THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDY OF N-NITROSAMINE FORMATION BY *ESCHERICHIA COLI* SEROTYPES ISOLATED FROM URINARY TRACT INFECTIONS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

JAMES E. JOHNSON

Oklahoma City, Oklahoma

1974

STUDY OF N-NITROSAMINE FORMATION BY ESCHERICHIA COLI SEROTYPES ISOLATED FROM URINARY TRACT INFECTIONS

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENTS

I wish to extend my thanks to all faculty members of the Department of Microbiology and Immunology for their efforts in furthering my education.

My sincere love and appreciation are expressed to my wife, Patti, for the many hours of personal sacrifice she endured and the moral encouragement she gave to me during my graduate education.

The photographs in this dissertation were taken and developed by Mr. M. Pittman, a graduate student in the Department of Microbiology and Immunology.

I would also like to thank Dr. J. T. Price of the Department of Pathology, who interpreted the kidney sections examined during this study.

Acknowledgment of the financial assistance which made this study possible goes to my wife, The Public Health Service-National Institutes of Health Training Grant T01 AI00162, (1970-71) and the Oklahoma Medical Research Foundation-Colin M. MacLeod Fellowship (1971-74).

TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF ILLUSTRATIONS	vi
Chapter	
I. INTRODUCTION AND LITERATURE REVIEW	1
II. MATERIALS AND METHODS	12
III. RESULTS	27
IV. DISCUSSION	63
V. SUMMARY	73
LITERATURE CITED	75

LIST OF TABLES

Table		Page
1.	Gas-liquid Chromatographic Conditions Used for the Detection of Volatile Amines and N-nitrosamines	22
2.	Biochemical confirmation of Presumptive Escherichia coli Isolates	29
3.	Agglutination Reaction of Escherichia coli Urinary Tract Isolates	30
4.	Type Specific Escherichia coli Urinary Tract Isolates According to Serogroup	31
5.	N-nitrosamine Formation by Escherichia coli Serotypes Isolated from Urine	33
6.	Gas-liquid Chromatographic Identification of Amines and N-nitrosamines	37
7.	Comparison of Detection Procedures for Analysis of Extracts Prepared During In Vitro N-Nitrosamine Formation by E. coli Isolates of Urinary Tract and Intestinal Origin	41
8.	Fluorometric Assay of Urine Containing Twenty Milligrams Diphenylamine	49
9.	Fluorometric Assay for Diphenylamine From the Urine of Rats Given Ten Milligrams Diphenylamine Every Eight Hours by Gastric Intubation	51
10.	The Volume of Urine Recovered from Normal Rats Before and During Diuresis Induced with 5% (w/v) Glucose Ad Libitum	52
11.	N-Nitrosodiphenylamine Recovery from Urine of Animals Infected with Escherichia coli and Intubated with Varying Concentrations of Diphenylamine	56

LIST OF ILLUSTRATIONS

Figure		Page
1.	Quantitation of Bacteria by the Calibrated Loop - Direct Streak Method.	16
2.	Diphenylamine Standard Curve Determined by Fluorometric Analysis.	24
3.	Thin Layer Chromatogram of Four Escherichia Coli Broth Extracts Demonstrating In Vitro Nitrosation of Diphenylamine	34
4.	Thin layer Chromatogram Demonstrating In Vitro N-nitrosation of Dibutylamine, Diethylamine, Diphenylamine, and Piperidine by Escherichia coli Strain 93.	36
5.	Gas-liquid Chromatogram of an Extract from a Broth Culture of Escherichia coli Strain 93 Containing Dibutylamine, 0.2% Glucose, and 0.2% Nitrate	39
6.	Gas-liquid Chromatogram of an Extract from a Broth Culture of Escherichia coli Strain 93 Containing Piperidine, 0.2% Glucose, and 0.2% Nitrate.	40
7.	The Effect of Glucose on the In Vitro Nitrosation of Diphenylamine by Escherichia coli Strain 93	43
8.	The Effect of Several Concentrations of Diphenylamine on Nitrosation and Diphenylnitrosamine Recovery from Extracts of Escherichia coli Broth Cultures.	44
9.	The Effect of Incubation Time on the Formation and Recovery of Diphenylnitrosamine from Extracts of Escherichia coli Strain 93 Broth Cultures.	46
10.	In Vitro Growth Curve for E. coli Strain 93 in Rat Urine (pH 7.0).	47
11.	Quantitative Plate Counts on Urine After Direct Injection of Escherichia coli Strain 93 into the Rat Kidney . . .	54
12.	Extract from Urine of an Animal Infected with Escherichia coli Strain 108 and Intubated with 20 mg diphenylamine and 40 mg Potassium Nitrate every 8 Hours	61
13.	Extracts of Urine from Several Animals Infected with Escherichia coli Strain 108, Intubated with 20 mg Diphenylamine and 40 mg Potassium Nitrate Every 8 Hours	62

STUDY OF N-NITROSAMINE FORMATION BY ESCHERICHIA COLI SEROTYPES ISOLATED FROM URINARY TRACT INFECTIONS

CHAPTER I

INTRODUCTION

Urinary tract infection is one of the most common infectious diseases encountered in medicine (34). Urine is normally free of microorganisms unless it is contaminated during collection or a urinary tract infection has occurred. Urinary tract infection is defined as an infection involving the collecting system and kidneys or the bladder and is recognized by a wide array of clinical symptoms such as chills, fever, dysuria, urinary frequency, pyuria and the presence of large numbers of microorganisms in a properly collected specimen (34). It was recognized as early as 1888 that urine specimens obtained from patients with renal infections contained large numbers of Escherichia coli in pure culture. Disease was produced when Escherichia coli suspensions were injected into experimental animals, thus fulfilling Koch's postulates (34). Escherichia coli is considered to be the most common etiologic agent encountered in urinary tract infections. Epidemiologic studies have shown that relatively few Escherichia coli serotypes are involved in urinary tract infections (13, 18, 23, 31, 52, 65, 66, 67). The ten most common O serotypes

involved are 01, 02, 04, 06, 07, 08, 018, 025, 050 and 075 (65), although differences in the common serotypes may be noted in different geographic locations.

Urinary tract infection is diagnosed when 10^5 organisms/ml or more are found in a clean-voided urine specimen (29). Urine is an excellent culture medium and if microorganisms are inoculated into normal urine they will double approximately every 45 minutes and rapidly approach 10^8 organisms/ml (5). This is the approximate number of bacteria present in the urine of most patients with urinary tract infection (56).

In recent years, N-nitrosamines have been demonstrated to be carcinogenic for many animal species. Precursors of N-nitroso compounds may be converted to nitrosamines in the presence of nitrite by a chemical reaction under acid conditions or by enzymatic reactions mediated by nitrate-reducing bacteria in the presence of nitrate. The frequency of urinary tract infections in the general population and the presence of secondary amines, nitrates, and nitrites in the environment leads to the speculation that carcinogenic N-nitrosamines may be synthesized during the course of acute or chronic urinary tract infections caused by members of the family Enterobacteriaceae which, by definition, must reduce nitrate to nitrite (16, 30). There have been few studies designed to demonstrate the nitrosating capability of Escherichia coli serotypes isolated from urinary tract infections.

The objectives of this study were to:

1. determine the common O antigenic types of Escherichia coli involved in urinary tract infections in this geographic locale,

2. study the frequently found serotypes for their ability to nitrosate secondary amines in the presence of nitrate *in vitro*, and

3. study the nitrosamine forming strains for their ability to nitrosate secondary amines *in vivo*.

HISTORICAL REVIEW

N-Nitrosamines

Over the past three decades many chemical compounds have been shown to be carcinogenic for laboratory animals and humans. Tumors have resulted from systemic application of a particular compound in question or through accidental or occupational contact with the carcinogen (17, 35, 39, 41, 70). N-nitroso compounds have been studied extensively and the carcinogenicity of this group of chemicals is well established (3, 8, 15, 39). Interest in the endogenous formation of carcinogenic N-nitrosamines has increased following the demonstration that these compounds may be chemically synthesized by the interaction of secondary amines and nitrite (55). The chemical formation of nitrosamines may occur in an acid environment, such as the stomach. Nitrosamines may be formed from secondary amines and nitrite in human and cat gastric juice. This nitrosation has been performed *in vitro* and *in vivo* (59). Other areas of the body are maintained at or near neutral pH, thus conditions rarely exist in which the pH is low enough for the non-enzymatic nitrosation reaction to occur.

N-nitroso compounds are found in the environment as natural ingredients of foodstuffs, plants, and tobacco (35, 39, 63, 70).

Carcinogenic nitrosamines, such as dimethylnitrosamine and nitrosopyrrolidine, have been identified in foods such as bacon, sausages, fish and mushrooms and they occur in the µg/kg range (57, 58). The role of naturally occurring nitrosamines may vary according to the frequency and duration of ingestion of these substances.

Nitrates and Nitrites

Nitrates are distributed in nature as natural constituents of plants and they are present in high concentration in beets, radishes, celery, lettuce, and spinach (17, 35, 70). They are also present in lower concentrations in fruits and as additives in cured meats. Nitrates may occur in varying concentrations in water as a result of sewage effluent, fertilizers, aquatic plants, and the leaching of nitrates from the soil (17, 35). They are not toxic in the concentrations normally ingested by man, however the health hazard of nitrates occurs when they are reduced to nitrites and coupled to amines to form carcinogenic N-nitroso compounds. Nitrites are used as preservatives in cured meats, fishes and cheese or may be found in green vegetables as the result of bacterial reduction of nitrate (48). Microorganisms in the oral cavity may account for the presence of nitrites in saliva. This is another example of the reduction of nitrates in body fluids (21).

Amines

Since nitrates and nitrites are available to most animals in their diet, the limiting factors in endogenous nitrosamine formation appear to be the availability of secondary amines and chemical or

enzymatic coupling agents. Dimethylamine, diethylamine, and nitrite have been recovered from fish meal, fish products, cereals, tea, and tobacco (35). The origin of these chemicals may be endogenous or result from natural contamination, bacterial growth or manufacturing processes. Other amines capable of nitrosation are drugs and pesticides which may be ingested (22). Preparation of meat and fish for consumption may result in the production of secondary amines by the conversion of cadaverine and putrescine into piperidine and pyrrolidine (35). Many foods contain lecithin and choline which may be degraded by intestinal bacteria to form dimethylamine which is eliminated as urinary dimethylamine (4). Amines are present, therefore, naturally in the diet or as degradatory products of bacterial action or food processing.

The stomach is considered to be relatively free of microorganisms since the pH is not conducive to the establishment of a resident flora (54, 55). Thus, nitrosation of amines could not be mediated through microbial enzymatic processes. Any formation of nitrosamines would be dependent on chemical combination at an acid pH. Rats and mice frequently develop stomach cancer when fed a large single dose or several small doses of N-nitrosamines (41). To date, no target organ susceptible to the carcinogenic affect of N-nitrosamines has been determined for man, however it has been suggested that synthesis of N-nitrosamines during an episode of bacteriuria may increase the risk of stomach cancer (27).

If bacteria are able to catalyze the conversion of amines to nitrosamines in the presence of nitrates or nitrites, the reaction

could occur only at sites where the necessary components are found. Such a location might be the mouth, although nitrate-reducing Enterobacteriaceae do not constitute a part of the normal flora (7). Nitrate reducing microorganisms are found regularly in the lower intestinal tract, however the endogenous production of carcinogenic compounds is unlikely because nitrates and nitrites are rapidly absorbed into the circulation leaving insufficient time for the interaction of bacteria, amines, and nitrates (26). Nitrate excretion studies in rats indicated rapid removal of nitrate in the urine, while little nitrate was recovered from the intestinal tract (26). This suggested that the most likely site for nitrosamine formation is in the bladder of individuals with urinary tract infections.

Microorganisms, Nitrate, and Amines

Sander (53) first demonstrated that bacteria were capable of forming nitrosamines from secondary amines and nitrate in vitro. The microorganisms used in the study were Escherichia coli, Proteus vulgaris and Serratia marcescens, all nitrate-reducing bacteria. These nitrate-reducing bacteria were added to a glucose-free culture system containing potassium nitrate and dipropylamine, diphenylamine, or N-methylaniline. The culture was maintained at a neutral pH and the conversion of the amines to nitroso compounds was demonstrated. The formation of nitrosamines was thought to be due to bacterial enzymatic activity since chemical conversion occurs only at an acid pH (45). The efficiency of nitrosamine formation was correlated with the chemical composition of the precursor amine, the more basic amines being less readily converted to nitrosamines than neutral amines.

Sander and Seif (54) investigated the formation of nitrosamines in the human stomach due to bacterial action. The stomach of achlorhydric individauls might be colonized by intestinal bacteria due to the reduced acidity and hence, microbial nitrosamine production might occur. Thirty-one achlorhydric subjects were tested for nitrosamine formation in vivo. The patients were given sodium nitrate and diphenylamine intragastrically. Subsequent examination of the stomach contents demonstrated the presence of diphenylnitrosamine, indicating that the bacteria colonizing the stomach had reduced nitrate to nitrite and subsequently catalyzed the nitrosation of diphenylamine to diphenylnitrosamine. Drasar and Hill (14) have proposed such a mechanism as being responsible for the development of stomach cancer in achlorhydric individuals.

Alam, Saporoschetz and Epstein (1, 2) studied the formation of N-nitrosopiperidine in the stomach and intestinal contents of normal rats. The stomach and intestinal contents contained microorganisms, however, the identity of these microorganisms was not established. Bacterial conversion of piperidine to N-nitrosopiperidine in the presence of either nitrate or nitrite was expected as the stomach of the rat was less acidic, thus encouraging microbial colonization. In vitro studies utilizing gastric juice containing nitrite, piperidine and normal microbial flora resulted in the demonstration of nitrosopiperidine. Similar experiments using contents of the small intestine did not reveal nitrosamine formation, although all precursor substances and microorganisms were present. The lack of nitrosamine formation in the small intestine was not explained (1). The in vivo administration

of nitrates and piperidine resulted in the recovery of nitrosopiperidine from gastric and small intestinal contents. The coprophagic nature of rats could account for the colonization of the entire digestive tract (26). When 25 mg of sodium nitrate and 1250 mg of piperidine hydrochloride were injected into ligated small intestinal loops, N-nitrosopiperidine was recovered in amounts ranging from 2-25 µg (1, 2). Results of these studies indicated the ability of nitrate-reducing micrcorganisms to form nitroso compounds in the presence of nitrates and amines.

The formation of ^{14}C dimethylnitrosamine by rat intestinal flora under anaerobic conditions was studied by Klubes, Cerna, Rabinowitz and Jondorff (33). Utilizing ^{14}C dimethylamine and sodium nitrite at neutral pH, the formation of ^{14}C dimethylnitrosamine was accomplished. The addition of neomycin to the incubation mixture, which inhibited microbial growth, resulted in lower yields of the nitroso compound. This antibiotic inhibits the facultative aerobic bacteria without affecting the anaerobes such as Bacteroides or Clostridium species. It was concluded that aerobic bacteria make a significant contribution to nitrosamine formation under these experimental conditions. Rabinowitz (51) then studied the formation of ^{14}C dimethylnitrosamine, substituting sodium nitrate for sodium nitrite, and recovered ^{14}C dimethylnitrosamine. These findings supported the conclusion that nitrate reducing microbes may catalyze the nitrosation of dimethylamine in vivo, although neither the enzyme system nor the microbial species responsible was identified. These experiments were repudiated by Collins-Thompson, Sen, Aris, and Schwinghamer (12).

