NITROFURAZONE ACTION ON BACTERIA

THE MODE OF ACTION OF NITROFURAZONE ON BACTERIA

By

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ABSTRACT

Studies are described which show that activated nitrofurazone is able to cause damage to covalently closed circular DNA in minicells from *Escherichia coli* strain χ1256. These results are discussed in terms of the nature of the damage and of the repair of this damage in minicells.

Studies on the effect of nitrofurazone on macromolecules in *Escherichia coli* B/r have shown that the activated nitrofurazone inhibits the synthesis of RNA and ribosomes and causes damage to the function of ribosomes. Studies have also shown that the activated nitrofurazone inhibits aerobic and anaerobic metabolism and lowers the ATP levels in bacteria. The mechanism by which these effects are produced are discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

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HISTORICAL INTRODUCTION

5-Nitrofurans are a group of synthetic chemicals which have found widespread use in human and veterinary medicine, and in agriculture. The use of these chemicals as pharmacological agents began with the 1944 report of Dodd and Stillman (1) on the effectiveness of nitrofurazone as a topical antibacterial drug. Since that time, several thousand 5-nitrofuran derivatives and related analogs have been synthesized, several of which have been used commercially.

I. Synthesis

Furfural (furaldehyde) is the starting point in the synthesis of many nitrofurans. Since furfural decomposes rapidly in the presence of concentrated acid, early researchers had little success in nitrating this compound. Through the efforts of Marquis (2) in 1901, a technique was developed for reacting furan directly with a mixture of fuming nitric acid and acetic anhydride. The intermediate obtained was then treated with base to remove the acetate group and form the nitro compound. It was not until 1930, however, that Gilman and Wright (3) succeeded in nitrating furfural as a step in the synthesis of certain aminofurans (3). A brief outline of the steps of the synthesis of four nitrofurans appears in Fig. 1.

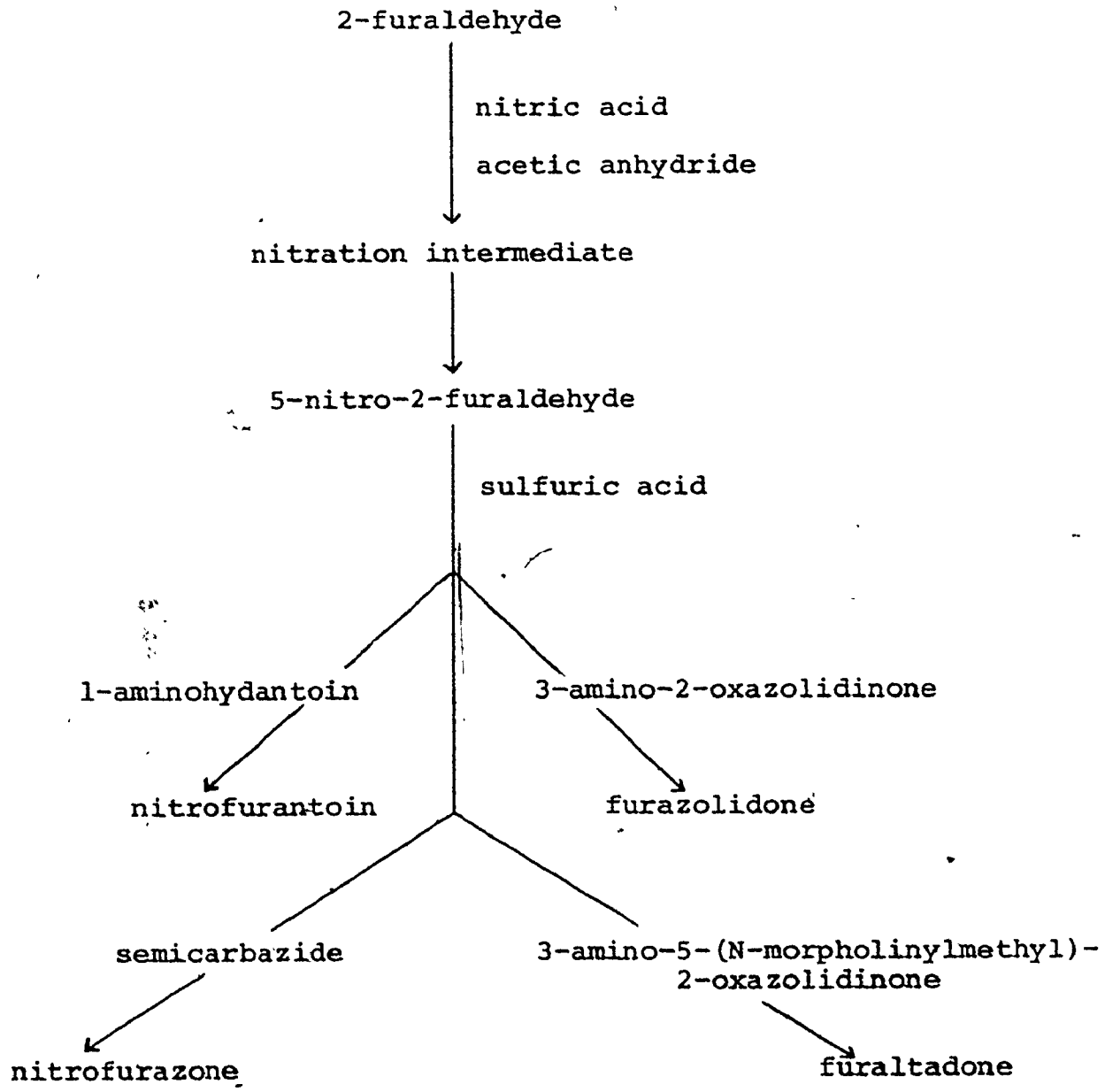


Figure 1. General scheme of nitrofuran synthesis.

II. Antibacterial Activity

The use of nitrofuran derivatives as antibacterial chemotherapeutic agents in the treatment of disease in man and domestic animals has been a development of the past 35 years. The activity of several nitrofurans against representative species of bacteria is shown in Table I. The eight compounds are active against six of the seven species tested. Except for nifuroxime and nitrofurfuryl methyl ether, the compounds are inactive against *Pseudomonas aeruginosa*. Quantitative difference in antibacterial activity between these compounds exist.

The relative antibacterial activity of certain compounds related structurally to nitrofurazone is shown in Table II. The non-nitrated furaldehyde semicarbazone (I) is devoid of antibacterial activity at concentrations 20 to 180 times those at which nitrofurazone (nitrofuraldehyde semicarbazone) (II) is effective. Dodd and Stillman compared 17 nitrofurans and their non-nitrated analogs and concluded that a nitro group in the 5-position conferred considerable antibacterial activity on derivatives of furan; 2-furaldehyde, 2-furfuryl alcohol, and 2-furoic acid. The antibacterial activity of the thiophene analog (III) in Table II is definitely lower than that of nitrofurazone. p-Nitrobenzaldehyde (IV) semicarbazone shows no antibacterial activity even in saturated solutions. The relative inactivity of the 2-nitro-4-furaldehyde semicarbazone (V) has been reported by Hayes (4).

TABLE I

Antibacterial Activity *In Vitro* by Nitrofurans

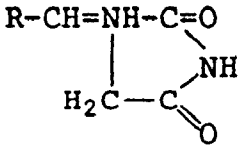
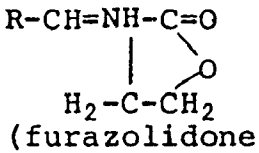
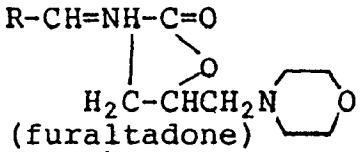
Compound[b]	Minimal Inhibiting Concentrations (μg/ml)[a]						
	E. coli	*Staph. aureus*	*S. typhosa*	*P. vulgaris*	*Ps. aeruginosa*	*Strept. pyogenes*	*A. aerogenes*
R-CH=NNHCONH$_2$ (nitrofurazone)	6	5	10	40	>200	10	13
R-CH=NNHCOCH$_2$ (nihydrazone)	5	5	5	22	>44	16	No data
R-CH=NNHCOCONH$_2$ (nifuraldezone)	17	10	23	45	>47	21	No data
 (nitrofurantoin)	3	12	12	100	>700	6	100
 (furazolidone)	1.5	5	0.7	55	>99	>55	5
 (furaltadone)	2	2	22	>390	>390	45	172
R-CH-NOH (nifuroxime)	10	37	1	125	194	23	No data

TABLE I (cont'd)

Compound[b]	Minimal Inhibiting Concentrations (μg/ml)[a]						
	E. coli	*Staph. aureus*	*S. typhosa*	*P. vulgaris*	*Ps. aeruginosa*	*Strept. pyogenes*	*A. aerogenes*
R-CH_2OCH_3 (nitrofurfuryl methyl ether)	14	67	17	22	48	72	No data

[a] Data from The Norwich Pharmacal Company.

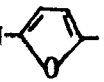
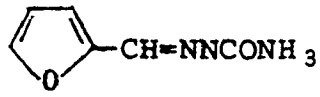
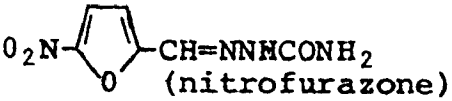
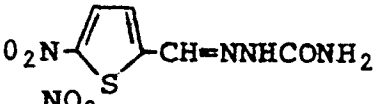
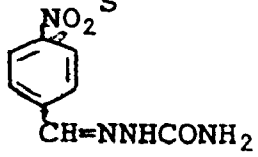
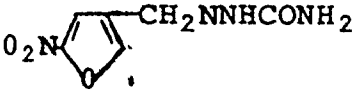
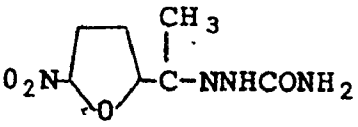
[b] R=O_2N- 

TABLE II

Antibacterial Activity *In Vitro* of Some Nitrofurans and Other Compounds

No.		Minimal Inhibiting Concentration (μg/ml)			
		E. coli	*Staph. aureus*	*S. typhosa*	*P. vulgari*
I		>750	>750	>750	>750
II	 (nitrofurazone)	6	5	10	40
III		9	35	35	>35
IV		>120	>120	>120	>120
V		>100	>100	>100	>100
VI		6	8	10	90

[a] Data from The Norwich Pharmacal Company.

The methyl ketone semicarbazone (VI) has antibacterial activity similar to that of nitrofurazone. Ward and Dodd (5) found that in a limited series of simple derivatives of furan, thiophene, and pyrrole, the antibacterial activity conferred on these derivatives by the introduction of a nitro group in this order: furan > thiophene > pyrrole.

The chemotherapeutic properties of nitroheterocyclic compounds have recently been reviewed by Greenberg and Titsworth (5a).

III. Development of Bacterial Resistance

A number of studies on the development of bacteria resistant to nitrofurans have been carried out. There appears to be little or no cross resistance between the nitrofurans and other antibiotics. H. E. Paul and co-workers (6) have shown that bacteria made resistant to chloramphenicol remain susceptible to the nitrofurans. These workers were able to develop strains with limited resistance to nitrofurans but observed that resistance to nitrofurans developed slowly and was seldom complete. Kefauver *et al.* (7) found that an antibiotic-susceptible strain of *Staphylococcus aureus* developed only a 12-fold increase in resistance to furaltadone after ten transfers in broth containing increasing drug concentrations. Although bacteria resistant to the nitrofurans can be isolated after appropriate laboratory techniques, there is little evidence in clinical studies of development

of resistance *in vivo* (Schneierson). Waisbren and Strelitzer (8) reported no loss of sensitivity to nitrofurantoin by strains of coagulase-positive *Staphylococcus aureus* isolated from patients treated with nitrofurantoin.

The biochemical mechanism underlying the development of resistance to nitrofurans has been studied by several workers and a number of papers on this subject have been published. Asnis *et al.* (9) studied bacterial cells which had been made resistant to nitrofurazone and found that resistance was not accompanied by any change in morphology, growth rate, rate of glycolysis or nutritional requirements of the bacteria. Nitrofurazone-resistant bacteria showed little growth inhibition under aerobic conditions but remained susceptible to nitrofurazone when grown under strict anaerobic conditions. In an extension of these studies, using cell-free extracts of nitrofurazone and the parental non-resistant *Escherichia coli* strain, Asnis (10) found that certain flavoprotein enzymes could catalyze the reduction of nitrofurazone. One enzyme was NADH-linked, while the second could use either NADH or NADPH. Extracts of susceptible *E. coli* organisms could carry out both types of nitrofurazone reduction, while extracts of the resistant organisms could carry out only the first type (i.e., NADH-linked), but not the second.

McCalla *et al.* (11) have isolated some nitrofurazone-resistant mutants and found that the enzyme (reductase I)

involved in the first type of reduction is lost in one or two mutational steps, depending on the strain of *E. coli* used. McCalla *et al.* (12) have further characterized the reductases. As shown in Table III, they found that *E. coli* contains at least three nitrofurazone-reducing enzymes. Reductase I (Mol. Wt. ∿ 50,000) is active in the presence of O_2 and uses either NADPH or NADH. In contrast, reductase IIa and reductase IIb have higher molecular weights, but do not reduce nitrofurans in the presence of O_2 and use only NADH. The resistant strain of *E. coli* contain only reductases IIa and IIb, while the sensitive strain contains reductase I as well. When resistant strains are incubated under anaerobic conditions, lethality, mutation and DNA breakage are all greatly increased. These results provide evidence that reductase II is able to activate nitrofuran derivatives yielding products that cause damage to cells.

IV. Mode of Action

The broad antimicrobial activity of the nitrofurans and their relatively low acute toxicity to animals including man has led to considerable speculation on their mode of action. The first studies of the properties of the nitrofurans responsible for their antibacterial activity were concerned with the general role of reduction of the nitro group. Cramer (13) suggested that nitrofurazone interferes with a normal metabolic process by virtue of its

Table III

Properties of Nitrofuran Reductases[a]

	Reductase I	Reductase II	
		a	b
Cofactor	NADPH or NADH	NADH only	
O_2 Sensitivity	No	Yes	Yes
Molecular Weight	~50,000	120,000	700,000
Presence in Resistant Mutant	No	Yes	Yes

[a] Date from reference 12.

capacity to be reduced. Dann and Möller (14) synthesized and compared the antibacterial activity of several nitrobenzene, nitrothiophene, nitronapthalene and nitrofuran compounds and concluded that ease of reduction played a leading role in determining the effectiveness of aromatic or heterocyclic nitro compounds.

