamino-2-furaldehyde acetylhydrazone (or a N,N-bis-acetylamino metabolite) and 5-amino-2-furaldehyde semicarbazone (Olivard et al., 1962). Rats and chickens fed 5-nitro-2-furaldehyde acetylhydrazone also excreted 5-acetamido-2-furaldehyde acetylhydrazone in their urine (Olivard et al., 1962). These studies indicate that 5-nitro-2-furaldehyde acetylhydrazone is enzymatically reduced to the amine analog which is enzymatically acetylated to form the acetylamido (or N-acetylamino) analog (Figure I-1, Pathways 1A and 1C) (Olivard et al., 1962).

The formation of polymers of high molecular weight is another consequence of nitrofuran reductive metabolism (Figure I-1, Pathway 1A; Figure I-2). Drug derived polymers have been isolated from the urine of rats fed nitrofurazone (H. Paul et al., 1960) as well as from the enzymatic reduction of nitrofurazone by nitroreductase II (Asnis, 1957). This area of nitrofuran metabolism has been little studied.

Tissue slices from various rat organs can reduce nitrofurans aerobically (Bender and Paul, 1951; H. Paul et al., 1960; McCalla et al., 1971a). Liver, kidney, and teste tissue slices can reduce nitrofurazone at higher rates than skeletal muscle, heart, lung, and spleen tissue slices (McCalla et al., 1971a). As a rule, however, oxygen

inhibits nitroreduction of nitrofurans in tissue homogenates and in most purified enzyme systems (e.g., xanthine oxidase and aldehyde oxidase) (Akao et al., 1971; Tatsumi et al., 1973; Wolpert et al., 1973).

Bacteria, including those from the gastrointestinal tract, are known to contain at least two different classes of nitroreductase. Nitroreductase I from E. coli uses either NADH or NADPH as an electron donor and is unusual in that it is not inhibited by oxygen (Asnis, 1957; McCalla et al., 1975). Nitroreducatse-II from E. coli can utilize only NADH as an electron donor and is inhibited by oxygen (Asnis, 1957; McCalla et al., 1975). Nitroreductase-I has been purified and has a molecular weight of approximately 50,000 (McCalla et al., 1975). Upon purification nitroreductase-II was resolved into nitroreductase IIa (molecular weight = 120,000) and nitroreductase-IIb (molecular weight = 700,000) (McCalla et al., 1975). Reduction of nitrofurazone by nitroreductase I from E. coli results in a brownish discoloration of the reaction mixture. This discoloration cannot be removed by dialysis, suggesting that an intermediate reduction product polymerizes to form a resin of high molecular weight (Asnis, 1957). Nitroreductase II does not cause discoloration upon reducing nitrofurazone but does cause a new absorption peak at 335 nm not observable in the reaction catalyzed by nitroreductase I (Asnis, 1957). The absorption maximum at 335 nm corresponds to the absorption maximum of 5-amino-2-furfuraldehyde semicarbazone

(Ebetino et al., 1962), the aminofuran analog of nitrofurazone, suggesting the formation of the aminofuran analog by nitroreductase I. These data suggest different mechanisms of nitroreduction for nitroreductase I and nitroreductase II.

There is strong evidence for the formation of reactive intermediate metabolite(s) upon the enzymatic nitroreduction of nitrofurans. Incubation of E. coli sensitive to the bactericidal action of nitrofurazone with ^{14}C-nitrofurazone causes the radiolabel to be covalently bound to the trichloroacetic acid insoluble fraction of intact cells. E. coli resistant bacteria, which lack nitroreductase I, do not show extensive binding of the radiolabel to the trichloroacetic acid insoluble fraction. Bacteria of intermediate sensitivity to nitrofurazone show intermediate nitroreducatse activity as well as intermediate binding of the radiolabel of ^{14}C-nitrofurazone to the trichloracetic acid insoluble fraction (McCalla et al., 1970). Treatment with ribonuclease and deoxyribonuclease to solubilize the purine and pyrimidine base analogs of RNA and DNA did not result in a measurable decrease in the bound radiolabel (McCalla et al., 1970), suggesting that the majority of the radioactivity bound to cellular material is associated with protein (McCalla et al., 1970). This may be due to the overwhelming amount of protein relative to DNA in the bacteria. However, bacteria which have no deficiency in nitroreductase I exhibit DNA breaks upon exposure to

nitrofurans whereas bacteria which lack nitroreductase I do not exhibit breaks in DNA upon the same treatment (McCalla et al., 1971b). Reduction of ^{14}C-nitrofurazone by purified nitroreductase from bacteria or xanthine oxidase will also cause binding of the radiolabel to serum albumin (McCalla et al., 1970). Nitroreduction of nitrofurans is a prerequisite for binding of metabolite to the trichloroacetic acid insoluble fraction of 9,000 x g rat liver supernatant and rat liver microsomes (McCalla et al., 1971a; Wang et al., 1975a).

Tissue slices from rats exhibited covalent binding of the radiolabel from ^{14}C-nitrofurazone upon aerobic incubation. There was good correlation between the ability of a given tissue to reduce nitrofurazone and to bind the radiolabel from ^{14}C-nitrofurazone. Liver, kidney, and testes exhibited greater binding than spleen (McCalla et al., 1971a).

Tissue binding has been demonstrated in vivo in animals administered nitrofurans (Stripp et al, 1973; Wang et al., 1975a). The radiolabel of ^{14}C-nitrofurazone became covalently bound in various tissues following ip injections in mice. Covalent binding occurred almost equally in liver, kidney, mammary tissues, testes, plasma, and red blood cells (0.5-1.0 nanomole/mg protein) one to two hours after doses of 600 to 1000 mg/kg (Stripp et al., 1973).

Certain sulfhydryl containing compounds, such as glutathione and cysteine, can protect against covalent

binding of nitrofurans to tissue macromolecules upon reduction. Glutathione and cysteine cause an eight to ten fold decrease in binding of the nitroreductive metabolites of N-[4-(5-nitro-2-furyl)-thiazolyl] acetamide to the trichloroacetic acid insoluble fraction of rat liver microsomes upon anaerobic incubation (Wang et al., 1975a). Upon reduction of nitrofurazone by xanthine oxidase, cysteine will protect against covalent binding to serum albumin by reduced nitrofurazone metabolite (McCalla et al., 1970). Depletion in vivo in the mouse of glulathione by diethyl maleate prior to administration of ^{14}C-nitrofurazone caused a doubling of binding of the radiolabel in most tissues (Stripp et al., 1973). Nitrofurazone also decreased the hepatic glutathione levels 50 percent two hours after ip administration of 600 to 1000 mg/kg to mice (Stripp et al., 1973). Also, covalent binding of the radiolabel of ^{14}C-nitrofurazone was inhibited by glutathione in 9,000 x g dialyzed supernatant of rat testes and liver. These data suggest that nitrofuran metabolite tissue binding can occur throughout the body and that labile sulfhydryl groups may be intimately involved (Stripp et al., 1973).

The observation that tissue binding of the reduced metabolites of nitrofurans occurs and that sulfhydryl containing compounds protect against tissue binding have led to speculation that nitrofurans may form conjugates with sulfhydryl containing compounds. Certain nitrofuran derivatives, such as 5-nitro-2-furaldehyde, and 5-nitro-2-

furanmethandiol diacetate (Table III-1; No. 1) appear to form glutathione conjugates catalyzed by glytathione S-transferase from rat liver and release the nitro group as inorganic nitrite (Boyland and Speyer, 1970). Certain nitrofurans will appear to spontaneously form glutathione conjugates with release of the nitro group as inorganic nitrite, but at a slower rate than observed when glutathione-S-transferase is present (Boyland and Speyer, 1970). None of the molecular structures of the glutathione conjugates have been determined (Boyland and Speyer, 1970). However 36 nitrofuran derivatives tested, including nitrofurazone and nitrofurantoin, did not form conjugates or release inorganic nitrite in the presence of glutathione and glutathione S-transferase (Boyland and Speyer, 1970). This is the only information published so far on conjugation reactions of nitrofurans with a sulfhydryl containing compound. The formation of the postulated conjugates of reduced nitrofurans with sulfhydryl compounds probably procedes by a totally different mechanism than that observed with glutathione and glutathione-S-transferase since nitroreduction is not involved in the latter reaction. It also should be noted that neither a glutathione conjugate nor any other sylfhydryl conjugate has ever been isolated or identified from in vivo studies in animals. In in vitro studies where glulathione or cysteine were shown to inhibit binding of nitrofuran metabolites to tissue macromolecules

there were no reports as to whether conjugates were formed (Stripp et al., 1973; Wang et al., 1975a).

Sulfhydryl group containg compounds, such as cysteine, have been shown to cause nitroreduction of nitrofurans (Matsuda, 1965; Matsuda and Nakanishi, 1965; Matsuda, 1966). Cysteine will cause significant nitroreduction upon heating with nitrofurans for 30 minutes (Matsuda, 1965). Nitrofurylacrylamide shows an increase in the rate of nitroreduction by cysteine with an increase in pH (Matsuda, 1965). Reduction of nitrofurylacrylamide and nitrofurazone by cysteine is increased by increasing the temperature, the length of time at elevated temperature, and the concentration of cysteine (Matsuda, 1965). Hemoglobin and ferrous iron (Fe^{++}) stimulate the reduction of nitrofurazone and furylfuramide by cysteine although neither hemoglobin nor ferrous iron reduce the nitrofurans by themselves (Matsuda and Nakanishi, 1965). Whale meat extract and fish meat extract will reduce nitrofurans upon boiling (Matsuda, 1966). Nitrofurylacrylamide and furylfuramide are somewhat more susceptible to nitroreduction by fish sausage meat than nitrofurazone (Matsuda, 1966). It is believed that sulfhydryl groups are responsible for the reduction of nitrofurans described (Matsuda, 1966).

Cysteine has been reported to greatly increase the rate of reduction of nitrofurazone by xanthine oxidase (Taylor et al., 1951). However, neither cysteine nor glutathione caused any alteration in the rate of nitroreduction of

N[4(5-nitro-2-furyl)-thiazolyl] acetamide by rat liver microsomes (Wang et al., 1975a).

A hypothesis has been proposed that upon enzymatic reduction of nitrofurans a reactive hydroxlamine intermediate is formed and is responsible for covalent tissue binding (McCalla et al., 1970; Wang et al., 1975a). Various investigators have claimed identification of hydroxylamine analogs formed upon enzymatic nitroreduction of nitrofurans (Taylor et al., 1951; H. Paul et al., 1960; Wolpert et al., 1974). However these hydroxylamine analogs of enzymatically reduced nitrofurans have never positively been identified in vitro or in vivo (Tatsumi et al., 1976).

A second possible pathway for the observed covalent binding of nitrofurans to tissue macromolecules is through the formation of nitroaromatic anion radicals. Nitroaromatic anion radicals formed by one-electron reduction of nitroaromatic and nitroheterocyclic compounds, including nitrofurans, upon enzymatic nitroreduction have been identified by electron spin resonance spectroscopy (Mason and Holtzmann, 1975; Wardman and Clarke, 1976). However, nitrobenzene did not covalently bind to any significant extent with protein upon anaerobic incubation with the 9,000 x g rat liver supernatant fraction, even though the nitroaromatic anion radical from nitrobenzene can be formed in such a system (Mason and Holtzmann, 1974). This would suggest that the formation of nitroaromatic anion radicals is not the mechanism by which the reduction

products of nitrofurans bind to tissue proteins. Nitrofurantoin has been proposed to cause lung toxicity through the formation of superoxide anion formed by oxygen interacting with the nitroaromatic anion radical (RNO_2^- + $O_2 \rightarrow RNO_2$ + O_2^-) (Mason and Holtzmann, 1975; Boyd et al., 1977).

A second route of metabolism for azomethine type nitrofurans is acid hydrolysis of the azomethine linkage in the gastrointestinal tract to yield 5-nitro-2-furaldehyde and a hydrazine derivative (Figure I-1, Pathway 2a) (Olivard et al., 1962). The 5-nitro-2-furaldehyde is oxidized and excreted as 5-nitro-2-furoic acid (Figure I-1, Pathway 2b). The cleaved hydrazine derivative may be further acetylated (Figure I-1, Pathway 2c). Rabbits fed 5-nitro-2-furaldehyde acetylhydrazone excreted 5-nitro-2-furoic acid and 1,2-diacetylhydrazine in their urine. However, if 5-nitro-2-furaldehyde acetylhydrazone was administered ip, neither of the preceeding metabolic products was demonstrable in the urine (Olivard et al., 1962). These data indicate that acid hydrolysis of azomethine-type nitrofurans can occur only in the gastrointestinal tract.

A third pathway of metabolism of nitrofurans involves oxidation of the furan ring (Figure I-1, Pathway 3a). Bright yellow, polar, labile metabolites absorbing near 415 nm have been isolated from the urine of animals fed different types of nitrofurans. Rabbits fed nitrofurazone excreted a crytallizable material which absorbed at 412.5 nm

(H. Paul et al., 1960). Rabbits, rats, dogs, and monkeys fed 5-nitro-2-furaldehyde acetylhydrazone excreted (in the urine) a similar metabolite absorbing at 415 nm (Olivard et al., 1962). A yellow, 417 nm absorbing metabolite was isolated from rats fed nitrofurantoin (Olivard et al., 1976) and was identified as the 4-hydroxy-5-nitrofuran derivative of nitrofurantoin (Olivard et al., 1976). The nitrofuran metabolites which absorb near 415 nm described by pervious investigators are believed to be the 4-hydroxy-5-nitrofuran derivatives of the parent compounds. The 4-hydroxy-5-nitrofuran derivatives exist in equilibrium with the corresponding aci-nitro tautomer forms (Figure I-1, Pathway 3b) (Olivard et al., 1976).

A second oxidative metabolic pathway for nitrofurans is oxidation of the side chain attached to the 2-position of the furan ring. An example of this would be oxidation of nifuradene to nitrofurantoin (Figure I-1, Pathway 4A).

The absorption, distribution, and excretion of nitrofurazone, furylfuramide, and nitrofurazone will be emphasized since these were the nitrofurans investigated in vivo in the rat for this dissertation problem.

Rats dosed orally with 100 mg nitrofurazone/kg in aqueous suspension had plasma levels of 4.5 μg nitrofurazone/ml (M. Paul et al., 1960). Approximately 34 percent of the nitrofurazone present in plasma is bound to plasma proteins (M. Paul et al., 1960).

Rats dosed orally with 100 mg nitrofurazone/kg (formyl-^{14}C, 0.12 μCi/mg) in 10 percent gum arabic excreted 61.2, 26.2, and 1.04 percent of the radiolabel after 24 hours and 66.1, 34.9, and 1.21 percent of the radiolabel after 96 hours in urine, feces, and respired carbon dioxide, respectively. Recovery in rats whose bile ducts were cannulated of the ^{14}C radiolabel in the bile amounted to 22.9 percent after 24 hours and 27.3 percent after 48 hours. Upon oral administration of the radioactive bile, 26.3 percent and 1.4 percent of radiolabel appeared in the urine and bile, respectively, after 24 hours, indicating only a slight potential for enterohepatic recirculation of the radiolabel. Only 0.08 percent, a trace, and 0.23 percent of the unchanged nitrofurazone was collected in the urine, feces, and bile, respectively, 24 hours after administration of nitrofurazone. This indicates that the vast majority of nitrofurazone undergoes biotransformation upon oral administration in rats (Tatsumi et al., 1971). In rats treated with Neomycin to kill gastrointestinal bacteria, the percentage of radioactivity in the urine and feces and the percentage of unchanged drug in the feces did not differ significantly from the pattern in rats with normal gastrointestinal flora (Tatsumi et al., 1973). This indicates that even though enteric bacteria of the rat can reduce nitrofurazone, they are not necessary for metabolic degradation in vivo. In vivo and in vitro studies indicate that nitrofurazone is degraded mainly in the mucosa of the

small intestine upon oral administration in the rat (Tatsumi et al., 1973). The absorption route of the radiolabel of ^{14}C-nitrofurazone upon oral administration is almost exclusively via the portal system after oral administration (Tatsumi et al., 1975).

Absorption and excretion studies of furylfuramide in rats were executed at the same time as the studies with nitrofurazone in the preceeding paragraph (Tatsumi et al., 1971; Tatsumi et al., 1973; Tatsumi et al., 1975). Rats dosed with furylfuramide (acrylamide-^{14}C, 0.15 μCi/mg) in 10 percent gum arabic excreted 19.5, 74.6, and 0.40 percent of the radiolabel after 24 hours and 21.9, 79.7, and 0.52 percent of the radiolabel after 96 hours in urine, feces, and respired carbon dioxide, respectively. Recovery of the ^{14}C radiolabel in the bile of rats whose bile duct was cannulated amounted to 29.4 percent after 24 hours and 38.6 percent after 48 hours. Upon oral administration of radioactive bile, 2.3 percent and only a trace of the radiolabel appeared in the urine and bile, respectively, after 24 hours, indicating essentially no potential for enterohepatic recirculation. Only 0.05 percent, a trace, and 0.07 percent of the unchanged furylfuramide was collected in the urine, feces, and bile, respectively, after 24 hours. This indicates that furylfuramide, like nitrofurazone, undergoes almost complete biotransformation upon oral administration to rats. In rats treated with Neomycin the percentage of radioactivity in the urine and

feces and the percentage of unchanged drug in the feces did not differ significantly from the corresponding data from rats with normal gastrointestinal flora (Tatsumi et al., 1973). As with nitrofurazone, this indicates that even though enteric bacteria of the rat can reduce furylfuramide, they are not necessary for metabolic degradation in vivo. Also like nitrofurazone, furylfuramide appears to be mainly degraded in the mucosa of the small intestine upon oral administration (Tatsumi et al., 1973). Finally, the absorption route of the radiolabel of ^{14}C-furylfuramide upon oral administration is almost exclusively via the portal system in the rat (Tatsumi et al., 1975).

Rats administered 100 mg nitrofurantoin/kg orally had plasma levels of 2.6 µg nitrofurantoin/ml at 4 hours after administration (M. Paul et al., 1960). Approximately 53 percent of the nitrofurantoin present in the plasma of the rat is bound to plasma proteins (M. Paul et al., 1960). Nitrofurantoin appears at a concentration of 2 to 5 µg/ml in blood after therapeutic oral doses of 400 to 800 mg/day (100 to 200 mg q.i.d.) in humans even if there is impaired renal function (M. Paul et al., 1960).

Rats administered 25 mg nitrofurantoin/kg orally excrete 52 percent of the dose as unchanged nitrofurantoin in 48 hours (M. Paul et al., 1960). Normal humans receiving 600 mg nitrofurantoin/day (150 mg q.i.d.) excreted an average of 44.3 percent of the nitrofurantoin unchanged in the urine within 24 hours (Beutner et al., 1954/55). Patients taking

nitrofurantoin for urinary tract infactions on the same regimen excreted an average of 34.1 percent of the nitrofurantoin as unchanged drug in the urine within 24 hours (Beutner et al., 1954/55). The maximum urine concentrations of nitrofurantoin was 420 µg/ml in the normal humans and 250 µg/ml in patients with urinary tract infections (Beutner et al., 1954/55).

Rats administered 25 mg nitrofurantoin/kg orally excreted only 2.0 percent of the dose as unchanged nitrofurantoin in the feces after 48 hours (M. Paul et al., 1960).

Nitrofurantoin is present in the milk of dogs at a concentration of 2 to 33 µg/ml four hours following an oral administration of 20 mg nitrofurantoin/kg (M. Paul et al., 1960). Dogs administered 15 mg nitrofurantoin/kg orally have saliva concentrations of 1.6 µg/ml one hour after administration (Buzard et al., 1961).

Nitrofurantoin has been identified in the bile of chickens, mice, rats, dogs, and humans after oral administration (M. Paul et al., 1960). A patient orally administered 100 mg nitrofurantoin/kg had a concentration of 34 µg/ml in the bile 2 hours after administration (M. Paul et al., 1960).

Nitrofurantoin has been shown to be rapidly absorbed upon oral administration in the rat (Buzard et al., 1961). The small intestine is the chief site of absorption (Buzard et al., 1961). Nitrofurantoin can be reabsorbed from the

urinary bladder into the blood stream (Buzard et al., 1961).

General Toxicity

Animal studies reveal that nitrofurans, given in sufficient quantities, can cause growth depression, emesis, neurotoxicity, interference with spermatogenesis, and changes in the adrenal glands (Paul and Paul, 1964). Nitrofurazone general toxicity will be used as a model in this discussion.

The oral LD_{50} during a 7-day observation period of nitrofurazone is 590 mg/kg body weight in Donryu rats and 640 mg/kg body weight in ICR/JCL mice (Miyaji, 1971).

Toxic effects of high doses of nitrofurazone in animals include growth retardation, neurotoxicity, renal tubular, hepatic, adrenal and testicular cytotoxicity and inhibition of immunocompetence (International Agency for Research on Cancer, 1974).

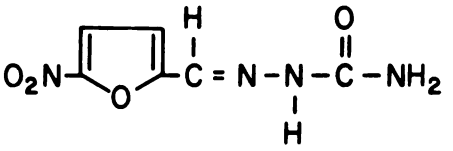
In oral studies with mice, hyperirritability is the most prominent sign when nitrofurazone is given in doses of 300 mg/kg or more. Lethal doses resulted first in hyperirritability, tremors, and convulsions. Death was apparantly from respiratory failure (Paul and Paul, 1964).