They showed that nitrosamines were not formed by enzymatic activity but that the chemical nitrosation of secondary amines could occur at pH 5.5 to 5.8, higher than that previously thought required for chemical nitrosation. In contrast to the reports of Sander (53) and Hawksworth and Hill (24, 25), Escherichia coli did not catalyze the conversion of dimethyl or diethylamine to the corresponding N-nitrosamine. Dimethylnitrosamine was recovered from cultures of streptococci which are non nitrate-reducing microorganisms. Collins-Thompson and coworkers (12) conducted a series of experiments to distinguish between enzymatic and chemical nitrosation of amines by streptococcal cultures. These experiments revealed that culture filtrates and media containing viable or autoclaved organisms contained comparable amounts of N-nitrosodimethylamine, suggesting that nitrosation was carried out by a nonenzymatic reaction.

Ayanaba and Alexander (6) tested various bacteria and fungi for their ability to convert diphenylamine to diphenylnitrosamine. Preliminary studies indicated that the greatest yields of diphenylnitrosamine were obtained using Pseudomonas sp. and Cryptococcus sp. Experiments were carried out to isolate the enzyme responsible for N-nitrosation. Cell extracts were prepared utilizing the French pressure cell to rupture the microorganisms. The supernatant fluid was shown to have the greatest amount of nitrosating activity. The nitrosating property of the cell extracts was heat-labile in contrast to the heat stable preparations of Collins-Thompson and coworkers (12), suggesting that microorganisms contribute to N-nitrosamine formation in nature. Microbial interactions in nature may include

synthesis of secondary amines, production of nitrite from nitrate or ammonia oxidation, or formation of an enzyme catalyzing N-nitrosation.

Thus, it appears that *in situ* formation of nitrosamines in humans could occur in any location where bacteria, nitrate or nitrite, and precursor amines are present. The two most likely sites are the large intestine and the bladder of individuals with urinary tract infections. Hawksworth and Hill (24, 25) carried out *in vitro* experiments with *Escherichia coli* to demonstrate the ability of the organism to nitrosate diphenylamine, diethylamine, piperidine, pyrrolidine, and N-methylaniline. Five of the ten strains studied were capable of nitrosating these amines under the conditions employed. Additional studies (26) verified the nitrosating capability of 27% of the *E. coli* strains tested. Other enteric bacteria appeared to possess nitrosating capability to a lesser degree.

Brooks, Cherry, Thacker, and Alley (10) demonstrated the presence of dimethylnitrosamine in the urine of a patient with cystitis due to *Proteus mirabilis*. This compound was not present in urine collected after treatment and elimination of the etiologic agent from the urinary tract. *In vitro* studies with the organism in autoclaved urine and in cooked meat medium indicated that *Proteus mirabilis* could nitrosate dimethylamine. Quantitation of dimethylnitrosamine formed during the course of this urinary tract infection indicated that sufficient nitrosamine for carcinogenesis (15, 41) was produced. Thacker and Brooks (64) studied the formation of N-nitrosodimethylamine in normal urine by three strains each of *Proteus mirabilis*, *Proteus vulgaris*, *Proteus rettgeri* and *Proteus morganii*. All strains of

Proteus mirabilis, Proteus rettgeri and Proteus morganii produced N-nitrosodimethylamine when incubated aerobically in urine. Proteus vulgaris strains failed to produce nitrosamine. Both dimethylamine and nitrate were shown to be present in low concentration as normal constituents of the urine used. The results indicated that N-nitroso-dimethylamine may be formed in the urine of people with bacteriuria due to common urinary tract pathogens.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains Used

Bacterial reference strains were obtained from several sources. *Escherichia coli* serotypes 06 and 075 were supplied by Dr. G. Dominique of Tulane Medical Center, New Orleans, Louisiana. These strains were used as positive controls to check the efficacy of the serotyping procedure and typing sera used. A nitrosamine producing strain, *E. coli* 555, was obtained from Dr. J. Hawksworth of St. Mary's Hospital, London, England. This organism was used as a positive reference strain for *in vitro* nitrosamine formation studies. *Proteus mirabilis* 40-512-1, known to form dimethylnitrosamine, was supplied by Dr. J. Brooks, Center for Disease Control, Atlanta, Georgia. This strain was also used as a known nitrosating control microorganism in the *in vitro* assays.

Other *E. coli* strains used for *in vitro* studies were isolated from human feces or urine. A total of 207 isolates from patients exhibiting symptoms of acute or chronic urinary tract infection were obtained from several hospitals in the Oklahoma City area. Twelve *E. coli* strains of intestinal origin were supplied by Dr. L. Bernard, Children's Memorial Hospital, Oklahoma City. Both enteropathogenic

and nonpathogenic strains were among the intestinal isolates. Thus, a variety of isolates was used for a comparison of nitrosating ability among E. coli strains of normal flora and those strains causing urinary tract infections and intestinal infections.

All strains of E. coli were stored at 4°C on stock culture agar slants (Difco). The strains were transferred at 3 month intervals throughout the course of the study. Fresh isolates were used for biochemical confirmation and serologic studies, as well as for all in vitro and in vivo studies.

E. coli strains typically demonstrate the following biochemical characteristics: they ferment glucose and lactose with the production of gas, split indol from tryptophan, produce an acid environment due to highly acid fermentation products, produce few neutral fermentation products such as acetylmethylcarbinol, cannot utilize citrate as a sole source of carbon, and are able to reduce nitrate to nitrite (16, 30). Utilizing these properties, all isolates were confirmed as E. coli by the following procedures:

1. appearance on eosin-methylene blue agar
2. utilization of glucose, lactose, and/or sucrose
3. positive indol test
4. positive methyl red test
5. negative Voges-Proskauer reaction
6. negative Simmon's citrate test, and
7. reduction of nitrate to nitrite when grown on nitrate agar.

Isolates conforming to the above criteria were serotyped by their O somatic antigen according to the slide agglutination method of

Kaufmann (30). The K envelope antigens of E. coli interfere with O agglutination, therefore all strains tested were boiled for one hour prior to typing in order to remove the K antigens. Brain heart infusion slants (Difco) were inoculated with E. coli strains and incubated at 35°C for 18 hours. The fresh cultures were washed from the slants with 0.7 ml sterile physiologic saline (0.9%, w/v). The saline suspensions were boiled for one hour in a water bath. One drop of each saline suspension was added to a series of agglutination wells on a premarked slide. Each well contained one drop of a monospecific O antiserum diluted 1:5 in a 1:10,000 merthiolate-water mixture. The O antisera used were types 01, 02, 04, 06, 07, 08, 018, 025, 050, and 075 (Difco). A saline control consisting of one drop of each E. coli suspension and one drop of physiologic saline was added to each slide. The slides were rotated until visible agglutination occurred or one minute had elapsed. If agglutination appeared in all the wells, including the saline control, the E. coli strain was designated as rough. If no agglutination was noted, the strain was termed untypable. If agglutination occurred in a well containing one of the antisera and not in the saline control, the strain was designated as a specific serotype. Reference cultures were typed as positive controls throughout the serotyping studies.

Experimental Animals

Male rats of Holtzman descent, weighing 240-260 grams, were used for the animal experiments. These animals were obtained from the rat colony of the Oklahoma Medical Research Foundation, Oklahoma City. Rats were housed individually in metabolic cages designed so that

urine could be collected free of fecal material. The animals were not fed solid food but were given 5% (w/v) glucose solution ad libitum. This method of feeding was carried out in an effort to prevent fecal contamination of the urine and to increase the urine volume (20). This procedure facilitated the extraction, concentration, and assay of nitrosamines.

In Vitro Studies

Urine as Culture Medium

Quantitation of Bacteria. A calibrated loop-direct streak method was used to quantitate E. coli organisms for growth curves in vitro. A loop, calibrated to deliver 0.01 ml, was dipped into a 6 hour broth culture and streaked on an eosin-methylene blue plate as shown in Figure 1. This procedure was also used to quantitate the inoculum for animal infections, the number of organisms excreted in the urine during infection, and the microorganisms recovered from infected animals at the termination of each experiment. This method yielded three dilutions on a single plate corresponding to 10^{-2}, 10^{-3}, and 10^{-4}.

Growth Curve. In vitro and in vivo growth curves for E. coli were determined using rat urine as the culture medium. The in vitro curve was determined by inoculating 0.1 ml of an E. coli broth culture containing 10^{7} organisms/ml into flasks containing 40 ml of filter-sterilized rat urine adjusted to pH 7.0. The flasks were incubated at 35°C in a shaking water bath (Aquatherm model R-86, New Brunswick Scientific). One ml samples of urine were removed for colony counts

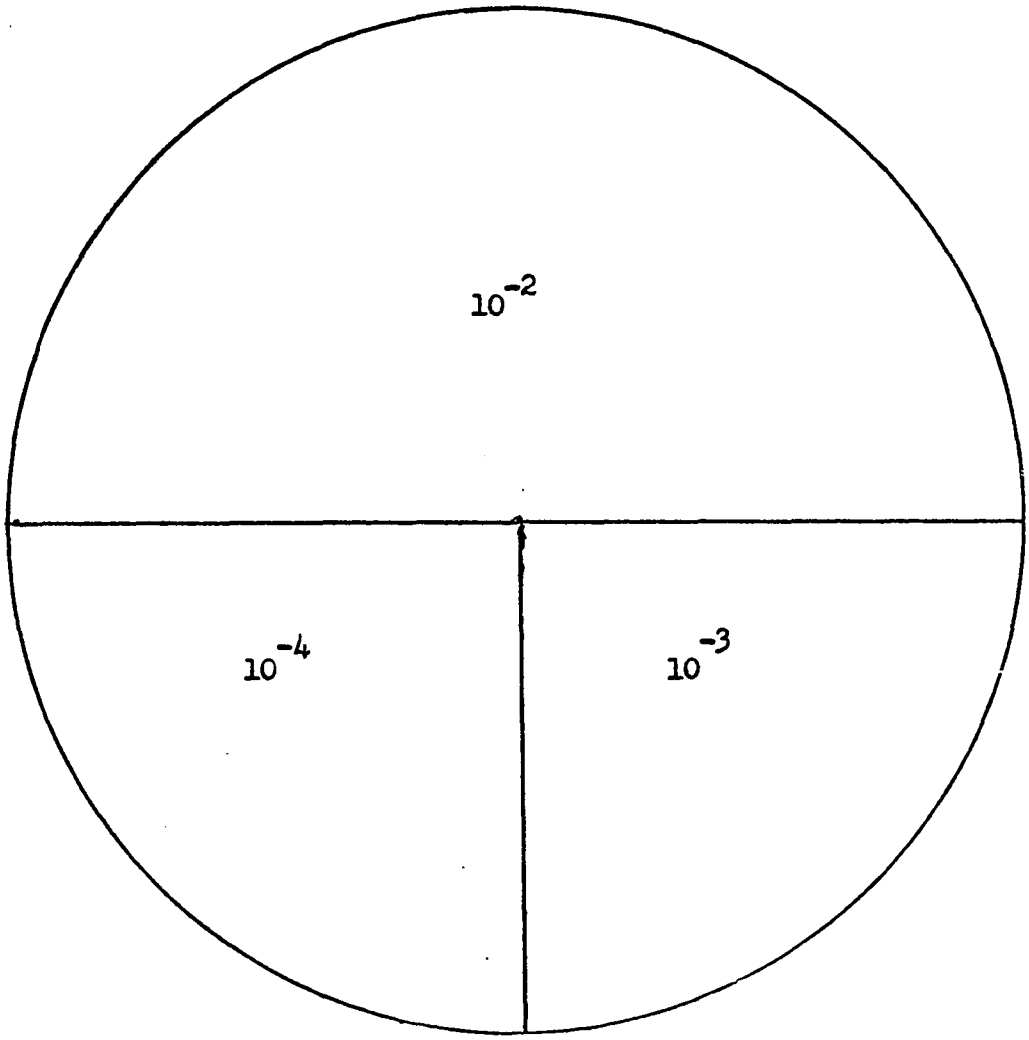

Fig. 1 - Quantitation of bacteria by the calibrated loop - direct streak method. Inoculum is spread over top one -half of plate then into lower right quadrant and finally into the lower left quadrant, yielding the dilutions noted.

at 0, 1, 2, 3, 4, 5, 6, 12, and 24 hours. Dilutions of the urine culture were made to obtain plates, streaked as shown in Figure 1., having approximately 30 colonies on one of the quadrants. All dilutions were plated in duplicate to obtain an average colony count for each sampling period. For in vivo nitrosation studies, it was necessary to prevent bacterial growth and metabolism after the organisms were excreted in the rat urine. For this reason, it was necessary to ascertain the minimal inhibitory concentration of an antibiotic to be added to the collection vessel. In addition, it was assumed that the antibiotic would prevent growth of any contaminating normal flora. All strains of E. coli tested were susceptible to a 10 µg disc of gentamycin (BBL) by plate testing, thus this antibiotic was used in animal experiments. The number of gentamycin discs (10 µg) needed to inhibit growth of E. coli was determined by adding 0, 1, 2, 3, 4, 5, 6, 7, or 8 discs to 20 ml urine samples containing 10^7 organisms/ml, the maximum number of microorganisms excreted during in vivo studies. According to the results of this assay, it was necessary to add 4 discs (40 µg) to each urine collecting vessel.

In vivo excretion curves were obtained by using broth cultures of E. coli containing 10^9 organisms/ml. The organisms were centrifuged, washed twice with sterile nutrient broth, and resuspended at the desired concentration in 10 ml of sterile broth. Rats were anesthetized by ether and an incision was made on the right dorsal area exposing the right kidney. The kidney was brought to the surface using forceps and 5 µl of the E. coli suspension was injected into two different sites. The incision was closed using clamps and the rat was

returned to its cage. The progress of the infection was monitered by quantitative plate counts of rat urine samples. The urine samples were collected in sterile disposable 4.5 ounce cups (Falcon Plastics) at 0, 2, 4, 6, 12, 24, 48, 72, 96, and 144 hours postinfection. The cages were disinfected with water, 5% (v/v) phenol, water, and ethanol prior to collection of the urine specimens. The rats were induced to urinate at the specific time intervals by excitation due to handling.

N-Nitrosation by Escherichia coli in Urine. To establish that the N-nitrosation of secondary amines occurred in urine, studies were done using filter-sterilized urine as the culture medium. The studies were performed utilizing human and rat urine to which 0.2% (w/v) glucose and 0.2% (w/v) potassium nitrate were added. Diphenylamine and piperidine were employed as the precursor amines to be converted to the corresponding N-nitrosamines.

N-Nitrosamine Assays

Diphenylamine has been shown to be more readily nitrosated than other amines and for this reason the conversion of diphenylamine to N-nitrosodiphenylamine was studied. Ten ml samples of nutrient broth containing 0.2% (w/v) glucose (Bacteriologic grade, Difco) and 0.2% (w/v) potassium nitrate (Baker Chemicals) were inoculated with the E. coli strain under study and incubated overnight at 35°C. One mmole of diphenylamine (Eastman-Kodak) was added aseptically to 90 ml of glucose-nitrate broth prior to the addition of the 10 ml overnight broth inoculum. These cultures were incubated at 35°C in a shaking water bath for 18 hours. The cultures were then centrifuged at 9,000 x g at 4°C (Sorvall RC2-B) for ten minutes to remove bacterial cells

and debris. The supernatant fluids were checked for the presence of nitrite using a qualitative sulfosalycylic acid-alpha naphthol reagent (61), and then alkalinized to pH 9.0. The broths were extracted twice with 50 ml of dichloromethane (Fisher Scientific) and since diphenylamine and diphenylnitrosamine are nonvolatile compounds, the extracts were concentrated to dryness in a rotary evaporator (Rinco Instrument Company). The residue was washed twice with 1.0 ml of dichloromethane (Fisher Scientific) and the washings were transferred to small vials and dried at 70°C. The residue in the vials was reconstituted with dichloromethane to 0.1 ml and diphenylamine and diphenylnitrosamine were assayed by thin layer chromatography according to the methods described below.