Green (15) found that nitrofurazone inhibits respiration of ~~*Staphylococcus*~~ *aureus* and ~~*Escherichia*~~ *coli*. At the enzyme level, Green and co-workers (16) determined the effect of nitrofurazone on malic, succinic, lactic and 5-glycerophosphate dehydrogenases of *E. coli* using either methylene blue reduction or oxygen uptake as a measure of activity. They found rather marked interference by nitrofurazone in those reactions but no interference in the formic dehydrogenase of *E. coli* or in the 3-glycerophosphate dehydrogenase of rabbit thigh muscle. From these results and from studies on urease they postulated that nitrofurazone inhibits some enzymes requiring an active sulfhydryl group. Brodie and Gots (17) found no effect of nitrofurazone on triose phosphate dehydrogenase of yeast, an enzyme which is considered to be "sulfhydryl" in nature. They further indicated that what Green and his co-workers found was not true inhibition of enzyme activity but rather reduction of the nitrofuran in preference to the dye.

Asnis *et al.* (18) reported no effect of nitrofurazone on formic dehydrogenase of *E. coli* in an aerobic system

when oxygen uptake was measured, but found complete inhibition of hydrogenase in an anaerobic system in which evolution of hydrogen was measured.

Asnis and Gots (19) reported that nitrofurazone inhibited the anaerobic glycolysis of *E. coli* as determined by measuring carbon dioxide production. Asnis (20) also demonstrated that aerobic oxidative dissimilation of glucose by resistant *E. coli* was not inhibited by nitrofurazone but that anaerobic dissimilations were still sensitive to inhibition.

While inhibition of glucose or pyruvate metabolism may contribute to the inhibition of growth and to killing, it is now known that nitrofurans have many other effects. Recently, it has become apparent that one of the principal targets of nitrofuran action may be DNA. Szybalski (21) first noted that radiation resistant *E. coli* B/r was cross resistant to nitrofurazone. This observation has been confirmed and extended to a variety of other nitrofurans (22). Endo *et al.* (23) showed that NFT [3-amino-6-(2-(5-nitro-2-furyl)vinyl)-1,2,4-triazine] specifically inhibits bacterial DNA synthesis and induces prophage development in lysogenic *E. coli*. Further work on the action of NFT *in vivo* has shown that it reduces the priming activity of the DNA template for DNA polymerase and, at high doses, causes the breakdown of DNA. On the other hand, nitrofurazone and nitrofurantoin do not produce mass lysis of typical

lysogenic strains of *E. coli* (24,25). Further, the Schiff's base nitrofurans not only inhibit the net increase of DNA but also of RNA and of protein in *E. coli* B/r (24,26). More recently, single strand breaks have been detected when DNA from nitrofuran-treated bacteria (27) or mammalian cells (28) is analysed on alkaline sucrose gradients. Subsequent incubation of treated cells in drug-free medium led to repair of these lesions. McCalla *et al.* (11) have recently shown that in cells and cell-free extracts containing nitrofuran-reductase radioactive nitrofurazone is reduced to form unstable compounds which in turn form stable and presumably covalent linkage to protein and possibly to other macromolecules. In cells lacking nitrofuran reductase, however, little radioactive nitrofurazone becomes bound to macromolecules, suggesting that reduction products or intermediates are more toxic than is nitrofurazone itself. Terawaki and Greenberg (26) observed that nitrofurazone does not inactivate transforming DNA *in vitro*. They also suggested that metabolic modification of the compound might be required for activity.

V. Nitrofurans as Mutagens

The production of mutations with chemicals had been extensively explored by many workers after the discovery of the mutagenic action of radiation. Zampieri and Greenberg (24) reported that nitrofurazone greatly increased

the rate of mutation of a lac^- *E. coli* mutant to lac^+. McCalla and Voutsinos (30) have tested 22 nitrofurans for their ability to induce revertants of *E. coli* WP2 and its *uvr*A^- derivative from trp^- to trp^+. They found that all the nitrofurans tested proved to be active while two furan analogues (lacking the nitro group) were inactive. Evidence for the involvement of an activated compound was provided by examining the reversion of two nitrofurazone-resistant strains which lack reductase I. These strains were not induced to revert to trp^+ in air but did revert under anaerobic conditions where reductase II was active. McCalla *et al.* (31) have found that the mutagenic activity of nitrofuran derivatives is easily demonstrated by using *E. coli* WP2 and its *uvr*A derivative but not by using *S. typhimurium* as the test strains. Similar results have been reported by Yahagi *et al.* (32). This may be due to the greater lethality of the nitrofuran compounds toward *S. typhimurium* than toward *E. coli*. Yahagi *et al.* also found that At-2(2-Furyl)-3-(5-nitro-2-furyl)acrylamine) which was used as a common food additive in Japan, is a potent mutagen.

VI. Nitrofurans as Carcinogens

Since 5-nitrofurans have been widely used for chemotherapeutic and other purposes, it was quite alarming when Stein *et al.* (33) reported in 1966 that FNT (formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide) fed

to female rats produced tumors of the breast, stomach, kidney and intestine. Morris *et al.* (34) subsequently confirmed these observations. Structure-activity relationship studies (35,36) on FNT and its analogs have implicated the 5-nitro group as being primarily responsible for the carcinogenicity of FNT. Changes in the side chain substituents at the 2-position of thiazole ring of FNT did not affect carcinogenic potency but did drastically alter organ specificity. FANFT (4-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide) caused almost exclusively bladder tumors in rats (38,39), mice (39) and dogs (40), while NFTA (N-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide) induced mammary and lung tumors in rats (41) and leukemia in mice (42).

All of the oncogenic 5-nitrofurans mentioned above have a heterocyclic substituent attached directly to the 2-position of the furan ring.

A large number of 5-nitrofurans having two heterocyclic rings separated by a vinyl bridge have been synthesized (43). The induction of tumors of the breast, intestine and lungs in rats by these compounds, e.g., trans-2-[(dimethylamino)methylimino]-5-[2-(5-nitro-2-furyl)vinyl]-1,3,4-oxadizole demonstrated that the direct attachment of the several heterocyclic rings to the furan ring was not required for oncogenic activity (44).

Morris *et al.* (34) reported that nitrofurantoin was not a rat carcinogen. This observation has been confirmed

in recent studies (45), and has raised the possibility that the -CH=N- bridge between the furan ring and the second heterocyclic ring might provide compounds devoid of carcinogenic activity. However, this possibility was questioned when 4-methyl-1-[(5-nitrofurfurylidene)amino]-2-imidazolidinone and other similar compounds proved to be potent rat carcinogens (45). The reasons for the lack of carcinogenic activity of nitrofurantoin has not been explained, however, it is known that this drug is rapidly excreted.

Some monocyclic nitrofurans have also been tested for oncogenic activity in rats. Nitrofurazone (34) induced a high incidence of mammary tumors which appeared benign histologically but which, however, gave rise to malignant tumors when transplanted (36). Devoid of carcinogenic activity were 5-nitro-2-furamidoxime (45) and 5-nitro-2-furanmethandiol-diacetate (34). More monocyclic substituted 5-nitrofurans should be tested to ascertain the relationship between the molecular structure and carcinogenic potential.

A large variety of aromatic amino compounds are known to be carcinogenic. Many years of intensive study by the Millers and others (46) have indicated that these compounds are first N-hydroxylated and then esterified to form highly reactive electrophilic compounds capable of reacting with tissue constituents and ultimately leading to formation of tumors.

A similar mechanism has been suggested in an attempt to explain the tumorigenic activity of nitrofurans. Evidence showing nitro-reduction of nitrofurans by mammalian systems has been presented. Paul *et al.* (47) detected the nitro-reduction products of nitrofurazone in urine samples from nitrofurazone-fed rats.

McCalla (48) reported that nitrofurazone undergoes reduction in animal tissues, and Akao *et al.* (49) showed that rat liver homogenate reduced several oncogenic 5-nitrofurans. Wang *et al.* (50) recently reported reduction of NFTA *in vitro*.

VII. Miscellaneous Properties

a. Induction of apoplastidic *Euglena*: McCalla (51) showed that many, but not all nitrofuran derivatives, convert normal green *Euglena gracilis* to permanently colourless forms which are viable provided they are supplied with an organic carbon source. Exposure of *Euglena* to low concentrations of these agents for about two generation times, followed by plating on drug-free medium, resulted in a high proportion of bleached colonies. Thus he concluded that the nitrofurans induce apoplastidity by causing permanent damage to the chloroplast system rather than by inhibiting its replication temporarily.

b. Nitrofurans as Radiosensitizers: More recently, Chapman *et al.* (52) indicated that some of the nitrofurans are radiosensitizers of hypoxic mammalian cells. They

found that at concentrations apparently not toxic to cells, some nitrofurans are as effective as oxygen in increasing the radiosensitivity of hypoxic cells. This property makes nitrofurans candidates for clinical use in the radiotherapy of those solid tumors which contain poorly oxygenated cells.

VIII. *E. coli* Minicells

Minicells are small spherical anucleate bodies produced by an aberrant cell division at the polar ends of certain mutant strains of bacteria (53,54,55,56,57). Unlike the normal bacterial cells, these spherical bodies fail to grow and divide. The first report of an apparent minicell-producing strain was made by Gardner (58) who examined an aberrant cell division process in a strain of *Vibrio chloera* that resulted in the production of spherical non-growing granules. Hoffman and Frank (59) followed the rare production of a minicell from one cell in a growing culture of *E. coli* by using time-lapse photomicroscopy.

Minicells are produced throughout the growth cycle of minicell-producing strains and contain RNA and protein but little or no bacterial DNA. As such, they have been very useful in studies on cell division, the localization of enzymes and constituents in cells, the synthesis and function of cell wall and membrane, and the mechanism of bacterial conjugation. The more recent discovery that plasmid DNA in plasmid-containing, minicell-producing,

strains segregates into minicells of *E. coli* (60,61,62,63) has provided a model system for studies on DNA repair of radiation-induced damage and transcription and translation of plasmid-specific genetic information.

IX. Objectives Of This Work

From the foregoing it should be obvious that our knowledge of the mechanisms by which nitrofurans damage cells is still of a very fragmentary nature. For example, some nitrofurans are known to be specific inhibitors of DNA synthesis while others are not. Given the known effect of some of the non-specific agents on bacterial respiration, it is reasonable to ask whether the fact that RNA and protein synthesis as well as DNA synthesis is inhibited by these agents might be a secondary effect of lack of ATP. Also, nothing is known about the effect of activated nitrofurans on RNA or on ribosomes.

While it is known that nitrofurans cause breaks in DNA which can be detected in alkaline gradients, it is not known whether the breaks exist before the DNA is exposed to alkali or whether they result from the action of OH^- on lesions in the DNA.

Therefore, the specific objectives of this work were:

1. To study the nature of the damage induced by activated nitrofurazone in DNA.

2. To examine the effects of a given sub-lethal dose of nitrofurazone on a variety of cellular processes such as glucose metabolism, ATP production, macromolecule synthesis and ribosome synthesis and function with a view to extending our knowledge of the action of the drug.

MATERIALS AND METHODS

I. Bacterial Strains

Escherichia coli was obtained from A.O. Olson, Atomic Energy of Canada Limited, Chalk River, Ontario and *E. coli* χ1256, a strain which produces minicells containing λdv plasmids, from R. Curtiss III, University of Alabama, Birmingham. Strain *nfr*-207, a mutant of B/r, which lacks nitrofurazone reductase I was isolated by D. R. McCalla and A. Reuvers (11). *E. coli* WP2 and its *exrA*$^-$, *uvrA*$^-$ derivative CM611 were obtained from B. A. Bridges, MRC Cell Mutation Unit, University of Sussex. Spray dried *Micrococcus luteus* cells were purchased from Miles Laboratories, Kankakee, Ill.

II. Media

Bacteria (except for *E. coli* χ1256) were normally grown in medium A (64). When glucose metabolites were to be analysed or ATP levels determined, cells were grown in medium B (65). Strain χ1256 cells were grown in medium C (66).

Medium A (grams per liter): Na_2HPO_4, 11.3; KH_2PO_4, 3.0; NH_4Cl, 1.0; $MgSO_4$, 0.01; $CaCl_2$, 0.001, $FeCl_3$, 0.002; glucose, 5.0.

Medium B (grams per liter): K_2HPO , 28.0; KH_2PO_4, 8.0; $MgSO_4 \cdot 7\ H_2O$, 0.10; $(2H_4)_2SO$, 1.0; casein hydrolysate, 2.5; glucose, 2.0.

Medium C (grams per liter): NH_4Cl, 5.0; NH_4NO_3, 1.0; Na_2SO_4, 2.0; K_2HPO_4, 9.0; KH_2PO_4, 3.0; $MgSO_4 \cdot 7H\ O$, 0.1; thiamine HCl, 0.002; glucose, 5.0; casamino acid, 5.0.

III. Chemicals

All chemicals for media and other solutions were reagent grade or the most highly purified available commercially.

N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide (FANFT) was a gift from Abbott Laboratories, Chicago, Ill. 5-Nitro-2-furaldehyde semicarbazone (nitrofurazone) and N-(5-nitro-2-furfuryl)idine)-1-aminohydantoin (nitro-furantoin) were gifts from Norwich Pharmacal, Norwich, N.Y. 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) was provided by Dr. T. Sugimura, National Cancer Research Institute, Tokyo. Glucose-6-phosphate, DNase, GTP, UTP, ATP, CTP and NADH were obtained from Sigma Chemical Co., St. Louis, Mo. and glucose-6-phosphate dehydrogenase and polyuridylic acid from Worthington Biochemical Corp., Freehold, New Jersey. The sources for radioactive chemicals are listed below:

Thymidine-methyl-H^3 (18 Ci/mmole); ethyl alcohol-C^{14} (2 mCi/mmole); acetate-C^{14} (1 mCi/mmole); pyruvate-C^{14} (2 mCi/mmole); formate-C^{14} (5 mCi/mmole); lactate-C^{14} (5 mCi/mmole) and succinate-C^{14} (5 mCi/mmole) New England Nuclear, Boston, Massachusetts.

Uracil-C^{14} (4.6 mCi/mmole); glucose-U-C^{14} (4.3 mCi/mmole); ATP-C^{14} (1.02 mCi/mmole); phenylalanine-L-C^{14} (10 mCi/mmole); Amersham/Searle Co., Arlington Heights, Ill.

Nitrofurazone-C^{14} (2 mCi/mmole) was prepared by Dr. C. Yu from nitrofurfural and semicarbazide-C^{14}.

IV. Labelling and Cultivation of Minicells

An overnight culture strain of X1256 was diluted 1:100 in 300 ml of fresh growth medium supplemented with 200 µg/ml deoxyadenosine and 75 µCi/ml [methyl-^{3}H] thymidine (67). The bacteria were grown for 6 to 8 hr at 37°C to a final concentration of about 8 x 10^8 cells per ml.

