Title
Analytical and metabolic studies with nitrofurantoin

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Publication Date
1978

Peer reviewed|Thesis/dissertation

ANALYTICAL AND METABOLIC STUDIES WITH NITROFURANTOIN

by

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B.S., University of California, Berkeley 1966
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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACOLOGY

in the

GRADUATE DIVISION

(San Francisco)

Date Librarian

Degree Conferred: APR - 2 1978

ACKNOWLEDGEMENTS

The author wishes to acknowledge the following persons:

Dr. Mary Vore (Research Advisor) -- For her constant support and encouragement during the course of the research. For her enthusiasm and erudition in the area of drug metabolism coupled with her unfailing human qualities.

Dr. Betty-ann Hoener -- For her willingness to share her expertise in the art of high-pressure liquid chromatography and for her joyful and positive approach to research.

Dr. E. Leong Way -- For his assistance by serving on the Research Committee and his help throughout my academic training.

Dr. John C. Craig -- For his useful comments in matters of mass-spectral analysis and his general enthusiasm for the study of nitrofurans.

Dr. Larry Gruenke -- For performing the electron ionization mass spectral analyses.

Jay d'Angona -- For performing the chemical ionization mass spectral analyses.

Mrs. Carol Horvath -- For her superior typing.

Mr. Charles Lee -- For his excellent illustrations.

This investigation was supported by Patent Funds from the Graduate Division of the University of California, San Francisco, Academic Senate Funds from the School of Medicine of the University of California, San Francisco, United States Public Health Service CA-18435. The NMR Facility used is supported by Grants No. RR 00892-01A1 from the Division of Research Resources, NIH, to UCSF.

ABSTRACT

Nitrofurantoin, an antibacterial agent, is used extensively in the treatment of urinary-tract infections. Nitrofurantoin is a potent mutagen in Salmonella typhimurium TA-100 and causes DNA damage in mammalian cell cultures. Pulmonary and hepatotoxicities and polyneuropathies have been observed in humans. Formation of toxic metabolites of nitrofurantoin may be responsible for the observed toxicities; however, metabolites of nitrofurantoin have not been previously identified.

A high-pressure liquid chromatographic (HPLC) method was developed for the determination of nitrofurantoin and its metabolites in biological fluids. The HPLC assay for nitrofurantoin, which requires only 0.2 ml of biological fluid and shows a linear relationship in the range of 0.02 to 200 μg/ml, can be performed in 9 min and is reproducible (coefficient of variation less than 2%). Results for nitrofurantoin so obtained correlate well with those obtained by the established Hyamine 10-X spectrophotometric method (coefficient of variation = 2%), but the HPLC method is more sensitive. With no modification, the HPLC procedure can also be used for other 5-nitrofurans, such as furazolidone or nitrofurazone. The sensitivity, accuracy, and convenience of the method make it suitable for clinical monitoring and pharmacokinetic/bioavailability studies with 5-nitrofuran derivatives.

The reductive metabolism of nitrofurantoin under anaerobic conditions was characterized in various tissues from control, germ-free, and germ-free acclimatized rats. Nitrofurantoin metabolism was highest in homogenates of cecum and colon contents of germ-free acclimatized and control rats (0.39 $\pm$ 0.02 nmol/min-mg protein) but was absent in cecum and colon contents of germ-free animals. Appreciable levels of activity were also present in homogenates of liver (0.21 $\pm$ 0.01 nmol/min-mg protein) and of small intestine walls (0.18 $\pm$ 0.02 nmol/min-mg protein) with lesser rates of metabolism observed in kidney homogenates (0.09 $\pm$ 0.01 nmol/min-mg protein).

As determined from HPLC, three metabolites were formed by the anaerobic incubation of nitrofurantoin with rat liver 9,000 xg supernatant fraction and in the isolated perfused rat liver. The major metabolite of nitrofurantoin which was isolated and purified by HPLC, was identified as 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione by chemical ionization mass-spectral analysis (MH^+ = 209) and Fourier transform proton nuclear magnetic resonance (δ, ppm = 2.76 triplet, 3.35 triplet, 4.20 singlet, 7.10 singlet). This finding was confirmed by comparative studies with an authentic specimen prepared by catalytic reduction of nitrofurantoin. This major metabolite was also identified in the urine of a patient on nitrofurantoin. A second, minor metabolite with

HPLC and ultraviolet absorption characteristics similar to 1-[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione (aminofurantoin) was detected in homogenates from rat cecum and colon contents. The mutagenicity of the two end-product metabolites of nitrofurantoin--aminofurantoin and 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione--was tested by using the Salmonella typhimurium TA-100 tester strain. Under the conditions used, neither metabolite was mutagenic.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	N,O-bis-(trimethylsilyl)-acetamide
CI	chemical ionization
CV	coefficient of variation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EI	electron ionization
eV	electron volt
FDA	Food and Drug Administration
fid	free induction decay
GLC	gas liquid chromatography
HPLC	high-pressure liquid chromatography
i.d.	inside diameter
IR	infrared
M	moles per liter
N	gram equivalents per liter
NADH	reduced nicotinamide adenine dinucleotide
NAPH	reduced nicotinamide adenine dinucleotide phosphate
nm	nanometer
NMR	nuclear magnetic resonance
o.d.	outside diameter
RF	radiofrequency
UV	ultraviolet
ε	molar extinction coefficient
λ_{max}	maximum absorption frequency

I. INTRODUCTION AND STATEMENT OF THE PROBLEM

The 5-nitrofurans, which do not occur in nature, are used clinically as antibacterials in human and veterinary medicine (Miura and Reckendorf, 1967). Furan compounds were first discovered in 1780 by Scheele, who obtained Furoic acid in the dry distillation of mucic acid. Furfural was discovered accidentally by Dobereiner (1832) in the course of preparing formic acid from sugar and manganese dioxide. Nitration of furfural was successfully accomplished in 1930, almost a century after its discovery. Several nitrated compounds were synthesized but the toxicity of these compounds prevented any further development until 1939 when a program was initiated by Eaton Laboratories (Norwich Pharmacal). This program led to the discovery of nitrofurazone by Dodd and Stillman in 1944, who made the important observation that a nitro group in the 5-position of 2-substituted furans conferred antibacterial activity on these compounds (Paul and Paul, 1964). A few years after the introduction of nitrofurazone, additional 5-nitrofurans were selected from several hundred synthesized compounds for use in human and veterinary medicine. Nitrofurantoin was introduced in 1952 for clinical trials as a urinary antibacterial; it was the first 5-nitrofuran recommended for peroral use. Many 5-nitrofurans have been introduced since the mid 1950s and their applications have been considerably expanded. In addition to antibacterial properties, certain

5-nitrofurans exhibit antifungal, antischistosomal, and antiprotozoan properties. On account of this broad spectrum of activity, 5-nitrofurans can keep animals disease-free and are used extensively in animal feeds as growth promoters (Federal Register, 1976). Residues of the 5-nitrofurans or their metabolites may therefore be present in tissues of animals having been fed such a diet. The Food and Drug Administration (FDA) issued a notice for hearing on a proposal to withdraw new drug applications for certain 5-nitrofurans in food producing animals (Federal Register, 1976). This action from the FDA was prompted by the finding in rats of oncogenic activity of certain 5-nitrofurans (e.g. nitrofurazone) and by the absence of comprehensive residue analysis of animals treated with the 5-nitrofurans.

Several 5-nitrofurans (e.g. furylfuramide) were, until 1974, used as food preservatives in Japan (Tazima et al., 1975). The strong mutagenic and carcinogenic activity of many 5-nitrofurans led to concern over their use as food preservatives and to a re-evaluation of their risk vs. benefit ratios. Because furylfuramide demonstrated mutagenicity and carcinogenicity, its use as a food preservative was withdrawn from the Japanese market (Tazima et al., 1975). Other 5-nitrofurans are used as pesticides (Tazima et al., 1975). More recently, 5-nitrofurans have been used as radio-sensitizers to enhance the efficacy of the radiotherapy of solid tumors (Reuvers et al., 1972; Chapman et al., 1974).

Nitrofurantoin has been used in various clinical conditions, but its primary use is for infections of the genitourinary tract. These conditions include pyelonephritis, pyelitis, cystitis, prostatitis, epididymitis, seminal vesiculitis and prophylactic use after cystoscopy and surgical procedures. At present, the major use of nitrofurantoin is for the treatment of pyelonephritis, pyelitis, and cystitis by oral administration.

Having a broad spectrum of activity, nitrofurantoin is effective against most strains of Escherichia coli, Staphylococcus aureus, and Enterococci. The sensitivity to nitrofurantoin of 2,154 strains of bacteria which were isolated from the urine of patients with urinary tract infections has been reviewed by Miura and Reckendorf (Miura and Reckendorf, 1967) and is summarized in Table I.1.

The long term use of nitrofurantoin has been associated with lung disease characterized by acute respiratory distress, interstitial pneumonitis and in some cases pulmonary fibrosis (Israel and Diamond, 1962; Hailey et al., 1969; Rosenow, 1972; Bone et al., 1976; Bottiger and Westerholm, 1977). Several cases of hepatitis (Strömberg and Wengle, 1976; Engel et al., 1975) and the development of polyneuropathies (Toole and Parrish, 1973; Jarknowitz et al., 1977) have been reported in occasional patients. Hyperlactatemia and hemolytic anemia have also been observed in patients with glucose-6-phosphate dehydrogenase deficiency (Lavelle et al., 1976).

In addition to this spectrum of clinical toxicity, nitrofurantoin has been shown to be mutagenic in Salmonella typhimurium tester strains TA-98 and TA-100 (Wang et al., 1975; Yahagi et al., 1976; Goodman et al., 1977), and to cause single-strand breaks in mammalian cells DNA in vitro (Olive and McCalla, 1975) or in bacteria (McCalla et al., 1971a). Nitrofurantoin has not, however, been shown to be carcinogenic in the rat when tested under conditions which detected carcinogenicity in other nitrofuran derivatives (Morris et al., 1969; Cohen et al., 1973).

The reductive metabolism of the aromatic nitro group to reactive intermediates has been implicated in the mediation of the toxicities of nitrofurantoin (Olive and McCalla, 1977; Bennett et al., 1974). Nitro reduction has been shown to be essential for the single-strand breaks in DNA (Olive and McCalla, 1977), mutagenesis (Rosenkranz and Speck, 1976), and the alkylation of bacterial (McCalla et al., 1971a) or pulmonary and hepatic protein (Boyd and Osborne, 1975). The products of the metabolic reduction of nitrofurantoin however, have not been definitively identified.

This study delineates two areas of investigation relating to 5-nitrofurans in general and nitrofurantoin in particular. The first area of research dealt with methods to improve the level of detection of 5-nitrofurans in biological fluids for clinical monitoring and

pharmacokinetic/bioavailability studies as well as for the analysis of residues of 5-nitrofurans present in animal tissues. The second area of research was designed to characterize the reductive metabolism of nitrofurantoin by various rat tissues as well as to isolate, purify and characterize the major metabolites of nitrofurantoin.

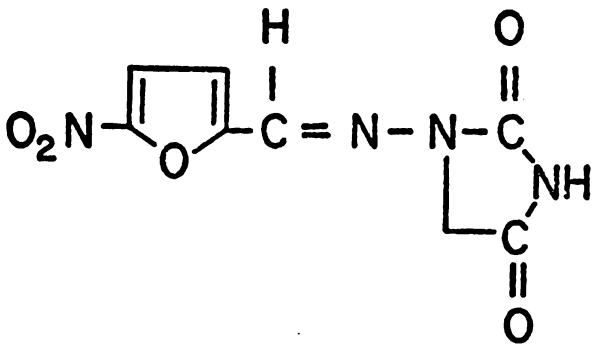
Table I.1

SENSITIVITY OF BACTERIA TO NITROFURANTOIN.

Microorganism	Sensitivity (%)			
	Legler (1962)	Groth-uesmann (1963)	Thompson and Rae (1964)	Naumann (1964)
Escherichia coli	96	78	91	92
Aerobacter aerogenes	83			
Proteus vulgaris	27	27	72	71
Enterococci	99	81	94	99
Staphylococcus aureus	92	84	100	
Staphylococcus albus	99			

II. PHYSICAL PROPERTIES OF NITROFURANTOIN

Nitrofurantoin is 1-[[(5-nitro-2-furanyl)methylene] amino]-2,4-imidazolidinedione. Additional names commonly encountered are: 1-[(5-nitrofurfurylidene)amino]hydantoin and N-(5-nitro-2-furfurylidene)-1-aminohydantoin. The most frequently used trade names are Furadantin• (Eaton) and Macrodantin• (Eaton).



Nitrofurantoin

The empirical formula for nitrofurantoin is $C_8H_6N_4O_5$ and its molecular weight is 238.16. Nitrofurantoin is odorless and appears as light yellow crystals.

A. Melting range

Nitrofurantoin (Sigma Chem. Co., St. Louis, MO 63178), Lot No. 100C-1300) melts at 260-262°C.

B. Ultraviolet absorption spectra

The ultraviolet (UV) absorption spectrum of a 10.0 μg/ml nitrofurantoin solution in distilled water is given in Fig. II.1. This spectrum was recorded with a Shimadzu model MPS-50L double-beam recording spectrophotometer using distilled water in the reference cuvette. The maximum absorption frequencies (λ_{max}) obtained for nitrofurantoin in various solvents were: dimethylformamide (380 and 270 nm), 0.01 N H_2SO_4 (365 and 265 nm), and 0.01 N NaOH (390 and 280 nm).

In distilled water, values of E(1%, 1cm) at 367 and 265 nm have been reported as 760 and 540, respectively (Cadwallader and Jun, 1976). The molar extinction coefficients (ε) at 367 and 265 nm are calculated to be 12,000 and 16,000, respectively, whereas the reported values are 13,100 and 17,300, respectively (Cadwallader and Jun, 1976). The effect of pH on the λ_{max} of nitrofurantoin is shown in Fig. II.2, KH_2PO_4-NaOH and boric acid-NaOH buffer systems were used. The spectral shift observed with alkalinization of the solution is reversible upon reacidification (Cadwallader and Jun, 1976).

C. Infrared absorption spectrum

The infrared (IR) spectrum of nitrofurantoin in a mineral oil mull is shown in Fig. II.3. The spectrum was obtained on a Perkin-Elmer model 457 grading infrared spectrophotometer. The band assignments are as follows:

3300 cm^{-1} (hydantoin NH stretch), 1750-1710 cm^{-1} (hydantoin C=O stretch), 1550 cm^{-1} (nitro), 1245 cm^{-1} (furan C-O-C), 1020 and 960 cm^{-1} (2,5-disubstituted furan).

D. Nuclear magnetic resonance spectrum

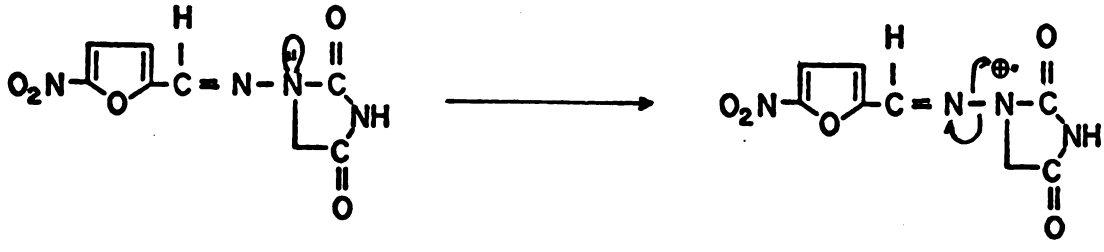
The proton nuclear magnetic resonance (NMR) spectrum of nitrofurantoin in D_2O (99.8 atom % D, Aldrich, gold label, Milwaukee, WI 53233) is shown in Fig. II.4. The NMR spectrum was obtained with a Varian XL-100 Fourier transform NMR spectrometer equipped with a Nicolet Instrument Corp. model TT-100 accessory. One thousand pulses (pulse width = 5 μsec, acquisition time = 3.4 sec, acquisition delay = 400 μsec) were used for the Fourier transform. The spectral assignments are presented in Table II.1.

E. Electron ionization mass spectrum

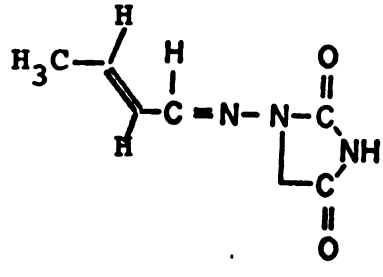
Approximately 0.1-0.2 mg of nitrofurantoin was used for electron ionization (EI) mass spectrometric analysis as a direct probe sample. EI mass spectra were taken on an Associated Electrical Industries model MS 12 mass spectrometer. Sample probe temperatures ranged from 20 to 300°C and the voltage of the ionizing electron beam was maintained at 70 eV.

The EI mass spectrum of nitrofurantoin is shown in Fig. II.5. The percent relative abundances and nominal molecular weights (m/e) for the molecular ion ($M^{+\cdot}$) and the various

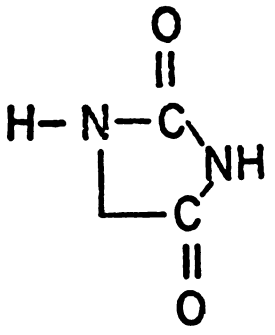
fragments are shown in Table II.2. The mass spectrum of nitrofurantoin is interpreted in terms of the fragments which result from ions where the site of electron deficiency and positive charge have been assumed to be on nonbonding electron orbitals of heteroatoms.



The homolytic fission of a bond adjacent to the positive charge provides a means of pairing the odd electron to form an even-electron ion, retaining the positive charge, which will be detected in the mass spectrometer. The following fragments are postulated:



at m/e = 167;



at m/e = 100;

and nitrofurantoin, m/e = 238. We attribute the high relative abundance of the 2,4-imidazolidinedione (m/e = 100) to the generally higher stability of molecular ions derived from cyclic compounds as opposed to aliphatic compounds.

F. Solubility

The reported solubility values for nitrofurantoin are presented in Table II.3 (Merck, 1976; Cadwallader and Jun 1976).

G. Stability

Nitrofurantoin in its crystalline form and in solution loses its yellow color, and decomposes upon exposure to light and contact with metals other than stainless steel and aluminum. Due to this photosensitivity, storage, incubation,

and analytical procedures with nitrofurantoin must be carried out under subdued light. All nitrofurantoin solutions (e.g. in water, plasma, or urine) prepared in our work, were stable for more than one month when stored in air-tight containers which were protected from the light.

Table II.1

^{1}H-NMR SPECTRAL ASSIGNMENTS FOR NITROFURANTOIN

Bond (δ,ppm)		Assignment
singlet	4.40	hydantoin $-CH_2-$
singlet	4.70	HDO (solvent)
doublet	7.10	furan 3H
doublet	7.60	furan 4H
singlet	7.85	-CH=N- azomethine proton

Table II.2

ELECTRON IONIZATION MASS SPECTRAL DATA FOR NITROFURANTOIN

m/e	(% relative abundance) of peaks above m/e = 60
79	(30)
100	(100)
167	(30)
238	(30)

Table II.3

SOLUBILITY VALUES FOR NITROFURANTOIN

Unless specified, ambient temperature is assumed.

Solvent		Solubility (mg/liter)
water	(pH 7, 25°C)	190
	(pH 7, 37°C)	312
ethanol	47.5%	185
	70%	712
	95%	510
peanut oil		21
glycerol		600
propylene glycol	20%	1,560
acetone		5,100
polyethylene glycol 300		15,100
dimethylformamide		80,000

Fig. II. 1 ULTRAVIOLET ABSORPTION SPECTRUM OF NITROFURANTOIN IN DISTILLED WATER.

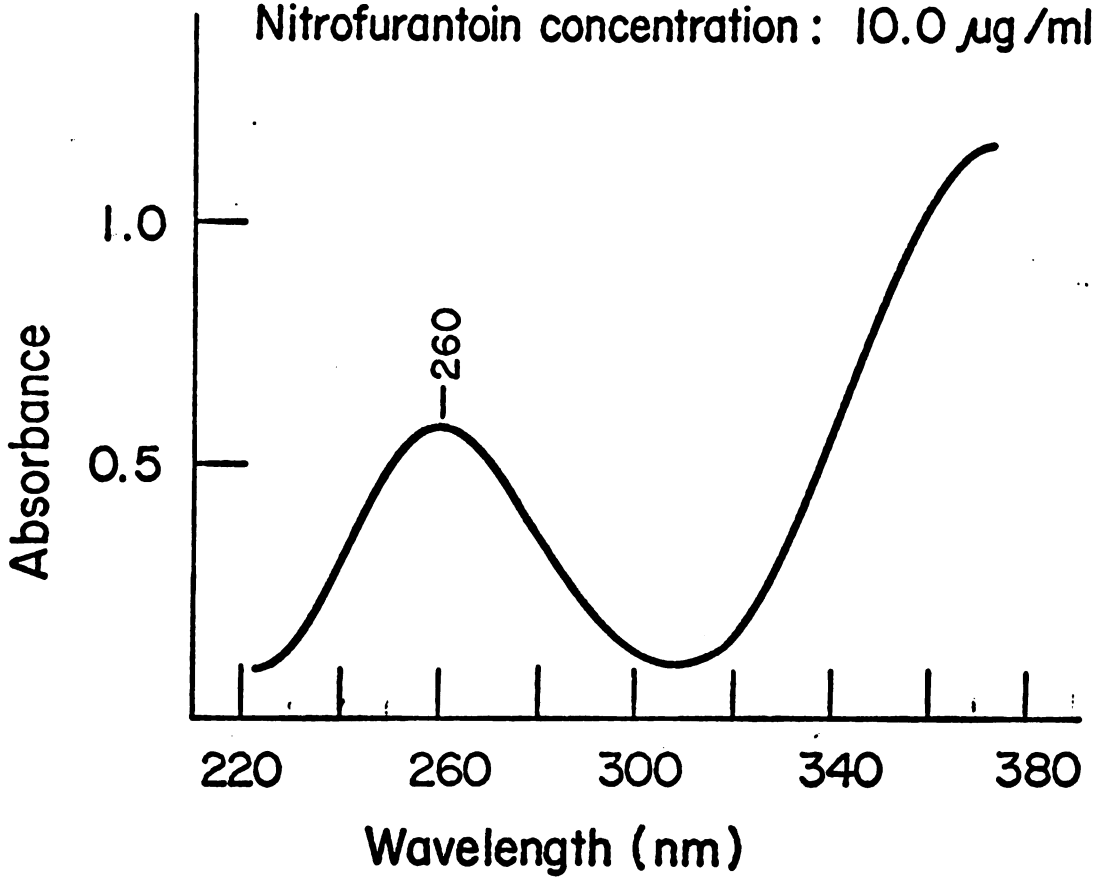


FIG. II.2 EFFECT OF pH ON THE ULTRAVIOLET MAXIMUM ABSORPTION FREQUENCY OF NITROFURANTOIN

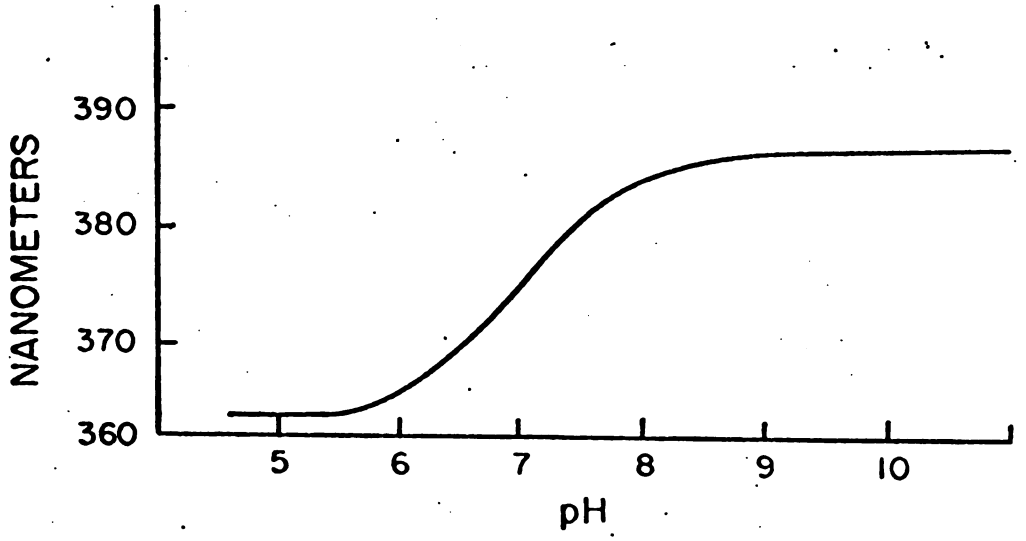


FIG. II.3 INFRARED ABSORPTION SPECTRUM OF NITROFURANTOIN

(Perkin Elmer model 457)