The first method used was specific for N-nitrosamines (50). Silica gel G plates were spread to 0.25 mm thickness, dried at room temperature for 30 minutes, stored in a dessicator, and activated at 110°C for 30 minutes before use. Ten µl samples were spotted 2 cm from the base of the plates. After the samples had dried, the plates were developed in a chromatography tank containing a solvent system composed of hexane:diethylether:dichloromethane (4:3:2). Following migration of the solvent front 10 cm from the sample application site, the plates were removed from the tank and air-dried. The presence of diphenylnitrosamine was detected by spraying the plate with a palladium chloride reagent with subsequent exposure of the plate to shortwave UV light (Mineralight Model R-51, Ultraviolet Products, Inc.) for 5 minutes. N-nitrosamines form a blue-violet complex when treated in this manner. An alternate detection method (61) utilized a solvent

system of benzene:petroleum ether (1:1). The plates were silica gel G containing 10% (w/w) zinc dust. Since this procedure was used to identify both diphenylamine and diphenylnitrosamine on the same plate, the solvent front was allowed to migrate 15 cm to achieve better separation of the two compounds. The color developing reagent used was a modified Enrlich's reagent containing 1 g of para-dimethylaminobenzaldehyde dissolved in 25 ml of 12 N hydrochloric acid and 75 ml of methanol. Reacted plates were sprayed with the reagent, air dried, and warmed in a hot air oven at 90°C for 5 minutes. This sequence was found to enhance color development, diphenylamine appearing yellow and diphenylnitrosamine appearing reddish-brown.

Preliminary in vitro studies were performed using diphenylamine, while subsequent studies employed volatile secondary amines such as dibutylamine, diethylamine, and piperidine (Eastman-Kodak). For detection of these compounds, an experimental design similar to that used for diphenylamine was employed. Since volatile secondary amines are less readily nitrosated than diphenylamine, 500 ml broth cultures were used instead of 100 ml cultures. Dibutylamine, diethylamine, and piperidine (0.05%, w/v) were added to designated culture vessels instead of the 1 mM concentration used in diphenylamine experiments. Due to the volatile nature of these amines and their N-nitrosamines, the solvent was not evaporated to complete dryness. Concentration was carried to less than 1 ml by rotary evaporation. The residue was transferred to small vials and held at room temperature until the remaining solvent had vaporized. The residue was then reconstituted to 0.1 ml with dichloromethane and assayed for N-nitrosamines using

thin layer and gas-liquid chromatography. The thin layer system utilized the palladium chloride reagent specific for N-nitrosamines. Samples for gas chromatographic analyses were prepared by spotting the extract remaining after the initial thin layer chromatographic analysis onto silica gel G plates with subsequent development in the hexane: ether:dichloromethane solvent system (50, 61). The right edges of the plates were sprayed to show the migration of a standard N-nitrosamine and sections with the same Rf value as the standard were scraped from the plates and washed twice with 1 ml dichloromethane to elute any N-nitrosamine from the silica gel. The eluates were concentrated to 0.1 ml and assayed by gas liquid chromatography. A Varian Aerograph (model 1700) equipped with a flame ionization detector was used employing a 6 foot stainless steel column packed with 28% Penwalt 223 and 4% potassium hydroxide on Gas Chrom Q. This column will separate amines and their derivatives. The conditions under which gas liquid chromatography were carried out are summarized in Table 1.

In vitro experiments were done using diphenylamine as the precursor amine in an effort to determine the kinetics of diphenylnitrosamine formation with respect to the time required for nitrosamine formation and the concentration of the substrate. The length of incubation required for formation of N-nitrosodiphenylamine was determined using a series of 50 ml broth cultures of E. coli containing 10 mg diphenylamine. These cultures were incubated for 0, 8, 16, 24, 32, 40, or 48 hours, centrifuged, alkalinized (pH 9.0), extracted, concentrated, and assayed for the presence of diphenylnitrosamine. In a similar manner, 50 ml broth cultures containing 0.2% (w/v) glucose,

TABLE 1

GAS-LIQUID CHROMATOGRAPHIC CONDITIONS USED FOR THE DETECTION OF VOLATILE AMINES AND N-NITROSAMINES

Flow rate (ml/min)		Temperature (^{o}C)	
Nitrogen	40	Column	160
Hydrogen	40	Detector	190
Oxygen	400	Injector port	195

Sensitivity : 2 x 10^{-10} milliamperes/millivolt
Sample size : 2 microliters

0.2% (w/v) potassium nitrate, and varying concentrations of diphenylamine were inoculated with 10^7 E. coli cells of the strain under study. Diphenylamine was added in concentrations of 0, 5, 10, 20, and 50 mg since these concentrations were considered as possible doses in in vivo experiments. The cultures were incubated overnight and assayed for diphenylnitrosamine.

In Vivo Studies

Recovery of Diphenylamine

Fluorometry was used to quantitate diphenylamine and diphenylnitrosamine present in rat urine. The procedure was found to be unsuitable for diphenylnitrosamine since the nitroso group quenched the fluorescence of the phenol rings. However, this method was useful for the quantification of diphenylamine. An Aminco-Bowman Spectrophotofluorometer (American Instrument Co.) was used with a maximum excitation wave length setting of 295 nm and maximum emission wave length of 370 nm. The instrument was calibrated using a 1 ug quinine sulfate standard. Dilutions of urine extracts were made in 95% ethanol and absolute values were established by comparison with a standard curve for diphenylamine in ethanol (Figure 2).

Recovery of N-nitrosodiphenylamine

Male rats, individually housed in metabolic cages, were given 5% (w/v) glucose solution ad libitum to increase their urine volume (20). After three days of diuresis, the animals were infected with a predetermined inoculum of E. coli strains showing nitrosating capability in in vitro experiments. Five µl of a 10^8/ml suspension of

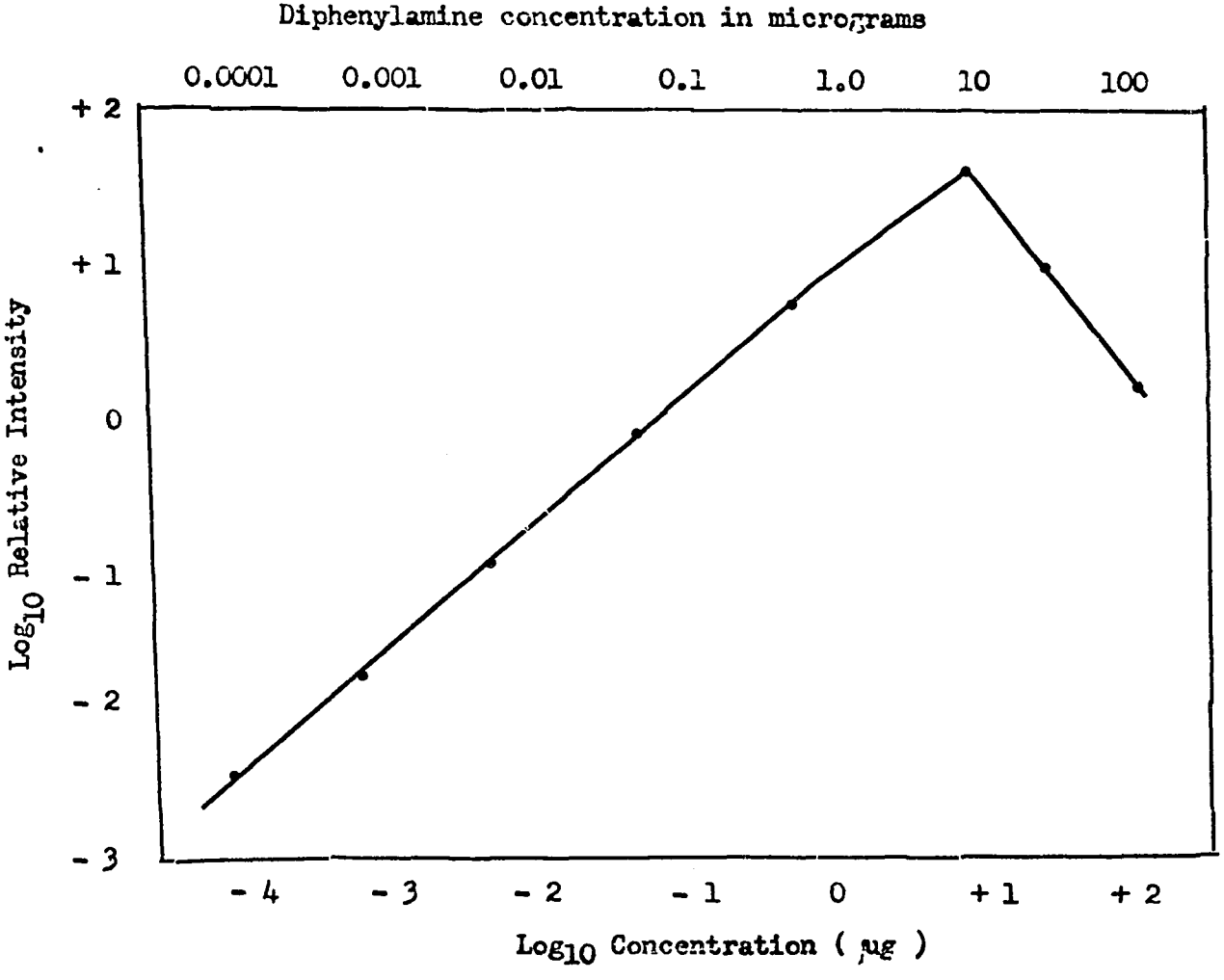

Fig. 2 - Diphenylamine standard curve determined by fluorometric analysis.

E. coli strain 93 or 108 was injected into 2 cortical areas of the kidney, using a 10 µl Hamilton syringe. Following the surgical procedure, the rats were allowed to recuperate for 24–48 hours. At this time the highest number of organisms was excreted in the urine. The animals were then given varying doses of diphenylamine in corn oil and potassium nitrate in distilled water by gastric intubation *per os*. The ratio of amine to nitrate was 1:2. *In vitro* studies have indicated this to be an optimal ratio for nitrosation (64). Intragastric intubations were given at 8 hour intervals and urine was collected and analyzed for the presence of nitrite, diphenylamine, and N-nitrosodiphenylamine. Aspiration of bladder contents and kidney homogenates were obtained for quantitative colony counts at the end of each experiment. Four normal and infected kidneys were recovered and fixed in 10% (v/v) neutral buffered formalin for tissue examination. Tissue sections were prepared and subsequently stained with hematoxylin-eosin (36) and Brown and Brenn's modified gram stain (11). These kidney sections were examined for the presence of microorganisms and indications of inflammatory processes compatible with an acute or chronic infection.

Urine specimens collected in the presence of gentamycin were refrigerated at 4°C until assayed. A portion was used to assay for the reduction of nitrate to nitrite and the remaining urine specimen was adjusted to pH 9.0. The alkalinized urine was extracted twice with one-half volume of dichloromethane. The extract was washed with an equal volume of glycine-hydrochloride buffer (pH 2.0) to remove interfering amines, followed with an equal volume of 20% (w/v) potassium carbonate to remove any buffer trapped in the solvent. The

extract was dried over anhydrous sodium carbonate for 30 minutes to eliminate residual water. The dichloromethane extract was then concentrated, dried, and reconstituted in the same manner used for the *in vitro* assay. Fifty µl samples were spotted on silica gel G plates containing 10% (w/w) zinc dust and developed utilizing a spray reagent specific for phenolic compounds (61).

CHAPTER III

RESULTS

Identification of E. coli Isolates

Biochemical Identification

Two hundred seven bacterial strains isolated from urine and 12 intestinal strains presumptively identified as E. coli were collected from hospitals in the Oklahoma City area. Biochemical confirmation of these strains was carried out and agreement or disagreement with the reactions listed in Table 2 was established. All 12 intestinal strains demonstrated reactions characteristic for Escherichia coli. The majority of the strains isolated from urinary tract infections also conformed. Ninety-eight % of the isolates from urine were lactose positive and had typical colony morphology when plated on eosin methylene blue agar. When grown on triple-sugar iron agar slants, all of the isolates fermented lactose and/or sucrose (acid slant) and dextrose (acid butt) with gas formation. This reaction is typical of E. coli. Other biochemical reactions commonly employed in the identification of E. coli are frequently summarized by the acronym IMVIC, referring to the production of indole from tryptophan, acid production as indicated by methyl red change, acetyl-methylcarbinol production (Voges-Proskauer test), and the utlization of citrate as a carbon

source. As seen in Table 2, 96% of the strains obtained from urine were indole positive, 97% were methyl red positive, while 98% were Voges-Proskauer negative and 96% did not utilize citrate as a carbon source. Genera of the family Enterobacteraceae must be capable of reducing nitrate to nitrite and it may be noted that all of these isolates conformed to this characteristic. It will be seen in Table 2 that 97% of the isolates investigated were confirmed as Escherichia coli. Two indole negative strains were designated E. coli and this was confirmed by serologic typing.

Serologic Identification

Common E. coli serogroups isolated from urinary tract infections are 01, 02, 04, 06, 07, 08, 018, 025, 050, and 075 (65). The 201 E. coli isolates were serotyped using monospecific antisera. Results of the serotyping experiments are shown in Table 3. Twenty-nine of the strains were designated as rough because agglutination occurred in all of the sera used as well as the saline control. Ninety-two strains were inagglutinable in the O antisera employed. Eighty strains showed type-specific agglutination within one minute and were designated typable.

The E. coli strains showing mono-specific agglutination are shown according to serotype in Table 4. Frequently isolated serotypes were 06, 075, and 04. These three serogroups represented 40, 19, and 13% respectively, of the typable strains. The other 28% typable E. coli strains were distributed among 01, 050, 07, 025, 02, and 018 serogroups. No strains of the 08 serogroup were isolated during the course of this study.

TABLE 2

BIOCHEMICAL CONFIRMATION OF PRESUMPTIVE *ESCHERICHIA COLI* ISOLATES

Reaction	Isolates demonstrating typical biochemical reactions for the genus *Escherichia*		
	Urine isolates (207 strains)		Intestinal isolates (12 strains)
	Number	Per cent	Number
Lactose fermentation: EMB	203	98	12
Lactose/Dextrose fermentation: TSI	207	100	12
Indol positive	199	96	12
Methyl-Red positive	201	97	12
Voges-Proskauer negative	203	98	12
Citrate negative	200	96	12
Nitrate reduction	207	100	12
Total number of strains identified as *E. coli*	201		12

TABLE 3

AGGLUTINATION REACTION OF *ESCHERICHIA COLI* URINARY TRACT ISOLATES

Type of reaction	Number of isolates	Per cent
Type specific	80	40.0
Rough	29	14.5
Untypable	92	45.5
Total	201	100.0

TABLE 4

TYPE SPECIFIC *ESCHERICHIA COLI* URINARY TRACT ISOLATES ACCORDING TO SEROGROUP

Serogroup	Number of type-specific isolates	Per cent
06	32	40
075	15	19
04	10	13
01	7	9
050	5	6
07	5	6
025	4	5
02	1	1
018	1	1
08	0	0
Total	80	100

In Vitro N-Nitrosamine Assays

Broth Culture Experiments

Thirty-seven strains of E. coli selected at random from the 201 confirmed isolates from urine were analyzed for their ability to form N-nitrosodiphenylamine from diphenylamine and nitrate. The reaction was carried out in nutrient broth cultures containing 0.2% (w/v) glucose, 0.2% (w/v) potassium nitrate, and 10 mg diphenylamine. As shown in Table 5, 87% of the E. coli strains tested nitrosated diphenylamine. There was no relationship between serotype and N-nitrosating ability since diphenylnitrosamine was demonstrated in 91% of the extracts from type-specific strains and 95% of extracts from untypable strains. Eighty-seven per cent of the isolates from urine produced the N-nitroso compound, whereas 100% of the intestinal isolates (6 enteropathogenic and 6 non-typable) were capable of nitrosating diphenylamine in the presence of nitrate.