V. Purification of Minicells

Cells and minicells were sedimented by centrifugating the culture at 10,000 rpm in a Sorvall SS34 rotor for 10 min (68). By use of a Vortex mixer for 2 min, the pellet was suspended in one-tenth the original volume in sterile buffered saline (69) with gelatin (containing NaCl, 0.85%; KH_2PO_4, 0.03%; Na_2HPO_4, 0.06%; gelatin, 100 µg/ml). Samples of 2.5 to 3 ml were then layered over a sterile 5 to 20% sucrose gradient (35 ml) and centrifuged at 5000 rpm for 15 min with an SW27 rotor in a Beckman L-2 ultracentrifuge. The minicell band was withdrawn from the gradient with a syringe and centrifuged at 15,000 rpm for 15 min in a Sorvall SS34 rotor to collect the minicells. The pellet was then suspended in buffer, layered over a second 35 ml, 5 to 20% sucrose gradient, and again centrifuged as described

above. After the minicell band had been removed and the minicells pelleted, the minicell fraction was suspended in the minicell growth medium at an absorbance of 0.2 at 620 nm. The purified minicell fraction usually contained less than 1 bacterial cell per 10^6 minicells (68).

VI. Velocity Sedimentation of DNA in Alkaline Sucrose

Minicells (10^9/ml) were incubated in medium containing nitrofurans. After incubation, samples were sedimented, washed once in buffer (pH 7) containing 0.01 M KH_2PO_4, 0.001 M $MgSO_4$, 0.0001 M $CaCl_2$ and 0.1 M NaCl and resuspended gently by manual agitation in 300 µl of lysis buffer (pH 8.1) containing 0.02 M tris, 0.05 M NaCl and 0.02 M EDTA (69). The suspensions were transferred at 23°C to a cuvette, in which cell lysis was achieved by slowly introducing 60 µl of lysing solution containing 0.8 M NaOH and 1% SDS into the stirred suspension. One tenth ml of the alkali-SDS lysate was then layered on a 5-20% alkaline sucrose gradient which was centrifuged at 40,000 rpm for 40 min in a Beckman SW50.1 rotor. The bottom of the tube was punctured and approximately 35 fractions were collected on paper discs by gravity. The fractions were treated with cold 5% TCA, washed twice in 95% ethanol, and dried, and the radioactivity was counted in a Beckman scintillation counter in a toluene fluor.

VII. Velocity Sedimentation of DNA in Neutral Sucrose

Minicells (10^9/ml) after treatment with nitrofurazone were washed and suspended in 50 µl of 0.1 M tris buffer (pH 8). Prior to neutral sucrose gradient sedimentation, 100 µl of a chilled buffer (pH 8) containing 0.03 M tris, 800 µg/ml lysozyme, 0.02 M EDTA and 0.01 M KCN was added to the 50 µl minicell suspension (67). After incubation for 15 min the resulting minicell protoplasts were immediately lysed by shaking manually with 150 µl of 2% SDS and placing the mixture in a water bath at 37°C. Minicell lysates were directly layered on 4.4 ml, 5-20% sucrose gradients (pH 7.4) containing 0.05 M tris, 0.7 M NaCl and 0.01 M EDTA. Gradients were centrifuged at 45,000 rpm for 100 minutes in an SW50.1 rotor. Fractions were collected and radioactivity determined as described above.

VIII. Isolation of λdv DNA as Covalently Closed Circles

After the minicell lysates were centrifuged in neutral sucrose gradients (see above), fractions containing Covalently closed, circular (CCC) DNA were pooled, placed in dialysis bags and dialysed for 30 min at room temperature against a one litre solution containing 50 mM Na_2HPO_4 and 1 mM EDTA (pH 6.5) (67). Dialysed DNA solutions could be stored at 4°C for at least 10 days without much change in the proportion of CCC-DNA.

IX. Assay for the Effect of Activated Nitrofurazone on CCC-DNA

Reaction mixtures contained [^{3}H]-labelled CCC-DNA, 20 µg NADPH, 0.24 mg glucose-6-phosphate and 0.2 units of glucose-6-phosphate dehydrogenase. Where indicated, 15 µg nitrofurazone and/or 0.5 µl of *E. coli* enzyme preparation were also added. The final volume was 200 µl. After incubation for 30 min, the DNA was analysed on gradients as described above.

X. Assay for the Effect of Micrococcal Nuclease on CCC-DNA

The reaction mixtures (67) contained CCC-DNA (specific activity 4.7 x 10^5 cpm/µg) isolated from either nitrofurazone-treated or untreated minicells and calf-thymus DNA (1.3 µg) suspended in a buffer (pH 6.5) containing 75 mM Na_2HPO_4, 1 mM EDTA, 10 mM 2-mercaptoethanol. The reaction was started by introduction of 3 µl of a partially purified extract from *Micrococcus luteus* (see section XI). After a 5 min incubation at 37°C, 200 µl of the mixture were added to 50 µl of 1 M NaOH. The alkaline suspension was layered on 5-20% alkaline sucrose gradients and centrifuged at 40,000 rpm for 40 minutes. The gradients were collected and radioactivity was counted as described above.

XI. Preparation of a Partially Purified Endonuclease from *Micrococcus luteus*

Spray-dried cells (12 g) were suspended by use of

a Waring blender in 0.01 M tris buffer (pH 8.0) and collected by centrifugation. The cells were resuspended in 250 ml of 0.2 M sucrose and 0.01 M tris (pH 8) and 50 mg of lysozyme was added. After 45 min at 30°C, 250 ml of cold water was added. The lysate was stirred vigorously until a uniform viscous gel formed, and was sonicated for 5 min. The sonicated solution was centrifuged at 30,000 x g for 30 min, and the supernatant fluid decanted. Ammonium sulfate was added to the supernatant to a final concentration of 65%. The solution was stirred for 1 hr and centrifuged for 30 min at 30,000 x g. The precipitate was dissolved in 1,000 ml of 0.005 M potassium phosphate buffer, pH 7.5 and used as endonuclease. This preparation corresponds to "fraction II" of Carrier and Setlow (70).

XII. Preparation and Sedimentation Analysis of RNA

Log-phase bacteria (1 to 2 x 10^9/ml, a cell density which was used throughout these experiments) were incubated with or without 50 μg/ml nitrofurazone followed by 0.1 mCi/ml of uracil-^{14}C for either 15 sec or for 30 min. In short pulse labelling experiments, cells were rapidly chilled with crushed ice and 0.01 M Na azide added. The RNA was prepared according to Hayes *et al.* (73). Radioactive bacteria were ground with chilled alumina. The bacteria-alumina paste was thoroughly mixed with 5 ml of 5 x 10^{-3} M tris-HCl buffer (pH 7.0) containing 0.01 M $MgCl_2$ and the

mixture centrifuged at 10,000 g for 5 min to remove alumina and cell debris. The supernatant fraction was removed, the pellet re-extracted with 2.5 ml of the same buffer and the combined aqueous extracts added to an equal volume of water-saturated phenol and shaken for 15 min in the cold. After centrifugation at 10,000 g for 5 min, the aqueous layer was removed, the phenol layer re-extracted with a half volume of buffer and the combined extracts precipitated with 2 volumes of ethanol in the presence of 0.1 M NaCl. After 15 min at $0^{\circ}C$, nucleic acids were collected by centrifugation, freed from phenol by repeated ethanol precipitation from 5×10^{-3} M tris-HCl (pH 7.4) containing 0.01 M $MgCl_2$ and 0.1 M NaCl and finally dissolved in 2 ml of 5×10^{-3} M tris-HCl (pH 7.4) containing 0.001 M $MgCl_2$ and 2 µg of purified DNase. The mixture was incubated at $4^{\circ}C$ for 15 min, treated with phenol as described above, and RNA reisolated by ethanol precipitation. DNA-free RNA was dissolved in 2 ml of 0.01 M sodium acetate-acetic acid buffer (pH 5.0) containing 0.1 M NaCl and 0.1 ml of a 2% purified bentonite preparation in 0.01 M acetate buffer. The suspension was kept at $0^{\circ}C$ for 10 min, the bentonite removed by centrifugation at 20,000 g for 20 min and the RNA recovered by ethanol precipitation. After two further ethanol precipitations, the RNA was resuspended in 0.01 M acetate buffer and layered on 5-20% sucrose gradients, which were centrifuged for 10 hr at 25,000 rpm

at 4°C in a Beckman SW25 rotor. Fractions were collected after puncturing the bottom of the tube, and the absorbance at 260 nm was measured. The precipitate formed in each tube after addition of trichloracetic acid (to a final concentration of 5%) was washed with TCA on a membrane filter, dried and counted in a scintillation counter with a toluene fluor.

XIII. Analysis of Ribosomes

Cells were collected, washed twice with 0.01 M tris-HCl buffer (pH 7.4) containing either 10^{-4} or 10^{-2} M magnesium acetate as indicated and ground in a mortar with chilled alumina. The cell extract was clarified by centrifuging at 20,000 x g for 15 min, layered on top of a 5-20% sucrose gradient and centrifuged at 25,000 rpm for 2 hr and 30 min in a Beckman SW25 rotor (71). Forty fractions were collected from the bottom of each tube. A sample of each fraction was taken and diluted for the measurement of the absorbance at 260 nm. Trichloroacetic acid was added to the remainder of each fraction to a final concentration of 5% and the precipitate collected on membrane filters, washed, dried and counted in a scintillation counter.

XIV. Assay of DNA-Depended RNA Polymerase Activity

Exponentially growing cells (20 ml) were collected, washed and sonicated in 1 ml of 0.04 M tris (pH 8) buffer.

The extract was centrifuged at 81,000 g for 70 min after which 0.1 ml of the supernatant fluid was assayed for DNA-depended RNA polymerase activity described by Summers and Sigel (72). The reaction mixture (0.25 ml) contained 0.2 M KCl, 0.04 M tris-Cl pH 7.9, 0.0046 M $MgCl_2$, 0.002 M $MnCl_2$, 0.07 mM EDTA, 0.11 mM GTP, 0.11 mM CTP, 0.15 mM UTP, 0.15 mM ^{14}C-ATP (1 μCi/μmole), 0.04 M mercaptoethanol and 25 μg calf thymus DNA. Incubation was for 15 min at 37°C. Samples (0.1 ml) were withdrawn and pipetted onto small filter paper discs which were then placed in ice cold 5% trichloroacetic acid; washed once in trichloroacetic acid, and twice in ethanol; counted in a scintillation counter with a toluene fluor.

XV. Polyuridylic Acid Directed Polyphenylalanine Synthesis

Ribosomes were isolated by the procedure described by Nirenberg and Mathaei (74). Cells were disrupted by grinding with alumina. The alumina paste was extracted with buffer containing 0.01 M tris, pH 7.8, 0.01 M magnesium acetate, 0.06 M KCl, 0.006 M mercaptoethanol (standard buffer). Alumina and cell debris were removed by centrifugation at 20,000 x g for 20 min. The supernatant was decanted, and 3 μg DNase per ml were added, which

rapidly reduced the viscosity of the suspension. The preparation was centrifuged again at 20,000 x g for 30 min to clear the extract of remaining debris. The liquid layer was centrifuged at 105,000 x g for 2 hr to sediment the ribosomes. The bulk of the supernatant (S-100 fraction) was retained and the solution just above the pellet was decanted and discarded. The pellet containing the ribosomes was washed by resuspension in the standard buffer and recentrifugation at 105,000 x g for 2 hr. The supernatant fluid was discarded and the ribosomes were suspended in standard buffer. The S-100 fraction and the ribosomes were dialysed against 60 volumes of standard buffer overnight at 5°C. The assay of poly-U-directed polyphenylalanine synthesis by ribosomes and S-100 fraction was also carried out as described by Nirenberg and Matthaei. The reaction mixtures contained the following (in μmoles/ml): 100,tris, pH 7.8; 10, magnesium acetate; 50, KCl; 6, mercaptoethanol; 5, phosphenolpyruvate; 0.03 each of ATP, GTP, CTP and UTP; 0.02 ^{14}C-L-phenylalanine; 10 μg polyuridylic acid, 20 μg phosphenolpyruvate kinase, 1 mg *E. coli* soluble RNA, 1 mg ribosome protein; and 1.8 mg S-100 protein. Samples were incubated at 35°C for 60 min and then deproteinized with 10% trichloroacetic acid. The precipitate was resuspended in 4% TCA, recentrifuged, and decanted twice. This process was then carried out once with hot (90°C) TCA for 15 min to remove nucleic

acids. The residue was centrifuged again, and washed once with warm 95% alcohol, twice with warm 2:2:1 alcohol-ether-chloroform (each time for a period of 10 min), once with warm ether, and then the residue was air dried. The dried polypeptide was suspended in acetone, deposited on filter paper disc and counted in a scintillation counter.

XVI. Analysis of Glucose Metabolites

Metabolites from 10 ml cultures of aerobically or anaerobically grown cells exposed to uniformly labelled glucose-C^{14} (1 μCi/ml) for 60 min at 37°C were analysed by silicic acid column chromatography according to Dobrogosz (65). Samples were prepared by mixing cell suspensions with an equal volume of a cold carrier containing the following components: ethyl alcohol, acetic acid, pyruvic acid, formic acid, fumaric acid, lactic acid, succinic acid and citric acid (all concentrations were 0.1 M except fumaric acid which was 0.05 M). Sufficient H_2SO_4 was added to adjust the pH to 1.5 to 1.8. This step served to stop any further reaction in addition to providing cold carrier materials. The mixture was centrifuged at 10,000 x g for 10 min, and the supernatant fraction was frozen until used. One ml of the supernatant was mixed with 1.8 g of dry silicic acid, transferred to the top of silicic acid column (40 cm x 1.2 cm dia.) with 5 ml of benzene. The colums were developed by elution with 50 ml of chloroform, followed by 600 ml of a linear

chloroform: 5% t-butanol in chloroform gradient. After gradient elution was completed, an additional 50 ml of 5% t-butanol in chloroform was added, followed by 100 ml of 10% t-butanol in chloroform. Ten ml fractions were collected. One ml of each fraction was mixed with 1 ml of t-butanol in a counting vial followed by 15 ml of scintillation fluor containing 0.4% PPO and 0.01% POPOP in toluene. Known samples of ^{14}C-labelled ethyl alcohol, acetate, pyruvate, formate, lactate and succinate were treated in the above manner to determine their elution position.

In some experiments, radioactivity released as $^{14}CO_2$ was determined. The evolved $^{14}CO_2$ was trapped in an alcoholic solution of hyamine or ethanolamine and the radioactivity was determined as described above.

XVII. Assay of Alcohol Dehydrogenase Activity

Anaerobically grown cells (2×10^9) were washed and sonicated in 0.01 M phosphate buffer. The extract was centrifuged at 81,000 g for 10 min after which 0.2 ml of the supernatant was assayed for alcohol dehydrogenase activity (75). The reaction mixture (total volume - 3.2 ml) contained: 1.5 ml of 0.032 M pyrophosphate buffer (pH 8.8), 0.5 ml of 2 M ethanol and 1 ml of 0.025 M NAD^+. Absorbance at 340 nm was measured at 1 min intervals.

XVIII. Measurement of ATP Levels

ATP was assayed using firefly extracts (76). Samples were prepared by sonicating 5 ml of cell culture. The bioluminescence induced by 0.1 ml samples of extracts was then measured in a Nuclear Chicago Mark I Scintillation counter. Concentrations were kept within the linear range.

