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BIOACTIVATION OF 1-NITRONAPHTHALENE

by

Dale Edward Johnson

A dissertation submitted in partial fulfillment
of the requirements of the degree of
Doctor of Philosophy
(Toxicology)
in The University of Michigan
1976

Doctoral Committee:

Professor Herbert H. Cornish, Chairman
Professor Gerald D. Abrams
Professor Rolf Hartung
Professor Joseph E. Sinsheimer

DEDICATION

To Alice

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LIST OF ABBREVIATIONS

1-NN	1-Nitronaphthalene
2-NN	2-Nitronaphthalene
1-NA	1- or α-Naphthylamine
2-NA	2- or β-Naphthylamine
AAF	2-Acetylaminofluorene
SKF-525A	β-Diethyl-aminoethyldiphenylpropylacetate
LD_{50}	Lethal Dose for 50 per cent of Animals
ED_{50}	Effective Dose for 50 per cent of Animals
TLC	Thin Layer Chromatography
GLC	Gas Liquid Chromatography
ip	Intraperitoneal
po	Oral
iv	Intravenous
sc	Subcutaneous
OSHA	Occupational Safety and Health Administration

I. INTRODUCTION

A. Chemical Carcinogenesis

In recent years, numerous chemicals later shown to be carcinogens have entered into the human environment as ingredients or impurities of common consumer items including foods, drugs, cosmetics, economic poisons and household and sanitary goods (Hueper and Conway, 1964). Additional carcinogenic substances contaminate the environment in the form of chemical pollutants of the air, water and soil (Hueper and Conway, 1964). Chemical carcinogenesis, once thought only to be associated with poor industrial working conditions, has become the serious concern of public health agencies, medical and health professions, legislatures, courts, compensation commissions, the legal profession, labor organizations, managements of industries and commercial establishments, and the general public (Hueper and Conway, 1964, Clayson, 1962, Conney, 1973, Rall, 1972).

Recent evidence based on industrial exposures, epidemiological studies and migrant population studies indicates that chemicals are a major cause of cancer in man (Heidelberger, 1973). Estimates suggest that anywhere from 15 to 90 per cent of all human cancers may be caused by chemicals (Higginson, 1972).

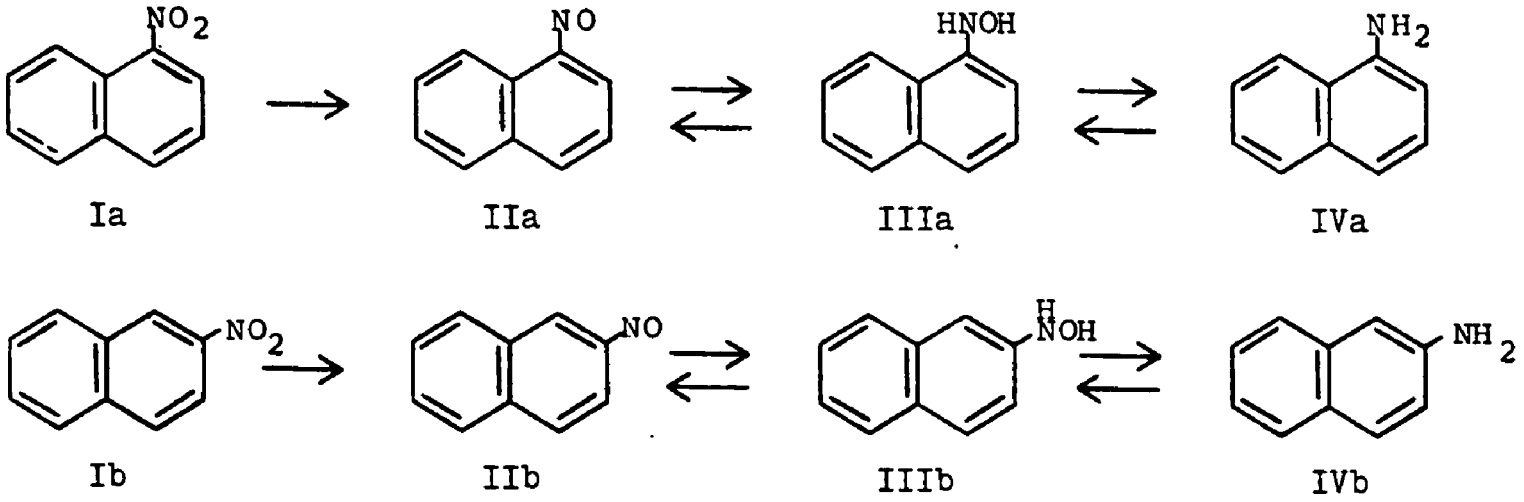
It is somewhat disconcerting to consider that approximately 300 to 500 new chemical agents are produced in significant quantities annually (Rall, 1972) and certain segments of the population are exposed to these untested agents. If chemicals are indeed a major cause of cancer in man, it is of enormous human value to develop adequate and economical screening systems to predict whether a substance may cause cancer (ed., Brit. Med. J., 1974) and to exert a rigid surveillance of the environment in order to exclude potential carcinogens (Heidelberger, 1973). Although some countries have legislation enforcing the testing of new drugs (ed., Brit. Med. J., 1974), pesticides and food additives (Rall, 1972), the control of new potential industrial hazards is less exact and has enormous implications for the industrial sector and technology in general. Most countries, including the United States, do not have the resources to adequately test each chemical substance produced to prove whether it does or does not cause cancer (Rall, 1972). Certainly technological development must not be stifled, and yet technology must not erode human health and well being(Rall, 1972). Man should not have to be the test animal of industrial and environmental hazards where retrospective epidemiological studies of increased human deaths finally warrant the testing of a substance in laboratory animals.

Research must attempt to provide the basis for predicting the potential biological actions and/or hazards of a substance based on its chemical structure, its metabolism, its acute toxic effects in appropriate animal models and its effects in in vitro test systems and shortened in vivo tests (Rall, 1972, ed., Brit. Med. J., 1974, Conney, 1973, Heidelberger, 1973). When important chemicals are shown to be potentially hazardous, 'safer' substitutes or processes to eliminate exposures should be sought.

B. The Nitronaphthalenes as Test Compounds

This research was initiated to assess the carcinogenic potential of certain industrial chemicals selected on the basis of their chemical structures and proposed metabolic pathways. The nitronaphthalenes were selected as test compounds since their amine analogs, the naphthylamines, are known carcinogens in man and several other aromatic nitro compounds have shown to be carcinogenic. Additionally, it is attractive to hypothesize that nitroreduction of the nitronaphthalenes may lead to the same highly reactive intermediates, N-hydroxy-naphthylamines and/or nitrosonaphthalenes, as does N-oxidation of the naphthylamines (See Figure 1). These intermediates have been shown to be carcinogenic and mutagenic, and the N-oxidation pathway has been proposed as a major contributing factor in the carcinogenesis of aromatic amines (Reviewed in sections I.C.2,3, and 4.)

Figure 1. Proposed Common Intermediates in the Metabolism of the Nitronaphthalenes and Naphthylamines



Ia = 1-nitronaphthalene; IIa = 1-nitrosonaphthalene; IIIa = N-hydroxy-1-naphthylamine; IVa = 1-naphthylamine; Ib = 2-nitronaphthalene; IIb = 2-nitrosonaphthalene; IIIb = N-hydroxy-2-naphthylamine; IVb = 2-naphthylamine.

Pathways Known to Exist

In Vivo		In Vitro	
IVa → (IIIa)* + IIa	(Radomski and Brill, 1970)	IVa → (IIIa)* + IIa	(Brill and Radomski, 1971)
IVb → (IIIb)** + IIb	(Radomski and Brill, 1970)	IVb → (IIIb)** + IIb	(Brill and Radomski, 1971)
Ib → IIb + (IIIb)**	(Radomski, et al., 1973)	Ia → IVa	(Poirier and Weisburger, 1974)
		Ib → IVb	(Poirier and Weisburger, 1974)
		Ia → IIIa	(Sternson, 1975)

* measured after conversion to IIa

** measured after conversion to IIb

Presently, 1-and 2-nitronaphthalene are not recognized as being hazardous to man. This view arises primarily from the lack of published research on any aspect of 1-nitronaphthalene toxicity and apparent negative results in two cancer studies with 2-nitronaphthalene utilizing mouse bladder implantation techniques (Bryan, et al., 1963 and 1964).

1-Nitronaphthalene is prepared commercially by nitrating naphthalene with mixed acid at 50-60°C. Technical grade 1-nitronaphthalene contains 0.5 to 1 per cent 2,4-dinitronaphthalene and 3 per cent 2-nitronaphthalene, which are easily removed by crystallization from ethyl alcohol or by partial melting. The chemical is used as a deblooming agent for petroleum and oils (2-3 parts/1000 parts oil), for the manufacture of dyes and intermediates, and is a component of commerical explosives. Most of 1-nitronaphthalene produced commercially is either sulfonated or reduced to 1-naphthylamine. 1-naphthylamine has been used for the production of several intermediates and azo dyes and also in the manufacture of certain rodenticides such as 1-naphthylthiourea. N-phenyl-1-naphthylamine, a condensation product, is used in the manufacture of diphenylnaphthylmethane dyes and as an antioxidant in rubber. The most economical method of producing the important intermediate 1-naphthol involves hydrolysis of 1-naphthylamine under pressure in an autoclave. 1-naphthol is used as an intermediate for 2,4-dinitronaphthol, hydroxynaphthalene sulfonic acids and in the

manufacture of several dyes. It is also used in the manufacture of the insecticide carbaryl and several synthetic perfumes. Production of 1-naphthol (also an indication of 1-nitronaphthalene usage) in the United States in 1964 was 896,000 pounds (Encyclop. Chem. Tech., 1967).

2-Nitronaphthalene is prepared by indirect means and is not of major technical importance either in total use or as an intermediate. 2-Naphthol, possibly the most important naphthalene derivative, is not produced from 2-nitronaphthalene or 2-naphthylamine. Interest in 2-nitronaphthalene in the past and in this study stems only from the fact that its amine analog, 2-naphthylamine, is such a potent bladder carcinogen in man.

Principal manufacturers of 1-nitronaphthalene are E. I. duPont de Nemours and Co., Inc. and General Aniline and Film Corp.. Major manufacturers of 1-naphthylamine are E. I. duPont de Nemours and Co., Inc., Allied Chemical Corp. and Toms River Chemical Company (Encyclop. Chem. Tech., 1967).

The following review presents evidence for the carcinogenicity of aromatic nitro compounds in general and gives a brief historical account of the carcinogenicity of the amine analogs of 1-and 2-nitronaphthalene. The role of N-hydroxy metabolites in mediating tissue lesions is also discussed as well as evidence that these intermediates do occur in the metabolism of both aromatic amines and nitro compounds. In addition, appendices A and B contain a compilation of the

animal studies which have confirmed the carcinogenicity of 2-naphthylamine and have led most investigators to conclude that 1-naphthylamine is a weak or even a non-carcinogen.

C. Literature Review

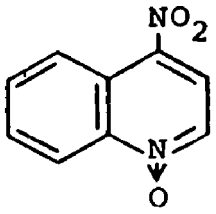
1. Carcinogenicity of Aromatic Nitro Compounds

Poirier and Weisburger (1974) have described three major categories of aromatic nitro compounds that give rise to tumor production: (a.) derivatives of 4-nitroquinoline-N-oxide, (b.) certain heterocyclic nitro compounds and nitrobenzene derivatives and (c.) the nitro analogs of classic aromatic amine carcinogens.

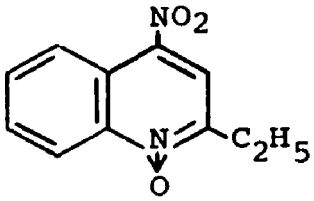
a. 4-Nitroquinoline-N-oxide and Derivatives

Endo, Ono and Sugimura (1971) have reviewed the chemistry and biological actions of 4-nitroquinoline-N-oxide including carcinogenicity studies of the parent compound and several derivatives. 4-Nitroquinoline-N-oxide itself has been demonstrated to be carcinogenic in mice, rats, birds, rabbits, guinea pigs and hamsters. Figure 2 illustrates the chemical structures of derivatives of 4-nitroquinoline-N-oxide shown to be carcinogenic in mouse skin painting experiments by Nakahara and co-workers (1958).

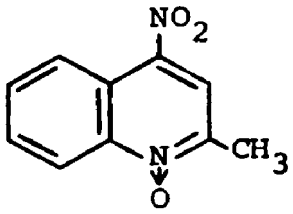
From the results of several *in vitro* and *in vivo* metabolism studies reviewed by Matsushima and Sugimura (1971) and studies on interactions with DNA, RNA and protein synthesis (Endo, *et al.*, 1971), the probable pathway of



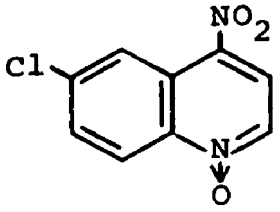
4-Nitroquinoline-N-oxide



4-Nitro-2-ethyl-quinoline-N-oxide



4-Nitroquinaldine-N-oxide



6-Chloro-4-nitroquinoline-N-oxide

Figure 2. Carcinogenic Derivatives of 4-Nitroquinoline-N-oxide

activation of 4-nitroquinoline-N-oxide has been elucidated. Apparently, nitroreduction to 4-hydroxyaminoquinoline-N-oxide (Kato, et al., 1970) is an obligatory step for the carcinogenic action of the compound.

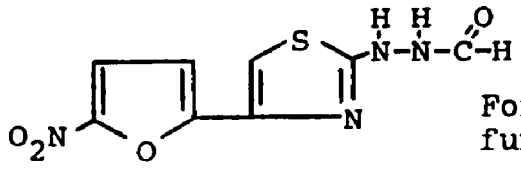
This theory is in agreement with results from Araki and co-workers (1970) who found that only 4-nitroquinoline-N-oxide derivatives which could be enzymatically converted to 4-hydroxyamino derivatives were carcinogenic.

b. Heterocyclic Nitro Compounds and Nitrobenzene Derivatives

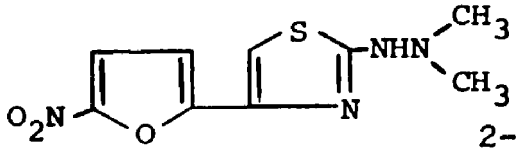
Cohen and co-workers (Cohen, et al., 1970) found several nitrofurans and related compounds to be potent carcinogens in the rat (Figure 3). In studies on the structure-activity-relationship of carcinogenic nitrofurans, nitrobenzene derivatives and aminobenzene derivatives, it was shown that the nitro or amino group was required for tumor production. It has been theorized that an N-hydroxy intermediate may be the reactive species (Cohen, et al., 1970) in these related compounds.

c. Carcinogenic Nitro Compounds with Carcinogenic Amine Analogs

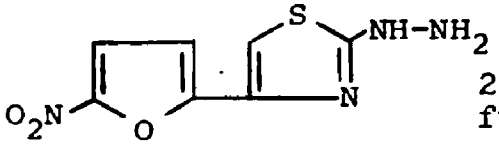
Several nitro analogs of aromatic amine carcinogens have been shown to have significant carcinogenic activity (Figure 4). Conspicuously missing from the list is 2-nitronaphthalene, the analog of the potent bladder carcinogen 2-naphthylamine. In two studies, 2-nitronaphthalene



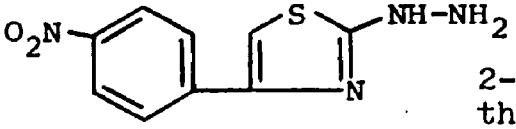
Formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazine



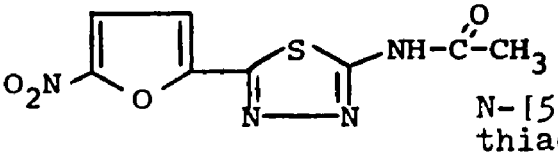
2-(2,2-Dimethylhydrazino)-4-(5-nitro-2-furyl)thiazole



2-Hydrazino-4-(5-nitro-2-furyl)thiazole

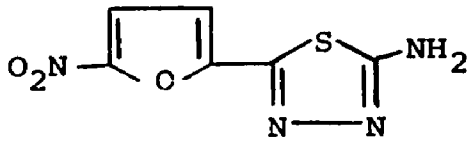


2-Hydrazino-4-(4-nitrophenyl) thiazole

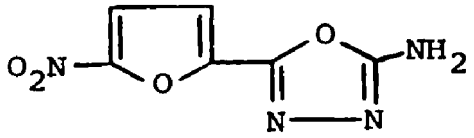


N-[5-(5-Nitro-2-furyl)-1,3,4-thiadiazol-2-yl]acetamide

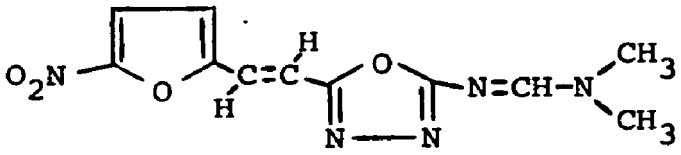
Figure 3. Carcinogenic Nitrofuran and Nitrobenzene Derivatives



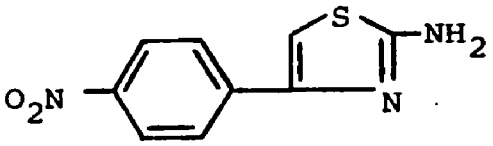
2-Amino-5(5-nitro-2-furyl)-1,3,4-thiadiazole



2-Amino-5-(5-nitro-2-furyl)-1,3,4-oxadiazole



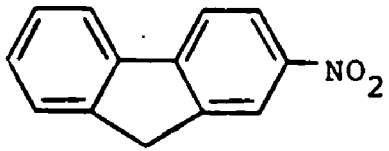
trans-2-[(Dimethylamino)methylimino]-5-[2-(5-nitro-2-furyl)vinyl]-1,3,4-oxadiazole



2-Amino-4-(p-nitrophenyl)thiazole

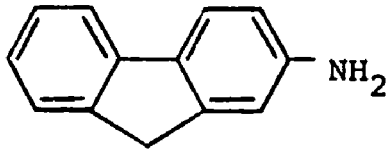
Figure 3 (continued). Carcinogenic Nitrofuran and Nitrobenzene Derivatives

(From: Cohen, et al., 1970; Cohen, et al., 1973 and Cohen, et al., 1975)



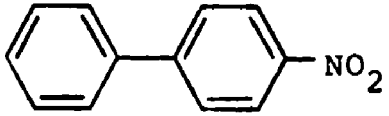
2-Nitrofluorene

Morris, et al., 1950



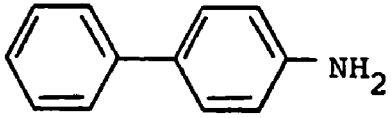
2-Aminofluorene

Bielschowsky, 1944



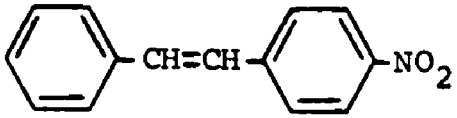
4-Nitrobiphenyl

Deichmann, et al., 1958



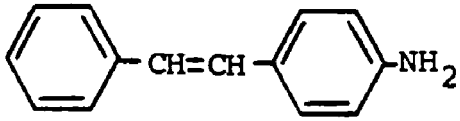
4-Aminobiphenyl

Walpole, et al., 1954



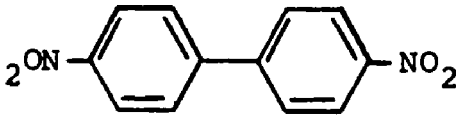
4-Nitrostilbene

Druckrey, 1955 in Poirier and Weisburger, 1974



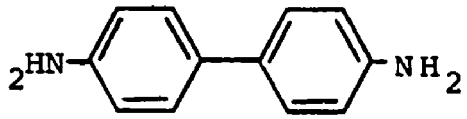
4-Aminostilbene

Haddow, et al., 1948



4-4'-Dinitrobiphenyl

Laham, et al., 1970



4-4'-Diaminobiphenyl

Case, et al., 1954

Figure 4. Carcinogenic Nitro Compounds with Carcinogenic Amine Analogs (References refer to first reports of carcinogenicity)

apparently did not show appreciable carcinogenic activity when implanted in mouse bladders (Bryan, et al., 1963 and 1964) although Conzelman and co-workers (1970) found bladder tumors in one monkey fed 2-nitronaphthalene. These studies are discussed in detail in the Discussion section. No reports of carcinogenicity studies with 1-nitronaphthalene were found in the literature.