Diphenylnitrosamine and diphenylamine were detected by thin layer chromatography utilizing a developing reagent specific for phenolic compounds (61). The addition of this reagent to a plate on which E. coli broth extracts, an uninoculated control extract, and 10 µg standards of diphenylamine and diphenylnitrosamine were spotted gave the typical results seen in Figure 3. Sample 1, a control broth, was extracted in the same manner as the inoculated cultures. The intense reactions (Rf 0.6) correspond to the reactions of the diphenylamine standards (Rf 0.6) that were applied at locations 5 and 7. Diphenylnitrosamine was not detected in the uninoculated control broth. The extracts of E. coli broth cultures (samples 3, 4, 6, 8) had large

TABLE 5

N-NITROSAMINE FORMATION BY *ESCHERICHIA COLI* SEROTYPES ISOLATED FROM URINE

Serotype	Number of isolates	Number forming N-nitrosamines	Per cent
Type-specific	22	20	90
Untypable	13	11	85
Rough	2	1	50
Total	37	32	87

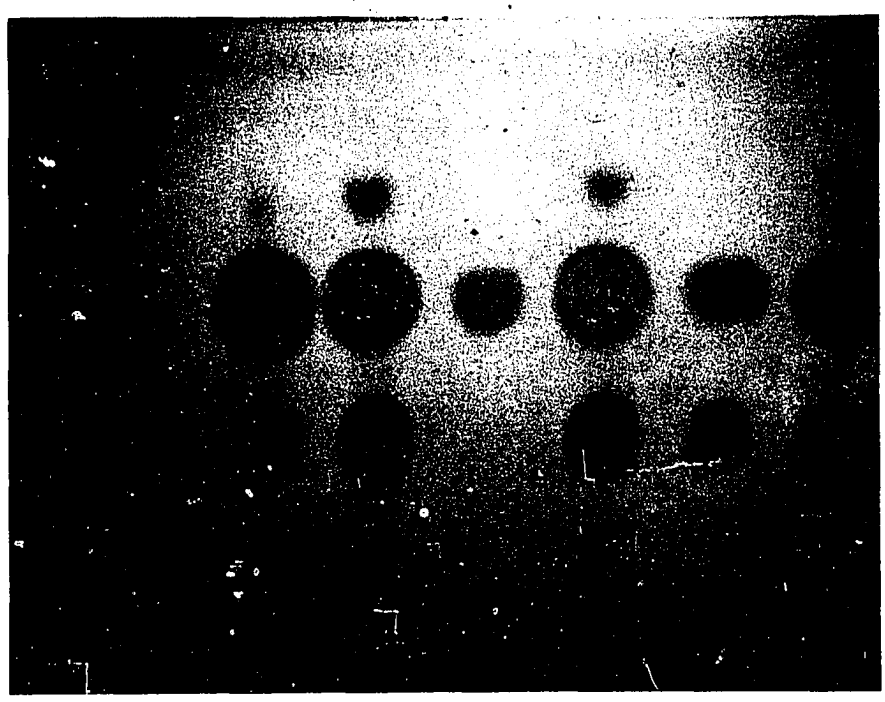

Fig. 3 -Thin layer chromatogram of four Escherichia coli broth extracts demonstrating in vitro nitrosation of diphenylamine.

1. Extract of an uninoculated control
   Rf 0.6 : diphenylamine
2. Diphenylnitrosamine standard (10 µg)
3. Extract of E. coli strain 93
4. Extract of E. coli strain 108
6. Extract of E. coli strain 116
8. Extract of E. coli strain 123
   Rf 0.8 : unknown diphenylamine derivative
   Rf 0.6 : diphenylamine
   Rf 0.4 : diphenylnitrosamine
5. Diphenylamine standard (10 µg)
7. Mixed standard (10 µg)
   Rf 0.6 : diphenylamine
   Rf 0.4 : diphenylnitrosamine

spots at Rf 0.6 confirming the presence of residual diphenylamine. Also present were reactions (Rf 0.4) which corresponded to the diphenylnitrosamine standard (samples 2 and 7). Culture extracts also demonstrated a small spot at Rf 0.8. The reagent used is specific for phenol rings and the compounds demonstrated at Rf 0.8 are unknown diphenylamine derivatives (61).

Following completion of studies demonstrating conversion of diphenylamine to its corresponding N-nitrosamine, the formation of volatile N-nitroso compounds was investigated. Two dialkylamines (dibutylamine and diethylamine) and one heterocyclic amine (piperidine) were employed as precursor compounds for the nitrosation reaction. Fifteen E. coli strains previously shown to nitrosate diphenylamine were used in these experiments. A thin layer chromatogram of extracts from E. coli strain 93 broth cultures grown in the presence of the amines listed above is shown in Figure 4. In each case, the N-nitrosamine corresponding to the precursor amine was formed (locations 5, 6, 7, 8). This is evidenced by comparison with the standard N-nitrosamine shown on the left side of the thin layer plate (locations 1, 2, 3, 4). The developing reagent used was specific for N-nitrosamines and no other compounds were detected in the extracts. Gas-liquid chromatography of the extracts was carried out to confirm identification. This procedure was selected in order that both amines and nitrosamines could be eluted from the same column. The elution times (Rst) and distances (Rf) for diethylnitrosamine, dibutylamine, dibutylnitrosamine, piperidine, and nitrosopiperidine are shown in Table 6. Diphenylamine and diphenylnitrosamine are nonvolatile diphenyl compounds and will

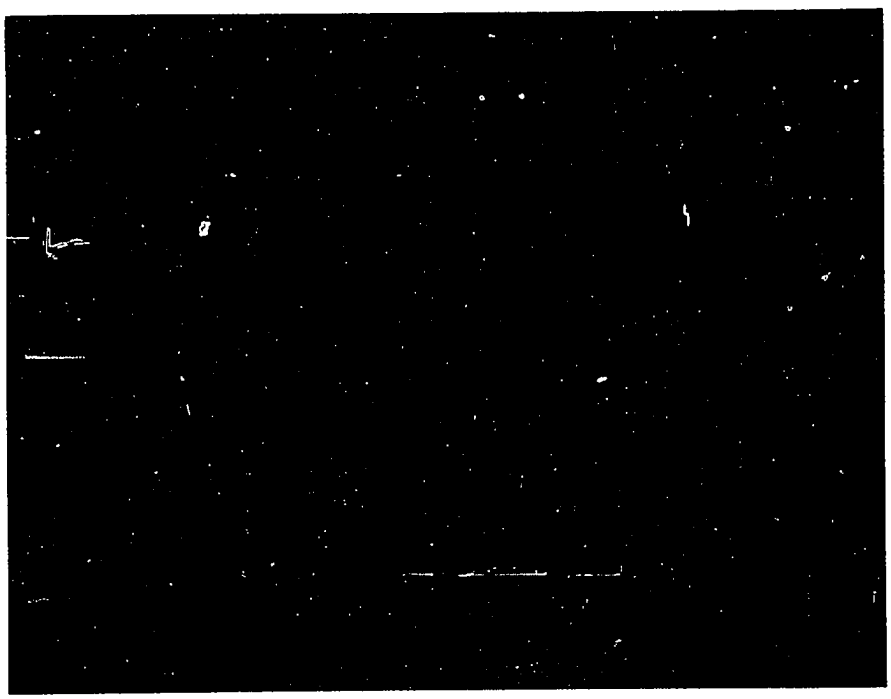

Fig. 4 - Thin layer chromatogram demonstrating in vitro N- nitrosation of dibutylamine, diethylamine, diphenylamine, and piperidine by Escherichia coli strain 93.

1. Dibutylnitrosamine standard : Rf 0.65
2. Diethylnitrosamine standard : Rf 0.55
3. Diphenylnitrosamine standard : Rf 0.71
4. Nitrosopiperidine standard : Rf 0.50
5. Extract of E. coli strain 93 grown in presence of dibutylamine Rf 0.65 : dibutylnitrosamine
6. Extract of E. coli strain 93 grown in presence of diethylamine Rf 0.55 : diethylnitrosamine
7. Extract of E. coli strain 93 grown in presence of diphenylamine Rf 0.71 : diphenylnitrosamine
8. Extract of E. coli strain 93 grown in presence of piperidine Rf 0.50 : nitrosopiperidine

TABLE 6

GAS-LIQUID CHROMATOGRAPHIC IDENTIFICATION
OF AMINES AND N-NITROSAMINES

Compound	Rf[a]	Rst[b]
Diethylamine	-[c]	-[c]
Diethylnitrosamine	3.5	4.0
Dibutylamine	2.9	3.5
Dibutylnitrosamine	21.5	26.0
Piperidine	2.5	3.0
Nitrosopiperidine	14.1	17.5

[a]Rf:distance a compound migrates, measured from injection point to peak maximum (cm)

[b]Rst:elution time of compound from injection to peak maximum (min)

[c]Diethylamine elutes with the solvent front

not elute from this column. Diethylamine elutes very rapidly so that the peak cannot be distinguished from the solvent front. Each of the other compounds has a characteristic retention time and migratory distance and it was possible to identify chromatographic peaks by comparison with standards. Representative gas chromatograms of extracts of E. coli broth cultures grown in the presence of dibutylamine and piperidine are presented in Figures 5 and 6, respectively. The first large peak seen represents the solvent front. The retention time for dibutylamine was 3.5 minutes, while N-nitrosodibutylamine was eluted at 26 minutes (Figure 5). Piperidine and nitrosopiperidine had retention times of 3.0 and 17.5 minutes, respectively (Figure 6). A summary of volatile and nonvolatile N-nitrosamine formation in broth cultures by E. coli isolates of intestinal and urinary tract origin is presented in Table 7. All of the intestinal strains formed N-nitrosodiphenylamine. The nitrosating ability of these strains was not studied further. Eighty-seven % of the E. coli strains isolated from urine nitrosated diphenylamine, giving a total of 90% nitrosating strains when intestinal isolates were included. The 15 strains studied with regard to N-nitrosation of diethylamine, dibutylamine and piperidine had previously been shown to form diphenylnitrosamine. All isolates studied formed diethylnitrosamine and dibutylnitrosamine while 93% of isolates formed detectable levels of nitrosopiperidine (Table 7).

The medium used in preliminary studies contained 0.2% (w/v) glucose, which enhanced the growth of E. coli and presented the possiblity of a decreased pH which may enhance nitrosation through

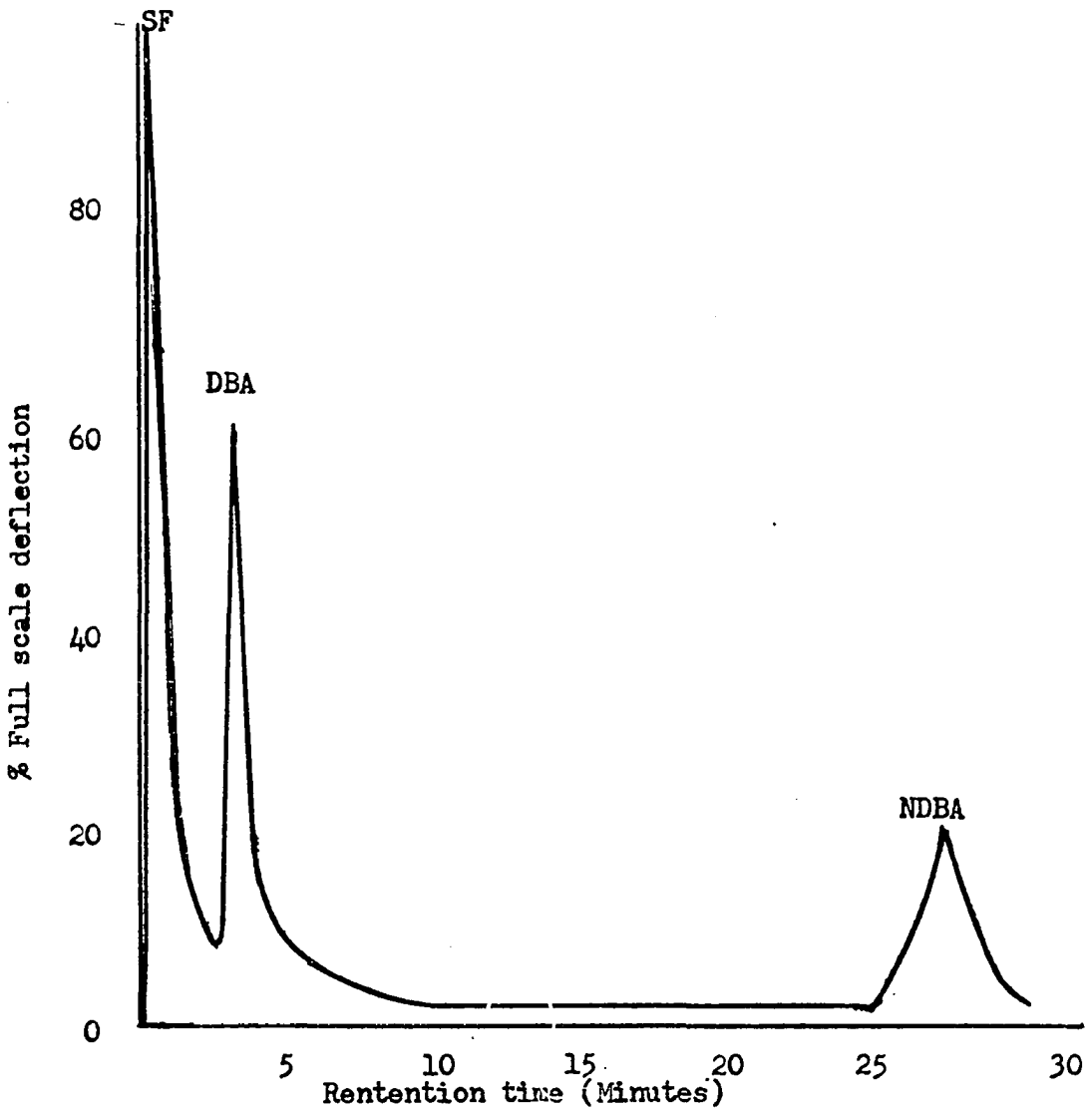

Fig. 5 - Gas-liquid chromatogram of an extract from a broth culture of Escherichia coli strain 93 containing dibutylamine, 0.2% glucose, and 0.2% nitrate. The peaks are the solvent front (SF), dibutylamine (DBA), and N-nitrosodibutylamine (NDBA).