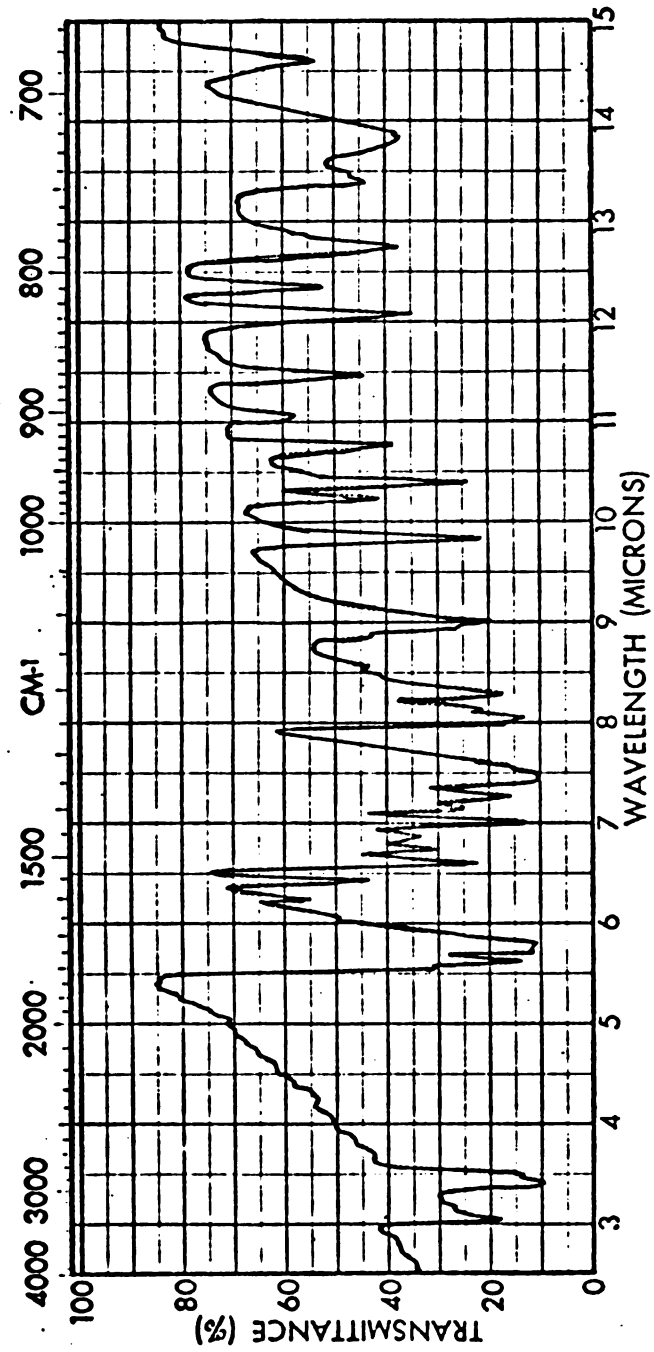


FIG. II.4 ^{1}H-NMR SPECTRUM OF NITROFURANTOIN in D_2O

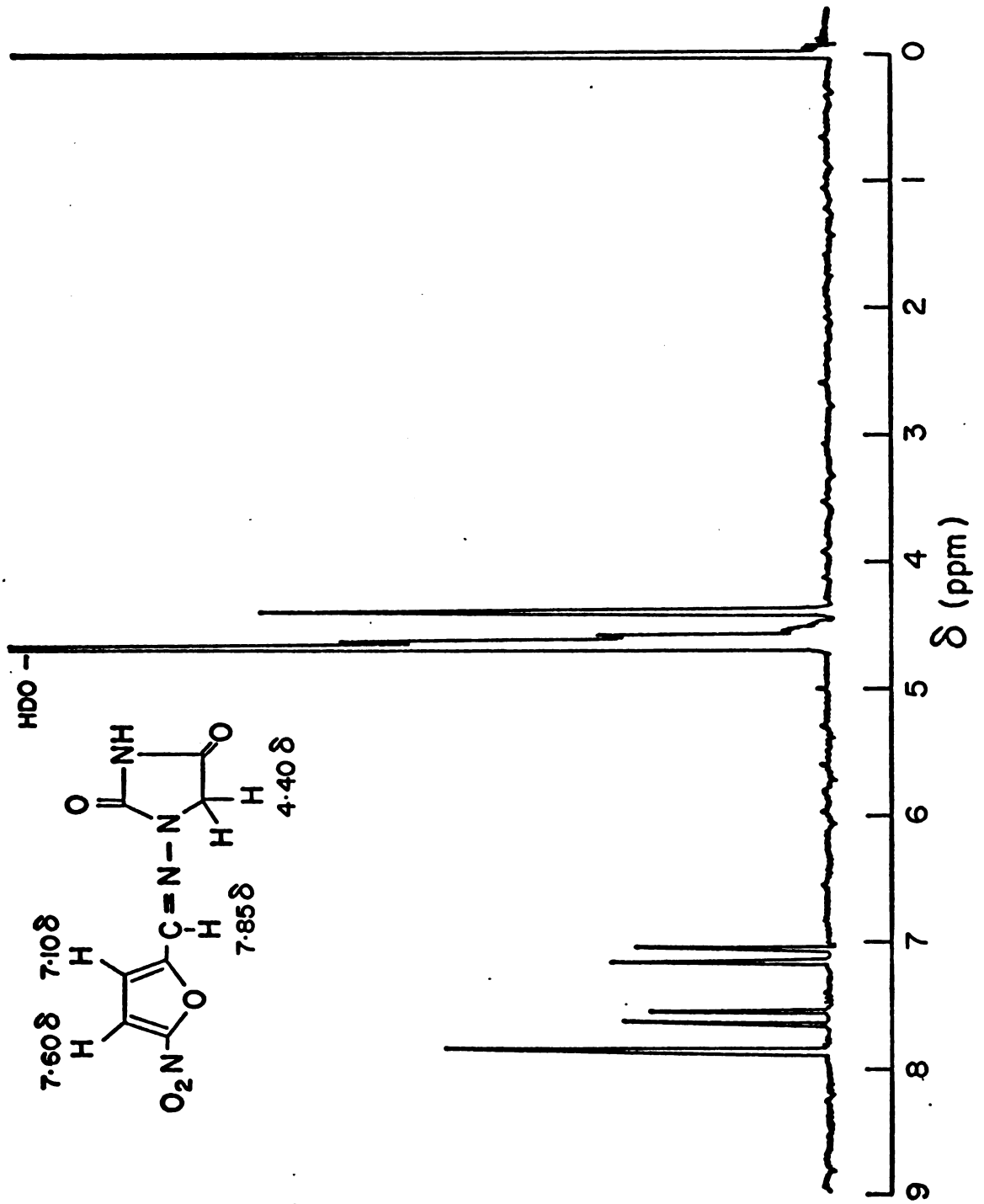
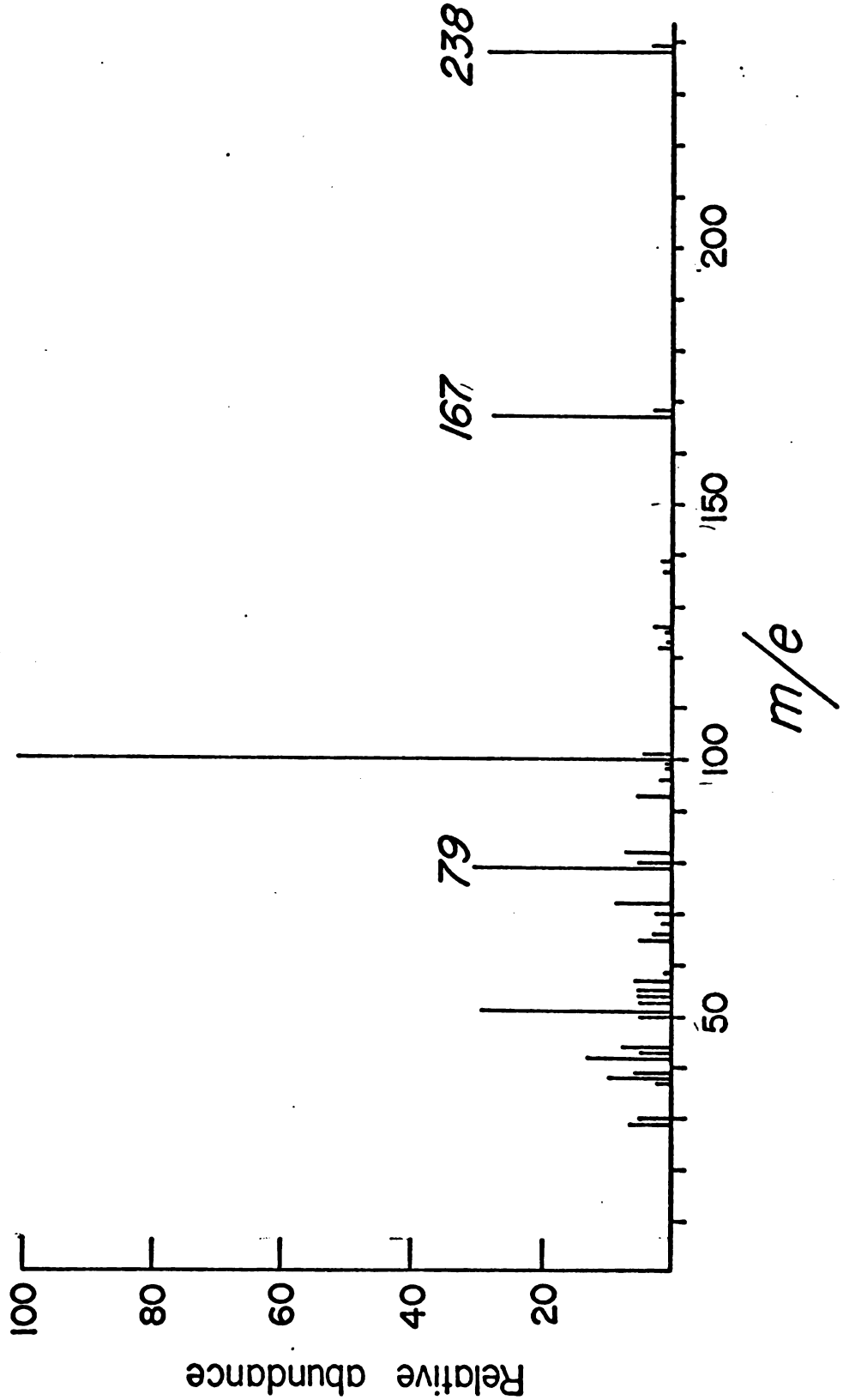


Fig. II·5 ELECTRON IONIZATION MASS SPECTRUM OF NITROFURANTOIN



III. ANALYTICAL STUDIES ON NITROFURANTOIN

A. Introduction

Quantitative determinations of nitrofurantoin in biological samples were first performed by Paul et al. (Paul et al., 1949). The method, based on the ultraviolet absorption characteristics of nitrofurantoin, was used to determine drug concentrations in rat urine following ether extraction. The absorption maximum was, however, pH dependent (Stoll et al., 1973). The method which has been established as the preferred assay for nitrofurantoin is the Hyamine 10X procedure developed by Conklin and Hollifield in 1965 (Conklin and Hollifield, 1965). This method is described in details in Section III.B. Various other methods have been reported among which is a polarographic procedure for the determination of nitrofurantoin in urine; the sensitivity limit of this assay is, however, 1 µg/ml (Jones et al., 1965). A microbiological procedure was also developed for the assay of nitrofurantoin in urine (Jones et al., 1965). A turbidimetric procedure for nitrofurantoin and other 5-nitrofuran derivatives in serum and urine samples was developed using Streptococcus faecalis as an indicator organism (Gang and Shaikh, 1972). The turbidimetric method lacks specificity since furazolidone was found to interfere. Moreover, the limit of the sensitivity of the method is 0.4 µg/ml. A colorimetric assay has also been reported for the analysis of

nitrofurantoin in plasma or serum of rats. This and other colorimetric methods involve conversion of the 5-nitrofuran derivatives to 5-nitrofurfuraldehyde phenylhydrazone, followed by solvent extraction and concentration by column chromatography (Buzard et al., 1956). These colorimetric techniques are elaborate and lack specificity.

The problems inherent in the existing methods were therefore two-fold, low sensitivity and lack of specificity. The poor sensitivity resulted in lack of pharmacokinetic data available since the peak concentration of nitrofurantoin which might be expected in blood after a therapeutic dose of 100 mg p.o, is 6 µg/ml assuming instantaneous absorption and rapid and uniform distribution of about 40% of the dose (Mattok et al., 1970). The actual concentrations encountered clinically range between 0.5 and 1.5 µg/ml. The noted exception to the sensitivity problem is the Hyamine 10X method as modified by Mattok et al. providing a sensitivity limit greater than 0.2 µg/ml (Mattok, et al., 1970). The lack of specificity on the other hand rendered all of the above methods unsuitable for metabolic studies since most 5-nitrofuran derivatives were found to interfere and all compounds sharing the 5-nitrofuran moiety would be detected as a single entity. It was, therefore, necessary to develop a method optimizing both sensitivity and specificity for the determination of nitrofurantoin as well as its metabolites.

B. Hyamine 10X assay

The preferred method of analysis of nitrofurantoin in biological fluids has been the Hyamine 10X assay. The method requires nitromethane extraction of nitrofurantoin from the biological fluid, followed by the formation of a yellow complex upon addition of the quaternary ammonium compound Hyamine 10X hydroxide. The concentration of nitrofurantoin is then determined spectrophotometrically from the absorbance of the complex at 400 nm (Conklin and Hollifield, 1965). This original method, which has a sensitivity of 5 µg/ml, was modified to assay the drug in whole blood or plasma and the sensitivity limit was increased to 2 µg/ml with recoveries of 95-100% (Conklin and Hollifield, 1966a).

However, this method was still inadequate for the quantitative determination of nitrofurantoin in blood of subjects who had received normal therapeutic doses. The Hyamine 10X method for the determination of nitrofurantoin in blood samples was further improved by Mattok et al. in that smaller volumes were required (0.8 ml) and the sensitivity was greater (0.2 µg/ml) (Mattok et al., 1970). The following assay is a modification of the Mattok method to suit our needs for the quantitation of nitrofurantoin from biological fluids and metabolic incubation mixtures.

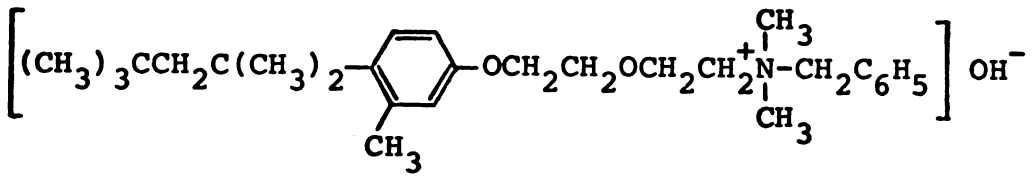
Materials and Methods

A stock reference solution of nitrofurantoin (100 µg/ml) was prepared by dissolving 10 mg of nitrofurantoin (Sigma Chem. Co.) in 5 ml N,N-dimethylformamide (Aldrich Chem. Co., Spectrophotometric grade) and diluted to 100 ml with distilled water in a volumetric flask. A range of standard nitrofurantoin solution (0.5 - 50 µg/ml) was prepared by diluting aliquots of the stock solution with the necessary volume of distilled water.

A stock solution of nitrofurantoin in plasma (20 µg/ml) was prepared using human plasma (AB type from an untransfused male donor; Blood Bank of the University of California, San Francisco). Nitrofurantoin plasma solutions (10, 5, 1, and 0.5 µg/ml) were obtained by diluting the plasma stock solution with an appropriate volume of plasma. The solutions in plasma were stable for at least a month when kept at 0-4°C.

Standard curves were obtained by treating solutions of nitrofurantoin (0.5 - 50 µg/ml in distilled water and 0.5 - 20 µg/ml in plasma) in the following manner. In a centrifuge tube, 0.8 ml of nitrofurantoin solution was mixed for 20 sec with 2.0 ml of nitromethane (Aldrich Chem. Co., Spectrophotometric grade). The tube was centrifuged at 1500 xg for 10 min and the aqueous upper layer discarded using a Pasteur pipette. A 1.5 ml aliquot of the clear nitromethane layer was

then transferred to a test tube and mixed with 0.15 ml of 0.01 M methanolic Hyamine 10X hydroxide (Benzyldimethyl[2-[2-(p-1,1,3,3-tetramethylbutylcresoxy) ethoxy]ethyl]ammonium hydroxide; ICN Chemical and Radioisotope Div., Irvine, CA 92715, Lot No. 2079.



Hyamine 10X hydroxide

After one minute the absorbance was read at 400 nm with a double-beam Shimadzu recording spectrophotometer model MPS-50L using nitromethane in the reference cuvette. The concentration of nitrofurantoin-Hyamine 10X complex in samples was then determined by comparison with absorbances from the standard curves.

Results

The standard curve for the nitrofurantoin-Hyamine 10X complex is shown in Fig. III.1. The regression line for this curve is $y = (0.033 \pm 0.001)x + (0.002 \pm 0.003)$. Beer's law was obeyed for nitrofurantoin concentrations up to 30 µg/ml.

If the nitrofurantoin samples were allowed to stay at room temperature for periods of one hour or longer after addition of the Hyamine 10X, a progressive increase in absorbance at 400 nm was observed. In the absence of nitrofurantoin, the addition of Hyamine 10X to nitromethane also produced such an increase in absorbance which, as discussed by Harrison et al. resulted from the formation of methazonic acid (O_2N-CH_2-CH=N-OH) from these reagents (Harrison et al., 1973).

Despite the reported specificity of the Hyamine 10X method, (Conklin and Hollifield, 1966b) other nitrofuran analogs (e.g. furazolidone) also formed a Hyamine 10X complex as readily as did nitrofurantoin. The absorbance of the Hyamine 10X complex formed was linearly related with the concentration of the 5-nitrofuran analog used. We concluded that the Hyamine 10X assay was not suited to our needs as a method to identify both nitrofurantoin and its metabolites. Since the Hyamine 10X method could not provide the necessary specificity and since encouraging results had been obtained with gas liquid chromatographic analyses of hydantoin derivatives (Kupferberg, 1970) we attempted to use gas liquid chromatography (GLC) for the quantitation of nitrofurantoin and its metabolites.

C. Gas Liquid Chromatography

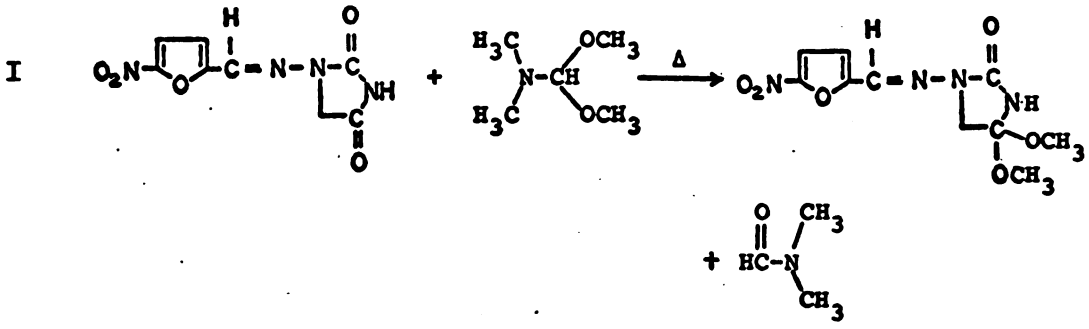
Materials and Methods

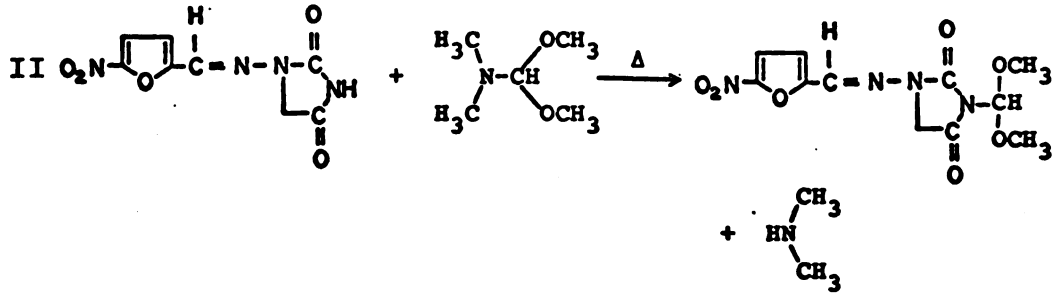
Experimental conditions for analysis:

A Varian model 2440 (Varian Aerograph, Walnut Creek, CA) equipped with a Perkin-Elmer Hitachi recorder, model 156 was used for gas liquid chromatographic analysis. A flame-ionization detector was used in all determinations. Various glass coiled columns, 1.83 m (6 ft) x 0.63 cm (0.25 in) o.d., were used. Most work was done with a column packed with either 3% OV-1 on 100-120 mesh Gas-Chrom Q, 3% OV-17 on Chromasorb W HP 8-100-mesh, or 3% SE-30 on Gas-Chrom Q (Applied Science Laboratories, State College, PA). Columns were conditioned at 260°C for 40 hr and injected periodically with trimethylsilyl donors (Silyl 8, Pierce Chemical Co., Rockford, IL). Nitrogen as a carrier gas was maintained at a flow rate of 30 ml/min, while the flow rates for air and hydrogen were 250 and 24 ml/min, respectively. The injector temperature was varied between 190 and 215°C while the detector temperature was maintained at 230°C. Column temperatures were usually programmed to vary from 100° to 200°C.

Attempted methylation with dimethylformamide dimethyl acetal:

We attempted to alkylate nitrofurantoin with dimethylformamide dimethyl acetal concentrate (Methyl-8™ concentrate, Pierce Chemical Co., Rockford, IL) according to the following possible schemes.





Dimethylformamide dialkyl acetals are useful methylating reagents because of the rapidity of derivatization. The reactions are generally complete

as soon as solution is achieved. Scheme II was proposed by Venturella et al. for the methylation of barbiturates (Venturella et al., 1973). In such cases, the mechanism of methylation using dimethylformamide dimethyl acetal depends on the presence of a rigid keto tautomer to prevent the slower rate reaction of the reagent with the enolic form.

Methyl-8™ (300 µl) in a dry syringe was added to 50 mg of anhydrous nitrofurantoin in a 5 ml vial (Reacti-Vial, Pierce Chemical Co., Rockford, IL). Alternatively, 50 mg of nitrofurantoin was dissolved in 300 µl of nitromethane to which 300 µl of Methyl-8™ concentrate was added. The vial was capped and allowed to stand at 60°C for 15 min, the initial straw color present upon the addition of Methyl-8™ quickly changed to a dark brown solution. Aliquots of the reaction mixture were then injected into the gas chromatograph.

Attempted flash-heater methylation with Hyamine 10X hydroxide:

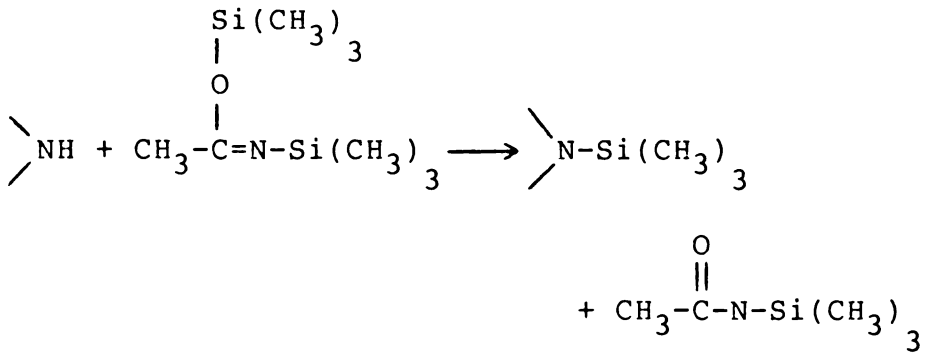
The second experiment consisted of attempts at on-column methylation of nitrofurantoin by the thermal elimination of a trialkylamine moiety from the tetralkylammonium salt Hyamine 10X hydroxide. Flash heater methylation of barbiturates (Brochmann-Hanssen, 1969) and phenytoin (Kupferberg, 1970) have been successfully accomplished with trimethylanilinium

hydroxide, and this method of derivatization employing an alkylammonium salt is now used routinely for urine and serum analyses of barbiturates and related drugs. On column methylation using the vaporizer of the gas-liquid chromatograph as a reactor presents the advantage of having no isolation procedures since the products are swept into the column.

To 50 mg of nitrofurantoin in 2 ml of benzene was added 0.5 ml of methanolic Hyamine 10X hydroxide (1 M, ICN Chemical and Radioisotope Div., Irvine, CA). After shaking for 30 sec and centrifugation for 1 min, aliquots of the methanolic phase (lower phase) were injected into the gas chromatograph.

Attempted silylation with N,O-bis-(trimethylsilyl)-acetamide:

A trimethylsilyl group can be introduced into a variety of organic compounds including imides. The replacement of active hydrogen by the silyl group reduces the polarity of the compound and diminishes the possibility of hydrogen bonding, with the resultant increase in volatility. In addition, stability is usualy enhanced by reduction of the number of reactive sites. The following silylation scheme was attempted:



To 5 mg of dry nitrofurantoin in a 5 ml Reacti-Vial was added 250 µl of N,O-bis-(trimethylsilyl)-acetamide (BSA, Pierce Chemical Co.). The vial was capped and heated at 60°C for 15 min. Alternatively, nitrofurantoin (5 mg) was dissolved in 250 µl of a solution of 50% BSA containing 1% trimethylchlorosilane as a catalyst in silation grade pyridine (Pierce Chemical Co.), and the reaction mixture allowed to stand for 15 min at room temperature. The reaction mixtures were then injected into the gas chromatograph.

Attempted acetylation with heptafluorobutyric acid anhydride:

Direct acetylation is usually possible for compounds having an alcoholic or phenolic group or for primary or secondary amines. Derivatization of amides via perfluoroacylation has also been described (Ehrsson and Mellstroem, 1972).

To 500 µl benzene containing 500 µg nitrofurantoin in a 5 ml Reacti-Vial, was added 300 µl of a 0.05 M trimethylamine in benzene solution. The use of

trimethylamine as a catalyst usually gives quantitative acylation reactions in a few minutes. The anhydride (25 µl of heptafluorobutyric acid anhydride, Pierce Chemical Co.) was then added to the Reacti-Vial mixture, the vial capped and heated for 20 min at 50°C. After cooling to room temperature, 1 ml of water was added and the vial shaken for one min. Aqueous ammonia (1.5 ml of a 5% solution) was added and shaking was continued for 5 min. The addition of water hydrolyzes the anhydride and the aqueous ammonia removes the heptafluorobutyric acid from the benzene phase into the aqueous phase to avoid any interference from this reagent during gas chromatography. After centrifugation, aliquots of the benzene phase were injected into the gas chromatograph.

Results

Direct injection of nitrofurantoin in a variety of solvents (e.g. nitromethane, DMSO) onto the GLC did not provide reproducible results under the conditions given. Direct GLC analysis of 60 nitrofuran derivatives, including nitrofurantoin, had previously been reported (Nakamura et al., 1966). However, we were unable to duplicate these experiments when using a similar method. The experimental conditions reported by Nakamura et al. include column and detector temperatures of 210°C and an injector temperature of

300°C. Since the melting point for nitrofurantoin (decomposition) occurs in the range 260 -262°C, in our hands the use of such high injector temperatures (300°C) resulted in the observation of pyrolysis products. A lowering of the injector temperature to 260°C and below resulted in the accumulation of nitrofurantoin on the column inlet. In order to increase the vapor pressure and improve the column behavior of nitrofurantoin, we attempted various derivatizations including methylation with dimethylformamide dimethyl acetal and Hyamine 10X hydroxide, silylation with N,O-bis-(trimethylsilyl)-acetamide, and acetylation with heptafluorobutyric acid anhydride. None of the derivatization schemes attempted resulted in a promising method for the GLC analysis of nitrofurantoin and its metabolites. When reproducible peaks were present, tailing and peak asymmetry rendered the method unsuitable for quantitation of nitrofurantoin and its possible metabolites. In most cases, decomposition appeared to be the major problem, leading to lack of reproducibility.

A second GLC method had been reported for the detection of residues of 5-nitrofurans in animal tissues (Frahm, 1975; Ryan, 1975). This method involves the hydrolysis of 5-nitrofurans to 5-nitro-2-furaldehyde in 4 N aqueous HCl at 70°C followed by extraction of

the 5-nitro-2-furaldehyde with benzene. The disadvantage of this procedure is its lack of specificity for any particular nitrofuran since the hydrolysis of all 5-nitrofurans containing a 2-azomethine side chain will form a 5-nitro-2-furaldehyde. Moreover, since our ultimate use of the gas chromatographic method was to identify nitrofurantoin metabolites, there would be no way of determining whether the 5-nitro-2-furaldehyde was generated by the hydrolysis of the parent compound or the hydrolysis of a metabolite in which the 5-nitrofuran moiety was still intact. We concluded that this hydrolysis method was not suited for our purpose and that in general GLC did not appear promising for the analysis of nitrofurantoin and its metabolites.

D. High-Pressure Liquid Chromatography

Materials and Methods

Instrumentation:

High-pressure liquid chromatography (HPLC) is a recently developed technique based on classical column liquid chromatography. HPLC appeared to be a suitable method for the rapid and sensitive quantitation of 5-nitrofurans in biological fluid without prior derivatization and/or extraction. All our work with 5-nitrofurans was performed with reverse-phase HPLC which signifies that the compounds are separated on the

basis of differential partition between the following two phases: an hydrophobic column packing (μBondapak C_{18}) and an hydrophilic solvent. Each compound will have a particular affinity for the non-polar hydrocarbon phase of the column and for the mobile phase. Elution characteristics can be changed (1) by changing the affinity of the compound for the stationary phase by making the compound more or less hydrophobic, e.g. via a change in the state of ionization of the compound by altering the pH of the solvent or (2) by altering affinity for the solvent, e.g. via changes in ionic strength or polarity of the solvent. For instance, a decrease in the polarity of an aqueous solvent by the addition of methanol or acetonitrile displaces the molecules from the stationary phase by competition or mass action effects.

A high-pressure liquid chromatograph (Model ALC/GPC 244, Waters Associates, Inc. Milford, MA 01757) was used. This model is characterized by a dual reciprocating pumping system consisting of two especially driven positive-displacement heads. Flow-rates are set by digital dials in increments of 0.1 ml/min to 9.9 ml/min at pressures ranging from 0 to 420 kg/cm^2. Solvent delivery is controlled by varying the frequency of the piston at full chamber discharge. Pulseless flow is obtained by superimposing the profiles of each piston so that one compensates for the other. The

pressure developed in the system is monitored with a flow-through sensor. The safe pressure limits can be set between 8 and 420 kg/cm^2. A constant volume can be delivered by the pump in spite of changes in flow resistance arising from changes in viscosity, density or line restriction. Internal control circuits maintain the flow-rates independent of fluctuations in line voltage or frequency. Fig. III.2 shows a diagram of the Waters liquid chromatograph with two pumps for gradient elution. When used in the isocratic (constant solvent composition) mode, only one pump is utilized.

The HPLC model includes a U6-K universal injector and a dual channel UV absorption detector. The instrument was fitted with a 30 cm x 3.9 mm i.d. μBondapak C_{18} reverse-phase column, consisting of a monomolecular layer of octadecyl trichlorosilane (10%) chemically bonded to silica micro-particles of 10 μm diameter (Water Associates). The chromatograph was operated isocratically at a flow rate of 2.0 ml/min at ambient temperature. The wavelength of detection was fixed at 365 nm. A dual-pen recorder was used (OmniScribe Model A5211-1, Houston Instruments, Austin, TX 78753).

Reagents:

Chemicals.--The purity of nitrofurantoin (Sigma Chem. Co., St. Louis, MO 63178) was assessed from

melting point (260-262°C), infra-red on NaCl disk (1343, 1435, 1525, 1724, 1785 cm^{-1}), UV (max_{H_2O} = 370, 266 nm; min_{H_2O} = 307 nm), and thin layer chromatography on Uniplate silica gel GF 250 μm (Analtech Inc., Newark, DE 19711) developed in ether: acetone (3:2) or benzene:acetone (3:2). Powdered samples of nitrofurazone and furazolidone were used as obtained (Eaton Laboratories, Morton-Norwich Products, Norwich, NY 13815, Lot Nos. P6335 and S3071, respectively).

The chromatographic solvents, methanol (glass distilled, Burdick and Jackson Lab., Muskegon, MI 49442) and distilled water (glass redistilled and stored in glass were filtered through a 0.45 μm filter prior to use (Catalog No. HAWPO 4700 and FHLPO 4700, Millipore Corp., Bedford, MA 01730). Spectrophotometric grade dimethyl sulfoxide and nitromethane (Aldrich Chem. Co., Milwaukee, WI 53233) were used as received. Methanolic Hyamine 10X (0.01 mole/liter) was prepared by dilution of a 1.0 mole/ liter solution (ICN Chemical and Radioisotope Div., Lot No., 2079).

Mobile Phase.--The mobile phase consisted of 20:80 methanol:0.01 mole/liter Na acetate pH 5.0. It was prepared by mixing 800 ml of water with 200 ml methanol. After addition of 0.6 ml glacial acetic acid, the pH of the solution was adjusted to 5.0 using

4 N NaOH. The mobile phase was degassed before use by applying a vacuum to the stirred solvent.

Biological Fluids.--Human plasma (AB type from an untransfused male donor) which had been stored frozen at -20°C for 4 weeks was obtained from the blood bank of the University of California, San Francisco. The plasma was thawed and allowed to come to room temperature before use. Human urine from a male donor was collected fresh daily.

Patient urine was obtained from the collection bag of a catheterized male patient receiving nitrofurantoin. Blood, from the same patient, was collected in a heparinized tube (Vacutainer, Becton-Dickinson; Rutherford, NJ 07070). It was centrifuged at 3,000 xg for 4 min and the plasma fraction decanted. This plasma and the urine were stored at -20°C for 4 days prior to analysis.

Stock solutions:

Plasma.--A stock solution of nitrofurantoin (10 µg/ml) was prepared by placing 1 ml of a 100 µg/ml aqueous nitrofurantoin solution in a 10 ml volumetric flask and then diluting to 10 ml with plasma. The 10 µg/ml stock solution was serially diluted with plasma to obtain nitrofurantoin concentrations of 5, 2, 1 and 0.5 µg/ml.

Urine.--A stock solution containing 200 µg/ml of nitrofurantoin was prepared by dissolving 2 mg of nitrofurantoin in 10 ml of urine in a volumetric flask. The stock solution wrapped in foil was shaken overnight to ensure proper dissolution. Alternatively, solubility difficulties were avoided by dissolving 2 mg of nitrofurantoin in 0.5 ml of dimethyl sulfoxide in a 10 ml volumetric flask; the solution was then diluted to 10 ml with urine to yield a 200 µg/ml solution. This 200 µg/ml stock solution was serially diluted with urine to obtain nitrofurantoin concentrations of 100, 50, 20, and 10 µg/ml.

Internal Standards.--Two internal standard solutions of furazolidone were used in order to cover both urine and plasma ranges of nitrofurantoin and still retain useful nitrofurantoin to furazolidone peak height ratios. A 5 mg/ml furazolidone solution in DMSO was used as internal standard for urine samples. For the plasma samples, a 0.05 mg/ml furazolidone solution was used; it was prepared by dissolving 5 mg of furazolidone in 3 ml of DMSO in a 100 ml volumetric flask and diluting to 100 ml with double distilled water.

Procedures

Standard curves:

Plasma.--To 200 µl of the nitrofurantoin plasma solution was added 10 µl of 0.05 mg/ml furazolidone internal standard solution. The solution was shaken, and 300 µl of methanol added. The resulting solution was mixed thoroughly and transferred to a 4 ml centrifuge tube. After centrifugation for 15 min at 10,000 xg to precipitate the proteins, the supernatant was poured into a 4 ml glass tube and injected on the HPLC. Using a 100 µl syringe, injection volumes of 15 to 60 µl were satisfactory for the entire range of plasma concentrations when the sensitivity of the 365 nm detector was set at 0.01 absorbance units full scale. The chromatograph was operated at a flow rate of 2.0 ml/min at room temperature using 20:80 methanol: 0.01 mole/liter Na acetate pH 5.0 as the mobile phase. The retention times for nitrofurantoin and furazolidone were 6 and 8 minutes, respectively. The standard curves were constructed by plotting nitrofurantoin/ furazolidone peak height ratios versus nitrofurantoin concentrations.