As mentioned earlier, the nitronaphthalenes were selected for this investigation primarily because they are the nitro analogs of the naphthylamines. Accordingly, a thorough literature review of the carcinogenicity of the naphthylamines is necessary. If the similar metabolic pathways proposed in Figure 1 are real, it must be established that the N-hydroxy and/or nitroso intermediates are highly reactive. The following sections will examine these points.

2. Carcinogenicity of the Naphthylamines

a. Industrial Bladder Cancer in Man

The carcinogenic potential of commerical grade 1-and 2-naphthylamine in man after prolonged industrial exposure is well established and has been reviewed by Scott (1962), Clayson (1962), and Deichmann (1967). While the carcinogenicity of purified 2-naphthylamine has been established in animals (Appendix A) similiar results have not been obtained with 1-naphthylamine (Appendix B).

1-Naphthylamine was first prepared by a Russian chemist, Zinin, in 1842 by the reduction of 1-nitronaphthalene, a process still used industrially. 2-Naphthylamine was first prepared in Germany by Lieberman and Schneiding in 1875 by the reduction of 1-bromo-2-nitronaphthalene with tin and hydrochloric acid. Up until the time its manufacture had been discontinued, the process had been refined by heating 2-naphthol with ammonia and ammonium sulfite at 150°C under pressure. The naphthylamines were used for the manufacture of azo dyes, condensation colors and for certain rubber processes (Scott, 1962). Additional uses of 1-naphthylamine were discussed earlier.

The first report of chemically caused industrial bladder cancer is usually credited to Rehn in a paper presented at the 24th meeting of the Congress of German Surgeons in 1895. Rehn's conclusions, which showed remarkable insight into the problem of industrial bladder cancer are worthy of noting: "(1) The vapors which develop during fuchsin manufacture produce disorders in the urinary system, (2) after working a number of years in the fuchsin plant, the patient may develop bladder tumors as a result of continuous irritation, and (3) the harmful effect is principally due to inhalation of aniline vapors" (Price, 1971). Because of Rehn's report and similar ones that followed, industrial bladder cancer was called 'aniline cancer' for several years until it was finally accepted in

the 1950's that aniline was not a significant cause of bladder tumors (Scott, 1962). The first report that attributed a bladder tumor to 'naphthylamine' was made by Leichtenstern in 1898 (Scott, 1962). In the ensuing years, several authors from dye producing countries reported additional cases of bladder cancer in dye workers and it became apparent that contact with benzidine and the naphthylamines was involved in a significant number of instances (Scott, 1962, Price, 1971).

The Association of British Chemical Manufacturers discontinued the production of 2-naphthylamine in 1952 (Scott, 1962) primarily on the basis of an epidemiological study by Case and co-workers (1954) who investigated bladder cancer in members of the British chemical industry between 1921 and early 1952. Their final report, published in 1954, indicated that death due to bladder cancer occurred thirty times more frequently in employees of companies engaged in the production of 2-naphthylamine than would be expected in the general British population. Benzidine and 1-naphthylamine were also implicated. Of the number of cases of bladder tumors where exposure to a single chemical occurred, 59 were attributed to 2-naphthylamine, 38 to benzidine and 28 to 1-naphthylamine. Additionally, 162 cases were considered due to mixed exposures (Case, et al., 1954). In another study including data up to 1962 from a United States based coal tar dye manufacturing plant, the incidence

of bladder tumors in 366 workers exposed to 2-naphthylamine, benzidine and combinations of these two with 1-naphthylamine was studied (Goldwater, et al., 1965). The incidence of malignant tumors of the bladder was 26.2 per cent with a time interval from first exposure to diagnosis of bladder malignancy ranging from 2 to 42 years with a mean of 22.9 years. A change in the 2-naphthylamine process in 1953 reduced the incidence of bladder tumors and the entire process was terminated in 1963 (Kleinfeld, 1967).

The case against 2-naphthylamine in man has been firmly established for several years, whereas even today the carcinogenic potential of 1-naphthylamine remains unknown. Several hold to the theory that the carcinogenicity of 1-naphthylamine from industrial exposures is actually due to its contamination with 2-naphthylamine (Scott, 1962). Commerical 1-naphthylamine was estimated to contain as high as 4 to 10 per cent 2-naphthylamine prior to 1960 (Scott, 1962). Kleinfeld (1967), reporting on a dye plant where 1-naphthylamine has been made since 1927, indicated that contamination levels were 3 to 6 per cent prior to 1959. After refining the process, the contamination was ultimately reduced to 0.3 per cent and the company no longer considered the 1-naphthylamine manufacturing areas to be hazardous locations (Kleinfeld, 1967).

Scott (1962) has expressed the view that the carcinogenicity of 1-naphthylamine per se has been estab-

lished by results of the study by Case and co-workers (1954) and extrapolation from experiments where pellets containing 1-amino-2-naphthol (a urinary metabolite of 1-naphthylamine) implanted into mouse bladders produced a significant tumor yield. However, Munn (1967) cautions against these conclusions since other investigators have shown that the vehicle exerts co-carcinogenic activity in bladder implantation experiments.

The eventual follow-up study (as yet unpublished) by Kleinfeld on workers who started their exposure to 1-naphthylamine after the 2-naphthylamine contamination had been reduced to 0.3 per cent will be most interesting and may shed light on the controversy.

b. Carcinogenicity in Animals

In 1937, Hueper presented a historic paper at the Meeting of the American Association of Pathologists (Hueper and Wolfe, 1937) relating preliminary results in producing bladder cancer in dogs with 2-naphthylamine. This was followed by publication of their results in 1938 marking it as the first time cancer had been induced in an animal model by a chemical suspected of producing the same type of cancer in humans (Hueper, et al., 1938). Several studies since have confirmed Hueper's findings in the dog as well as in other species (a compilation of animal carcinogenic studies done with 2-naphthylamine appears in Appendix A). Animal studies using 1-naphthylamine apparently

have not produced the results seen with 2-naphthylamine (a compilation of animal carcinogenic studies done with 1-naphthylamine appears in Appendix B). In dogs, 2-naphthylamine has been reported to induce a 100 per cent incidence of bladder tumors in one to two years (Belman, et al., 1968), whereas 1-naphthylamine - in the only 'positive' dog study published - produced one bladder papilloma in one of three dogs after 8.75 years (Bonser, et al., 1958). Since the 1-naphthylamine used was commercial grade - 4 to 10 per cent 2-naphthylamine - the significance of this result is unknown. The reasons for the apparent difference in the biological action of 1- and 2-naphthylamine are difficult to explain. An interesting observation was made by both Case and co-workers (1954) and Kleinfeld (1967). Both groups found the latency period for commercial grade 1-naphthylamine induced tumors in man was substantially longer than for 2-naphthylamine. Case, et al. (1954) reported 16 years for 2-naphthylamine and 22 years for 1-naphthylamine while Kleinfeld's figures were 20.3 years and 23.1 years respectively. Also, in Kleinfeld's study, the shortest incubation time was 11 and 6 years for 1- and 2-naphthylamine respectively.

The failure of many of the animal studies with 1-naphthylamine may have been due to an inadequate exposure time, exposure level or latency period. Also, in Appendix B, the low number of published studies done in dogs is

apparent when compared to 2-naphthylamine. This point will be examined in more detail in the discussion on the excretion of N-hydroxy metabolites of 1- and 2-naphthylamine.

c. Mechanism of Action and Metabolism

Miller and Miller have reviewed the mechanisms of chemical carcinogenesis in several papers (Miller and Miller, 1969 and 1970 for example) emphasizing the view that aromatic amines are actually 'precarcinogens' that are metabolically activated to ultimate carcinogenic forms. According to this theory, these activated intermediates are electrophilic reactants which react with critical cellular nucleophilic targets including the bases of the nucleic acids and certain protein-bound amino acids and thus initiate the neoplastic transformation.

Strong evidence has accumulated indicating that metabolites of 2-naphthylamine excreted in the urine are responsible for inducing bladder cancer, that N-hydroxylation may be the activating metabolic pathway and that N-hydroxy metabolic products may be the ultimate carcinogens.

(1). The Role of Urinary Metabolites in the Induction of Bladder Tumors

The retention of urine itself in the bladder is thought by some to be a contributing factor in bladder cancer in man (Price, 1971). A study by Perelman and Schabad (quoted in Price, 1971) indicated that 92 of 140 men (65.7 per cent) with bladder tumors showed signs of

urinary retention or obstruction of the bladder outlet. Other studies have shown that regression of bladder tumors has occurred after diversion of the urine (Price, 1971), indicating that the urine may be involved in the persistence of established tumors. Also, Chapman (quoted in Price, 1971) showed that transitional cell papillomas developed in 76 of 125 (60.8 per cent) rat bladder pouches containing paraffin pellets and exposed to urine, whereas no tumors developed in 18 other pouches also containing pellets but not exposed to urine. On the other hand, Wood (1970) did not find an accelerated induction of ureteric tumors in rabbits fed 2-acetylaminofluorene under conditions of a partial, unilateral ureteric stenosis.

Hueper and co-workers (1938) had believed that the carcinogenic substance derived from 2-naphthylamine in their classic dog experiments reached the urinary bladder via the urine, and emphasized this by showing the presence of aromatic amines and other metabolites in the urine. Two important studies clearly showed that the carcinogenic material reaches the bladder via the urine. Scott and Boyd (1953) fed 2-naphthylamine to three groups of dogs and observed neoplastic changes. The first group had normal bladders and developed several bladder tumors. The second group had the ureter of one kidney implanted into the sigmoid colon while the other ureter was left in its normal position. Tumors arose in the bladders but not the

colons of this group. The third group had both ureters diverted to the sigmoid and no tumors were found in either the bladders or colons, however there were tumors in the ureters and metaplastic changes in the pelvis of the kidneys of some of the dogs. McDonald and Lund (1954) surgically transformed the dome of the bladders of dogs into a blind pouch so that urine would not contact the mucosa of the pouches. After feeding 2-naphthylamine, they found that only that part of the bladder in contact with urine would develop tumors. In Scott's and Boyd's study the bladders survived as did McDonald's and Lund's pouches indicating intact blood supplies and reinforcing the notion that the carcinogen reaches the bladder mucosa via the urine and not by the blood supply to the organ.

Attempts by several investigators to find the actual carcinogenic metabolite excreted in the urine have led to various theories on how metabolism affects the carcinogenicity of 2-naphthylamine. 2-Amino-1-naphthol was shown to be a metabolite of 2-naphthylamine in several studies (Scott, 1962) in man, dogs and rats. Bonser and colleagues,(1951) found an approximate correlation between the amount of 2-amino-1-naphthol conjugates excreted and the relative susceptibility to induced bladder tumors in dogs, mice, rats and rabbits. Dogs, a susceptible species, excreted up to 70 per cent of 2-naphthylamine as 2-amino-1-naphthol derivatives; whereas rabbits, a resistant species,

excreted very small amounts of the metabolites. In a later study, Bonser's group found that 2-amino-1-naphthol implanted into the bladder of mice induced local tumors whereas 2-naphthylamine did not (Bonser, et al., 1952). Thus began the ortho-hydroxy-amine theory (Deichmann, 1967, ed., Food Cos. Tox., 1964) which Clayson has reviewed extensively in his book on chemical carcinogenesis (Clayson, 1962). The theory relies on the premise that aromatic amines are ortho-hydroxylated in the liver, conjugated and excreted in the urine where they are subject to the action of hydrolytic enzymes resulting in the liberation of free ortho-hydroxy-amines, the actual carcinogenic metabolites (Clayson, 1962, ed., Food Cos. Tox., 1964). The theory held that only aromatic amines which had the para position blocked (such as 2-naphthylamine) were carcinogenic. Since aromatic amines preferentially hydroxylate in the 4-position (para), blockage of this position would result in an increased hydroxylation in the position ortho to the amine group (Clayson, 1962). Best evidence in favor of this hypothesis is that some of the ortho-hydroxy-amines are carcinogenic on bladder implantation and that they can be enzymatically oxidized to quinone imines which can combine with proteins (Clayson, 1962). The theory gained additional support by the finding that patients with bladder cancer had increased amounts of β-glucuronidase in their urine (Boyland, et al., 1957). The theory fails to account for

the non-carcinogenicity of several aromatic amines which form ortho-hydroxy metabolites including aniline and O-toluidine and the weak or nonactivity of 1-naphthylamine (ed., Food Cos. Tox., 1964). Also, the amounts of ortho-hydroxy-amines liberated in the urine are minimal, and according to Clayson (1962) the pattern of metabolites obtained in cases in which cancer is or is not obtained do not always differ greatly. Also, the ortho-hydroxy-amines are highly unstable and the methods used for testing their carcinogenic potential, bladder implantation, has recently been subject to varying criticism (Clayson, 1974).

Despite its recent unpopularity, Clayson has stated (1962) that prior to 1960, the ortho-hydroxy-amine theory was the only hypothesis which seemed capable of accounting for the majority of the known facts.

(2). The Role of the N-hydroxylation Pathway and N-hydroxy Metabolites

The first evidence that N-hydroxylation might be the initial activation step in the metabolism of carcinogenic amines and amides was obtained by Cramer, Miller and Miller (1960) with their work on the carcinogen, 2-acetylaminofluorene (AAF). The increased carcinogenicity of the metabolite N-hydroxy-AAF as compared to AAF (Miller, 1970) and the higher yields of macromolecule-bound fluorene derivatives in the livers of rats given N-hydroxy-AAF rather than AAF implicated the derivative as an interme-

diate in the in vivo reactivity of AAF (Miller, 1970). Other evidence includes the finding that the guinea pig has little or no ability to N-hydroxylate AAF and accordingly AAF is not carcinogenic in this species whereas N-hydroxy-AAF does produce tumors (Miller, 1970).

Nelson and Troll (1961) first reported in 1961 that 2-naphthylamine was metabolized by N-hydroxylation. This along with the finding that N-hydroxy-2-naphthylamine was a potent bladder carcinogen (Bonser, et al., 1963), even more potent than 2-naphthylamine (Boyland, et al., 1963), ushered in the N-hydroxylation theory of the activation of aromatic amines (ed., Food Cos. Tox., 1964). Recent evidence such as carcinogenicity and mutagenicity studies with N-hydroxynaphthylamines (discussed more fully in the following sections) indicates that N-hydroxylation is the most likely activating metabolic pathway of the naphthylamines. The N-hydroxylation pathway has also been implicated as the activating step in several other carcinogenic aromatic amines and amides including 4-aminobiphenyl (Radomski and Brill, 1971), 7-fluoro-AAF, 4-acetylaminostilbene, 2-acetylaminophenanthrene, 4-acetylaminobiphenyl, 4-nitroquinoline-N-oxide, N,N-dimethyl-4-aminoazobenzene and 4-aminoazobenzene (Miller and Miller, 1969). Opponents of this theory have pointed to one inconsistent finding, namely that 1-naphthylamine, only weakly or even non-carcinogenic, is N-hydroxylated to N-hydroxy-1-naphthylamine which has been shown to be a more potent carcinogen in rats

than N-hydroxy-2-naphthylamine (Radomski, et al., 1971).

A third theory is a combination of the other two concepts, N-hydroxylation and ortho-hydroxylation. Troll and Belman (1967) have shown evidence that an N-hydroxy-ortho-hydroxy metabolite may fill the requirements of the actual carcinogenic material. This carcinogenic metabolite, identified as bis(2-hydroxylamino-1-naphthyl)-phosphate, occurs only in man and dog, the species affected by 2-naphthylamine induced bladder cancer, and the analogous metabolite of 1-naphthylamine has not been found (Troll and Belman, 1967).

3. Carcinogenicity Studies with N-hydroxy Derivatives of the Naphthylamines

Boyland and co-workers (Boyland, et al., 1963) in 1963 reported that N-hydroxy-2-naphthylamine was more carcinogenic in rats than 2-naphthylamine after twice weekly ip injections for three months. Later, Boyland's group found N-hydroxy-1-naphthylamine also to be carcinogenic after implantation into mouse bladders (Belman, et al., 1968). Belman, et al. (1968) again in a three month-twice weekly injection study in rats, showed that N-hydroxy-1-naphthylamine was actually a more potent carcinogen than N-hydroxy-2-naphthylamine. Radomski and co-workers (1971) confirmed these results in a study of the same design, but found the reverse - the N-hydroxy derivative of 2-naphthylamine to be more potent - when the two compounds were administered to newborn mice by subcutaneous injection

(Radomski, et al., 1971). Radomski, et al. (1971) also tested 1- and 2-nitrosonaphthalene and found both of these compounds to be carcinogenic in rats.

The results of these studies with respect to 2-naphthylamine are in accord with the results of studies carried out with other carcinogenic aromatic amines whose N-hydroxy metabolites are carcinogenic (Belman, et al., 1968). The fact that the N-hydroxy derivative was found to be more carcinogenic in rats than 2-naphthylamine itself (Belman, et al., 1968) coupled with Radomski and coworkers' finding that N-hydroxy-2-naphthylamine solutions instilled into the bladders of dogs produced tumors whereas solutions of 2-naphthylamine did not (Radomski, et al., 1971), indicate that the N-hydroxy derivative of 2-naphthylamine is a proximate bladder carcinogen. The comparative carcinogenicity of N-hydroxy-1-naphthylamine (shown to be a more potent carcinogen that N-hydroxy-2-naphthylamine) is perplexing when the carcinogenicity of 1- and 2-naphthylamine are compared; 1-naphthylamine being at most only a weak carcinogen. Such results have led some investigators to doubt that N-hydroxylation, by itself, is the carcinogenic-activating pathway. Troll and Belman, in particular, have used this argument to add credence to their proposal that bis(2-hydroxylamino-1-naphthyl)-phosphate is the ultimate carcinogenic substance (Troll and Belman, 1967).