RESULTS

I. Effect of Nitrofurans on DNA

Nitrofurans are able to induce single strand breaks in bacterial(27) and mammalian DNA (28). These single strand breaks in DNA are usually measured by velocity sedimentation in alkaline sucrose gradients by means of the procedure developed by McGrath and Williams (79). Bacteria are converted to protoplasts which are then lysed in a thin overlayer of sodium hydroxide, which is usually at a final concentration of at least 0.2 M on top of a linear 5-20% alkaline sucrose gradient. The alkaline treatment denatures double-helical DNA into linear single-strand DNA macromolecules so that the extent of strand breakage may be estimated by the relative sedimentation velocities of the DNA molecules through the gradient. However, as noted in the introduction, this approach does not tell whether these breaks are formed by the direct action of "activated" nitrofurans, as a consequence of the first step in excision repair or, indeed, whether breaks exist at all before the DNA is exposed to alkali. Minicells derived from *E. coli* strain X1256 were therefore used to study the mechanism of nitrofurazone-induced breakage of DNA. This was possible

because minicells contain λdv covalently closed circular DNA (CCC-DNA) (supercoiled). The introduction of a single-strand nick, irrespective of the mechanism, is sufficient to convert a closed-circular DNA molecule into an "open" or "relaxed" circular molecule (77). This conversion is readily detected in neutral gradients (in sharp contrast to the situation with linear bacterial double-helical DNA) by a clear shift to a lower sedimentation coefficient. In alkaline sucrose, denatured CCC-DNA sediments about 3 times as rapidly as the two alkali denatured components of open circles - linear and closed-circular single-strand molecules.

A. Reduction of nitrofurazone by minicells

Since metabolic activation of nitrofurazone is necessary for its biological effects, it was necessary to examine whether or not minicells contain nitrofurazone reductase prior to studying any effect of the drug on DNA in minicells. Fig. 2 shows the kinetics of the reduction of nitrofurazone by minicells and by *E. coli* B/r cells. Minicells metabolized nitrofurazone at a rate only moderately slower than that found with *E. coli* strain B/r. Since the amount of minicells (10^9/ml) used in this experiment was roughly equivalent to that of B/r cells (10^8/ml) (the size of minicell is approximately one tenth of *E. coli* B/r), the results show that minicells do contain substantial amounts of nitrofurazone reductase.

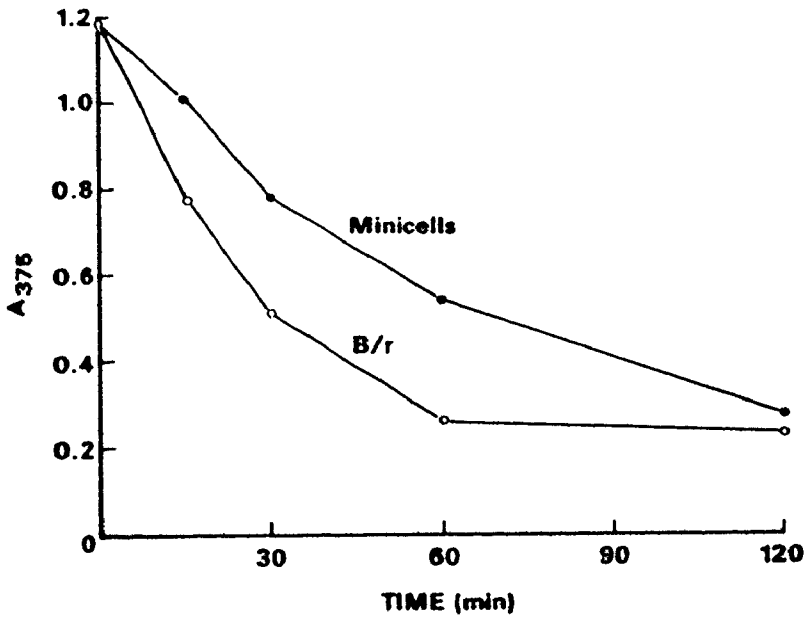


Figure 2. Reduction of nitrofurazone by *E. coli* χ1256 minicells and *E. coli* B/r cells.

Initial nitrofurazone concentration was 17.5 μg/ml. The concentration of the cell suspensions was adjusted to give a final absorbance (620 nm) of 0.2 (this represents 10^8 and 10^9 cells/ml for B/r and "minicell" respectively).

B. Sedimentation analyses of minicell lysates

Fig. 3 shows a typical sedimentation profile obtained with labelled DNA from control and nitrofurazone-treated minicells on neutral sucrose gradients. The more rapidly sedimenting component contained 22% of the total radioactivity in the form of covalently closed circles (CCC) while the more slowly sedimenting component contained "relaxed" circles in which at least one phosphodiester bond had been cleaved. The profile from nitrofurazone-treated minicells (75 µg/ml for 60 min) showed that only 16% of the total radioactivity sedimented to the position expected for CCC material indicating that about 30% of the closed circles had been "nicked" as a consequence of exposure of the minicells to nitrofurazone.

When samples of the same control minicells were run on alkaline gradients (Fig. 4), it was found that the CCC-DNA contained 30% of the total radioactivity while in drug-treated minicells only 13% of the total radioactivity sedimented as CCC-DNA. Thus, about 55% of the CCC-DNA must have been "nicked" by treatment of the minicells with nitrofurazone plus exposure of minicell lysates to alkali. This represented a 25% greater loss of CCC-DNA than was detected in the neutral gradient.

These data show that almost twice as many breaks were detected in alkali as in neutral gradients and suggest that two types of lesions must be present in the DNA when

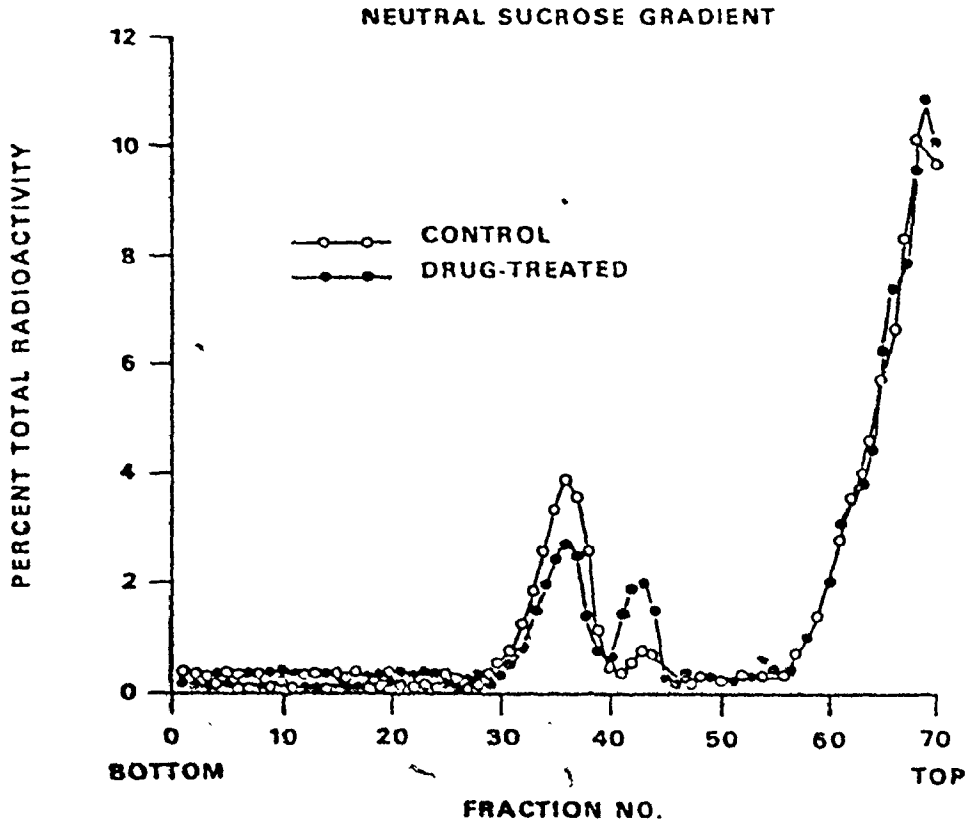


Figure 3. Neutral sucrose sedimentation profile of CCC-DNA from lysates of nitrofurazone-treated and control minicells.

The minicells incubated with nitrofurazone (75 μg/ml) for 1 hr were lysed and subjected to the centrifugation on neutral sucrose gradients. The faster sedimenting component contains CCC-DNA while the slower component contains relaxed circles having one or more single strand breaks.

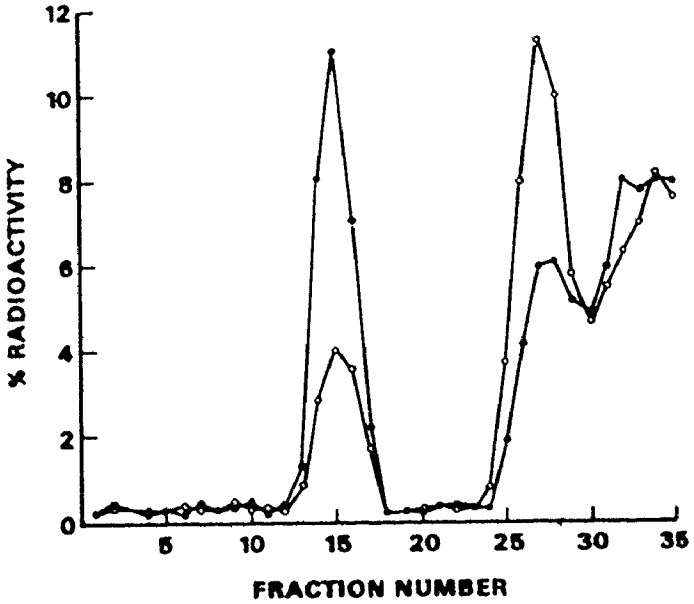


Figure 4. Alkaline sucrose sedimentation profile of CCC-DNA from lysates of nitrofurazone-treated and control minicells.

The minicells incubated with nitrofurazone (75 μg/ml) for 1 hr were lysed and subjected to the centrifugation on alkaline sucrose gradients. ●———●, control; o———o, nitrofurazone.

the cells are lysed: pre-existing breaks (detected in both kinds of gradients) and "alkali labile lesions" which are not breaks in the phosphodiester bonds as would be detected in neutral gradient but are converted to breaks in alkaline gradient. Figure 5 shows that these effects were seen over a wide range of nitrofurazone concentrations and both lesions were formed with first-order kinetics as indicated by the exponential decline in the surviving fraction of covalently closed-circular molecules as a function of concentration of nitrofurazone.

C. Proof that alkali-labile lesions are induced by nitrofurazone

The existence of alkali-labile lesions was confirmed directly by isolating CCC-DNA from drug-treated minicells on a neutral gradient and then re-examining the sedimentation of this material under both neutral and alkaline conditions. Table IV shows that when CCC-DNA prepared from untreated minicells using neutral gradient was resedimented, the recovery of radioactivity in CCC-DNA was 20-25% less than that obtained with neutral gradients. This result has been attributed to the presence of alkali labile lesions in normal λdv DNA (67). When similar DNA preparations from drug-treated minicells were resedimented in alkali, the recovery of CCC-DNA was much lower than that obtained with untreated minicells. This, together with the *in vivo* results described above leads to the conclusion that nitrofurazone induces alkali labile lesions in DNA.

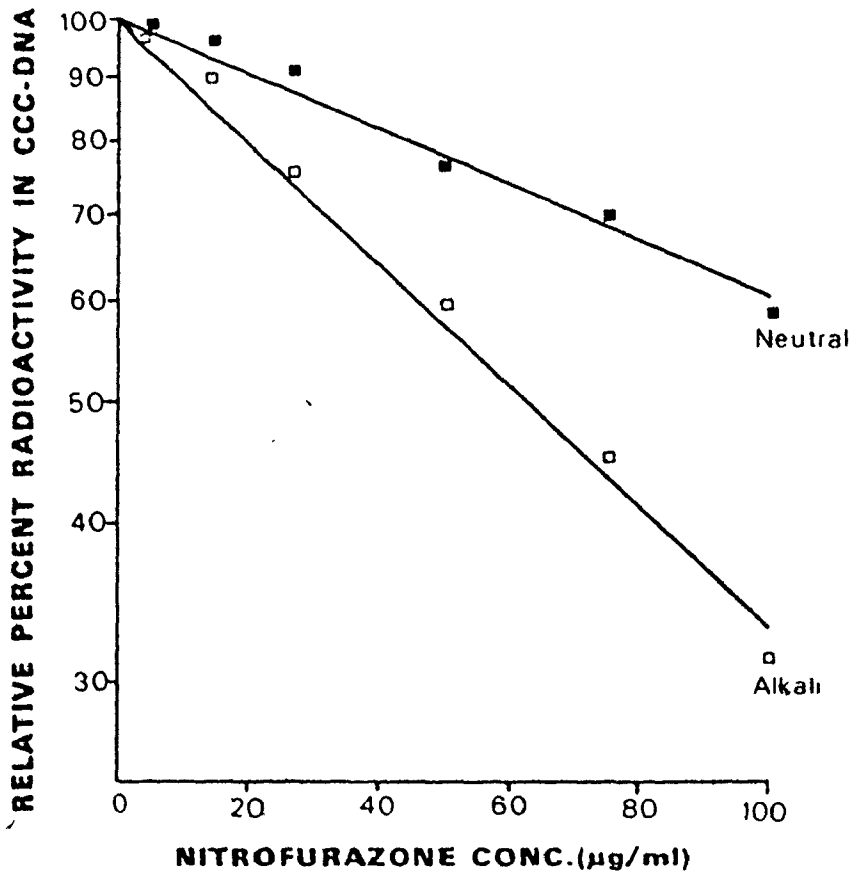


Figure 5. Dose-response curve for the breakage by nitrofurazone of DNA in intact minicells.

Each point was obtained by sedimenting lysates of drug-treated minicells on neutral or alkaline gradients to determine the percentage of total radioactivity in the CCC-form. The data were then expressed as a percentage of the total counts found in CCC-DNA in the treated minicells compared to those in CCC-DNA from untreated cells. Points represent the average of duplicate experiments with a standard deviation within 5%.

TABLE IV

Breaks in DNA of Nitrofurazone-treated and Control Minicells

Exp. No.	Treatment	% of Total Radioactivity in CCC-DNA	
		Neutral	Alkali
1	control	82	57
	nitrofurazone-treated	70	30
2	control	76	56
	nitrofurazone-treated	66	27

Minicells were treated with nitrofurazone (75 µg/ml) for 1 hr at 37°C. CCC-DNA was then isolated and analysed on gradient as described in the text.