Urine.--To 200 µl of the nitrofurantoin urine solution was added 3 µl of 5 mg/ml furazolidone internal standard solution. The solution was mixed thoroughly and injected directly on the HPLC. Using a 10 µl

syringe, injection volumes of 4 to 10 µl were satisfactory for the entire range of urine concentrations when the sensitivity of the 365 nm detector was set at 0.1 absorbance units full scale. The chromatographic conditions were identical to those described above for the plasma standard curves. The urine standard curves were constructed by plotting nitrofurantoin/furazolidone peak height ratios versus nitrofurantoin concentrations.

Stability studies:

Plasma.--Solutions of nitrofurantoin (10 and 1 µg/ml plasma) were prepared as described above. Each solution was divided into 20 vials each containing 200 µl of nitrofurantoin solution; the vials were capped and stored at -10°C, protected from light. Analyses were performed on 10 separate days over a period of 26 days: days 0 (not frozen), 1, 2, 4, 5, 7, 10, 15, 22 and 26. Two vials of each concentration were thawed on the day of analysis and 10 µl of 0.05 mg/ml furazolidone internal standard solution was added to each vial. The analyses were performed as described above for the plasma standard curves. On each day of analysis a plasma standard curve was constructed and the nitrofurantoin concentrations of the stability study solutions were determined by comparing their

nitrofurantoin/furazolidone peak height ratios with those of the standard curve.

Urine.--Solutions of nitrofurantoin (200 and 18 µg/ml urine) were prepared as described above. Each solution was divided into 36 vials each containing 200 µl of nitrofurantoin solution. The capped vials were stored protected from light, half of them at -10°C the remainder at room temperature. Analyses were performed on 9 separate days over a period of 22 days: days 0 (not frozen, 1, 2, 4, 5, 7, 10, 15 and 22). Two of the vials of each concentration were thawed on the day of analysis. Samples containing 200 µg/ml nitrofurantoin had crystallized upon freezing and in this case 200 µl of DMSO was added while thawing the solution. Samples which had been kept at room temperature were used directly. To each vial 3 µl of 5 mg/ml furazolidone internal standard solution were added, and the analyses were performed as described above for the urine standard curves. On each day of analysis a urine standard curve was constructed and the nitrofurantoin concentrations of the stability solutions was determined by comparing their nitrofurantoin/furazolidone peak height ratios with those of the standard curve.

Repeated injections:

A 2 µg/ml plasma solution and a 50 µg/ml urine solution of nitrofurantoin were prepared and analysis were performed as described above for the standard curves.

Effect of various solvents on the method:

Solutions of nitrofurantoin at concentrations of 100, 50, 20, 10 and 5 µg/ml were prepared in double-distilled water, DMSO, plasma and urine. The analyses were performed as described above for the urine standard curves except that with the plasma solutions, proteins were first precipitated with methanol prior to HPLC injection. Curves were constructed for each solvent by plotting nitrofurantoin/furazolidone peak height ratios versus nitrofurantoin concentrations.

Sensitivity:

Solutions of nitrofurantoin at concentrations of 0.4, 0.2, 0.1, 0.05, and 0.02 µg/ml in plasma were prepared. To 400 µl of the nitrofurantoin solution, 10 µl of a 5 µg/ml furazolidone internal standard solution was added. The solution was mixed and 600 µl of methanol added to precipitate the proteins. After thorough mixing, the solution was centrifuged for 15 min at 10,000 xg, and the supernatant poured into a 4 ml glass tube and injected on the HPLC.

Comparison with Hyamine 10X assay:

Solutions of nitrofurantoin at concentrations of 20, 10, 5, 1 and 0.5 µg/ml in plasma were prepared. Each solution was subjected to both the HPLC method described above for the standard curves and to the Hyamine 10X method (Conklin and Hollifield, 1966a; Mattok et al., 1970). With the use of standard curves obtained for each method, the actual concentration of the solutions was determined. A correlation curve was then obtained by plotting the nitrofurantoin concentrations obtained by the HPLC method against the nitrofurantoin concentrations obtained from the Hyamine 10X method.

Nitrofurazone plasma standard curve:

Solutions of nitrofurantoin at concentrations of 10, 5, 2, 1 and 0.5 µg/ml in plasma were prepared. To 200 µl of nitrofurazone solution was added 10 µl of 0.05 mg/ml furazolidone internal standard solution. The analyses were then performed as described above for the nitrofurantoin standard plasma curves, injecting five samples for each nitrofurazone concentration. The retention times for nitrofurazone and furazolidone were 7 and 8 minutes respectively. Concentration curves were constructed by plotting nitrofurazone/ furazolidone peak height ratios versus nitrofurazone concentrations.

Data handling:

With all curves, a straight-line fit of the data was made by least-square linear regression analysis using the PROPHET system. The PROPHET system is a specialized computer resource developed by the Chemical/ Biological Information Handling Program of the National Institutes of Health. The results of the regression analysis were expressed throughout in the following form: y = (slope of the line $\pm$ standard deviation of the slope) x + (intercept of the line $\pm$ standard deviation of the intercept).

Results

In human volunteers, a single 100 mg oral dose of nitrofurantoin results in nitrofurantoin blood concentrations near 1 µg/ml (Mattok et al., 1970). When administered intravenously or intramuscularly, blood concentrations of nitrofurantoin reach 5 µg/ml. On the other hand, maximum urinary nitrofurantoin concentrations of 158 to 372 µg/ml were reported in nine normal individuals, each of whom received a 100 mg tablet every 4 hr (Paul et al., 1967). Since plasma concentrations are several hundred times smaller than urine concentrations, two standard curves were used; the urine standard curve covers the range 10-200 µg/ml whereas the plasma standard curve covers the range 0.5-10 µg/ml nitrofurantoin. In addition, plasma

concentrations ranging from 0.02 to 0.4 μg/ml were used to test the sensitivity of the present method.

The standard curves were constructed by adding known amounts of nitrofurantoin and internal standard (furazolidone) to urine and plasma and plotting the peak height ratio of nitrofurantoin to furazolidone ($\underline{y}$) against the concentration of nitrofurantoin in μg/ml ($\underline{x}$) (Fig. III.3). Over a period of 26 days, ten plasma standard curves were constructed each with five nitrofurantoin concentrations. With 50 points, the regression line for plasma was $\underline{y} = (0.469 \pm 0.009)\ \underline{x} + (0.097 \pm 0.047)$, with a coefficient of variation (CV) of the slope of 1.9% and a correlation coefficient of 0.99. For urine, over a period of 22 days, nine standard curves were constructed each with five nitrofurantoin concentrations. With 45 points, the regression line for urine was $\underline{y} = (0.0176 \pm 0.0001)\ \underline{x} + (0.0127 \pm 0.0165)$ with a CV of the slope of 0.6% and a correlation coefficient of 0.99.

Stability studies were performed over a period of 26 days for plasma and 22 days for urine. Concentrations of nitrofurantoin in biological fluids were obtained by comparing their nitrofurantoin/ furazolidone peak height ratios with those of a standard curve obtained the same day. Plasma samples had been kept frozen at -10°C and were thawed on the day of analysis. The two concentrations studied 10 and

1 µg/ml were stable over the period of 26 days tested, giving 10.06 ± 0.59 and 1.07 ± 0.08 respectively (Fig. III.4). Two concentrations of nitrofurantoin in urine were selected comparable to those typically encountered clinically, 200 and 18 µg/ml. Urine samples kept frozen at -10°C and thawed on day of analysis were stable over the period of 22 days tested, giving 198.42 ± 8.17 and 17.98 ± 0.54 respectively. If, however, the samples were kept at room temperature, both urine solutions rapidly degraded in such a way that at day 22 the 18 µg/ml solution no longer contained any detectable nitrofurantoin. These results indicate that biological fluids awaiting nitrofurantoin analysis need to be stored frozen.

Analysis of the samples was performed at 365 nm. This wavelength increases the sensitivity of the assay since the nitro group is determined near its absorption maximum (370 nm for nitrofurantoin). Moreover, at 365 nm most of the extraneous peaks resulting from the biological fluids were eliminated. As can be seen from control human urine and plasma (Figs. III.5 and III.6), we do not suspect any interference by endogenous substances with this assay since under the conditions used for analysis, all the extraneous peaks had retention times less than 3 minutes, whereas the retention times for nitrofurantoin and furazolidone were 6 and 8 minutes, respectively. Moreover, as

discussed later, metabolites obtained from the incubation of nitrofurantoin with various rat tissue homogenates do not interfere with the detection of the parent compound (Aufrère et al., 1977a).

With urine samples where no precipitation is necessary, analyses can be performed in 9 minutes per sample. In the case of plasma, centrifugation after protein precipitation is necessary since injections of plasma on the HPLC results in increases in operating pressures due to the build-up of proteins at the head of the column. Plasma samples require 15 minutes for protein precipitation after which analyses can be performed in 9 minutes per sample. Neither the urine samples nor the methanol from the plasma samples were filtered prior to HPLC injection.

The reproducibility of the method was determined from repeated injection experiments. Two concentrations were used, 2 µg/ml in plasma and 50 µg/ml in urine, each was injected nine times in the chromatograph. The 2 µg/ml plasma solution gave a CV of 1.5% (1.96 $\pm$ 0.03 µg/ml) whereas the 50 µg/ml urine solution gave a CV of 0.6% (51.00 $\pm$ 0.29 µg/ml).

The effect of various solvents on the present method was determined by preparing solutions of nitrofurantoin 100, 50, 20, 10 and 5 µg/ml in double-distilled water, DMSO, plasma and urine. Concentration curves were constructed and the regression lines for

each solvent were calculated: water, y = (0.0178 ± 0.0001) x - (0.0007 ± 0.0023); dimethyl sulfoxide, y = (0.0176 ± 0.0002) x + (0.0085 ± 0.0127); plasma, y = (0.0183 ± 0.0003) x - (0.0050 ± 0.0188); and urine, y = (0.0176 ± 0.0001) x + (0.0102 ± 0.0083). The correlation coefficients in all cases were greater than 0.99 (Fig. III.7). The particular biological fluids or solvents used had, therefore, no effect on the quantitative aspect of the method. Moreover, it was concluded that the use of DMSO for increasing the solubility of standard solutions having high nitrofurantoin concentrations (>100 µg/ml) did not interfere with the method. Following protein denaturation and centrifugation, whole blood and milk were also used, without modification of the present assay, and were found to be suitable biological tissues for the determination of nitrofurantoin.

Chromatograms of urine and plasma samples from a male patient receiving nitrofurantoin (50 mg every 6 hours) are shown in Fig. III.5 and III.6, respectively. Biological samples were taken 90 min following the administration of this patient's fifth 50 mg oral dose of nitrofurantoin. This patient was also receiving the following drugs: codeine, dextropropoxyphene, aluminum hydroxide gel, bisacodyl, ascorbic acid, a multiple vitamin and oxycodone with aspirin phenacetin and caffeine. From the nitrofurantoin to furazolidone peak

height ratio in the patient's chromatograms, the nitrofurantoin urinary concentration was 26.1 µg/ml, whereas the plasma concentration was 0.72 µg/ml.

In order to determine the sensitivity of the method, nitrofurantoin solutions in plasma were used over a concentration range from 0.02 to 0.4 µg/ml. For each of the concentrations, two to three injections were made. The curve was constructed by plotting the peak height ratio of nitrofurantoin to furazolidone against the concentration of nitrofurantoin. With 11 points the regression line was $\underline{y}$ = (10.40 $\pm$ 0.18) $\underline{x}$ + (0.08 $\pm$ 0.04). A similar low concentration curve was obtained with aqueous nitrofurantoin solutions in the range from 0.01 to 0.2 µg/ml. The regression line for this curve was $\underline{y}$ = (11.12 $\pm$ 0.19) $\underline{x}$ + (0.05 $\pm$ 0.02) (Fig. III.8).

A comparison was made between the present method and the Hyamine 10X method. Solutions of nitrofurantoin in plasma covering the range of 0.5 to 30 µg/ml were prepared and for each concentration both methods were used to determine actual concentrations of the solution. Concentrations obtained by the HPLC method were plotted against those obtained from the Hyamine 10X method giving a regression line $\underline{y}$ = (1.015 $\pm$ 0.019) $\underline{x}$ - (0.560 $\pm$ 0.314) (Fig. III.9). As compared to the Hyamine 10X method, the HPLC method offers the advantage of easier sample preparation, since it

requires only the addition of the internal standard and no extraction is necessary. The sensitivity is greater with the HPLC method, 0.02 µg/ml being easily detected, whereas in the original Hyamine 10X method the sensitivity is reported to be 2 µg/ml (Conklin and Hollifield, 1966a) and in the improved Hyamine 10X method the sensitivity is 0.2 µg/ml (Mattok et al., 1970). In addition, solutions for HPLC analysis are stable since there is no progressive loss of sensitivity as is observed with the Hyamine 10X method due to the degradation of methazonic acid formed by the addition of Hyamine 10X to nitromethane (Harrison et al., 1973). However, when the absorbance of the Hyamine 10X complex is measured rapidly after its formation, as is recommended by the authors of these publications, nitrofurantoin concentrations can be measured at the reported sensitivities (Conklin and Hollifield, 1966a; Mattok et al., 1970).

With no modification in the procedure, the HPLC assay can be extended to other 5-nitrofurans. A standard plasma curve with nitrofurazone is presented in Fig. III.10. The regression line for the standard plasma curve with nitrofurazone is $y = (0.730 \pm 0.005)\ x - (0.051 \pm 0.015)$, (CV = 0.7%, r = 0.99). Since nitrofurazone is used in animal feeds for the prevention of a variety of diseases, the method lends

itself to analysis of residues of 5-nitrofuran derivatives present in tissues of animals having been fed such a diet.

It is concluded that the present assay offers a sensitive, accurate, stable and convenient method for the analysis of nitrofurantoin and nitrofurazone in biological fluids. The HPLC method is linear over a nitrofurantoin concentration range from 0.02 µg/ml to 200 µg/ml and is sufficiently accurate and sensitive for clinical monitoring of therapeutic levels in patients and for pharmacokinetic/bioavailability studies.

Fig. III.1 STANDARD CURVE FOR NITROFURANTOIN - HYAMINE 10X COMPLEX

Absorbances at 400 nm are plotted against nitrofurantoin - Hyamine 10X complex concentrations. Regression line: $\underline{y} = (.033 \pm .001)\underline{x} + (.002 \pm .003)$.

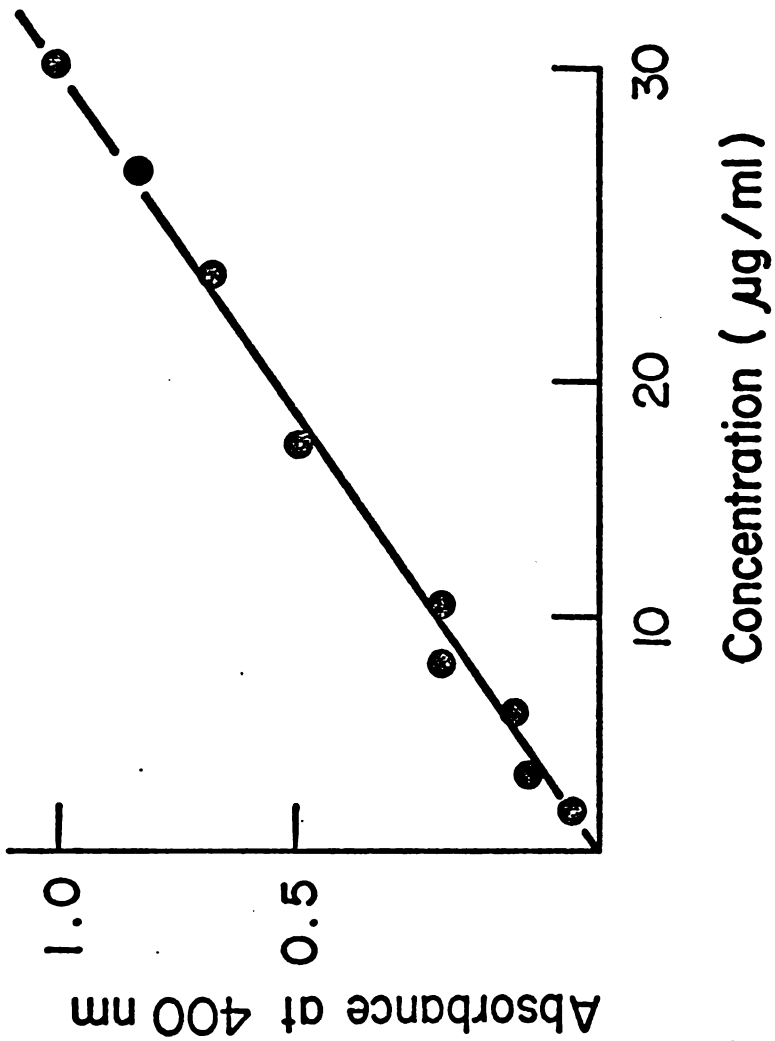

Absorbance at 400 nm
1.0
0.5
10
20
30
Concentration (µg/ml)

Fig. III.2 BLOCK DIAGRAM OF THE WATERS ALC/GPC 244 SYSTEM

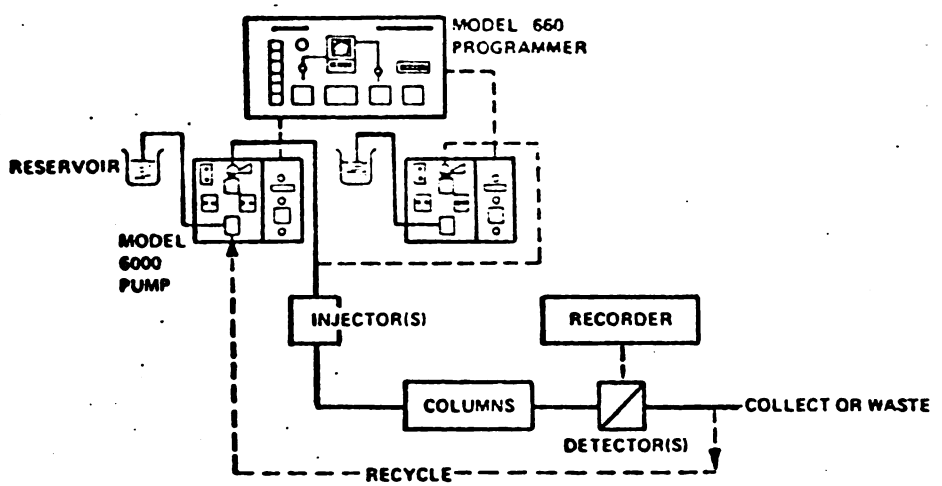


Fig. III.3 NITROFURANTOIN PLASMA STANDARD CURVE

Nitrofurantoin/furazolidone peak height ratios are plotted against nitrofurantoin concentrations. Regression line:

$\underline{y} = (0.469 \pm 0.009)\ \underline{x} + (0.097 \pm 0.047)$; $r = 0.99$.

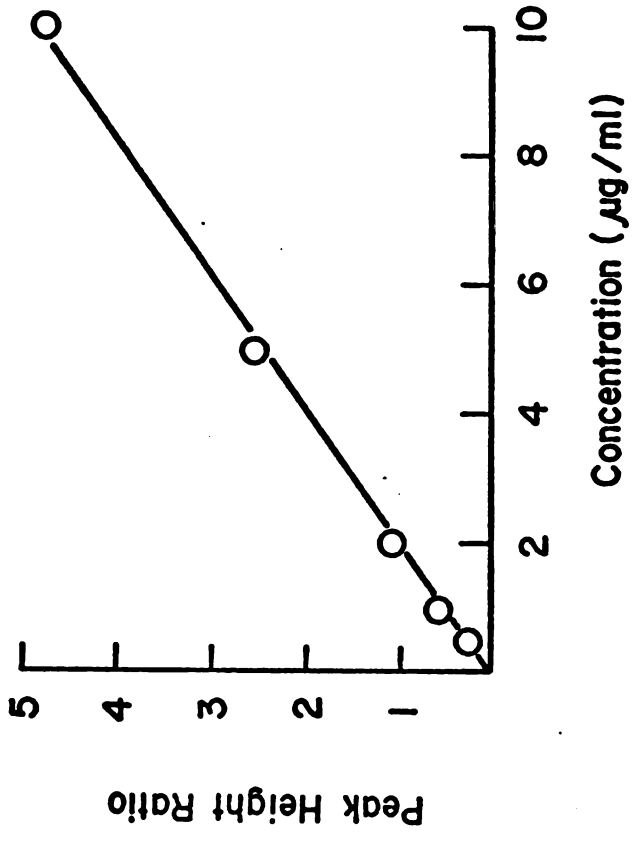

Peak Height Ratio
5
4
3
2
1
2
4
6
8
10
Concentration (µg/ml)

Fig. III.4 NITROFURANTOIN STABILITY STUDY

Samples containing either 1, 10 or 18 µg/ml of nitrofurantoin were prepared, divided into capped vials and frozen at -10°C. Vials were thawed and assayed on the day indicated. Concentrations of nitrofurantoin are plotted against day of assay.

△ 1 µg/ml in plasma (1.07 ± 0.08)

□ 10 µg/ml in plasma (10.06 ± 0.59)

○ 18 µg/ml in urine (17.98 ± 0.54)

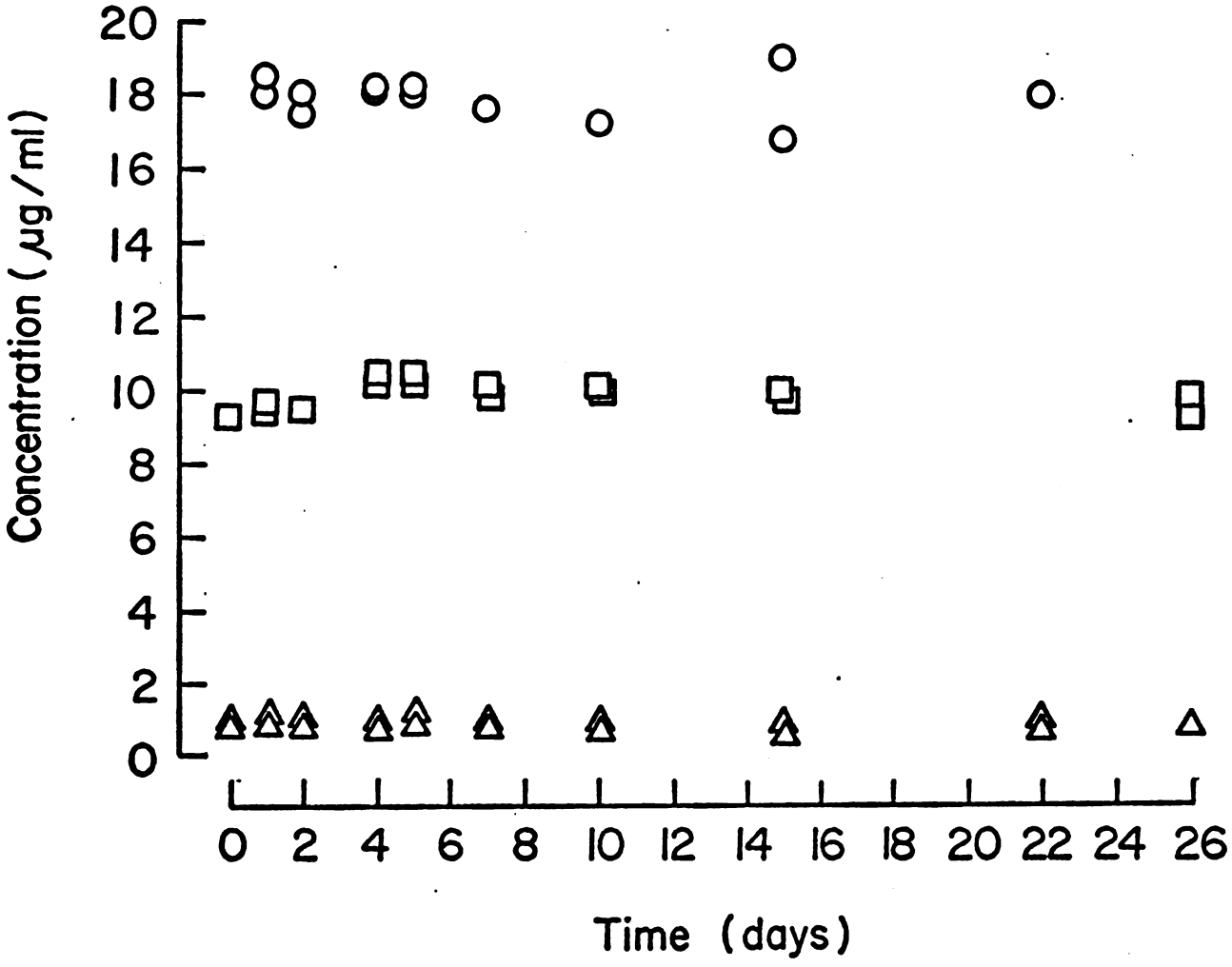

Concentration (µg/ml)
20
18
16
14
12
10
8
6
4
2
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time (days)

Fig. III.5 CHROMATOGRAMS OF a) CONTROL URINE AND b) URINE FROM PATIENT ON NITROFURANTOIN.
HPLC injection (↓), retention times for nitrofurantoin (1) and furazolidone (2) are 6 and 8 min, respectively. The nitrofurantoin to furazolidone peak height ratio indicates a nitrofurantoin urinary concentration of 26.1 µg/ml.

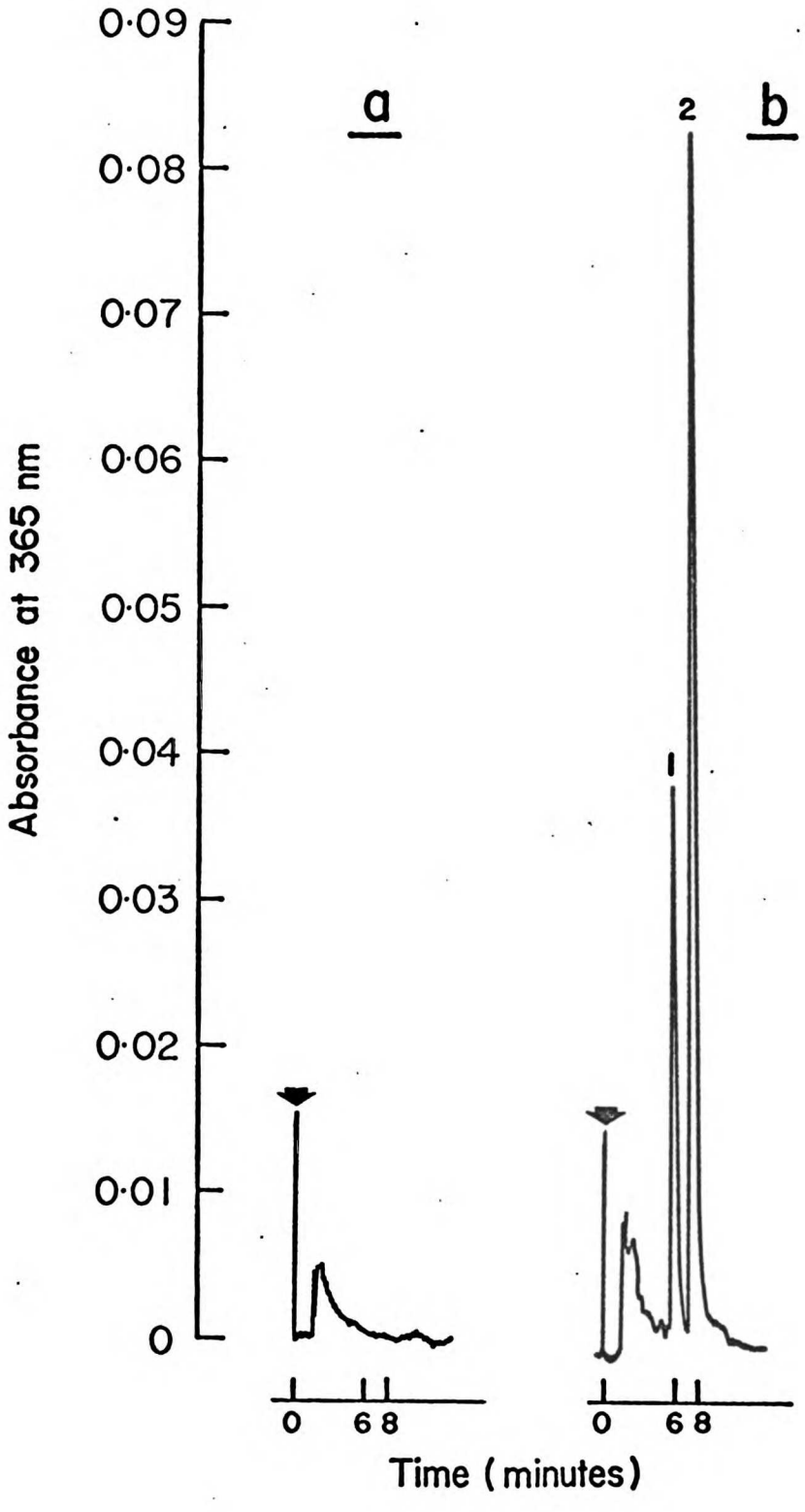

0·09
0·08
0·07
0·06
0·05
0·04
0·03
0·02
0·01
0
Absorbance at 365 nm
a
b
2
1
0
6
8
0
6
8
Time (minutes)

Fig. III.6 CHROMATOGRAMS OF a) CONTROL PLASMA AND b) PLASMA FROM PATIENT ON NITROFURANTOIN.
HPLC injection (↓), retention times for nitrofurantoin (1) and furazolidone (2) are 6 and 8 min, respectively. The nitrofurantoin to furazolidone peak height ratio indicates a plasma nitrofurantoin concentration of 0.72 μg/ml.