A possible explanation for the differences in the biological activities of 1- and 2-naphthylamine may be a result of the differences in the quantity of N-oxidized derivatives excreted. Radomski and Brill (1970) found that at the maximally tolerated acute dose (70 mg/kg) in dogs, there was not a significant difference in the excreted quantities of N-oxidized products of 1- and 2-naphthylamine. However, at 5 mg/kg, a dose at which 2-naphthylamine is carcinogenic in dogs and 1-naphthylamine apparently is not, negligible quantities of N-oxidation products were found in the urine of dogs given 1-naphthylamine, whereas considerable quantities (0.2 per cent of the administered dose) were found in the urine of dogs given 2-naphthylamine (Radomski and Brill, 1970). Consistent with this is the finding by Ziegler, et al. (1971) that the microsomal fraction from pig livers N-oxidizes 2-naphthylamine at a considerably faster rate than 1-naphthylamine. From these findings, one might hypothesize that although 1-naphthylamine is N-hydroxylated and N-hydroxy-1-naphthylamine is carcinogenic, negligible amounts are excreted in the urine at doses usually given to dogs or to which humans would be exposed (Radomski and Brill, 1970). Since Radomski and Brill (1970) only measured N-hydroxy-naphthylamines and nitroso-naphthalenes in their assay, their findings may not be a true indication of N-oxidation products available for interaction with the bladder mucosa. For example, conjugates of N-hydroxy-2-naphthylamine, identified as urinary meta-

bolites in the dog (Conzelman, et al., 1969), may be subject to hydrolysis by various enzymes in the urine, such as β-glucuronidase, thereby liberating the free reactive species. Also, Uehleke (1966) has shown that the bladder mucosa has N-hydroxylation capabilities of its own, indicating that amines in the urine may be a source of N-oxidation products.

It should be pointed out that while N-hydroxylation has been clearly implicated as a first activation step in the carcinogenicity of several aromatic amines and amides, N-hydroxylation per se does not confer carcinogenic activity on all aromatic amines and amides (Miller and Miller, 1969). This has been shown by the lack of carcinogenicity of N-phenylhydroxylamine, N-ethyl-N-phenylhydroxylamine, N-benzoyl-N-phenylhydroxylamine and N-hydroxyl-1-acetyl-aminofluorene (Miller and Miller, 1969). It is apparent that the nature of the ring structure and its substituents play a significant role in the carcinogenicity of aromatic amines (Miller and Miller, 1969).

It should be apparent from this review thus far that the search for the ultimate carcinogenic form of 2-naphthylamine is still continuing. Also, it can be concluded that several carcinogenic metabolites, rather than a single derivative, are responsible for the carcinogenic acitivity of 2-naphthylamine. The following metabolites,

with a carcinogenicity spectrum ranging from highly active to weakly active, have all demonstrated carcinogenic activity in some testing system(s), in some species and toward some tissue. These metabolites are N-hydroxy-2-naphthylamine and/or an O-ester thereof, bis(2-hydroxylamino-1-naphthyl)-phosphate, bis(2-amino-1-naphthyl)-phosphate and 2-amino-1-naphthol free and/or conjugated (Arcos and Argus, 1974).

4. Mutagenicity Studies with N-hydroxy Derivatives of the Naphthylamines

The hypothetical correlation between carcinogenesis and mutagenesis, first introduced by Boveri in 1895 (Corbett, et al., 1970) and as yet still unresolved, has led to several investigations on the mutagenicity of the naphthylamines and their N-oxidized metabolites. Perez and Radomski (1965) found N-hydroxy-1-naphthylamine to be an active mutagen in purine dependent E. coli whereas N-hydroxy-2-naphthylamine was found to be weakly mutagenic. Compounds which were found to be devoid of mutagenic activity included 2-naphthylamine, 1-amino-2-naphthol, 2-amino-1-naphthol, di-(2-amino-1-naphthyl) hydrogen phosphate, 1-amino-2-naphthyl sulfate and 2-amino-1-naphthyl sulfate. Belman and colleagues (1968) also found the N-hydroxy derivatives of 1- and 2-naphthylamine to be mutagenic (with the same relative potency) in E. coli mutants (measured by induction of reverse mutations). The parent naphthylamines as well as N-hydroxy-aniline and 2-amino-1-

naphthol were not mutagenic in this test system. Ong and deSerres (1972) found identical results with the ad-3 system of Neurospora crassa (measured by the induction of forward recessive lethal mutations) and, additionally, found that 2-naphthylamine was only mutagenic when grown with vegetative cultures (metabolically active). Ong and deSerres concluded that metabolism, presumably to N-hydroxy-2-naphthylamine, converted 2-naphthylamine to a mutagenic form.

Mayer (1972) used the UDENFRIEND hydroxylation medium to incubate 1- and 2-naphthylamine with Saccharomyces cerevisiae. The resulting oxidative products were mutagenic for the yeast cells when tested for petite mutants and genic canavanine-resistant mutants. In another study, Mayer (1973) reported mitotic crossing over in yeast using the same system. Tagashira and co-workers (1974) have shown that 2-nitrosonaphthalene inactivates the colony forming ability of Salmonella typhimurium Q and its repair-deficient mutants and can be nonenzymatically converted to a reduced form that inactivates bacteriophage. Identical studies using 1- and 2-nitronaphthalene gave negative results whether or not vegetative cultures were used indicating that these bacteria may lack the appropriate nitroreductase activity. In an earlier study, Corbett, et al. (1970) had found that the N-hydroxy derivatives of 1- and 2-naphthylamine were toxic but not mutagenic to intracellular T_4 phage.

While the mutagenicity of the N-hydroxy derivatives of 1- and 2-naphthylamine is firmly established, it appears that metabolic activation must take place in order for the naphthylamines to acquire mutagenic activity. This correlates well with the carcinogenicity studies previously discussed.

5. Interactions of Naphthylamine Metabolites with Macromolecules

Miller, J. and Miller, E. (1969) have stated that it is axiomatic that carcinogenic agents induce cancer through interaction with tissue components, and that some permanent or quasipermanent alteration in cellular structure must occur. This alteration must be such that a cell and its descendants are no longer responsive to the forces controlling growth as are the normal cells of the tissues involved. As stated earlier, ultimate carcinogenic forms of chemical agents are thought to be electrophilic reactants which react with critical cellular nucleophilic targets. These nucleophilic centers include the bases of nucleic acids and certain protein bound amino acids (Miller, E. and Miller, J., 1969).

Several studies (reviewed by Miller and Miller, 1966) have shown some preferential binding of small amounts of radioactivity from administered ^{14}C-2-naphthylamine in the urinary bladders of several different species. The retention of the radioactivity was seen for up to several weeks, although none of the metabolites was

identified (Miller and Miller, 1966). Roberts and Warwick (1966) studied the *in vivo* binding of ^{3}H-2-naphthylamine to rat liver, spleen and kidney and found that binding occurred to DNA, ribosomal RNA and protein. Hughes' and Pilczyk's (1969/70) results were in agreement in their studies in mice. However, they also found that the level of radioactivity bound in the livers of mice depended on the strain. Highest levels were found in the CBA strain which is susceptible to hepatocarcinogenesis from 2-naphthylamine, and lowest levels occurred in C_{57} mice, a resistant strain. These results showing reactivity to all three macromolecules are consistent with most of the carcinogens which have been studied adequately (Miller and Miller, 1966).

The evidence presented thus far implies that N-hydroxylation is an activating pathway in the metabolism of aromatic amines, and that N-hydroxy derivatives are certainly proximate if not ultimate carcinogenic forms. Also, it can be concluded that 1-naphthylamine is definitely not innocuous. In species known to N-hydroxylate aromatic amines, low levels of N-hydroxy-1-naphthylamine could conceivably result in mutagenesis. Although the threat of bladder cancer in man appears less with 1-naphthylamine than with 2-naphthylamine, its carcinogenic potential must still be considered as unknown.

6. Evidence for N-hydroxy Intermediates in the Metabolism of Aromatic Nitro Compounds

Critical to this research is the notion that nitroreduction is an activating pathway, which in the

case of the nitronaphthalenes, may lead to the same highly reactive N-hydroxy derivatives derived from 1- and 2-naphthylamine (See Figure 1). Best evidence for this bio-activation comes from work done on two classes of compounds; nitrobenzene and derivatives and 4-nitroquinoline-N-oxide and related compounds. The biological actions of nitro-benzenes have been reviewed by Kaplan (1974) and the activity of 4-nitroquinoline-N-oxide by Endo, et al. (1971). Experimental evidence indicates that nitrobenzenes are re-duced stepwise to aminobenzenes via nitroso and hydroxyl-amino derivatives (Weisburger and Weisburger, 1973). It is generally held that methemoglobin formation is often a good index of the production of N-hydroxy derivatives, particularly of the nitrobenzenes (Weisburger and Weisburger, 1973). As discussed earlier, nitroreduction of 4-nitro-quinoline-N-oxide to 4-hydroxyaminoquinoline-N-oxide is considered an obligatory metabolic step for the carcino-genic activity of 4-nitroquinoline-N-oxide (Kato, et al., 1970). p-Nitrobenzoic Acid (Kato, et al., 1970) and 4-nitro-biphenyl (Uehleke, 1967) are other aromatic nitro compounds which have been shown to form hydroxylamine intermediates in vitro during enzymatic nitroreduction.

Poirier and Weisburger (1974) have shown the 1- and 2-nitronaphthalene are slowly reduced to 1- and 2-naphthylamine in vitro using rat liver preparations. The

N-oxy derivatives, however, were not detected as products or intermediates of the reaction. Radomski et al. (1973) detected trace amounts of N-oxy derivatives in the urine of monkeys given 2-nitronaphthalene (50 mg/kg po) but not in urine of dogs given the same dose. Recently, Sternson (1975) used a sensitive electrochemical method to detect N-hydroxy-1-naphthylamine as an in vitro metabolic product (using rabbit liver microsomal suspensions) of 1-nitronaphthalene. These findings represent the first evidence that N-hydroxy derivatives may exist in the metabolism of the nitronaphthalenes.

If the hypothetical reductive steps in the metabolic pathway in Figure 1 are correct in vivo and the N-hydroxy or nitroso intermediates do occur, then 1- and/or 2-nitronaphthalene must be considered as 'precarcinogens' and/or 'premutagens'. This potential bioactivation of the nitronaphthalenes warrants the current study.

D. Objectives and Plan of the Present Investigation

The overall plan of the present study is to find evidence that bioactivation of the nitronaphthalenes occurs *in vivo*. Major emphasis will be given to three main pursuits: (1) correlating acute target organ toxicity with metabolism to show that an 'activating' step is involved in the metabolism of the nitronaphthalenes, (2) proving that nitroreduction of the nitronaphthalenes occurs *in vivo* indicating that metabolism may lead to the identical intermediates produced in the N-oxidation of the naphthylamines and (3) assessing the carcinogenic potential of the nitronaphthalenes.

Treon and Cleveland (1960) have reported on aspects of the acute toxicity of 2-nitronaphthalene, however analogous studies with 1-nitronaphthalene have not appeared in the literature. Therefore, this investigation will also involve the assessment of a wide variety of biologic responses of rats to 1-nitronaphthalene including LD_{50} determinations, target organ toxicity, effects on fertility and preliminary assessment of teratogenic potential.

II. Materials and Methods

A. Animals

1. Source and Maintenance

Sprague-Dawley rats ranging in weight from 200 to 550 gm were utilized in this investigation. All animals were purchased from Spartan Research Laboratories, Haslett, Michigan. Rats were housed in stainless steel cages in pairs or singly with Teklad® Rat and Mouse Chow and tap water available *ad libitum*.

2. Weights

Body weights of rats were obtained on a Torbal Balance Model PL-2 from The Torsion Balance Co. and wet organ weights were obtained on a Mettler K7T platform balance.

B. Chemicals

1. Commercial Sources

1-Nitronaphthalene (1-NN), 2-nitronaphthalene (2-NN) and 1-naphthylamine (1-NA) were purchased from Aldrich Chemical Company, Inc.. 2-Naphthylamine (2-NA) was purchased from Sigma Chemical Company. Sodium phenobarbital was purchased from Mallinckrodt Chemical Works and SKF-525A (β-diethyl-aminoethyldiphenylpropylacetate) from Smith, Kline and French. All other chemicals and organic solvents were Reagent or Analytical Grade except

the following: p-dimethylaminobenzaldehyde (Eastman Organic Chemicals), alizarin Red S (Matheson Coleman & Bell) and formaldehyde solution (American Drug Industries, Inc.).

Distilled water was prepared in a Corning Glass Works Model AG-2 distillation apparatus. Peanut oil was commercial Planters^{R} oil.

2. Purity

1-NN, 2-NN and 1-NA were checked for purity by melting points (Fisher-Johns apparatus), analysis by thin layer chromatography (TLC) in five solvent systems (see section II.C.1.d.) and by gas-liquid chromatography (GLC). Additionally, 1-NA and 2-NA were analyzed by a combination of GLC and mass spectrometry. All samples were found to be pure by these criteria.

3. Weights and Dosage Calculations

Chemicals were weighed on a Mettler Analytical Balance Model B6 (accuracy of $\pm$ 0.02 mg.) and a Mettler P120 Platform Balance (accuracy of $\pm$ 3 mg.). All dosages were calculated on a mg/kg of body weight basis for each individual animal.

4. Preparation of Material for Injection

Solutions for injections were made fresh each day by dissolving the compound in an appropriate amount of ether and dissolving this in peanut oil. The ether was allowed to evaporate (aided by a stream of nitrogen) leaving the

compound dissolved in peanut oil. Vehicle for control injections was prepared in the same manner using ether and peanut oil. Solutions were injected with disposable plastic syringes and needles.

C. Analytical Techniques for Metabolite Detection

1. Thin Layer Chromatography (TLC)

a. TLC Plates

Prekotes^{R} (Silica gel GF), 250 micron, 5x20 cm and 20x20 cm with a fluorescent indicator were purchased from Applied Science Laboratories, Inc. and used in the identification and isolation of metabolites. These glass backed plates were activated for 30 minutes at 100^{o}C prior to use. Pre-coated TLC sheets (Silica gel 60F-254), 250 micron, 20x20 cm on aluminum from EM Laboratories, Inc. were used in the initial screening of urinary extracts. These sheets were cut to appropriate sizes with a standard paper cutter and not activated prior to use.

b. Application of Solutions to Plates

Solutions of standard compounds and urinary extracts were prepared in absolute methanol and applied as spots using TLC glass capillary tubes from Applied Science Laboratories, Inc. Disposo^{R} pipets (Pasteur type) from Scientific Products were drawn to a thin tip after heating and used for applying solutions in long streaks for preparative TLC.

c. Detection of Compounds and Metabolites

Standard compounds and metabolites from urinary extracts were visualized on TLC plates with short-wave ultraviolet light or by color complex formation with various spray reagents. A Mineralight^{R} multi-band UV lamp, UVSL-58 from Ultra-Violet Products, Inc. was used for the UV detection. The following spray reagents were used: (1) Ninhydrin reagent from Sigma Chemical Company, (2) Ehrlich's reagent (Laham, et al., 1970): p-dimethylaminobenzaldehyde 2 per cent in 95 per cent ethanol: 6N hydrochloric acid (4:1), and (3) Gibb's reagent (Laham, et al., 1970): first spray; sodium carbonate 0.125M: second spray; N-2,6-trichloro-p-quinone imine in 100 per cent ethanol (0.4 per cent).

d. Solvent Systems

The following solvent systems were used in 4x12x10 inch rectangular glass developing tanks.

Solvent System	Components
A	petroleum ether (Bp 30-60):acetone (4:1)
B	benzene: ethanol (95%) (19:1)
C	chloroform: methanol (absolute) (19:1)
D	benzene: acetone (8:1)
E	n-butanol: n-propanol: ammonium hydroxide (equivalent to 0.2N NH_3) (2:1:1)

On the day of use, 100 ml. of the mixture were added to a tank which was allowed to equilibrate for at least one hour prior to use.

2. Gas-Liquid Chromatography (GLC)

Analyses for purity were done on a Varian Aerograph Model 600-D gas chromatograph equipped with a flame ionization detector. Two columns were prepared from materials from Applied Science Laboratories, Inc.: (1) 5 ft., 3% OV-17 on Gas Chrom Q and (2) 5 ft., 3% SE 30 on Gas Chrom Q. Injections were made with a micro-syringe from Hamilton using methanol or ethanol solutions.

3. GLC-Mass Spectrometry

GLC-mass spectral analyses on standard solutions and isolated metabolites were done by the Physical Instrumentation Laboratory, Department of Environmental and Industrial Health, School of Public Health, University of Michigan. Mass spectral analyses were performed on a AEI MS 30 interfaced to a PYE Model 104 GLC unit and a digital pdp 8/m data output system incorporating a Tektronix 4010-1 visual display unit. A 5 ft. column of 3% OV-17 on Gas Chrom Q was used for the GLC separation.

D. Special Procedures and Equipment

1. Methemoglobin Determination

The method for determining methemoglobin was kindly supplied by A.M.Kaplan from his doctoral dissertation

(Kaplan, 1974). A Beckman Model DUR spectrophotometer equipped with a Gilford Model 200 absorbance indicator was used to determine the per cent methemoglobin. The determination is based on the spectrophotometric measurement of absorbance of a hemolyzed solution of blood at 620 nm, a wavelength where the absorbance of methemoglobin does not vary with pH and is distinct from that of oxyhemoglobin and carboxyhemoglobin. By measuring the absorbance before and after adding cyanide, which converts methemoglobin to cyanomethemoglobin, the relative amount of methemoglobin was determined. The total amount of hemoglobin was determined by adding potassium ferricyanide (100% methemoglobin), reading the absorbance, then forming cyanomethemoglobin and again reading the absorbance.

Kaplan's method for sample analysis was as follows: a) 0.2 ml of blood were added to 9.6 ml of distilled water and 0.2 ml of Triton X-100-sodium tetraborate solution, mixed well and allowed to stand for five minutes for complete hemolysis. b) 3 ml of the clear hemolysate were placed in a 1-cm path length cuvette and read at 620 nm against distilled water. This was reading A_1. c) Two drops of a 10 per cent solution of sodium cyanide were added, mixed well and read again. This was reading A_2. d) To a second 3 ml portion of the hemolyzed blood, two drops of a 10 per cent solution of potassium ferricyanide were added, mixed well and read at 620 nm against distilled

water. This reading was A_3. e) To this second solution, two drops of 10 per cent sodium cyanide were added, mixed well and read at 620 mμ against distilled water. This reading was A_4.

Calculation: $\frac{A_1-A_2}{A_3-A_4} \times 100 = \%$ methemoglobin

2. Hepatic Microsomal Analyses

The methods for determining hepatic microsomal hydroxylase and N-demethylase activity were kindly supplied by D. Bechtel and are standard procedures of the Toxicology Laboratory, Department of Environmental and Industrial Health, School of Public Health, University of Michigan. Hydroxylase activity was evaluated by measurement of N-acetyl-p-aminophenol formation from acetanilide. The procedure involved the acid hydrolysis of N-acetyl-p-aminophenol to p-hydroxyaniline, ether extraction, water solubilization by addition of HCl, and reaction with benzoquinone to form a colored complex. N-demethylase activity was evaluated by measurement of 4-aminoantipyrine formation from aminopyrine. The procedure entailed a diazotization reaction of 4-aminoantipyrine with nitrous acid and α-naphthol to form a colored product. Colorimetry was performed on a B and L Spec 20 and concentrations ascertained from predetermined slope factors supplied by D. Bechtel from his dissertation research. Activities were expressed as μg product formed/20 minutes/g liver.