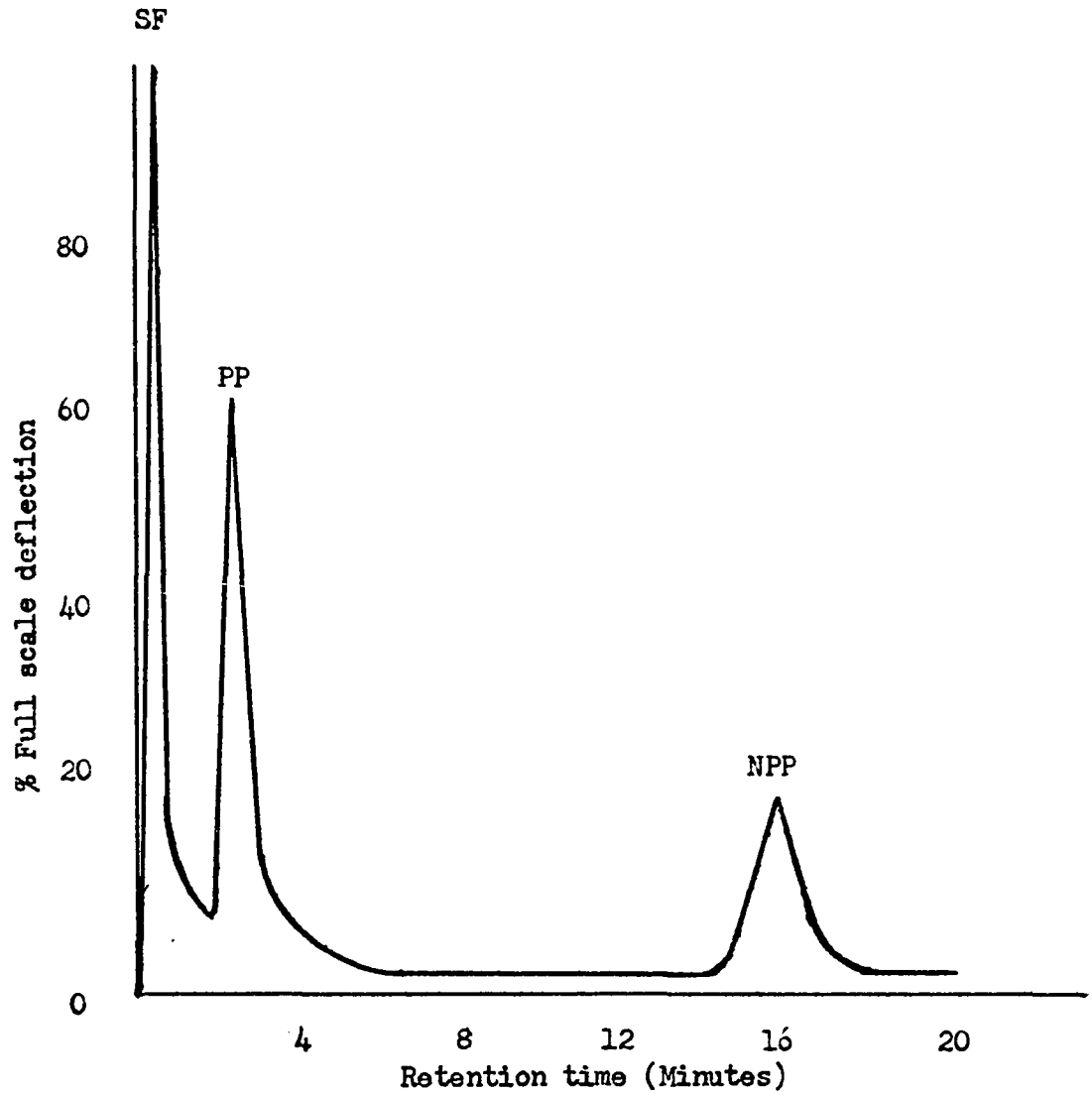

Fig. 6 - Gas-liquid chromatogram of an extract from a broth culture of Escherichia coli strain 93 containing piperidine, 0.2% glucose, and 0.2% nitrate. The peaks are the solvent front (SF), piperidine (PP), and N-nitrosopiperidine (NPP).

TABLE 7

COMPARISON OF DETECTION PROCEDURES FOR ANALYSIS OF EXTRACTS PREPARED DURING *IN VITRO* N-NITROSAMINE FORMATION BY *E. COLI* ISOLATES OF URINARY TRACT AND INTESTINAL ORIGIN

Origin of Isolates N-nitrosamine assayed	Per Cent Nitrosation	
	Thin-layer chromatography	Gas-liquid chromatography
Intestinal tract		
diphenylnitrosamine	100	not tested
Urinary tract		
diphenylnitrosamine	87	not tested
diethylnitrosamine	100	0
dibutylnitrosamine	100	100
nitrosopiperidine	93	93

chemical catalysis. Experiments were designed to study the effect of glucose on the formation of nitrosodiphenylamine *in vitro*. These experiments related to planned *in vivo* studies because normal rat urine contains little or no glucose. The effect of removing glucose from the test system is shown in Figure 7. Cultures grown in the absence of glucose gave a low yield of diphenylnitrosamine while the cultures grown in the presence of 0.2% (w/v) glucose demonstrated increased formation of diphenylnitrosamine. Although the yield of nitrosodiphenylamine was low in the glucose free extracts, this compound was present in detectable concentrations. These experiments demonstrated the feasibility of using diphenylamine for animal studies.

Upon selecting diphenylamine as the substrate for *in vivo* N-nitrosation experiments, the phenomenon of substrate inhibition was investigated. Results of these experiments are shown in Figure 8. Nutrient broth cultures of *E. coli* strain 93 were grown in the presence of 0, 5, 10, 20, or 50 mg diphenylamine. N-nitrosodiphenylamine was recovered from all culture extracts except the negative control culture which contained no diphenylamine. Increasing amounts of the nitroso compound were recovered as the concentration of the amine increased. This is evident as larger and more intense reactions on the chromatography plate.

Experiments were conducted to determine if increasing amounts of nitrosamines would be synthesized with longer periods of incubation. Broth cultures containing 10 mg diphenylamine, 0.2% (w/v) glucose, and 0.2% (w/v) potassium nitrate were inoculated with *E. coli* strain 93 and incubated for 0, 8, 16, 24, 32, 40, and 48 hours (Figure 9). Con-

Fig. 7 - The effect of glucose on the *in vitro* nitrosation of diphenylamine by Escherichia coli strain 93.

1. Extract of E. coli broth culture without glucose
   Rf 0.65 : diphenylamine
   Rf 0.45 : diphenylnitrosamine
2. Extract from uninoculated broth
   Rf 0.65 : diphenylamine
3. Mixed standard
   Rf 0.65 : diphenylamine
   Rf 0.45 : diphenylnitrosamine
4. Same as sample 1
5. Extract of E. coli broth culture with 0.2 % glucose
   Rf 0.80 : unknown diphenylamine derivative
   Rf 0.65 : diphenylamine
   Rf 0.45 : diphenylnitrosamine

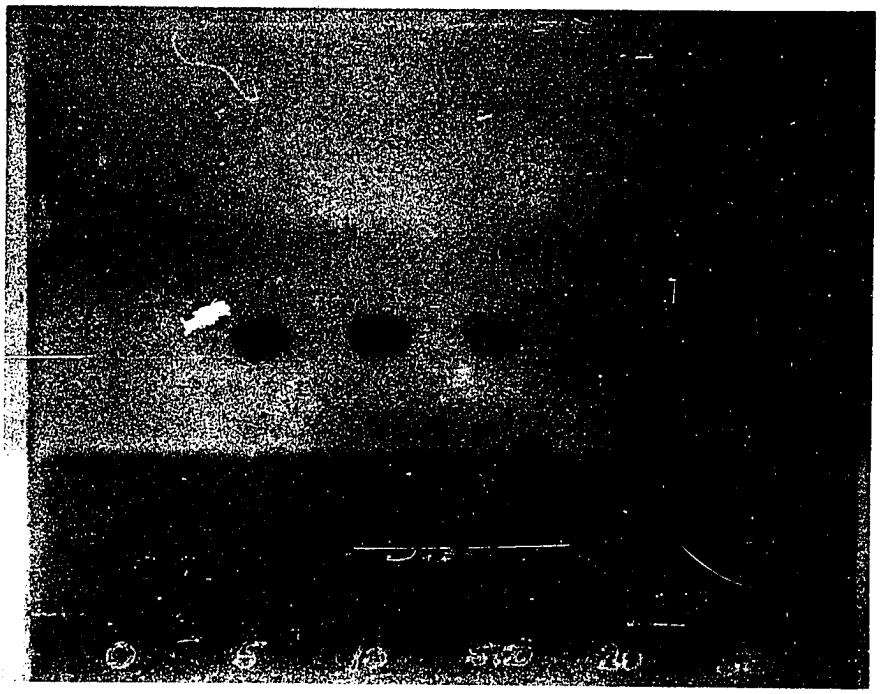

Fig. 8 - The effect of several concentrations of diphenylamine on nitrosation and diphenylnitrosamine recovery from extracts of Escherichia coli broth cultures.

1. Without diphenylamine
2. Culture contained 5 mg diphenylamine
3. Culture contained 10 mg diphenylamine
4. Diphenylnitrosamine standard: Rf 0.6
5. Culture contained 20 mg diphenylamine
6. Culture contained 50 mg diphenylamine

version of diphenylamine to diphenylnitrosamine was noted at all time intervals in approximately equal amounts, except immediately post-inoculation. This sample, processed at the beginning of the experiment, served as a negative control and diphenylnitrosamine was not recovered. A non-reacting area was noted at the same Rf value as the standard diphenylnitrosamine and subsequent studies, using developing reagents for diphenylamine, indicated that this spot was diphenylamine. Chromatography of diphenylamine and the nitrosamine in hexane:ether: dichloromethane did not resolve these compounds and chromatograms of extracts obtained from animal studies were processed with a petroleum ether and benzene solvent system. This solvent system was satisfactory for separating diphenylamine and its derivatives.

Rat Urine Experiments

Forty ml of filter-sterilized rat urine (pH 7.0) were inoculated with 0.1 ml of an E. coli strain 93 broth suspension in order to study the growth kinetics of E. coli in rat urine (Figure 10). The experiment was performed in quadruplicate and the points on the growth curve represent an average of the values obtained. Following a 3 hour lag phase, exponential growth was apparent and a concentration of 2.6×10^8 microorganisms/ml was attained at 12 hours. The results of this experiment indicated that rat urine, in the absence of infection and inflammation, would serve as a satisfactory culture medium.

In vitro N-nitrosamine studies were subsequently carried out using filter-sterilized urine instead of nutrient broth as a culture medium. E. coli strains 93, 555, 98, and 103 were inoculated into flasks containing 50 ml sterile rat urine, 0.2% potassium nitrate, and

Fig. 9 - The effect of incubation time on the formation and recovery of diphenylnitrosamine from extracts of Escherichia coli strain 93 broth cultures.

1. Immediate extraction of culture
2. Extract of broth culture 8 hours post - inoculation
3. Extract of broth culture 16 hours post - inoculation
4. Diphenylnitrosamine standard : Rf 0.6
5. Extract of broth culture 24 hours post - inoculation
6. Extract of broth culture 48 hours post - inoculation

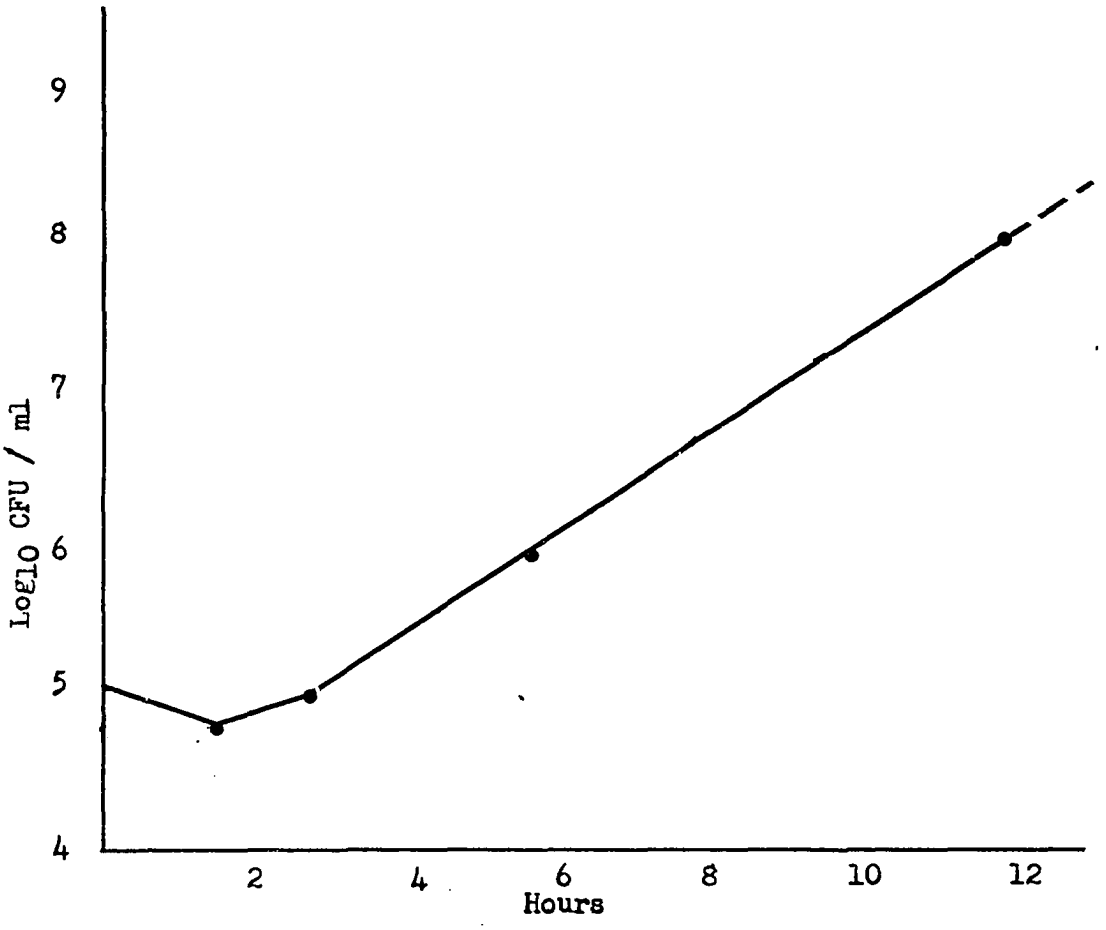

Fig. 10- In vitro growth curve for E. coli strain 93 in rat urine (pH 7.0). The points on the curve represent the mean of four samples.

10 mg diphenylamine. Thin layer chromatography of extracts of the urine cultures demonstrated diphenylnitrosamine formation by E. coli strains 93, 103, and 555. Results were the same whether human or rat urine replaced the broth. When 0.1% piperidine was used as the precursor amine, N-nitrosopiperidine was recovered. Diphenylnitrosamine and/or N-nitrosopiperidine were not detected in the urine extracts containing E. coli strain 98. This strain served as a negative control because in vitro broth culture experiments had revealed this nitrate-reducing strain to be incapable of carrying out the nitrosation reaction.

Animal Experiments

Fluorometric Diphenylamine Assays

Prior to in vivo N-nitrosation studies, it was necessary to determine if orally administered diphenylamine was excreted in rat urine. Preliminary studies using thin layer chromatography assay procedures indicated small amounts were being excreted or recovered by the extraction procedure. A standard curve for diphenylamine in ethanol was prepared by plotting the $\log_{10}$ of the relative intensity against the $\log_{10}$ of the diphenylamine concentration (Figure 2). The curve was linear from 0.0001 µg to 5.0 µg. Quenching was noted at higher concentrations of diphenylamine with the greatest decrease in fluorescence occurring between 10 and 100 µg. The recovery of 20 mg diphenylamine added to rat urine and immediately extracted is shown in Table 8. Six internal standards were included with an average recovery of 17.8 ± 2.8 mg diphenylamine for an 89.4% recovery rate.

TABLE 8

FLUOROMETRIC ASSAY OF URINE CONTAINING
TWENTY MILLIGRAMS DIPHENYLAMINE

Pooled urine sample	Milligrams recovered	Per cent
1	19.2	96.0
2	19.6	98.0
3	21.6	108.0
4	17.5	87.5
5	15.6	78.0
6	13.8	69.0
Average	17.8±2.8	89.4

Results of fluorometric analysis of urines from rats given 10 mg diphenylamine by intragastric intubation every 8 hours for 48 hours are shown in Table 9. Three animals were used and the average values were plotted. Assays were carried out on pooled urine specimens collected during the first 8 hours (10 mg diphenylamine given), the second 16 hours (20 mg diphenylamine given), and the second day (30 mg diphenylamine given). The diphenylamine concentration was calculated by converting the $\log_{10}$ concentration value obtained from the standard curve to an antilog and then converting the antilog to a real number. The average recovery rate was 15 µg diphenylamine/10 mg diphenylamine intubated, which is 0.15%, a very low recovery. These results indicated that formation of small amounts of N-nitrosodiphenylamine (1-10 µg) could be expected in vivo as only small amounts of diphenylamine were being excreted in the urine. However, the N-nitrosamine assay procedure will detect as little as 1.0 µg of an N-nitroso compound, indicating that the expected amounts of diphenylnitrosamine from in vivo experiments would be detectable.