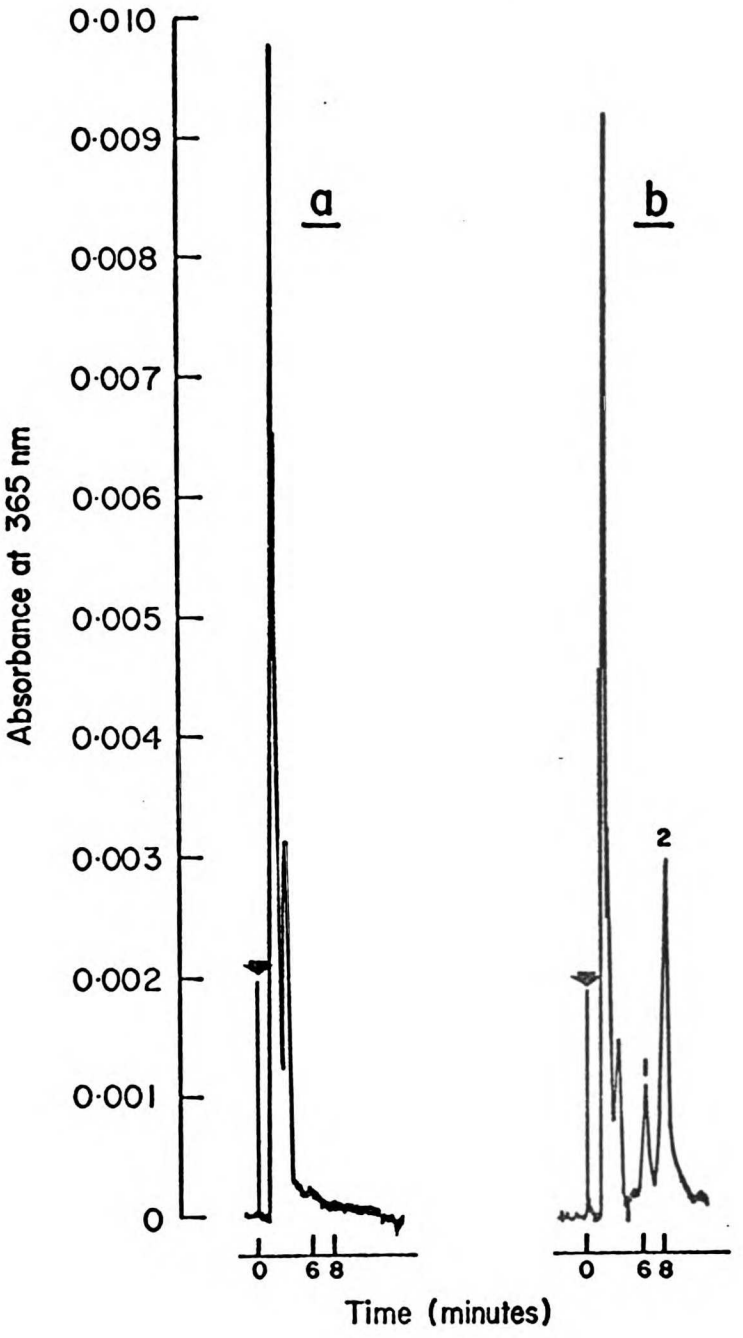

0·010
0·009
0·008
0·007
0·006
0·005
0·004
0·003
0·002
0·001
0
Absorbance at 365 nm
a
b
2
0
6 8
0
6 8
Time (minutes)

Fig. III.7 RECOVERY OF NITROFURANTOIN FROM VARIOUS SOLVENTS

Nitrofurantoin/furazolidone peak height ratios are plotted against nitrofurantoin concentrations.

● water [$\underline{y}$ = (0.0178 $\pm$ 0.0001) $\underline{x}$ - (0.0007 $\pm$ 0.0023)]

□ DMSO [$\underline{y}$ = (0.0176 $\pm$ 0.0002) $\underline{x}$ + (0.0085 $\pm$ 0.0127)]

△ plasma, [$\underline{y}$ = (0.0183 $\pm$ 0.0003) $\underline{x}$ - (0.0050 $\pm$ 0.0188)]

○ urine, [$\underline{y}$ = (0.0176 $\pm$ 0.0001) $\underline{x}$ + (0.0102 $\pm$ 0.0083)]

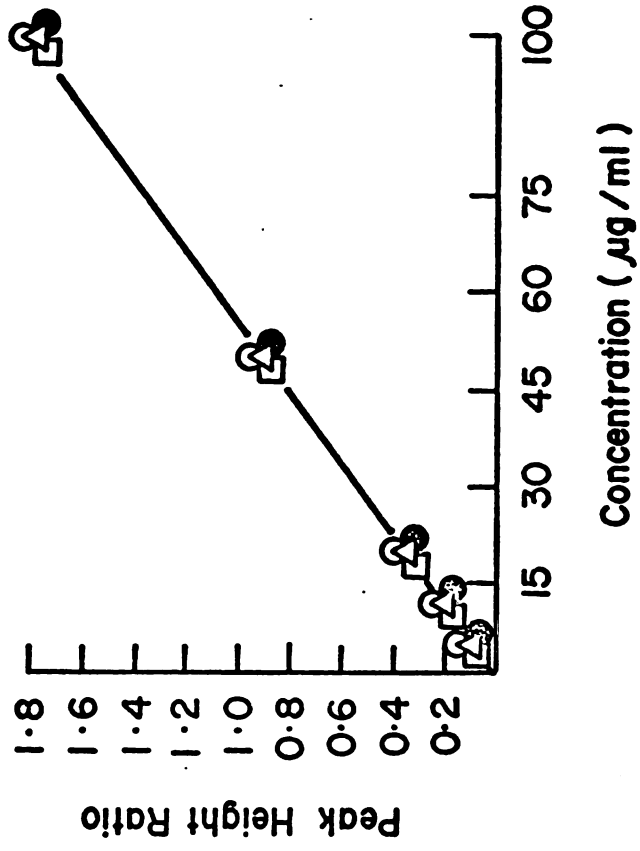

Peak Height Ratio
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
15
30
45
60
75
100
Concentration (µg / ml)

Fig. III.8 STANDARD CURVE FOR LOW CONCENTRATIONS OF NITROFURANTOIN

Nitrofurantoin/furazolidone peak height ratios are plotted against nitrofurantoin concentrations. Regression line:

$\underline{y} = (11.12 \pm 0.19)\ \underline{x} + (0.05 \pm 0.02)$; $r = 0.99$.

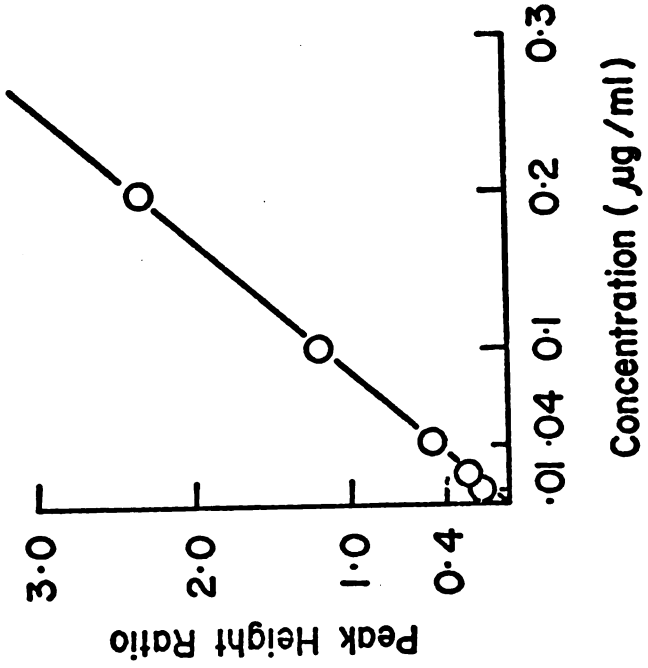

Peak Height Ratio
3·0
2·0
1·0
0·4
·01 ·04 0·1 0·2 0·3
Concentration (µg/ml)

Fig. III.9 COMPARISON BETWEEN HPLC METHOD AND HYAMINE 10X METHOD

Nitrofurantoin concentrations obtained by the HPLC method are plotted against those obtained from the Hyamine 10X method, giving the regression line:

$$\underline{y} = (1.015 \pm 0.019)\ \underline{x} - (0.560 \pm 0.314);\ r = 0.99.$$

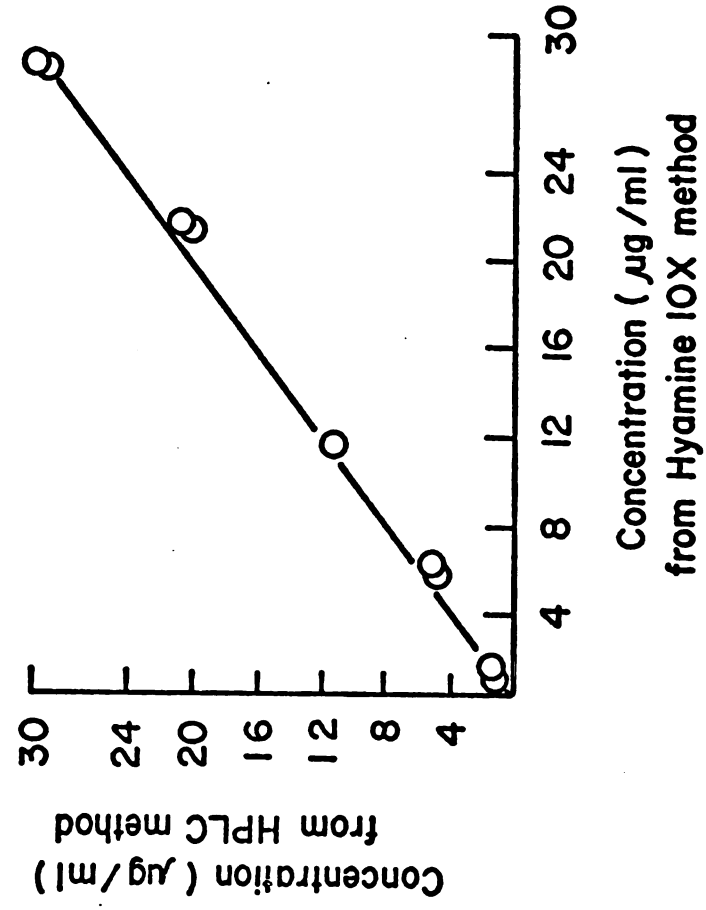
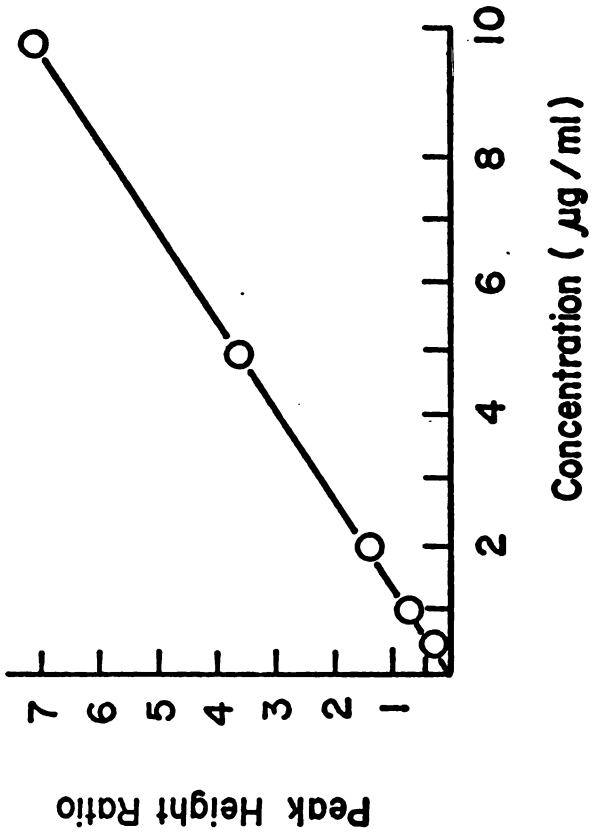

Concentration (µg/ml)
from HPLC method
30
24
20
16
12
8
4
Concentration (µg/ml)
from Hyamine 10X method
4
8
12
16
20
24
30

Fig. III.10 NITROFURAZONE PLASMA STANDARD CURVE

Nitrofurazone/furazolidone peak height ratios are plotted against nitrofurazone concentrations. Regression line:

$\underline{y} = (0.730 \pm 0.005)\ \underline{x} - (0.051 \pm 0.015)$; $r = 0.99$.


Peak Height Ratio
1
2
3
4
5
6
7
2
4
6
8
10
Concentration (µg / ml)

IV. METABOLIC STUDIES ON NITROFURANTOIN

A. Rates of metabolism in germ-free, germ-free acclimatized, and control rats

The toxicity of certain aromatic and heterocyclic nitrocompounds has been associated with the formation of reactive reduction products (McCalla et al., 1971b; Weisburger and Weisburger, 1973). For example, the methemoglobinemia produced by nitrobenzene results from the simultaneous oxidation of hemoglobin and phenylhydroxylamine to methemoglobin and nitrosobenzene, respectively (Kiese, 1974; Reddy et al., 1976). The carcinogenic and mutagenic activity of nitrofurazone (McCalla et al., 1971b) and 4-nitroquinoline-1-oxide (Poirier and Weisburger, 1974) is thought to result, at least in part, from the reduction of these compounds to hydroxylamines. Certain aromatic and heterocyclic N-hydroxylamines have been shown to be cytotoxic, mutagenic and carcinogenic in mammalians (Weisburger and Weisburger, 1973). It appears, therefore, that the biological reduction of aromatic nitrocompounds plays an important role in the toxicity of these compounds, however, the specific tissues and particular enzymes involved in this nitroreductase activity remain uncertain.

The liver is the major site for the biotransformation of xenobiotic compounds in the body. Other tissues, however, such as intestine, lung, and skin may contribute

to the metabolism--and thus the metabolic activation--of chemicals in the body. Among extrahepatic tissues, enteric tissue and its microflora play a significant role in the metabolism of certain chemicals including nitro compounds (Reddy *et al.*, 1976; Zachariah and Juchau, 1974).

Mammalian enzyme systems show a much higher activity for the reduction of aromatic nitro compounds in the absence of oxygen (Gilette *et al.*, 1968). This observation has raised some questions as to the validity of the results obtained from anaerobic *in vitro* experiments since outside of the enteric lumen milieu it is unlikely that anaerobic conditions exist in the various tissues *in vivo*. The intestinal microflora can reduce heterocyclic and aromatic nitro compounds via N-hydroxylamine intermediates (Scheline, 1973). In contrast to mammalian nitroreductase, the bacterial nitroreductase has been shown to reduce nitro groups even in the presence of oxygen (Scheline, 1973). Bacteria are known to contain at least two different classes of nitroreductase. Nitroreductase-I from *E. coli* uses either NADH or NADPH as an electron donor and is not inhibited by oxygen (Asnis, 1957; McCalla *et al.*, 1971a). On the other hand, nitroreductase-II from *E. coli* uses only NADH as an electron donor and is inhibited by oxygen (Asnis, 1957; McCalla *et al.*, 1971a).

It has been shown that the *in vitro* metabolic activation of nitrofurantoin by rat liver enzymes to an active mutagen and the binding of ^{14}C-nitrofurantoin to pulmonary and hepatic proteins are processes which are maximal under anaerobic conditions (Rosenkranz and Speck, 1976; Boyd and Osborne, 1975). In view of the high content of anaerobic and facultative anaerobic bacteria in the intestinal tract along with its favorable anaerobic conditions, this organ represents the most likely locus of *in vivo* formation of the toxic molecular species. The rate of metabolism of nitrofurantoin under anaerobic conditions in enteric tissues was therefore examined. To determine the contribution of the gut flora to the overall metabolism of nitrofurantoin, the rates of disappearance of nitrofurantoin in germ-free rats, germ-free rats acclimatized to the normal animal room, and control rats were measured.

Materials and Methods

Nitrofurantoin solutions (100 μg/ml) were prepared by dissolving 10 mg of nitrofurantoin in 5 ml N,N-dimethylformamide (spectrophotometric grade, Aldrich Chem. Co., Milwaukee, WI 53233) and diluted to 100 ml with distilled water in a volumetric flask. Male germ-free rats (Sprague-Dawley, 60 g; Charles River Breeding Labs) were sacrificed by decapitation immediately upon arrival. Male litter mates of the germ-free animals were allowed to acclimatize in the

animal room for fifteen days (germ-free acclimatized rats) in the same cage with a control (i.e. not germ-free) rat prior to sacrifice.

All tissues (liver, kidney, small intestine walls, small intestine contents, and cecum and colon contents) were homogenized in four parts (w/v) of 1.15% KCl-0.01 M potassium phosphate buffer (pH 7.7) (buffer A) in a Potter-Elvehjem homogenizer with a Teflon pestle. All tissue preparations were performed at 4°C. Livers were perfused with Buffer A prior to homogenization. Small intestine contents were obtained by squeezing contents out of the intestinal lumen one cm below the pylorus to one cm above the ileocecal valve. Small intestine walls were prepared from the segments which provided small intestine contents. Intestinal segments were washed thoroughly with Buffer A., opened longitudinally, and the exposed mucosal surface wiped dry to minimize bacterial contamination. The 9000 xg supernatant fraction was prepared by centrifugation of the homogenate for 20 min at 9,000 xg followed by filtration of the supernatant through cheezecloth. Protein was determined by the method of Lowry et al., using bovine serum albumin as a standard (Lowry et al., 1951).

Typical incubation mixtures contained 2.2 ml of 0.01 M potassium phosphate buffer (pH 7.35) and

1.8 ml of an NADPH generating system consisting of $NADP^+$ (1.0 µmole), glucose-6-phosphate (30 µmole) and $MgCl_2$ (15 µmole). The incubation mixtures were saturated with nitrogen which was passed through a deoxygenizer system consisting of a 0.4% sodium hydroxide solution containing 0.5% sodium dithionite and 0.05% of the sodium salt of 2-anthraquinone-sulfonic acid. One ml of the tissue homogenate (corresponding to 200 mg of tissue) or one ml of the 9,000 xg supernatant fraction (corresponding to 250 mg of tissue) was added to the anaerobic incubation mixture. The incubation was started by addition of 3 ml of 100 µg/ml nitrofurantoin solution (1.26 µmole) bringing the total volume to 8.0 ml. All procedures were performed in subdued light due to the photosensitivity of nitrofurantoin solutions. Incubations were performed in a Dubnoff metabolic incubator at 37°C. To insure anaerobic conditions, incubation vessels were capped with a perforated rubber septum which allowed maintenance of a constant flow of nitrogen.

The disappearance of nitrofurantoin was measured using a modificaton of the Hyamine 10X method (Mattok et al., 1970). Aliquots (0.8 ml) were removed from the incubation mixture at 0, 6, 12, 18, 24, and 45 min and immediately added to centrifuge tubes containing 2.0 ml of nitromethane, mixed thoroughly for 20 sec and placed

on ice. The samples were then centrifuged for 15 min and at 1500 xg, the aqueous layer removed, and 1.5 ml of the nitromethane layer was treated with 0.15 ml of 0.01 M methanolic Hyamine 10X hydroxide as described previously. The absorbance at 400 nm was read within 5 min in a Shimadzu recording spectrophotometer, model MPS-50L, with nitromethane in the reference cuvette. The concentration of nitrofurantoin-Hyamine 10X complex was determined by comparison with absorbances from a calibration curve obtained from standard solutions of nitrofurantoin.

Results

To determine the role of the intestinal microflora in the metabolism of nitrofurantoin, the rates of anaerobic metabolism by various enteric tissues in germ-free, germ-free acclimatized, and control rats were measured. As shown in Table IV.1, metabolism, as measured by the rate of disappearance of nitrofurantoin was highest in the homogenate of cecum and colon contents of germ-free acclimatized and control rats and in the liver in all animal groups. No metabolism was detected in the cecum and colon contents of germ-free rats, indicating that the metabolism observed in germ-free acclimatized and control rats was due to the bacteria present. These data provide evidence that

the intestinal microflora may play a role in the biodisposition of nitrofurantoin.

The distribution of microorganisms throughout the gastrointestinal tract is such that the total number of microorganisms increases exponentially from the duodenum to the lower colon. Within the cecum the anaerobic microorganisms outnumber the aerobic and facultative flora by a factor of 10^3 to 10^4 (Scheline, 1973). Metabolism in other tissues of germ-free or germ-free acclimatized rats was not significantly different from that of control rats. The rate of metabolism of nitrofurantoin in the small intestine wall homogenate was comparable to that obtained in liver homogenate. It is unlikely that this activity could be due to bacterial contamination since activity was unchanged in germ-free animals and no activity was detected in homogenates from small intestine contents.

The finding that under anaerobic conditions, small intestine walls and cecum and colon contents metabolize nitrofurantoin indicate that a fraction of an oral dose of nitrofurantoin may be metabolized before entering the systemic circulation. Orally administered nitrofurantoin has been shown to be rapidly absorbed from the small intestine and the colon (Buzard et al., 1961; Veronese et al., 1974). Secretion of nitrofurantoin from the blood to the

intestinal lumen has also been reported, but quantitative aspects of this contribution to the overall distribution of nitrofurantoin are uncertain (Buzard et al., 1961). In addition, a small fraction (2% of the dose) of nitrofurantoin appears in the feces in the rat when orally administered (25 mg/kg) (Paul et al., 1960).

B. Determination of apparent K_m and V_{max} of nitrofurantoin metabolism in 9,000 xg supernatant fraction of liver.

Materials and Methods

Solutions of nitrofurantoin were prepared in water (0.039, 0.050, 0.078 mM) or in 5% DMSO in water (0.126 and 0.157 mM) to increase nitrofurantoin solubility. Incubation of nitrofurantoin with 9,000 xg supernatant fraction was carried out at 37°C in a Dubnoff metabolic incubator. Maintenance of the anaerobic conditions was as described previously. Incubation mixtures contained 0.01 M potassium phosphate buffer pH 7.4 (200 µmole), $NADP^+$ (1 µmole), glucose-6-phosphate (30 µmole), $MgCl_2$ (15 µmole), liver 9,000 xg supernatant (from 250 mg tissue), and nitrofurantoin in a total volume of 8.0 ml. The disappearance of nitrofurantoin was measured at 3, 6, 9 and 12 min using the modified Hyamine 10X method (Mattok et al., 1970) described

previously. Nitrofurantoin concentrations were determined by comparison with absorbance values from a calibration curve obtained from standard solutions of nitrofurantoin. Protein was determined by the method of Lowry et al. using bovine serum albumin as a standard (Lowry et al., 1951).

Results

The Lineweaver-Burk plot for the anaerobic metabolism of nitrofurantoin by the 9,000 xg supernatant fraction of rat liver is shown in Fig. IV.1. The apparent K_m was calculated to be 59 μM while the V_{max} was 0.49 nmoles of nitrofurantoin metabolized per mg protein per min. The concentrations of nitrofurantoin used (39-157 μM) spanned the K_m.

C. Characterization of metabolism by liver, lung, kidney, small intestine walls, small intestine contents, and cecum and colon contents.

Materials and Methods

Male Sprague-Dawley rats (250 g) were sacrificed by decapitation, the livers perfused with 1.15% KCl-0.01 M potassium phosphate buffer pH 7.7 and the liver, lung, kidney, small intestine walls, small intestine contents, and cecum and colon contents homogenized in four parts (w/v) of the buffer as

described. Homogenates of liver, lung, kidney, small intestine walls, small intestine contents, and cecum and colon contents were incubated with nitrofurantoin as described previously. Incubation mixtures contained potassium phosphate buffer pH 7.4 (200 µmole), $NADP^+$ (1 µmole), glucose-6-phosphate (30 µmole), $MgCl_2$ (15 µmole), tissue homogenate (from 200 mg of tissue), and nitrofurantoin (1.26 µmole) in a total volume of 8.0 ml. Aliquots (1 ml) were removed from the incubation mixture at 0, 10, 30, and 45 min, mixed with 1 ml of methanol and centrifuged at 9,000 xg for 30 min to precipitate the proteins.

Aliquots (10 µl) of the supernatant were then analyzed by HPLC. The separation of metabolites formed by the various tissues was carried out by a modification of the HPLC assay for nitrofurantoin in plasma and urine (Aufrère et al., 1977b). A high-pressure liquid chromatograph (Model ALC/GPC 244, Waters Associates, Inc. Milford, MA 01757) characterized by a constant flow of solvent at working presures up to 420 bar was used. This model includes a U6-K universal injector and a 660 solvent flow programmer. A dual-channel fixed-wavelength ultraviolet absorption detector was used with the wavelengths of detection fixed at 280 and 365 nm. A dual-pen recorder (OmniScribe Model A5211-1; Houston Instruments, Austin, TX 78753) was used. The

instrument was fitted with a 30 cm x 7 mm (i.d.) μBondapak C_{18} reverse-phase column (Waters Associates). The mobile phase was distilled water/methanol and was programmed for 0-30% methanol in twenty min, using program 10. The distilled water (redistilled and stored in glass) and methanol (glass distilled, Burdick and Jackson, Lab.; Muskegon, MI 49442) were filtered (0.45 μm filter, Catalog No. HAWPO 4700 and FHLPO 4700, Millipore Corp.; Bedford, MA 01730) and then degassed before use. The chromatograph was operated at a flow rate of 4.0 ml/min, at ambient temperature. The gradient elution program was started at the time of injection.

Results

The HPLC elution profiles of metabolites of nitrofurantoin formed by incubation for 10, 30, and 45 min with rat liver homogenates are shown in Fig. IV.2. Similar patterns of metabolites were observed in the HPLC elution profiles in samples taken at 10 and 30 minutes.

For simplicity, difference chromatograms for the 45 min sample were constructed by subtracting the corresponding control chromatograms (i.e., incubations carried out in the absence of nitrofurantoin) (Fig. IV.3.A).

The HPLC elution profiles of metabolites formed by incubation for 45 minutes with other rat tissue homogenates are shown in Fig. IV.3.B and C. Three metabolites, M-1, M-2, and M-3, with retention times of 21.0, 5.0 and 3.5 min, respectively were formed in the liver (Fig. IV.3.A). Only M-1 and M-2 were formed in homogenates of lung, kidney or small intestine walls. M-1 and a new metabolite, M-4, with a retention time of 24 min, were formed by the bacteria from the cecum and colon contents. Unlike the other metabolites, M-4 did not absorb at 280 nm and was detected at 365 nm. Incubation with small intestine contents did not result in any observable metabolism of nitrofurantoin.

D. Preparative-scale incubations with 9,000 xg supernatant fraction of liver

Materials and Methods

Preparative anaerobic incubations:

In experiments designed to isolate large quantities of metabolites for further characterization, nitrofurantoin (10 µmole in 0.2 ml DMSO) was added to an incubation mixture containing potassium phosphate buffer pH 7.4 (0.1 mmole), $NADP^+$ (6 µmole), glucose-6-phosphate (180 µmole), $MgCl_2$ (90 µmole), and the 9,000 xg supernatant fraction from 500 mg of liver in a

total volume of 8.6 ml. Incubation conditions were as described previously for the characterization of metabolism by various rat tissue homogenates. The reaction was terminated at 60 min by the addition of 10 ml of methanol, the mixture centrifuged at 9,000 xg for 30 min to precipitate the proteins, and the supernatant taken to dryness under reduced pressures.

HPLC purification:

The purification of metabolites was carried out by a modification of the HPLC conditions described for the characterization of metabolites of nitrofurantoin from various rat tissue homogenates. Conditions for the gradient elution program of the high-pressure liquid chromatograph were identical to those already described. The wavelengths of detection were fixed at 254 and 280 nm.

The dried sample from the preparative incubation with 9,000 xg supernatant fraction of liver was dissolved in 6 ml of distilled water, filtered (0.45 μm filter, HAWPO 1300, Millipore Corp.) and 0.6 ml aliquots of the filtrate injected onto the HPLC. The fractions containing the metabolites were collected as they eluted from the HPLC column. The HPLC fractions eluting between 21.0 and 21.5 min (Fig. IV.4) corresponding to the major metabolite, designated as M-1, were collected from ten successive HPLC injections,

combined and taken to dryness under vacuum. Further studies characterizing M-1 were then carried out on the dried samples.

Ultraviolet spectroscopic characterization of the metabolites:

As the fractions from the preparative incubation with the liver 9,000 xg supernatant eluted from the HPLC column, they were collected, and their UV absorption spectra recorded in a Shimadzu model MPS-50L, double-beam recording spectrophotometer (Fig. IV.5).

The dried samples of M-1 were dissolved in distilled water and the UV absorption spectrum recorded. In order to check the stability of M-1, aqueous samples were placed into capped vials and frozen at -20°C. The vial contents were thawed and the UV spectra recorded daily over a period of six days.

Mass spectral analysis:

Electron ionization mass spectrometry--The samples for mass spectral analysis were obtained from collection of the eluent from the preparative HPLC analysis (Fig. IV.4). Fractions were collected between 21.0 and 21.5 min (M-1) and between 22.0 and 22.5 min for ten successive HPLC injections. The purity of the fractions was checked by UV absorption spectroscopy (Fig. IV.5) and the fractions combined and lyophilized. Approximately 0.1 mg of the dried samples

was used for mass spectral analysis as direct probe samples. EI mass spectra were taken on an Associated Electrical Industries model MS 12 mass spectrometer. Sample probe temperatures ranged from 20° to 300°C and the voltage of the ionizing electron beam was maintained at 70 eV.

Chemical ionization mass spectrometry--
Approximately 0.2 mg of dried M-1 obtained from the HPLC fraction eluting between 21.0 and 21.5 min (Fig. IV.4) was subjected to chemical ionization (CI) mass spectral analysis with an Associated Electrical Industries model MS-902 high resolution mass spectrometer. Isobutane was used as the reagent gas at 0.5 Torr ion chamber pressure and at 320°C. Sample introduction was by direct insertion probe in which the sample was carried into the ion chamber by the isobutane gas flow.

Nuclear magnetic resonance spectrometry:

M-1 resulting from the lyophilization of the HPLC fraction eluting between 21.0 and 21.5 min was dissolved in approximately 0.6 ml of D_2O (99.8 atom % D, Aldrich, gold label, Milwaukee, WI 53233, Lot No. 041947). Care was taken not to expose the dried M-1 to air which would result in rapid oxidation of the sample.

The proton nuclear magnetic resonance (NMR) spectra were measured with a Varian XL-100 Fourier transform NMR spectrometer equipped with a Nicolet Instrument Corp. Model TT-100 accessory. One thousand pulses (pulse width = 5 μsec, acquisition time = 3.4 sec, acquisition delay = 400 μsec) were used for the Fourier transform.

Results

Since M-1 appeared to be the major metabolite of nitrofurantoin and was formed by all tissues with metabolic activity (Fig. IV.3), further studies were carried out to isolate and identify this product. In order to optimize the yield of M-1, nitrofurantoin (1.16 mM) was incubated for 60 min under anaerobic conditions with the 9,000 xg supernatant fraction of liver. The HPLC elution profile of the reaction mixture is shown in Fig. IV.4. All fractions representing endogenous materials, added co-factors, nitrofurantoin and its metabolites were collected as they eluted from the column. The retention times for the major metabolites were 21.0 to 21.5 min for M-1, 5.0 to 5.5 min for M-2, and 3.5 to 4.0 min for M-3.