3. Isolation of Metabolites

a. Urine Collection

Rats were injected with 1-NN or 2-NN and immediately placed in a stainless steel metabolism cage two at a time with water and food available ad libitum. Urines were collected for 24 hours through a glass wool filter into a beaker resting on a freezing/stirring unit by Thermoelectronics Unlimited, Inc. These urine samples, frozen immediately throughout the 24 hours, were stored at 0°C until further use. Several samples were pooled at the time of thawing to allow for a sufficient quantity of metabolic products for isolation.

b. Extraction and Isolation Procedure

The extraction and isolation procedure, presented in outline form in Figure 5, was as follows. A sufficient number of urine samples were thawed at room temperature, pooled and four volumes of distilled water added. This was extracted three times with ether (same volume as original urine volume). The ether layers and interfacial emulsion were pipeted off, pooled, and placed in 50 ml glass stoppered centrifuge tubes which were sealed with Parafilm^{R}. To break the emulsion, the tubes were placed in an acetone-dry ice bath and quick-frozen. After or while thawing, the tubes were centrifuged on an IEC Model UV centrifuge at half speed for 30 minutes. If the emulsion was not sufficiently broken at this point, the

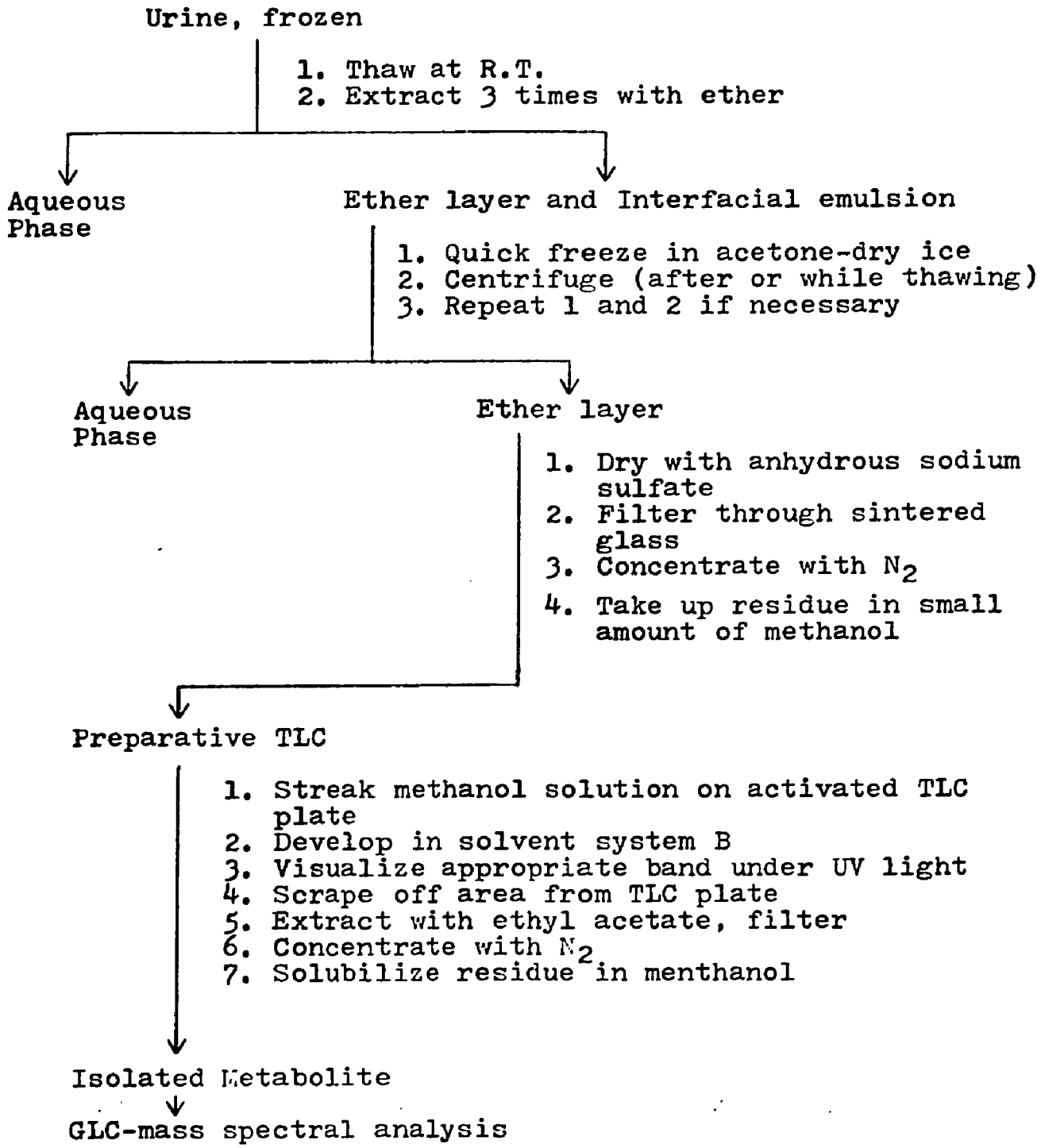


Figure 5. Metabolite Extraction and Isolation Procedure

freezing and centrifugation steps were repeated. The ether layers were pipeted off and dried over anhydrous sodium sulfate. The anhydrous ether extract was then filtered by gentle suction through a fine sintered glass funnel. It was then concentrated by blowing a stream of nitrogen gas over the surface. The residue was taken up in a small amount of absolute methanol and streaked (applied in a thin line across the origin) onto several TLC plates. The amount of solution and width of the streak applied to the plate is critical. Too heavy an application will result in poor separation and resolution of bands will not be consistent. Too wide a streak will result in gross overlapping of bands after development and makes purification difficult. On the other hand, too little makes detection of trace quantities under UV light difficult if not impossible. The plates were developed in solvent system B for approximately 45 minutes, removed from the tanks, and viewed under short wave UV light in a dark room. The appropriate areas on the plates were marked and the absorbant scraped off the plate with a razor blade. The silica gel was placed in a disposable glass pipet fitted at the small end with a glass wool plug and eluted with a small quantity of ethyl acetate into a scintillation vial. The extract was concentrated by using a stream of nitrogen and the residue taken up in a small amount of methanol. This methanol solution was used directly for the GLC-mass spectral analysis.

4. Preparation of Fetuses in Teratogenicity Study

a. Fixation and Sectioning

Pregnant female rats were anesthetized with ether and litters removed by caesarian section approximately 24 hours prior to term. Fetuses were placed in neutral buffered formalin prepared as follows:

NaH_2PO_4 (monobasic · H_2O)	9.2	g
or (monobasic anhydrous)	8.0	g
Na_2HPO_4 (dibasic anhydrous)	13.0	g
Formaldehyde solution 37%	200	ml
Distilled water qs ad	2000	ml

Fetuses selected for skeletal staining were removed from the formalin, eviscerated, skinned and the fat pads were excised. Fetuses selected for whole mount sagittal sections remained in the formalin for two weeks, then were sliced longitudinally with a razor blade and submitted to the Pathology Department for histological preparation.

b. Alizarin Red S Staining of Skeletons

The following modification of a procedure by Burdi (1965) was used for staining the fetuses.

1. Fix 40 minutes in formalin 37%: glacial acetic acid: absolute ethanol (1:1:8)

2. Wash in water for 30 minutes

3. Dry and transfer to a freshly prepared 1% aqueous solution of KOH to which has been added 2-3 drops of 0.1% alizarin Red S per 100 ml of solution.

4. Each day for 5 days transfer specimens to a fresh KOH-alizarin Red S mixture.

5. Rinse in water and dry on paper towels.

6. Place in a 1:1 mixture of glycerin and 95% ethyl alcohol for 1 to 2 hours, then transfer to a fresh glycerin-alcohol mixture for storage and final clearing.

E. Histopathology

Tissues were rapidly excised from sacrificed animals (ether anesthesia and heart puncture exsanguation), sliced when appropriate and fixed in neutral buffered formalin. Fixed tissue specimens were processed by the Pathology Department, University of Michigan Medical Center and stained with hematoxylin and eosin. In the carcinogenicity assay, the bladders of treated and control animals were fixed in a distended state (either filled with urine or injected with normal saline, then tied off and removed). Multiple step sections at intervals of 250 microns were made of all bladders and any grossly abnormal tissue. Photomicrographs were made on a Zeiss Photomicroscope I.

F. Statistical Analysis and Data Presentation

The Student's t test (Steel and Torrie, 1960) was used in analyzing various data; p values are indicated in the Results section. Fisher's Exact Test (Steel and Torrie,

1960) was used in analyzing the results from the dominant lethal assessment of the effects of long-term 1-NN administration on male fertility. LD_{50} determinations were made utilizing a computerized adaptation of Logistic analysis adapted from Berkson (1944) and made available by Dr. R. Hartung. Probit analyses (Finney, 1947) were made utilizing a Scientific Subroutine Package computer program. Calculations of means and standard errors of the means were done utilizing the DESCRIBE command in MIDAS. Growth curves were computer generated by using the Cal-comp plotting programs.

III. Experimental Designs

A. Acute Studies

1. Range-finding Study (1-NN)

A range-finding study was conducted in order to narrow the range of dosages to be administered during the LD_{50} determination and also to estimate organ toxicity. 1-NN was administered to several groups of 2 rats each in the following sequence: 50mg/kg ip; 100mg/kg ip; 200mg/kg ip; 1g/kg ip; 2g/kg po; 34mg/kg iv and 120mg/kg iv. Symptomatology, deaths and histopathology were studied and recorded during 14 day periods after dosing.

2. LD_{50} Determination (1-NN)

Twenty four rats were divided into 4 groups of 5 rats each and 1 group of 4 rats. The groups with 5 rats received 50, 75, 100 and 150 mg 1-NN/kg ip and the group of 4 rats received 200 mg 1-NN/kg ip. Toxic signs, especially respiratory distress, and deaths were recorded for 14 days after dosing.

3. Target Organ Toxicity Determination (1-NN)

Eighteen rats were given 100 mg 1-NN/kg ip. Nine of the rats, in respiratory distress, were sacrificed at 24 hours(3), 48 hours(3), and 72 hours(3) after the injection. Organ to body weight ratios were calculated and tissue samples prepared for histopathological examina-

tion. Additionally, nine rats which received 100 mg 2-NN/kg ip and nine control animals which were given vehicle injections were processed in the same manner.

4. ED_{50} for Respiratory Distress Determination (1-NN)

Rats in the LD_{50} study were observed for respiratory distress along with 18 rats receiving 100 mg 1-NN/kg ip in the target organ toxicity study and 20 rats receiving 25 mg 1-NN/kg ip from the carcinogenicity study. Animals were considered reactors if they developed grossly detectable dyspnea lasting for 24 hours or more.

B. Acute Toxicity Studies after Phenobarbital or SKF-525A Pretreatment

Target organ toxicity determination, LD_{50} determination and ED_{50} determination for respiratory distress were repeated with 1-NN (100mg/kg ip) after pretreatment with either phenobarbital or SKF-525A. Target organ toxicity parameters were also measured in phenobarbital pretreated animals which received 100 mg 2-NN/kg ip. Two vehicle control groups were included; SKF-525A and phenobarbital pretreated.

1. Phenobarbital Pretreatment

A sodium phenobarbital solution (1mg/ml) was substituted for drinking water for a four day period prior to the 1-NN injections. Individual totals for pretreatment were calculated by consumption.

2. SKF-525A Pretreatment

An aqueous solution of SKF-525A (80mg/kg ip) was administered to each rat one hour prior to the intraperitoneal injection of 1-NN.

C. Methemoglobin Determination

Four rats per group were dosed as follows: 100mg 1-NN/kg ip; 1g 1-NN/kg ip; 100mg 2-NN/kg ip; 2g 1-NN/kg po; 82.7mg 1-naphthylamine/kg ip and 100mg 1-nitroso-2-naphthol/kg ip. Tail vein blood was collected at 0, 30, 60, 120, 180 and 240 minutes after the dose was given and per cent methemoglobin was estimated.

D. Preliminary Hepatic Microsomal Enzyme Evaluation

Three rats received 25 mg 1-NN/kg ip on three successive days while three control rats were given vehicle only. On the fourth day, the rats were sacrificed and liver samples obtained and hydroxylase and N-demethylation activity evaluated (See Section II.D.2.).

E. Carcinogenicity Assay (1-NN and 2-NN)

Sixty rats, born on the same day, were purchased and acclimated to the animal laboratory for two weeks. During this time all the animals were checked for normal weight gain and any signs of disease. The rats were randomly divided into three groups of 20 rats each. Twice weekly

body weights (Monday and Thursday) were obtained and from these twice weekly injections (Tuesday and Friday) were calculated. The treated group received 25 mg 1-NN/kg ip twice weekly. Control group A received vehicle injections (volume calculated on a weight basis) ip twice weekly and the control group B received nothing. Twenty additional rats receiving 25 mg 2-NN/kg ip twice weekly were added to the study after 3 weeks. Injections were continued for 12 weeks followed by a 12 week latency period where weekly body weights only were recorded. At sacrifice, tissue samples were collected and fixed in neutral buffered formalin and processed and evaluated by the Pathology Department of the University of Michigan Medical Center. Multiple sections were made of bladders and any grossly abnormal tissue. Gross observations at autopsy were recorded and photographed.

F. Effect of Long-Term Administration of 1-NN on Male Fertility

Ten rats from the carcinogenicity assay group and ten from the injected control group were randomly selected one month after the last injection. These 20 males were mated with 20 normal females for one week then returned to the carcinogenicity study. The resulting pregnant females were sacrificed at midterm and the standard parameters used in a dominant lethal mutagenesis assay were noted and recorded.

G. Teratogenicity and Embryotoxicity Study (1-NN)

Twenty timed pregnant rats were purchased on day one of gestation and housed in individual cages. The treated group (10) received 50 mg 1-NN/kg subcutaneously on days 6, 8, 10, 12 and 14 whereas the control group received vehicle injections on the same schedule. On the 20th day (one day prior to term), the litters were removed by caesarian section, weighed and observed for gross abnormalities. The normal parameters for assessing embryotoxicity were noted and recorded. Two fetuses from each litter were processed for clearing of the soft tissues and stained with Alizarin S and the skeletal structure studied. Two fetuses from each litter were fixed in neutral buffered formalin, sectioned with a razor blade, and checked for cleft palates and gross internal abnormalities. Saggital section mounts of the whole body were prepared for histological examination. The remaining fetuses were stored in neutral buffered formalin pending the outcome of the above studies. After careful evaluation, it was decided to process the remaining specimens by the Alizarin Red S staining procedure.

H. Metabolism Studies (1-NN and 2-NN)

Twenty rats were given 100 mg 1-NN/kg ip and 9 rats received 100 mg 2-NN/kg ip. The pooled 24 hour urines

of 14 rats in the 1-NN group and the 9 rats in the 2-NN group were processed separately according to the procedures outlined in Section II.D.3.

IV. Results

A. Acute Studies

1. Range-finding Study (1-NN)

The range-finding study was undertaken to narrow the range of dosages to be administered during the LD_{50} determination and also to estimate organ toxicity. All animals receiving 200 mg or 1 g/kg ip died within 24 hours in severe respiratory distress. Both rats receiving 100 mg/kg ip developed acute dyspnea within 24 hours. A yellow, gummy exudate from the nasopharynx was abundant and wet the surrounding hair. At 48 hours one rat began to recover and resumed a limited water and food intake. The other animal's condition became worse and it expired within 72 hours. Hematocrits obtained from tail vein blood at 48 hours were elevated indicating a moderate degree of dehydration - correlating with a substantial decrease in water consumption. One rat at 50 mg/kg also developed acute respiratory distress but recovered within 48 hours while the other animal exhibited no apparent signs of toxicity. Rats receiving oral and iv doses also developed acute respiratory distress. The animals receiving iv injections also exhibited marked ptosis and a mild degree of trembling shortly after the infusion.

Initial histopathological examination of various organs indicated that 1-NN induced liver necrosis in the

centrilobular region and caused severe pulmonary congestion (discussed in detail in Section IV.A.3. Target organ toxicity determination).

2. LD_{50} Determination

The LD_{50} value was calculated using a computerized adaptation of Logistic analysis, adapted from Berkson (1944). The ip LD_{50} was calculated to be 86.3 mg/kg with 95 per cent fiducial limits of 54.5 to 136.6 mg/kg.

3. Target Organ Toxicity Determination (1-NN)

Relative organ weights (organ weight/body weight ratios) have been described by Benitz (1970) as the first morphological signs of dystrophic or dysplastic changes. Peck (1966) however, claims that these data are only of value if the chemical has a specific effect on a target organ. As an initial assessment of target organ toxicity, several organs were weighed in a specific time course after 1-NN, 2-NN or control injections. One hundred mg/kg was selected as an appropriate dose as a majority of animals receiving 100 mg 1-NN/kg ip developed the respiratory distress syndrome and liver toxicity. As can be seen in Table 2, the relative lung weights increased markedly at 24, 48 and 72 hours after the 1-NN injection (graphically displayed in Figure 6). In comparing the histological sections of the lungs of 1-NN treated animals and controls (see Figures 7 and 9) 24 hours after the injections, it can

TABLE 1

14 Day Mortality Following a Single ip Injection of 1-NN

Dose (mg/kg)	Dead/Total
200	4/4
150	4/5
100	3/5
75	0/5
50	1/5

TABLE 2

Organ to Body Weight Ratios[a] in 1- and 2-Nitronaphthalene (100mg/kg ip) Treated Rats

		Time of Sacrifice after Single Dose					
		24 Hours		48 Hours		72 Hours	
Group	N	Lung	Liver	Lung	Liver	Lung	Liver
Control[b]	9	0.46±.03	3.97±.01	0.46±.01	3.79±.17	0.56±.08	3.97±.19
1-NN	9	0.63±.01[c]	3.79±.09	0.92±.06[c]	4.54±.18	1.06±.03[c]	4.83±.15[c]
2-NN	9	0.45±.02	4.31±.13	0.44±.02	3.87±.35	0.49±.03	4.04±.12

[a] Expressed as organ weight/body weight x 100 ± SE

[b] Vehicle injection

[c] Difference from control group statistically significant ($p < 0.05$), Student's t test

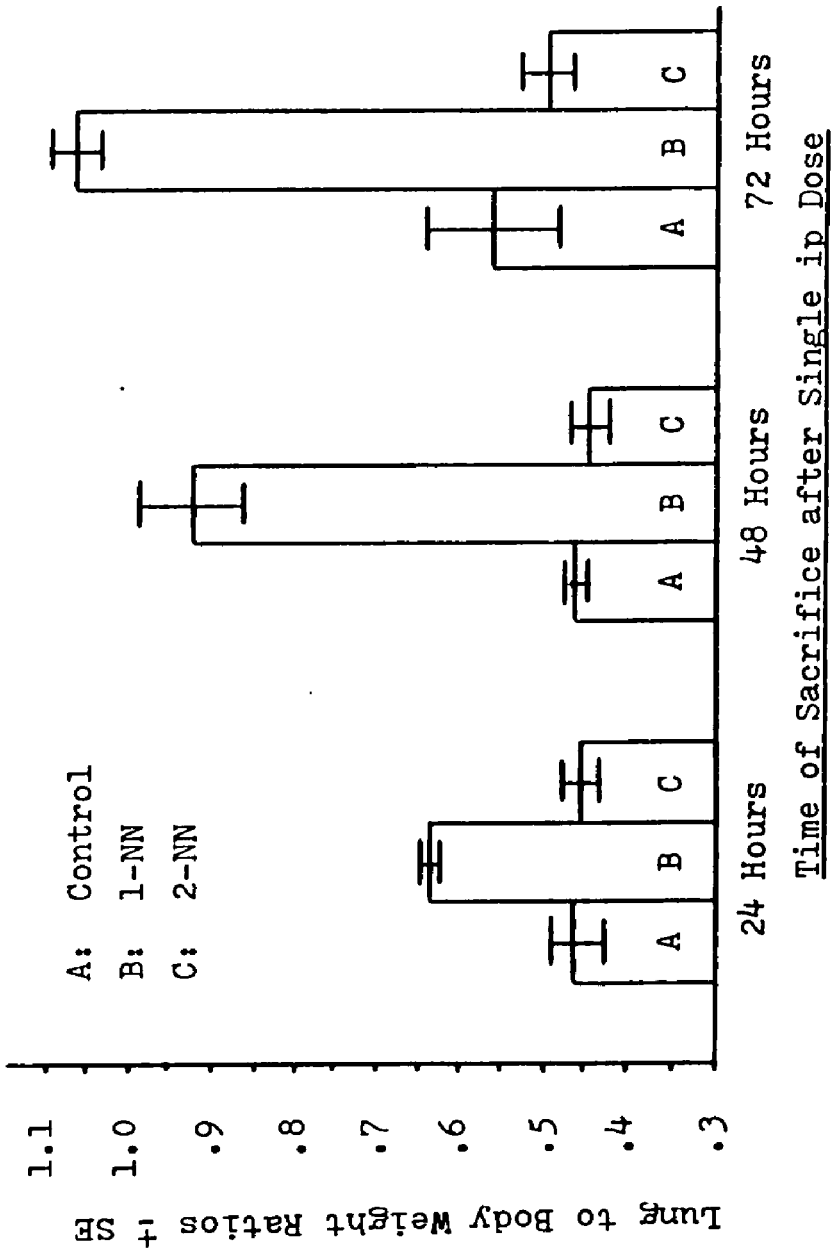


Figure 6. Lung to Body Weight Ratios in 1- and 2-Nitronaphthalene (100 mg/kg ip) Treated Rats

be assumed that the increased lung weight was a result of pulmonary congestion. These photomicrographs also reveal the focal atelectasis seen in all 1-NN treated animals developing acute dyspnea. Interestingly, 2-NN in the equimolar 100 mg/kg dosage, failed to induce the respiratory distress, the pulmonary changes or liver toxicity (Figures 11 and 12). Table 2 also reveals the increase in relative liver weight (only significant at 72 hours) which apparently accompanied the centrilobular liver necrosis induced by 1-NN (See Figure 10). Benitz (1970) states that increased liver weights may be a result of hyperemia, hyperplasia, hypertrophy or enzyme induction phenomena. Here, hyperemia and enzyme induction appear to be good possibilities, and the latter was studied in a preliminary fashion and reported in Section III.D.