Nitrofurazone causes spermatogenic arrest in many animals, including mice, rats, and dogs (Paul and Paul, 1964). The testes of animals treated with nitrofurazone atrophy due to spermatogenic arrest (Paul and Paul, 1964). Other nitrofurans, e.g. nidroxyzone, also cause spermatogenic arrest in animals.

Appendix I-1

I. Chemical and Physical Properties of Nitrofurazone

A. Chemical Formula --



B. Molecular Weight - 198.14

C. Synonyms (International Agency for Research on Cancer, 1974)

1. Chemical - There are at least 12 chemical synonyms for nitrofurazone. The most common are 5-nitro-2-furaldehyde semicarbazone and nitrofurazone (generic name).

2. Trade Names - There are over 60 trade names for nitrofurazone, the most well known being Furacin^{R} (Norwich Pharmacal).

3. Chem. Abstr. Name - hydrazinecarboxamide, 2-[(5-nitro-2-furanyl)methylene]. Chem.. Abstr. No. - 59-87-0.

D. Description

1. A microcrystalline, lemon yellow solid, appearing also in polymorphous forms (International Agency for Research on Cancer, 1974).

2. Pale yellow needles (Paul and Paul, 1964).

E. Melting Point

1. 236-240°C (decomposition) (International Agency for Research on Cancer, 1974).

2. 227-241°C (decomposition) (Paul and Paul, 1964).

F. U V Absorption Spectrosopy (International Agency for Research on Cancer, 1974).

1. (In methanol) - λmax 365 nm (log e=4.5707)
λmax 260 nm (log e=4.5515)
λmin 302 nm

2. (In ethanol - water (.005%)) - λmax 375 nm (log e=4.5150)
λmax 260 nm (log e=4.5150)
λmin 306 nm

G. Solubility of Nitrofurazone per Liter of Solvent

Grams Nitrofurazone	Solvent (1 Liter)
67	Dimethyl Formamide[1]
11.6	Polyethylene Glycol[1]
2.41	Acetone[1]
0.92	Ethanol (95%)[2]
0.238	Water[1]
0.210	Water[2]
0.037	Chloroform[1]
0.022	Chloroform[2]
0.023	Benzene[1]
0.015	Peanut Oil[2]

1. International Agency for Research on Cancer, 1974.
2. Paul and Paul, 1964.

H. Stability

Nitrofurazone is stable if protected from light. Nitrofurazone undergoes acid hydrolysis at pH 1.6 (Paul et al., 1960).

Table I-1

Principle Uses of Nitrofurans in Human Medicine

Compound	Clinical Usage
Nitrofurazone	Used topically for treatment of surface bacterial infections and infections of conjuctiva and cornea. Used in urethral inserts for urethral inflammations and in vaginal suppositories for bacterial vaginitis and cervicitis. Used in solutions for bacterial otitis externa, bacterial otitis media and in nasal decongestant combinations.
Nitrofurantoin	Used orally for treatment of acute and chronic infections of the genitourinary tract, including pyelonephritis, pyelitis, cystitis and prostatitis.
Furazolidone	Used orally for infectious gastroenteritis of various etiologies. Used in treatment of cholera.
Nifuroxime	Used combined with furazolidone in suppositories for treatment of vaginitis due to certain fungi, bacteria, and protozoa.
Nitrofurfuryl Methyl Ether	Used topically as a fungicide, sporacide and bactericide. Used in treatment of dermatomycoses

(Paul and Paul, 1964; Miura and Reckendorf, 1967; International Agency for Research on Cancer, 1974; Goodman and Gilman, 1975).

Table I-2

Principle Veterinary Uses of Nitrofurazone

Compound	Veterinary Usage
Nitrofurazone	Used topically against bacterial infections of surface lesions of the skin, eye, ear, udder and genital tract. Used in combination with penicillin in treatment of bovine mastitis. Used orally in treatment of porcine necrotic enteritis, gray diarrhea in mink, avian coccidiosis, canine coccidiosis, coccidiosis in sheep, avian typhoid and avian pullorum.
Nitrofurantoin	Used orally for urinary tract infections. Also indicated in canine epizootic tracheobronchitis (kennel cough).
Furazolidone	Used orally for a number of avian diseases such as coccidiosis, typhoid, pullorum, paratyphoid, blackhead, paracolon, sinusitis, hexamitiasis, blue comb, chronic respiratory distress syndrome, vibrionic hepatitis, synovitis, histomoniasis and non-specific enteritis. Used orally for a number of porcine diseases, such as bacterial enteritis, infectious hemorrhagic enteritis and vibrionic dysentery. Used orally as a growth promoter in poultry and hogs. Used to increase egg production in chickens.
Nihydrazone	Used in treatment of avian diseases such as coccidiosis, chronic respiratory disease syndrome, pullorum, typhoid, paratyphoid and histomoniasis.
Furaltadone	Used in treatment of bovine mastitis. Also used in avian disease such as typhoid, pullorum, paratyphoid and chronic respiratory disease syndrome.
Nifuraldezone	Used to treat bacterial enteritis in calves (scours).

Nidroxyzone	Used as growth promoter in chickens and hogs. Used to increase egg production in chickens.
Nifuroxime	Used topically against surface infections.
Nitrofurfuryl Methyl Ether	Used topically in surface infections and in dermatomycoses.

(Paul and Paul, 1964; Merck & Co., 1967; Miura and Reckendorf, 1967; International Agency for Research on Cancer, 1974)

Figure I-1. Metabolic Pathways in the Biotransformation of Azomethine-Type Nitrofurans.

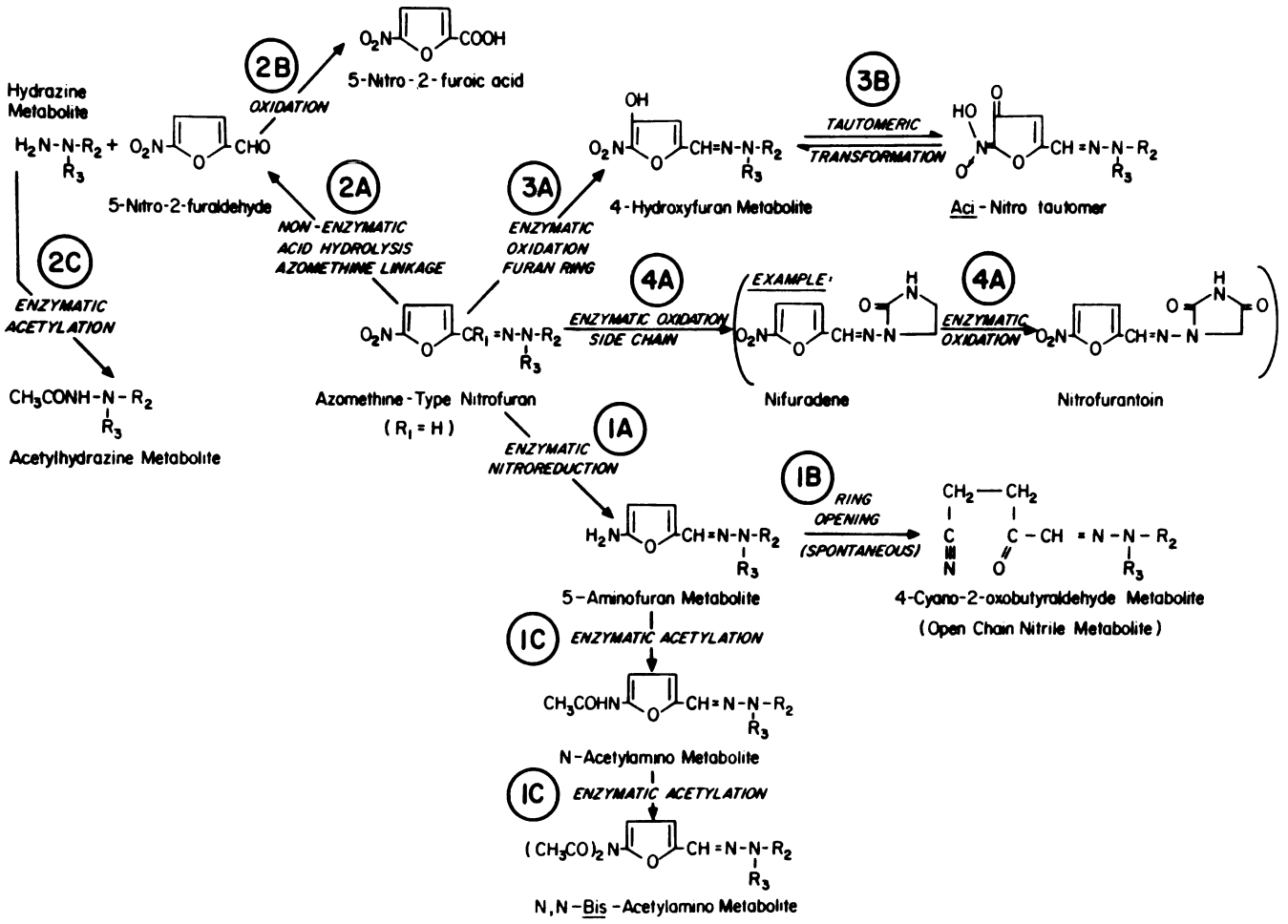
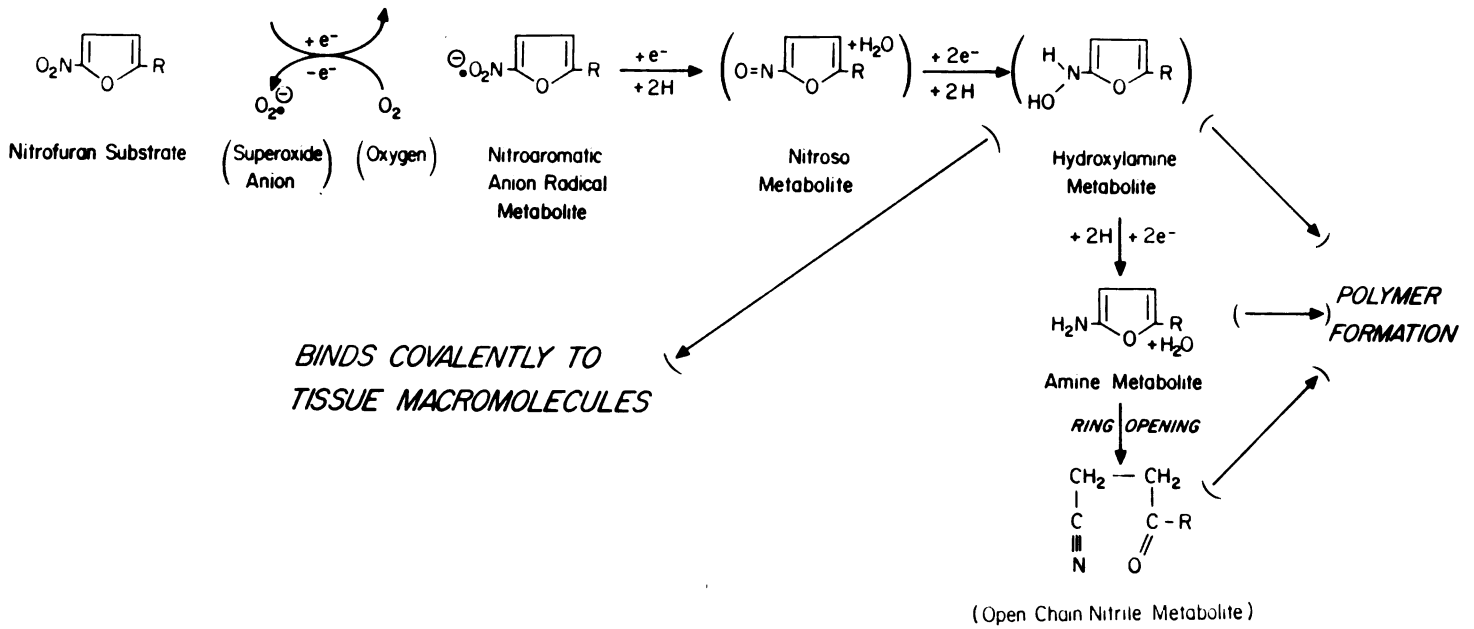


Figure I-2. Enzymatic Nitroreductive Pathways of Nitrofuran Compounds.



II. GENERAL CONCEPTS IN MUTAGENESIS

The term mutation was originally coined by the Dutch botanist Hugo de Vries in the 1880's. He used the term mutation to describe sudden hereditary changes in Lamarck's evening primrose, Oenothera lamarckiana. Although the changes de Vries observed were not mutations in the strictest sense, the term mutation has been retained to define any heritable change in genetic material (Auerbach, 1976; Gardner, 1968).

Since DNA is the principal genetic material, mutagens must alter DNA directly or indirectly. This includes not only a chemical transformation of an individual gene, called a gene or point mutation, but also structural and numerical changes in chromosomes which contain, as a rule, the vast majority of DNA within a cell (Gardner, 1968; Freeze, 1971; Strauss, 1971; Grice and DaSilva, 1973; Legator et al., 1973; Goldstein et al., 1974). Geneticists will sometimes restrict the term mutation to point mutations only (Gardner, 1968; Goldstein et al., 1968). However, it is not always possible in actual practice to distinguish between point mutations and structural changes. Chromosome aberrations vary in size from gross structural changes to those at the border of visibility with the light microscope to those at the molecular level of magnitude not visible with the light microscope (Gardner, 1968).

Several kinds of mutations have been identified. 1) Base Pair transformation: A given base pair may be replaced in the DNA molecule to give rise to a base pair transformation. Transitions and transversions are the two types of base pair transformations. A transition occurs in the DNA molecule if the original purine is replaced by another purine and the original pyrimidine is replaced by another pyrimidine. A transversion occurs if a purine is replaced by a pyrimidine or if a pyrimidine is replaced by a purine in the DNA molecule. 2) Frameshift mutation: An addition or deletion of a given base pair can occur in the DNA molecule. This is called a frame shift mutation because the ordered translation of the codons will be dramatically changed. A frame shift mutation therefore usually has more serious consequences. 3) Rearrangements, deletions, and breaks: These effects in the DNA molecule are visible at the microscopic level. Breaks and gaps may be observed in the chromosomes. Segments of chromosomes may be inverted or material may be translocated from one chromosome to another. Exchanging of material between two different chromosomes can also be observed. 4) Non-disjunction: An unequal partition of chromosomes between daughter cells creating the gain or loss of extra chromosomes into the daughter cells (Grice and DaSilva, 1973; Legator et al., 1973; Goldstein et al., 1974).

Mutations may occur in any cell and be expressed upon division. In higher animals two general types of cells are

of interest in mutagenic evaluation. These are germinal cells, which are used for the propagation of the organism, and somatic cells, or all the cells which do not contribute to the progeny of the organism (Freeze, 1971). Mutations in either germinal or somatic cells can be of great consequence. Mutation of a germ cell, if severe, may result in cell death. The consequences of this are usually minor since the cell can usually be replaced, provided that cell death is not extensive. However, if the cell survives, the consequences can be severe, since mutations that leave the germ cells viable can lead to an early tetal death or abnormally developed offspring. Germinal mutations may also affect development in the progeny at a later stage of life. Finally, if the mutation is recessive heterozygous in the progeny it will not be expressed phenotypically in that individual but may be expressed in his progeny if the mutation becomes homozygous. The phenotype may take many generations to express itself, but the recessive trait will be passed from generation to generation. As can be seen, the impact on the human gene pool is greatest for recessive mutations. Dominant viable mutations usually have a slight impact by comparison on the human gene pool. This is usually because of decreased reproductive capacity or of the recognition of a genetic trait that discourages willingness to have progeny. Lastly, dominant lethal mutations have no impact on the human gene pool (Freeze, 1971).

The consequences of a mutation in a somatic cell depends on the developmental state of the organism, the type of cell affected, the number of cells affected, and the type of genetic alteration that has been produced. Cell death, as a rule, is usually least harmful to the organism except in early differentiation during embryogenesis of the organism. At this stage the death of a few cells can be disastrous for normal development and a teratogenic effect will occur. Naturally, death of many cells in the adult organism can be detrimental (Freeze, 1971).

Somatic mutations, presumably by affecting duplicating cells leading to abnormal cell types with uncontrolled duplication, may result in a cancer. This is the somatic-cell mutation theory of carcinogenesis. This theory has recently gained support through the observation that many known carcinogens have been shown to be mutagens (Miller and Miller, 1971; Bartsch, 1976). Over 80 percent of a wide variety of carcinogens have been detected as mutagens (Ames et al., 1975; de Serres, 1976).

Chemical mutagens are thought to cause mutations through reactive elecrophilic derivatives, such as reactive carbonium ions or free radicals which bind or react with DNA. Certain of these chemicals are electrophiles without further modificiation, e.g., nitrogen mustards, imines, and epoxides. Other chemicals need to be metabolized to form mutagenic electrophilic compounds, e.g., polycyclic aromatic hydrocarbons, aromatic amines, and aminoazo dyes. However,

not all chemicals cause mutations through electrophilic derivatives, e.g., intercalating agents and base analogs. The formation of electrophilic intermediates, however, is believed to be the most common mechanism of chemical mutagenesis (Freeze, 1971; Grice and DaSilva, 1973). Even ionizing radiation is believed to cause mutations through the formation of free radicals in the surrounding media and not through direct radiation hits in DNA (Freeze, 1971).

A large number of test systems are used in evaluating chemicals for mutagenicity. Although direct monitoring of mutations in the DNA molecule may be the most relevant indicator of mutagenicity, it is seldom feasible. Thus, a variety of endpoints have been developed to test for mutagenicity (Grice and DaSilva, 1973). An endpoint may be a phenotypic change, such as a change in nutritional requirements in bacteria. Death may be an endpoint in mutagenicity assays, such as the selective chemical toxicity in DNA repair deficient bacteria as opposed to normal bacteria. Chromosome aberrations can also be monitored and related to DNA alteration. Compounds may interact in vitro with DNA and base adducts of the compounds tested can be isolated from the DNA. Most importantly, each mutagenicity assay has an endpoint that can theoretically be related to a mutation. The theory behind each of the mutagenicity tests used in evaluating nitrofurans will be discussed in the next section.

III. MUTAGENICITY STUDIES OF NITROFURAN DERIVATIVES

Bacterial Testing

Theory of Test

The *Salmonella typhimurium* tester strains, developed by Dr. Bruce Ames, are specifically selected for sensitivity and specificity for reversion from histidine auxotrophs to prototrophs (*his* → *his*+ reversions) by mutagens (Ames *et al.*, 1973; Ames *et al.*, 1975; McCann *et al.*, 1975). The test system was able to detect 85 percent of a wide variety of carcinogens as mutagens (McCann *et al.*, 1975). Chemicals which must be metabolically activated to become mutagens may be tested by the addition of the 9000 x g supernatant fraction of liver homogenate containing the microsomal mixed function oxidase system to the test system (Ames *et al.*, 1973; Ames *et al.*, 1975; McCann *et al.*, 1975).

TA1535 and TA1538, two of the basic tester strains, are used to detect mutagens which cause base pair substitutions (*hisG46* histidine mutation) and frameshift mutations (*hisD3052* histidine mutation), respectively. In addition, each tester strain contains two additional mutations to increase their sensitivity to mutagens. One causes the loss of the excision repair system (*ΔuvrB* mutation) responsible for repairing damaged DNA; the other causes loss of the lipopolysaccharide barrier that coats the surface of the

bacteria (rfa LPS mutation) facilitating easier absorption of chemical mutagens (Ames et al., 1973; Ames et al., 1975; McCann et al., 1975).

TA100 and TA98, two new tester strains, were developed by the addition of an R-factor plasmid, pKM101, to the standard tester strains TA1535 and TA1538, respectively (Ames et al., 1975; McCann et al., 1975). This made the new strains more sensitive to certain mutagens (Ames et al., 1975; McCann et al., 1975). The term R-factor designates plasmids which carry antibiotic resistance genes. The pKM101 plasmid carries ampicillin resistance genes. The mechanism by which the pKM101 plasmid enhances mutagenesis detection of chemicals is not fully understood, but there are indications that its presence interferes with error prone - recombinational repair in the bacteria (McCann et al., 1975). Furylfuramide (AF-2), a nitrofuran, is mutagenic in the new tester strains (McCann et al., 1975; Wang et al., 1975). The old tester strains, TA1535 and TA1538, were not reverted by furylfuramide (McCann et al., 1975). It was decided to pursue the testing of nitrofuran derivatives utilizing TA100 and TA98 tester strains based on their ability to be reverted by furylfuramide.

Materials and Methods

Six nitrofurans (No.2,8-12 - Table III-1) were donated by Abbott Laboratories, three (No.4,6,7 - Table III-1) by Norwich Pharmacal Laboratories, and one (No.13 - Table III-

1) by Dr. Gordon Newell of the Stanford Research Institute. Two nitrofurans were purchased from Aldrich (No.1,3 - Table III-1), one from ICN Pharmaceuticals (No.4 - Table III-1) used only for animal testing, and one from Sigma (No.5 - Table III-1). Dimethyl sulfoxide was purchased from Aldrich. All other chemicals were of reagent grade.