D. The breakage of CCC-DNA by nitrofurazone *in vitro*

When CCC-DNA was exposed to the nitrofurazone-reductase preparation alone (i.e., without drug) (Table V) 32-35% was converted to open circles, presumably as a result of endonucleases present in the preparation. In this case, both alkali and neutral gradients gave the same result. When CCC-DNA was exposed to nitrofurazone plus the reductase and then analyzed on neutral gradients, it was found that an additional 20-22% had been converted to relaxed circles. When samples of the same treated DNA were analysed on alkaline gradients, a significantly larger amount of damage (33-44%) was found. These results indicate that both breaks and alkali-labile lesions are introduced into DNA by nitrofurazone treatment *in vitro*, as well as *in vivo*. Table V also shows that when CCC-DNA was exposed to 75 µg of nitrofurazone per ml of buffer for 30 min *in vitro*, none of the DNA was converted to the relaxed form. This demonstrates that it was metabolites of nitrofurazone and not the drug itself that caused the lesions in DNA.

Table VI shows that when CCC-DNA treated with nitrofurazone using reductase prepared from *E. coli* strain CM611 (which lacks "repair endonuclease" (78)), the percentage of radioactivity recovered as CCC-DNA is substantially higher than when *E.coli* strain WP2 which possesses "repair endonuclease: served as reductase source. This suggests that the "repair endonuclease" might play a role in the drug-induced breakage of DNA.

TABLE V

Breakage of DNA by Activated Nitrofurans *In Vitro*

Exp. No.	Nitrofurazone	Enzyme	Neutral Gradients		Alkaline Gradients	
			% of Total Radioactivity in CCC-DNA	% Circles nicked by treatment	% of Total Radioactivity in CCC-DNA	% Circ. nicked treatmer
1	-	-	68	0	49	0
	+	-	70	0	51	0
	-	+	46	32	32	35
	+	+	31	54	16	68
2	-	-	81	0	59	0
	+	-	80	2	56	5
	-	+	55	32	40	33
	+	+	39	52	19	77

Reaction mixtures contained 4,000 cpm (sp. act. 4.7×10^5 cpm/µg) of CCC-DNA isolated from ^3H-labelled minicells, 20 µg NADPH, 0.24 mg glucose-6-phosphate and 0.2 units of glucose-6-phosphate dehydrogenase. Where indicated, 15 µg nitrofurazone and/or 0.5 µl of *E. coli* enzyme preparation were also added. The final volume was 200 µl. After incubation for 30 minutes at 37°C, the DNA was analysed on gradients as described in the text.

TABLE VI

Breakage of DNA Induced by Nitrofurazone Activated by Different Reductase Sources

Exp. No.	Nitrofurazone	Enzyme Source	% of Radioactivity Recovered as CCC-DNA	% of control
1	-	WP2	42	100
	+	WP2	10	24
	-	CM611	50	100
	+	CM611	34	68
2	-	WP2	53	100
	+	WP2	11	20
	-	CM611	61	100
	+	CM611	43	70

The reaction components and conditions were the same as described in Table V.

E. Action of *Micrococcus luteus* endonuclease on CCC-DNA isolated from drug-treated minicells

CCC-DNA isolated from drug-treated minicells was incubated with a partially purified extract of *Micrococcus luteus* and analysed on alkaline gradients. Table VII shows that treatment of DNA from control minicells with extract of *M. luteus* did not reduce the percentage of radioactivity recovered as CCC-DNA, indicating that the extract is free of endonuclease active toward normal λdv DNA. However, it is evident that the extract possess endonucleotic activity toward DNA isolated from drug-treated minicells since, approximately 32-35% of the CCC-DNA from drug-treated minicells was converted to relaxed circles. This suggests that at least that fraction of the CCC-DNA molecules contained endonuclease-sensitive lesions.

F. Repair of damage to CCC-DNA induced by nitrofurazone

McCalla *et al.* (27) reported that nitrofuran-induced breaks in bacterial DNA can be repaired. This led to an examination of whether or not minicells can repair damaged CCC-DNA. Minicells treated with nitrofurazone (75 μg/ml for one hour) were washed and reincubated in drug-free medium for various periods. Lysates were then analysed on either neutral or alkaline gradients. Figure 6 shows that under neutral conditions, the amount of CCC-DNA in drug-treated minicells returned to the control value in 60 min, implying that repair of such lesions was complete in that time. Under

TABLE VII

The Action of *Micrococcus luteus* Endonuclease toward DNA from Nitrofurazone-treated Minicells

Exp. No.	DNA Source (Minicells)	*Micrococcus luteus* Extracts	% of Total Radioactivity Recovered as CCC-DNA	% of Control
1	nitrofurazone-treated	-	26	100
	nitrofurazone-treated	+	18	68
	control	-	53	100
	control	+	52	98
2	nitrofurazone-treated	-	29	100
	nitrofurazone-treated	+	19	65
	control	-	56	100
	control	+	58	103

CCC-DNA was isolated from drug-treated (75 μg/ml for 1 hr) or untreated minicells as described in the Methods. The reaction mixture contained 4,000 cpm of CCC-DNA, calf thymus DNA (1.3 μg) suspended in a buffer (pH 6.5) containing 75 mM Na_2HPO_4 -1 mM EDTA- 10 mM 2-mercaptoethanol and 3 ml of extract of *Micrococcus luteus*. The final volume was 200 μl. After incubation for 5 minutes at 37°C, the DNA was analysed on alkaline sucrose gradients.

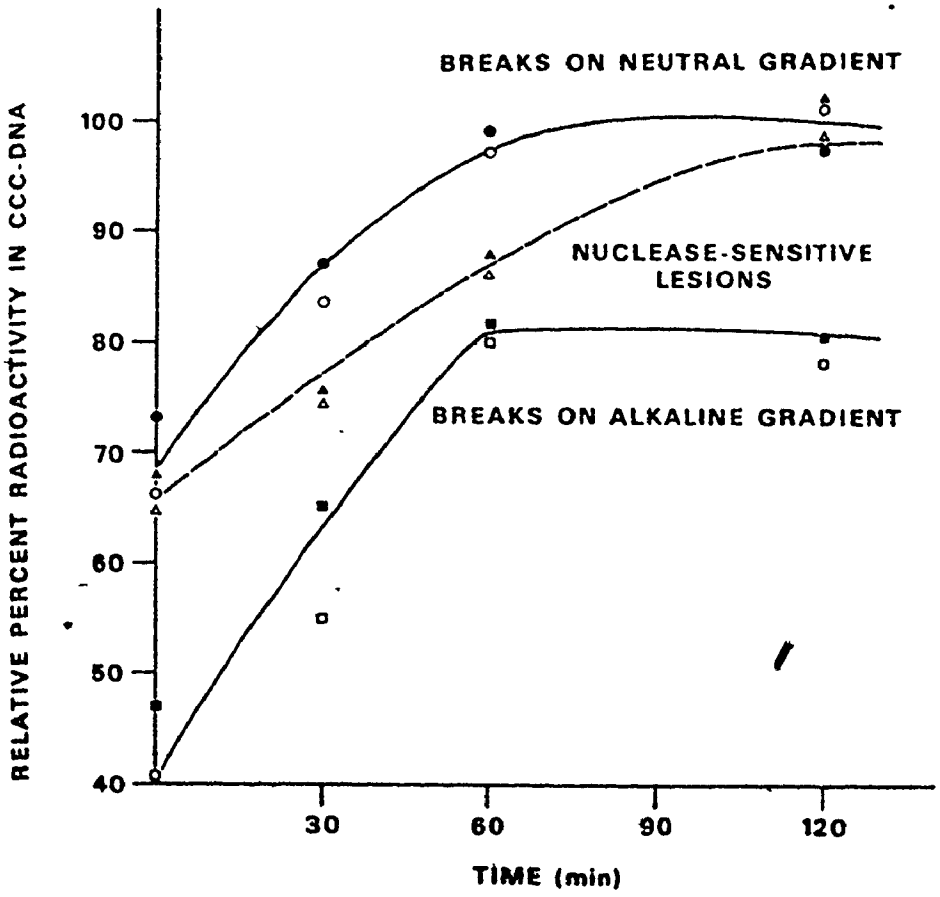


Figure 6. Time course for the repair of DNA in *E. coli* minicells after exposure to 75 µg/ml nitrofurazone.

The times indicated represent the period of reincubation in drug-free medium. The circles and squares show data from minicells which were lysed and analysed on neutral and alkaline gradients, respectively. The triangles represent the percentages of the total of control radioactivity found in CCC-DNA after isolated CCC-DNA from drug-treated minicells on neutral gradients was exposed to *Micrococcal* endonuclease and was analysed on alkaline gradients relative to that found in a parallel DNA sample isolated from control minicells.

alkaline conditions, however, about 20% of the DNA remained broken even after two hours. Since even prior to reincubation of the minicells, about 25% of breaks detected in alkaline gradient could be attributed to alkali-labile lesions (see Sec. B), it can be concluded that little, if any, repair of such lesions took place.

To see whether or not lesions which were sensitive to *Micrococcus luteus* endonuclease can be repaired, minicells treated with nitrofurazone were washed and reincubated in drug-free medium for various times. CCC-DNA was then isolated from minicell lysates on neutral gradients, treated with an extract of *M. luteus* and resedimented on alkaline gradients. Figure 6 shows that after two hours of reincubation, the drug-induced lesions had completely disappeared, suggesting that minicells are capable of repairing the Micrococcal endonuclease-sensitive lesions produced by nitrofurazone treatment. The endonuclease preparations had essentially no effect on CCC-DNA from untreated minicells.

G. Breakage of CCC-DNA by other nitrofurans

The action of nitrofurazone on CCC-DNA was compared with that of three other nitrofuran derivatives. FANFT and AF-2 are highly carcinogenic and mutagenic compounds while nitrofurantoin is weakly mutagenic but appears to be noncarcinogenic (34). Table VIII shows that exposure of minicells to concentrations of FANFT or AF-2 as low as 5 μg/ml

TABLE VIII

Breakage of DNA by Different Nitrofurans

Exp. No.	Nitrofurans	Concentration (μg/ml)	% of Radioactivity Recovered as CCC-DNA	% of control
1	-	-	32	100
	AF2	5	15	49
	FANFT	5	13	43
	nitrofurantoin	75	31	97
2	-	-	28	100
	AF2	5	12	44
	FANFT	5	13	47
	nitrofurantoin	75	26	95

The minicells treated with nitrofurans for 30 minutes were lysed and subjected to the centrifugation on alkaline sucrose gradients as described in text.

for 30 min decreased the recovery of CCC-DNA by over 50%, while nitrofurantoin concentrations as high as 75 µg/ml for 30 minutes resulted in breakage of no more than 5% of the CCC-DNA. These results indicate that the ability of these three compounds to induce single strand breaks in CCC-DNA parallels their carcinogenicity and mutagenicity.

II. Effects of Nitrofurazone on RNA and Ribosomes

A. RNA synthesis

The kinetics of the labelling with[^{14}C]- uracil in control and nitrofurazone (50 µg/ml) treated cells are shown in Figure 7. The incorporation of [^{14}C] -uracil into cellular RNA was strongly inhibited in the nitrofurazone-treated cells after the first two minutes. This observation may indicate that two minutes is the length of time required for penetration and activation of the drug and for the accumulation of cellular damage.

In order to determine if the synthesis of all species RNA was inhibited by nitrofurazone, control and drug-treated cells were exposed to [^{14}C]-uracil for either 15 seconds or 30 minutes. From other work (71,73,79), rapidly labelled RNA sedimenting between 16S and 4S was considered to represent mainly mRNA. Figure 8 clearly demonstrates that the labelling of this mRNA was greatly decreased by nitrofurazone.

Figure 9 shows that the synthesis of 23S, 16S and 4S RNA were inhibited by about 92, 70 and 65% respectively

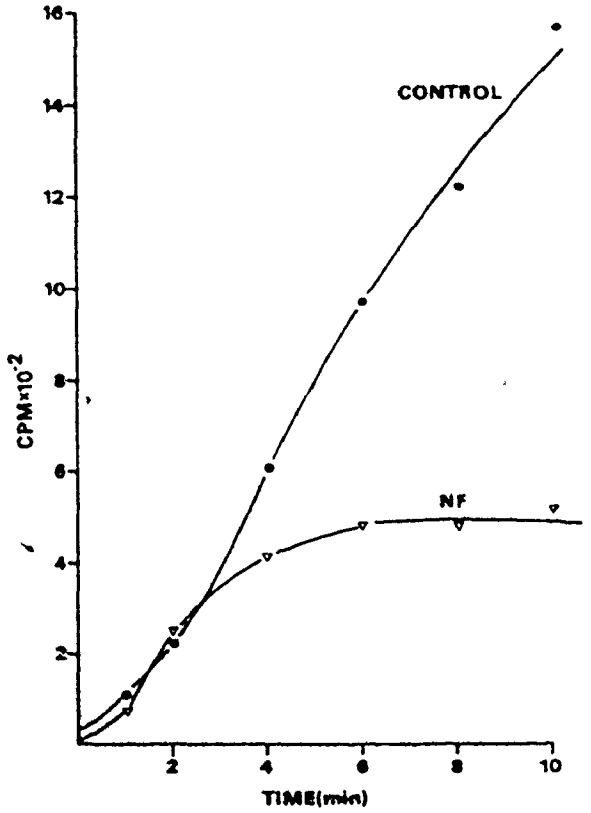


Figure 7. Effect of nitrofurazone on incorporation of uracil-^{14}C into the acid-insoluble fraction of *E. coli* strain B/r cells.

Cells were incubated with nitrofurazone (50 μg/ml) in the presence of uracil-^{14}C. At intervals portions of the suspension were removed and assayed for TCA-insoluble radioactivity: ●, control; ▽, nitrofurazone.

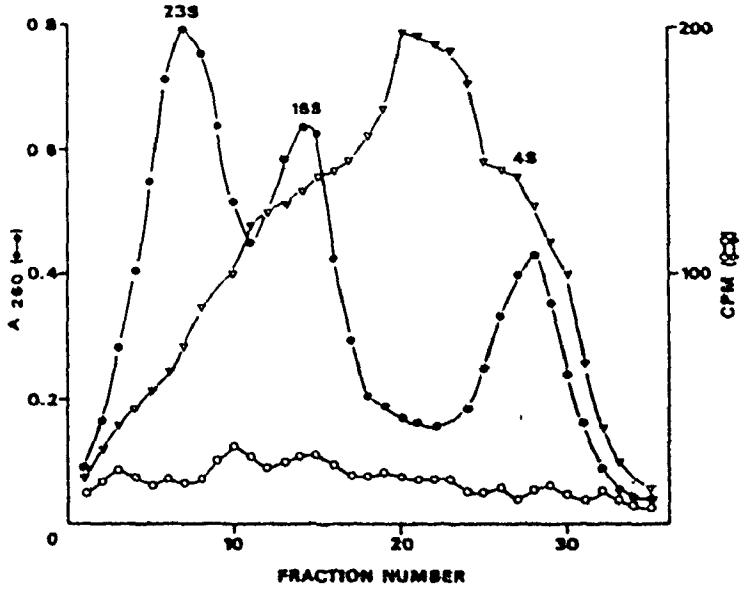


Figure 8. Sedimentation of short pulse-labelled RNA extracted from nitrofurazone-treated or control *E. coli* B/r.