4. ED_{50} for Respiratory Distress Determination (1-NN)

The respiratory distress induced by 1-NN was found to be dose related with an ED_{50} of 59.8 mg/kg ip and 95 per cent fiducial limits of 43.5 to 82.1 mg/kg. The data on responders appears in Table 3.

A comparison of ED_{50} and LD_{50} values of the respiratory response and mortality indicates a seemingly close association. Tables 4 and 5 list the results of probit analyses of 1-NN dose vs. mortality and the dose related respiratory distress respectively. Comparative probit estimates of dose vs. response appear in Figure 13. The differences in the slopes of the dose response curves

Figure 7

Lung from control rat given vehicle injection ip, 24 hours prior to sacrifice. H&E, 143X.

Normal lung.

Figure 8

Liver from control rat given vehicle injection ip, 24 hours prior to sacrifice. H&E, 143X.

Normal liver.

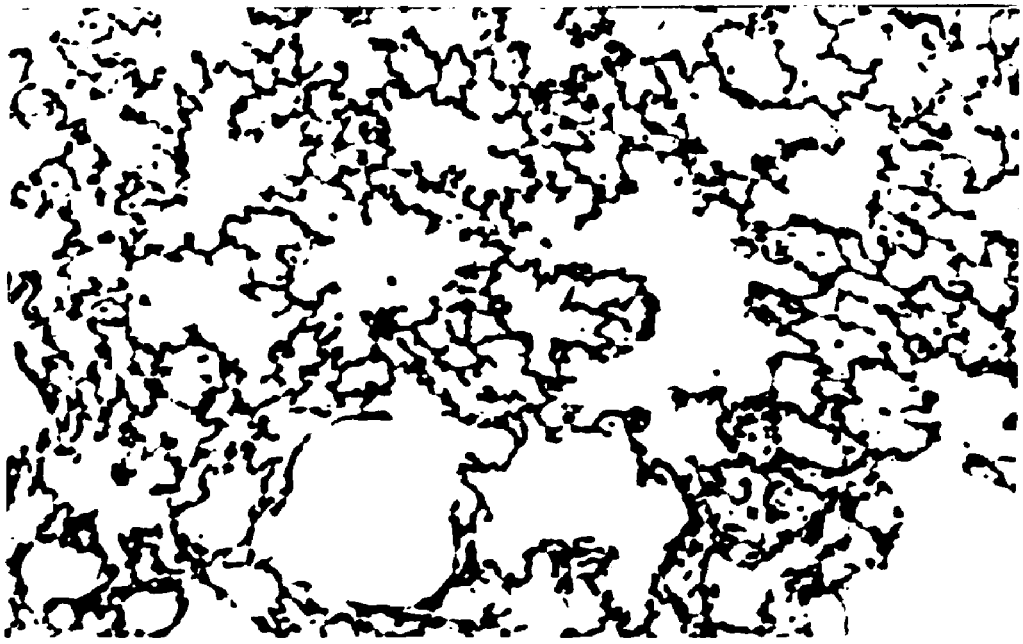


Figure 7

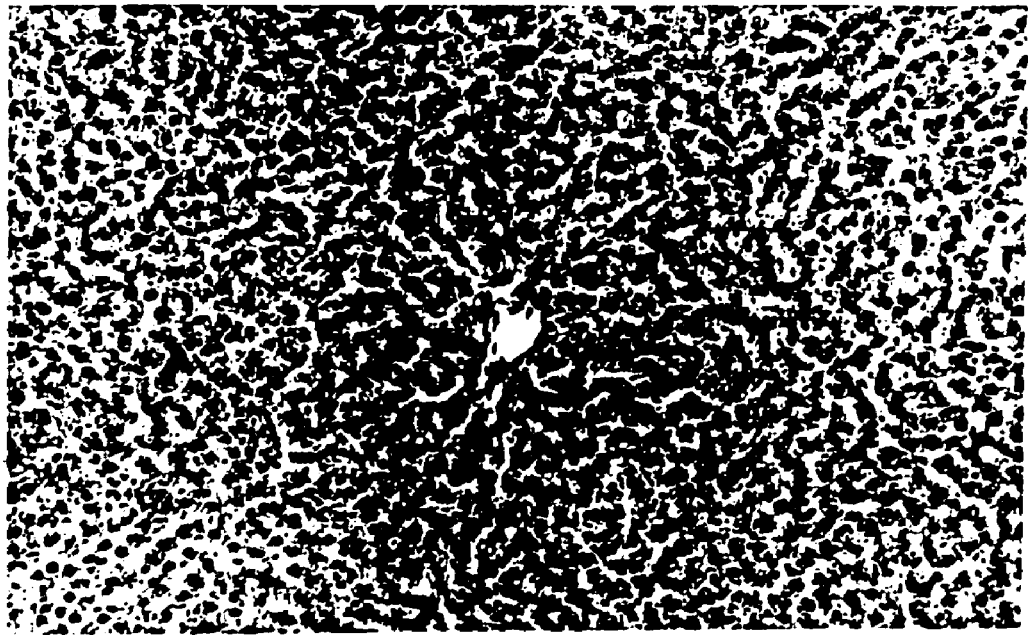
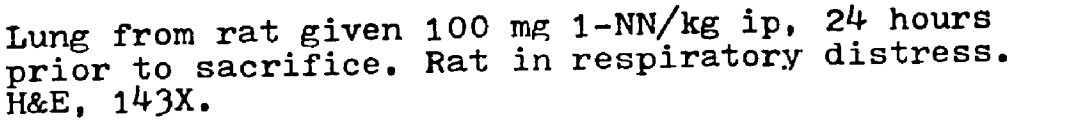


Figure 8

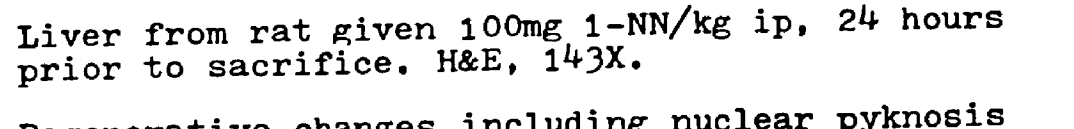
Figure 9



Lung from rat given 100 mg 1-NN/kg ip, 24 hours prior to sacrifice. Rat in respiratory distress. H&E, 143X.

Septal capillary congestion and focal atelectasis. Small amounts of edema fluid are seen.

Figure 10



Liver from rat given 100mg 1-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Degenerative changes including nuclear pyknosis and hydropic change in hepatocytes surrounding central vein. A small amount of lipid accumulation is evident. A few cells appear anuclear which may indicate early centilobular necrosis.

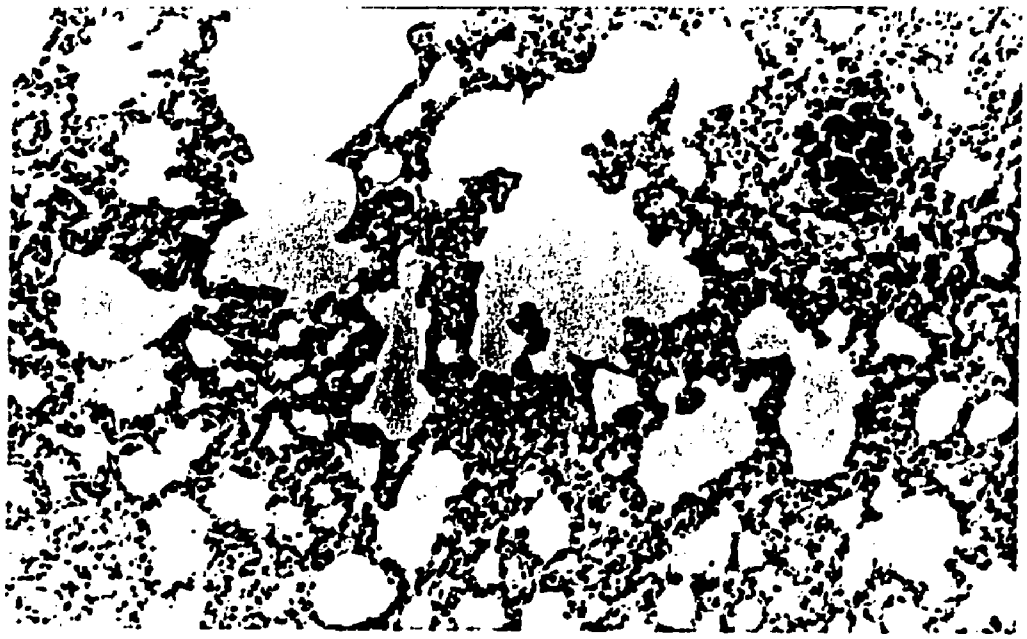


Figure 9

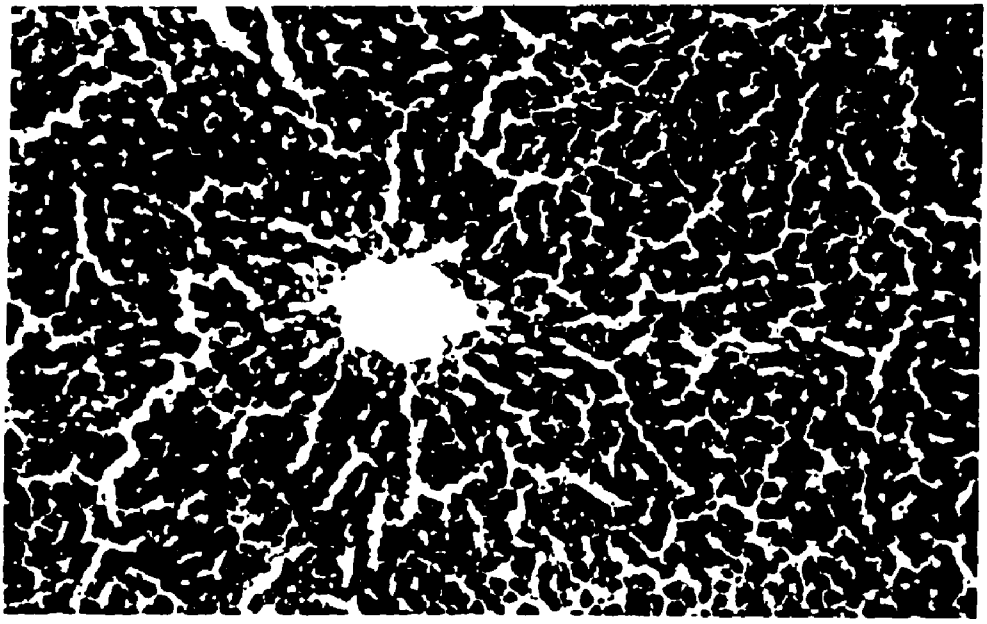


Figure 10

Figure 11

Lung from rat given 100 mg 2-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Normal lung.

Figure 12

Liver from rat given 100 mg 2-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Normal liver.

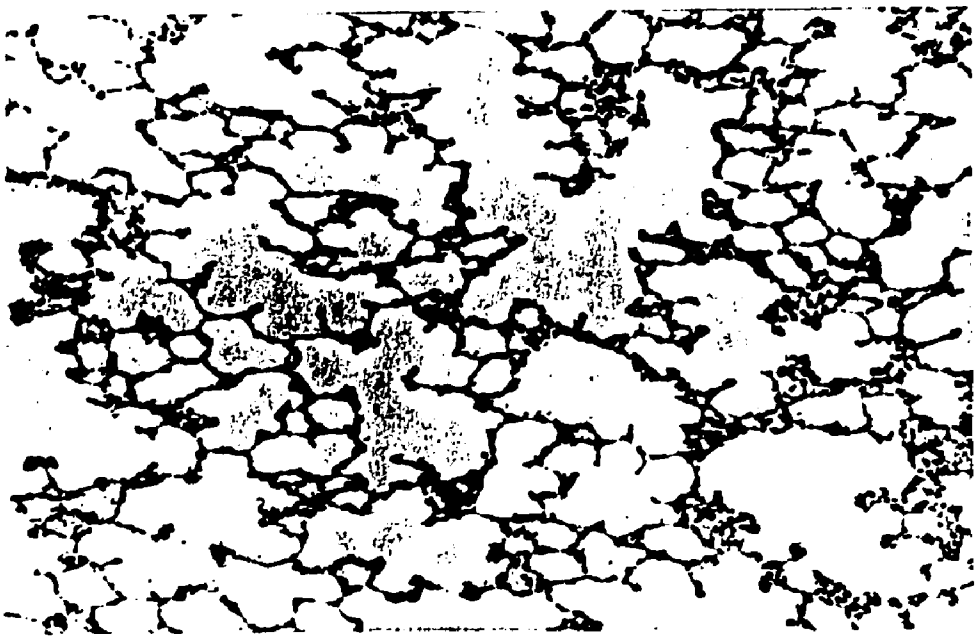


Figure 11

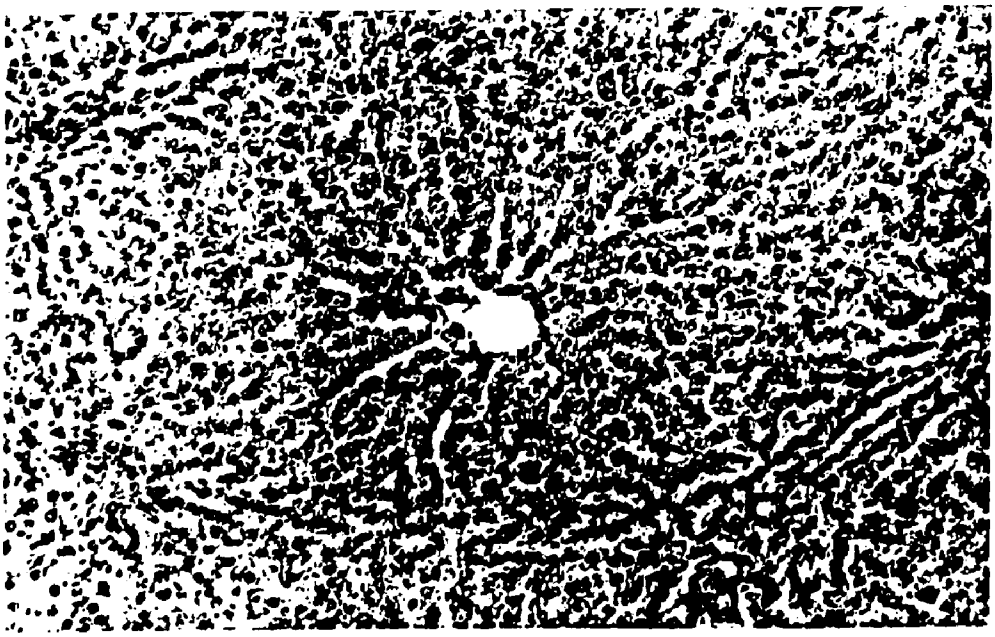


Figure 12

TABLE 3

Acute Toxicity (ED_{50} for Respiratory Distress)
of 1-Nitronaphthalene(ip)

Dose (mg/kg)	Responding/Total	ED_{50}(mg/kg)
200	4/4	59.8
150	5/5	
100	17/23	
75	3/5	95% Fiducial Limits
50	2/5	43.5 - 82.1
25	3/20	

TABLE 4

Probit Analysis[a]: LD_{50} for 1-NN

Dose (mg/kg ip)	Log Dose	N	Number Responding	Per Cent Responding	Expected Probit
50	1.699	5	1	20	3.521
75	1.875	5	0	0	4.409
100	2.000	5	3	60	5.039
150	2.176	5	4	80	5.927
200	2.301	4	4	100	6.557

probit equation: $y = 5.045 + 5.042\ x$

chi square for significance of probit = 3.646 (df=3)

[a] Graphically displayed in Figure 13

TABLE 5

Probit Analysis[a]: ED_{50} for Respiratory Distress induced by 1-NN

Dose (mg/kg ip)	Log Dose	N	Number Responding	Per Cent Responding	Expected Probit
25	1.398	20	3	15	3.884
50	1.699	5	2	40	4.820
75	1.875	5	3	60	5.367
100	2.000	23	17	73.9	5.756
150	2.176	5	5	100	6.303
200	2.301	4	4	100	6.692

probit equation: $y = 0.46 + 3.11\ x$

chi square for significance of probit = 1.006 (df=4)

[a] Graphically displayed in Figure 13

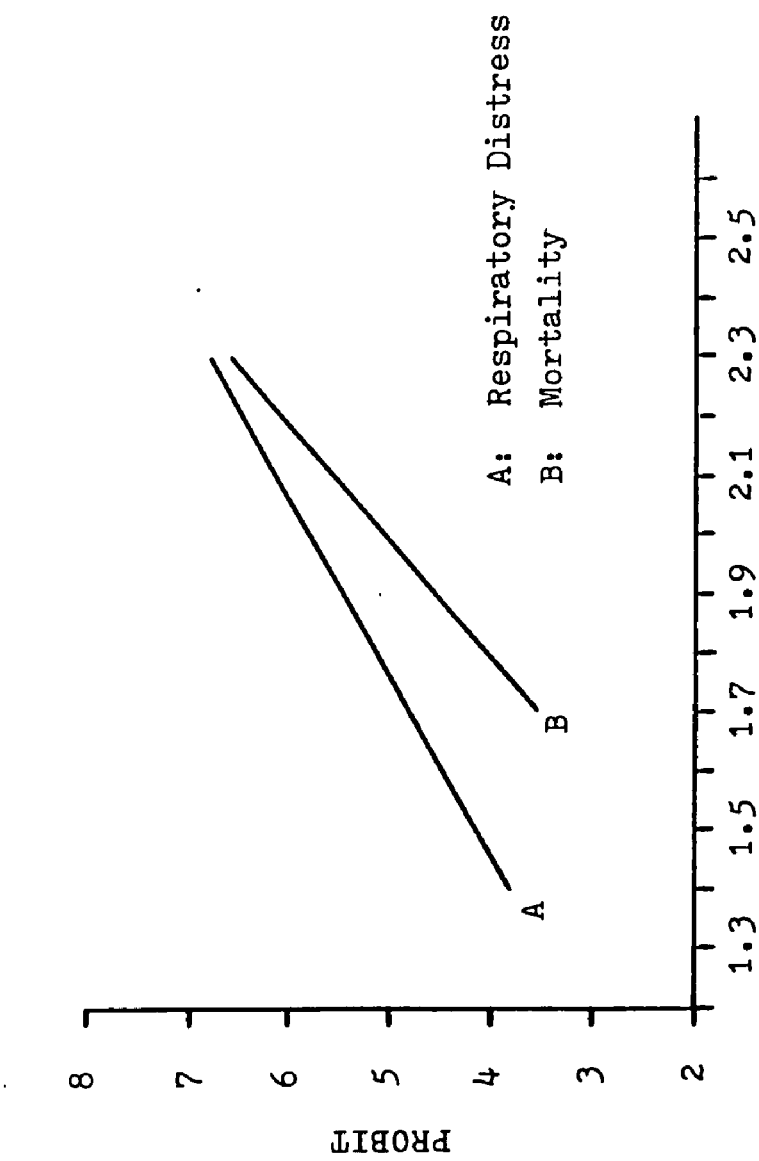


Figure 13. Probit Analyses of 1-NN Induced Acute Toxicity: Respiratory Distress vs. Mortality

indicate that while this acute toxic response with ensuing dramatic decrease in food and water consumption leading to dehydration may be a contributing factor, it is not the major cause of acute mortality in 1-NN treated rats. The respiratory distress was not altered by pretreating the animals with atropine eliminating the possiblity of cholinergic stimulation. Histological examination of the heart did not reveal any significant abnormality suggestive of myocardial failure.