Salmonella typhimurium tester strains TA100 (TA1535/pKM101) and TA98 (TA1538/pKM101) were provided by Dr. Bruce Ames of the University of California, Berkeley. The bacterial tester strains were cultured and stored at -80° C (Appendix III-1). Autoclaved wooden sticks were used to inoculate sterile test tubes containing 5 ml of nutrient broth (Appendix III-1) from the frozen cultures. Care was taken not to let the frozen cultures get too warm when used for inoculation lest they melt causing the bacteria to lyse. The inoculated tubes were capped and incubated at 37°C with agitation in a water bath overnight (12 hours). The top agar overlay was prepared according to the following proportions: a 100 ml portion of the top agar (Appendix III-1) was autoclaved in a flask and placed at least to the agar line in a water bath at 45°C to prevent hardening. Five ml of fresh culture of the tester strain was added to the top agar. The bacteria can remain at 45°C for several minutes without harm (Ames et al., 1975). Ten ml of filter sterilized 0.05 mM histidine·HCl - 0.05 mM biotin solution (Appendix III-1) was added to the top agar. The trace of histidine in the top agar allows all the bacteria on the

plate to undergo several divisions; once histidine has been depleted, all growth stops. This early growth may be necessary for mutagenesis to occur (Ames et al., 1975). Aliquots of the inoculated top agar (2.3 ml) were pipetted into autoclaved and empty culture tubes at 45° C. The nitrofurans were dissolved in dimethyl sulfoxide, added to the culture tube with a Schwartz-Mann pipette, and mixed uniformly. The volume of dimethyl sulfoxide used ranged between 0.01 to 0.05 ml per plate. Less than 0.5 ml of dimethyl sulfoxide does not interfere with mutagenesis (Ames et al., 1975). Dimethyl sulfoxide did not induce reversions at 0.05 ml per plate in either TA100 or TA98 tester strains. The contents were then poured on minimal glucose agar plates (Appendix III-1). Uniform distribution of the top agar was accomplished by tilting and rotating the plate immediately after pouring. If this step is not done quickly, the top agar will not spread uniformly over the bottom agar before hardening. The plates were then put upside down in a dark, 37°C incubator. Revertant histidine prototroph colonies were counted after 48 hours (Ames et al., 1975).

All but two of the nitrofuran compounds were tested at 0.1, 0.5, 1.0, 5.0, 10.0, and 25.0 ug/plate (Table III-2). Nitrofurazone (No. 4) and nitrofurantoin (No. 5) were tested at appropriate doses (Table III-2) to obtain a linear dose-response curve and were tested at 4 plates per dose (Figure III-1 a&b).

Results

All nitrofurans tested in both TA100 and TA98 (No.2,4-12) were mutagenic in both strains (Table III-2). Compounds 1 and 3 appear to be only slightly mutagenic in TA100 (Table III-2). The dose-response curve obtained for nitrofurazone best illustrates the effects of nitrofurans in TA100 and TA98 (Figure III-1a). Low doses of nitrofurazone elicit a linear dose-response curve in TA100, followed by a leveling, presumably due to a counterbalancing between killing and his- → his+ reversions. At 5.0 µg nitrofurazone per plate massive killing causes a net decrease in his- → his+ revertants compared to the previous dose. TA98 is less sensitive both to reversion and to the bactericidal properties of nitrofurazone. Nitrofurantoin shows similar properties (Figure III-1b) except killing doses were not reached in testing. These results are typical of the mutagenic properties of all nitrofurans tested in TA100 and TA98.

The Micronucleus Test

Theory of Test

The micronucleus test is an *in vivo* assay which assesses mutagenicity by quantitating the percentage of micronucleated bone marrow reticulocytes (Schmid, 1973; Schmid, 1975). Mitotic cells with chromatid breaks or

chromatid exchanges suffer from disturbances in the anaphase distribution of the chromatin. At anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes, as well as centric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too, but a considerable proportion is transformed into one or several secondary nuclei which are, as a rule, much smaller than the principal nucleus and are therefore called micronuclei. Similar events occur if the functioning of the spindle apparatus is impaired. In this event, however, the main nucleus is often replaced by a whole group of small nuclei, which are usually larger than typical micronuclei (Schmid, 1973; Schmid, 1975).

Micronuclei are found in a variety of different cell types, including myeloblasts, myelocytes, and erythroblasts. The majority, however, is observed in reticulocytes (polychromatic erythrocytes or immature erythrocytes), a cell type particularly well suited for this test. A few hours after completion of the last mitosis, the erythroblast expels its nucleus. For unknown reasons, micronuclei present in the same cells remain behind and are easily detected. Young erythrocytes are unique from other cell types because they stain differently from older forms. For the duration of their immature life, lasting approximately 24 hours, they stain not red but bluish and are therefore

referred to also as polychromatic erythrocytes. If scoring is restricted to these cells, virtually all anomalies that may be observed are known to have arisen during a 30 hour period following exposure to the agent undergoing testing (Schmid, 1973; Schmid, 1975).

Materials and Methods

The micronucleus test was conducted, with minor modifications, in the manner described by Dr. W. Schmid (1973, 1975), developer of the test. The animals used in testing were either male Sprague-Dawley (wt.$\pm$S.D.=208$\pm$14gm) or Long-Evans (wt.$\pm$S.D.=83$\pm$gm) rats. Each treatment group consisted of 5 animals. Compounds to be tested were administered ip either as a solution in dimethyl sulfoxide or as a suspension in physiological saline. Half the dose was given 30 hours and the remainder 6 hours prior to sacrifice. The theoretical reason for dose splitting was to subject as great a proportion of the cells to the action of the possible test agent during two DNA synthetic periods (Schmid, 1973). In some tests the entire dose was given 6 hours prior to sacrifice as noted (Tables III-3 through III-6). This was done specifically for two furylfuramide test groups since Sugiyama et al. (1975) reported that maximal chromosome aberrations in the bone marrow of male Long-Evans rats occurs 6 hours after dosing with furylfuramide. Dimethyl sulfoxide or physiological saline served as negative controls (Tables III-3 through III-6). Aqueous

triethylenemelamine (1.0 mg/kg or 0.5 mg/kg) served as a positive control (Tables III-3 through III-6).

The original dosages of nitrofurazone and nitrofurantoin in dimethyl sulfoxide considered for testing in male Sprague-Dawley rats were too toxic. A single 300 mg/kg ip injection of nitrofurazone at a concentration of 200 mg/ml dimethyl sulfoxide killed a group of 5 male Sprague-Dawley rats within 24 hours. A single 150 mg/kg ip injection of nitrofurazone at a concentration of 200 mg/kg dimethyl sulfoxide killed 2 out of 5 male Sprague-Dawley rats within 24 hours; the other 3 rats were near death. It was decided from these preliminary data to dose the rats with 15, 30, and 60 mg/kg of nitrofurazone for testing purposes. At 60 mg/kg of nitrofurazone the rats exhibited bizarre central nervous system effects. These effects included fighting and irritability. The rats were observed to have a "piano player syndrome". Rats affected by this syndrome were observed to stand on their hind legs and move their front paws up and down. A single 200 mg/kg ip injection of nitrofurantoin at a concentration of 149.2 mg/ml dimethyl sulfoxide left all of a group of 5 male Sprague-Dawley rats alive; a subsequent dose of 200 mg/kg nitrofurantoin killed all 5 rats within 6 hours. Therefore, nitrofurantoin was tested at 50, 100, and 200 mg/kg. Furylfuramide was tested at 60, 120, and 240 mg/kg in physiological saline according to the protocol of Sugiyama et al. (1975). However, if dimethyl sulfoxide was used as a solvent, the dose was

reduced to 120 mg/kg because of excessive toxicity to the rats possibly due to increased absorption.

Nitrofurazone and nitrofurantoin were originally tested in male Sprague-Dawley rats. Male Long-Evans rats were used later for testing nitrofurazone and furylfuramide to study strain differences and to conform to the protocol of Sugiyama et al. (1975) where furylfuramide was reported to cause an increase only in gaps and breaks in the bone marrow metaphases of male Long-Evans rats.

Differences due to the effects of aqueous or dimethyl sulfoxide solvents used for administration of nitrofurazone and nitrofurantoin were tested in male Long-Evans rats. Dimethyl sulfoxide was used to administer the compounds in solution; physiological saline was used to administer the compounds as a suspension. Sugiyama et al. (1975) reported on the cytogenetic effects of furylfuramide administered as a suspension in physiological saline.

The micronucleus test was executed according to the following procedure. After sacrificing the rat, the femur was exposed by dissection of the surrounding tissue about the femur, pelvis, and tibia. The head of the femur was exposed by popping it from the socket joint of the pelvis by hand. The femur was freed from the surrounding muscle and bones using scissors and fingers. Distally, the epiphysial portion of the femur was torn off together with any tibia fragments. Excess muscle was removed with scissors and the femur cleaned. For each rat, a 13 ml centrifuge tube filled

with 5 ml fetal calf serum (Microbiological Associates) was provided and 1 ml fetal calf serum pulled into a syringe. The distal portion of the disarticulated femur was clipped at an angle exposing the bone marrow channel. The head of the femur was clipped off exposing a small hole to the bone marrow channel and an unmounted needle placed in the hole. The syringe containing the fetal calf serum was next mounted on the needle, the femur held over the fetal calf serum in the centrifuge tube, and the plunger depressed flushing the bone marrow out the distal end of the femur and into the fetal calf serum. The fetal calf serum was then centrifuged at 1000 rpm for 5 minutes and the supernatant removed by a vacuum suction flask, leaving behind an equal volume of fetal calf serum and packed cells. The remaining packed cells and fetal calf serum were then mixed by a vortex. A small drop of the cell suspension was transferred by Pasteur pipet to the end of a slide and smeared out by pulling the cells behind a polished cover glass at a 45 degree angle.

The slide smears were air dried and stained within 24 hours with Giemsa and May-Greenwald stains prepared in the following ordered procedure: 1) staining 3 minutes in undiluted May-Greenwald solution (Appendix III-2), 2) staining 2 minutes in May-Greenwald solution diluted with distilled water 1:1, 3) rinsing briefly in distilled water, 4) staining 10 minutes in Giemsa solution (Appendix III-2) diluted with distilled water 1:6, 5) rinsing briefly under tap water, 6) blotting dry with filter paper, 7) cleaning

back of slides with methanol, 8) clearing in xylene for 5 minutes, and 9) mounting of slides with cover slips, xylene, and Pro-Texx^{R}. Because the commercial supply of May-Greenwald stain became depleted it was necessary to use in some experiments a Giemsa-Wrights stain combination (Rossi and MacGregor, 1975). The slides stained by this method were prepared in the following ordered procedure: 1) staining 4 minutes in undiluted Wrights-Giemsa solution (Appendix III-2), 2) staining 4 minutes in .10 M aqueous phosphate buffer at pH 6.8 with 3.4 percent undiluted Wrights-Giemsa solution, 3) rinsing in tap water, 4) blotting dry with filter paper, 5) cleaning back of slides with methanol, and 6) mounting of slides with cover slips, xylene, and Pro-Texx^{R}. After mounting, the slides were left to dry for 72 hours.

The slides were read in a double blind fashion. For each rat 4 to 8 slides were made of the bone marrow. The experiment and rat number were etched at the end of the slide. A second party coded the slides by putting tape over the etchings, assigning a random number per rat, and arranging the slides in random order in the slide boxes. The number of reticulocytes counted ranged from 1,000 to 3,000 (Tables III-3 through III-6). The data were analyzed by the unpaired t-test and by analysis of variance.

Results

Neither nitrofurazone at 15, 30, and 60 mg/kg nor nitrofurantoin at 50, 100, and 200 mg/kg (all doses tested) significantly increased the percentage of reticulocytes containing micronuclei in male Sprague-Dawley rats (Tables III-3 and III-4).

Sugiyama et al. (1975) reported that furylfuramide a carcinogenic nitrofuran (Nomura, 1975) in doses of 15 to 240 mg/kg ip, increased cytogenetic aberrations in male Long-Evans rats (70-90 gm); they reported maximal increases only in chromatid breaks and gaps in bone marrow cells 6 hours after administration of furylfuramide and a return to negative control levels after 24 hours. For direct comparison male Long-Evans rats were given furylfuramide and nitrofurazone and the occurrence of micronuclei in reticulocytes observed.

Nitrofurazone (60 mg/kg), either in dimethyl sulfoxide or physiological saline, did not significantly increase the percentage of reticulocytes containing micronuclei in male Long-Evans rats (Table III-5). Furylfuramide, when administered as a suspension in physiological saline at 240 mg/kg total in two doses 30 and 6 hours prior to sacrifice, showed a statistically significant increase ($P<.05$, unpaired t-test) in the percentage of reticulocytes containing micronuclei when compared to the negative control (Table III-6). Examination of the data by analysis of variance between negative control, 60, 120, and 240 mg/kg given in physiological saline showed statistical significance at

$P<.05$. However, neither a) single doses of furylfuramide administered 6 hours prior to sacrifice at 240 mg/kg in saline or 120 mg/kg in dimethyl sulfoxide nor b) split doses of furylfuramide at 120 mg/kg in dimethyl sulfoxide caused a significant increase in the percentage of reticulocytes containing micronuclei.

The triethylenemelamine positive control treated rat groups exhibited a statistically significant increase of $P<.01$ in the percentage of reticulocytes with micronuclei over negative control rat groups in all tests (Tables III-3 through III-6). In a separate test, there was no statistical difference in the percentage of reticulocytes with micronuclei in male Sprague-Dawley rats (wt. $\pm$ S.D. = 205 $\pm$ 8 gm) administered ip either 4.00 ml water/kg (percentage reticulocytes with micronuclei $\pm$ S.D. = .47 $\pm$ .13) or 4.00 ml dimethyl sulfoxide (percentage reticuloctyes with micronuclei $\pm$ S.D. = .46 $\pm$ .19) under standard testing procedure.

These results indicate that furylfuramide is a weak mutagen in the male Long-Evans rats and do not agree quantitatively with the data of Sugiyama *et al.* (1975) which state that 8.9 percent of metaphase cells contained chromatid breaks and gaps after ip administration of furylfuramide at 240 mg/kg by *in vivo* cytogenic testing. Our results suggest that the micronucleus test may not always parallel *in vivo* cytogenetics in detecting mutagens. Nitrofurazone was therefore further examined by *in vivo* cytogenetic testing.

In Vivo Cytogenetic Testing

Theory of Test

It has been previously stated that the definition of a mutation includes structural and numerical changes in chromosomes (Evans, 1976; Grice and DaSilva, 1973; Legator et al., 1973). Agents which induce chromosomal aberrations are assumed also to induce point mutations (Schleiermacher, 1971). Chromosomal aberrations should be regarded as one part of a spectrum of events produced by mutagens, and point mutations as a part of this spectrum (Legator et al., 1973). However, the ability of agents which cause point mutations at specific loci in the eukaryotic genome also to cause chromosomal aberrations is an area of considerable controversy. Evans (1976) claims that agents which cause point mutations will invariably cause chromosomal aberrations. However, Schleiermacher (1971) states that if chromosomal aberrations are not induced by a given agent it does not mean that no point mutations were induced. It has been pointed out that discussion of the genetic implications of induced chromosomal aberrations involves more speculation than fact (Grice and DaSilva, 1973). However, cytogenetic analysis is one of the few practical methods of monitoring mutagenic events in the intact animal.

Cytogenetic analysis can be performed in vivo or in vitro. Cytogenetic testing in vivo has the advantage of determining if the intact animal can interact, detoxify, or potentiate a chemical mutagen in a manner not observable by

in vitro testing. Thus, *in vivo* testing better parallels actual human exposure and can better predict a potential health hazard in humans than *in vitro* testing (Legator *et al.*, 1973).

Cytogenetic analysis can be performed with either somatic or germinal tissue. Gonadal mutagenicity is of concern for the population as a whole since it would be indicative of inheritable events transmitted to the offspring (Evans, 1976; Legator *et al.*, 1973). It is assumed compounds that can produce somatic mutations can also produce germinal mutations if they reach gonadal tissues (Legator *et al.*, 1973). From a practical standpoint, however, cytogenetic analysis of somatic cells is a preferable procedure to gonadal analysis (Legator *et al.*, 1973). Bone marrow is the tissue of choice for somatic cytogenetic analysis from the standpoint of technical simplicity and number of mitotic cells (Grice and DaSilva, 1973).

The mechanism by which chromosome aberrations are formed is open to speculation. There are presently two major theories. The first theory is the classical breakage-first hypothesis. This theory assumes that the initial lesion is a break in the chromosomal backbone generating broken ends. Three things could then happen to the broken ends: 1) The ends could repair normally and rejoin to form a normal chromosome configuration, 2) the ends could not be repaired at all giving rise to a break, or 3) they could misrepair by

joining with another lesion on another chromosome to cause an exchange figure. The second theory is the exchange hypothesis. This theory assumes that the initial lesion is not a break and that the lesion can be either repaired directly or may interact with another lesion by a process called exchange initiation. Subsequently, the chromatid may exchange with a sister chromatid. If this exchange is incomplete a chromosome break may result. If the exchange occurs with a chromatid from another chromosome an exchange figure will result (Heddle and Bodycote, 1970; Comings, 1974).

Materials and Methods

The *in vivo* cytogenetic testing of nitrofurazone utilized the serial biopsy technique as developed by Nemenzo (1975).

An advantage of the serial biopsy technique is that the animal need not be sacrificed to take a bone marrow sample. Therefore, the serial biopsy technique allows the animal to be sampled many times in a time sequence analysis. Also, the bone marrow can be sampled before the animal is exposed to the chemical being tested. Therefore, the animal can serve as its own negative control along with a separate negative control group (Nemenzo *et al.*, 1975). This technique utilizes metaphase cell spreads from rat bone marrow cells to evaluate the effect of a potential mutagen on the formation of chromosomal aberrations.

The rat from which a bone marrow sample was to be taken was anesthetized in a jar with vaporized methoxyflurane (Penthrane^{R}). The rat, immobilized, was then placed in a supine position in preparation for bone marrow extraction. This was accomplished by grasping the distal epiphyseal end of the femur firmly with the thumb and middle finger. The whole shaft was then flexed at a right angle to the long axis of the body. While holding the femur in a flexed position between the thumb and middle finger, the center of the patellar groove was located by palpation of the medial and lateral condyle by the free index finger. This serves as a landmark for the site of the needle puncture for the bone marrow tap (Figure III-2). A heparinized spinal needle (20 gauge x 1 inch) with the stylus in place was used for puncture. The initial puncture was made in the skin at the center of the patellar groove. The needle was then inserted into the bony structure in a straight downward direction accompanied with twisting and firm pressure. As the tip of the needle traversed the cortical and cancellous portion of the bone structures, an initial gritty sensation, followed by a gradual loss of resistance, indicated entry into the proximal end of the bone marrow channel. The needle stylus was removed and a 5 ml disposable syringe containing about 0.1 ml of heparin (1000 units/ml) was fitted tightly into the needle adaptor. The plunger was retracted gradually until 0.2 to 0.3 ml of brownish red material (bone marrow) entered the syringe and mixed with the heparin. This sample

was immediately flushed into 5 ml of pre-warmed (37° C) minimum essential medium (Microbiological Associates) containing 10 micrograms/ml of colcimed (Demecolcine^{R}) in a septum - covered 13 ml centrifuge tube. The tube was then inverted to enhance mixing and the cell suspensions incubated for 2 hours at 37 °C. At the end of the 2 hour incubation period the cell suspensions were centrifuged at 1000 rpm for 5 minutes and the supernatant liquid was removed by vacuum suction. The packed cells were resuspended with 5 ml of pre-warmed aqueous 0.075 M potassium chloride (37 ° C) and left to stand at room temperature for 15 minutes. Carnoy's solution (4 drops) (freshly prepared, 1:3 acetic acid to methanol) was added, the suspension centrifuged and the supernatant removed. The packed cells were then resuspended with 5 ml of Carnoy's solution and allowed to stand for 30 minutes before being recentrifuged at 1000 rpm for 5 minutes. The supernatant was removed, and the packed cells resuspended by gradually adding Carnoy's solution and flicking the tube with the index finger. The process of changing the fixative was repeated twice until a final suspension was made for smear preparation. The metaphase spread was prepared by placing a few drops of the preparation onto a precleaned microscope slide previously dipped in distilled water. Immediately after the spread was made the slide was tilted to one side to remove excess fluid and air dried in a vertical position. After drying the slides were stained in 3.0 percent

undiluted Giemsa staining solution (Appendix III-2) in distilled water for 10 minutes. The slides were then air dried for 48 hours and mounted with Pro-Texx^{R}. Six slides were made for each bone marrow sample from each animal. The slides were coded by etching with a diamond pencil at the ends of the slides.