Ultraviolet absorption spectroscopy:

The UV absorption spectra of all the fractions present in the metabolic incubation mixture with liver

9,000 xg are shown in Fig. IV.5. The UV absorption spectra for the major metabolites showed the following absorption maxima (in H_2O): M-1 (278 nm), M-2 (271 nm) and M-3 (239 and 288 nm).

The aqueous solution of M-1 stored at -20°C over a period of six days appeared to be stable since the sample exhibited no shift in absorption maximum (λ_{max} = 278 nm), no decrease in absorbance, and no appearance of new bands (Fig. IV.6).

The UV absorption spectrum of aminofurantoin (II in Fig. IV.7) produced from the catalytic reduction of nitrofurantoin has a λ_{max} (H_2O) at 348 nm (Ebetino et al., 1962). In contrast, the open-chain nitrile metabolites of two 5-nitrofurans have absorption maxima between 270 and 290 nm (Tatsumi et al., 1976; Chatfield, 1976). These data suggested that M-1 could be the open-chain nitrile derivative of nitrofurantoin.

Mass spectral analysis:

Electron ionization mass spectrometry--The electron ionization (EI) process generates odd-electron molecular ions:

$$M + e^- \longrightarrow M^{+\bullet} + 2e^-$$

These molecular ions ($M^{+\bullet}$) are in an unstable, excited state due to the excess energy absorbed during ionization and also on the account of the unpaired

electron. The odd electron ion can achieve a lower energy state by homolytic fission of certain bonds to pair the unpaired electron. Irrespective of which electron is expelled from a molecule at the time of ionization, the site of electron deficiency rapidly equilibrates to a few favorable sites in the molecular ion. Nonbonding electron orbitals of heteroatoms provide the most likely sites of electron deficiency in the molecular ion (Watson, 1976).

The EI mass spectrum of the HPLC fraction collected between 22.0 and 22.5 min is shown in Fig. IV.8. Table IV.2 gives the percent relative abundances and nominal molecular weights for the various ions observed. From mass spectral data, this compound was recognized as nicotinamide. This finding corresponds with the UV absorption data which had been obtained with this fraction. Shelley and Umberger (Shelley and Umberger, 1959) reported the UV absorption spectrum of nicotinamide with a λ_{max} of 262 nm and a minimum at 240 nm, corresponding to our UV data (Fig. IV.5). The nicotinamide observed in our incubation mixture results from the addition of the reducing co-factor NADPH to the liver 9,000 xg supernatant fraction.

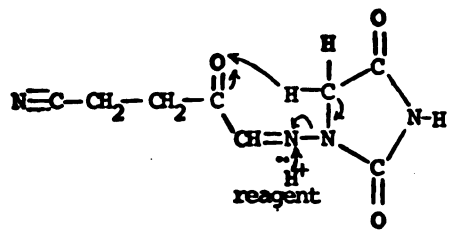
Due to the low volatility of M-1, this compound was not amenable to EI mass spectral analysis. At a source temperature of 300°C, however, a major peak was

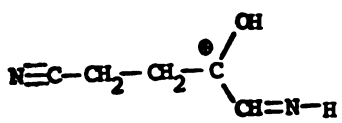
observed at m/e = 100 which is suggestive of an intact 2,4-imidazolidinedione ring.

Chemical ionization mass spectrometry--Chemical ionization (CI) mass spectrometry differs from electron ionization mass spectrometry in that sample molecules are ionized by interaction or collision with ions of a reagent gas rather than electrons. With alkyl ion reagent gases, less fragmentation is obtained in CI than in EI mass spectroscopy. In our case, the production of the reagent gas ion involved electron ionization of isobutane, leading to $C_4H_9^+$ as the predominant reactant ion. The adduct ions, MH^+, $(M+C_4H_9)^+$, produced in CI are therefore more stable in terms of internal energy than the analogous molecular ion, $M^{+\bullet}$, obtained in EI. In addition, the use of relatively high pressure (0.5 Torr) in CI provides a means of dissipating excess internal energy (Watson, 1976). The site of protonation is most likely on the heteroatom of greatest proton affinity, and in many cases, CI with isobutane as the reagent gas generates ions comprising the intact sample molecule thereby providing an indication of the molecular weight (Weinkam, 1976). The degree of fragmentation of a given compound under chemical ionization is a function of the Brønsted acid strength of the reagent-gas ion. Decreasing strength as a Brønsted acid indicates

increasing magnitude of proton affinity and thus decreasing effectiveness in causing fragmentation. Therefore, of the reagent gases commonly used, the t-butyl ion from isobutane produces the least fragmentation, protonated hydrogen produces the most extensive fragmentation, and the ions from methane are of intermediate potency in producing fragmentation.

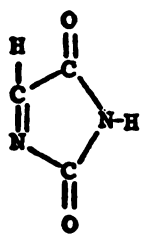
The CI mass spectrum for M-1 using isobutane as a reagent gas is shown in Fig. IV.9. Due to the mild ionization characteristics of the CI source, the protonated molecular ion [MH^+] was detected at m/e = 209. In addition, two major fragments were present at m/e = 101 and 111. On the basis of mass-spectral analysis alone, the structure for M-1 could not be unambiguously ascertained since 1-[[(5-amino-2-furanyl) methylene]amino]-2,4-imidazolidinedione (II in Fig. IV.7) and 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2, 4-imidazolidinedione (III in Fig. IV.7)) are isomeric with a molecular weight of 208 ($C_8H_8N_4O_3$). To differentiate between these two isomers, M-1 was analyzed by nuclear magnetic resonance (vide infra), allowing us to ascribe structure III to this compound. The generation of the fragment at m/e = 111 may be explained according to the following pathway:



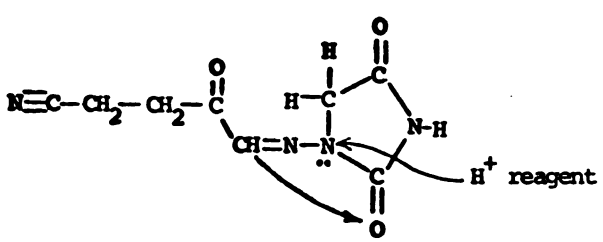


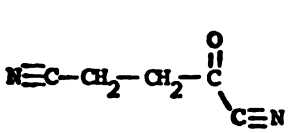
m/e = 111

+

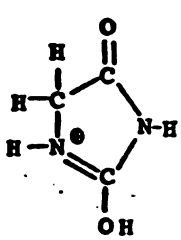


The generation of the fragment at m/e = 101 may result from the interaction of the reagent gas proton at two sites on the 2,4-imidazolidinedione moiety, according to the following pathways:

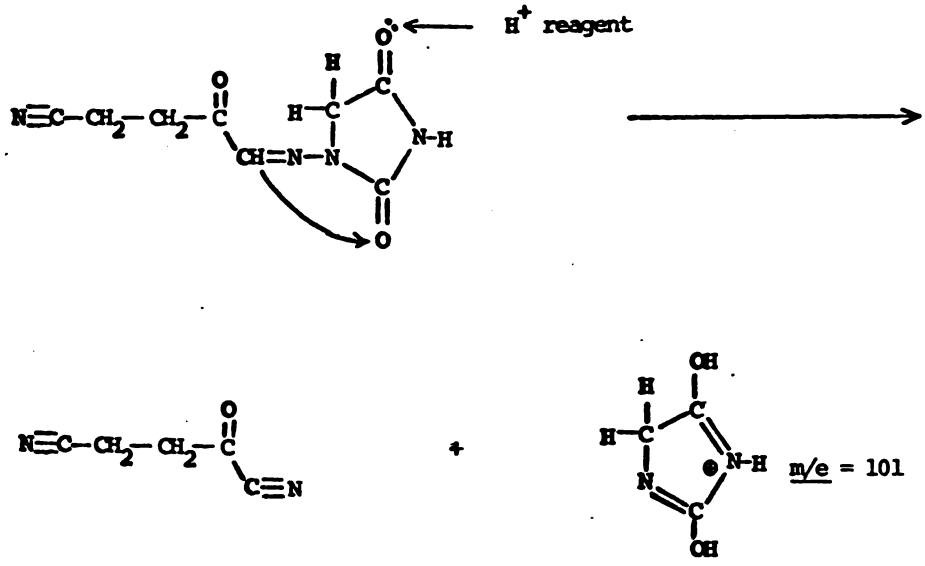




+



m/e = 101



Nuclear magnetic resonance spectrometry:

The use of Fourier transform NMR made it possible to measure conventional high-resolution spectra of samples at millimolar concentrations. Fig. IV.10 shows the basic equipment required to perform the NMR experiment. In the normal mode of NMR operation, spectra require several minutes to record because each transition is induced in succession by continuous scan from low field to high field (so-called continuous wave operation). However, when all transitions are stimulated simultaneously, as is done in pulse NMR, the excitation requires only a fraction of a second to execute. The sample is irradiated (at fixed fields) with a strong pulse of radiofrequency (RF) energy containing all the frequencies over the ^{1}H range

(i.e., a spread around 100 MHz at 2.3 T). The RF pulse duration used was 5 μsec; when the RF pulse is terminated, nuclei undergo relaxation processes and re-emit the absorbed energies and coupling energies. These re-emitted energies are emitted simultaneously as a complex pattern called the free induction decay (fid) which decays rapidly. The output is digitalized in a computer and each individual frequency is filtered out from the complex decay pattern. Each of these frequencies is plotted on a linear frequency scale corresponding to the conventional NMR spectrum shown in Fig. IV.11. The fid in Fig. IV.11(a) which is time based, is in the time domain. The transformation to Fig. IV.11(b) which is frequency based (i.e., in the frequency domain) is performed mathematically by Fourier transform. The use of Fourier transform NMR permits analysis at very low concentrations in ^{1}H NMR. This is important since only microgram quantities of the metabolite were available. Using this method, an entire spectrum can be recorded, computerized and transformed in a few seconds, and by repetition every four seconds, 1,000 spectra can be accumulated in 66 minutes, thereby providing sufficient signal enhancement for meaningful spectra.

Fig. IV.12 shows the NMR spectrum of M-1. Table IV.3 summarizes the chemical shifts data and their assignments. The NMR spectrum of the parent compound

nitrofurantoin in D_2O is shown in Fig. II.4 for reference purposes. The methylene protons from the hydantoin ring shifted from δ = 4.40 in nitrofurantoin to 4.20 in M-1, and the azomethine proton shifted from δ = 7.80 in nitrofurantoin to 7.10 in M-1. The aromatic furan resonances (δ = 7.15 and 7.62) seen in nitrofurantoin were no longer present in M-1 but were replaced by a doublet of triplets (δ = 2.76 and 3.35). The disappearance of the aromatic protons and the appearance of two vicinal methylene groups strongly suggest that M-1 is the open-chain nitrile derivative of nitrofurantoin (III in Fig. IV.7).

E. Metabolism in the isolated perfused rat liver

Materials and Methods

Adult male Sprague-Dawley rats (300-350 g) were used as liver donors. A total of four livers were studied. The liver was isolated and perfused using the method of Brauer et al. (Brauer et al., 1951). Blood for the liver perfusate was obtained from retired male Sprague-Dawley breeders. Livers were perfused via the portal vein with 25% donor blood in Krebs-Ringer bicarbonate buffer containing 1% (w/v) glucose. Heparin (0.9 ml per 100 ml of perfusate) was added. An extracorporeal plexiglass perfusion box maintained at 37°C was used for the experiments. Livers were

perfused at a flow rate of 2.0 ml of perfusate per minute per g of liver. The perfusate was aerated with a mixture of 95% O_2 and 5% CO_2 (Matheson Gas Products) using a membrane oxygenator made from 15 feet of Silastic® tubing of 1.47 mm i.d. and 0.25 mm wall thickness (Dow Corning Corp., Midland, MI) (Hamilton et al., 1974). The liver and perfusate were equilibrated for 30 min prior to the addition of 1.2 mg of nitrofurantoin in 1 ml of DMSO to the reservoir containing 100 ml of perfusate giving an initial concentration of 0.05 mM nitrofurantoin in the perfusate. One-half ml samples of perfusate were taken from the reservoir outflow at times 5, 10, 15, 20, 30, 45, and 60 min, and added to one ml of cold methanol. In order to determine the HPLC characteristics of the perfusate alone, a one-half ml sample was removed from the perfusate reservoir five min prior to the addition of nitrofurantoin.

Samples for time zero were prepared by adding 0.12 mg of nitrofurantoin in 0.1 ml of DMSO to 9.9 ml of fresh perfusate giving a 0.05 mM solution of nitrofurantoin in perfusate. In order to determine the recovery of nitrofurantoin from the perfusate, a 0.05 mM solution of nitrofurantoin was prepared in distilled water and analyzed in a manner identical to that of the perfusate samples.

Bile was collected, at timed intervals, from a cannula in the bile duct. The amount of nitrofurantoin in the bile was determined by adding 300 µl of bile to 600 µl of cold methanol and performing the HPLC analysis as described below. After completion of the experiment, the livers were dried and weighed.

Analyses of bile and perfusate were performed using a modification of the HPLC assay for nitrofurantoin in plasma and urine (Aufrère et al., 1977b). Following deprotination with methanol as described for studies in tissue, the samples were mixed and centrifuged at 10,000 xg for 20 min. The supernatant was decanted and 100 µl samples injected into the HPLC. The following conditions were used: a reverse-phase µBondapak C_{18} preparative column (Waters Associates; 30 cm x 7 mm) with a flow rate of 4 ml/min of a mobile phase consisting of 30% methanol/70% water. Ultraviolet detection was monitored at 365 nm.

Results

In preliminary experiments, initial perfusate concentrations were 0.5 mM nitrofurantoin. Under these conditions, the kinetics obtained were non-linear and dose-dependent, with a half life of approximately 20 min in the linear portion of the

curve observed at low substrate concentrations. All subsequent experiments were therefore performed with an initial substrate concentration of 0.05 m$\underline{M}$ in the perfusate. Under these conditions, the kinetics obtained for the disappearance of nitrofurantoin followed a first-order process with a half-life of 16 minutes. The disappearance of nitrofurantoin from the perfusate of the isolated perfused rat livers is shown in Fig. IV.13. The linearity of the kinetics was maintained throughout the period tested, corresponding to more than three half-lives. The livers were perfused with 25% male rat blood at a flow rate of 2.0 ml/min - g liver. Under these conditions, the oxygen supply to the liver was calculated to be approximately 0.10 ml O_2/min - g liver.

Secretion of nitrofurantoin into the bile occurred throughout the duration of the experiment. At any given time following equilibration of nitrofurantoin in the perfused rat liver, concentrations of nitrofurantoin in the bile were higher than those in the perfusate. This effect was for the most part due to the rapid metabolism of nitrofurantoin present in the perfusate since once excreted in the bile, the collected nitrofurantoin was no longer exposed to the metabolizing hepatic enzymes. As the experiment progessed there was a linear increase in the ratio of

bile to perfusate nitrofurantoin concentrations. Fig. IV.14 depicts this apparent concentrating effect in biliary secretion, the ratio of bile/perfusate being unity at the start of the experiment, whereas after 45 min the bile/perfusate ratio was four.

The water recovery experiments consistently indicated that the nitrofurantoin samples in water contained only 90% of the nitrofurantoin concentrations found in the perfusate at time zero. This reproducible effect was attributed to the precipitation of proteins from the perfusate. The volume occupied by the precipitated plasma proteins resulting in the apparent increase in nitrofurantoin concentration.

Using the HPLC elution program already described (0-30% methanol in 20 min, program 10) M-1 the major metabolite of nitrofurantoin was detected and collected in both the perfusate and the bile (retention time = 21 min). The ultraviolet spectrum for M-1 which was recorded in a Cary spectrophotometer, model 118, was identical to that of M-1 obtained during the anaerobic incubation of nitrofurantoin with the 9,000 xg supernatant fraction of rat liver homogenate (Fig. IV.6).

F. Preparation of 1-[[(3-cyano-1-oxopropyl)methylene] amino]-2,4-imidazolidinedione.

Materials and Methods

Nitrofurantoin (10 mmole) was added to a mixture of 1.0 g of 10% palladium on carbon catalyst (ICN, K and K Lab., Inc., Irvine, CA, Lot No. 13416-A) in 20 ml of methanol. Catalytic hydrogenation was performed in a Parr pressure-reaction apparatus (Parr Instrument Co., Inc., Moline, IL) in subdued light. The hydrogenation was stopped after 4 hours, the catalyst filtered and the filtrate stored overnight at 4°C. The crude preparation of 1-[[(3-cyano-1-oxopropyl)methylene] amino]-2,4-imidazolidinedione (III in Fig. IV.7) was purified by silicic acid column chromatography (Unisil, 100-200-mesh; Clarkson Chem. Co., Williamsport, PA) using chloroform:methanol (80:20) as solvent. The purified fraction was then taken to dryness in a rotary evaporator. Final purification of III was achieved by preparative HPLC using the water:0-30% methanol program described for the purification of M-1. About 3 mg of III in Halocarbon oil 11-14 was placed on a KBr disk and the infrared spectrum obtained on a Perkin-Elmer, model 457, grating infrared spectrophotometer. The UV absorption spectrum of III in distilled water and its CI mass spectrum were recorded as described previously.

Approximately 5 mg of III was used for elemental analysis (C,H,N).

The molar absorptivity of III was determined by preparing solutions in distilled water in the concentration range 2.1×10^{-5} to 2.1×10^{-4} M. The absorbance of each solution at 278 nm was plotted against its concentration and the molar absorptivity was obtained from Beer's law.

Results

The progress of the catalytic hydrogenation of nitrofurantoin was monitored by withdrawing 0.5 ml samples from the mixture at hourly intervals and recording their UV spectra. The disappearance of nitrofurantoin was rapid as determined by the absence of absorption bands at 265 and 370 nm after one hour. Two new UV bands appeared with absorption maxima at 348 and 278 nm. The absorption band at 348 nm suggested the presence of aminofurantoin (II in Fig. IV.7) previously synthesized and characterized by Ebetino et al. (Ebetino et al., 1962). After four hours of hydrogenation, no absorption at 348 nm was detected. The crude product was purified by silicic acid column chromatography and then lyophilized, yielding a tan-colored powder. Further purification was obtained by HPLC using the same elution program described for the isolation of M-1. The purified reduction product gave

retention times identical to those of the biologically formed M-1, eluting between 21.0 and 21.5 min. Lyophilization of this fraction yielded a white powder. The UV absorption spectrum of this powder in distilled water was identical to that of M-1 with a λ_{max} at 278 nm. Aqueous solutions of the product appeared stable, based on the UV absorption spectrum. The dry purified white powder obtained from lyophilization was stable under reduced pressures but its exposure to air resulted in the formation of a dark brown resinous material, suggesting the formation of polymers as oxidation products. We were therefore unable to determine the melting point of this material. Elemental analysis was therefore also unsatisfactory (Anal. calculated for $C_8H_8N_4O_3$: C,46.15; H,3.85; N,26.92; found: C,45.95; H,4.58; N,26.07).

With isobutane as the reagent gas, the CI mass spectrum of the purified product gave a protonated molecular ion at m/e = 209, and was identical to the CI mass spectrum of M-1 (Fig. IV.9). Infrared analysis of the synthetic product gave a weak band at 2250 cm^{-1} which is characteristic of nitriles. The absence of the bands in the range 1500-1550 cm^{-1} indicated the loss of the nitro group and the absence of bands in the region of 1020 cm^{-1} represented the loss of the furan ring. Additional bands occurred at 1600 cm^{-1} (C=N) and 1720 cm^{-1} (C=0). From these

data, it was concluded that the product formed by catalytic hydrogenation of nitrofurantoin was III, and that this material was identical to the biologically produced M-1.

The molar absorptivity of III was obtained from the absorbance-concentration data shown in Fig. IV.15. In accordance with Beer's law, a straight line passing through the origin was observed. Table IV.4 gives the data used for Fig. IV.15 and includes the molar absorptivities calculated for each solution, the calculated molar absorptivity ε_{278} is 1.99×10^3 liter/mole-cm.

G. Preparation of 1-[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione

Materials and Methods

Nitrofurantoin was hydrogenated according to the method of Ebetino et al. (Ebetino et al., 1962). The crude 1-[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione (II in Fig. IV.7) was separated from the palladium on carbon catalyst by stirring the mixture in DMSO, filtering the catalyst and precipitating II by diluting the filtrate with water. Crude crystals of II were then washed twice with nitromethane. Recrystallization was effected by dissolving II in 1 N HCl, filtering any remaining

impurities, and alkalinizing the filtrate with 1 N NaOH to pH 5.5-6.0. The purity of the reprecipitated II was checked by UV absorption spectroscopy, HPLC and CI mass spectral analyses.

Results

The UV absorption spectrum of II in water showed a λ_{max} at 348 nm, similar to what had been reported previously (Ebetino et al., 1962). With isobutane as the reagent gas, the CI mass spectrum of II gave a protonated molecular ion at m/e = 209, with additional fragments at m/e = 101, 109, and 111 (Fig. IV.16). With the use of the same HPLC elution program described for the characterization of metabolites in various tissues (Fig. IV.3) II had a retention time (24.0-24.5 min) and UV absorption characteristics identical to the biologically formed M-4. From these data, it was concluded that the two metabolites formed during the incubation of nitrofurantoin with cecum and colon contents -- M-1 and M-4 -- are III and II, respectively.

H. Mutagenicity of nitrofurantoin, 1-[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione, and 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione in Salmonella typhimurium.

Developed by B. Ames, the Salmonella typhimurium tester strains were specifically selected for sensitivity

and specificity for reversion from histidine auxotrophs to prototrophs ($his^- \rightarrow his^+$ reversions) by mutagens (Ames et al., 1975; McCann et al., 1975a). The test system was able to detect 85 percent of a wide variety of carcinogens as mutagens (McCann et al., 1975b). Chemicals which need to be metabolically activated to become mutagens may also be tested if the 9,000 xg supernatant fraction of liver homogenate is added to the test system (Ames et al., 1975).

Two of the basic tester strains, TA-1535 and TA-1538, are used to detect mutagens causing base pair substitutions (hisG46 histidine mutation) and frameshift mutations (hisD3052 histidine mutation), respectively. Each tester strain also contains two additional mutations to increase their sensitivity to mutagens. One mutation results in the loss of the excision repair system responsible for repairing damaged DNA, the other results in the loss of the lipopolysaccharide coat on the bacterial surface thereby increasing the permeability of chemical mutagens through the bacterial walls (Ames et al., 1975; McCann et al., 1975a. The two new tester strains, TA-100 and TA-98, were developed by the addition of an R-factor plasmid to the standard tester strains, TA-1535 and TA-1538, respectively, thereby making the new strains more sensitive to certain mutagens (Ames et al., 1975; McCann et al., 1975a). The R-factor designates plasmids which carry antibiotic resistant genes; in the case of TA-100 and TA-98 the plasmid carries ampicillin resistant genes.

Furylfuramide (AF-2), a nitrofuran, is mutagenic in the new tester strains (McCann et al., 1975b; Wang et al., 1975), whereas the TA-1535 and TA-1538 tester strains were not reverted by furylfuramide (McCann et al., 1975b). Other nitrofuran derivatives, including nitrofurantoin, are mutagenic when tested with the TA-100 and TA-98 tester strains (Goodman et al., 1977).

McCalla et al. (McCalla et al., 1971b) and Wang et al. (Wang et al., 1975) have proposed that following enzymatic reduction of 5-nitrofurans a reactive N-hydroxylamine intermediate is formed and is responsible for covalent tissue binding. It has also been argued that mutagenesis occurs via a reduced metabolite (presumably the N-hydroxylamine intermediate) since metabolic reduction of the 5-nitro group appears to be necessary for mutagenicity (Rosenkranz and Speck, 1976). It was therefore decided to test the mutagenicity of the two metabolites of nitrofurantoin--aminofurantoin and 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione (II and III, respectively in Fig. IV.7)--by using the TA-100 tester strain which is the most sensitive strain to 5-nitrofuran derivatives. Previous work had demonstrated that of the wide variety of nitro heterocyclics which were mutagenic in Salmonella typhimurium and E. coli, the corresponding amino heterocyclic derivatives were not reported as mutagens in these same strains (Wang et al., 1975; McMahon, 1976).

Similarly, the 5-aminofurans are probably not directly mutagenic in S. typhimurium. However, these compounds may become positive mutagens in S. typhimurium if enzymatically activated. Since the mixed function oxidase system may be able to oxidize a 5-aminofuran to the N-hydroxylamino derivative, we tested the mutagenicity of the compounds with and without the addition of the 9,000 xg supernatant fraction of rat liver homogenate (S-9 mix). This oxidation may render the 5-aminofuran compound mutagenic by a mechanism similar to the enzymatic reduction of a 5-nitrofuran to its N-hydroxylaminofuran metabolite.

Materials and Methods

Nitrofurantoin (Sigma Chem. Co.) was used without further purification. 1-[[(5-Amino-2-furanyl)-methylene]amino]-2,4-imidazolidinedione, and 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione (II and III, respectively) were prepared from the catalytic hydrogenation of nitrofurantoin as already described.

The purity of II and III was determined by HPLC. Based on the conditions used for HPLC analysis (sensitivity of the 365 nm detector set at 0.1 A full scale) the purity was determined to be greater than 99.6 percent.

Salmonella typhimurium tester strains TA-100 (TA-1535/pKM101) and TA-98 (TA-1538/pKM101) were

provided by B. Ames (Department of Biochemistry, University of California, Berkeley). The bacterial tester strains were cultured and stored at -80°C (Appendix I). Autoclaved wooden sticks were used to inoculate sterile test tubes containing 5 ml of nutrient broth (Appendix I) from the frozen cultures. The inoculated tubes were capped and incubated at 37°C while shaking in a water bath overnight (12-16 hours). The top agar overlay was prepared according to the following proportions: a 100 ml portion of the top agar (Appendix I) was autoclaved in a flask and placed in a water bath at 40°C to prevent hardening. Ten ml of 0.05 m*M* histidine•HCl - 0.05 m*M* biotin solution sterilized by filtration (Appendix I) was added to the top agar. The trace of histidine in the top agar allows all the bacteria on the plate to undergo several divisions; once histidine has been depleted, all growth stops. This early growth may be necessary for mutagenesis to occur (Ames *et al.*, 1975).

In experiments in which the S-9 mix and the test compound were poured onto the plate without prior incubation, 2.0 ml aliquots of the top agar were pipetted into autoclaved and empty culture tubes at 40°C. Aliquots (0.1 ml) of the fresh culture of the tester strain were added to the top agar mix, to which 0.4 ml of the S-9 mix (Apendix I) was added.

Nitrofurantoin and II were dissolved in DMSO, whereas III was dissolved in distilled water and the compounds were added to the culture tube. The volume of DMSO used ranged between 0.01 to 0.5 ml per plate. Less than 0.5 ml of DMSO does not interfere with mutagenesis (Ames et al., 1975). The contents of the tubes were mixed and then poured on minimal glucose agar plates (Appendix I). Uniform distribution of the top agar was accomplished by gently tilting and rotating the plate immediately after pouring. The plates were left to harden for 20 minutes and then put upside down in a dark 37°C incubator. The colonies, which represent bacteria which had reverted to histidine prototrophy, were counted after 48 hours (Ames et al., 1975).

In experiments in which the S-9 mix and the test compound were incubated prior to being poured onto the plate, the following solutions were added to culture tubes which were kept on ice (in order): 0.4 ml of the S-9 mix, 0.1 ml of the fresh culture of the tester strain, and the sample to be tested (usually in 0.1 ml or less). The culture tube was then incubated for 15 min at 37°C. The reaction was stopped by the addition of 2 ml of top agar and the contents of the tube mixed and poured on minimal glucose agar plates (Appendix I) as previously described. The plates were left to harden for 20 minutes, and then put upside down in a

dark 37°C incubator, and the colonies were counted after 48 hours.

Results and Discussion

Approximately 85 percent of a wide variety of compounds which are carcinogenic in mammals have been shown to revert Salmonella typhimurium histidine auxotrophs (Ames et al., 1975). The carcinogenicity of nitrofurantoin, which is mutagenic in TA-100 at doses of less than 0.25 µg per plate (Goodman et al., 1977), has not been demonstrated in feeding studies in rats (Morris et al., 1969; Cohen et al., 1973; Tazima et al., 1975). The bacterial tester strains are particularly sensitive to reversion by aromatic nitro compounds possibly because these compounds can be metabolically activated (presumably to the N-hydroxylamine intermediate) by nitroreductases in the Salmonella system (McCann and Ames, 1976). Nitroreductases present in mammalian liver and gut bacteria may also play a role in the metabolic activation of nitro carcinogens (Rosenkranz and Speck, 1976). It appears that all nitrofurans tested can revert TA-100 and this could be a general property of the nitrofurans (Tazima et al., 1975; Yahagi et al., 1976).

Preliminary work was performed both with the TA-98 and TA-100 tester strain to determine the sensitivity of both of these strains to the mutagenicity of nitro compounds. Using nitrofurazone and nitrofurantoin as test compounds, the TA-100 tester strain was selected for further work since it provided greater sensitivity to the reversion of histidine auxotrophs to prototrophs by nitro compounds (Table IV.6). These results are typical of the mutagenic properties of all nitrofurans tested in TA-100 and TA-98. For instance, TA-98 was less sensitive both to reversion and to the bactericidal properties of nitrofurazone (Goodman et al., 1977).

The sterility of the agar and of the S-9 mix was tested by addition of the S-9 mix in the absence of the bacterial tester strain. In no case was there any reversion noted, indicating that both the agar and the S-9 mix had not been contaminated. Nitrofurantoin was found to be strongly mutagenic to TA-100 tester strain at doses of less than 0.25 μg per plate, in good agreement with the results of Goodman et al. (Goodman et al., 1977). The addition of the S-9 mix did not alter the pattern of mutagenicity for nitrofurantoin. 1-[[(3-Cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione (III) when tested at 2.5 μg per plate showed a doubling in the number of revertants as compared with background (Table IV.6). There were

however, no significant changes in the number of revertants over the concentration range of 2.5 to 100 µg per plate. This compound was therefore not considered to be mutagenic in TA-98 or TA-100. Moreover, since the addition of the S-9 mix did not alter the number of revertants seen with III, it was concluded that III was not metabolically activated to a mutagen.