This dose related respiratory distress, caused by ip injections of a hepatotoxic chemical, appears to be unique to the literature.

B. Acute Toxicity Studies with 1-NN after Phenobarbital or SKF-525A Pretreatment

It is well known that the ability of hepatic microsomal enzymes to produce toxic metabolites is altered by enzyme inducers and inhibitors as well as by nutritional status (Mitchell, et al., 1973). For instance, the hepatotoxicity of carbon tetrachloride is nearly abolished if the animal is receiving a low protein diet, but this effect is reversed by administration of phenobarbital. Similarly, the hepatotoxicity of chloroform is increased in rats if the animals are pretreated with phenobarbital. Other examples of drugs causing hepatotoxicity presumbably via toxic metabolites include the commonly used analgesic acetaminophen and the diuretic furosemide (Mitchell, et al.

1973). A clue that the hepatotoxicity of 1-NN might be due to a metabolite came from a study by Radomski and Brill (1971) where the N-hydroxynaphthylamines and nitrosonaphthalenes were tested in rats for possible carcinogenicity. The treatment regimen for the nitrosonaphthalenes had to be abbreviated and finally terminated due to their hepatotoxic effects.

In this study, stimulation and inhibition of microsomal metabolism were used as tools to determine if metabolism of 1-NN resulted in detoxification or 'activation'. Phenobarbital, a stimulator of microsomal metabolism and SKF-525A, an inhibitor of microsomal metabolism (Conney, 1971), were used as <u>in vivo</u> microsomal effectors. The inhibitory effects of SKF-525A on nitroreductase activity has not been clearly shown, however, Kaplan (1974) has shown a decrease in methemoglobin production from nitrobenzene in SKF-525A pretreated rats.

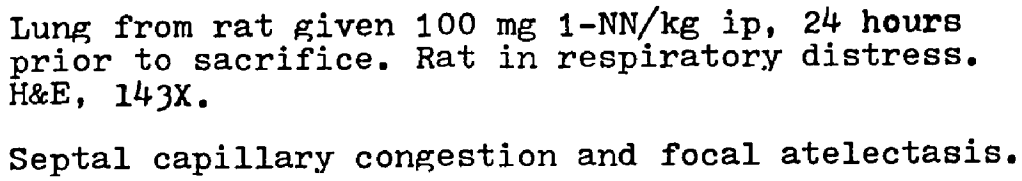
The effect of stimulating metabolism on the 1-NN induced respiratory distress was quite dramatic. The data in Table 6 indicate that the 1-NN induced dyspnea was abolished by phenobarbital pretreatment but apparently unchanged by pretreatment with SKF-525A. Figures 14-16 further illustrate this remarkable protection. Histological sections of the lungs of phenobarbital pretreated rats given 1-NN are essentially normal, whereas lungs of 1-NN treated rats (with or without SKF-525A pretreatment) exhibit septal capillary congestion and focal atelectasis.

TABLE 6

Effect of Phenobarbital or SKF-525A Pretreatment on 1-Nitronaphthalene Induced Respiratory Distress

Treatment	N	Per Cent Animals with Respiratory Distress
1-NN(200mg/kg ip)	4	100
1-NN(150mg/kg ip)	5	100
1-NN(100mg/kg ip)	23	73.9
1-NN(75mg/kg ip)	5	60
1-NN(50mg/kg ip)	5	40
1-NN(25mg/kg ip)	20	15
PB + 1-NN(350mg/kg ip)	1	0
PB + 1-NN(300mg/kg ip)	5	0
PB + 1-NN(250mg/kg ip)	5	0
PB + 1-NN(200mg/kg ip)	5	0
PB + 1-NN(150mg/kg ip)	5	0
PB + 1-NN(100mg/kg ip)	11	0
SKF-525A + 1-NN(100mg/kg ip)	9	77.7

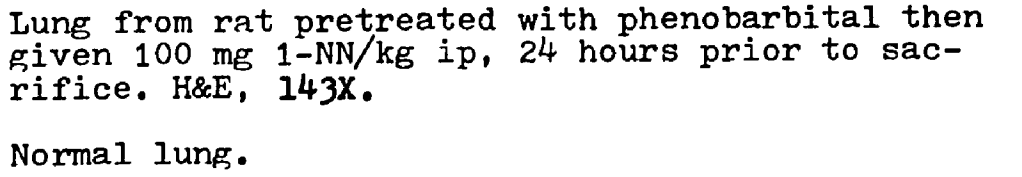
Figure 14



Lung from rat given 100 mg 1-NN/kg ip, 24 hours prior to sacrifice. Rat in respiratory distress. H&E, 143X.

Septal capillary congestion and focal atelectasis.

Figure 15



Lung from rat pretreated with phenobarbital then given 100 mg 1-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Normal lung.

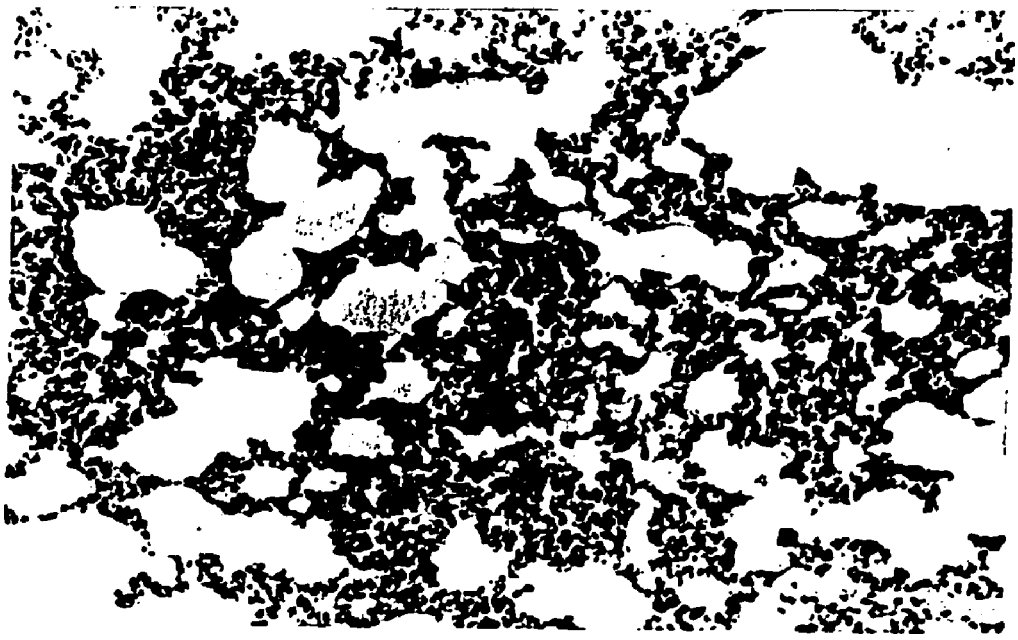


Figure 14

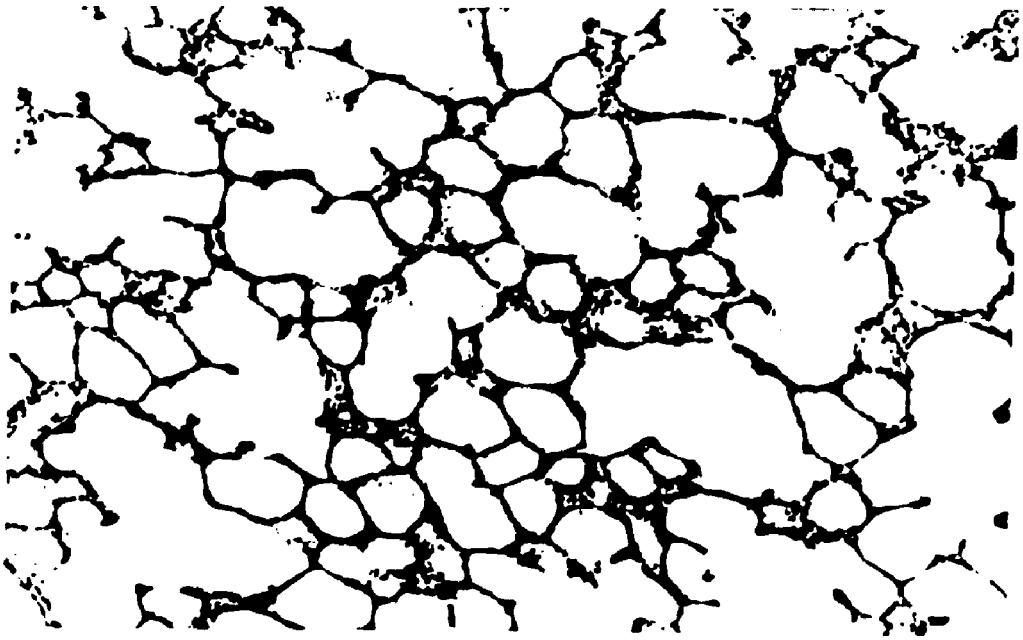


Figure 15

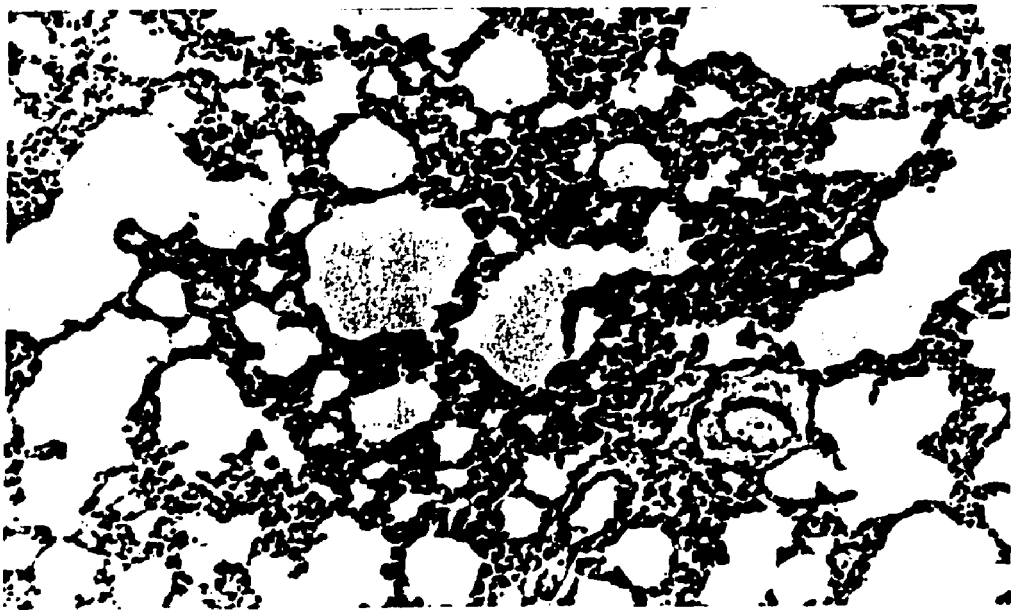


Figure 16

Lung from rat pretreated with SKF-525A then given 100 mg 1-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Septal capillary congestion and focal atelectasis.

In Table 7, it can be seen that the phenobarbital pretreated rats which had normal appearing lungs 24 hours after a 100 mg 1-NN/kg dose, did not shown an increase in lung weight. Curiously, neither did the SKF-525A pretreated rats where a substantial number (77.7 per cent) of animals developed respiratory distress.

Remarkable results were again encountered when the effects of phenobarbital or SKF-525A pretreatment on 1-NN induced liver toxicity were assessed. The severity of the centrilobular liver necrosis induced by 100 mg 1-NN/kg ip was substantially increased after phenobarbital pretreatment, whereas SKF-525A pretreatment protected against 1-NN induced liver necrosis. These differences were seen at both 24 and 48 hours after the injection. This is best illustrated by histological sections as seen in Figures 10, 17 and 18.

Interestingly, these data are consistent with both the notion that metabolism of 1-NN results in detoxification and the notion that it results in bioactivation. Since phenobarbital pretreatment abolished the 1-NN induced respiratory distress, a dose related response which is an apparent factor in mortality, metabolism of 1-NN apparently may lead to detoxification. However, phenobarbital pretreatment also leads to an exacerbation of 1-NN induced liver necrosis which would support the contention that metabolism of 1-NN is an 'activating' pathway. These

TABLE 7

Organ to Body Weight Ratios[a] in 1- and 2-Nitronaphthalene (100 mg/kg ip) Treated Rats after Pretreatment with Phenobarbital (PB) or SKF-525A

		Time of Sacrifice after Single Dose					
		24 Hours		48 Hours		72 Hours	
Group	N	Lung	Liver	Lung	Liver	Lung	Liver
Control[b]	6	0.47±.01	5.03±.46	0.44±.01	4.75±.02	0.44±.01	4.28±.05
PB + 1-NN	6	0.45±.04	5.60±.17	0.51±.02	5.16±.47	0.50±.02	6.04±.12[d]
PB + 2-NN	6	0.37±.01	5.65±.23	0.49±.05	4.48±.17	0.50±.05	3.79±.08
Control[c]	9	0.38±.01	3.72±.17	0.41±.02	3.85±.05	0.45±.03	2.96±.28
SKF-525A+ 1-NN	9	0.43±.03	4.17±.23	0.48±.05	4.48±.25	0.45±.02	4.12±.36

[a] Expressed as organ weight/body weight x 100 ± SE

[b] Phenobarbital pretreatment + vehicle injection

[c] SKF-525A pretreatment + vehicle injection

[d] Difference from control group statistically significant ($p < 0.05$), Student's t test

Figure 17

Liver from rat pretreated with phenobarbital then given 100 mg 1-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Severe centilobular necrosis.

Figure 18

Liver from rat pretreated with SKF-525A then given 100 mg 1-NN/kg ip, 24 hours prior to sacrifice. H&E, 143X.

Normal liver. Mild nuclear pyknosis.

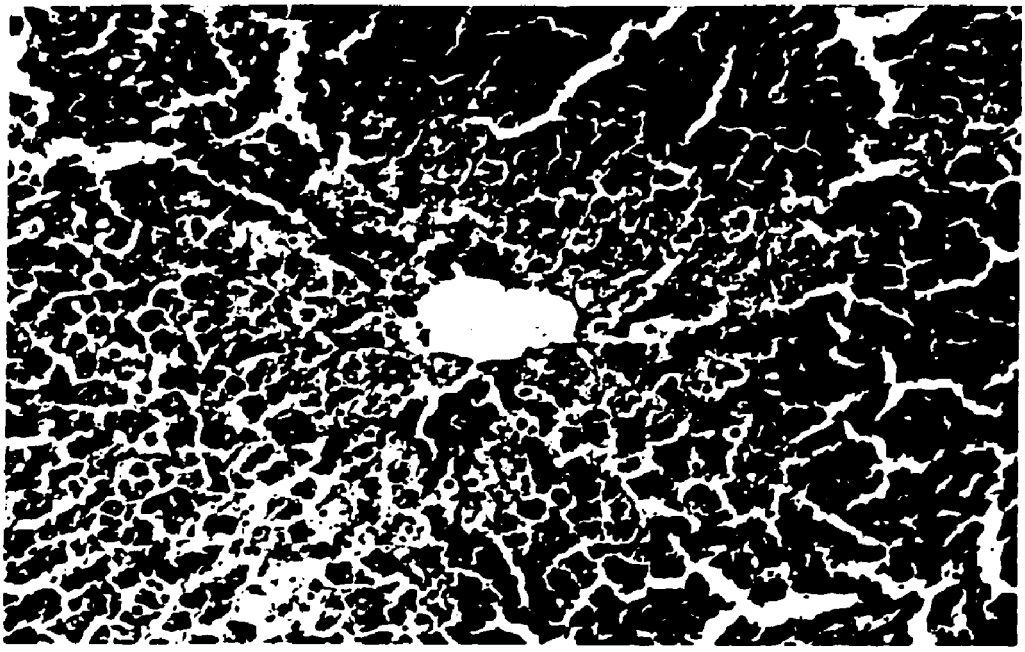


Figure 17

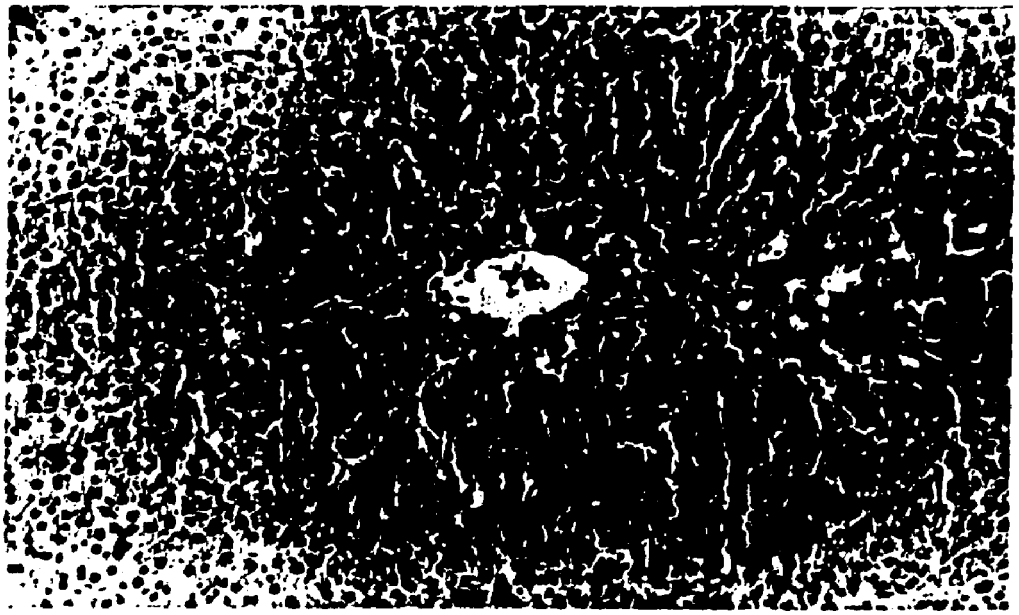


Figure 18

disparate impressions led to an assessment of the effect of phenobarbital pretreatment on mortality from a single injection of 1-NN. Mortality data from an LD_{50} study using phenobarbital pretreated rats are presented in Table 8. As can be seen, the data no longer appear to represent mortality as a simple dose related response as in the case without phenobarbital pretreatment. Since there were no fatalities at 100 mg 1-NN/kg, a dose where a majority of the animals died without phenobarbital pretreatment, it appears that increased metabolism does detoxify 1-NN in respect to mortality. However, mortality in the phenobarbital pretreated animals may be the result of another or possibly more than one mechanism. A comparison of the dose response curves of the mortality data (Table 8 and Figure 19) indicates that the phenobarbital induced protection against mortality is actually slight. This substantiates the conclusion that respiratory distress is not a major cause of mortality after 1-NN treatment in non-induced rats. The difference in slopes (Figure 19) leads to the assumption that a different mechanism may take primary responsibility for 1-NN induced mortality in phenobarbital pretreated rats.