Male Sprague-Dawley rats (wt.$\pm$S.D.=188$\pm$14gm) were used to test nitrofurazone for mutagenicity by the serial biopsy technique just described. There were 3 treatment groups with 5 animals per group. Each treatment group was sampled at given times. One treatment group was injected ip with nitrofurazone at 60 mg/kg ip as a single acute dose in a distilled water suspension at a concentration of 60 mg/ml. The nitrofurazone treatment group was sampled at a pre-treatment baseline, 6 hours post-treatment, and 24 hours post-treatment. The 6 hour time sample was picked because Sugiyama *et al*. (1975) observed that the nitrofuran furylfuramide (AF-2) caused a peak in chromosomal aberrations in the bone marrow of male Long-Evans rats 6 hours post-treatment. The 24 hour time sample was chosen because it is often used in standard *in vivo* cytogenetic testing. Triethylenemelamine in distilled water at a concentration of 0.2 mg/ml was administered ip as a positive control at 0.2 mg/kg. The positive control group was sampled at a pre-treatment baseline and at 24 hours post-treatment. At 24 hours post-treatment, triethylenemelamine is known to cause a marked increase in chromosomal

aberrations in the bone marrow of rats sampled by this method (Nemenzo et al., 1975). A negative control group of rats treated ip with 1.0 ml/kg distilled water was sampled for bone marrow at a pre-treatment baseline, 6 hours post-treatment, and 24 hours post-treatment. In all groups fifty metaphase cell spreads from each rat, or 250 from each time-sampled treatment group, were scored and photographed.

Several types of chromosome aberrations were monitored: gaps, breaks, exchanges, and double minutes. These aberrations were scored since furylfuramide has been reported to induce only gaps and breaks in chromosomes of rats in vivo (Sugiyama et al., 1975) and also exchange figures in human metaphase spreads in vitro (Tonomura and Sasaki, 1973). Aberrations were scored using the criteria of the Hine Laboratory Cytogentics Unit. 1) Gaps were defined as achromatic lesions less than the width of the chromatid with chromatid continuity. Gaps may vary in their incidence and are many times found in significant numbers in cells not exposed to known mutagens. It is possible that gaps comprise a mixture of phenomena which may represent mutagenic events in DNA causing breaks and non-mutagenic events such as localized regions of uncoiling (Evans, 1976). 2) Breaks were scored as achromatic lesions greater than the width of the chromatid or as chromatid discontinuity. Breaks are thought to represent breaks in the DNA molecule (Comings, 1974). 3) Exchanges are those chromosome figures showing one chromosome joined or attached to another

chromosome giving rise to bizarre metaphase spreads. 4) Double minutes (interstitial, isodiametric, or dot deletions) are pairs of acentric fragments, characteristically appearing as paired spheres of chromatin (Evans and O'Riordan, 1975). Double minutes are a particular classification of break (Evans, 1974). The metaphase cell spreads were analyzed in a double-blind fashion. To accomplish this a second party taped over the identification etchings on the slides, inscribed a random number for each rat on the tape, and arranged the slides randomly as to rat number. The metaphase cell spreads were analyzed once under the microscope while being photographed and a second time with the photographs alone. An experienced cytogeneticist (Dr. Jesus Nemenzo) rendered a second opinion in analyzing the photographs.

Results

The results clearly demonstrated that 60 mg nitrofurazone/kg administered as a suspension in distilled water ip did not cause an increase in chromosomal aberrations at 6 or 24 hours after dosing (Table III-7). The triethylenemelamine positive control treatment group demonstrated uniquely exchange figures in metaphase cell spreads and exhibited a dramatic increase in metaphase cell spreads with more than one chromatid break over negative controls (Table III-7).

Conclusions

Approximately 85 percent of a wide variety of compounds which have been shown to be carcinogenic in mammals revert Salmonella typhimurium histidine auxotrophs (Ames et al., 1975). The mutagenic and carcinogenic properties of the nitrofurans were not always positively correlated. Compounds 6-12 have been shown by Cohen et al. (1973a, 1973b) to be moderate to potent carcinogens in rats and mice. These compounds are also strong mutagens inducing reversion at doses of less than 1 ug per plate (Table 2). Nitrofurazone (No.4), which is a carcinogen in the rat (Erturk et al., 1970; Morris et al., 1969) also induced a high number of reversions at less than 1 ug per plate. By comparison, compound 1, which has so far been shown not to cause cancer in animals (Tazima et al., 1975), appears to be a weak mutagen in TA100, as does compound 3. Nitrofurantoin (No. 5) and compound 2, neither of which has demonstrable carcinogenic effects so far (Cohen et al., 1973a), have both been shown to be mutagenic in TA100 at doses of less than 1 ug per plate. Indeed, it appears that all nitrofurans tested can revert TA100 and this could be a general property of the nitrofurans (Tazima et al., 1975; Wang et al., 1975b; Yahagi et al., 1976).

The absence of in vivo mutagenic activity of nitrofurazone and nitrofurantoin in the rat is in agreement with the studies of Tonomura and Sasaki (1973) demonstrating that these compounds did not induce chromosomal aberrations

in cultured human lymphocytes nor induce unscheduled DNA repair synthesis in cultured fibroblasts from skin biopsies of a normal person. Further consonant evidence comes from the work of McCalla *et al*. (1971b) who reported that exposure of *E. coli* B/r to the highly carcinogenic N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide caused a large number of single-stranded breaks in DNA whereas nitrofurantoin caused relatively few breaks. Nitrofurazone was intermediate in causing breaks.

It may be that the TA100 and TA98 tester strains are intrinsically more sensitive in detecting the mutagenicity of the nitrofuran class of compounds than the *in vivo* mammalian tests used. Point mutations of the particular type caused by those nitrofurans tested in rats may not be manifested in the mammalian test systems used. Thus, although nitrofurazone and nitrofurantoin are mutagens in *Salmonella typhimurium* TA100 and TA98, they are not mutagenic *in vivo* in the rat as determined by the micronucleus test and by cytogenetic tests.

APPENDIX III-1

Bacterial Testing

Solutions and Media

Nutrient Broth (Ames et al., 1975)

Difco-Bacto^{R} nutrient broth	8 gm
Sodium chloride (NaCl)	5 gm
Distilled water	1000 ml

Dissolve the ingredients and dispense into capped culture tubes. Autoclave and store at room temperature.

Storage of R-factor tester strains (Ames et al., 1975)

Freshly grown up nutrient broth culture	0.8 ml
Dimethyl sulfoxide	0.07 ml

Store at -80° C in 2 ml (0.5 dram) sterile glass vials.

Minimal Glucose Agar Plates (Ames et al., 1975)

A.	VB salts (See below)	20 ml
	Distilled water	500 ml
B.	Agar (Difco^{R})	15 gm
	Distilled water	500 ml
C.	40 percent glucose in distilled water	50 ml

Autoclave solutions A, B, and C in separate flasks; glucose will caramalize if autoclaved in solution with A or B. While still hot after autoclaving mix solutions together and pour plates (30 ml/plate).

VB Salts (Vogel and Bonner, 1956)

Warm distilled water (45°C)-pre-warmed	670 ml
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	10 gm
Citric acid monohydrate	100 gm
Potassium phosphate, dibasic, anhydrous (K_2HPO_4)	500 gm
Sodium Ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$)	175 gm

Add each of the components to the distilled water in the order indicated above. Allow each component to dissolve completely before adding the next component. When the components are all dissolved, shore up the solution to 1000 ml with distilled water, then let the solution cool to room temperature. Add 5 ml of chloroform to inhibit mold growth and store in a capped bottle at room temperature.

APPENDIX III-1 (continued)

Top Agar (Ames et al., 1975)

Agar (Difco^R)	0.6 gm
Sodium chloride (NaCl)	0.5 gm
Distilled water	100 ml

Dissolve ingredients and autoclave. Do not allow top agar to drop below 45°C in temperature or else it will harden.

Histidine - Biotin Solution (Ames et al., 1975)

Dissolve L-histidine · HCl (0.5 mM) and biotin (0.5 mM) in distilled water. Sterilize by filtration with a Millipore^R filter unit with a 0.22 μ Millipore filter. Never autoclave the histidine-biotin solution, or else it will be destroyed.

Appendix III-2

Biological Stains

May-Greenwald Stain Preparation

Mix 0.3 grams of May-Greewald dye in 100 ml methanol, warm to 50°C, then cool to room temperature. Let stand with occasional shaking for 24 hours then filter. Store shielded from light in dark bottle. Seal with parafilm.

Giemsa Stain Preparation

Mix 1.0 gram of Giemsa dye in 66 ml of glycerine. Heat to 60°C for two hours and cool. Add 66 ml of methanol, stir for 24 hours, then filter. Store shielded from light in dark bottle. Seal with parafilm.

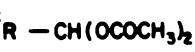
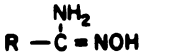
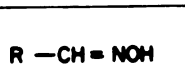
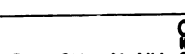
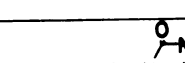
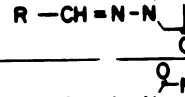
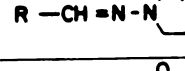
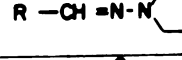
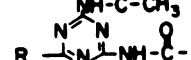
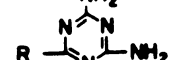
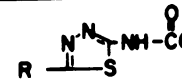
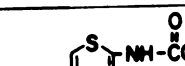
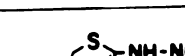
Wrights-Giemsa Stain Preparation (Rossi and MacGregor, 1975)

Absolute Methanol	473 ml (one pint)
Wrights Dye	1.7 gm
Giemsa Dye	0.17 gm

Add dyes together then add methanol and mix. Let stand at least one week before using. Store shielded from light in dark bottle. Seal with parafilm.

TABLE III-1

CHEMICAL STRUCTURES AND NAMES OF NITROFURANS TESTED.

NUMBER	STRUCTURE	NAME
1		5-Nitro-2-furanmethandiol diacetate
2		5-Nitro-2-furamidoxime
3		5-Nitro-2-furaldehyde oxime (Nifuroxime)
4		5-Nitro-2-furaldehyde semicarbazone (Nitrofurazone)
5		N-(5-Nitro-2-furfurylidine)-1-aminohydantoin (Nitrofurantoin)
6		1-(2-Hydroxyethyl)-3-[(5-nitrofurfurylidine)-amino]-2-imidazolidinone
7		4-Methyl-1-[(5-nitrofurfurylidine)amino]-2-imidazolidinone
8		N-N'-[6-(5-Nitro-2-furyl)-s-triazine-2,4-diyl]bisacetamide
9		4,6-Diamino-2-(5-nitro-2-furyl)-s-triazine
10		N-[5-(5-Nitro-2-furyl)-1,3,4-thiadiazol-2-yl]acetamide
11		2,2,2-Trifluoro-N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide
12		2-(2,2-Dimethylhydrazino)-4-(5-nitro-2-furyl) thiazole (DMNT)
13		2-(2-Furyl)-3-(5-nitro-2-furyl)acrylamide (Furylfuramide or AF_2)

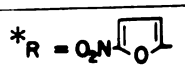
*R = 

TABLE III-2

Results of Nitrofuran Tests in *Salmonella typhimurium* TA 100 and TA 98

No.	Dose μg/Plate	His+ Revertants/Plate TA 100	TA 98	Carcinogenicity (Reference) Specie
1	0	74,76	N.D.	negative (Morris et al., 1969) Rat
	0.1	74		
	0.52	55		
	1.0	61		
	5.0	64		
	10.0	130		
	25.0	0		
2	0	81,85,60,61	29,18,27,31	negative (Cohen et al., 1973a) Rat
	0.1	97	28	
	0.5	205	48	
	1.0	318	74	
	5.0	756	291	
	10.0	732	399	
	25.0	375	482	

TABLE III-2 (continued)

No.	Dose µg/Plate	His+ Revertants/Plate TA 100	TA 98	Carcinogenicity (reference) Specie
3	0	74,76	N.D.21	Not reported
	0.1	86		
	0.5	63		
	1.0	80		
	5.0	105		
	10.0	169		
	25.0	100		
4	0	73,54,74,58	32,20,28	positive (Erturk et al., 1970) Rat
	0.10	115,88,86,81	23,24,30	
	0.25	108,144,107,131	28,26,28,25	(Morris et al., 1969) Rat
	0.50	293,287,323,296	28,26,31,33	
	0.75	449,456,379,410	41,53,29,44	
	1.0	530,493,530,537	57,41,57,41	
	1.5	432,496,540,311	61,71,67,55	

TABLE III-2 (continued)

No.	Dose µg/Plate	His+ Revertants/Plate TA 100	TA 98	Carcinogenicity (reference) Specie
4	2.0	457,512,612,261	73,104,91,102	
	3.0	267,577,467,382	107,109,119,104	
	5.0	23,47,131,1	296,205,203,196	
5	0	72,80,80,113	30,21,25,25,21	negative (Cohen et al., 1973a) Rat
	0.25	231,249,261,240	36,47,52,40	
	0.50	429,408,526,475	42,48,42	(Morris et al., 1969) Rat
	0.75	704,553,722,634	39,47,48,43	
	1.0	1386,1383,1609,1397	49,58,50,66	
	1.25	1144,1468,1373,1291	91,89,89,94	
	1.50	1254,1590,1734,1670	114,116,103,93	
6	0	52,52,70,44	36	positive (Cohen et al., 1973a) Rat
	0.1	118	38	
	0.5	321	34	
	1.0	580	56	
	5.0	249	208	

TABLE III-2 (continued)

No.	Dose µg/Plate	His+ Revertants/Plate TA 100	TA 98	Carcinogenicity (reference)	Specie
6	10.0	0	62		
	25.0	0	4		
7	0	52,52,70,44	36	positive (Cohen et al., 1973a)	Rat
	0.1	213	38		
	0.5	261	63		
	1.0	74	99		
	5.0	0	31		
	10.0	0	2		
	25.0	0	2		
8	0	81,85,60,81	29,18,27,31	positive (Cohen et al., 1973a)	Rat
	0.1	237	100		
	0.5	638	378		
	1.0	1232	870		
	5.0	190	1384		
	10.0	0	171		
	25.0	0	5		

TABLE III-2 (continued)

No.	Dose µg/Plate	His+ Revertants/Plate TA 100	TA 98	Carcinogenicity (reference) Specie
9	0	81,85,60,81	29,18,27,31	positive (Cohen et al., 1973a) Rat
	0.1	462	75	
	0.5	537	207	
	1.0	52	107	
	5.0	0	0	
10	0	81,85,60,81	29,18,27,31	positive (Cohen et al., 1973b) Mouse
	0.1	320	33	
	0.5	287	75	
	1.0	35	43	
	5.0	0	0	
11	0	81,85,60,81	29,18,27,31	positive (Cohen et al., 1973b) Mouse
	0.1	768	34	
	0.5	1460	92	

TABLE III-2 (continued)

No.	Dose µg/Plate	His+ Revertants/Plate TA 100	TA 98	Carcinogenicity (reference)	Specie
11	1.0	184	134		
	5.0	0	2		
12	0	81,85,60,81	29,18,27,31	positive (Cohen et al., 1973a)	Rat
	0.1	658	87		
	0.5	0	137		
	1.0	0	19		
	5.0	0	11		

The bacterial assay was performed as described in Materials and Methods. Each value represents the his+ revertants for a single plate. N.D., not determined. No., compound number as described from Table III-1. positive, indicates positive carcinogen; negative, indicates negative carcinogen.

Figure III-1 a and b

His^+ Revertants of S. typhimurium TA 100 and TA 98 by Nitrofurazone (a) and Nitrofurantoin (b).

Values indicated by circles for revertant colonies represent the mean values calculated from Table II (compound 4, nitrofurazone; compound 5, nitrofurantoin) for a particular dose. The bacterial assay was performed as described in Materials and Methods. ●, represent data in TA 100; o, represent data in TA 98; I, represent standard error; se, standard error.

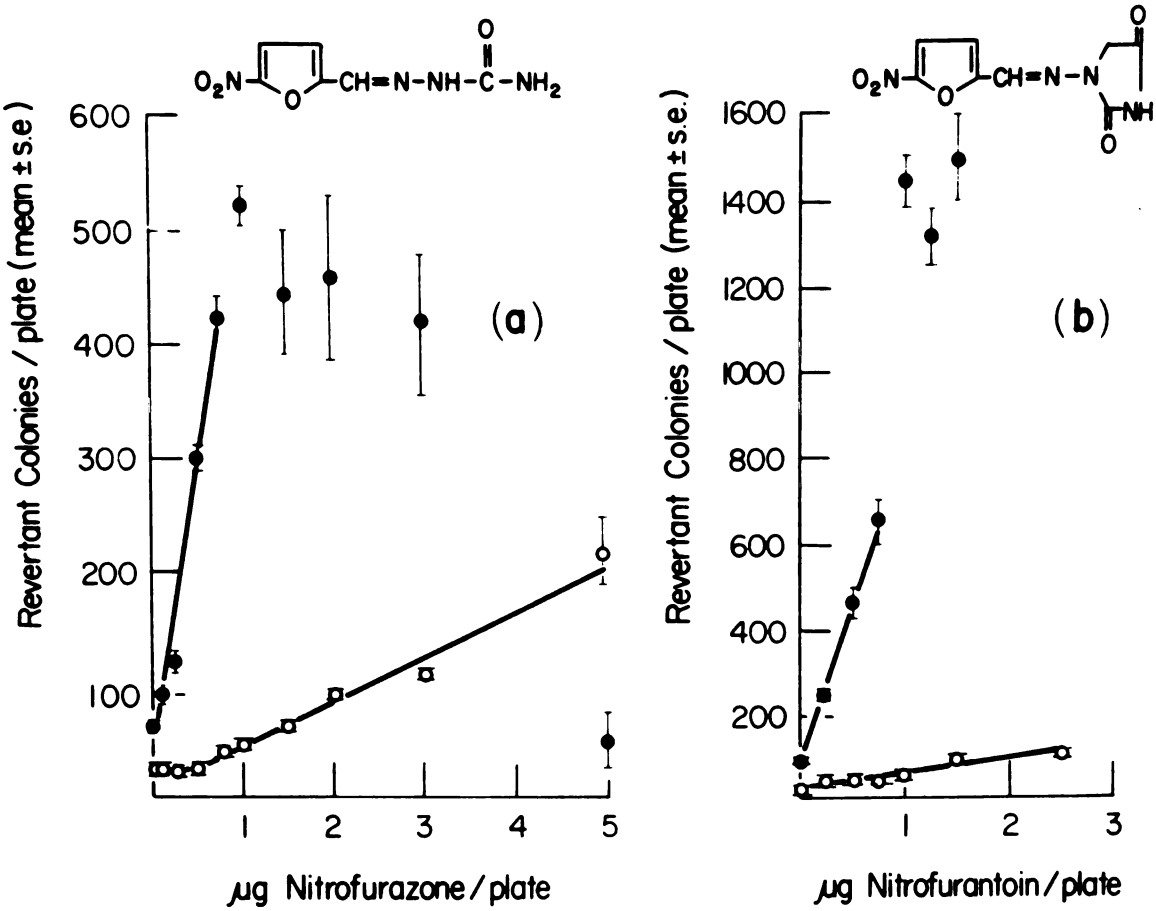


TABLE III-3

Nitrofurazone Micronucleus Test in Male Sprague-Dawley Rats

Compound	Dose	% Reticylocytes Containing Micronuclei Mean ± SD(N)
Dimethyl Sulfoxide	1.2 ml/kg	.83±.24(5)
Triethylenemelamine	1.0 mg/kg	7.48±.76(4)*
Water	1.0 ml/kg	
Nirofurazone	15 mg/kg	.64±.15(5)
Dimethyl Sulfoxide	0.3 ml/kg	
Nirofurazone	30 mg/kg	.58±.21(5)
Dimethyl Sulfoxide	0.6 ml/kg	
Nitrofurazone	60 mg/kg	.58±.15(5)
Dimethyl Sulfoxide	1.2 ml/kg	

*Positive control significantly elevated above negative control DMSO treated group ($P<.01$; unpaired t-test); 1000 reticulocytes counted in each animal in this group. Each animal received one-half the dose 30 hours and one-half 6 hours before sacrifice. The micronucleus test was performed as described in Materials and Methods. SD, standard deviation; N, number of animals in treatment group. 3000 reticulocytes were counted for each animal unless otherwise indicated. May-Greenwald and Giemsa stains were used in staining.

TABLE III-4

Nitrofurantoin Micronucleus Test in Male Sprague-Dawley Rats

Compound	Dose	% Reticylocytes Containing Micronuclei Mean ± SD(N)
Dimethyl Sulfoxide	4.0 mg/kg	.29±.05(5)
Triethylenemelamine	.50 mg/kg	2.98±.91(5)*
Water	4.0 ml/kg	
Nitrofurantoin	50 mg/kg	.34±.11(5)
Dimethyl Sulfoxide	.90 ml/kg	
Nitrofurantoin	100 mg/kg	.37±.14(5)
Dimethyl Sulfoxide	1.8 ml/kg	
Nitrofurantoin	200 mg/kg	.40±.17(5)
Dimethyl Sulfoxide	3.6 ml/kg	

*Positive controls significantly elevated above negative control DMSO treated group ($P<.01$, unpaired t-test). Each animal received one-half the dose 30 hours and one-half 6 hours before sacrifice. The micronucleus test was performed as described in Materials and Methods. SD, standard deviation; N, number of animals in treatment group. 2000 reticulocytes were counted for each animal. May-Greenwald and Griemsa stains were used in staining.