Effects of Diuresis on Urine Volume

The average volume of urine excreted by normal rats was determined to be 5 ml/24 hours. The N-nitrosamine assay procedures employed required a larger volume of fluid. Diuresis with 5% (w/v) glucose was employed to increase the urine volume (Table 10). After 72 hours consumption of 5% (w/v) glucose, the average urine volume increased to 155±28.4 ml/24 hours. This level of urine output was maintained until rats were given 20 mg of diphenylamine. The diphenylamine was intubated in the stomach every 8 hours. Following 3 doses

TABLE 9

FLUOROMETRIC ASSAY FOR DIPHENYLAMINE FROM THE URINE OF RATS GIVEN TEN MILLIGRAMS DIPHENYLAMINE EVERY EIGHT HOURS BY GASTRIC INTUBATION

Animal Number	Time Period					
	0-8 hours (10 mg diphenylamine intubated)		8-24 hours (20 mg diphenylamine intubated)		24-48 hours (30 mg diphenylamine intubated)	
	micrograms recovered	%	micrograms recovered	%	micrograms recovered	%
1	10.0	0.1	21.0	0.1	100.0	0.33
2	7.5	0.075	20.0	0.1	50.0	0.17
3	20.0	0.2	-	-	30.0	0.1

Average diphenylamine recovery/10 mg diphenylamine intubated = 15.0 µg (0.15%)

TABLE 10

THE VOLUME OF URINE RECOVERED FROM NORMAL RATS BEFORE AND DURING DIURESIS INDUCED WITH 5%(W/V) GLUCOSE AD LIBITUM

Animal	Urine Volume (ml/24 hours)			
	Before Diuresis	72 hrs Diuresis	Diuresis with 20 mg diphenylamine given intragastrically every 8 hours	
			3 doses	6 doses
1	5	160	75	50
2	2	120	140	70
3	8	200	180	80
4	3	140	130	40
5	4	150	45	15
6	7	150	100	20
7	3	130	60	30
8	2	200	150	40
9	6	120	50	50
10	8	190	110	50
11	7	140	70	30
12	5	160	100	20
Mean ± standard deviation	5±2.2	155±28.4	101±42.8	41±20

of diphenylamine, urine volume decreased approximately 35%. Additional diphenylamine (6 doses total) caused the urine volume to drop to an average of 41 ml in the second 24 hour period. The decreased urine output and the presence of hematuria indicated toxicity of this level of diphenylamine for rats.

Kidney Histology after Infection with E. coli

The experimental animal model chosen for this study required injection of E. coli into the right kidney to induce a unilateral pyelonephritis. When 2×10^6 cells of E. coli strain 93 were injected into 2 sites of a surgically exposed kidney, 10^4 organisms/ml were recovered in the urine within 2 hours postinfection. The average number of organisms recovered from the urine of 4 infected animals is shown in Figure 11. The number of E. coli excreted in the urine was determined by quantitative culture and the peak number of organisms (7×10^6/ml) was reached at 2 days postinfection. After 2 days, the urine colony count slowly decreased, representing recovery from the acute stage of the infection. Six days postchallenge 2×10^3 cells/ml were recovered from the urine. When urine counts were performed 14 and 21 days after infection, less than 10^2 organisms/ml were cultured. Homogenates of the infected kidneys removed after 21 days had an average of 10^6 organisms/kidney showing that many organisms were still present in kidney abcesses but were not being excreted in the urine. Possible formation of antibodies which facilitated the phagocytosis and destruction of organisms in the bladder may explain, in part, the low recovery rate of viable organisms in the urine.

Tissue sections of infected kidneys from rats receiving

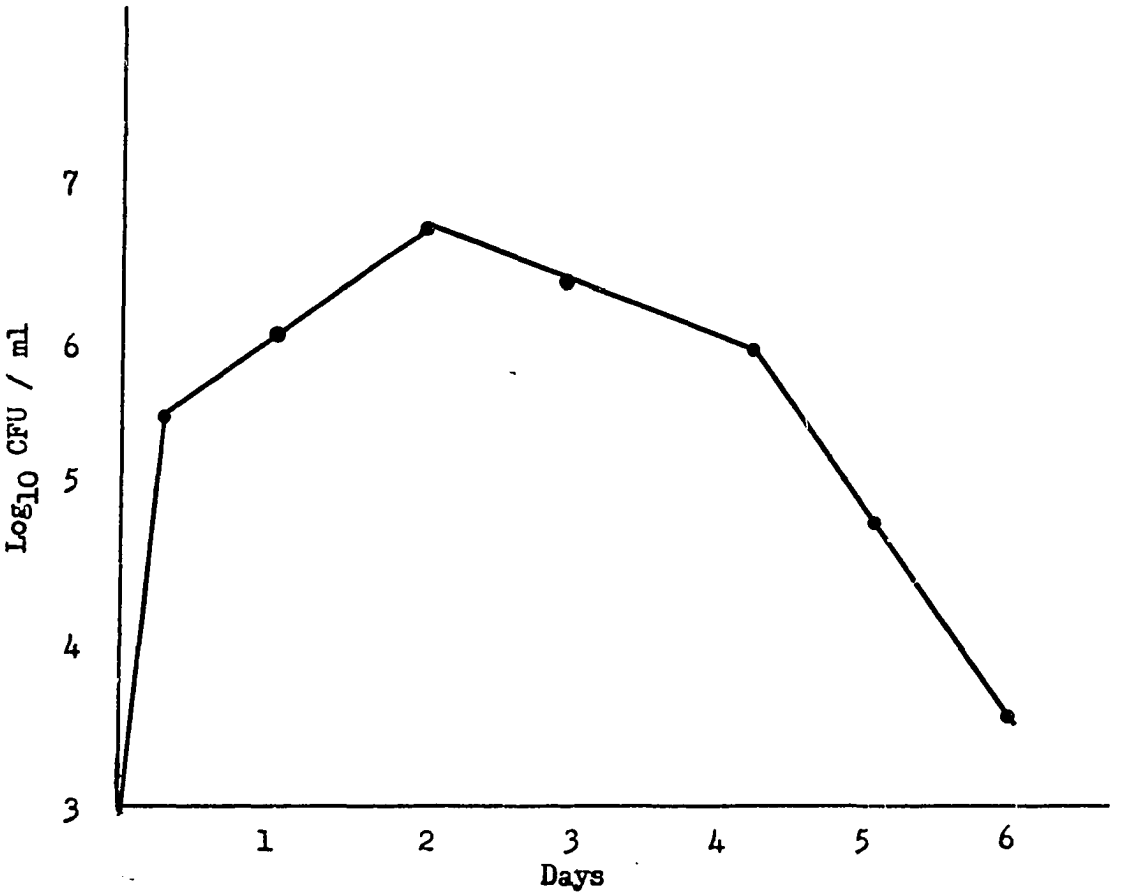

Fig. 11- Quantitative plate counts on urine after direct injection of Escherichia coli strain 93 into the rat kidney. The points on the curve represent the mean number of organisms cultured from urine specimens of four infected animals.

diphenylamine were stained using hematoxylin-eosin and the Brown and Brenn procedures (11, 36). Microscopic examination of the hematoxylin-eosin stained sections demonstrated the presence of an acute inflammatory process typical of cortical abcess formation. There were focal abcesses with large numbers of polymorphonuclear leukocytes and tubular infiltration. Organisms were not seen upon examination of the Brown and Brenn stained section. This was not unexpected because tissue homogenates yielded 10^6 organisms/kidney and greater numbers must be present before organisms can usually be detected microscopically (11). No pathology attributable to diphenylamine was noted. Tissue sections of kidneys from uninfected animals receiving diphenylamine were not prepared and the degree of kidney pathology due to diphenylamine alone cannot be assessed, however signs of toxicity were evidenced by dysuria and hematuria.

In Vivo Nitrosamine Formation

Table 11 is a summary of *in vivo* N-nitrosodiphenylamine formation in relation to the concentration of diphenylamine intubated. Eight out of 21 animals tested were suspected of excreting N-nitrosodiphenylamine when 10 or 50 mg amounts of diphenylamine were used as the substrate. When 20 mg of diphenylamine were given, 1 positive was noted out of 11 infected animals. When *E. coli* was grown in the presence of diphenylamine prior to injection into the animal, the formation of N-nitrosodiphenylamine occurred in 5 of 9 animals. N-nitrosodiphenylamine was not recovered from any of the control animals. The controls consisted of: untreated animals; non-infected but intubated animals; infected but not intubated animals; and sham operated

TABLE 11

N-NITROSODIPHENYLAMINE RECOVERY FROM URINE OF ANIMALS INFECTED WITH ESCHERICHIA COLI AND INTUBATED WITH VARYING CONCENTRATIONS OF DIPHENYLAMINE

	Concentration of diphenylamine intubated			
	50 mg	20 mg	20 mg[a]	10 mg
Number of animals	3	11	9	18
Number of animals forming N-nitrosodiphenylamine	2	1	5	6
Per cent	67	9	56	33

[a] Escherichia coli grown in presence of diphenylamine prior to infecting animals

(non-infected) and intubated animals.

Infected Rats given 50 mg diphenylamine/8 hours. Animal studies were carried out using E. coli strain 93, an in vitro nitrosating organism isolated from urine. Rats were unilaterally infected in the right kidney, allowed to recuperate for 24-48 hours, and then 50 mg of diphenylamine in corn oil and 50 mg of nitrate in distilled water were given by intragastric intubation every 8 hours throughout the experiment. Urine samples collected before and after infection but prior to administration of the amine and nitrate did not yield detectable amounts of nitrite, diphenylamine, or diphenylnitrosamine. Urine collected during each 8 hour dose period was assayed in the same manner. All animals given diphenylamine had detectable amounts of diphenylamine in extracts of urine samples. Urines from control animals did not yield diphenylnitrosamine. Two out of 3 infected animals given diphenylamine and nitrate intragastrically had nitrite in their urine samples. Thin layer chromatographic analysis of the extracts from the two nitrite positive urine specimens demonstrated a small purple spot at the same Rf as the N-nitrosodiphenylamine standard. These spots were observed in the extracts from 8-16 hour urine collections. The rats given diphenylamine (50 mg/8 hours) appeared sick and had dysuria between 24 and 32 hours after the start of the experiment. Due to this toxicity, the diphenylamine concentration was reduced and, in subsequent experiments, all urines collected from a given rat were pooled. Thus, in later experiments, a 24 or 48 hour urine specimen was assayed rather than an 8 hour specimen as described above.

Infected rats given 10 mg diphenylamine/8 hours. The next

series of experiments was performed according to the established protocol except that 10 mg of diphenylamine and 20 mg of nitrate were administered every 8 hours for a 48 hour period. Proper control animals were included in each series. Diphenylamine was recovered from the urine of all animals receiving this compound. Nitrite was present in the urine of only 1 of the 4 infected animals and none of the urine extracts had detectable amounts of diphenylnitrosamine. The extracts were prepared from 24 hour pooled urines.

Other experiments were initiated using the same conditions as the previous study except that the entire 48 hour urine specimen from each animal was pooled and an extract prepared. Ten µl samples of these extracts did not have detectable levels of diphenylnitrosamine. When the remaining 90 µl samples were assayed by thin layer chromatography, large bleached areas with a purple periphery were observed in 6 out of 10 urine extracts from infected and intubated animals. The Rf of this reaction corresponded to that of the diphenylnitrosamine standard. The stained portion was heaviest at the base of the bleached areas. The color developing reagent used was specific for N-nitrosamines in that a purple color (N-nitrosamine) develops after spraying the chromatogram and exposure to ultraviolet light. These results suggested that diphenylnitrosamine had been formed in vivo in the E. coli infected animals instilled with diphenylamine and nitrate because the purple stain was not observed in the urine extract of the non-infected control receiving diphenylamine. A bleached area, similar to that described above, was noted and this substance was proven to be diphenylamine.

Other preliminary experiments, using the same conditions as above, were performed and the chromatograms of urine extracts were developed using a spray reagent which differentiated diphenylamine from its derivatives (61). The pooled extracts from 4 infected animals were assayed. Again, nitrite was detectable in only 1 of the 4 urines prior to extraction. All extracts had detectable amounts of diphenylamine but diphenylnitrosamine was not detected.

Infected rats given 20 mg of diphenylamine/8 hours. Since nitrite was not consistently recovered from urine of infected animals, the concentration of nitrate was increased to 40 mg per gastric intubation. At the same time, the diphenylamine dose was increased to 20 mg per 8 hours. Rats were diuresed, infected, and intubated as in previous experiments. The urine from each animal, collected over the 48 hour period of diphenylamine and nitrate administration, was pooled. Nitrite was present in low amounts in 2 out of 3 of the pooled urines from infected animals and in higher concentration in the third. Chromatography of the entire urine extract from the latter animal revealed a reddish-brown spot compatible with the color of the diphenylnitrosamine standard. However, the Rf value of the experimental extract was less than the Rf of the standard. The reason for this may be the presence of other compounds or overloading of the chromatographic plate, resulting in the tailing observed.

Subsequent experiments were performed to eliminate interfering amines and other compounds from the urine extracts. An extract of 24 hour pooled urines was made, resulting in 2 extracts per animal. Nitrite was present in the urine of 1 out of 3 infected animals.

Chromatographic analysis of a 50 µl sample from the first urine extract of this animal revealed a compound giving the same color reaction as the standard diphenylnitrosamine and at the same Rf value (Figure 12). Urine extracts from the other 2 animals contained only diphenylamine. In order to confirm that the compound in question was diphenylnitrosamine, thin layer chromatographic analysis was performed and a developing reagent specific for N-nitrosamines was employed (50). Fifty µl of the extract in question was analyzed. Upon developing the plate, a purple spot appeared which had an Rf value of 0.62 compared with the Rf 0.65 of the diphenylnitrosamine standard. This spot was directly below a pale bleached area (diphenylamine) which appeared to inhibit the migration of the N-nitrosodiphenylamine compound.

In an attempt to enhance *in vivo* nitrosamine formation, additional experiments were performed. In these experiments, *E. coli* strain 108 was grown in the presence of diphenylamine prior to use as an infecting inoculum. All other conditions such as the concentration of diphenylamine (20 mg), nitrate (40 mg), and the infecting dose were the same as in prior experiments. Nitrite was detected in the urine of 4 out of 5 infected animals. Fifty µl samples of the extracts of pooled urine were applied to thin layer chromatography plates for analysis (Figure 13). Diphenylamine was demonstrated in all extracts. Extracts from 3 out of the 5 infected animals (samples 1, 5, 6) contained detectable amounts of diphenylnitrosamine. Three of the extracts (samples 1, 3, 6) also contained unknown diphenylamine derivatives although these were not the same extracts as those containing diphenylnitrosamine. The experiment was repeated using *E. coli* strain 93. Diphenylnitrosamine was detected in the urine of 2 out of 4 infected animals.