The possiblity that metabolism of 1-NN involves an 'activating' step will be examined in detail in Section V. Discussion.

TABLE 8

Probit Analysis[a]: LD_{50} for 1-NN in Rats Pretreated with Phenobarbital

Dose (mg/kg ip)	Log Dose	N	Number Responding	Per Cent Responding	Expected Probit
100	2.000	5	0	0	4.408
150	2.176	10	7	70	4.974
200	2.301	10	6	60	5.376
250	2.398	10	9	90	5.688
300	2.477	10	7	70	5.942
350	2.544	6	5	83.3	6.157

probit equation: $y = 2.02 + 3.214\ x$

chi square for significance of probit = 6.155 (df=4)

[a] Graphically displayed in Figure 19

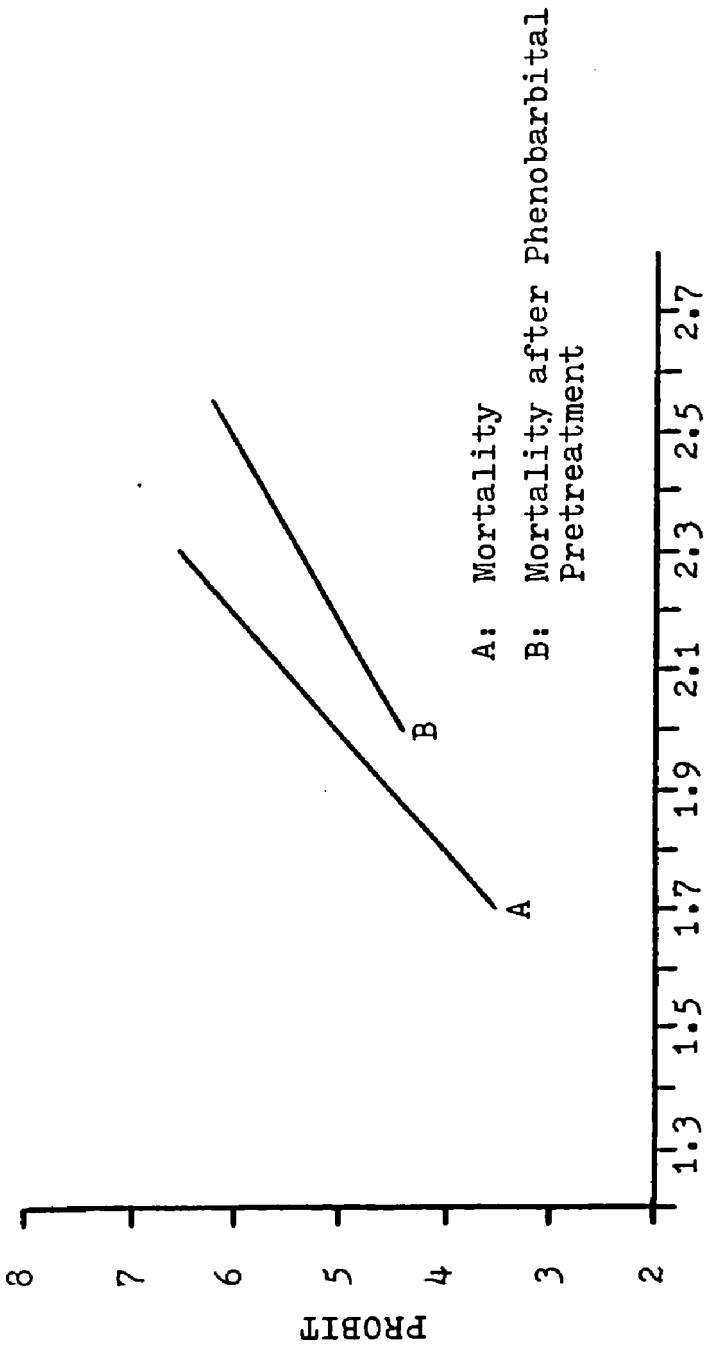


Figure 19. Probit Analyses of 1-NN Induced Acute Toxicity: Mortality vs. Mortality after Phenobarbital Pretreatment

C. Methemoglobin Determination

1-NN, 2-NN or 1-NA did not elicit a methemoglobinemia significantly higher than that in controls in any of the groups tested. This is in agreement with Kaplan (1974) and others, but still curious if one considers the ease at which monocyclic aromatic nitro compounds such as nitrobenzene produce a significant methemoglobinemia. On the other hand, 1-nitroso-2-naphthol induced a peak methemoglobin level of 17 per cent within 30 minutes suggesting that N-oxy-naphthylamine derivatives are capable of inducing methemoglobin formation. It is attractive to speculate that since N-oxy derivatives of the naphthylamines have a greater affinity for binding to tissue macromolecules than their monocyclic analogs, that this binding would supercede methemoglobin production. However, this supposition was not pursued further in this investigation.

D. Preliminary Hepatic Microsomal Enzyme Evaluation

An assessment of marker hepatic microsomal enzyme activity after 1-NN treatment was conducted to investigate one explanation for increased relative liver weight after treatment. The possibility that aromatic nitro compounds could stimulate their own metabolism is also an interesting possibility. If nitro compounds such as 1-NN could be detoxified by a stimulation of metabolism, the acute toxic effects would be bypassed (for instance the

respiratory distress from 1-NN) and the more insidious effects - mutagenesis and/or carcinogenesis - might occur as a result of increased 'bioactivation'. In the long run this could be a more destructive toxic effect since the mutant gene would be able to enter into the gene pool.

The preliminary data in Table 9 suggest that 1-NN may indeed stimulate its own metabolism by induction of hydroxylase enzymes and this may in part account for the increased liver weight seen in Table 7.

E. Carcinogenicity Assay (1-NN and 2-NN)

The experimental design and treatment schedule, 25 mg/kg ip for the twelve week injection period followed by a twelve week latency period, was selected for the following reasons. First, several studies of similar duration (3 months treatment period) have shown that N-oxidized derivatives of the naphthylamines to be carcinogenic (reviewed in the Introduction, Section I.C.3.). A dose of 1-NN equimolar to the compounds previously tested was not possible due to the acute toxicity of 1-NN. As it was, 3 out of 20 rats developed respiratory distress after the first injection, but all recovered. Because of the relatively low ED_{50} for respiratory distress, 25 mg/kg ip was thought to be the maximum dose that would allow maintenance of animals in a reasonably healthy state. The shortened

TABLE 9

Effect of Three Day Treatment (25mg/kg ip/day) of 1-Nitronaphthalene on Liver Microsomal N-Demethylation and Hydroxylation in the Rat

Group	Hydroxylation Activity (μg product formed/20 min./g liver[a]	N-Demethylation Activity (μg product formed 20/ min./g liver[a]
Control	118 ± 8.11	75 ± 9.8
1-NN	208 ± 33.4[b]	83 ± 8.4

[a] Results expressed as mean ± SE (N=3)

[b] Difference from control group statistically significant ($p<0.05$), Student's t test

overall length of the study, as well as the use of male rats, decreases the number of spontaneous tumors arising in both controls and treated animals (Davis, et al., 1956) and is more consistent with the challenge of finding shortened in vivo carcinogenicity tests. This abbreviated test, however, does not allow for the tabulation of increased tumor incidence but places major emphasis on early changes. Intraperitoneal administration was selected over oral since Zacheriah, et al. (1974) have shown the gastrointestinal contents in rats to have high nitroreductase activity.

It was considered of utmost importance to carefully study the effects of 1-NN and 2-NN on the bladder, since preliminary findings indicated that the naphthylamines may be excreted as metabolites (See Section IV.H.). Also, Uehleke and co-workers have shown both the absorption of 2-naphthylamine by the bladder mucosa (Uehleke, 1968) and the N-hydroxylating capability of the bladder epithelium (Uehleke, 1966). This suggests that the N-hydroxy or nitroso derivatives of the naphthylamines may in fact have contact with the bladder mucosa after the animal is exposed to the nitronaphthalenes. This theoretically could occur even if the intermediate or a conjugate of the intermediate were not excreted in the urine per se, since the reactive derivative could arise in the bladder, under appropriate conditions, by the N-oxidation of the excreted amine.

Other organs and tissues were also studied in detail for signs of early neoplasia or other pathologic changes.

1. Weight Gain and Growth Curves

Body weights were taken twice weekly during the 12 weeks of treatment and weekly during the 12 week latency period. The growth curves during the treatment period for the two control groups and 1-NN group appear in Figure 20. Of interest is the drastic weight loss in the 1-NN group initially with subsequent recovery but continued over-all weight depression. At 12 weeks (84th day), the 1-NN group showed a 8.54 per cent reduction in expected weight gain compared with the control group A injected with vehicle (Table 10). This shows good correlation with the NCI Fredrick Cancer Research Center (Gordon, et al., 1975) parameters for dose selection where a 10 per cent weight reduction after 12 weeks is considered along with certain parameters of acute toxicity including LD_{50} values in determining doses for carcinogenicity studies. As can be seen in Figure 21, the 1-NN group did not show a recovery to normal values even during the following 12 week latency period. The body weight data from the 2-NN group which was started at a later date than the controls was normalized to the control group B and the growth curves are shown in Figure 22 for the treatment period and in Figure 23 for the latency period. These curves suggest that 25 mg/kg ip, although equimolar with 1-NN dosage, may not have been

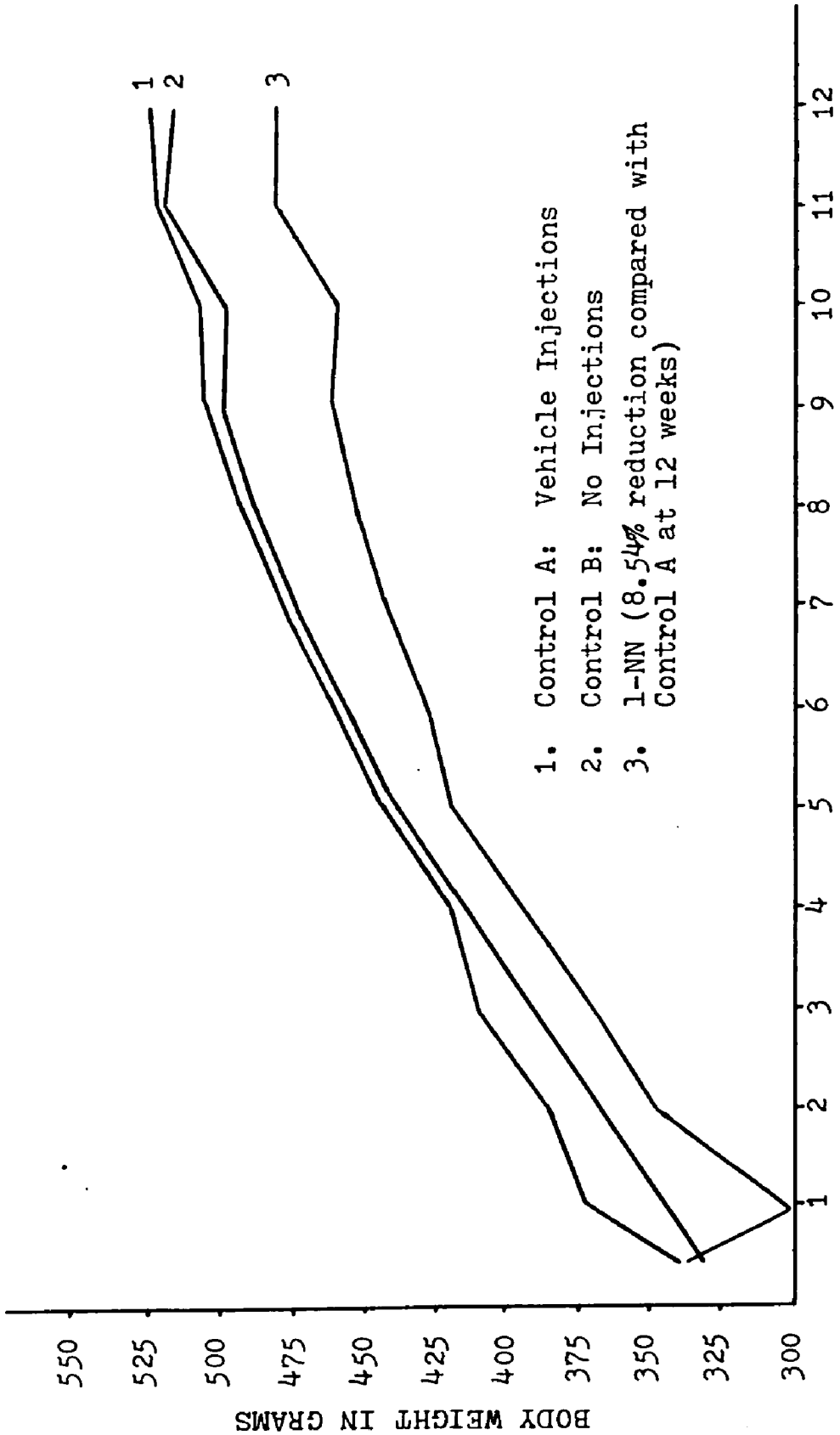


Figure 20. Carcinogenesis Assay. Growth Curves During Treatment Period: 1-NN vs. Controls

TABLE 10

Body Weight[a] Depression after Long-Term 1-Nitronaphthalene Treatment[b]

Group	N	Day of Study 1	84	168
1-NN	20	338 ± 2.9	484 ± 5.5*	580 ± 6.9
Control A[c]	20	341 ± 3.7	529 ± 8.7	612 ± 11.0
Control B[d]	20	331 ± 2.8	519 ± 5.3	604 ± 6.4

[a] Expressed as mean body weight in gm ± SE

[b] Twice weekly ip injections of 1-NN (25mg/kg) for 12 weeks, followed by a 12 week latency period

[c] Control group A received twice weekly vehicle injections for 12 weeks, followed by a 12 week latency period

[d] Control group B received nothing during 24 weeks

* Shows a 8.54% reduction at 12 weeks compared to control group A

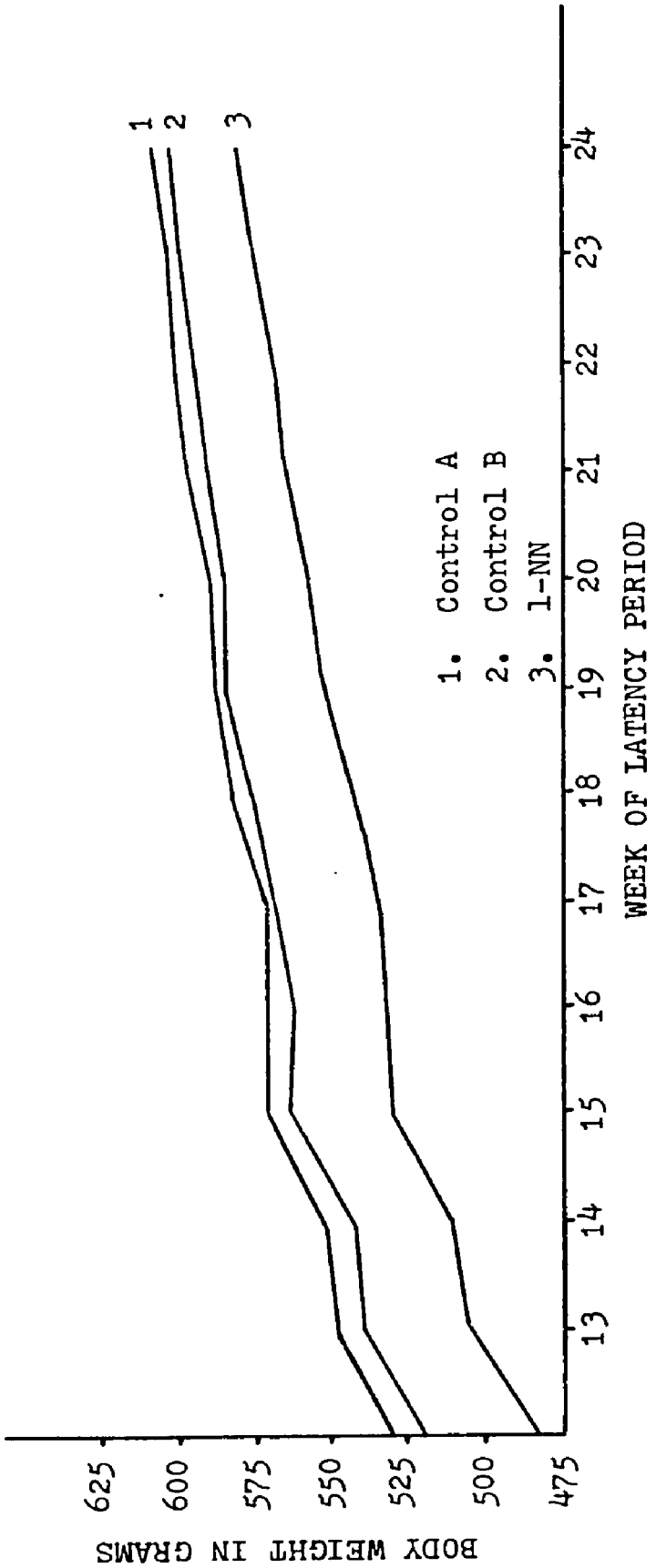


Figure 21. Carcinogenesis Assay. Growth Curves During Latency Period: 1-NN vs. Controls