TABLE III-5

Nitrofurazone Micronucleus Test in Male Long Evans Rats

Compound	Dose	% Reticulocytes Containing Micronuclei Mean ± SD(N)
Physiological Saline	8.00 ml/kg	.13±.06(5)
Triethylenemelamine	.50 mg/kg	2.75±.91(5)*
Physiological Saline	8.00 mg/kg	
Nitrofurazone	60 mg/kg	.14±.11(5)
Physiological Saline	4.00 ml/kg	
Nitrofurazone	60 mg/kg	.18±.09(5)
Dimethyl Sulfoxide	4.00 mg/kg	

*Positive control significantly elevated above negative control DMSO treated group ($P<.01$, unpaired t-test). Each animal received one-half the dose 30 hours and one-half 6 hours before sacrifice. The micronucleus test was performed as described in Materials and Methods. SD, standard deviation; N, number of animals in treatment group. 2000 reticulocytes were counted for each animal. A Wrights-Giemsa staining combination was used in staining.

TABLE III-6

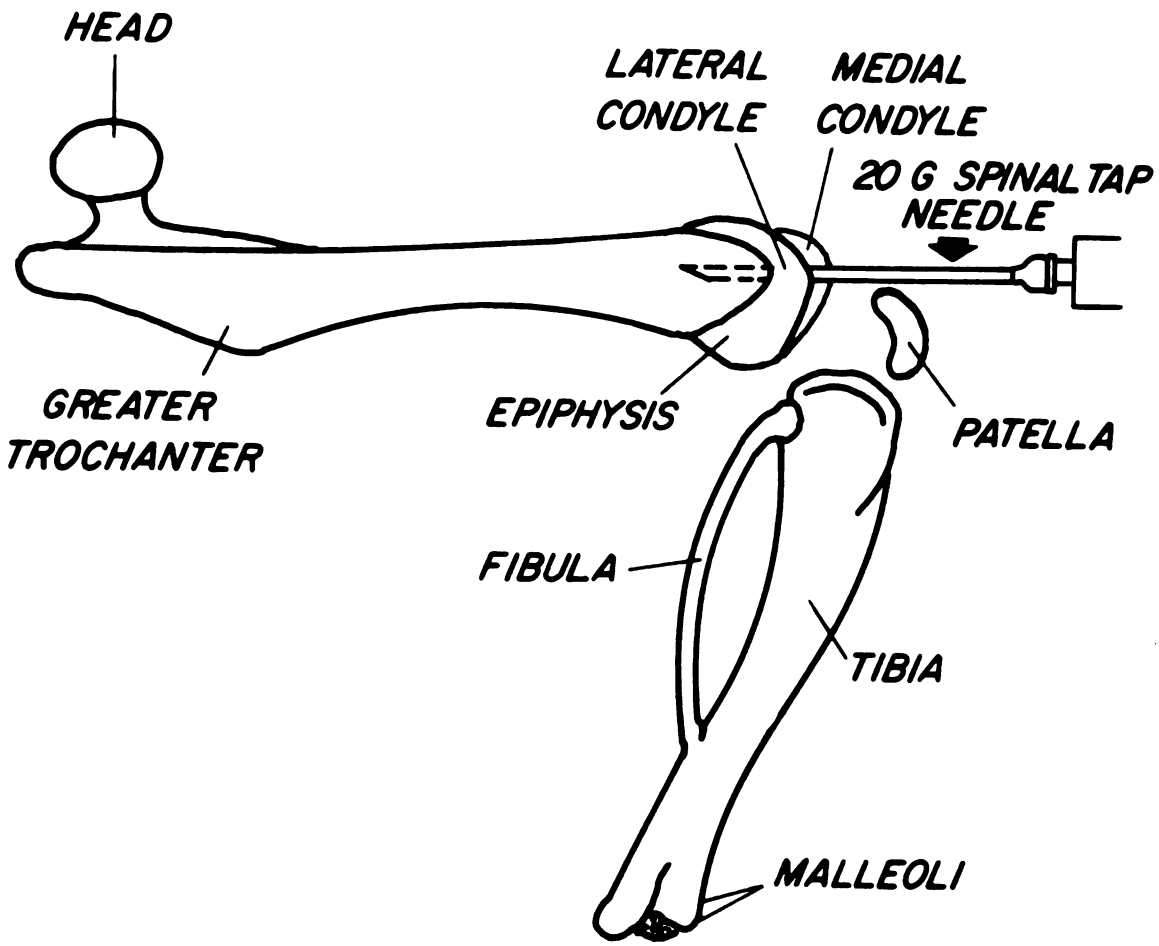
Furylfuramide (AF-2) Micronucleus Test in Male Long Evans Rats

Compound	Dose	% Reticylocytes Containing Micronuclei Mean ± SD(N)
Physiological Saline	8.00 ml/kg	.13±.06(5)
Triethylenemelamine Physiological Saline	0.50 mg/kg 8.00 ml/kg	2.75±.91(5)*
Furylfuramide (6 hrs) Physiological Saline	240 mg/kg 8.00 ml/kg	.33±.23(5)
Furylfuramide (6 hrs) DMSO	120 mg/kg 4.00 ml/kg	.15±.11(5)
Furylfuramide DMSO	120 mg/kg 4.00 ml/kg	.19±.12(5)
Furylfuramide Physiological Saline	60 mg/kg 2.00 ml/kg	.20±.13(5)
Furylfuramide Physiological Saline	120 mg/kg 4.00 ml/kg	.20±.15(5)
Furylfuramide Physiological Saline	240 mg/kg 8.00 ml/kg	.49±.32(5)#

*Positive control group significantly elevated above negative control physiological saline treated group ($P<.01$, unpaired t-test). #AF-2 treated group significantly elevated above negative control physiological saline treated group ($P<.05$, unpaired t-test). Each animal received one-half the dose 30 hours and one-half 6 hours before sacrifice except as noted in parentheses where a single dose was administered and the rat sacrificed 6 hours later. The micronucleus test was performed as described in Materials and Methods. SD, standard deviation; N, number of animals in treatment group. 2000 reticulocytes were counted for each animal. A Wrights-Giemsa staining solution was used in staining.

Figure III-2

Anatomical Illustration Demonstrating
Biopsy of Bone Marrow from Rat Femur



Courtesy of Dr. Jesus Nemenzo.

TABLE III-7

Nitrofurazone Study-Cytogenetic Analysis of
Rat Bone Marrow Cells by Serial Biopsy

Treatment	Time (Hrs)	*Aberrant cell metaphases per 250 cell metaphases				
		Gaps	One Break Cells	Multiple Break Cells	Exchanges	Double Minutes
Water (1 ml/kg)	Baseline	21	7	0	0	0
	6	12	9	0	0	0
	24	10	8	0	0	0
Triethylene-melamine (.200 mg/kg)	Baseline	16	13	1	0	0
	24	8	10	14	13	1
Nitrofurazone (60 mg/kg)	Baseline	18	7	1	0	0
	6	11	5	1	0	0
	24	5	5	0	0	0

*Each treatment group consisted of 5 rats; 50 cell metaphases from each rat were analyzed per sampling and pooled to give a total of 250 cell metaphases analyzed per sampling of each treatment group. The cytogenetic test was performed as described by Nemenzo (14). All rats in treatment groups received water (1 ml/kg) administered either alone in the negative control group or as a solvent with triethylenemelamine or nitrofurzaone. One break cells are cells with only one chromatid break. Multiple break cells are cells with two or more chromatid breaks. Baseline, indicates sampling of bone marrow metaphases before treatment of rat group with chemicals.

IV. BIOTRANSFORMATION STUDIES WITH NITROFURAZONE

Theory of Metabolic Study

There is evidence that the reduced metabolites of nitrofurazone may interact with compounds that contain sulfhydryl groups, such as cysteine and glutathione. Reduction of nitrofurazone by xanthine oxidase results in covalent binding of a reduction product with serum albumin *in vitro* (McCalla *et al.*, 1970); cysteine will protect against this covalent binding (McCalla *et al.*, 1970). In addition, glutathione protects against the covalent binding to tissue macromolecules of reduced metabolites of nitrofurazone in rat tissue homogenates (Stripp *et al.*, 1973). If glutathione is depleted by pretreatment of mice with diethyl maleate prior to administration of nitrofurazone a doubling of covalent binding of nitrofurazone metabolites is seen in most tissues (Stripp *et al.*, 1973). Nitrofurazone will also deplete glutathione *in vivo* in the mouse (Stripp *et al.*, 1973).

There is speculation among investigators that a hydroxylamine metabolite may be responsible for the covalent binding with tissue macromolecules upon enzymatic reduction of nitrofurans (McCalla *et al.*, 1970; Wang *et al.*, 1975a). However, a hydroxylamine derivative has never been positively indentified upon reduction of nitrofurans *in vivo* or *in vitro* (Tatsumi *et al.*, 1976). Identification of a cysteine adduct may provide indirect evidence for the

formation of a hydroxylamine metabolite upon the enzymatic reduction of nitrofurazone.

If a hydroxylaminofuran derivative is responsible for the observed covalent binding upon reduction of nitrofurazone the mechanism of binding may be analogous to that observed with the N-oxidation of acetaminophen (N-(4-hydroxyl)acetamide). Enzymatic oxidation of acetaminophen by the mixed function oxidase system generates an N-hydroxy metabolite. The hydroxyl group has the potential to disassociate to form a hydroxyl anion and an electrophilic species which is stabilized through electron donation by the p-oxygen functionality (Figure IV-1) (Mitchell et al., 1974). The hydroxylaminofuran derivative may form a stabilized electrophilic cation in a similar manner through electron donation by the oxygen of the furan ring (Figure IV-1). A glutathione adduct of acetaminophen has been indicated through the isolation of a mercapturic acid adduct of acetaminophen in the urine of humans treated with this drug (Goldstein et al., 1974; Mitchell et al., 1974).

The use of cysteine to trap electrophilic reactive metabolic intermediates through adduct formation has been used successfully with several compounds, e.g. acetylhydrazine ($CH_3CONHNH_2$) (Nelson et al., 1976).

Materials and Methods

Chemicals. Nitrofurazone (5-nitro-2-furaldehyde semicarbazone) was donated by Norwich Pharmacal Corporation.

Purified buttermilk xanthine oxidase (No. X-1875, 12.6 units/ml), hypoxanthine (No. H-9377), and L-cysteine hydrochloride (No. C-7880) were purchased from Sigma Chemical Company. The ^{14}C-nitrofurazone (5-nitro-2-furaldehyde semicarbazone [formyl-^{14}C], Figure IV-2) was donated by Dr. Taijiro Matsushima, University of Tokyo, Japan. The ^{14}C-nitrofurazone was synthesized by the Daiichi Pure Chemicals Company, Tokyo, Japan (specific activity, 1.39 mCi/mmole; radiochemical purity, 97-99 percent). The radiochemical purity was rechecked by HPLC (Figure IV-4) and found to be 98 percent. The ^{14}C-nitrofurazone (45.6 μCi) was dissolved in acetone, divided into aliquots and evaporated to dryness in vials. The vials were stored at room temperature in the dark. For metabolism studies, ^{14}C-nitrofurazone from a vial was redissolved in acetone to form a stock solution which was stored in the dark at 4° C. An appropriate aliquot from this stock solution was added to the reaction vial and evaporated for each metabolism experiment. The ^{14}C-cysteine (DL-[3-^{14}C] cysteine hydrochloride; Figure IV-2) was purchased from Amersham/Searle Corporation (specific activity, 52 mCi/mmole; radiochemical purity, 95 percent). The radiochemical purity was 89 percent as determined by high pressure liquid chromatography (Figure IV-11). The ^{14}C-cysteine was dissolved in methanol (0.05 M HCl) and stored as a stock solution (27.4 μCi/ml) in a septum covered vial under nitrogen. The acidic and anaerobic conditions of

storage of ^{14}C-cysteine were necessary to protect against oxidation of cysteine to cystine. Before each metabolism experiment, an appropriate aliquot of the ^{14}C-cysteine stock solution was added to a reaction vial and evaporated before the reaction mixture was added.

Thin Layer Chromatography. In preliminary studies, thin layer chromatography was used in an attempt to separate the metabolites of nitrofurazone. Cellulose plates (Analtech; precoated with MN 300F cellulose, thickness = 250 microns) were developed in a solvent system of 1-butanol-acetic acid-water (12:3:5, by volume) to separate metabolites of nitrofurazone (Rf of nitrofurazone = .77). However, this technique did not give a satisfactory resolution of metabolites of nitrofurazone. Therefore, high pressure liquid chromatography was utilized to separate the reduced metabolites of nitrofurazone.

High Pressure Liquid Chromatography (HPLC). A Model ALC/GPC 244 High Pressure Liquid Chromatograph (Waters Associates) was utilized for the separation of the reduced metabolites of nitrofurazone. The methanol (Burdick and Jackson Laboratories, Muskegon, Michigan) and water solvents were double distilled, filtered, and degassed before use in the high pressure liquid chromatograph. A μBondapak C_{18} reverse phase column (7mm ID x 30 cm, Waters Associates) was employed with a solvent flow rate of 4 ml/minute. Three different solvent-gradient systems were utilized in the separation of nitrofurazone metabolites: 1) pump A, 5

percent methanol/95 percent water (50 mM acetate buffer, pH 4.0); pump B, 100 percent methanol; Gradient: 0 percent pump B to 25 percent pump B in 15 minutes, curve 10 (Figures IV-4 through IV-7), 2) pump A, 100 percent water; pump B, 100 percent methanol; Gradient: 0 percent pump B to 30 percent pump B in 30 minutes, curve 10 (Figures IV-8 and IV-9), 3) pump A, 5.0 mM heptane sulfonic acid (PIC Reagent B-7, Waters Associates) in 1 percent acetic acid/99 percent distilled water; pump B, 5.0 mM heptane sulfonic acid in 1 percent acetic acid/99 percent methanol; Gradient: 0 percent pump B to 30 percent pump B in 30 minutes, curve 10 (Figures IV-10 through IV-12 and IV-14 a & b).

Hexane sulfonic acid is utilized in an analytical technique called paired ion chromatography and was specifically employed to lengthen the elution time of cysteine. At acidic pH, cysteine exists in the $-NH_3^+$ cationic form which will form a complex with the hexane sulfonic acid anion ($(SH)CH_2CH(COOH)-NH_3^+ + {}^-SO_3(CH_2)_6CH_3 \rightarrow [(SH)CH_2C(COOH)-NH_3^+ \; {}^-SO_3(CH_2)_6CH_3]^\circ$). This complex is less polar than the unpaired ionic form and will be eluted Later under reverse phase conditions (Figure IV-13 a & b).

Enzymatic Reduction of Nitrofurazone. The reaction mixtures (3.0 ml) contained 0.5 mM nitrofurazone, 2.0 mM hypoxanthine, 0.1 M phosphate buffer (pH 7.4) and 5.0 or 0.5 mM cysteine when used. The reactions were carried out in 22 mm glass vials. Nitrogen was bubbled through the reaction mixture for 15 minutes after which the nitrogen tube

(Polyethylene tubing -1.77 mm x 2.8 mm) was pulled out of the reaction mixture to a position over the reaction solution (Figure IV-3). The reaction was started by addition in an injection through the septum of xanthine oxidase (.76 units or .25 units/ml reaction mixture). The reaction was incubated at 37°C in a water bath for 80 or 90 minutes at which time all nitrofurazone was reduced (Figure IV-3). A 0.5 ml aliquot of the reaction mixure was added to double distilled methanol (reaction mixture: methanol, 1:1) to precipitate the protein and then centrifuged for 10 minutes at 10,000 rpm. A .200 ml aliquot of the supernatant was injected into the high pressure liquid chromatograph column and 30 second aliquots (2 ml) were collected in 22 mm glass scintillation vials in experiments employing radiolabelled compounds. The DPM of each aliquot were determined on a Hewlitt-Packard Scintillation Counter Model 3325 with Onmifluor^{R} aqueous scintillation cocktail.

Results

The first experiment was designed to determine if cysteine would effect the HPLC radioactive elution profile of the reduced metabolites of ^{14}C-nitrofurazone. Two parallel reactions were carried out; one reaction contained cysteine (5.0 mM), the other reaction did not. If the presence of cysteine were to be associated with the formation of a new metabolite it would appear as a new peak or a change in metabolite formation as monitored in the HPLC

radioactive elution profile. The reaction carried out in the absence of cysteine demonstrated radioactive peaks eluting at 4 minutes, 13 minutes, and (as a shoulder) at 15 minutes (Figure IV-5). The reaction carried out in the presence of cysteine had more than a two fold increase in the radioactivity eluting at 4 minutes, a disappearance of the radioactive peak eluting at 13 minutes, and a peak eluting at 15 minutes corresponding to the shoulder eluting at 15 minutes in the reaction without cysteine (Figure IV-5). Two new radioactive peaks were observed at 6 minute and 18 minute elution times. In the reaction without cysteine, monitored spectrophotometrically at 365 nm on the liquid chromatograph, a strong absorbance peak at an elution time of 13 minutes and a shoulder eluted at 15 minutes were observed; the reaction with cysteine exhibited a disappearance in the absorbance peak eluting at 13 minutes but an absorbance peak was present at 15 minutes (Figure IV-6). This parallels the observation of the radioactive elution profile for the same reaction. The same reactions monitored at 280 nm on the liquid chromatograph were somewhat less clear because of the strong absorption of hypoxanthine and oxidative products, such as xanthine. However, an absorbance peak was eluted at 13 minutes in the reaction without cysteine (Figure IV-7). In the reaction with cysteine the peak at 13 minutes disappeared and new peaks appeared at 6-7 minutes and at 18 minutes (Figure IV-

7). This again parallels the radioactive elution profiles of the same reactions.

The conclusion from the preceding data is that cysteine does indeed change the pattern of reductive metabolite formation of nitrofurazone in the xanthine oxidase-hypoxanthine system. Two new metabolite peaks appear in the reaction with cysteine. Also, there is an increase in radioactivity at an elution time of 4 minutes at the expense of radioactivity at 13 minutes.

The second experiment was designed to determine if the change in the reduced metabolite pattern of nitrofurazone in the presence of cysteine was due to the formation of an adduct with cysteine. It was therefore necessary to run two parallel reactions: one reaction contained ^{14}C-nitrofurazone and unlabeled cysteine; the other reaction contained ^{14}C-cysteine and unlabelled nitrofurazone. If an adduct were formed, a peak of radioactivity of the same elution time would be present upon HPLC analysis of both reactions. Analysis of the above reactions revealed no superimposable peaks at elution times longer than 4 minutes (Figures IV-8 and IV-9). However, unchanged cysteine is eluted at 4 minutes, the same time as a principal reductive metabolite of nitrofurazone. In addition, ^{14}C-cysteine present in the reduction of nitrofurazone by xanthine oxidase-hypoxanthine also eluted at 4 minutes. Thus, it was possible that a cysteine adduct of a reductive metabolite of nitrofurazone was present and eluted at 4 minutes.

To determine if there was a cysteine adduct of nitrofurazone present in the 4 minute peak it was necessary to separate ^{14}C-cysteine from the reductive metabolites of ^{14}C-nitrofurazone. This was accomplished by paired ion chromatography. The reduction of ^{14}C-nitrofurazone by xanthine oxidase-hypoxanthine plus cysteine revealed two major peaks of radioactivity eluting at 3 1/2 minutes and 5 minutes on the liquid chromatograph (Figures IV-10b through IV-12). Unchanged ^{14}C-cysteine is eluted at 4 to 4 1/2 minutes by the same system (Figure IV-11). The resolution between these peaks was not dramatic due to the limitation of the 30 second aliquots used for analysis. However, in a control experiment designed to determine if cysteine could reduce nitrofurazone, ^{14}C-nitrofurazone was incubated with cysteine and xanthine oxidase in the absence of hypoxanthine for 2 hours at 37° C and then 10 hours at 25° C under anaerobic conditions. Two major metabolites of ^{14}C-nitrofurazone were detected on the HPLC radioactive elution profiles. These were eluted at precisely the same times as the metabolites of ^{14}C-nitrofurazone in the xanthine oxidase-hypoxanthine system (Figure IV-10b). Some nitrofurazone (42 percent), remained unchanged. Resolution of the two major metabolites of ^{14}C-nitrofurazone and cysteine, was excellent when monitored at 254 nm (Figure IV-10a) under these conditions. However, hypoxanthine and its oxidation products absorb strongly at 254 nm (Figure IV-14 a&b) and elute at 5 minutes, the same time as a major metabolite of

nitrofurazone. Therefore, this metabolite cannot be monitored at 254 nm in typical xanthine oxidase-hypoxanthine reactions.

A third set of experiments was designed to increase the sensitivity of detection of a ^{14}C-cysteine adduct. Two reactions were carried out. The first contained the xanthine oxidase-hypoxanthine system and a 10:1 ratio of cysteine (5.0 m$\underline{M}$) to nitrofurazone (0.5 m$\underline{M}$). The second reaction contained the same reduction system and equimolar concentratons of cysteine (0.5 m$\underline{M}$) and nitrofurazone (0.5 m$\underline{M}$). The rationale for these ratios of nitrofurazone and cysteine is that in the reaction with a 10 fold excess of cysteine to nitrofurazone only a maximum of 10 percent of the total cysteine could form an adduct with nitrofurazone metabolites. In the reaction with equimolar concentrations of nitrofurazone and cysteine, however, 100 percent of the cysteine could theoretically form an adduct with nitrofurazone metabolites. Thus, for example, if 50 percent of the nitrofurazone reduced formed a cysteine adduct, only 5 percent of the cysteine would be conjugated in the first reaction; however, in the second reaction with equimolar concentrations of cysteine and nitrofurazone, 50 percent of the cysteine would be conjugated. Therefore, the radioactive HPLC elution profile of ^{14}C-cysteine would be significantly altered in the second reaction if cysteine formed a conjugate with reduced nitrofurazone in the manner described. However, the radioactive elution profile of ^{14}C-

cysteine did not present a significantly different pattern in either the equimolar reaction or the reaction with 10 fold excess cysteine from the radioactive elution profile of unchanged ^{14}C-cysteine (Figure IV-11 and IV-12 a & b). Thus, ^{14}C-cysteine radioactivity was always eluted as a peak between the two major nitrofurazone reductive metabolites (Figure IV-11 and IV-12 a & b). These data indicate that a reduced nitrofurazone cysteine adduct is not formed since no significant pattern change in the elution of ^{14}C-cysteine radioactivity by HPLC can be detected in the reaction mixtures as compared to ^{14}C-cysteine alone.