Addition of 1-[[(5-amino-2-furanyl)methylene] amino]-2,4-imidazolidinedione, (aminofurantoin, II) at doses between 0.5 and 10.0 µg/plate did not significantly alter the number of revertants as compared with background (Table IV.5). However, at high dose levels of 100 to 500 µg/plate, aminofurantoin markedly increases the number of histidine revertants. As determined from HPLC, the purity of aminofurantoin was greater than 99.6%. At the high dose levels of aminofurantoin used, the nitrofurantoin present (less than 0.4%) will be detected by the bacterial system due to the high mutagenic potency of nitrofurantoin. In a series of 44 aromatic amines tested for mutagenicity by McCann et al., the test compounds were not considered mutagenic when more than 250 µg of the aromatic amine added per plate gave negative results since calculation of the number of revertants per nmole was very low (McCann et al., 1975b). The addition of the S-9 mix

did not change the number of revertants seen at a given dose of aminofurantoin. From the lack of effect of low doses of aminofurantoin with or without the S-9 mix, we concluded that aminofurantoin was not a mutagen under the conditions tested.

APPENDIX I

Solutions and media for the mutagenicity bacterial testing (Ames et al., 1975).

Nutrient Broth

Difco-Bacto® nutrient broth	8 gm
Sodium chloride	5 gm
Distilled water	1000 ml

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

Storage of Tester Strains

Freshly grown up nutrient broth culture	0.80 ml
Dimethyl sulfoxide	0.07 ml

Store at -80°C in 2 ml sterile glass vials.

Minimal Glucose Agar Plates

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

Autoclave solutions A, B, and C in separate flasks; glucose will caramelize if autoclaved in solution with A or B. While still hot after autoclaving mix solutions together and pour plates (25 ml/plate).

VB Salts (Vogel and Bonner, 1956)

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

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

Top Agar

Agar (Difco®)	0.6 gm
Sodium chloride	0.5 gm
Distilled water	100 ml

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

Histidine - Biotin Solution

Dissolve L-histidine•HCl (0.5 mM) and biotin (0.5 mM) in distilled water. Sterilize by filtration using a 0.22 μm Millipore filter. Do not autoclave the histidine-biotin solution, since it is heat labile.

S-9 mix

The rat liver enzymes were obtained from male rats (Sprague Dawley derived, about 200 g). Induction of the liver enzymes was obtained by treating the rats with a single i.p. injection of Aroclor 1254 (500 mg/kg) four days prior to sacrifice).

The livers (10-15 g each) were weighed and then placed in beakers containing two volumes of 1.15% KCl-0.01 M phosphate buffer pH 7.7 (2 ml/g wet liver), minced with scissors, and homogenized in a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for twenty minutes at 9,000 xg and the supernatant (the S-9 fraction) decanted and filtered through cheesecloth. The 9,000 xg fraction was then centrifuged at 100,000 xg for 60 min. The supernatant discarded, the microsomal pellet suspended in the buffer and centrifuged a second time at 100,000 xg for 60 min. The supernatant was discarded and the microsomal pellet homogenized in buffer. Microsomes were stored at -80°C. On the day of the experiment, sufficient amounts of the microsomal pellet were thawed at room temperature, kept on ice, and the S-9 mix prepared.

The S-9 mix contains per plate: 15 µl of 0.1 M $MgCl_2$, 5 µl of 0.1 M EDTA, 25 µl of 0.005 M pH 7.4 phosphate buffer, 10 µl of 0.05 M $NADP^+$, 50 µl of

0.05 M glucose-6-phosphate, 25 µl of 0.02 M glucose-6-phosphate dehydrogenase, 70 µl of water, and 200 µl of the microsome mix (obtained by diluting 1 ml of microsomal fraction with 12.5 ml of water). Possible bacterial contaminants were removed by filtration of the liver S-9 mix through a sterile disposable filter unit (Nalge, 0.45 µm pore size).

TABLE IV.1. ANAEROBIC INCUBATION OF NITROFURANTOIN BY VARIOUS RAT TISSUES.

Results are the means ± SE of duplicate determinations of three different preparations, the tissue from each preparation was obtained from two rats, representing a total of six rats per group. Incubation mixtures contained potassium phosphate buffer pH 7.4 (200 µmole), $NADP^+$ (1 µmole), glucose-6-phosphate (30 µmole), $MgCl_2$ (15 µmole), tissue homogenate (from 200 mg of tissue) or tissue 9,000 xg supernatant (from 250 mg of tissue), and nitrofurantoin (1.26 µmole) in a total volume of 8.0 ml. The reactions were linear with time.

Preparations	Nitroreductase Activity : Nitrofurantoin Disappearance (nmole/mg protein-min)		
	Germ-free	Germ-free Acclimatized	Control
Tissue homogenate			
Liver	0.20 ± 0.02	0.20 ± 0.02	0.21 ± 0.01
Small intestine walls	0.19 ± 0.04	0.16 ± 0.03	0.18 ± 0.02
Small intestine contents	∿0.00	∿0.00	∿0.00
Cecum and colon contents	∿0.00	0.37 ± 0.02	0.39 ± 0.02
Kidney	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.01
Tissue 9,000 xg supernatant fraction			
Liver	0.42 ± 0.02	0.41 ± 0.03	0.43 ± 0.01
Small intestine walls	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Small intestine contents	∿0.00	∿0.00	∿0.00
Cecum and colon contents	∿0.00	∿0.00	∿0.00
Kidney	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01

Table IV.2

ELECTRON IONIZATION MASS SPECTRAL DATA FOR THE HPLC FRACTION COLLECTED BETWEEN 22.0 AND 22.5 MIN (NICOTINAMIDE).

Molecular Weight	m/e (% relative abundance) of peaks above m/e = 60	
122	78	(94)
	106	(72)
	122	(100)

Table IV.3

^{1}H-NMR SPECTRAL ASSIGNMENTS FOR M-1

Band (δ, ppm)		Assignment
triplet	2.76	<u>a</u> in $N\equiv C-\underline{\overline{a}}H_2-C\underline{\overline{b}}H_2-$
triplet	3.35	<u>b</u> in $N\equiv C-\underline{\overline{a}}H_2-C\underline{\overline{b}}H_2-$
singlet	4.20	hydantoin $-CH_2-$
singlet	4.70	HDO (solvent)
singlet	7.10	-CH=N-
doublet 1.3; broad singlets 3.6, 3.8, 5.35		impurities

Table IV.4

ULTRAVIOLET ABSORPTION DATA FOR 1-[[(3-CYANO-1-OXOPROPYL) METHYLENE]AMINO]-2,4-IMIDAZOLIDINEDIONE IN WATER[a]

$10^4 C$ (M)	A_{278}	$10^{-3} \varepsilon_{278}$
2.10	0.372	1.77
1.26	0.255	2.02
1.05	0.208	1.98
0.84	0.169	2.01
0.63	0.131	2.07
0.21	0.045	2.14

$\varepsilon_{287} = 1.99 \times 10^3$ liter/mole-cm

[a]At the absorption maximum, 278 nm.

Table IV.5

MUTAGENICITY TESTING FOR AMINOFURANTOIN IN SALMONELLA TYPHIMURIUM TA-100.

Nitrofurantoin is included as a positive control.

Dose µg/plate	S-9 mix[1]	Incubation[2]	Revertants per plate[3]
Aminofurantoin			
0	-	-	78
0.5	-	-	171
1.0	-	-	90
1.5	-	-	87
2.0	-	-	149
10.0	-	-	221
0	+	-	119
0.5	+	-	155
1.0	+	-	151
1.5	+	-	162
2.0	+	-	117
10.0	+	-	165
0	-	+	160
0.5	-	+	232, 260
1.0	-	+	220, 193
2.0	-	+	198, 265
10.0	-	+	293, 357
100.0	-	+	1032, 954
500.0	-	+	1080, 948

Table IV.5, continued

Dose µg/plate	S-9 mix[1]	Incubation[2]	Revertants per plate[3]
Aminofurantoin			
0	+	+	182
0.5	+	+	174, 161
1.0	+	+	174, 205
2.0	+	+	158, 306
10.0	+	+	254, 247
100.0	+	+	912, 932
200.0	+	+	708
500.0	+	+	864
Nitrofurantoin			
0	-	-	79, 78
0.5	-	-	315, 578
1.0	-	-	795, 1024
0	+	-	119
0.5	+	-	504
1.0	+	-	1364
0	-	+	160
0.5	-	+	940, 920
1.0	-	+	462, 736

Table IV.5, continued

Dose µg/plate	S-9 mix[1]	Incubation[2]	Revertants per plate[3]
Nitrofurantoin			
0	+	+	182
0.5	+	+	590
1.0	+	+	328

1. The S-9 mix was added (+) or not (-) as indicated.
2. Incubation of S-9 and test compound prior to pouring onto the plate, as described in Materials and Methods.
3. Each value represents the *his*$^+$ revertants for a single plate.

Table IV.6

Mutagenicity testing for 1-[[(3-cyano-1-oxopropyl) methylene]amino]-2,4-imidazolidinedione in Salmonella typhimurium TA-100 and TA-98.

Nitrofurantoin is included as a positive control.

Dose µg/plate	Tester Strain	S-9 mix[1]	Incubation[2]	Revertants per plate[3]
1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione				
0	98	-	-	6
5.0	98	-	-	18
10.0	98	-	-	17
50.0	98	-	-	19
100.0	98	-	-	21
0	100	-	-	79, 78
1.0	100	-	-	104
2.5	100	-	-	167
5.0	100	-	-	145, 129
10.0	100	-	-	154, 158
20.0	100	-	-	152
50.0	100	-	-	109
100.0	100	-	-	121
0	100	+	-	119
1.0	100	+	-	126
2.5	100	+	-	137
5.0	100	+	-	130
10.0	100	+	-	134
20.0	100	+	-	161

Table IV.6, continued

Dose µg/plate	Tester Strain	S-9 mix[1]	Incubation[2]	Revertants per plate[3]
Nitrofurantoin				
0	98	-	-	6
0.5	98	-	-	27
1.0	98	-	-	17
0	100	-	-	79, 78
0.5	100	-	-	315, 578
1.0	100	-	-	795, 1024
0	100	+	-	119
0.5	100	+	-	504
1.0	100	+	-	1364
0	100	-	+	160
0.5	100	-	+	940, 920
1.0	100	-	+	462, 736
0	100	+	+	182
0.5	100	+	+	590
1.0	100	+	+	328

1. The S-9 mix was added (+) or not (-) as indicated.
2. Incubation of S-9 and test compound prior to pouring onto the plate, as described in Materials and Methods.
3. Each value represents the *his*$^+$ revertants for a single plate.

Fig. IV.1 LINEWEAVER-BURK PLOT FOR THE ANAEROBIC INCUBATION OF NITROFURANTOIN WITH RAT LIVER 9,000 xg SUPERNATANT FRACTION.

The values represent the mean of three different experiments. The rate of the reaction (V) is expressed as nmol per min per mg protein. Units of S, m$\underline{M}$. Incubation mixtures contained potassium phosphate buffer pH 7.4 (200 µmole), $NADP^+$ (1 µmole), glucose-6-phosphate (30 µmole), $MgCl_2$ (15 µmole), liver 9,000 xg supernatant (from 250 mg tissue), and nitrofurantoin in a total volume of 8.0 ml.

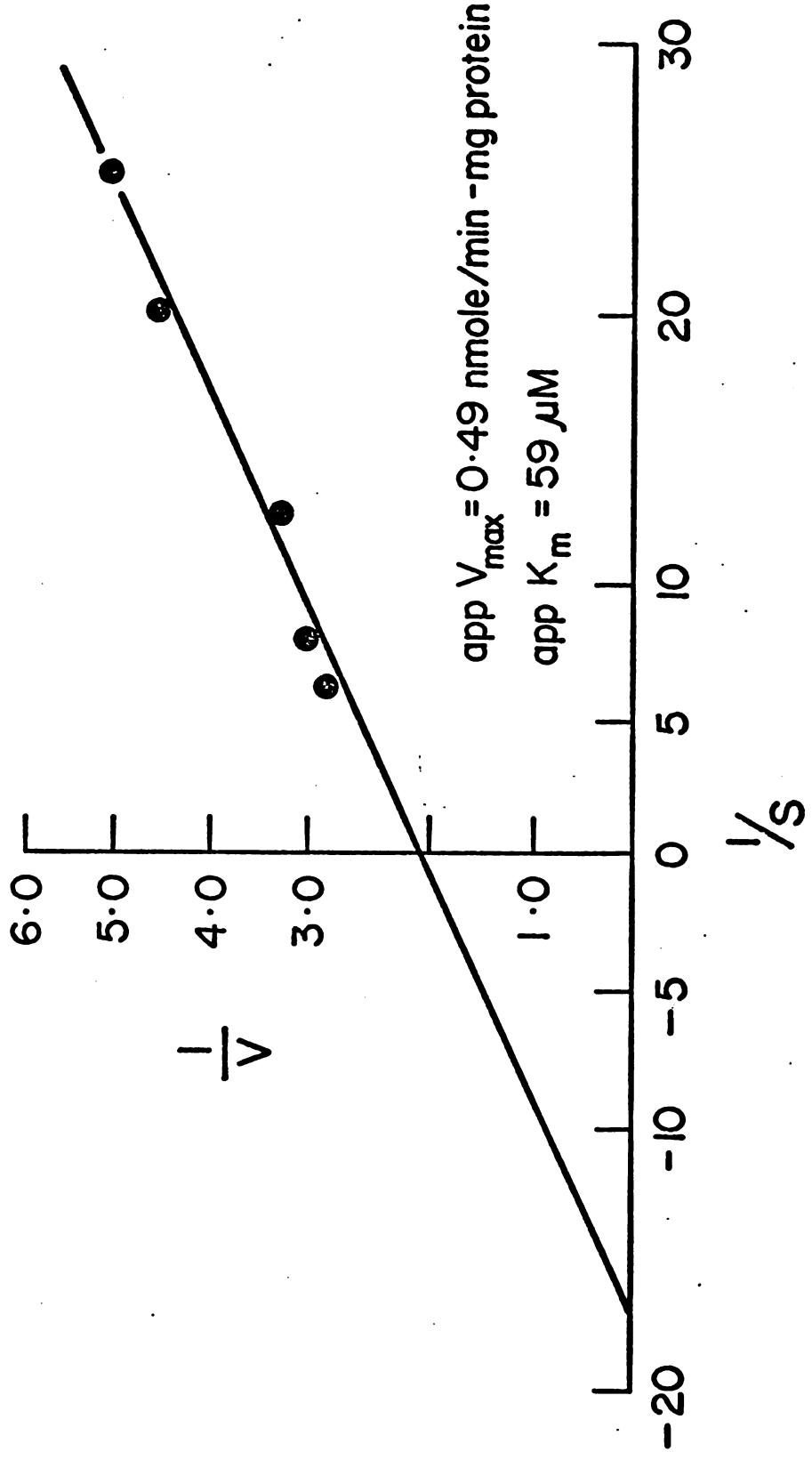

app V_{max} = 0·49 nmole/min-mg protein
app K_m = 59 μM
$\frac{1}{V}$
$\frac{1}{S}$
6·0
5·0
4·0
3·0
1·0
0
5
10
20
30
-5
-10
-20

Fig. IV.2 HPLC ELUTION PROFILE OF THE ANAEROBIC REDUCTION OF NITROFURANTOIN BY RAT LIVER HOMOGENATES.

Incubation mixtures contained potassium phosphate buffer pH 7.4 (200 μmole), $NADP^+$ (1 μmole), glucose-6-phosphate (30 μmole), $MgCl_2$ (15 μmole), tissue homogenate (from 200 mg of tissue), and nitrofurantoin (1.26 μmole) in a total volume of 8.0 ml. Samples were removed after 0, 10, 30, and 45 min of incubation. HPLC conditions were as follows: column: μBondapak C_{18}; flow rate: 4 ml/min; gradient: water:methanol (100:0) to water:methanol (70:30) in 20 min, program 10; the wavelength of detection was 280 nm. Elution times (min) were: nitrofurantoin (26.0-26.5), M-1 (21.0-21.5), M-2 (5.0-5.5), M-3 (3.5-4.0).

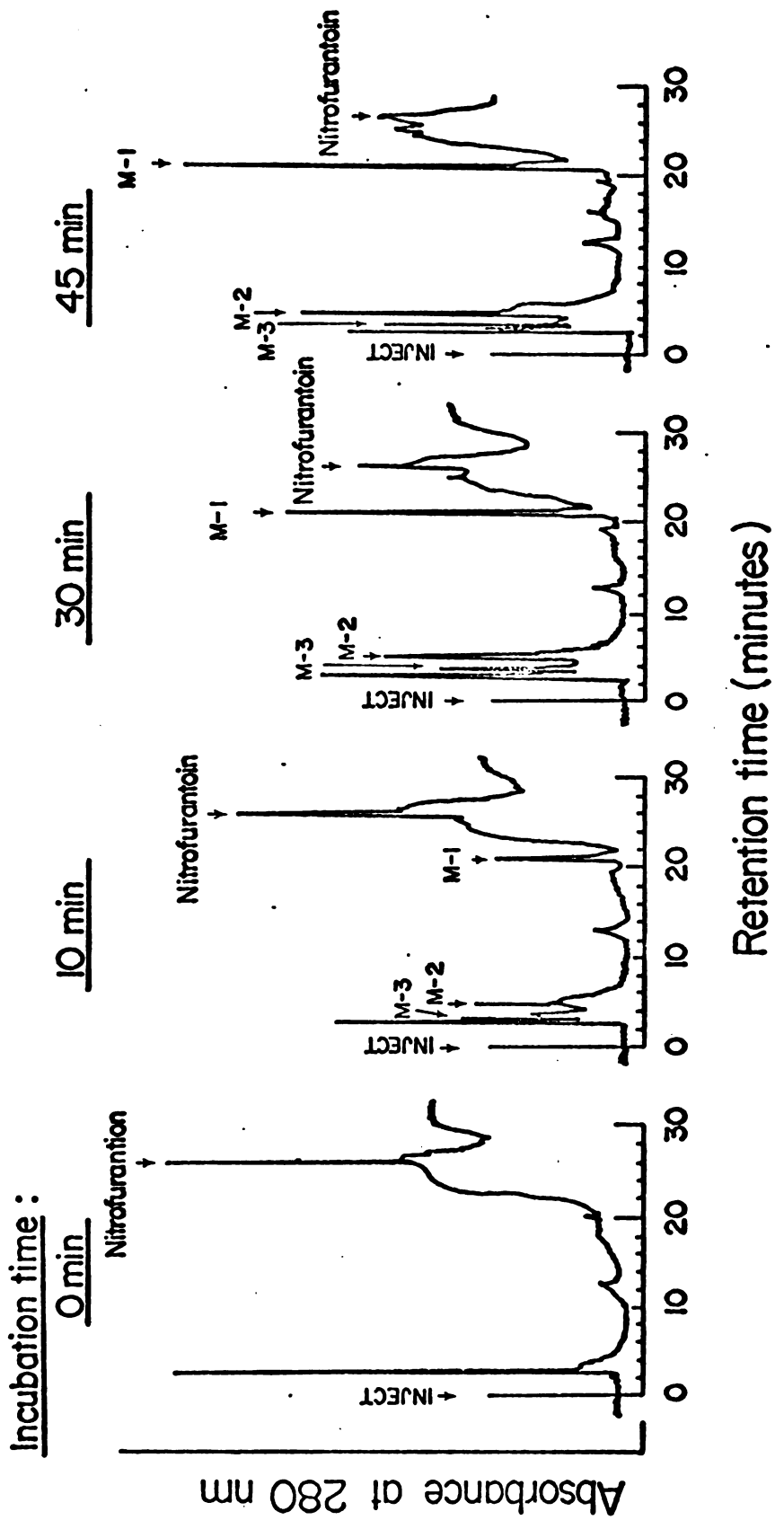

Incubation time :
0 min
10 min
30 min
45 min
Nitrofurantoin
M-1
M-2
M-3
INJECT
Absorbance at 280 nm
Retention time (minutes)
0
10
20
30

Fig. IV.3 HPLC ELUTION PROFILES OF METABOLITES OF NITROFURANTOIN FORMED BY RAT TISSUE HOMOGENATES.

Incubation mixtures contained potassium phosphate buffer, pH 7.4 (200 µmole), $NADP^+$ (1 µmole), glucose-6-phosphate (30 µmole), $MgCl_2$ (15 µmole), tissue homogenate (from 200 mg of tissue), and nitrofurantoin (1.26 µmole) in a total volume of 8.0 ml. Samples were removed after 45 min of incubation. For simplicity, these chromatograms were constructed by subtracting the corresponding control chromatograms (i.e., no nitrofurantoin present). A representative chromatogram of kidney, lung, and small intestine walls, is shown in B. The ratios of M-1, M-2, and nitrofurantoin peaks were similar in each of these three preparations. HPLC conditions were as follows: column: µBondapak C_{18}; flow rate: 4 ml/min; gradient: water: methanol (100:0) to water:methanol (70:30) in 20 min, program 10; the wavelength of detection was 280 nm. Elution times (min) were: nitrofurantoin (26.0-26.5), M-1 (21.0-21.5), M-2 (5.0-5.5), M-3 (3.5-4.0), M-4 (24.0-24.5). M-4 was only observed in cecum and colon contents and was detected at 365 nm. No metabolism was observed in small intestine contents.

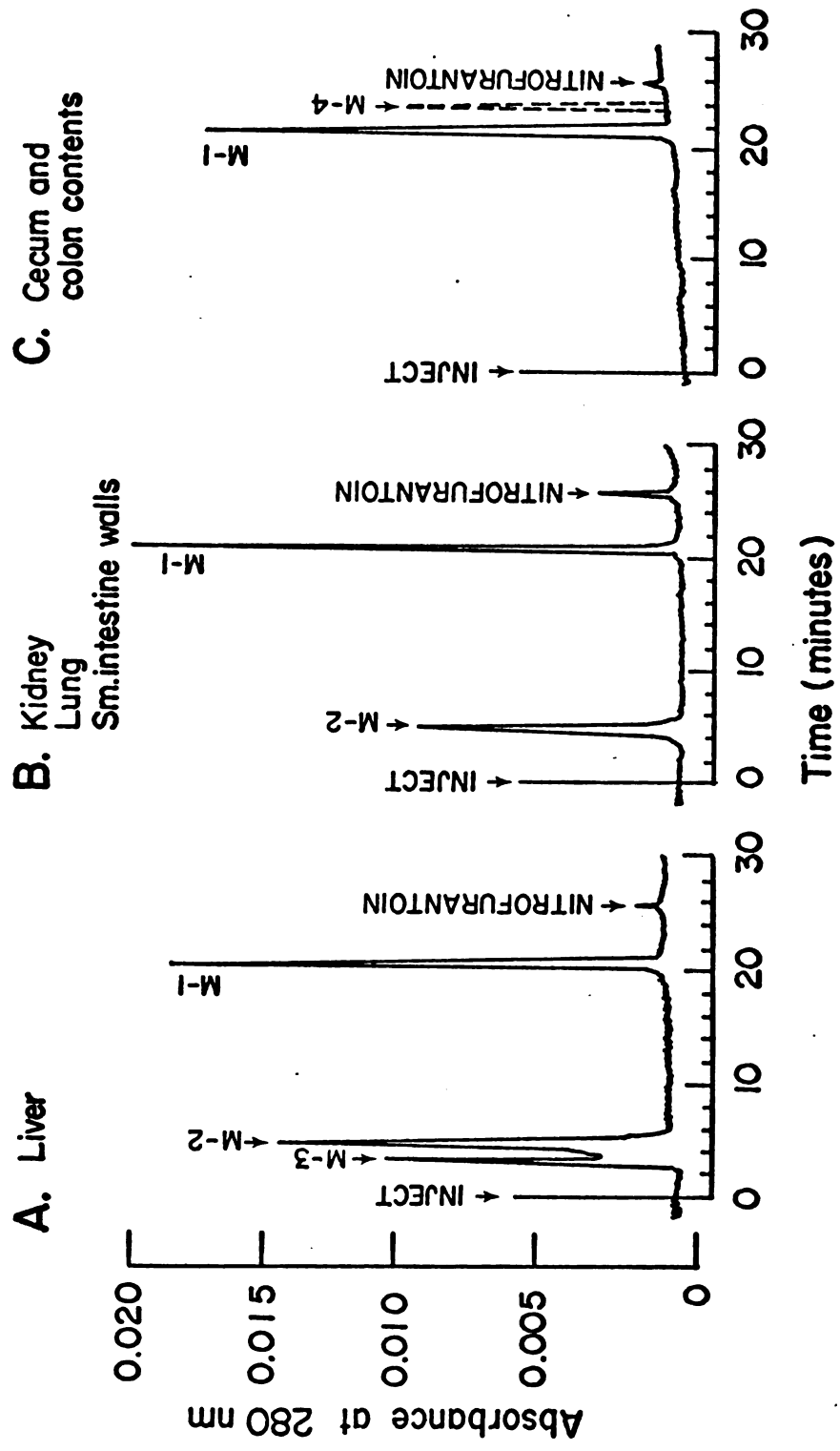

A. Liver
B. Kidney
Lung
Sm.intestine walls
C. Cecum and
colon contents
INJECT
M-1
M-2
M-3
M-4
NITROFURANTOIN
Absorbance at 280 nm
0.020
0.015
0.010
0.005
0
0
10
20
30
Time (minutes)

Fig. IV.4. CHROMATOGRAM FROM HPLC ANALYSIS OF THE PREPARATIVE ANAEROBIC INCUBATION MIXTURE OF NITROFURANTOIN WITH RAT LIVER 9,000 xg SUPERNATANT FRACTION.

HPLC conditions were as follows: column: μBondapak C_{18}; flow rate: 4 ml/min, gradient: water:methanol (100:0) to water:methanol (70:30) in 20 min, program 10; wavelength of detection was 280 nm. Elution times (min) were: nitrofurantoin (26.0-26.5), M-1 (21.0-21.5), M-2 (5.0-5.5), M-3 (3.5-4.0).

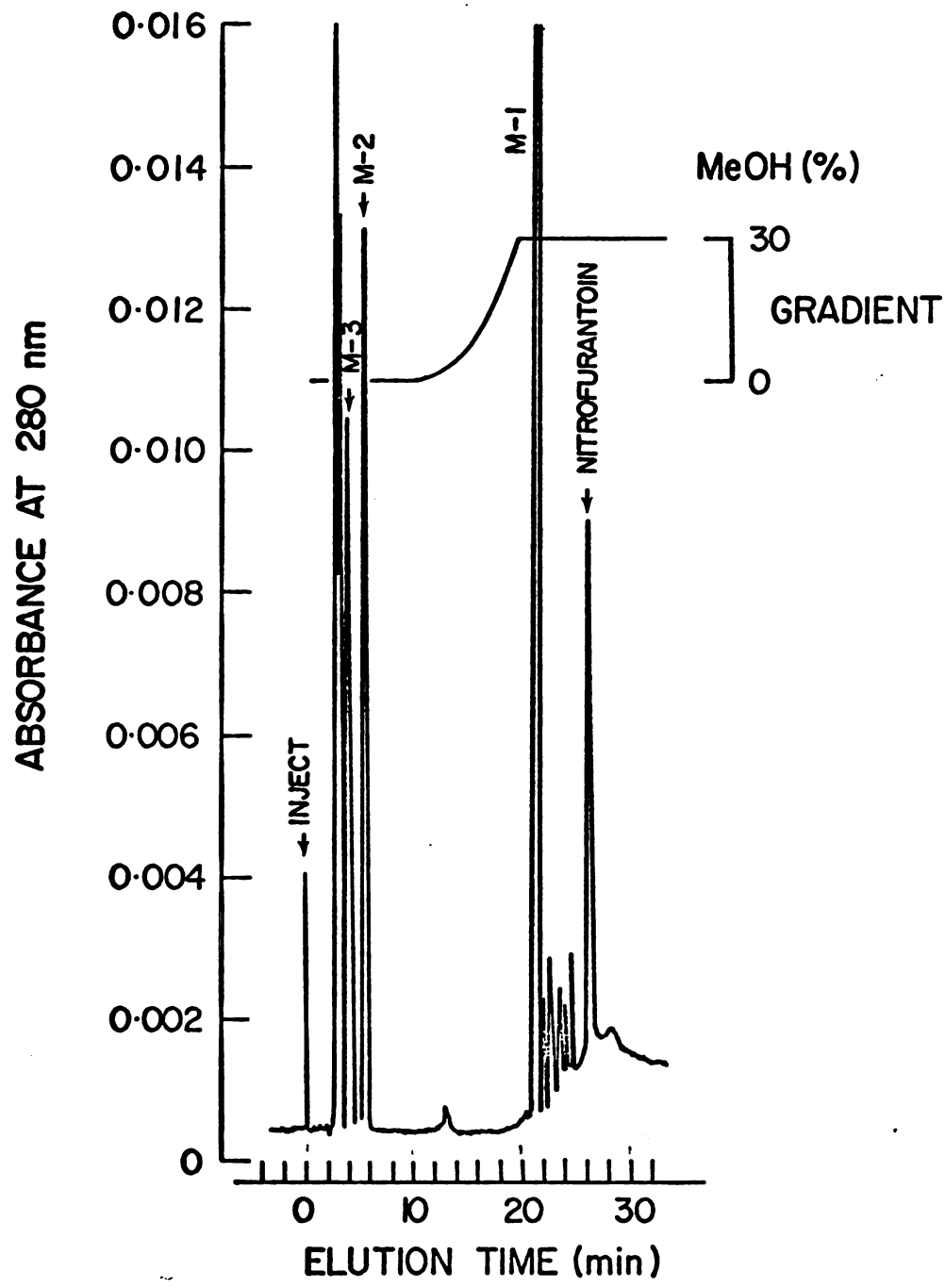
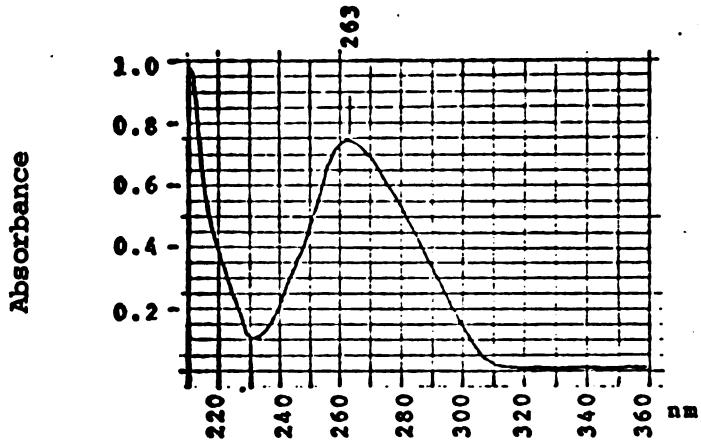
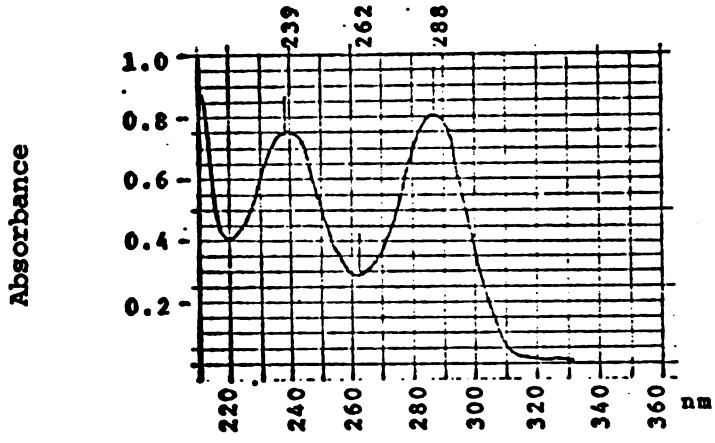

0·016
0·014
0·012
0·010
0·008
0·006
0·004
0·002
0
ABSORBANCE AT 280 nm
INJECT
M-3
M-2
M-1
NITROFURANTOIN
MeOH (%)
30
GRADIENT
0
0
10
20
30
ELUTION TIME (min)

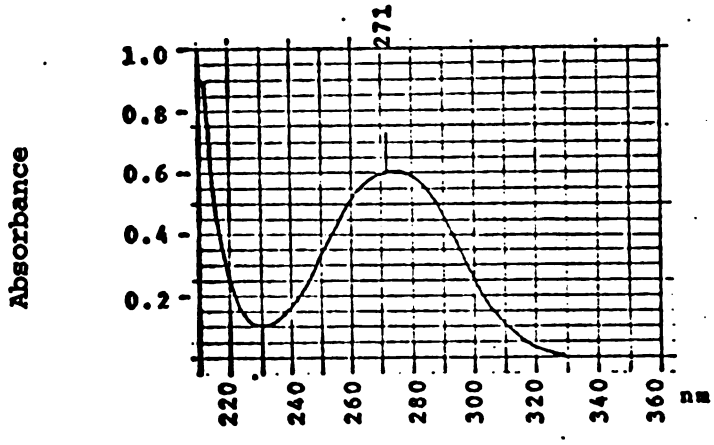
Fig. IV.5 ULTRAVIOLET ABSORPTION SPECTRA OF FRACTIONS COLLECTED FROM HPLC OF NITROFURANTOIN METABOLISM BY RAT LIVER 9,000 xg SUPERNATANT FRACTION.