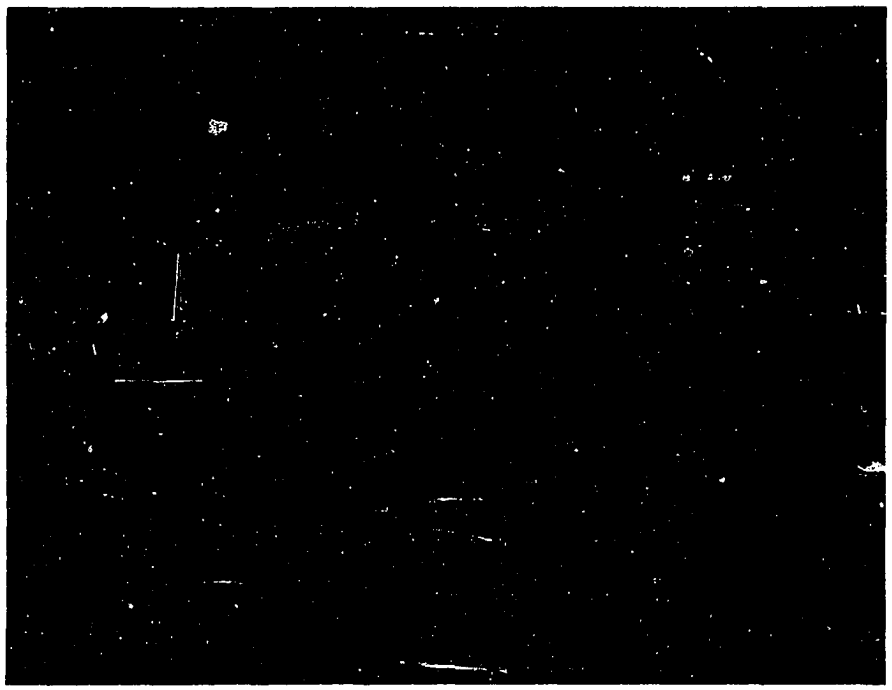

Fig. 12 - Extract from urine of an animal infected with Escherichia coli strain 108 and intubated with 20 mg diphenylamine and 40 mg potassium nitrate every 8 hours.

1. Extract of urine from an intubated but noninfected animal
   Rf 0.8 : diphenylamine
2. Diphenylnitrosamine standard : Rf 0.6
3. Extract of urine from an infected and intubated animal
   Rf 0.8 : diphenylamine
   Rf 0.6 : diphenylnitrosamine
4. Diphenylamine standard : Rf 0.8
5. Mixed standard
   Rf 0.8 : diphenylamine
   Rf 0.6 : diphenylnitrosamine

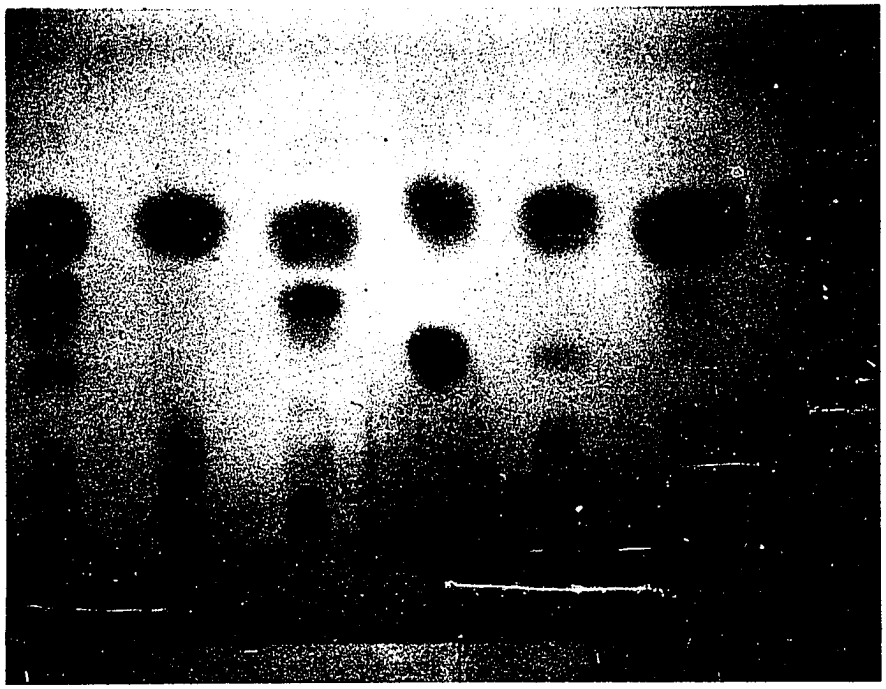

Fig. 13 - Extracts of urine from several animals infected with Escherichia coli strain 108, intubated with 20 mg diphenylamine and 40 mg potassium nitrate every 8 hours.

1. Extract of urine from an infected and intubated animal
   Rf 0.8 : diphenylamine
   Rf 0.7, 0.65 : unknown diphenylamine derivatives
   Rf 0.6 : diphenylnitrosamine
2. Extract of urine from an intubated but non-infected animal
   Rf 0.8 : diphenylamine
3. Extract of urine from an infected and intubated animal
   Rf 0.8 : diphenylamine
   Rf 0.7, 0.65 : unknown diphenylamine derivatives
4. Mixed standard
   Rf 0.8 : diphenylamine
   Rf 0.6 : diphenylnitrosamine
5. Extract of urine from an infected and intubated animal
   Rf 0.8 : diphenylamine
   Rf 0.6 : diphenylnitrosamine
6. Extract of urine from an infected and intubated animal
   Rf 0.8 : diphenylamine
   Rf 0.7 : unknown diphenylamine derivative
   Rf 0.6 : diphenylnitrosamine
7. Extract of urine from an infected and intubated animal
   Rf 0.8 : diphenylamine

CHAPTER IV

DISCUSSION

Certain O serogroups of Escherichia coli are more frequently involved in urinary tract infections than others. Turck and coworkers (65, 66) found that the predominant serotypes isolated from urine of people living in the western United States were 04, 06, and 075. E. coli serotypes 01, 07, 025, and 050 were also isolated but the incidence of these types in urinary tract infections was reduced. Studies from other sections of the United States and Europe reveal that some variations in serotype may occur in different geographic locations. In Europe, the most common E. coli serotypes found in urine are 01, 02, and 04 (13, 23, 37, 38). It is not unexpected to note differences on a continental scale, but it is interesting to note differences within a smaller geographic locality. Results of a recent study of 7 geographic locations in the United States showed that E. coli 09 was the most prevalent serotype isolated from urinary tract infections in California (43). This serotype is not common to other sections of America. The serotypes most frequently noted in all the geographic areas were 04, 06, and 075 (43). The two most common serotypes from studies in Missouri were 06 and 07, which accounted for 22% of the typable strains (43). The results of serotyping strains of E. coli

isolated in the present study compare well with those of previous studies in that the most frequent serotypes were 04, 06, and 075, which accounted for 72% of the typable strains. Winterbauer, Turck, Kennedy, and Petersdorf (69) surveyed E. coli isolated from urinary tract infections and found 70% of the typable strains were 04, 06, and 075 serogroups. There appears to be little difference in the E. coli serotypes isolated from this section of the country and other areas of the United States. The percent of typable strains (40%) was considerably lower than that observed in other investigations (65%) (13, 18, 37, 38, 43). This could be due to the omission of antisera specific for strains prevalent in the Oklahoma City area or to the limited scope of strains subjected to serotyping. Most investigations have been more comprehensive in the antisera used and the number of strains serogrouped (13, 43).

Sander (53) first demonstrated that E. coli and other members of the family, Enterobacteriaceae, could nitrosate secondary amines in the presence of nitrate. The addition of glucose to the medium resulted in increased yields of an N-nitrosamine. The results shown in Figure 7 confirm this phenomenon. The addition of glucose to the medium results in acid formation, providing an environment conducive to chemical nitrosation of the secondary amine present. However, the pH of the in vitro system containing glucose was never less than 5.6. This level of acidity has been regarded as being too high for the chemical conversion of amines (26). In the present study, control broth solutions at pH 5.6 were incubated with secondary amines and nitrate without being inoculated with a nitrosating strain of E. coli.

Assays of extracts from these solutions did not reveal nitrosamines. Further evidence against chemical nitrosation of amines in this study is the fact that E. coli strains incapable of nitrosating amines were isolated. E. coli strain 98 was used as a negative control throughout this investigation and did not nitrosate secondary amines under any of the cultural conditions employed. Glucose will also enhance microbial growth with increased metabolic activity. The possiblity of inducing the nitrosating enzyme or increasing the specific activity and concentration of the enzyme in the medium cannot be overlooked. No attempt was made to isolate the enzyme responsible for nitrosating secondary amines. Most of the strains (87%) investigated had the capability of nitrosating precursor amines and it would appear that these isolates actively nitrosate secondary amines and are not merely functioning to reduce nitrate to nitrite and generate an acid environment suitable for the acid catalysis of nitrosamines.

Sander (53) demonstrated the dependence of nitrosamine formation on the chemical composition of the precursor amine. The yield of nitrosamine was significantly lower when highly basic amines were utilized, compared to more neutral amines. Diphenylamine, a neutral amine, should be nitrosated with greater facility than other amines studied (dibutylamine, diethylamine, piperidine). Such was the case in experiments carried out in this study. The reason for the decreased nitrosation of basic amines lies in the protonated nature of the amine group (10, 25). Investigations regarding the carcinogenicity of nitrosamines have indicated that the nitrosamines derived from highly basic amines have greater carcinogenic potential (15, 41). This is

interesting from an environmental standpoint, as highly basic precursor amines would have to be present in greater concentration to produce detectable levels of N-nitrosamines. The function of the kidney, however, is to concentrate the components of a solution (plasma) and to excrete this concentrated solution (urine). Considering this natural role of a normally functioning kidney, the possiblity of having a concentration of basic secondary amines sufficient for nitrosation would be conceivable.

Healthy animals are normally resistant to urinary tract infections. To induce infection of the kidney in such animals, it is usually necessary to obstruct the urinary tract by implanting a foreign body or to traumatize the kidney. Diuresis, induced with glucose, has been demonstrated to have little effect on renal cortical infections but will enhance the invasive nature of E. coli and allow this microorganism to spread throughout the urinary tract. Plate counts of urine demonstrated 10^4-10^5 organisms per ml in urine specimens 4-5 days after injection of 10^4 organisms. Diuresis also increased the susceptibility of healthy rats to bacteriuria when as few as 10 E. coli cells were injected into the bladder lumen (20). A method has been reported for inducing experimental pyelonephritis in rats, with only minor kidney damage, by using a micropipette to deliver organisms directly into the kidney (44). This procedure produced a chronic pyelonephritis in rats lasting 100 days. The infecting microorganism (E. coli) was excreted in the urine for the entire period. Animal infections induced during the course of experiments reported in this investigation combined diuresis and direct kidney inoculation

in order to obtain large quantities of urine and to induce pyelonephritis with a minimum of tissue destruction. Animals infected in this manner developed an acute infection that resolved, over several days, into a chronic infection. The chronicity of the infection was verified by recovery of the infecting E. coli serotype 21 days after the surgical implantation of the inoculum. In vivo experiments reported here utilized animals only in the acute phase of the bacterial infection. This model would be feasible, however, for long-term feeding experiments designed to study tumorigenesis due to endogenous N-nitrosamine formation.

N-nitrosodimethylamine has been isolated from the urine of two humans with bacteriuria due to Proteus mirabilis (10). The strain of P. mirabilis was found to be capable of nitrosating dimethylamine in vitro. The present investigation represents the first controlled experiments demonstrating in vivo formation of N-nitroso compounds in an animal model. The prerequisites necessary for in vivo nitrosamine formation were present as a nitrate-reducing microorganism (E. coli), nitrate, and a precursor amine were present simultaneously in the bladder and/or kidney. The low level of nitrosation found may be related to several factors. Diphenylamine was demonstrated to be readily nitrosated in the presence of glucose. Although diuresis was induced by supplying the animals with 5% (w/v) glucose ad libitum, Freedman (20) presented evidence that the urine of animals undergoing glucose diuresis does not contain glucose in concentrations higher than that of normal animals. The lack of glucose in the urine of animals maintained on 5% (w/v) glucose solution was probably due to

tubular reabsorption which was not affected by the renal cortical abcesses that were induced. The difficulty in demonstrating nitrosamines *in vivo* in this study may have been due to the low levels of nitrite present. This could be a result of rapid urine formation due to diuresis and the flushing action of the large urine volume induced. Bacteria and nitrate must be present in the bladder of infected individuals for at least 4 hours in order for sufficient nitrite formation as evidenced by a positive nitrate reduction test (60). Most of the infected animals did not have demonstrable nitrite in their urine. Those urine samples positive for nitrite usually contained diphenylnitrosamine. Diuresis decreases the retention time of urine in the bladder and this results in a shorter reaction time between the microorganisms, secondary amines, and nitrite. *In vitro* studies were performed with 10^9 or greater microorganisms/ml, while the maximum number of organisms in urine of infected rats reached 10^7 organisms/ml. The *in vitro* system was a closed system and the microorganisms were not diluted by excretion from the system. The opposite situation existed in the *in vivo* system, where the agent responsible for nitrosation was constantly being excreted and, once excreted, growth of the organisms was stopped by antibiotics.

The potential for nitrosamine production in an infected human bladder would be increased because 75% of individuals with urinary tract infections have bacteria in excess of 10^9 organisms/ml of urine (29). Humans also retain urine for longer time intervals and are not subjected to glucose diuresis. Thus, conditions favoring bacterial interactions with nitrate and amines exist in man, whereas unfavorable conditions

existed in the animal model used. Bacterial strains may vary greatly in their nitrate-reducing ability. All E. coli used in these experiments reduced nitrate to nitrite, but variation in the amount of nitrite formed was noted. This could be of particular importance in human infections, as the nitrosating ability of infecting bacteria may determine whether or not enough nitrite is formed to react with secondary amines present in the urine and, thereby, present a carcinogenic threat (64).

Due to the low concentration of diphenylamine recovered from the urine of experimental animals, future studies should be designed using amines such as dimethylamine, piperidine, and pyrrolidine, which are naturally found in urine. Piperidine was employed in in vitro experiments, but could not be dissolved in a solution suitable for in vivo administration. This amine is readily nitrosated in the presence or absence of glucose, however its basic nature results in lower yields of its corresponding N-nitrosamine than those found with diphenylamine (25). The level of N-nitrosopiperidine formed during in vitro experiments carried out in the course of this study was near the limits of sensitivity of the assay techniques used. New techniques, such as gas-liquid chromatography using an electron capture detection system capable of detecting picogram quantities of nitrosamines and a thermo electron analyzer, which specifically identifies N-nitroso compounds, have recently been designed (10, 19). The analysis of urine extracts by these techniques would probably detect nitrosamines formed from secondary amines which are nitrosated to a lesser extent than diphenylamine.

Secondary amines are normally present in the urine as a result of absorption from the diet or through derivation from amine precursors (4, 70). Nitrates are also present in urine, depending on the concentrations ingested in food and water. Hawksworth and Hill (24, 25) have reported that nitrates must be present in higher concentration than the amine precursors in order for N-nitrosamine formation to occur and they postulated that individuals living in areas in which the water has a high nitrate content may be placed at greater risk than the general population. A retrospective study of such a population indicated a significant increase in gastric cancer incidence as opposed to people living under similar conditions but consuming less nitrate (27). The results supported the hypothesis that a high nitrate intake was conducive to nitrosamine formation in the bladder and that these nitrosamines were reabsorbed, giving rise to stomach cancer. Low nitrate concentrations (1 mM) may be sufficient for N-nitrosamine synthesis in vivo if the molecular ratio of amine:nitrate is between 1:1 and 1:4 (33,64). The amine:nitrate ratio was maintained at 1:2 thoughout the current study and the demonstration of nitrosamines supports such reports in the literature.