Conclusions

Three conclusions can be reached from the reductive biotransformation studies with nitrofurazone: 1) Cysteine will change the pattern of reductive metabolite formation of nitrofurazone in the xanthine oxidase-hypoxanthine system; 2) the change in the reductive metabolite formation of nitrofurazone with cysteine in the xanthine oxidase-hypoxanthine system cannot be due to the formation of a cysteine adduct with a reduced nitrofurazone metabolite; 3) the reduction of nitrofurazone by cysteine-xanthine oxidase probably produces the same reductive metabolites as the reduction of nitrofurazone by xanthine oxidase-hypoxanthine plus cysteine.

Figure IV-1

Hypothetical Electrophilic Cation Formation
from N-Hydroxy Acetaminophen and
a Hydroxylamine Furan Derivative

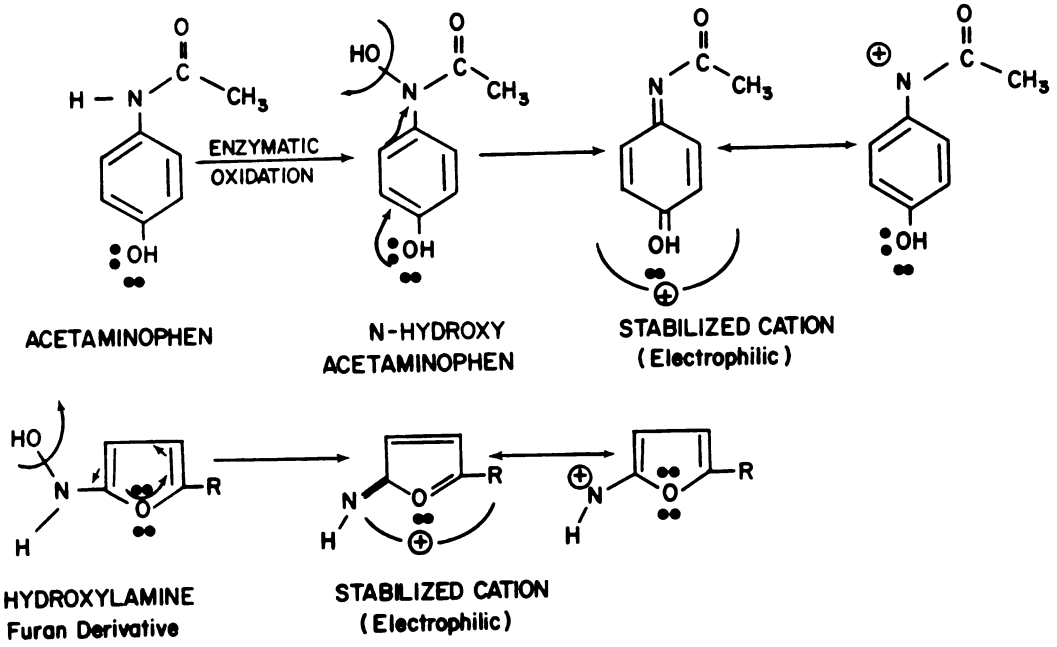
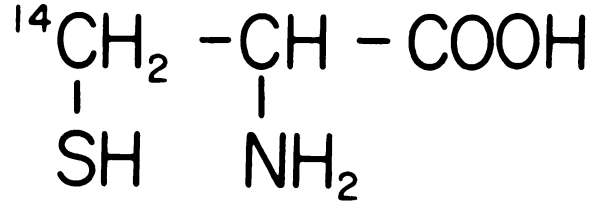
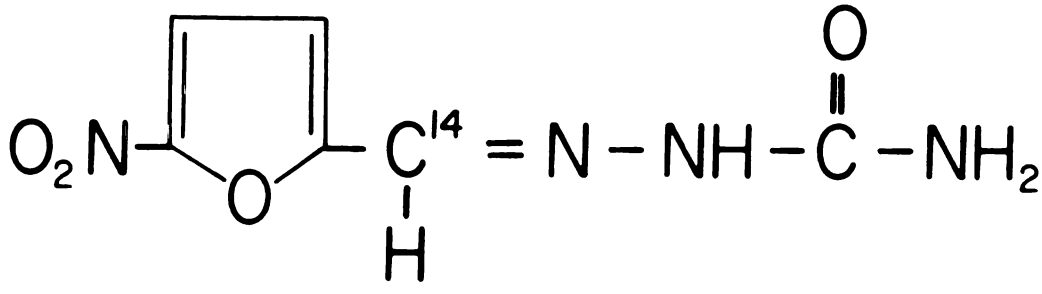


Figure IV-2

^{14}C-Cysteine (DL-[3-^{14}C] Cysteine Hydrochloride) and ^{14}C-Nitrofurazone (5-Nitro-2-Furaldehyde Semicarbazone [formyl-^{14}C])



^{14}C - Cysteine



^{14}C - Nitrofurazone

FIGURE IV-3. SCHEMATIC OF APPARATUS USED IN THE REDUCTION OF NITROFURAZONE BY XANTHINE OXIDASE.

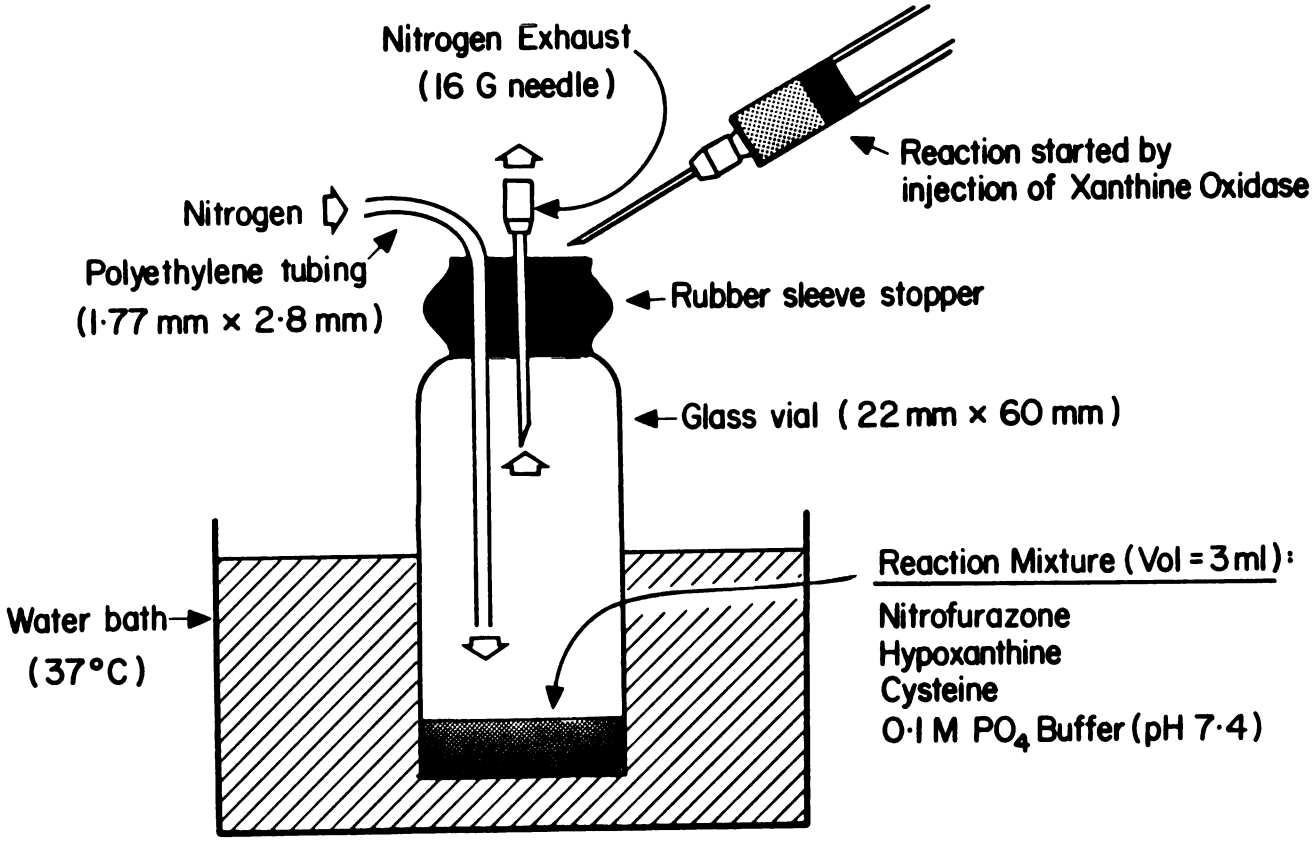


Figure IV-4

HPLC Radioactive Elution Profile of ^{14}C-Nitrofurazone

Solution Mixture (Aqueous) --

Solution volume: 3 ml
^{14}C-Nitrofurazone: 0.5 mM (0.30 μCi/ml solution mixture)
(0.005 ml dimethyl sulfoxide/ml solution mixture)
Hypoxanthine: 2.0 mM
Phosphate Buffer: 0.10 M - pH 7.4

HPLC Conditions --

Flow rate: 4 ml/minute
Pump A) 5 percent methanol/95 percent water
(50 mM acetate buffer - pH 4.0).
Pump B) 100 percent methanol.
Gradient: 0 percent B to 25 percent B, 15 minutes,
curve 10
Aliquot Sample size: 2 ml (30 second samples)

Percent Recovery of Radioactivity from HPLC --

104 percent

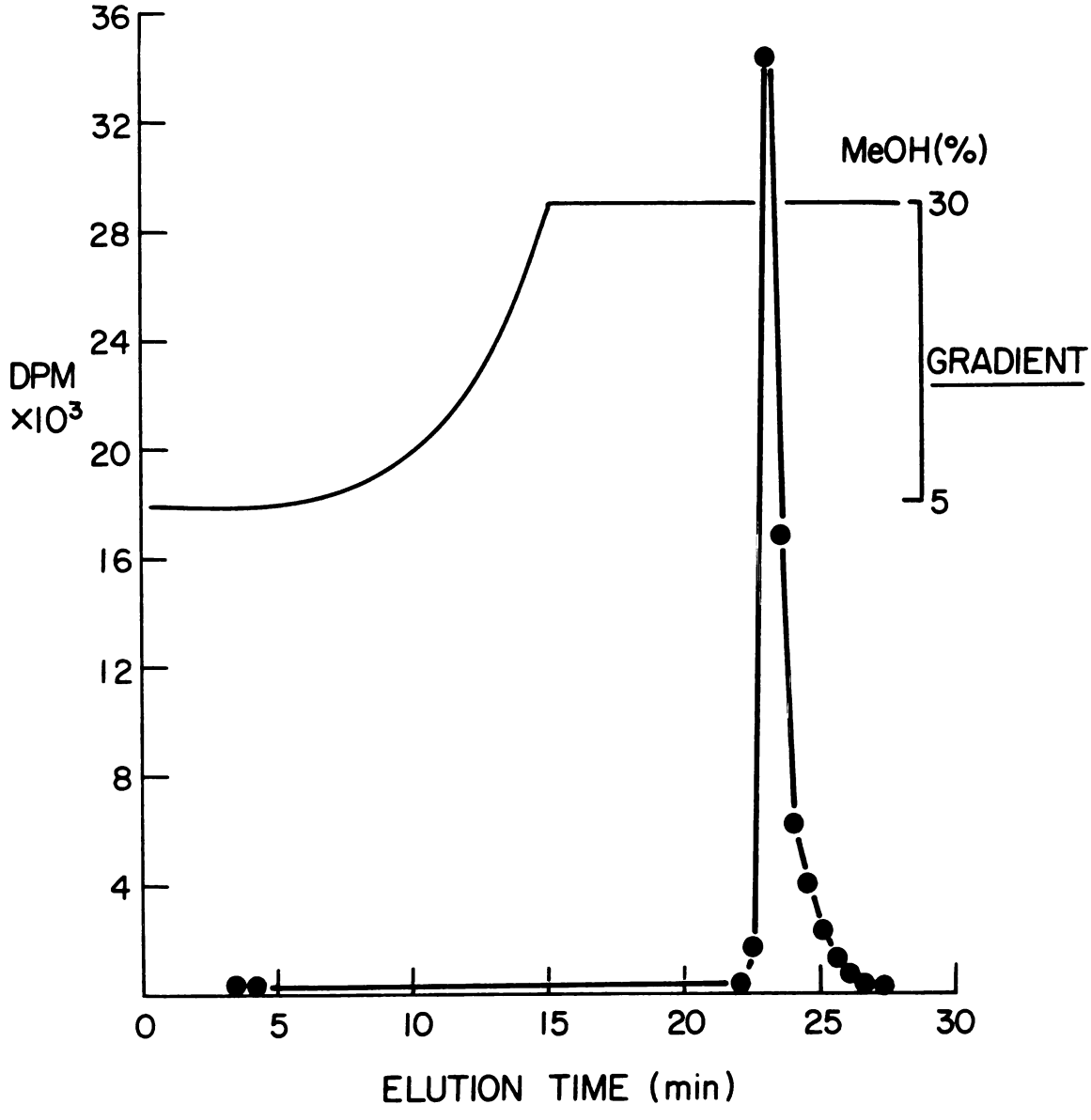

36
32
28
24
20
16
12
8
4
DPM
×10³
MeOH(%)
30
GRADIENT
5
0
5
10
15
20
25
30
ELUTION TIME (min)

Figure IV-5

Effect of Cysteine on the HPLC Radioactive Elution Profile of ^{14}C-Nitrofurazone Reduced by Xanthine Oxidase

Reaction Mixture -- Same as presented for Figure IV-4 except

Xanthine Oxidase: 0.25 units/ml (added to start reduction of nitrofurazone)
Cysteine (where indicated): 5.0 mM
Anaerobic Conditions: Reactions run under nitrogen
Reaction Time: 80 minutes

HPLC Conditions -- Same as Figure IV-4

Percent Recovery of Radioactivity from HPLC --

without Cysteine: 103 percent
with 5 mM Cysteine: 104 percent

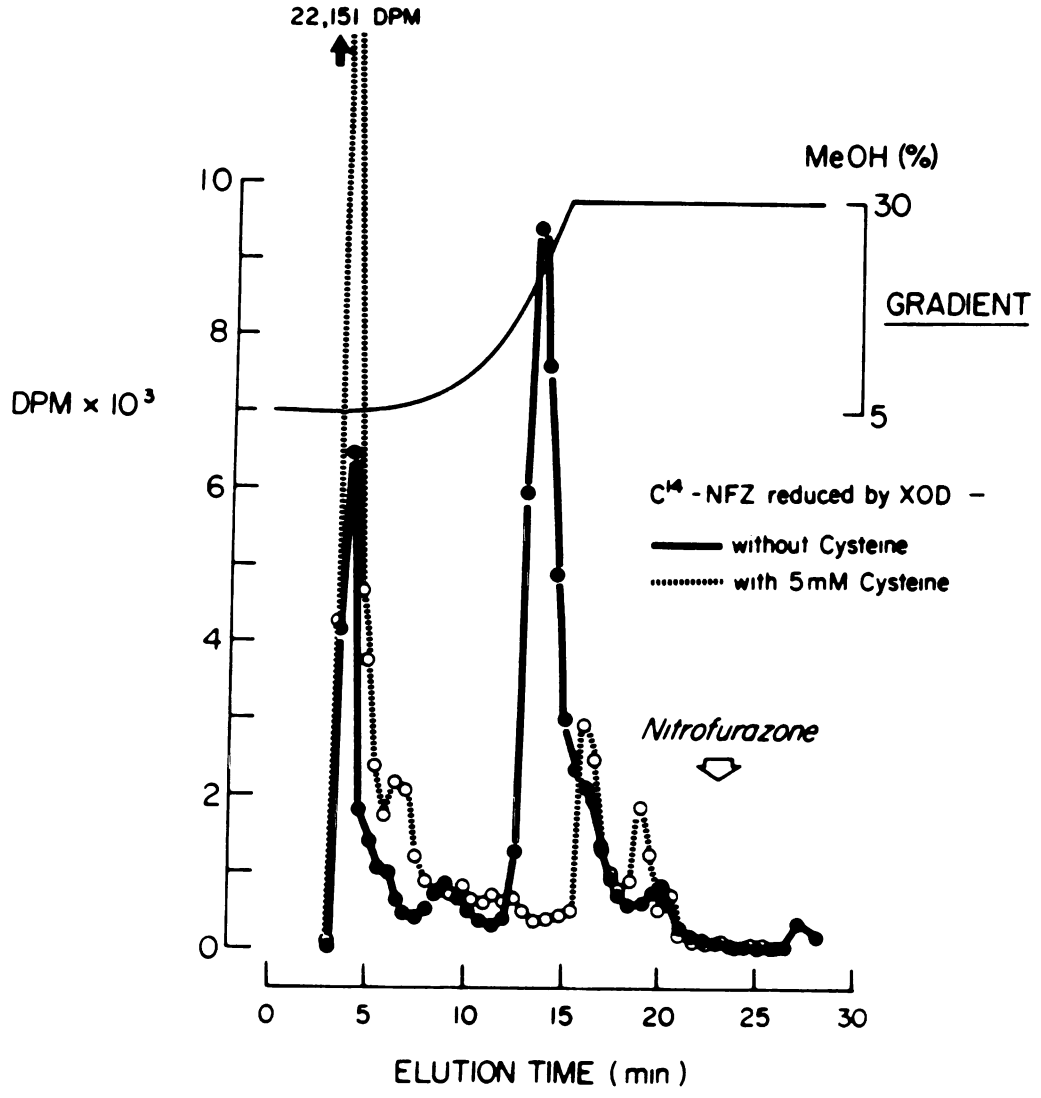

22,151 DPM
MeOH (%)
30
GRADIENT
5
DPM x 10³
10
8
6
4
2
0
C¹⁴ - NFZ reduced by XOD –
without Cysteine
with 5mM Cysteine
Nitrofurazone
0
5
10
15
20
25
30
ELUTION TIME (min)

Figure IV-6

Effect of Cysteine on the HPLC Elution Profile of Nitrofurazone Reduced by Xanthine Oxidase as Determined Absorbance at 365 λ (nm)

All conditions the same as presented for Figure IV-5, except spectroscopic monitoring at 365 λ (nm) instead of radioactivity monitoring.