Cells were pre-incubated with (o-o) or without (▽-▽) nitrofurazone for 30 min and then labelled with [^{14}C]-uracil for 15 seconds. RNA were extracted and sedimented on 5-20% sucrose gradient as described under Methods. Absorbance profile of control (●-●) is similar to that of RNA from treated bacteria.

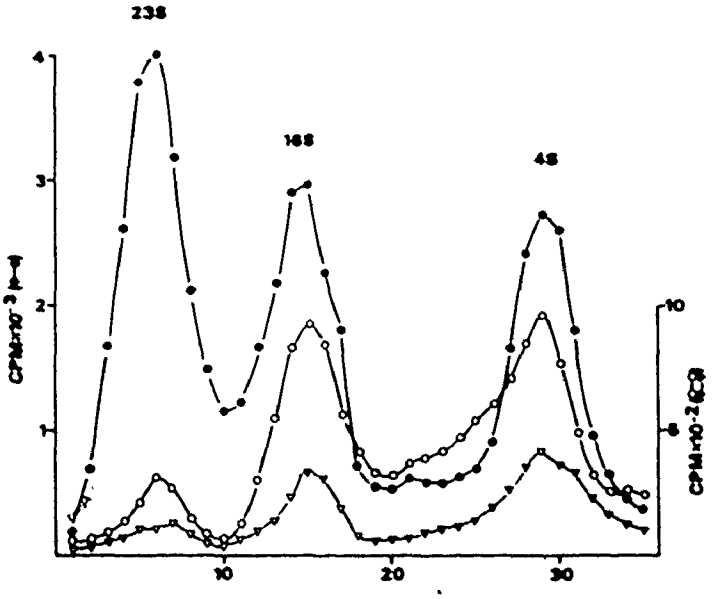


Figure 9. Sedimentation of labelled RNA extracted from nitrofurazone-treated or control *E. coli* B/r.

Cells were incubated with (o—o) or without (●——●) nitrofurazone (50 μg/ml) in the presence of [^{14}C]-uracil for 30 minutes. Symbols (▽—▽) refer to RNA from cells which were incubated with nitrofurazone for 30 minutes before the addition of [^{14}C]-uracil. Incubation of this sample was continued for an additional 30 minutes. RNA was extracted and sedimented as described under Methods.

by a 30 minute nitrofurazone treatment and the inhibitory effect was even more pronounced in cells which had been pre-incubated with drug for 30 minutes before addition of [^{14}C]-uracil. However, the interesting effect shown in Figure 9 is that the synthesis of 23S RNA was somewhat more sensitive to the drug than that of other two kinds of RNA. Figure 10 shows that in a drug-resistant strain (*nfr*-207) which lacks soluble nitrofuran-reductase, the synthesis of RNA was not affected by the treatment with nitrofurazone.

B. Effect of nitrofurans on DNA-dependent RNA polymerase

The observations described above raise the question as to how the drug affects RNA synthesis. Since nitrofurazone can inhibit the activity of some enzymes (16,18), the effect of the drug on the activity of DNA-dependent RNA polymerase was next examined. Table IX shows that the RNA polymerase activity was just as high in preparations from drug-treated cells as in those from untreated controls. Thus, the inhibition of RNA synthesis found in treated cultures is not due to permanent inactivation of the enzyme by nitrofurazone.

C. Effect of nitrofurans on the synthesis and function of ribosomes

As expected, inhibition of ribosomal RNA synthesis was reflected in decreased synthesis of ribosomal subunits. Figure 11 shows that after 30 minutes of drug treatment,

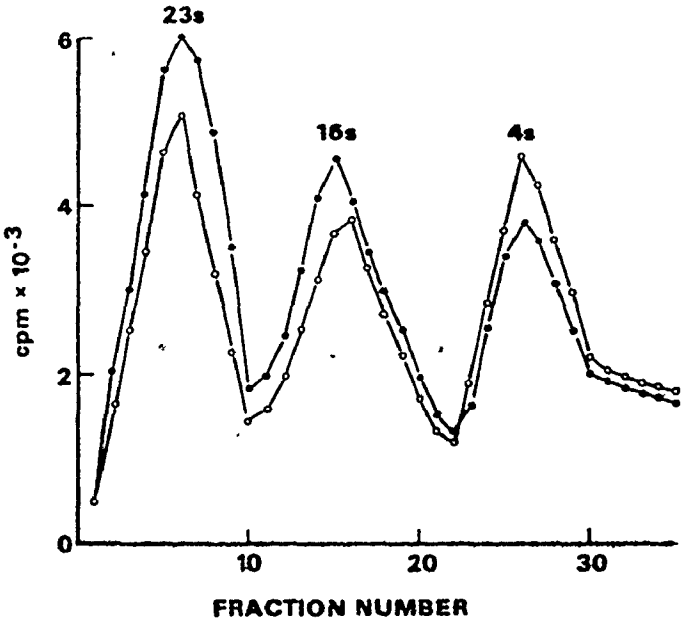


Figure 10. Sedimentation of labelled RNA extracted from nitrofurazone-treated or control *E. coli* 207 cells.

Cells were incubated with (o-o) or without (●-●) nitrofurazone (50 µg/ml) in the presence of [^{14}C]-uracil for 30 minutes.

TABLE IX

Effect of Nitrofurazone on the DNA-dependent RNA Polymerase Activity

Strain	Source of Enzyme	^{14}C Incorporated into RNA CPM
B/r	control	287
	NF-treated	272
207	control	269
	NF-treated	290

Cells were treated with nitrofurazone (50 µg/ml) for 1 hr. The cell extracts were prepared as described in Materials and Methods, section X. The reaction mixture (0.25 ml) contained 0.2 M KCl, 0.04 M tris-Cl pH 7.9, 0.0046 M $MgCl_2$, 0.002 M $MnCl_2$, 0.07 mM EDTA, 0.15 mM GTP, 0.15 mM CTP, 0.15 mM UTP, 0.15 mM ^{14}C-ATP (1 µCi/mMole), 0.04 M mercaptoethanol and 25 µg calf thymus DNA, and was incubated for 15 minutes at 37°C.

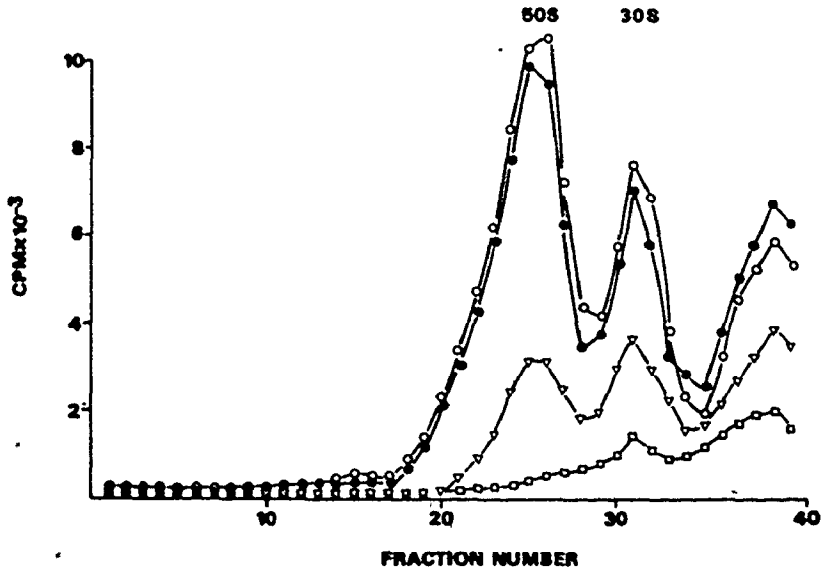


Figure 11. Sedimentation analyses of cell extracts at 10^{-4} M Mg^{2+} from nitrofurazone-treated or control *E. coli* B/r.

Cells were incubated at 37°C with (●—●; 5 μg/ml; Δ–Δ; 50 μg/ml) or without (o—o) nitrofurazone in the presence of ^{14}C-uracil for 30 minutes. The curve labelled (□ –□) was obtained with cells of which were incubated with nitrofurazone for 60 minutes and labelled with ^{14}C-uracil for 30 minutes. The absorbance profile from both drug-treated and control cells was similar to -o-o-.

the synthesis of both 50S and 30S subunits was inhibited by about 57% and 83% respectively. The inhibition was more severe in cells which were exposed to drug for 60 min prior to addition of labelled uracil. It is noteworthy that the synthesis of the 50S subunit was more sensitive to the drug than that of 30S subunits, thus confirming the observed effect of drug on ribosomal RNA synthesis.

It was of interest to know whether nitrofurazone has any effect on the formation of polysomes. Figure 12 shows that most of the absorbance (12a) and radioactivity (12b) in untreated cells were located in the 70S and 100S regions and that only a small proportion appeared at 50S and 30S regions. However, polysomes from drug-treated cells contained less radioactivity, most of the label being found in the 50S and 30S regions (Fig. 12b). Moreover, the absorbance peak corresponding to polysomes was much smaller in preparations from drug-treated cells (Fig. 12a). These data suggest that nitrofurazone is able to suppress the formation of polysomes. Figures 13 and 14 show that nitrofurazone has no effect on the synthesis of ribosomal subunits and formation of polysomes in the reductase-I-less strain *nfr*-207.

Since unstable intermediates formed during the reduction of nitrofurazone are known to react with protein to form stable derivatives (11), it was of interest to see if the reaction intermediates are able to bind to ribosomal

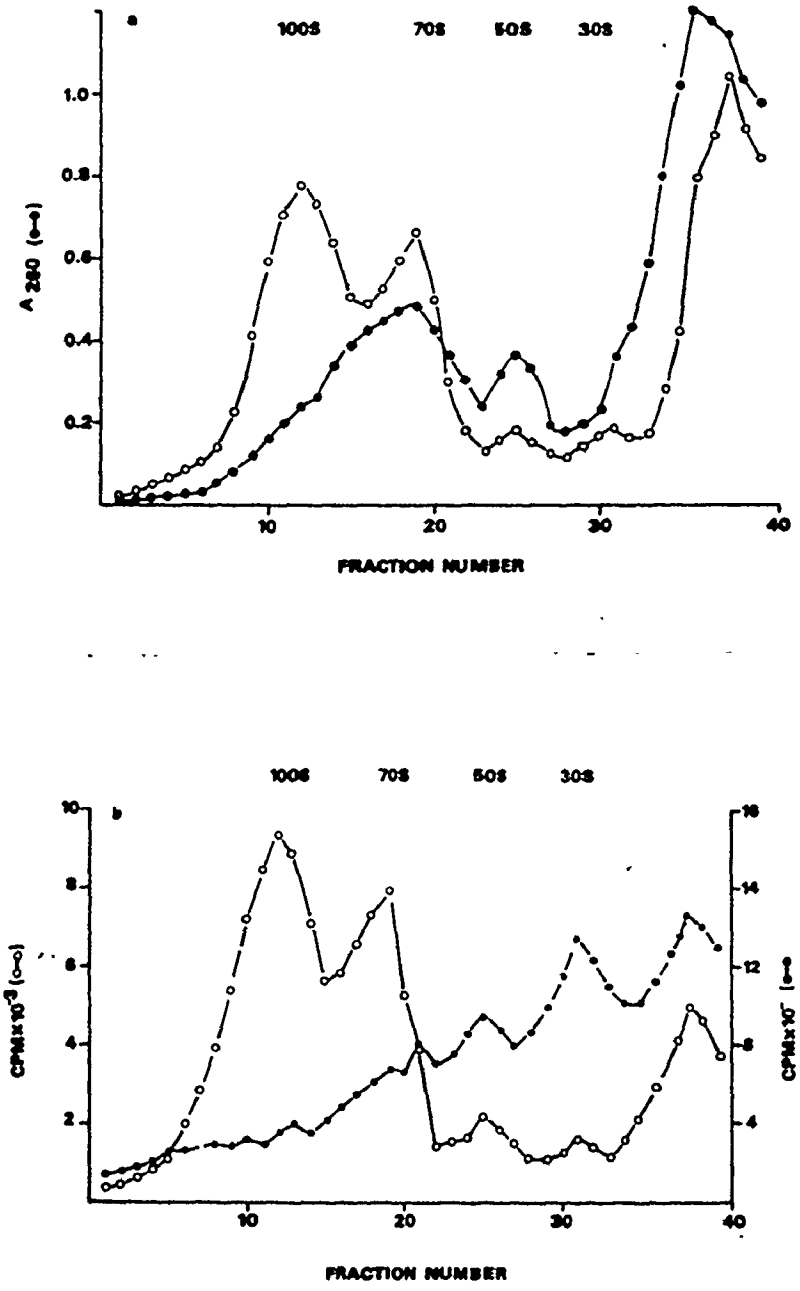


Figure 12. Sedimentation analyses of cell extracts at 10^{-2} M Mg^{2+} from control and drug-treated cells.

Cells were incubated at 37°C with (●-●) or without (o-o) nitrofurazone (50 µg/ml) for 30 minutes and followed by ^{14}C-uracil for 30 minutes. (a) profile of absorbance (260 nm) and (b) of radioactivity.

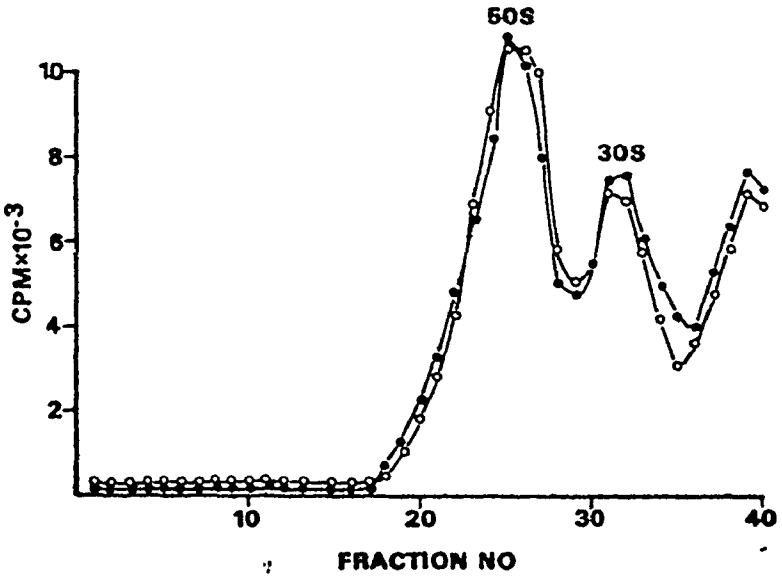


Figure 13. Sedimentation analyses of cell extracts at 10^{-4} M Mg^{2+} from nitrofurazone-treated or control *E. coli* 207.

Cells were incubated at 37°C with (●-●; 50 μg/ml) or without (o-o) nitrofurazone in the presence of ^{14}C-uracil for 30 minutes. The absorbance profile from both drug-treated and control cells was similar to -o-o-.