It is estimated that 10-20% of the female population in the United States has one or more urinary tract infections at some time during their life (62). Approximately 8 million Americans have a urinary tract infection at any given time (62, 68). Pregnant women are more susceptible to urinary tract infections than the general population (62). The incidence of complicated pregnancies or birth defects in humans due to N-nitrosamines is unknown but the teratogenic

effect of N-nitrosamines has been shown in many laboratory animals (28, 46). Animals are also quite susceptible to the carcinogenic action of N-nitrosamines (8, 9, 15, 32) and experiments utilizing a tissue culture system indicated that human liver metabolizes N-nitroso compounds in the same manner as rat liver (47), suggesting that man may well be susceptible to the carcinogenic effect of N-nitrosamines. Tumors may be formed by compounds such as dimethylamine, piperidine, and pyrrolidine after conversion to their respective nitrosamines in the bladder. Reabsorption of these compounds may lead to tumor formation at sites other than the bladder. Target organs have not been identified in man but one can assume that organs such as the liver and stomach could well be target sites due to the carcinogenic mechanisms of N-nitroso compounds (27). Experiments have shown that nitrosamines are not ultimate carcinogens but that metabolic derivatives of these compounds are the carcinogenic agents (3, 15, 42). The site where the nitrosamine is metabolized may be the site of tumorigenesis. It is currently believed that nitrosamines, or their metabolic derivatives, alkylate nucleic acids, resulting in base-pair mutations (41, 42, 49). Although the current studies were not concerned with demonstrating a carcinogenic effect of N-nitrosamines, the formation of diphenylnitrosamine in rats with urinary tract infections lends credence to the hypothesis that nitroso compounds, produced during urinary tract infections in man, may increase the risk of tumorigenesis. The population at greatest risk are young women of childbearing age and men over 50 years of age (68), an age at which bacteriuria first becomes noticable in the male population.

Small amounts of diphenylnitrosamine were recovered from extracts of urine from infected animals, an observation which should be regarded as a potential health hazard. The formation of N-nitrosamines *in vivo* during urinary tract infection implies a carcinogenic hazard to man and no exposure to a carcinogen should be regarded as safe (40). Further studies would be required to clarify the dose-response relationships between N-nitrosamines and carcinogenesis in humans and the role of *E. coli* in nitrosamine synthesis.

CHAPTER V

SUMMARY

Urinary tract infections due to Escherichia coli are common in humans and may involve up to 20% of the female population at some period of life. Serotypes most frequently isolated from urinary tract infections are 04, 06, and 075 and these serotypes have been identified as the most common isolates recovered from patients in the Oklahoma City area.

Results of in vitro nitrosamine formation studies using E. coli strains isolated from urine and feces demonstrated that the majority of strains tested (90%) could reduce nitrate and catalyze the conversion of diphenylamine to diphenylnitrosamine. Further investigations in vitro, using dibutylamine, diethylamine, and piperidine, confirmed the nitrosating ability of selected E. coli isolates.

The results of animal studies, using 2 of the in vitro nitrosamine producing E. coli strains, indicated that conversion of diphenylamine to diphenylnitrosamine may occur in E. coli infected animals when an amine:nitrate ratio of 1:2 is administered by the intragastric route. Growth of the E. coli inoculum in the presence of diphenylamine prior to infection enhanced the recovery of diphenylnitrosamine from urine extracts.

These experiments indicate that many strains of *E. coli* isolated from patients with bacteriuria may catalyze the formation of N-nitrosamine compounds *in vitro* and *in vivo*. A discussion of the health hazard to man and the population at risk has been presented.

LITERATURE CITED

1. Alam, B. S., Saporochetz, I. B., and Epstein, S. S. 1971. Formation of N-nitrosopiperidine from piperidine and sodium nitrite in the stomach and the isolated intestinal loop of the rat. Nature (London) 232:116-8.

2. Alam, B. S., Saporochetz, I. B., and Epstein, S. S. 1971. Synthesis of N-nitrosopiperidine from nitrate and piperidine in the gastrointestinal tract of the rat. Nature (London) 232:199-200.

3. Argus, M. F. and Hoch-Ligeti, C. 1961. Comparative study of the carcinogenic activity of nitrosamines. Jour. Nat. Cancer Inst. 27:695-709.

4. Asatoor, A. M. and Simenhoff, M. L. 1965. The origin of urinary dimethylamine. Biochem. Biophys. Acta III:384-92.

5. Asscher, A. W., Sussman, M., Waters, W. E., Davis, R. H. and Chick, S. 1966. Urine as a medium for bacterial growth. Lancet 2: 1037-41.

6. Ayanaba, A. and Alexander, M. 1973. Microbial formation of nitrosamines in vitro. Appl. Microbiol. 25(6):862-8.

7. Bailey, W. R. and Scott, E. G. 1970. Diagnostic Microbiology. C. V. Mosby Company, St. Louis.

8. Barnes, J. M. and Magee, P. N. 1956. The production of malignant primary hepatic tumors in the rat by feeding dimethylnitrosamine. Brit. Jour. Cancer 10:114-22.

9. Bertram, J. S. and Craig, A. W. 1970. Induction of bladder tumors in mice with dibutylnitrosamine. Brit. Jour. Cancer 24: 352-9.

10. Brooks, J. B., Cherry, W. B., Thacker, L. and Alley, C. C. 1972. Analysis by gas chromatography of amines and nitrosamines produced in vivo and in vitro by Proteus mirabilis. Jour. Inf. Dis. 126(2):143-53.

11. Brown, J. H. and Brenn, L. A. 1931. A method for the differential staining of gram-positive and gram-negative bacteria in tissue sections. Bull. John Hopk. Hosp. 48:69-73.

12. Collins-Thompson, D. L., Sen, N. P., Aris, B. and Schwinghamer, L. 1972. Non-enzymic in vitro formation of nitrosamines by bacteria isolated from meat products. Can. Jour. Microbiol. 18:1968-71.

13. Dootson, P. H., Maclaren, D. M. and Titcombe, D. H. M. 1972. Urinary Tract Infections, eds. W. Brumfitt, and A. W. Ascher. Univ. Oxford Press, London, pp. 139-45.

14. Drasar, B. S. and Hill, M. J. 1972. Intestinal bacteria and cancer. Amer. Jour. Clin. Nutrition 25:1399-1404.

15. Druckrey, H., Preussmann, R., Ivankovic, S. and Schmahl, D. 1967. Organotrope carcinogene wirking bei 65 rerscheiden N-nitrosoverbindungen an BD-ratten. Zeit. Krebsforch 69:103-201.

16. Edwards, P. R. and Ewing, W. H. 1955. Identification of Enterobacteriaceae. Burgess Pub. Co., Minneapolis.

17. Environmental Nitrosamines. 1973. Lancet 2:1243-4.

18. Ewing, W. H. and Davis, B. R. 1961. O antiserum pools for preliminary examination of Escherichia coli cultures. CDC Publication, Center for Disease Control, Atlanta, Georgia.

19. Fine, D. H., Rufeh, F., and Lieb, D. 1974. Group analysis of volatile and non-volatile N-nitroso compounds. Nature 247: 309-10.

20. Freedman, L. R. 1967. Experimental pyelonephritis, XIII. On the ability of water diuresis to induce susceptibility to E. coli bacteriuria in the normal rat. Yale Jour. Biol. and Med. 39:255-66.

21. Goaz, P. W. and Biswell, H. A. 1961. Nitrate reduction in whole saliva, Jour. Dent. Res. 41:355-65.

22. Greenblatt, M., Kommineni, V., Conrand, E., Wallcave, L., and Lijinsky, W. 1972. In vivo conversion of phenmetrazine into its N-nitroso derivative. Nature (New Biology) 236:25-6.

23. Grunsberg, R. N., Leigh, D. A., and Brumfitt, W. 1968. Escherichia coli serotypes in urinary tract infection: studies in domiciliary, ante-natal and hospital practice. Urinary tract infection. Eds. F. W. O'Grady and W. Brumfitt, London pp 68-79.

24. Hawksworth, G. M. and Hill, M. J. 1971. The formation of nitrosamines by human intestinal bacteria. Biochem. Jour. 122: 28P-29P.

25. Hawksworth, G. M. and Hill, M. J. 1971. Bacteria and the N-nitrosation of secondary amines. Brit. Jour. Cancer 25: 520-6.

26. Hill , M. J. and Hawksworth, G. 1972. N-nitrosocompounds: analysis and formation. IARC 3:116-21.

27. Hill, M. J., Hawksworth, G. M., and Tattersall, G. 1973. Bacteria, nitrosamines and cancer of the stomach. Brit. Jour. Cancer 28:562-7.

28. Ivakovic, S. and Bucheler, J. 1968. Leber-und blasen-carcinome beim meerschweinchen nach Di-n-butylnitrosamin. Zeit. Krebsforsch. 71:183-5.

29. Kass, E. H. 1956. Asymptomatic infections of the urinary tract. Trans. Assoc. Amer. Phys. 69:56-63.

30. Kauffman, F. 1966. The Bacteriology of Enterobacteriaceae. Williams and Wilkins Co., Baltimore.

31. Kennedy, R. P., Plordi, J. J., and Petersdorf, R. G. 1965. Studies on the epidemiology of Escherichia coli infections, IV. Evidence for nosocomial flora. Jour. Clin. Invest. 44: 193-201.

32. Khudoley, V. V. and Petrov, N. N. 1973. Morphologic changes in the liver of a fish (Lebistes reticulatus) induced by diethyl- and dimethylnitrosamines. Vop. Onkol. 19(1):88-94.

33. Klubes, P., Cerna, I., Rabinowitz, A. D., and Jondorf, W. R. 1972. Factors affecting dimethylnitrosamine formation from simple precursors by rat intestinal bacteria. Food. Cosmet. Toxicol. 10:757-67.

34. Lang, G. R. and Levin, S. 1971. Diagnosis and treatment of urinary tract infections. Med. Clin. N. A. 55(6):1439-56.

35. Lijinsky, W. and Epstein, S. S. 1970. Nitrosamines as environmental carcinogens. Nature 225:21-3.

36. Lillie, R. D. 1954. Histopathologic Technique and Practical Histo-chemistry. The Blakiston Div., McGraw-Hill Book Co., New York, N. Y.

37. Mabeck, C. E. Orskov, F., and Orskov, I. 1971. Studies in urinary tract infections VIII. Escherichia coli O:H serotypes in recurrent infections. Acta. Med. Scand. 190:279-82.

38. McGeachie, J. 1965. Grouping of urinary Escherichia coli. Jour. Clin. Path. 18:428-31.

39. Magee, P. N. 1971. Toxicity of nitrosamines: their possible human health hazards. Food Cosmet. Toxicol. 9:207-18.

40. Magee, P. N. 1973. Nitrosamines: ubiquitous carcinogens? New Scientist 59(860):432-4.

41. Magee, P. N. and Barnes, J. M. 1967. Carcinogenic nitroso-compounds. Adv. Cancer Res. 10:163-246.

42. Magee, P. N. and Swann, P. F. 1969. Nitroso compounds. Brit. Med. Bull. 25:240-4.

43. Marsik, F. J., and Parisi, J. T. 1971. Bacteriophage types and O antigen groups of Escherichia coli from urine. Appl. Microbiol. 22(1):26-31.

44. Miller, T. E., and Robinson, K. B. 1973. Experimental pyelonephritis: A new method for inducing pyelonephritis in the rat. Jour. Inf. Dis. 127(3):307-10.

45. Mirvish, S. S. 1970. Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. Jour. Nat. Cancer Inst. 44:633-9.

46. Mohr, U. Althoff, J., Emminger, A., Bresch, H. and Spielhoff, R. 1972. Effect of nitrosamines on nursing syrian golden hamsters and their offspring. Zeit. Krebsforsch. and Klin. Ohkologie 78(1):73-7.

47. Montesano, R. and Magee, P. N. 1970. Metabolism of dimethylnitrosamine by human liver slices in vitro. Nature (London) 228:173-4.

48. Nason, A. 1962. Enzymatic pathways of nitrate, nitrite, and hydroxylamine metabolisms. Bact. Rev. 26:16-42.

49. O'Connor, P. J., Capps, M. J., and Craig, A. W. 1973. Comparative studies of the hepatocarcinogen NN-Dimethylnitrosamine in vivo: reaction sites in rat liver DNA and the significance of their relative stabilites. Brit. Jour. Cancer 27(2):153-66.

50. Preussmann, R., Daiber, D. and Hengy, H. 1964. A sensitive colour reaction for nitrosamines on thin-layer chromatograms. Nature 201(4918):502-3.

51. Rabinowitz, D. 1972. Dimethylnitrosamine formation from sodium nitrate and dimethylamine by bacteria of rat intestine. Texas Rep. Biol. Med. 30(2):230.

52. Rantz, L. A. 1962. Serologic grouping of Escherichia coli: study in urinary tract infection. Arch. Intern. Med. 109:37-42.

53. Sander, J. 1968. Nitrosaminsynthese durch bakterien. Hoppe-Seyler's Zeit. Physiol. Chem. 349:1691-7.

54. Sander, J. and Seif, F. 1969. Bakterielle Reduktion von nitrat im magen des menschen als ursache liver nitrosamin-bildung. Arzneimittel-forsch. 19:1091-3.

55. Sander, J., Schweinsberg, F., and Menz, H. P. 1968. Untersuchungen uber die Entstenhung cancerogener nitrosamine in magen. Hoppe-Seyler's Zeit. Physiol. Chem. 349:1691-7.

56. Savage, W. E., Hajj, S. N., Kass, E. H., et al. 1967. Demographic and prognostic characteristics of bacteriuria in pregnancy. Medicine 46:385-407.

57. Sen, N. P., Donaldson, B., Iyengar, J. R., and Panalaks, T. 1973. Nitrosopyrrolidine and dimethylnitrosamine in bacon. Nature 241:473-4.

58. Sen, N. P., Miks, W. F., Donaldson, B., Panalaks, T., and Iyengar, J. R. 1973. Formation of nitrosamines in a meat curing mixture. Nature 245(5420):104-5.

59. Sen, N. P., Smith, D. C., Schwinghamer, L. 1969. Formation of N-nitrosamines from secondary amines and nitrite in human and animal gastric juice. Food Cosmet. Toxicol. 7:301-7.

60. Smith, L. E., Thayer, W. R., Malta, E. M., and Utz, J. P. 1961. Relationship of the Griess nitrite test to bacterial culture in the diagnosis of urinary tract infection. Ann. Intern. Med. 54:66-72.

61. Stahl, E. 1972. Thin Layer Chromotography. Academic Press, Inc., New York.

62. Stamey, T. A. and Pfau, A. 1970. Urinary infections: a selective review and some observations. Calif. Med. 113(6):16-35.

63. Stavrou, D. 1972. Bedeutung und Problematik der nitrosamine als umwelt kanzerogene. Fortschr. Med. 90(7):249-51.

64. Thacker, L. and Brooks, J. B. 1974. In vitro production of N-nitrosodimethylamine and other amines by Proteus species. Inf. and Immun. 9(4):648-53.

65. Turck, M., Ronald, A. R., Clark, H., Winterbauer, R. H., Atlas, E., Silverblatt, F. and Petersdorf, R. G. 1969. Studies on the epidemiology of Escherichia coli, 1960-68. Jour. Inf. Dis. 120:13-16.

66. Turck, M., and Petersdorf, R. G. 1962. The Epidemiology of non-enteric Escherichia coli infections: prevalence of serological groups. Jour. Clin. Invest. 41:1760-5.

67. Vosti, K. L., Goldberg, L. M., Monto, A. S., and Ram, L. A. 1964. Host-parasite interaction in patients with infections due to Escherichia coli. I. The Serogroups of E. coli from intestinal and extra-intestinal sources. Jour. Clin. Invest. 43:2377-85.

68. Wallace, J. F. and Petersdorf, R. G. 1971. Urinary tract infections. Postgrad. Med. 50:138-44.

69. Winterbauer, R. H., Turck, M., Kennedy, R. P., and Petersdorf, R. G. 1965. Studies on the epidemiology of hospital acquired Escherichia coli infections. Jour. Clin. Invest. 44:1112-3.

70. Wolff, I. A. and Wasserman, A. E. 1972. Nitrates, nitrites, and nitrosamines. Science 177:15-19.