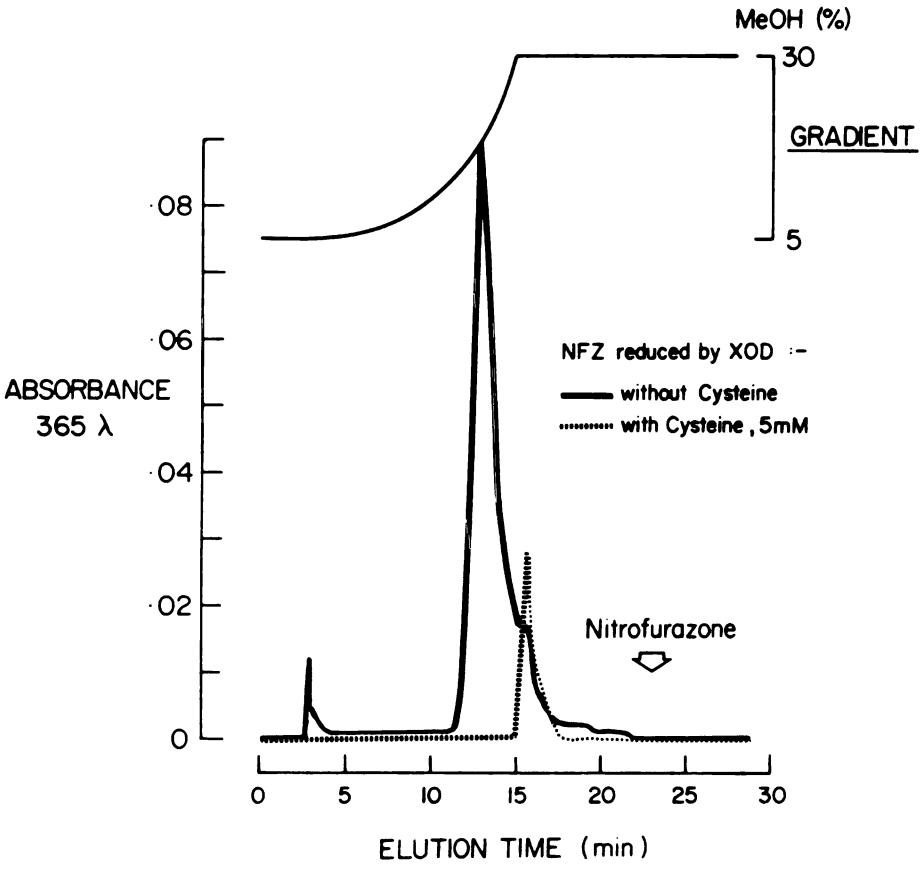

MeOH (%)
30
GRADIENT
5
·08
·06
ABSORBANCE
365 λ
·04
·02
0
NFZ reduced by XOD :-
without Cysteine
with Cysteine , 5mM
Nitrofurazone
0
5
10
15
20
25
30
ELUTION TIME (min)

Figure IV-7

Effect of Cysteine on the HPLC Elution Profile of Nitrofurazone Reduced by Xanthine Oxidase as Determined by Absorbance at 280 λ (nm)

All conditions the same as presented as for Figure IV-6

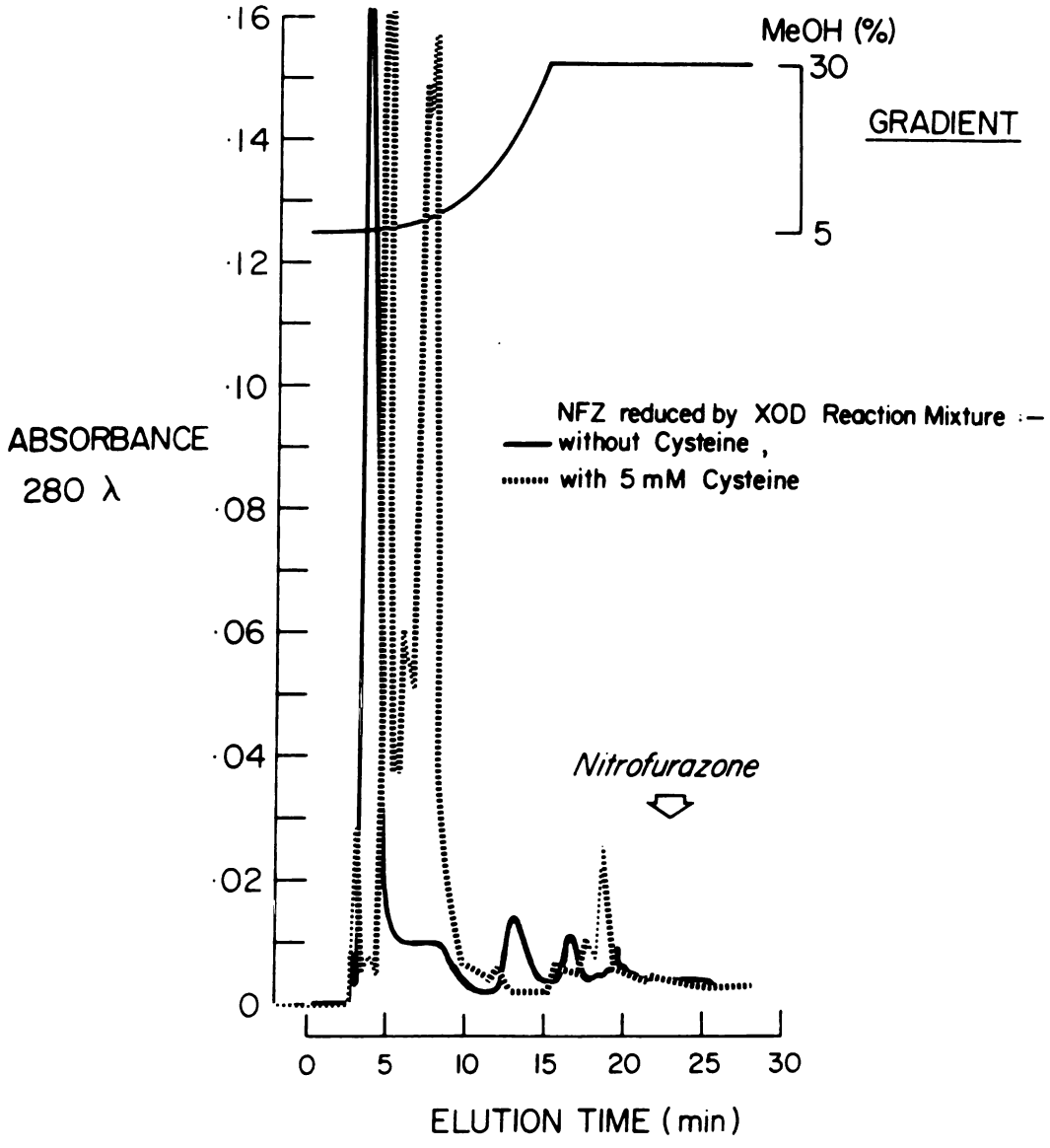

·16
·14
·12
·10
·08
·06
·04
·02
0
ABSORBANCE
280 λ
MeOH (%)
30
5
GRADIENT
NFZ reduced by XOD Reaction Mixture :—
without Cysteine ,
with 5 mM Cysteine
Nitrofurazone
0
5
10
15
20
25
30
ELUTION TIME (min)

Figure IV-8

Effect of Cysteine on the HPLC Radioactive Elution Profile of ^{14}C-Nitrofurazone Reduced by Xanthine Oxidase

Reaction Mixure: Same as presented for Figure IV-5 except

^{14}C-Nitrofurazone: 0.5 mM (0.38 μCi/ml reaction mixture)
Reaction Time: 90 minutes

HPLC Conditions --

Flow Rate: 4 ml/minute
Pump A) 100 percent water
Pump B) 100 percent methanol
Gradient: 0 percent B to 30 percent B, 30 minutes, curve 10
Aliquot Sample Size: 2 ml (30 second samples)

Percent Recovery of Radioactivity from HPLC --

without Cysteine: 117 percent
with 5 mM Cysteine: 104 percent

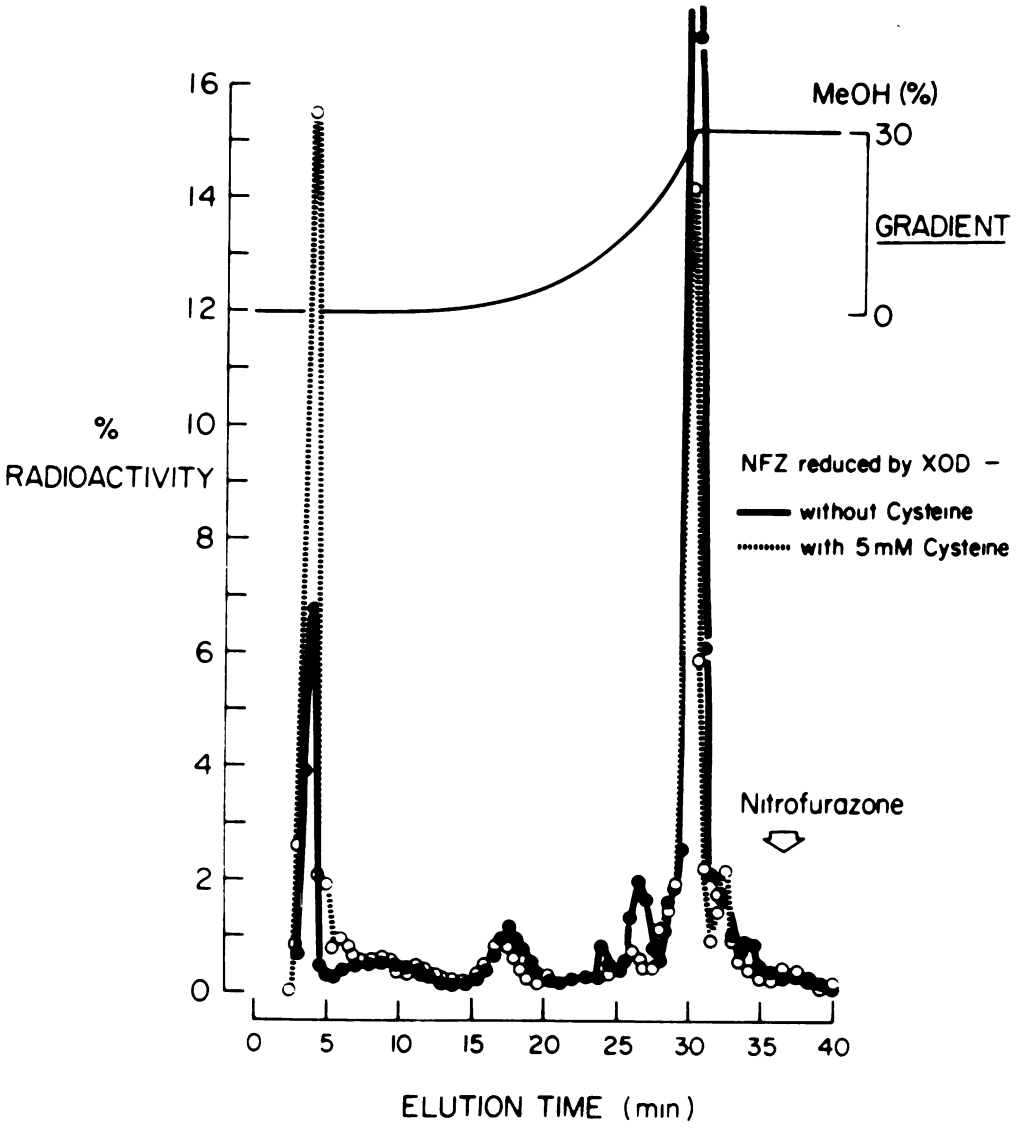

16
14
12
10
8
6
4
2
0
%
RADIOACTIVITY
MeOH (%)
30
GRADIENT
0
NFZ reduced by XOD –
without Cysteine
with 5 mM Cysteine
Nitrofurazone
0
5
10
15
20
25
30
35
40
ELUTION TIME (min)

Figure IV-9

Effect of Reduction of Nitrofurazone by Xanthine Oxidase on the HPLC Radioactive Elution Profile of ^{14}C-Cysteine.

Reaction Mixtures -- Same as presented for Figure IV-8 except

Unlabelled Nitrofurazone: 0.5 mM
^{14}C-Cysteine with XOD reaction mixture: 5.0 mM (0.74 µCi/ml reaction mixture)
^{14}C-Cysteine with NFZ and XOD Reaction Mixture: 5.0 mM (1.81 µCi/ml reaction mixture)

HPLC Conditions -- Same as presented for Figure IV-8.

Percent Recovery of Radioactivity from HPLC--

^{14}C-cysteine with XOD Reaction Mixture: 92 percent
^{14}C-cysteine with NFZ and XOD Reaction: 91 percent

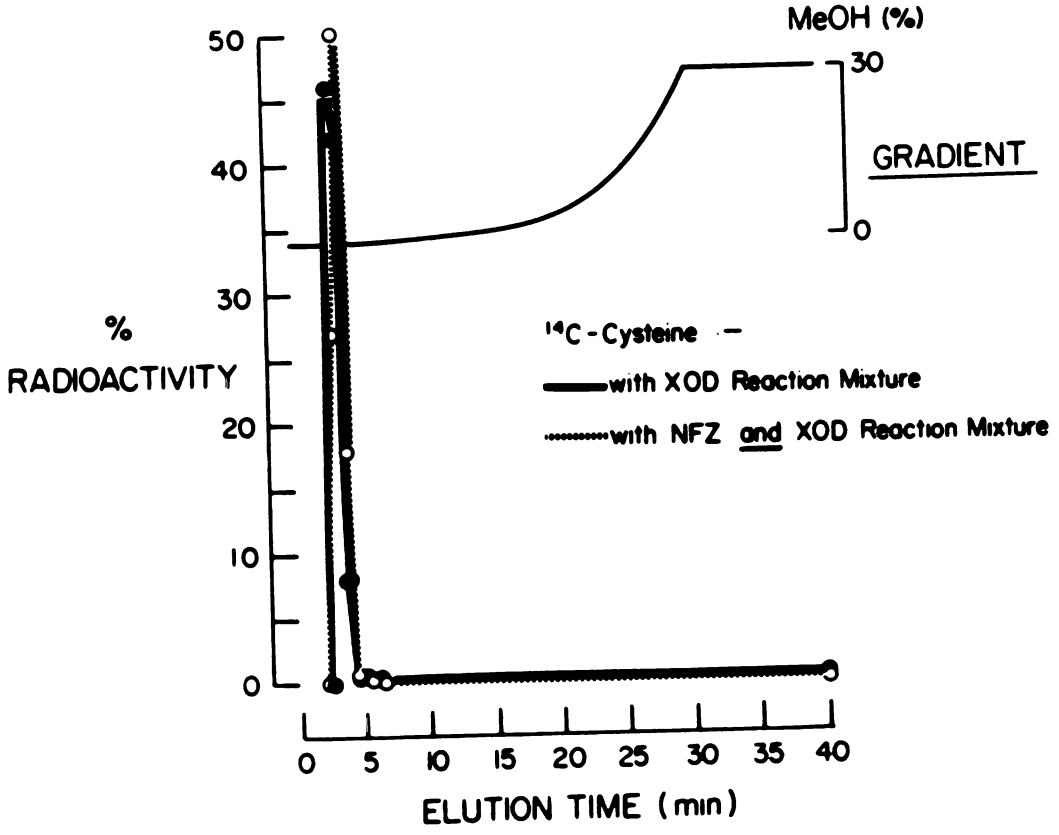

MeOH (%)
30
GRADIENT
0
50
40
30
20
10
0
%
RADIOACTIVITY
^{14}C-Cysteine
with XOD Reaction Mixture
with NFZ and XOD Reaction Mixture
0
5
10
15
20
25
30
35
40
ELUTION TIME (min)

Figure IV-10 a and b

HPLC Elution Profiles of Nitrofurazone Reduced by Xanthine Oxidase in the Presence of Cysteine or Hypoxanthine-Cysteine.

Reaction Mixtures --

NFZ reduced by XOD plus Cysteine without Hypoxanthine --
^{14}C-Nitrofurazone: 0.5 mM (0.29 μCi/ml reaction mixture)
Xanthine Oxidase: 0.25 units/ml solution mixture
Phosphate Buffer: 0.10 M - pH 7.4
Cysteine: 5.0 mM
Anaerobic Conditions: Reaction run under nitrogen
Reaction Time: 2 hours at 37°C, then 10 hours at 25°C

NFZ reduced by XOD plus Cysteine with Xypoxanthine --
Same as above except

Hypoxanthine: 2.0 mM
Reaction Time: 90 minutes at 37°C

HPLC Conditions --

Flow Rate: 4 ml/minute
Pump A) 5.0 mM heptane sulfonic acid (PIC Reagent B-7, Waters Associates) in 1 percent acetic acid/99 percent water.
Pump B) 5.0 mM heptane sulfonic acid in 1 percent acetic acid/99 percent methanol.
Gradient: 0 percent Pump B to 30 percent Pump B, 30 minutes, Curve 10
Aliquot Sample Size: 2 ml (30 second samples)

Percent Recovery of Radioactivity from HPLC --

without Hypoxanthine: 104 percent
with Hypoxanthine: 102 percent

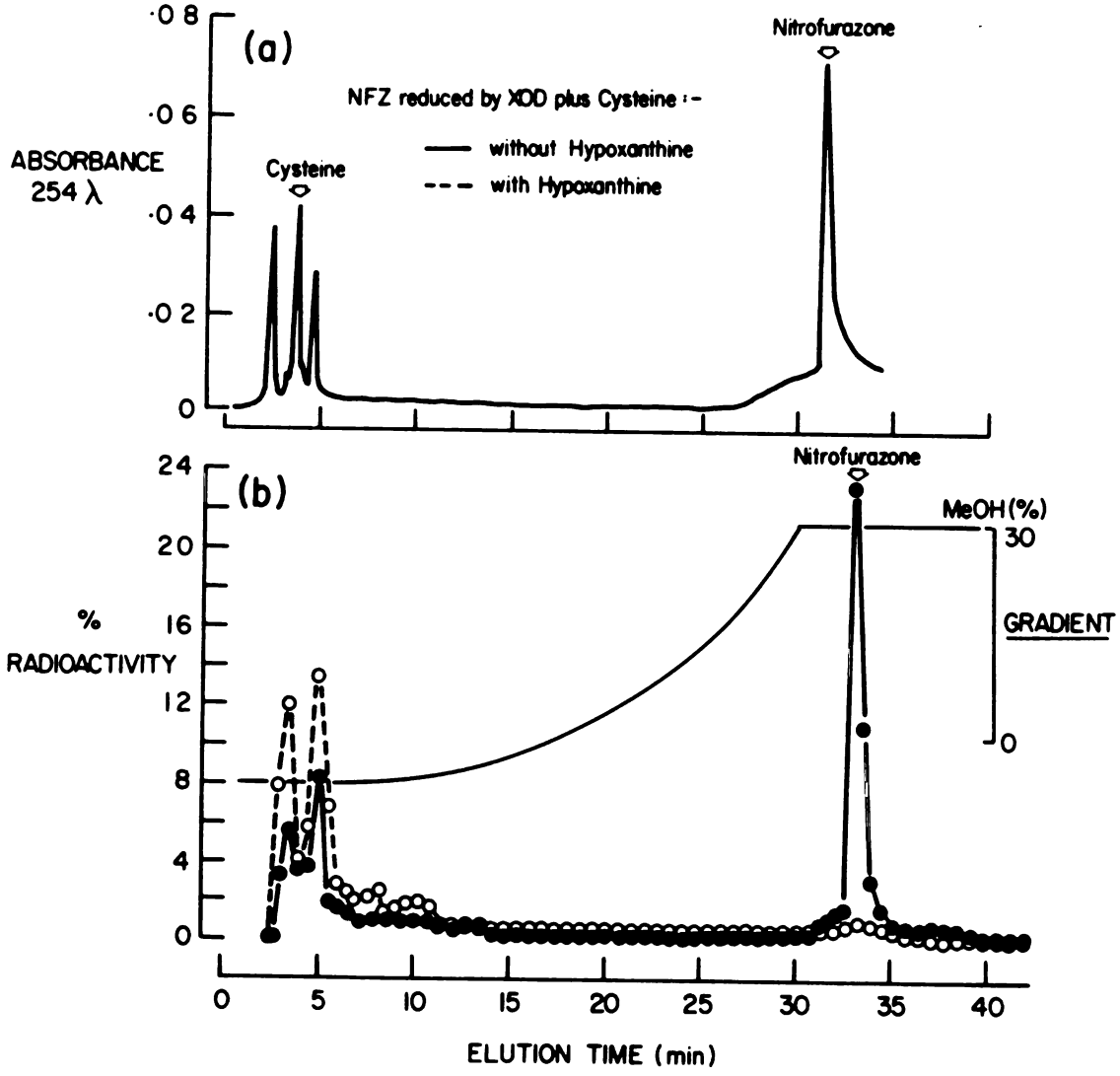

(a)
NFZ reduced by XOD plus Cysteine :-
without Hypoxanthine
with Hypoxanthine
Cysteine
Nitrofurazone
ABSORBANCE
254 λ
·08
·06
·04
·02
0
(b)
Nitrofurazone
MeOH(%)
30
0
GRADIENT
% RADIOACTIVITY
24
20
16
12
8
4
0
0
5
10
15
20
25
30
35
40
ELUTION TIME (min)

Figure IV-11

HPLC Elution Profiles of ^{14}C-Cysteine Alone and of ^{14}C-Nitrofurazone Reduced by Xanthine Oxidase-Hypoxanthine in the Presence of Cysteine

Reaction Mixtures --

^{14}C-cysteine alone --
^{14}C-cysteine: 16 mM (.14 μCi/ml solution mixture)
(water:methanol, 1:1)

^{14}C-NFZ Reduced by XOD plus cysteine --
Same as presented for NFZ reduced by XOD plus Cysteine with Hypoxanthine in Figure IV-10 a and b

Percent Recovery of Radioactivity from HPLC --

^{14}C-Cysteine alone (only) - 105 percent
^{14}C-NFZ reduced by XOD plus 5 mM Cysteine - 102 percent

Note: Inner Ordinate for ^{14}C-Nitrofurazone
Outer Ordinate for ^{14}C-Cysteine

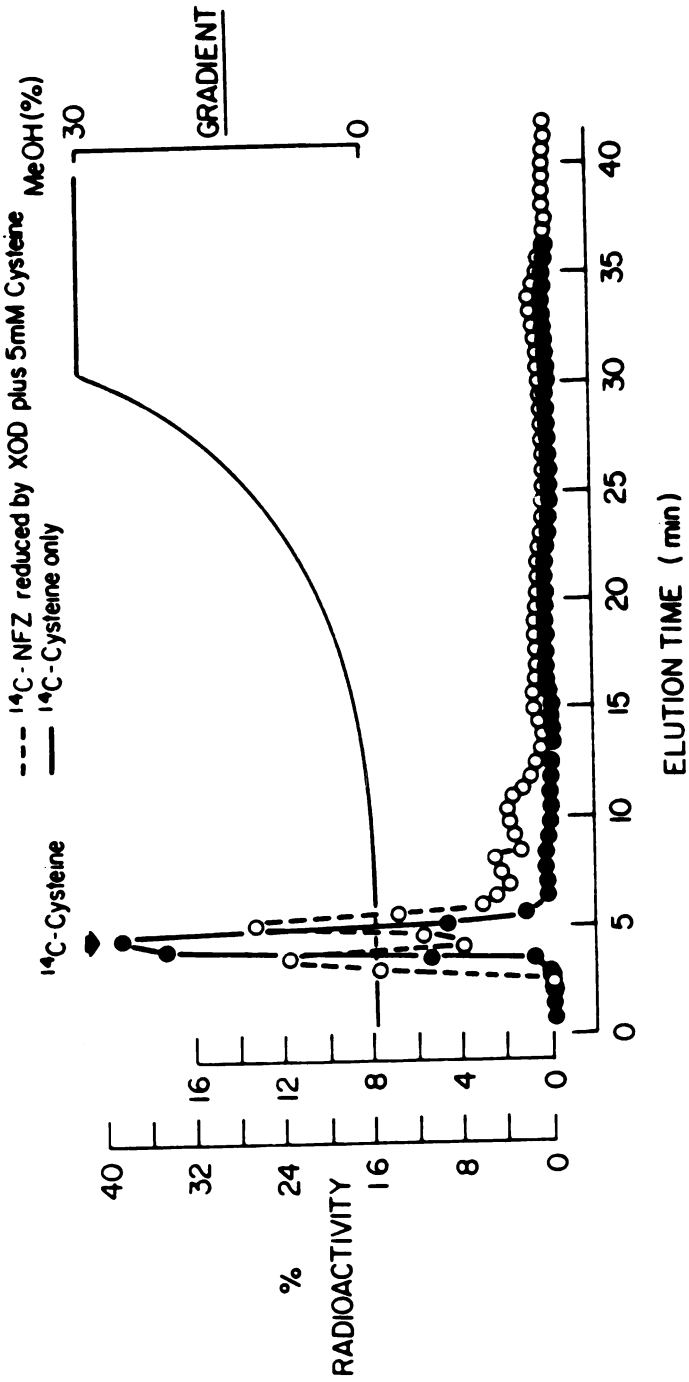

--- ^{14}C-NFZ reduced by XOD plus 5mM Cysteine
— ^{14}C-Cysteine only
MeOH(%)
30
0
GRADIENT
^{14}C-Cysteine
% RADIOACTIVITY
40
32
24
16
8
0
16
12
8
4
0
0
5
10
15
20
25
30
35
40
ELUTION TIME (min)

Figure IV-12 a and b

Reduction of Nitrofurazone (0.5 mM) by Xanthine Oxidase-Hypoxanthine plus Cysteine (5.0 mM or 0.5 mM) - HPLC Elution Profiles

Reaction Mixtures --

^{14}C-NFZ -- Same as presented for NFZ reduced by XOD plus Cysteine with Hypoxanthine in Figure IV-10 a and b.