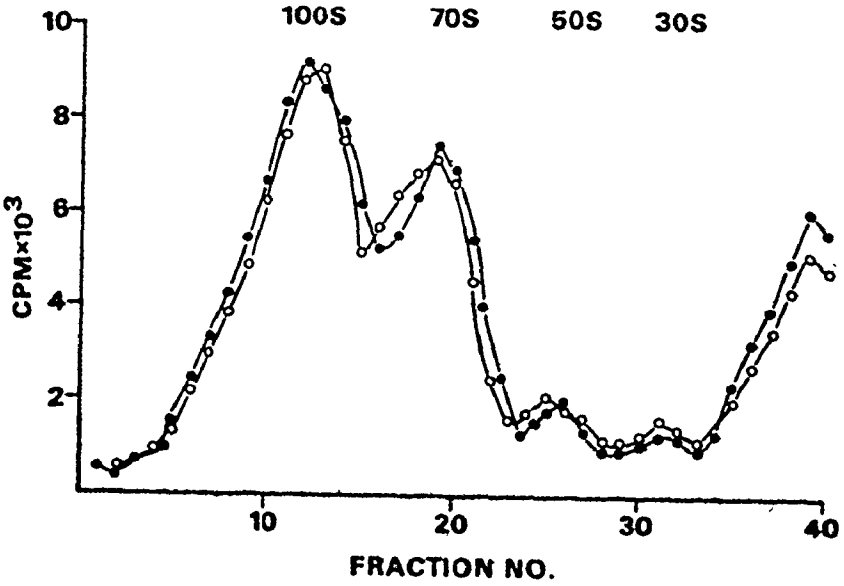


Figure 14. Sedimentation analyses of cell extracts at 10^{-2} M Mg^{2+} from nitrofurazone-treated and control *E. coli* 207.

Cells were incubated at 37°C with (●-●) or without (o-o) nitrofurazone (50 μg/ml) for 30 minutes and followed by ^{14}C-uracil for 30 minutes.

subunits. Figure 15 shows there was considerable radioactivity in the 50 and 30S regions of the gradient but that considerable radioactivity was also bound to slower sedimenting material.

The possibility that nitrofurazone treatment *in vivo* affects the function of ribosomes was investigated by examining the ability of ribosomes from drug-treated cells to support poly U-directed polyphenylalanine synthesis *in vitro*. Table X shows that the ability of ribosomes from treated cells to support polyphenylalanine synthesis was reduced to about 40% of that obtained with ribosomes from untreated cells. Results of reconstruction experiments show it is the ribosomes themselves and not the S-100 fraction that is damaged.

III. Effect of Nitrofurazone on Glucose Metabolism and ATP Levels

The effects reported here and elsewhere (24,28,30,31) make it clear that activated nitrofurazone reacts directly with cellular macromolecules causing breaks in DNA, mutations and damage to ribosomes. While direct damage to DNA might inactivate its template activity and thus account for the observed inhibition of nucleic acid and protein synthesis, it is also possible, given the known ability of nitrofurazone to inhibit glucose metabolism and respiration, that the primary cause of these effects is merely lack of energy for macromolecule synthesis. There has been no attempt to compare the relative effects of nitrofurazone on

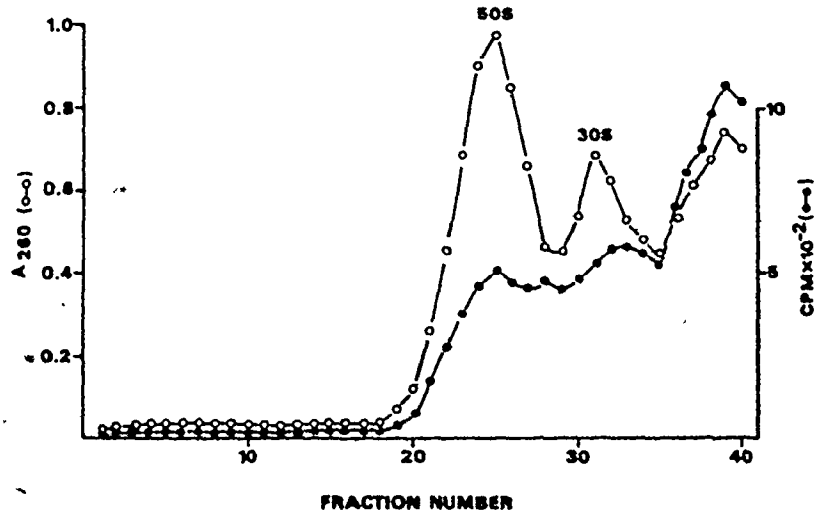


Figure 15. Sedimentation analyses of cell extracts at 10^{-4} M Mg^{2+} from *E. coli* B/r cells.

Cells were treated with ^{14}C-nitrofurazone (15 µg (0.15 µCi) ml) for 30 minutes. o——o, absorbance; •——•, radioactivity.

TABLE X

Activity of Ribosomes and S-100 Fraction from Nitrofurazone-treated *E. coli* B/r

Experiment	Source of Ribosomes	Source of S-100 Fraction	CPM	%
1	control	control	71,000	100
	NF	NF	27,000	37
	control	NF	69,000	97
	NF	control	31,000	47
	control	-	680	-
	-	control	270	-
2	control	control	64,000	100
	NF	NF	26,000	41
	control	NF	61,000	93
	NF	control	30,000	46
	control	-	580	-
	-	control	200	-

The reaction mixtures contained the following (in μmoles/ml): 100 Tris, pH 7.8; 10 magnesium acetate; 50 KCl; 6.0 mercaptoethanol; 5.0 phosphenolpyruvate; 1.0, ATP; 0.03 each of GTP, CTP and UTP; 0.02 M ^{14}C-L-phenylalanine; 10 μg polyuridylic acid, 20 μg phosphoenolpyruvate kinase; 1 mg *E. coli* soluble RNA, 1 mg ribosome protein; and 1.8 mg S-100 protein. Samples were incubated at 35°C for 60 minutes.

the synthesis of nucleic acids and on glucose metabolism using identical conditions. It was, therefore, of interest to examine the action of nitrofurazone on glucose metabolism and ATP production using the same concentration of drug and the same cell density as was used in the experiments reported above.

The non-gaseous, [^{14}C]-labelled end products accumulating during growth of bacteria on uniformly labelled glucose-[^{14}C] were analysed by silicic acid column chromatography. Figure 16 shows that under aerobic conditions, production of acetate, the major nonvolatile product formed in untreated cell,was strongly inhibited. Figure 16 also shows that pyruvate which was undetectable in the normal cells, became a major product in cells treated with nitrofurazone. Thus, the alteration of aerobic glucose metabolism is the replacement of acetate by pyruvate as the main product, indicating that the aerobic catabolism of glucose by drug-treated cells continued normally as far as pyruvate, but that further conversion of pyruvate to acetate was strongly inhibited. Table XI summarizes data obtained from end product analysis of aerobic and anaerobic glucose metabolism separated by silicic acid columns. Under anaerobic conditions, normal cells gave the typical products of *E. coli* fermentation: ethyl alcohol, acetate and formate: with small amounts of lactate and succinate. Nitrofurazone, however, significantly changed the pattern of label in fermentation products with a very striking reduction in amounts of ethanol, demonstrating

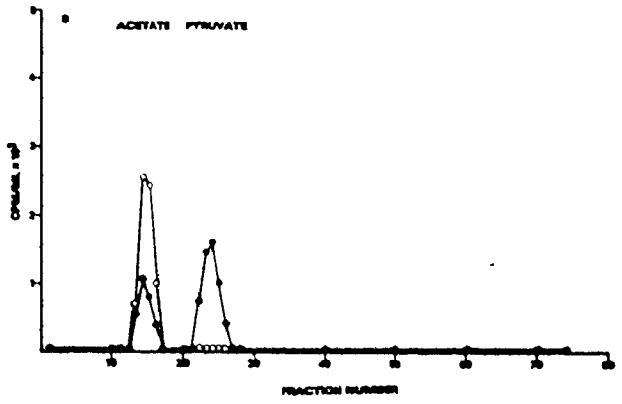


Figure 16. Chromatographic separation of soluble products from aerobic utilization of [^{14}C]-glucose by *E.coli* B/r.

Cells were aerobically incubated with nitrofurazone in the presence of [^{14}C]-glucose (1 μCi/ml) for 1 hr at 37°C. The samples were prepared and fractionated on a silicic acid column as described in text. o——o, control; ●——●, nitrofurazone.

TABLE XI

Effect of Nitrofurazone on Glucose Metabolism by *E. coli* B/r

Strain	Conditions		CPM x 10^3 Recovered in						
	O_2	NF	CO_2	EtOH	Acetate	Formate	Pyruvate	Lactate	Succinate
B/r	+	-	188	0	420	0	0	0	0
	+	+	97	0	86	0	248	0	0
	-	-	177	166	246	82	0	5	4
	-	+	91	10	252	40	0	2	2
nfr-207	+	-	176	0	362	0	0	0	0
	+	+	163	0	325	0	0	0	0
	-	-	181	152	264	76	0	6	4
	-	+	186	148	270	68	0	5	5

Cells of *E. coli* B/r or 207 were aerobically or anaerobically incubated with nitrofurazone (50 µg/ml) in the presence of ^{14}C -glucose (1 µCi/ml) for 1 hr at 37°C. Samples were then prepared and fractionated on a silicic acid column as described in text. The effect of drug on CO_2 production was conducted in the separated experiments and was measured as described in text.

that nitrofurazone (or a metabolite) blocks the reduction of acetate to ethanol. Furthermore, Table XI shows that formation of CO_2 was inhibited about 50% by nitrofurazone under both aerobic and anaerobic conditions. Since nitrofurazone treatment blocked the reduction of acetate to ethanol, it was of interest to see if the activity of alcohol dehydrogenase in cells is inhibited by nitrofurazone. Cells treated with nitrofurazone for 1 hour were washed and sonicated. Samples of cell extract were assayed for activity of alcohol dehydrogenase. Figure 17 shows that nitrofurazone had no inhibitory action on the activity of alcohol dehydrogenase.

In cells of strain *nfr*-207, nitrofurazone had no apparent effect on the glucose metabolism (Table XI).

The inhibition of glucose metabolism would be expected to decrease the amount of ATP generated via glycolysis and oxidative pathways. Therefore, the ATP level in the normal and drug-treated cells were determined by means of an assay based on firefly bioluminescence. As shown in Figure 18, the ATP content of the growing control culture increased, whereas the ATP content of the drug-treated culture declined. In contrast to nitrofurazone, NFT, a nitrofuran bacteriostatic compound, has shown at modest dosage inhibition on DNA synthesis, but not on ATP synthesis (unpublished data, McCalla).

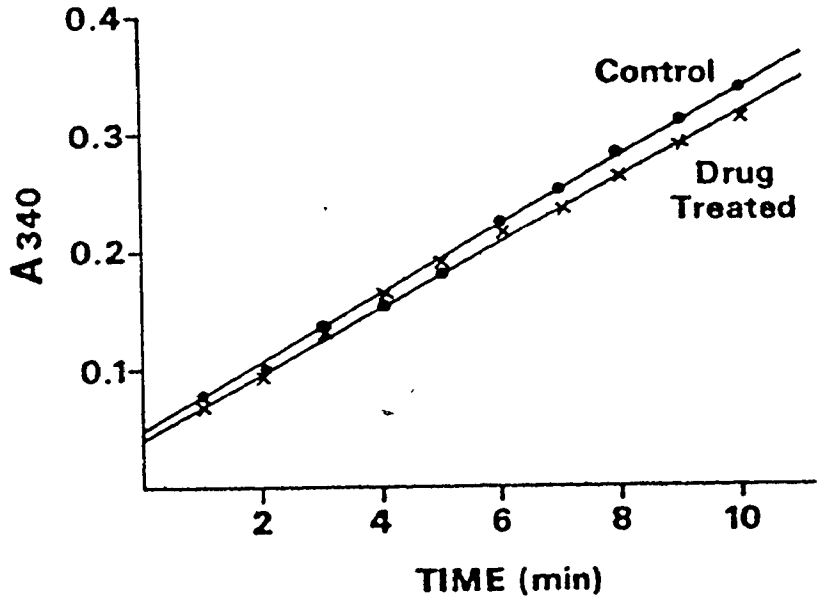


Figure 17. Effect of nitrofurazone on activity of alcohol dehydrogenase from *E. coli* B/r.

Cells treated with nitrofurazone (50 μg/ml) for 1 hr were collected, washed and sonicated. The cell extracts were then assayed for the activity of alcohol dehydrogenase as described in text.

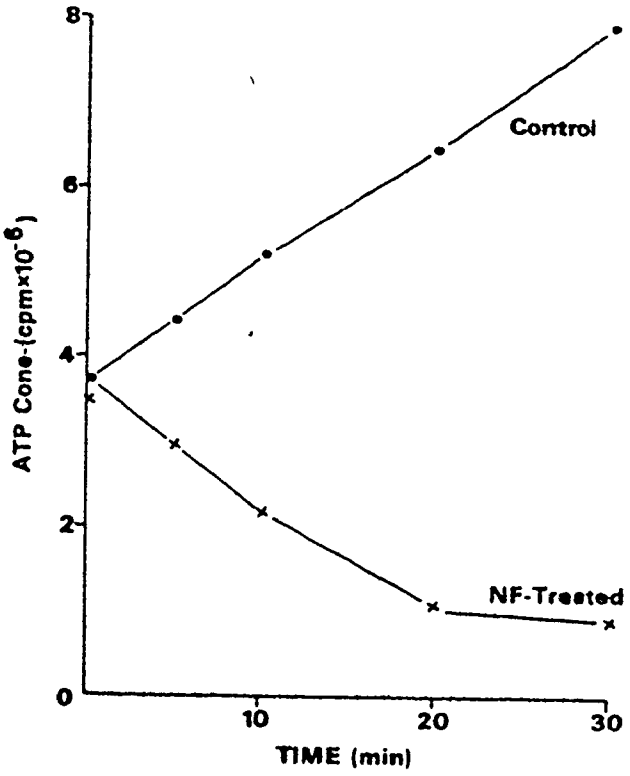


Figure 18. Effect of nitrofurazone on ATP levels of *E. coli* strain B/r.

Fifty ml of log-phase cells were incubated aerobically with nitrofurazone (50 µg/ml) at 37°C. At intervals indicated, 5 ml of culture were withdrawn and sonicated. One tenth ml of cell extracts were assayed for ATP by means of firefly method: ●, control; x, nitrofurazone. Under the same assay conditions, samples of 10^{-6} M ATP gave about 1.1 x 10^6 CPM.

DISCUSSION

I. Damage to DNA

The results presented in this thesis show that at least two and perhaps three kinds of lesions are introduced into DNA when minicells are incubated with nitrofurazone. First, there are alkaline labile lesions, i.e., lesions which show up as breaks only after exposure to alkali. Second, there are overt breaks which are seen on neutral as well as alkaline gradients. While the relationship between the breaks and the lesions which became converted to breaks by treatment with *Micrococcal* endonuclease is not entirely clear, it is certainly possible that the overt breaks are produced by the action of repair nucleases, present in the minicells, on modified groups in the DNA. As incubation in drug-free medium continued the breaks became closed. Thus the yield of breaks seen on neutral gradients may represent those which have been formed by nuclease action but not yet completely repaired.