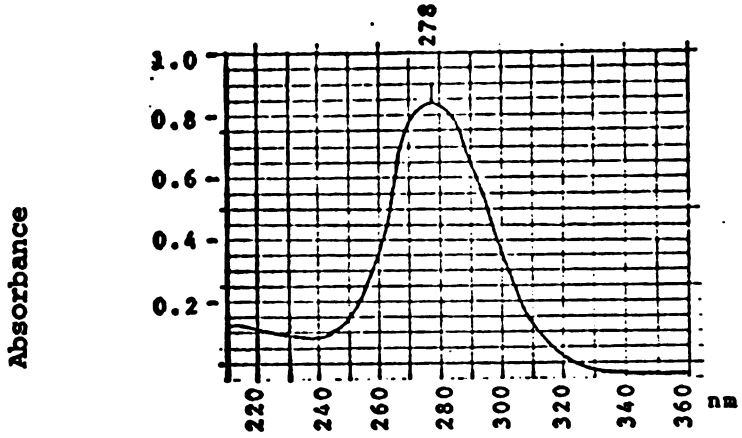
Fraction collected between 3.0-3.5 min.



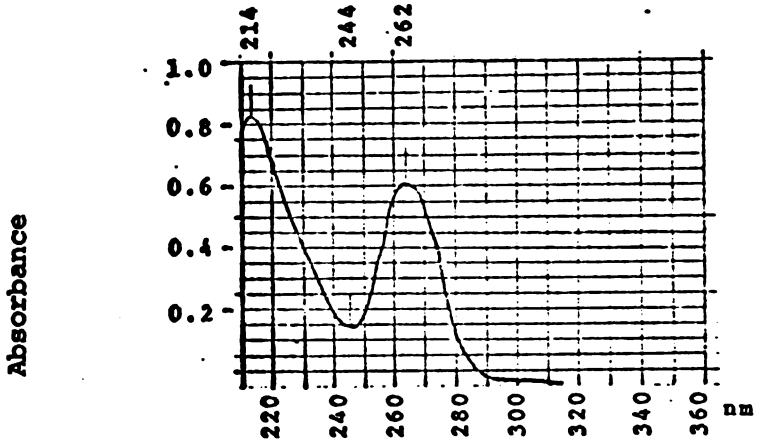
Fraction collected between 3.5-4.0 min (M-3).



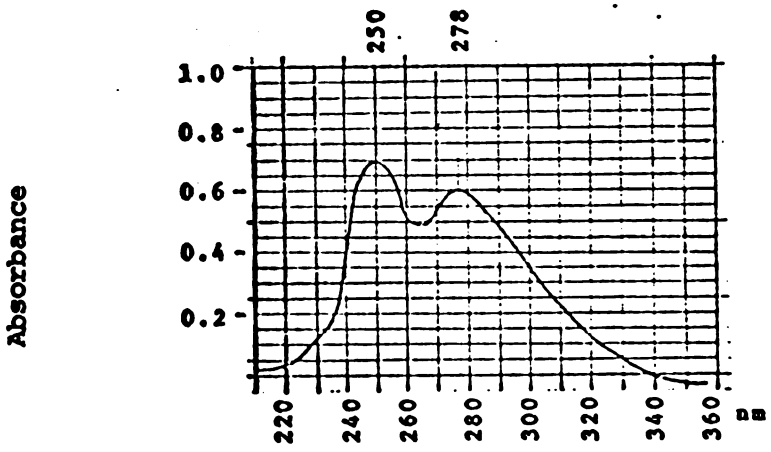
Fraction collected between 5.0-5.5 min (M-2).



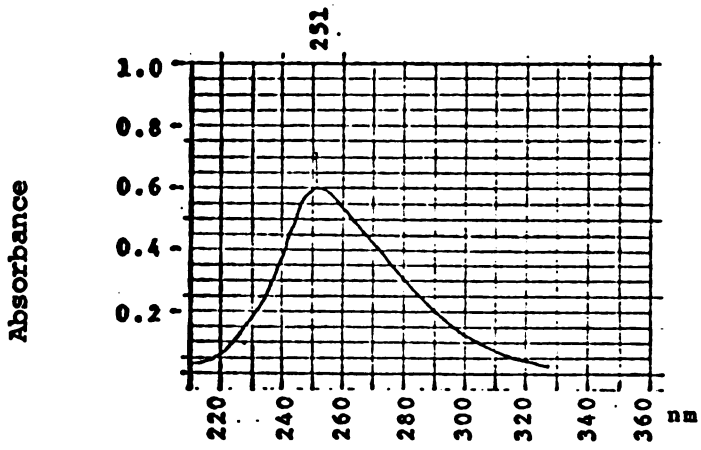
Fraction collected between 21.0-21.5 min (M-1).



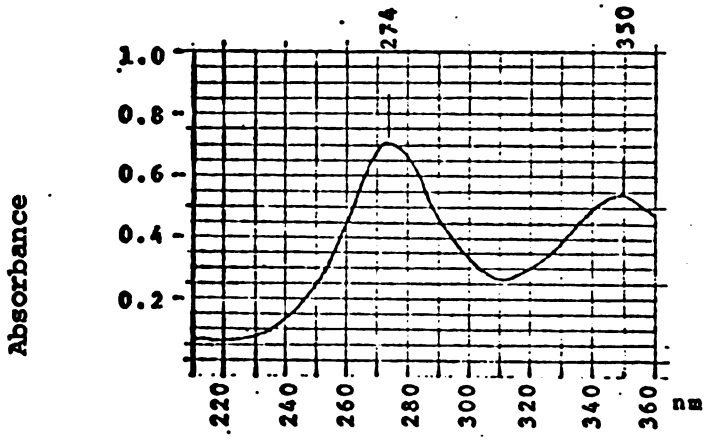
Fraction collected between 22.0-22.5 min.



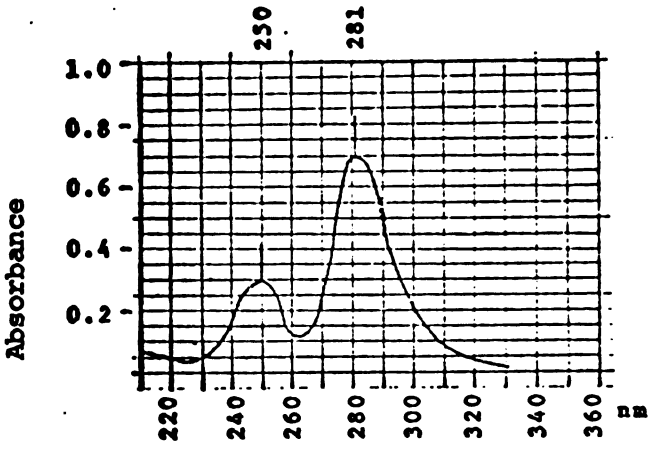
Fraction collected between 23.0-23.5 min.



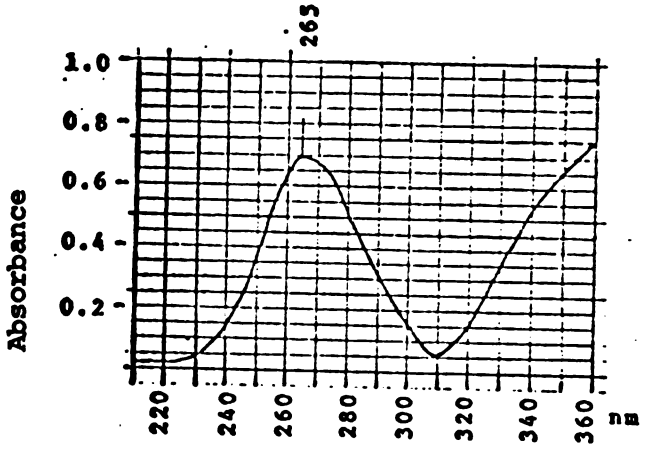
Fraction collected between 24.0-24.5 min.



Fraction collected between 24.5-25.0 min.



Fraction collected between 25.0-25.5 min.



Fraction collected between 26.0-26.5 min (nitrofurantoin).

Fig. IV.6 ULTRAVIOLET ABSORPTION SPECTRUM OF M-1.

M-1 in distilled water pH 5.6 (____); and after 6 days storage in distilled water at -20°C (-----).

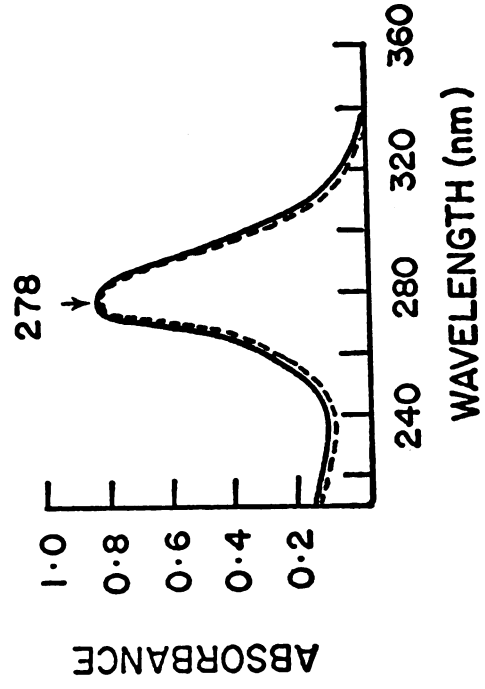
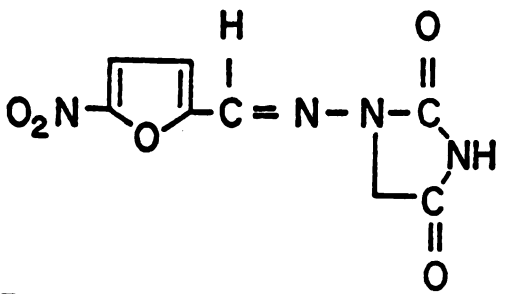
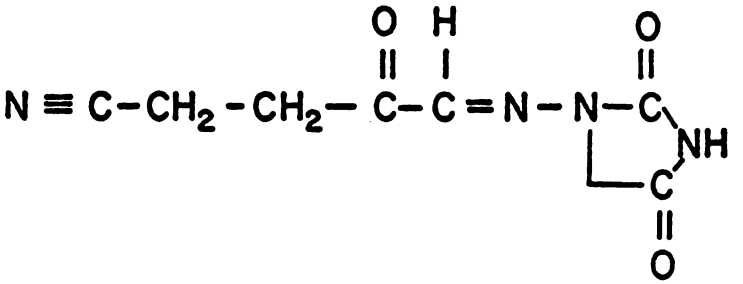


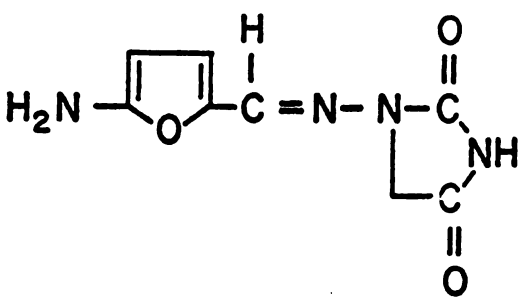
Fig. IV.7 Nitrofurantoin (I); 1-[[(5-amino-2-furanyl) methylene]amino]-2,4-imidazolidinedione (aminofurantoin) (II); 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione (III); general structure for azomethine 5-nitrofurans (IV).



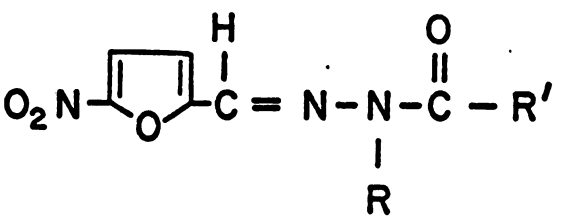
I



III



II



IV

Fig. IV.8. ELECTRON IONIZATION MASS SPECTRUM OF THE HPLC FRACTION COLLECTED BETWEEN 22.0 AND 22.5 MIN (NICOTINAMIDE).

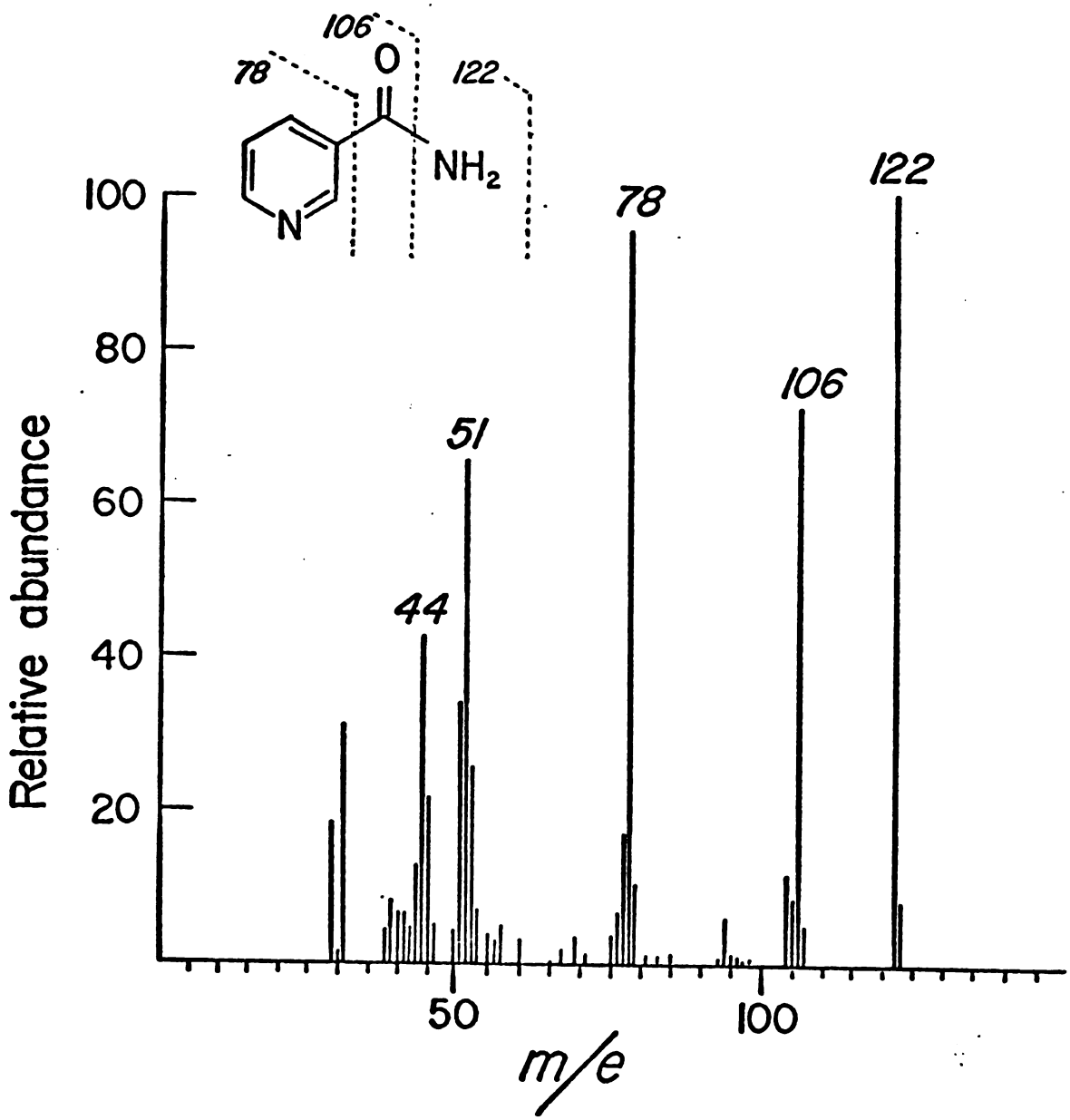


Fig. IV.9 ISOBUTANE CI MASS SPECTRUM OF M-1.

The peak at m/e = 57 corresponds to the t-butyl ion from the reagent gas. The mass spectrum of authentic sample (III in Fig. IV.7) is identical to that of M-1.

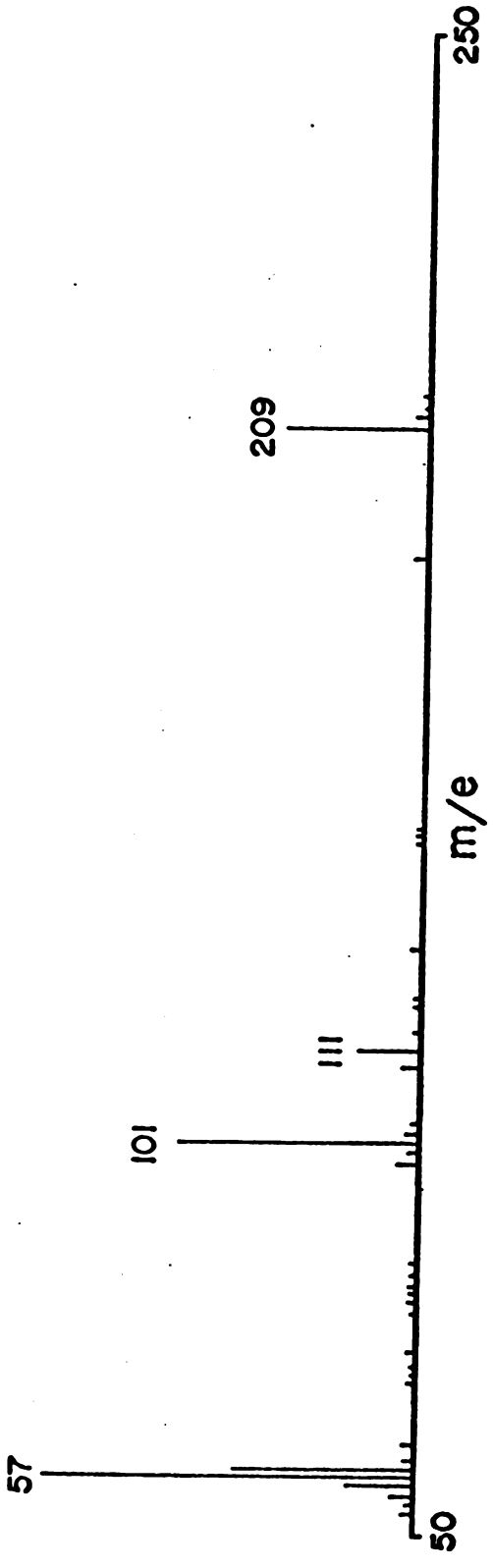

250
209
111
101
57
50
m/e

Fig. IV.10 SCHEMATIC DIAGRAM OF THE BASIC COMPONENTS OF A FOURIER TRANSFORM NMR EXPERIMENT.

(after James, 1975)

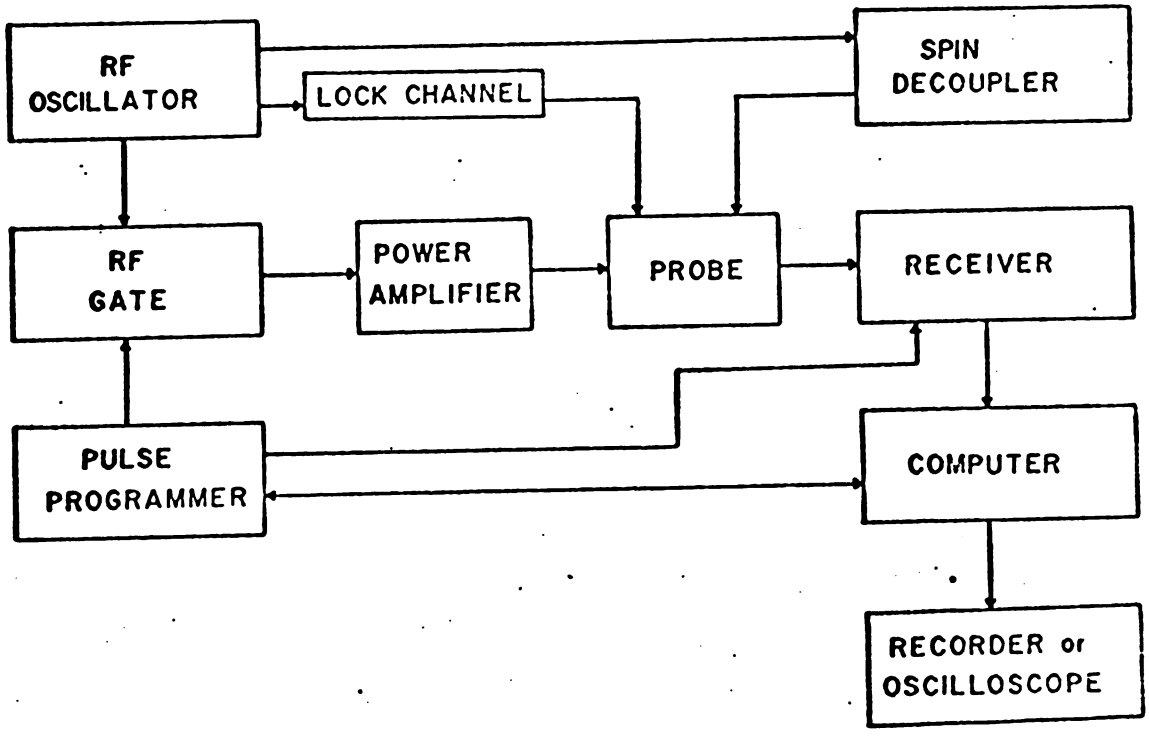
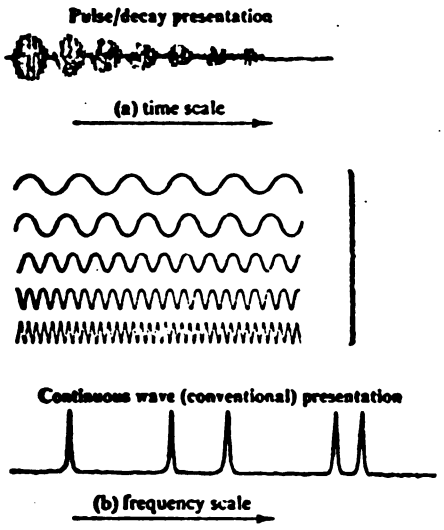


Fig. IV.11 SCHEMATIC REPRESENTATION OF PULSED NMR.
The output in the time domain is converted to the frequency domain by Fourier transform.



Exponential decay from all nuclei undergoing relaxation...

consists of a composite of several individual frequencies, which can be separated and...

presented on a linear frequency scale.

Fig. IV.12 ^{1}H-NUCLEAR MAGNETIC RESONANCE SPECTRUM OF M-1 IN D_2O. (chemical shifts in ppm from TSS).

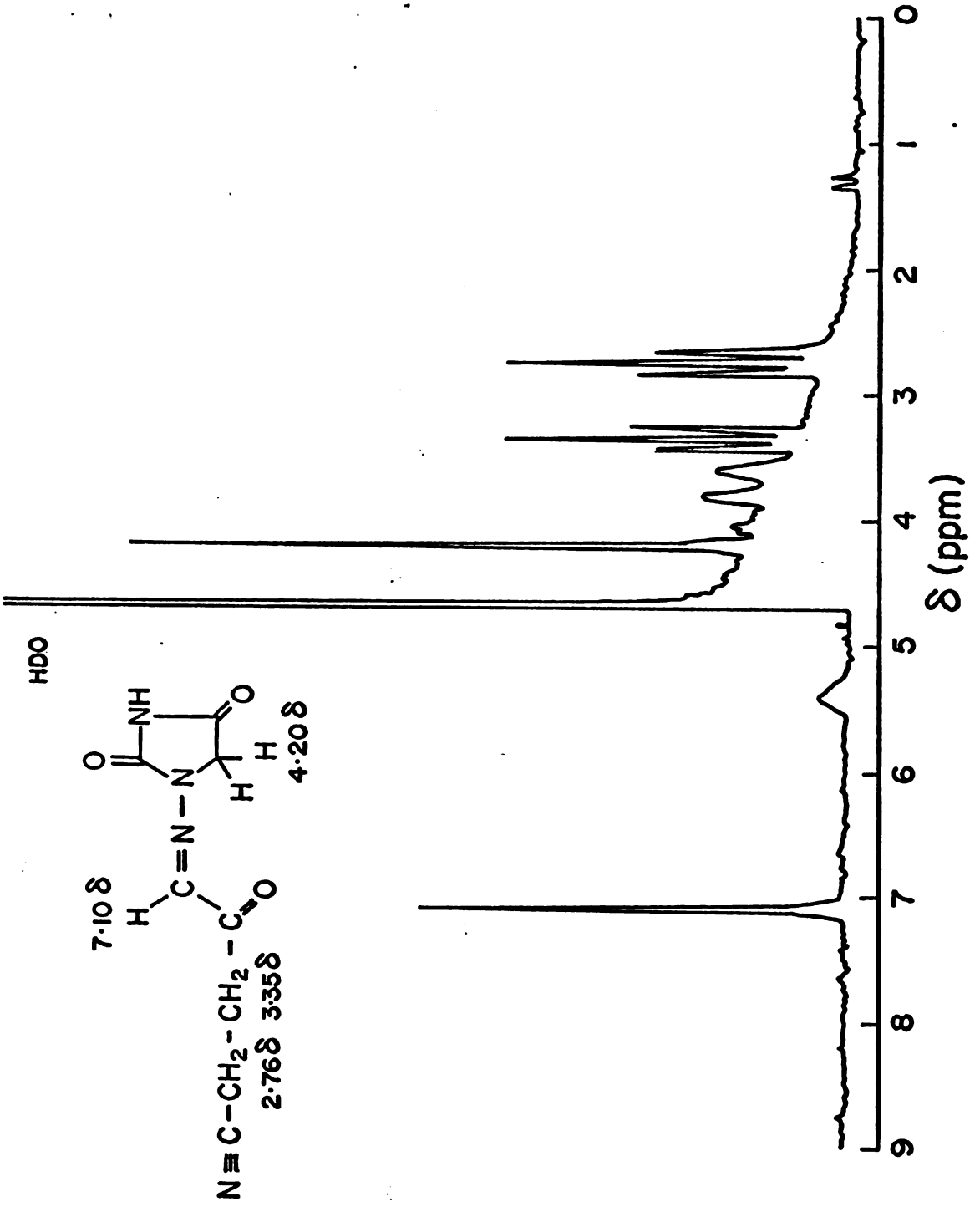

HDO
δ (ppm)
0
1
2
3
4
5
6
7
8
9
NH
O
O
N
H
H
4·20δ
C≡N
H
C
C=O
7·10δ
N≡C–CH₂–CH₂–C=O
2·76δ 3·35δ

Fig. IV.13 METABOLISM OF NITROFURANTOIN IN THE ISOLATED PERFUSED RAT LIVER.

The half-life of nitrofurantoin in the perfusate which had an initial concentration of 0.05 m$\underline{M}$ is 16 minutes. Each point represents the mean ($\pm$ SE) of the concentrations of nitrofurantoin in the perfusate obtained from four livers. The disappearance of nitrofurantoin approximates a first order process.