^{14}C-Cysteine (5.0 mM Cysteine) -- Same conditions as presented above except

Unlabelled Nitrofurazone: 0.5 mM
^{14}C-Cysteine: 5.0 mM (0.91 μCi/ml reaction mixture)

^{14}C-Cysteine (0.5 mM Cysteine) -- Same conditions as presented above except

Unlabelled Nitrofurazone: 0.5 mM
^{14}C-Cysteine: 0.5 mM (0.30 μCi/ml reaction mixture)

Percentage Recovery of Radioactivity by HPLC

^{14}C-NFZ: 102 percent
^{14}C-Cysteine (5.0 mM Cysteine): 101 percent
^{14}C-Cysteine (0.5 mM Cysteine): 95 percent

Note: Inner Ordinate for ^{14}C-Nitrofurazone
Outer Ordinate for ^{14}C-Cysteine

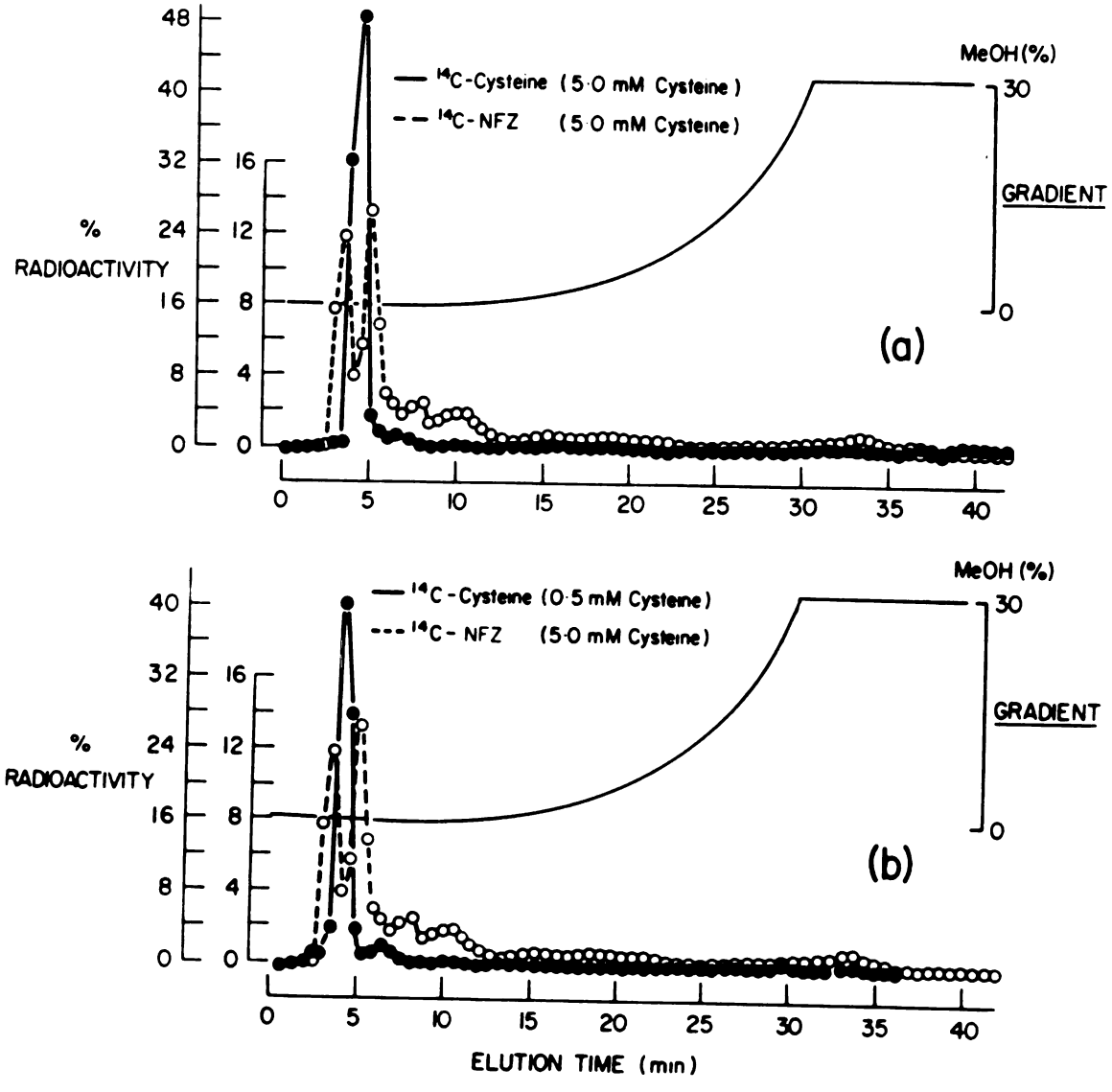

48
40
32
16
%
24
12
RADIOACTIVITY
16
8
8
4
0
0
0
5
10
15
20
25
30
35
40
^{14}C-Cysteine (5·0 mM Cysteine)
^{14}C-NFZ (5·0 mM Cysteine)
MeOH(%)
30
GRADIENT
0
(a)
40
32
16
%
24
12
RADIOACTIVITY
16
8
8
4
0
0
0
5
10
15
20
25
30
35
40
^{14}C-Cysteine (0·5 mM Cysteine)
^{14}C-NFZ (5·0 mM Cysteine)
MeOH (%)
30
GRADIENT
0
(b)
ELUTION TIME (min)

Figure IV-13 a and b

HPLC Elution Profiles of Cysteine as
Determined by Absorbance at 280 λ (nm)

Cysteine Concentration: 0.075 M (methanol: water, 1:1)
Injection Volume: .050 ml

(a) Eluting with 1 percent Acetic Acid/99 percent water.

(b) Eluting with 0.5 mM Heptane Sulfonic Acid (1 percent Acetic Acid/99 percent water).

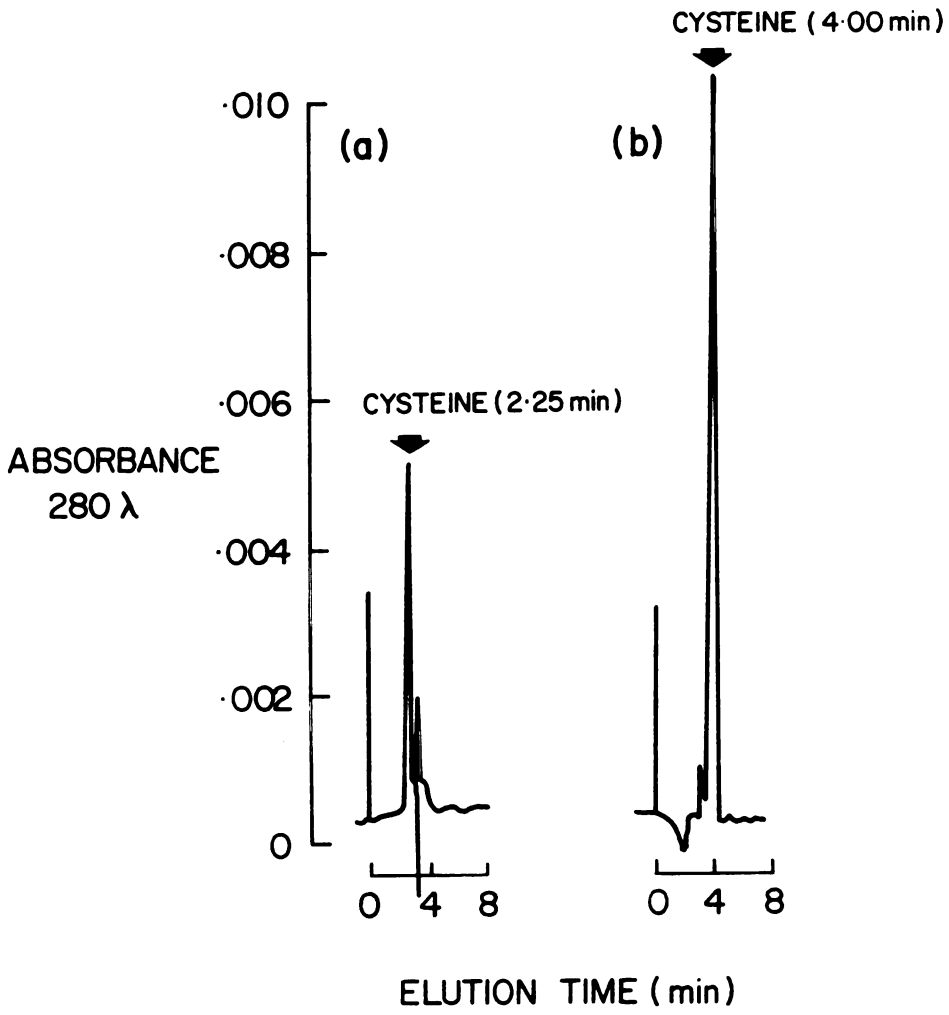

CYSTEINE (4·00 min)
·010
(a)
(b)
·008
·006
CYSTEINE (2·25 min)
ABSORBANCE
280 λ
·004
·002
0
0 4 8
0 4 8
ELUTION TIME (min)

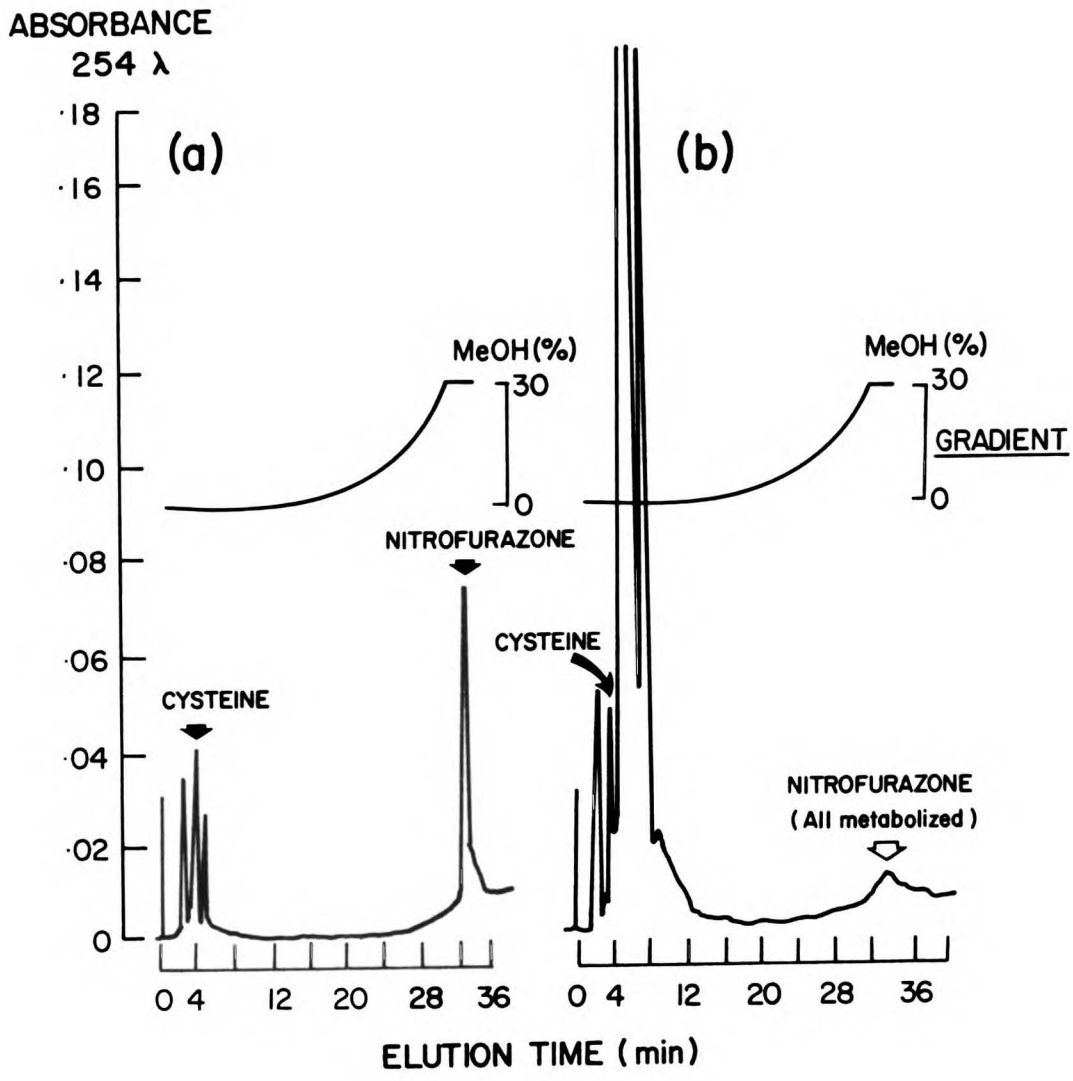
Figure IV-14 a and b

HPLC Elution Profiles of Reduction Products of Nitrofurazone Determined by Absorbance at 254 λ (nm)

(a) Partial Reduction of Nitrofurazone by Xanthine Oxidase-Cysteine only.

(b) Complete Reduction of Nitrofurazone by Xanthine Oxidase-Hypoxanthine plus Cysteine

Reaction and HPLC Conditions the same as presented for Figure IV-10 a and b.


ABSORBANCE
254 λ
·18
·16
·14
·12
·10
·08
·06
·04
·02
0
(a)
(b)
MeOH (%)
30
0
GRADIENT
NITROFURAZONE
CYSTEINE
NITROFURAZONE
(All metabolized)
0 4 12 20 28 36
ELUTION TIME (min)

V. DISCUSSION

Mutagenicity of Nitrofurans

The fact that nitrofurazone is positive in one mutagenicity test system (*Salmonella typhimurium*) but not in two other test systems (the micronucleus test and the *in vivo* cytogenetic test in rats) demonstrates the need for a battery of tests rather than reliance on one test system in evaluating potential mutagens. A conclusion from the testing of nitrofuran compounds alone could be that the *Salmonella typhimurium* test system is superior in detecting mutagens when compared to the micronucleus test and *in vivo* cytogenetic testing in mammals. However, this reasoning is specious, since compounds (*e.g.* benzene) can be indicated as mutagens in the latter two tests, but not in *Salmonella typhimurium* (Lyon, 1975).

There are several possible reasons why nitrofurazone is negative in the micronucleus test and by *in vivo* cytogenetic testing. One possibility is that nitrofurazone does not cause mutations in the bone marrow cells of the rats used for testing. A second possibility is that nitrofurazone causes point mutations in the bone marrow cells of rats, but that these mutations are not expressed in the form of chromosomal aberrations. Finally, nitrofurazone may not reach the bone marrow cells in high enough concentration to cause mutagenic events.

The number of investigations on the mutagenicity of nitrofurans have increased explosively over the past several years (Tazima et al., 1975). However, certain areas of investigation may still be fruitful. One area would be the investigation of the mutagenicity of individual metabolites of the nitrofurans. The aminofurans, isolated as reductive metabolites from the nitrofurans, should be evaluated in the Salmonella typhimurium TA100 and TA98 tester strains. It has been demonstrated that a wide variety of nitroheterocyclics are mutagenic in Salmonella typhimurium and E. coli and that aminoheterocyclic derivatives of these compounds are not indicated as mutagens in these same strains (Wang et al., 1975b; McMahon et al., 1976). Likewise, the aminofurans are probably not directly mutagenic in Salmonella typhimurium. However, these compounds may be positive mutagens in Salmonella typhimurium if enzymatically activated. The mixed function oxidase system may be able to oxidize an aminofuran derivative to the hydroxylaminofuran metabolite. This may render the aminofuran derivative mutagenic by a similar mechanism as the enzymatic reduction of the a nitrofuran to a theoretical hydroxylaminofuran metabolite.

Biotransformation of Nitrofurazone

The dramatic change in the pattern of metabolite formation of nitrofurazone upon reduction in the presence of cysteine may be due to a synergistic action of cysteine on

nitroreduction by xanthine oxidase-hypoxanthine. It has been previously reported that cysteine can enhance the reduction of nitrofurazone by xanthine oxidase (Taylor et al., 1951). Perhaps cysteine can interact strongly with a reduced intermediate metabolite of nitrofurazone and thereby increase its rate of reduction by xanthine oxidase. Such an interaction could account for the protection cysteine affords against the covalent binding of reduced nitrofurazone to serum albumin in the xanthine oxidase system (McCalla et al., 1970). The cysteine could rapidly interact with a reactive metabolite, e.g. a hydroxylaminofuran derivative, that was responsible for the covalent binding with protein.

There is some corroborating evidence that sulfhydryl containing compounds can stimulate certain enzymatic reactions. Glutathione and cysteine stimulate the metabolism of trihalomethanes (haloforms) to carbon monoxide by a rat liver microsomal fraction requiring NADPH as a cofactor and molecular oxygen (Ahmed et al., 1977). Glutathione alone did not serve as a cofactor for this reaction (Ahmed et al., 1977). These data parallel evidence that cysteine may increase the rate of reduction of nitrofurazone by xanthine oxidase (Taylor et al., 1951).

The effect of cysteine on the rate of metabolite formation upon reduction of nitrofurazone with xanthine oxidase-hypoxanthine should be done using high pressure liquid chromatography since previous studies have simply

monitored the decrease in absorbance of nitrofurazone at 365 nm as a measure of nitroreduction. However, metabolites of nitrofurazone, such as 5-amino-2-furaldehyde semicarbazone, absorb significantly in this region (Tatsumi et al., 1976). High pressure liquid chromatography could provide discrete quantitation of the rate of metabolite formation not obtainable by regular spectroscopic techniques.

The metabolites of nitrofurazone which were separated by high pressure liquid chromatogrph should be collected and analyzed by mass spectroscopy, infra-red spectroscopy, and ultra-violet spectroscopy. Standards of known nitrofurazone metabolites, such as 5-amino-2-furaldehyde semicarbazone and 4-cyano-2-oxobutyraldehyde semicarbazone, should also be analyzed to help confirm structures of the metabolites isolated by high pressure liquid chromatography.

A final area of needed investigation would be to examine the effect of cysteine on the nitroreduction of nitrofurazone by the 9,000 x g rat liver supernatant fraction. Contrary to the reported stimulatory effect of cysteine on the reduction of nitrofurazone by xanthine oxidase, cysteine does not stimulate nitroreduction of N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide when added to rat liver microsomes (Wang et al., 1975a). However, cysteine does protect against protein binding of the reduced nitrofuran metabolite to protein. It may be that the 9,000 x g rat liver supernatant fraction can reduce nitrofurazone in a different manner than xanthine oxidase. Also, the

9,000 x g rat liver supernatant fraction can esterify hydroxylamine derivatives, which facilitates the formation of an electrophilic cation which can conjugate with cysteine. This may occur with a hydroxylaminofuran derivative.

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