The fact (Fig. 5) that essentially all of the lesions which are sensitive to *Micrococcal* endonuclease have disappeared from the DNA of minicells at the time repair is complete (i.e., when all breaks have disappeared) is

consistent with this possibility. So is the observation that when a reductase I preparation obtained from a *uvr*A$^-$, *exr*$^-$ strain which lacks the "repair endonuclease" (78) was used to activate nitrofurazone *in vitro*, less of the CCC-DNA was converted to the relaxed form than when a *uvr*A$^+$, *exr*$^+$ strain served as the source of enzyme (Table 6). Alternatively, the breaks and nuclease sensitive lesions could be distinct types of damage induced by nitrofurazone in CCC-DNA. It is possible that the breaks result from a chemical reaction between DNA and reduced nitrofurazone. These interactions might induce structural instability in the backbone of the CCC-DNA and result in single strand breakage of the molecules without the participation of any endonuclease. To distinguish between these two alternatives will require purification of nitrofurazone reductase free of endonuclease. If breaks are observed when CCC-DNA is incubated with the purified reductase plus nitrofurazone, this will definitely demonstrate that activated nitrofurazone itself is capable of inducing breaks in DNA.

No attempt was made to identify the mode of repair responsible for disappearance of endonuclease lesions from DNA during incubation of minicells, but excision repair is a reasonable possibility since minicells contain UV-specific endonuclease (67). (This type of enzyme plays a major role in excision repair.) Although the activity of this enzyme in minicells is low, it is sufficient to

repair the lesions induced by gamma rays (67). Recombination repair (79), a second repair mechanism, is an unlikely candidate for repair of endonuclease-sensitive lesions in the minicells, since X1256 minicells are *recA*$^-$ and thus lacking a component required for recombination repair.

The available evidence suggests that the alkali labile lesions have a different molecular basis from the lesions that result in breaks which are observed under neutral conditions. This is most clearly seen from the observation (Fig. 6) that the alkali labile lesions remain in minicell DNA for at least two hours during incubation in drug-free media, whereas virtually all the breaks plus the lesions sensitive to *Micrococcal* endonuclease have disappeared in that time.

The lack of repair of alkali labile lesions by the minicells seems to be somewhat different from the situation in *E. coli* B/r in which most of the DNA returned to the control value of the sedimentation constant on alkaline gradients in 30 minutes (27). The difference could be due to lack of some repair enzymes in the minicells as a result of unequal distribution of cellular material during minicell formation (69), to the fact that the minicell-producing strain used was *recA*$^-$ or to the relative insensitivity of the technique used earlier. While many chemical and physical agents are known to cause single strand breaks in DNA under alkaline conditions, in relatively few cases

has it been determined whether or not breaks are present before exposure to alkali. Patterson *et al.* (80) showed that both breaks and alkali labile lesions were formed in gamma-irradiated DNA and that the latter, like alkali labile lesions induced by nitrofurazone, are not repaired.

Beerman and Goldbert (81) found that treatment of CCC-DNA with neocarzinostatin *in vitro* in the presence of 2-mercaptoethanol caused breaks which could be seen on neutral gradients. In contrast, Abelson and Penman (82) showed that camptothecin caused breaks only when DNA was exposed to the drug (or a derivative) when alkali is also present.

II. RNA and Ribosome Synthesis

It is clear from the results that the syntheses of all different classes of RNA are inhibited by nitrofurazone. This raises a question as to how nitrofurazone affects transcription. The evidence (Table IX) shows that the activity of DNA-dependent polymerase is not inhibited by the treatment of cells with nitrofurazone. This, together with the fact that nitrofurazone induces single strand breaks in DNA (27,28) would suggest that the mechanism of inhibition of transcription could be damage of DNA as a template. To test this possibility would require the isolation of DNA from drug treated cells and assay its activity as a template for transcription *in vitro*.

Alternatively or additionally, the inhibition could be due to the shortage of ATP caused by the suppression of bacterial respiration by nitrofurazone noted in the previous chapter.

A striking effect of nitrofurazone (Fig. 9) on RNA synthesis is the difference in the sensitivity of the synthesis of 16S and 23S RNA toward nitrofurazone treatment. There are several reports in the literature indicating that the formation of 23S RNA is preferentially suppressed by a variety of agents including 5-fluorouracil (83), chloramphenicol (83), mitomycin c (84) and UV radiation (85,86,87). Therefore, it appears that the synthesis of 23S RNA is generally more vulnerable than that of 16S RNA. The base sequence (88), and to a lesser extent the base composition (89) also appear to be significantly different between 16S and 23S RNA of *E. coli*. This would indicate that 16S and 23S RNA are formed from two separated cistrons. The different sensitivity of these two RNAs toward chemicals and radiation is consistent with such a notion.

The synthesis of mRNA is severely suppressed by nitrofurazone treatment (Fig. 8). Possibly this effect might explain the observed effects of the drug on polysome formation. It is well established (71,83) that the ribosomal profile in a bacterial extract is dependent on the magnesium concentration. At 10^{-4} M Mg^{2+}, 50S and 30S ribosomal sub-units prevail whereas at 10^{-2} M Mg^{2+}

predominantly 100S polysomes and 70S ribosomes are found. In extracts from drug treated cells in 10^{-4} M Mg^{2+} concentration, the 50S peaks were considerably lower than the 30S peaks in comparison with extracts prepared from untreated cells. The 50S ribosomal sub-units contain 23S RNA whereas 30S ribosomal sub-units contain 16S RNA (90,91). Thus, the fact that relatively fewer 50S particles than 30S are formed is likely due to the sensitivity of synthesis of the 23S RNA to the drug.

NFT, a potent carcinogenic and mutagenic nitrofuran, and mitomycin C, a bactericidal compound, have been shown to cause degradation of 50S and 30S ribosomal sub-units (84,92) as demonstrated by their ability to change the optical absorption profile of the sedimentation pattern. In the present work, nitrofurazone had no apparent effect on the absorption profile, indicating that its mechanism of action is different from that of NFT and mitomycin C.

The 70S ribosomes and 100S polysomes are involved in protein synthesis (93). Figure 12 shows that the formation of 70S and 100S particles was suppressed by nitrofurazone treatment. This observation is not unexpected since the synthesis of their basic components, 50S and 30S subunits and mRNA were inhibited by the drug.

The observation (Figure 12) that nitrofurazone causes an accumulation of 50S and 30S particles in cells extracted

with 10^{-2} M Mg^{2+} may also be the result of the inhibitory effect of drug on the synthesis of mRNA. As the availability of mRNA is reduced, ribosomal sub-units accumulate rather than reassemble to form functioning particles of 70S, 100S or larger.

III. Ribosomal Function

The fact that ribosomes isolated from nitrofurazone treated cells carry out poly U directed polyphenylalanine synthesis at only half of the control rate indicates that the ribosomes are themselves damaged by nitrofurazone treatment. The possibility that some other component of the protein synthesizing system was damaged was eliminated by the discovery that the S100 fraction from nitrofurazone treated bacteria permitted normal rates of polyphenylalanine synthesis when used with ribosomes from untreated cells. The damage to ribosomes may contribute, along with lack of mRNA, to the observed inhibition of protein synthesis. The molecular mechanism of the "malfunction" of ribosomes is not known. However, from the evidence presented, it may be due to the binding of reduced nitrofurazone to ribosomal protein (Fig. 15). Other agents such as streptomycin (94,96) and N-methyl-N'-nitro-N-nitrosoguanidine (96) are known or suspected to damage ribosomal function by binding to ribosomal proteins.

IV. Glucose Metabolism and ATP Synthesis

Glucose can be metabolized by *E. coli* either in the presence of oxygen (aerobic) or in the absence of oxygen (anaerobic). While in this thesis all the experiments with DNA, RNA and ribosomes were carried out under aerobic conditions, those concerned with glucose metabolism were done under both aerobic and anaerobic conditions.

Three sets of findings emerge with regard to the effects of nitrofurazone on glucose metabolism: 1) there is accumulation of pyruvate after nitrofurazone treatment and corresponding failure to convert pyruvate to acetate, 2) nitrofurazone lowers the ATP level, 3) a decrease in the amount of ethyl alcohol formed under anaerobic conditions is observed with the cells treated with nitrofurazone. The first finding suggests a direct block in pyruvate dehydrogenase of cells treated with nitrofurazone. This is in agreement with the early report that nitrofurazone inhibits the reactions mediated by "pyruvate oxidase" (97). The accumulation of pyruvate can itself lower the ATP level, since the amount of acetyl CoA formed is greatly reduced. This in turn reduces the amount of ATP formed by the action of the tricarboxylic acid cycle and oxidative phosphorylation. However, the alternative possibility is that the effect may be simply an inhibition of adenylate kinase or the activation of an adenosine triphosphatase.

The shortage of ATP in cells would result in blocking most energy required reactions including the formation of nucleotides and their triphosphates. This would directly decrease the nucleotide triphosphate pool and in turn decrease the rate of synthesis of nucleic acids.

The decrease in the amount of ethyl alcohol produced under anaerobic conditions indicates that nitrofurazone is able to change the pattern of anaerobic catabolism of glucose. Since the activation of nitrofurazone by reductase is required before it can exert its maximum antibacterial effect (11), this process may compete for the reduced cofactors which are normally utilized for the production of ethyl alcohol. Support for this idea comes from the recent finding (98) that nitrofurazone is able to lower the total content of NAD^+ and NADH. Similar observations have also been made on the effect of nitrate on glucose metabolism under anaerobisis (65). When bacterial cells were anaerobically incubated with nitrate, the latter was reduced at the expense of the reduction of acetate to ethyl alcohol. It is unlikely that decrease in the production of ethyl alcohol is due to the permanent inhibition of alcohol dehydrogenase by nitrofurazone, because the activity of this enzyme in extracts of cells treated with nitrofurazone was approximately the same as that in extracts of control cells (Fig. 16).

V. Requirement for Activation of Nitrofurazone

Development of strains resistant to nitrofurans has been carried out with the purpose of helping us understand the mode of action of nitrofurans on bacteria. Strain *nfr*-207 lacks the major component of nitrofurazone reductase (reductase I) but retains reductases IIa and IIb (12). Earlier work has shown that survival, DNA breakage, mutation and binding of labelled nitrofurazone to protein are all decreased in *nfr*-207 in air compared to its reductase-I-containing parent (12). This presumably is due to lack of activation of the drug. In the course of this investigation, the action of nitrofurazone on *E. coli* *nfr*-207 and B/r was compared. It was found that in *nfr*-207 the drug has essentially no effect on RNA or ribosome synthesis or on glucose metabolism. These results are in strong contrast to those presented for B/r. Taken at face value they indicate that nitrofurazone metabolites rather than the drug itself are required to inhibit energy production as well as to damage DNA, ribosomes and the like.

However, since the *nfr* strains have not yet been analysed genetically, the possibility that the resistance of some of these processes to nitrofurazone under aerobic conditions is due to additional mutations which affect permeability or other processes cannot be completely eliminated.

VI. Concluding Remarks

From the present work and data in the literature, it is clear that nitrofurans have many effects on cellular constituents and processes. Killing of bacteria by these agents or their metabolites may well be a complex process in which the most important target depends upon the genotype and physiological condition of the bacteria used and the particular nitrofuran administered.

Under some conditions damage to DNA is the important effect. This is shown by the observation that *E. coli* B/r is more resistant to killing than is *E. coli* B and that mutant strains which are deficient in excision repair of DNA are more sensitive to killing by many nitrofurans than are their normal counterparts.

At the same time, different agents differ widely in their ability to damage DNA. Thus, FANFT and AF-2 at .5 μg/ml cause as extensive DNA damage as nitrofurazone at 50 or 70 μg/ml. In comparison, nitrofurantoin has only weak mutagenic activity but kills *E. coli* at the same concentrations as nitrofurazone.

It is likely that at one end of the spectrum are compounds like FANFT and AF-2 which have a very powerful action on nucleic acids and that prime effect of such compounds at the low concentrations required to kill bacteria is that of damaging DNA. This damage leads to

rapid inhibition of DNA replication but not of RNA synthesis. Lethal doses of these agents do not appreciably affect energy production.

At the other extreme are compounds like nitrofurantoin which have weaker mutagenic activity. Higher concentrations of these agents are required to achieve comparable amounts of killing. At these concentrations ATP production is inhibited. This in turn results in the slowing of many synthetic processes.

Nitrofurazone would seem to be intermediate in its effects since sublethal doses of the agent cause damage to DNA and ribosomes as well as lowering ATP levels and inhibiting synthesis of macromolecules.

Given the efficacy of nitrofurans and nitroheterocyclic compounds as antibacterial and antiprotozooal agents, it seems likely that such compounds will remain in general use for some time. It therefore seems well worthwhile to try to select derivatives with the lowest possible mutagenic activity to mammals for use in chemotherapy or as food additives.

SUMMARY

Enzymically activated nitrofurazone reacts with covalently closed circular DNA (derived from *E. coli* minicells carrying λ dv) to give at least two kinds of damage: breaks which are detected on neutral sucrose gradients and alkali labile lesions in DNA which are converted to breaks when the DNA is subsequently treated with alkali. DNA, isolated from minicells exposed to the drug, also contains lesions which are converted to breaks upon treatment with endonuclease preparations obtained from *Micrococcus luteus*. Minicells repaired both breaks and nuclease susceptible lesions within 2 hours but did not repair alkali labile lesions within that time.

Experiments with three nitrofurans show that there are considerable differences in the degree to which DNA is damaged by activated metabolites of various derivatives and that the potency of the compounds as mutagens and carcinogens is correlated with the amount of damage caused to minicell DNA.

Nitrofurazone has been shown to inhibit the synthesis of all classes of RNA with the 23S rRNA being more strongly affected than 16S RNA. There is correspondingly greater

inhibition of synthesis of 50S ribosomal subunits. The drug also suppresses the synthesis of polysomes. However, the most striking effect of nitrofurazone is a marked decrease in the ability of ribosomes to support protein synthesis *in vitro*. The drug has no effect on the activity of DNA-dependent RNA polymerase.

Nitrofurazone affects glucose metabolism of bacterial cells in three ways: 1) under aerobic conditions the drug causes the accumulation of pyruvate by blocking the conversion of pyruvate to acetate, 2) the drug lowers the ATP level, 3) under anaerobic conditions a decrease of ethyl alcohol production is observed in cells treated with the drug.

Experiments with the strain *nfr*-207 (a mutant of B/r which is resistant to nitrofurans because it lacks nitrofuran reductase activity) show that nitrofurazone has essentially no effect on RNA or ribosomal synthesis or on glucose metabolism in this strain. These results probably indicate that it is the activated nitrofurazone rather than nitrofurazone itself that causes cellular damages on bacteria.

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