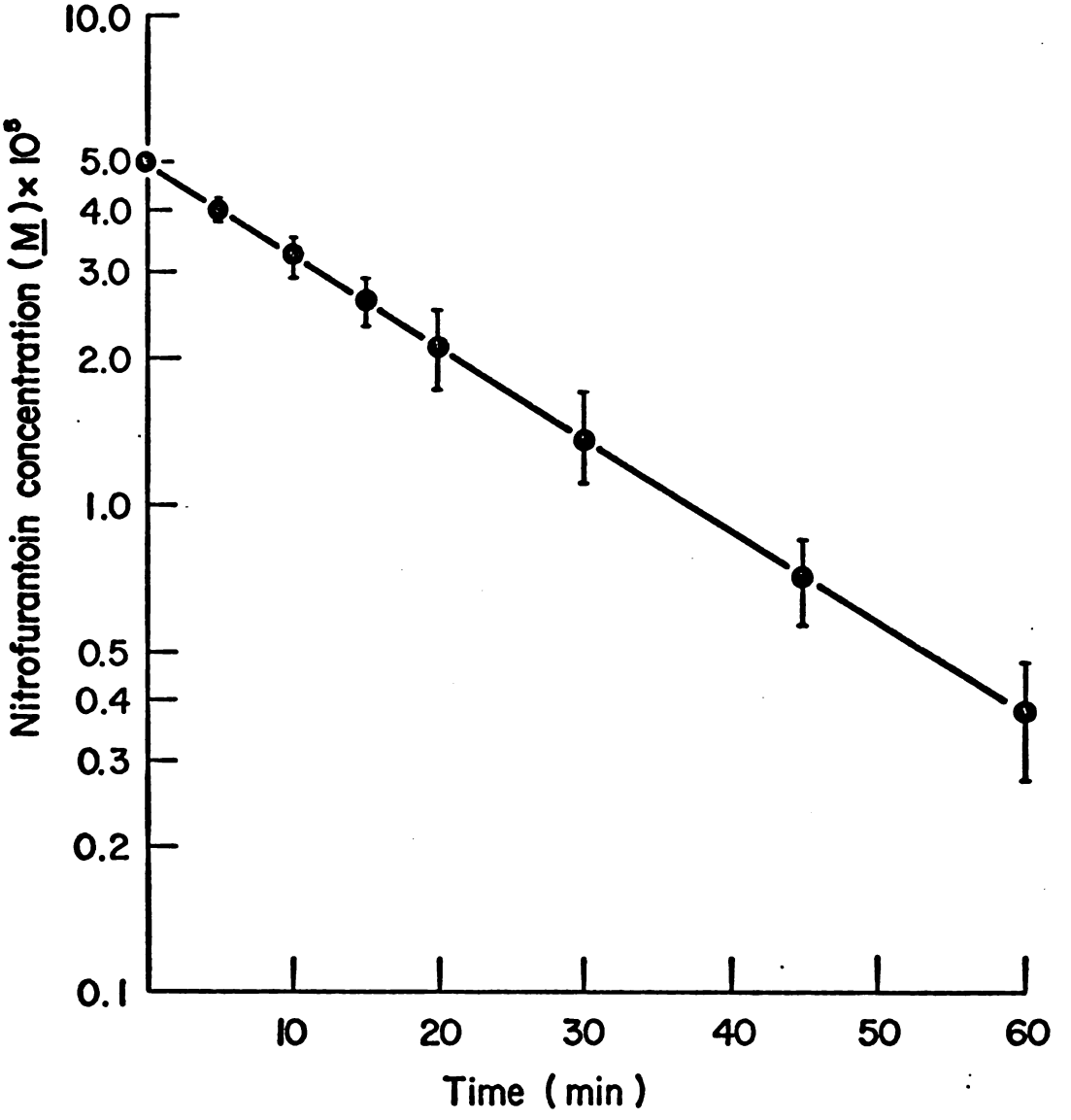

10.0
5.0
4.0
3.0
2.0
1.0
0.5
0.4
0.3
0.2
0.1
Nitrofurantoin concentration (M) x 10⁵
10
20
30
40
50
60
Time (min)

Fig. IV.14 APPARENT CONCENTRATING EFFECT IN THE BILE DURING PERFUSION OF NITROFURANTOIN IN ISOLATED PERFUSED RAT LIVERS.

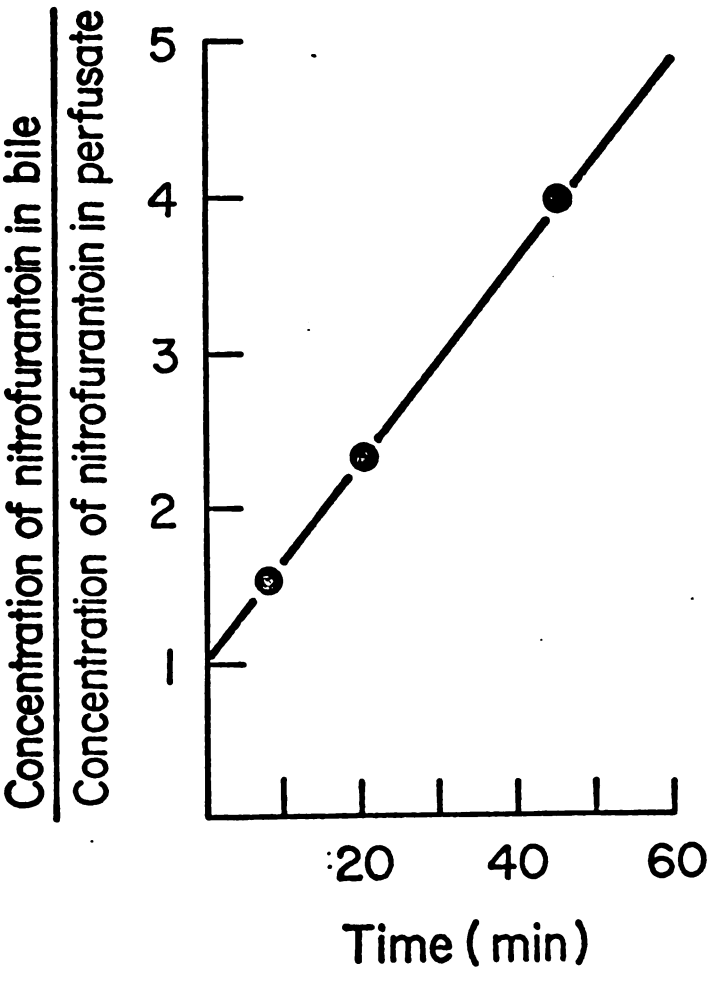


Fig. IV.15 MOLAR ABSORPTIVITY FOR 1-[[(3-CYANO-1-OXOPROPYL)METHYLENE]AMINO]-2,4-IMIDAZOLIDINEDIONE.

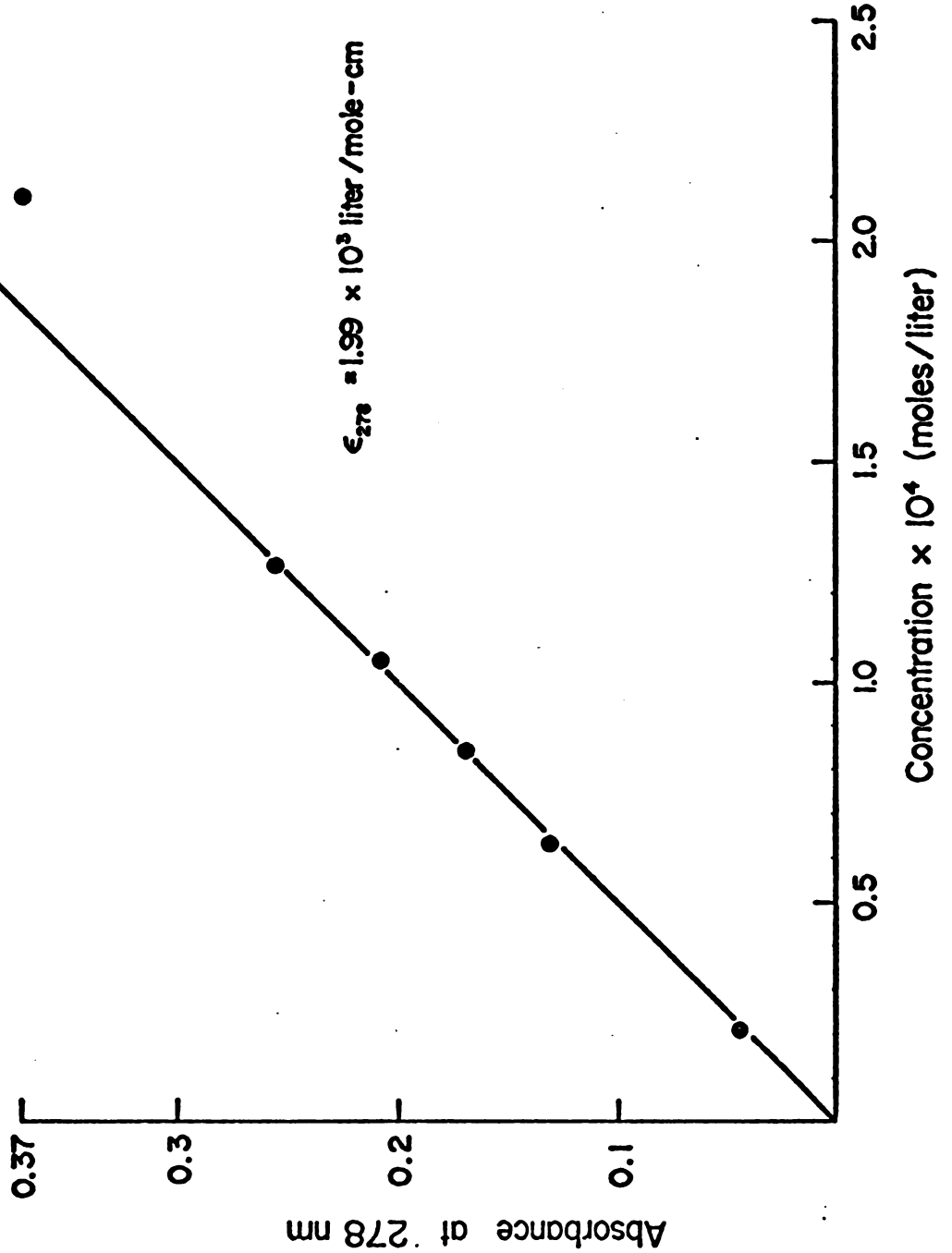
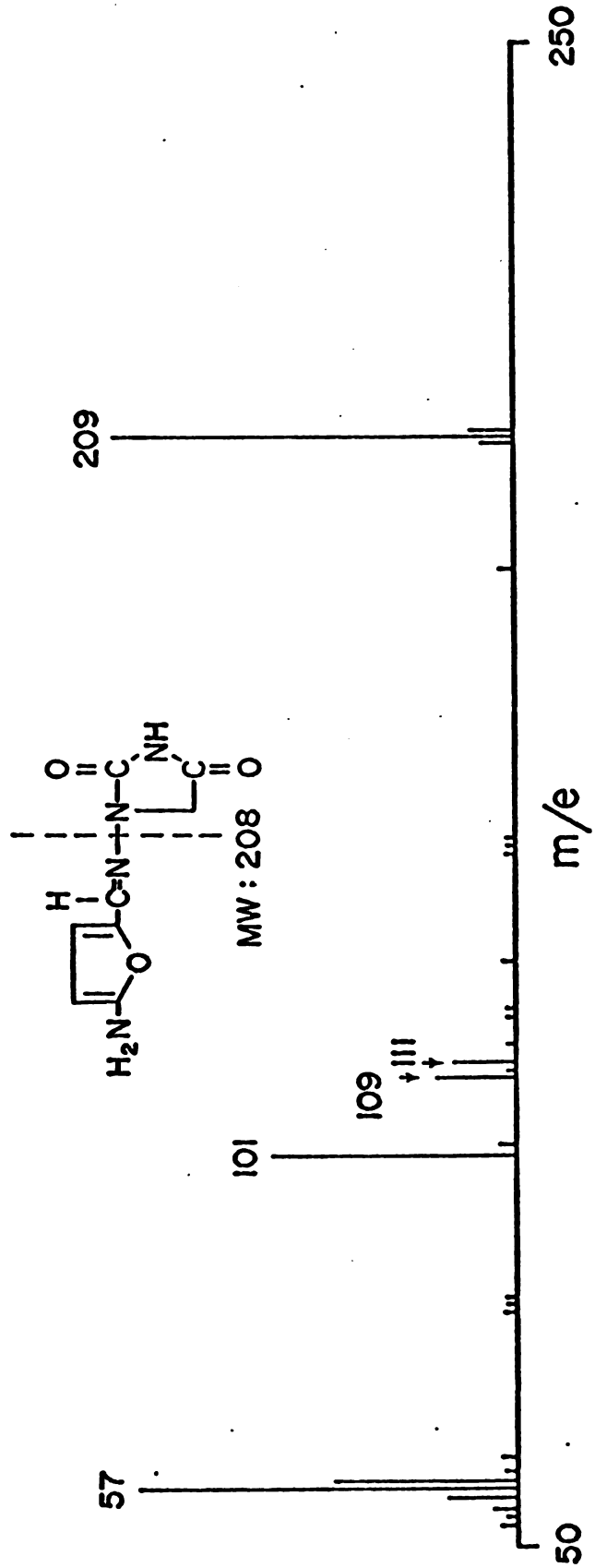


Fig. IV.16 ISOBUTANE CI MASS SPECTRUM OF 1-[[(5-AMINO-2-FURANYL)METHYLENE]AMINO]-2,4-IMIDAZOLIDINEDIONE. The peak at m/e = 57 corresponds to the t-butyl ion from the reagent gas. Source temperature: 220°C; pressure: 0.5 Torr.


250
209
m/e
MW : 208
101
109
111
57
50

V. DISCUSSION

Analytical studies on nitrofurantoin.

The methods presently used for the analysis of nitrofurantoin in biological fluids include ultraviolet, colorimetric, polarographic and microbiological assays (Cadwallader and Jun, 1976). The problems encountered with the existing analytical methods were either low sensitivity or lack of specificity. We attempted to develop a gas-liquid chromatographic method but this was unsuccessful possibly because the high temperatures that were required (>200°C) lead to rapid on-column decomposition of nitrofurantoin. With the use of high-pressure liquid chromatography, however, we obtained a rapid and sensitive quantitation of nitrofurantoin in biological fluids without prior derivatization and extraction (Aufrère et al., 1977b). In the HPLC method, peak heights and nitrofurantoin concentrations are linearly related in the range of 0.02 µg/ml to 200 µg/ml. The HPLC method is sufficiently accurate and sensitive for clinical monitoring of therapeutic concentrations in patients and pharmacokinetics/bioavailability studies. Moreover, with no modification in the procedure, the HPLC assay was extended to other 5-nitrofuran derivatives, such as nitrofurazone and furazolidone. Since these compounds are used in animal feeds for the prevention of a variety of animal diseases,

this HPLC method lends itself to analysis of residues of 5-nitrofuran derivatives present in the tissues of these animals.

Metabolic studies on nitrofurantoin.

The bacterial metabolism, by Aerobacter aerogenes, of 5-nitrofurans sharing the general structure IV shown in Fig. IV.7, has been described by Beckett and Robinson (Beckett and Robinson, 1959a). Based on UV spectral changes, it was postulated that with nitrofurans in which R is a hydrogen atom, the product of bacterial metabolism is the aminofuran which on storage, gradually undergoes cleavage of the furan ring. However, with IV in which R is an alkyl group or part of a cyclic system involving R', the spectrophotometric changes observed during the bacterial metabolism gave no evidence for the formation of the aminofuran, rather, cleavage of the furan ring occurred leading to the formation of the open-chain nitrile (Gavin et al., 1966). With nitrofurantoin, the evidence for the formation of this open-chain nitrile was limited to UV spectral changes (Beckett and Robinson, 1959b).

In mammalian tissues, several pathways have been determined for the metabolism of 5-nitrofurans. The first pathway is the reduction of 5-nitrofurans to the corresponding 5-aminofurans (Tatsumi et al., 1976; Wang et al., 1975). In this transformation, radical anions of the nitro-aromatic compounds appear to be obligate intermediates

in the enzymatic reduction to hydroxylamines or amines (Mason and Holtzman, 1975). The formation of the 5-aminofuran may be followed by acetylation to the N-acetyl- or N,N-bis-acetylaminofuran (Olivard et al, 1962). The second pathway is the reduction of the 5-nitrofuran followed by ring cleavage to the 3-cyanopropionyl compound (Tatsumi et al., 1976; Chatfield, 1976). In the third pathway, there is a transformation of the 5-nitrofuran to a 5-methylthiofuran (Ou, 1977). The fourth pathway is the hydroxylation of the furan ring resulting in the tautomeric mixtures of 4-hydroxy-5-nitro-2-furancarboxaldehyde derivatives and the corresponding aci-nitro forms (Olivard et al., 1976). The fifth pathway is the oxidation of the side chain of nitrofuraldehyde derivatives, e.g., oxidation of nifuradene to nitrofurantoin (Pugh et al., 1972). The final pathway is the acid hydrolysis of the azomethine bond in the gastrointestinal tract to yield 5-nitro-2-furan-carboxaldehyde which is excreted as 5-nitro-2-furoic acid and a hydrazine derivative (Olivard et al., 1962). These various pathways are depicted in Fig. V.1 and V.2.

Metabolic incubations of nitrofurantoin with various rat tissue preparations were carried out under anaerobic conditions because oxygen has been shown to inhibit reduction of the nitro group, and the in vitro toxicity of nitrofurantoin is enhanced by anaerobic conditions (Olive and McCalla, 1977; Boyd and Osborne, 1975; Rosenkranz and

Speck, 1976). The carcinogenic and mutagenic activity of certain aromatic and heterocyclic nitro compounds is believed to result from the formation of N-hydroxylamines as reduction products. The biological reduction of aromatic nitro compounds is important in the toxicity of these compounds; however, the particular tissue localization of nitroreductase activity and the identification of metabolites for nitrofurantoin remained uncertain.

Enteric tissue and its microflora have been shown to contribute significantly to the overall metabolism of nitro compounds (Zachariah and Juchau, 1974; Reddy et al., 1976). We therefore examined the rate of metabolism of nitrofurantoin under anaerobic conditions in enteric tissues in germ-free, germ-free acclimatized and control rats. As shown in Table IV.1, metabolism, as measured by the rate of disappearance of nitrofurantoin, was highest in the homogenate of cecum and colon contents of germ-free acclimatized and control rats. Appreciable rates of metabolism were also present in liver, small intestine walls, lung and kidney in all animal groups. No metabolism was detected in the cecum and colon contents of germ-free rats, indicating that the metabolism observed in germ-free acclimatized and control rats was due to the bacteria present. Metabolism in other tissues of germ-free or germ-free acclimatized rats was not significantly different from that of control rats. The rate of metabolism of nitrofurantoin in the small intestine wall homogenate was

comparable to that obtained in liver homogenate. It is unlikely that this activity could be due to bacterial contamination since activity was unchanged in germ-free animals and no activity was detected in the homogenates from small intestine contents. Centrifugation of the tissue homogenates to prepare the 9,000 xg supernatant fraction removed most of the activity from kidney and small intestine walls and all activity due to the bacteria of cecum and colon contents.

The pattern of formation of metabolites was investigated in various rat tissue homogenates including liver, lung, kidney, small intestine walls, small intestine contents, and cecum and colon contents. Three metabolites, M-1, M-2, and M-3, were formed in the liver (Fig. IV, 3A). Only M-1 and M-2 were found in homogenates of lung, kidney, and small intestine walls (Fig. IV, 3B). M-1 and a new metabolite, M-4, were formed by the bacteria from the cecum and colon contents (Fig. IV, 3C). Incubation with small intestine contents did not result in any observable metabolism of nitrofurantoin. In order to isolate metabolites, nitrofurantoin (1.16 m$\underline{M}$) was incubated for 60 min under anaerobic conditions with the 9,000 xg supernatant fraction of liver and the incubation mixture injected onto the HPLC. The metabolites were collected as they eluted from the HPLC column and their UV absorption spectra measured. Characterization of the major metabolite M-1 was obtained by chemical ionization mass-spectral analysis

(MH^+ = 209) and proton nuclear magnetic resonance (δ, ppm = 2.76 triplet, 3.35 triplet, 4.20 singlet, 7.10 singlet). M-1 was identified as 1-[[(3-cyano-1-oxopropyl)methylene] amino]-2,4-imidazolidinedione (III in Fig. IV.7). This finding was confirmed by comparative studies with an authentic specimen prepared by the catalytic hydrogenation of nitrofurantoin with 10% palladium on carbon catalyst. The open-chain nitrile derivative M-1 resulted from the reduction of the nitro group with subsequent opening of the furan ring. A second, minor metabolite with HPLC and UV absorption characteristics identical to those of aminofurantoin (II in Fig. IV.7) was detected in the metabolic mixture following the incubation of nitrofurantoin with cecum and colon contents (M-4 in Fig. IV.3). Our data, therefore, strongly suggests that II is a product of the bacterial metabolism of nitrofurantoin.

The kinetics of the anaerobic metabolism of nitrofurantoin were studied in the 9,000 xg supernatant fraction of rat liver using the Lineweaver-Burk method. The apparent K_m was 59 μM while the V_{max} was 0.49 nmoles of nitrofurantoin metabolized per mg protein per minute. Further kinetic characterization was done by using the isolated perfused rat liver preparation. In this preparation the metabolism of nitrofurantoin follows a first-order process with a half-life of 16 minutes.

Several enzyme systems are reported to be responsible for the reductive metabolism of aromatic nitro compounds. They are NADPH-cytochrome P-450 reductase (Gilette et al., 1968), NADPH-cytochrome c reductase (Wang et al., 1974), xanthine oxidase (Wang et al., 1974; Tatsumi et al., 1976); DT diaphorase (Kato et al., 1969), and aldehyde oxidase (Wolpert et al., 1973). Preliminary experiments in which nitrofurantoin was incubated with liver homogenate in the presence of allopurinol (10 μM) resulted in the complete inhibition of M-2, thereby suggesting the involvement of hepatic xanthine oxidase in the formation of this metabolite. The rapid degradation of nitrofurantoin by small intestine walls may be due to the high activity of xanthine oxidase present in this tissue (Wang et al., 1974). The extent of nitro reduction occurring in vivo is not known since, with the exception of cecum and colon contents, all tissues are perfused with oxygenated blood. Our data indicate, however, that the open-chain nitrile derivative is also formed in the isolated perfused rat liver preparation, being present both in the perfusate and in the bile. A product with an identical HPLC retention time of 21.0 min was also detected in the urine from a patient treated with nitrofurantoin (Aufrère et al., 1978a).

Isolation of a nitrile derivative of 5-nitrofurans was first reported by Gavin et al. (Gavin et al., 1966) following aerobic metabolism of 1-(5-nitrofurfurylidene-amino)-2-imidazolidinone by Escherichia coli. Recently,

Tatsumi et al. (Tatsumi et al., 1976) have demonstrated that nitrofurazone is metabolized in vitro by xanthine oxidase to the nitrile derivative, and Chatfield (Chatfield, 1976) reported that 2-(5-nitro-2-furyl)-4-thiomorpholino-iminomethyl)thiazole-1',1'-dioxide was metabolized in vivo in the mouse to the nitrile derivative. It is concluded that the formation of the open-chain nitrile derivative appears to be a general pathway for the metabolism of certain nitrofurans under various conditions.

The mutagenicity of the two metabolites of nitrofurantoin--aminofurantoin and 1-[[(3-cyano-1-oxopropropyl)methylene]amino]-2,4-imidazolidinedione--was tested by using the Salmonella typhimurium TA-100 tester strain which is the most sensitive strain to 5-nitrofuran derivatives. Previous work had demonstrated that the 5-aminoheterocyclic derivative corresponding to the 5-nitroheterocyclic compounds were not mutagenic in these same strains (Wang et al., 1975; McMahon, 1976). It was considered that aminofurantoin may become a positive mutagen in Salmonella typhimurium if enzymatically activated by the mixed function oxidase system. However, addition of aminofurantoin at low to medium doses did not significantly alter the number of histidine revertants as compared with background and the addition of the mixed function oxidase system did not change the number of revertants seen at a given dose of aminofurantoin. Negative

results were also obtained with 1-[[(3-cyano-1-oxopropyl) methylene]amino]-2,4-imidazolidinedione in the Salmonella typhimurium TA-100 tester strain. It was concluded that neither metabolite of nitrofurantoin was mutagenic under the conditions tested.

Fig. V.1 METABOLIC PATHWAYS OF 5-NITROFURANS RESULTING FROM THE BIOTRANSFORMATION OF THE 5-NITRO GROUP.

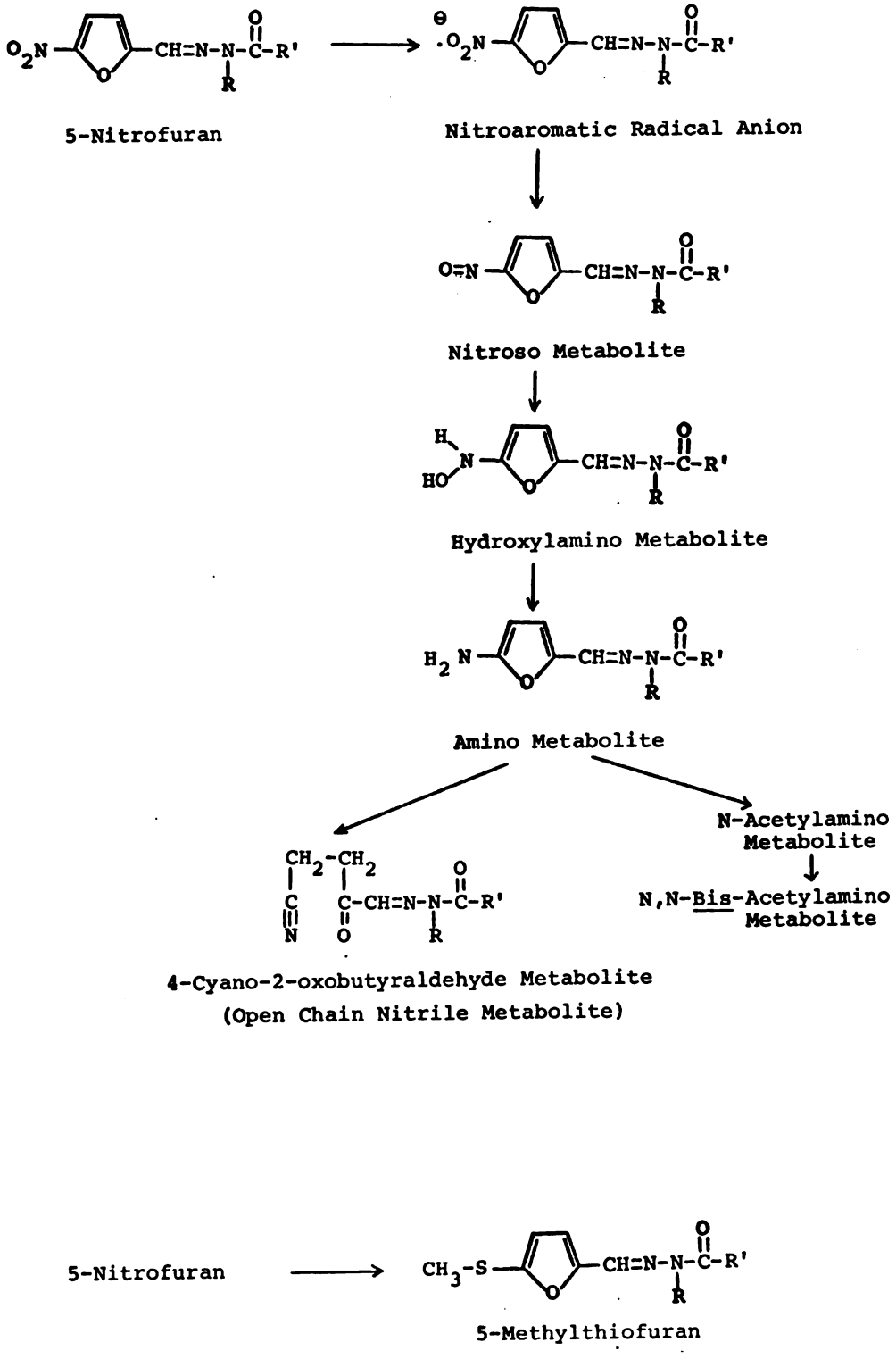
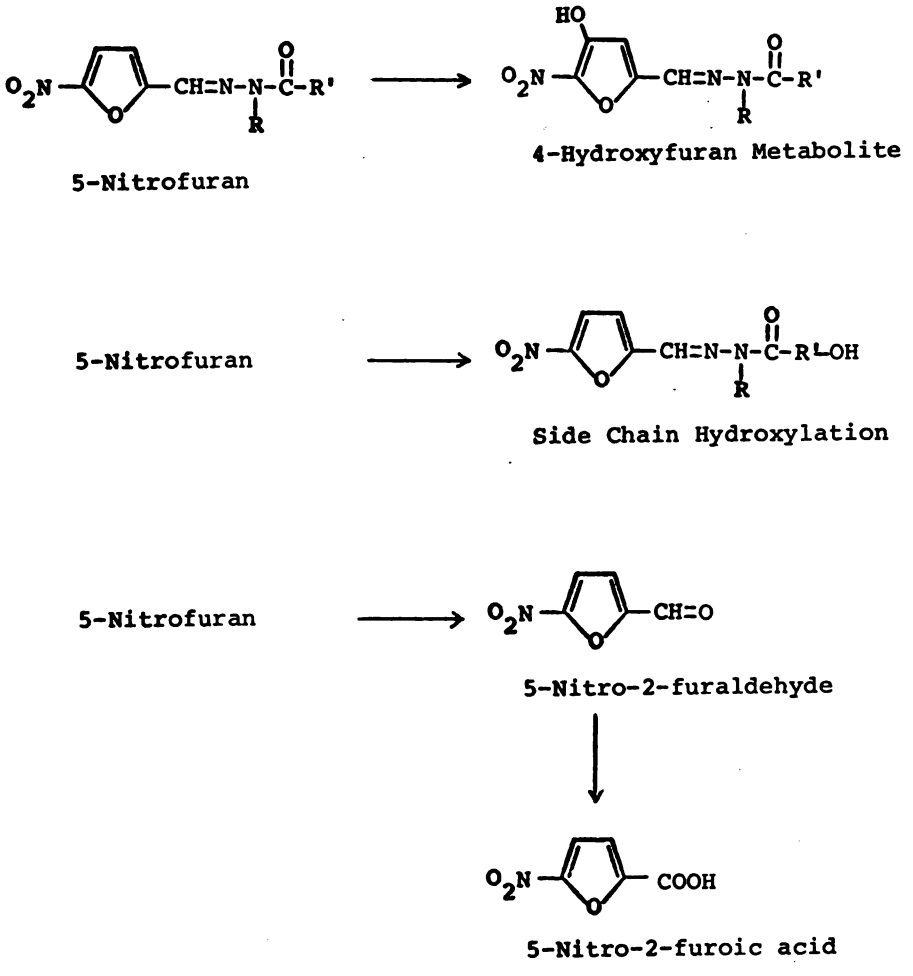


Fig. V.2 METABOLIC PATHWAYS OF 5-NITROFURANS WHICH DO NOT INVOLVE THE 5-NITRO GROUP.



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