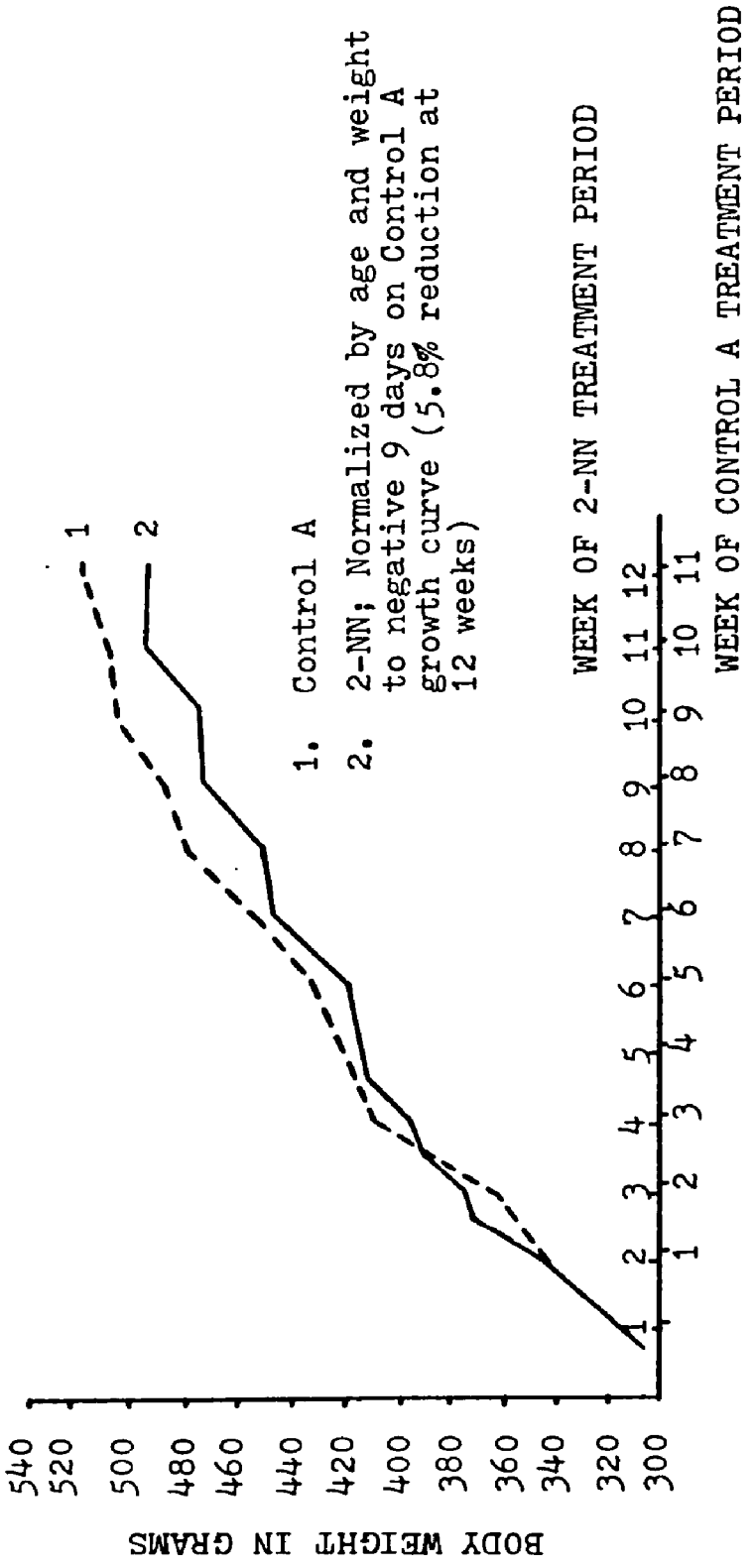


Figure 22. Carcinogenesis Assay. Growth Curves During Treatment Period:
2-NN Normalized to Control Group A

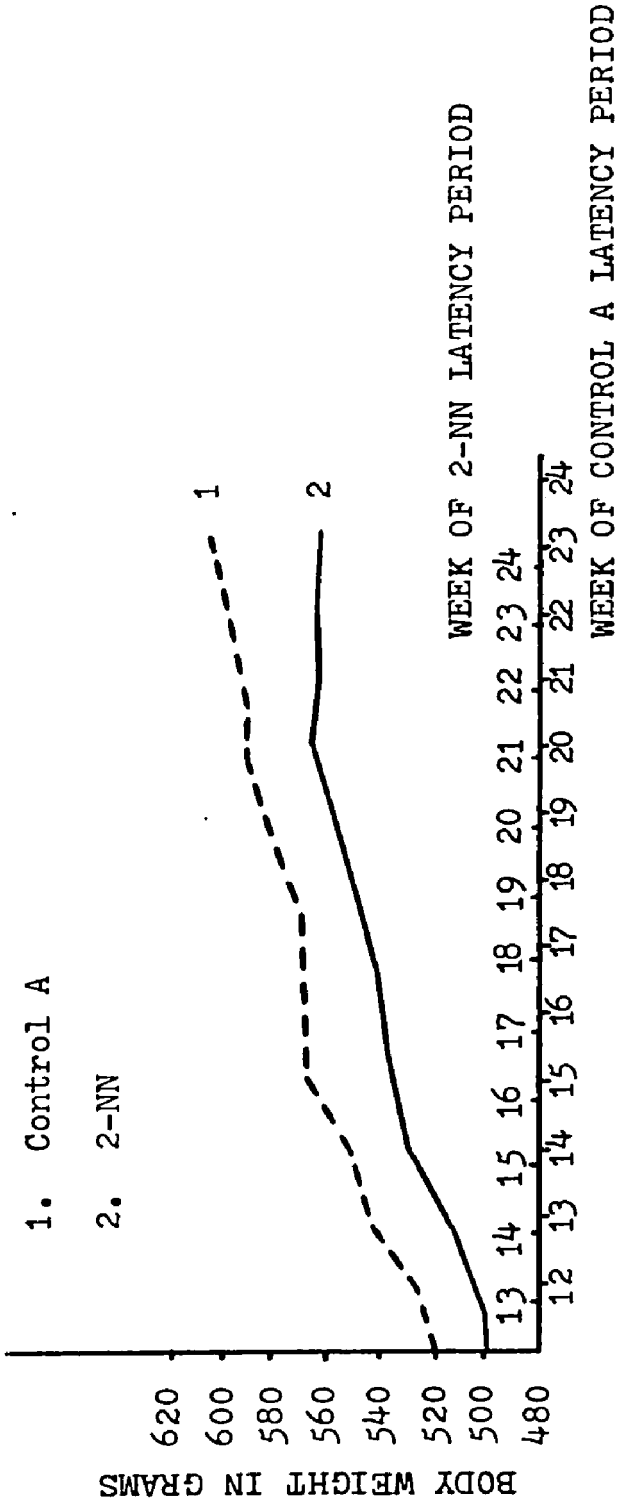


Figure 23. Carcinogenesis Assay. Growth Curves During Latency Period: 2-NN Normalized to Control Group A

appropriate for 2-NN as only a 5.8 per cent weight reduction was achieved at 12 weeks.

2. Relative Organ Weights

Organ to body weight ratios for liver, lung, heart, kidney and spleen were obtained on all four groups at autopsy and are listed in Table II. The slightly elevated kidney weights in the 2-NN group were due to two rats with hydronephrosis, and the increased lung weights of the 2-NN group were due to two rats were severe pneumonia (the weights are not significantly different if these rats are dropped from the sample).

3. Bladder Abnormalities

Spontaneous bladder tumors in rats are extremely rare. Snell (1965) reported having never seen one in research specimens at the National Cancer Institute. Davis, et al. (1956), Crain (1958) and Ratcliffe (1940) did not uncover any bladder tumors either in their own work or in the literature. Thompson and co-workers (1961) reported the same findings specifically in the Sprague-Dawley strain. The induction of bladder tumors in rats by chemicals is firmly established (Ito, 1973, Maltry, 1971), however, several investigators feel that other factors may be involved. Clayson (1974) reports that the presence of bladder stones, infestation with the rat bladder thread worm, Trichosomoides crassicauda, or induced inflammation may be factors acting as co-carcinogens with chemicals and therefore

TABLE 11

Carcinogenicity Assay of 1-NN and 2-NN: Organ to Body Weight Ratios[a]

Group	N	Liver	Lung	Heart	Kidney	Spleen
1-NN		3.675	0.334	0.297	0.676	0.165
2-NN		3.663	0.375	0.300	0.689	0.168
Control A[b]		3.598	0.319	0.306	0.670	0.167
Control B[c]		3.666	0.310	0.292	0.661	0.165

[a] Expressed as organ weight/body weight x 100

[b] Vehicle injection

[c] No injections

affecting the outcome of studies on bladder carcinogenesis. (These factors and how they relate to this study will be examined in detail in the Discussion Section). Since these other factors have only been suggested as co-carcinogens, it is apparent that any bladder tumors appearing in rats treated with various chemicals must be viewed as having been potentially induced by the presence of the compound or its metabolites in the urine.

In this study, at the time of autopsy, bladders were ligated with string at the urethral opening to keep the bladder in a distended state. Prior to removal, the bladders were either injected with normal saline or left full of urine. The bladders were then fixed in neutral buffered formalin. This in itself led to some interesting results. The mucosa of bladders with small amounts of urine would contract when placed in contact with the fixative resulting in a psuedo-papillomatous state. Figure 24 illustrates the typical mucosa of a non-distended bladder. This is compared to the distended bladder in Figure 25. For uniformity, future studies should include the removal of urine and injection of normal saline in every bladder with the volume based on body weight of the animal. The urine should be examined for pH, signs of inflammation and parasite infestation.

Figure 24

Bladder mucosa from rat in control group A, fixed in a nondistended state. H&E, 143X.

Psuedopapillomatous projections due to contractions during fixation.Normal bladder epithelium.

Figure 25

Bladder mucosa from rat in control group A, fixed after distending with normal saline. H&E, 143X.

Normal bladder epithelium of 2 to 4 cells thickness.

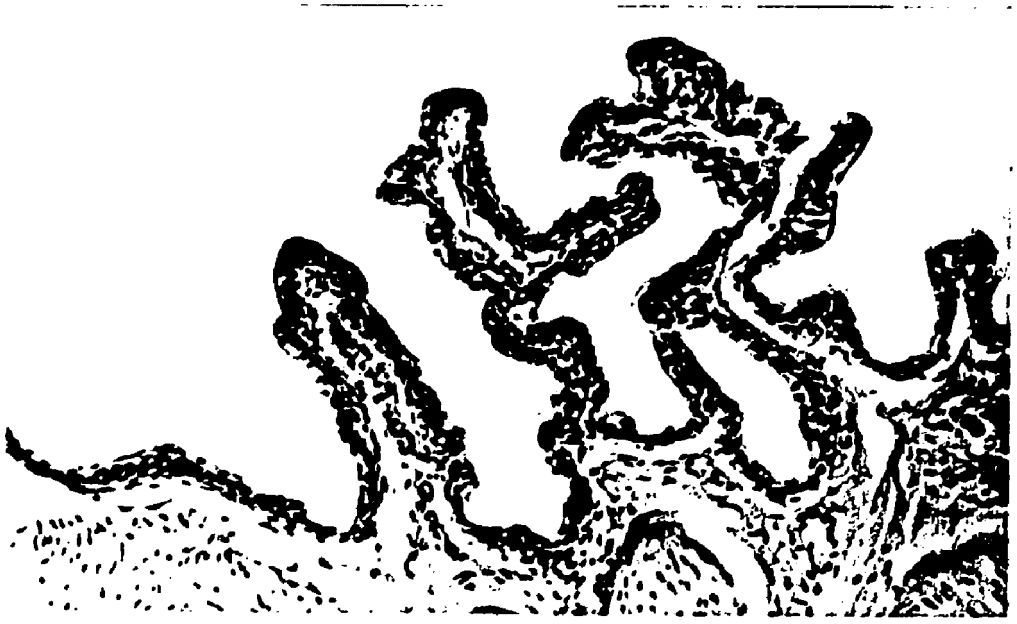


Figure 24



Figure 25

a. Control Group B - No Injections

There were no structural abnormalities in any of the bladders examined. None of the rats had urinary calculi in the bladder.

b. Control Group A - Vehicle Injections

No bladder from this group had any significant histological abnormality. Specifically, there was no hyperplasia of the transitional epithelium. Three of 20 rats had vesical calculi, but even in these the bladders were histologically unremarkable.

c. 1-NN Group

Sixteen out of 20 bladders in this group were histologically normal. Three bladders, from rats #DJ-1-NN8, DJ-1-NN9 and DJ-1-NN14, exhibited a focal hyperplasia of the transitional epithelium characterized by localized patches in which the thickened mucosa consisted of more cell layers than did the adjacent epithelium. This focal hyperplasia was considered orderly. One bladder, in rat #DJ-1-NN2, exhibited a generalized thickening of virtually the entire mucosal surface with at least a 2 to 3 fold increase in numbers of cells (Figure 26). Coarse folds of epithelium were prominent as were areas where mitotic figures were easily found. Intense proliferation resulted in the development of confluent masses of cells with a general loss of polarity. Several papillary projections were present with an irregular branching pattern (Figure 27). This was considered to be a low-grade non-

Figure 26

Bladder mucosa from rat # DJ-1-NN2. H&E, 143X. Lowgrade transitional cell carcinoma. Note coarse folding with intense proliferation resulting in confluent masses of cells. Mitotic figures are seen throughout the mucosal layer.

Figure 27

Bladder mucosa from rat # DJ-1-NN2. H&E, 143X. Lowgrade transitional cell carcinoma. Papillary projection with irregular branching pattern. (Compare with normal projections due to contraction as seen in Figure 24).



Figure 26

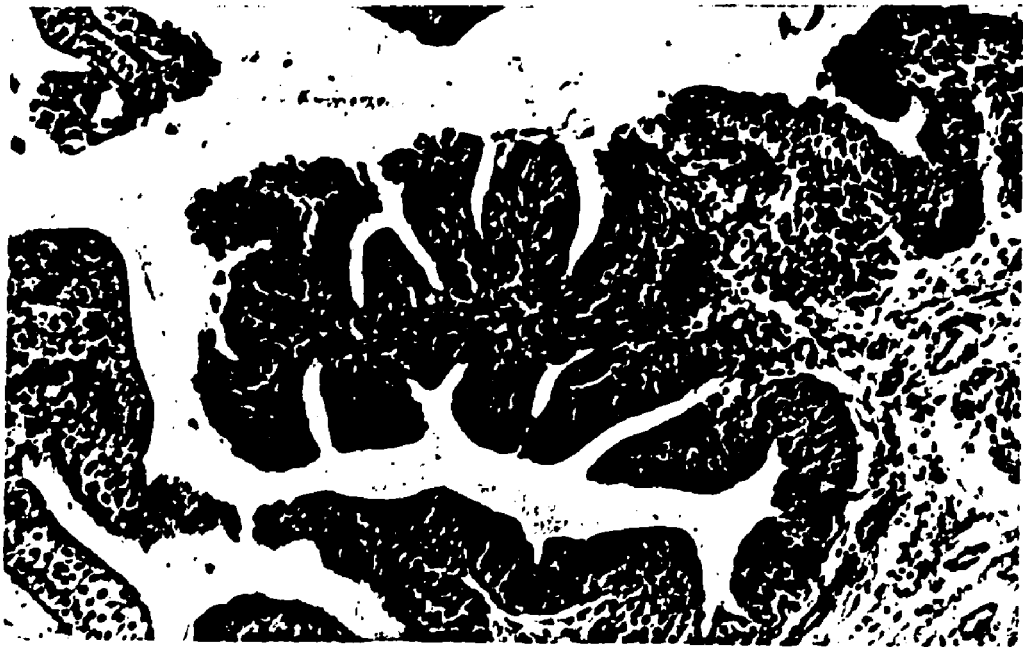


Figure 27

invasive transitional cell carcinoma. Interestingly, rat #DJ-1-NN2 was the only rat in the 1-NN group with bladder stones.

d. 2-NN Group

Seventeen out of 20 bladders in this group were histologically normal. Two bladders, in rats #DJ-2-NN4 and DJ-2-NN5, exhibited a focal hyperplasia of the transitional epithelium similar in intensity to that found in the bladders of rats #DJ-1-NN8, DJ-1-NN9 and DJ-1-NN14 from the 1-NN group. This was characterized by localized patches of thickened mucosa. One bladder, in rat #DJ-2-NN6, exhibited a marked hyperplasia with diffuse thickening of the mucosal surface. Plaque-like thickenings of cell layers occurred in some areas as did beginnings of small papillary projections. The intensity of this proliferative change was considered to fall somewhere in between the focal hyperplasia as seen in the 3 bladders in the 1-NN group and the 2 in the 2-NN group and the transitional cell carcinoma in rat #DJ-1-NN2. Four rats in this group were found to have urinary calculi. Two of these had normal bladders while the other two, rats #DJ-2-NN5 and DJ-2-NN6 had varying degrees of hyperplasia.

4. Other Significant Pathologic Findings

a. Control Group B - No Injections

Two of the 20 rats died during the study and a third rat was sacrificed in a moribund state. The left kidney of the dead rat #DJ-NTC15 was replaced by a mass weighing 32 g. Parts of it were composed of a yellowish-white glistening and moderately firm tissue and the rest was dark red, soft - resembling clotted blood. This was diagnosed as an infiltrating neoplasm characteristic of a nephroblastoma (Figure 28). Rat #DJ-NTC10 expired with a similar mass also replacing the left kidney. The mass measured 4x5x6 cm and weighed 50.8 g. This was again diagnosed as a nephroblastoma (Figure 29). Rat #DJ-NTC15 was 149 days old and rat #DJ-NTC10 was 163 days old at death. This is interesting since both rats were born on the same day and may have been litter mates. If true, this would represent the first report of nephroblastomas occurring in litter mates. In the literature, Snell (1967) had described a nephroblastoma in a 14 month old male BUF rat but thought perhaps it was chemically induced. Olcott (1950) found a spontaneously occurring nephroblastoma in a male rat less than 6 months old from an inbred Sherman-Mendel strain. And, Ratcliffe (1940) described four spontaneously occurring nephroblastomas in male Wistar rats; three had an average age of 200 days and one was 270 days old. Although a few investigators,

Figure 28

Kidney from rat # DJ-NTC10. H&E, 143X.
Nephroblastoma. Immature glomeruli present.

Figure 29

Kidney from rat # DJ-NTC15. H&E, 143X.
Nephroblastoma. Immature glomerular pattern.



Figure 28



Figure 29

including Ito (1973), have implicated various chemicals in the induction of nephroblastomas, these findings along with the other studies cited, indicate that nephroblastomas occurring in young male rats may well be spontaneous lesions.

Rat #DJ-NTC14 was sacrificed in a moribund state with a large tumor protruding from the chest wall. The mass measured 4.5x2x5 cm and weighed 26.9 g. It consisted of a low-grade malignant neoplasm of mesenchymal origin, probably a fibrosarcoma. In some areas the neoplasm had an epithelioid appearance blending with more spindled areas with a moderate amount of collagen production (Figure 30). Rat #DJ-NTC14 was 182 days old when near death and sacrificed.

b. Control Group A - Vehicle Injection

There were no tumors in any of the rats of this control group. Rat #DJVC-9 had focal dystrophic calcification in the testis and rat #DJVC-15 had a well developed lobular pneumonia. Rat #DJVC-11 had an abscess in the bulbourethral gland.

c. 1-NN Group

One other neoplasm was found in this group. Rat #DJ-1-NN12 had a benign soft tissue tumor within the subcutaneous tissue and lower dermis of the chest area weighing 200 mg. It was composed of a well circumscribed nodule of endothelial cells with numerous vascular channels

Figure 30

Tumor from rat #DJ-NTC14. H&E, 143X.

Low-grade fibrosarcoma. Moderate amount of collagen production amidst spindle-shaped cancer cells.

Figure 31

Tumor from rat #DJ-1-NN12. H&E, 143X.

Sclerosing hemangioma. Circumscribed nodule of fibroblasts and endothelial cells with numerous vascular channels. This is accompanied by a dense collagenous stroma.

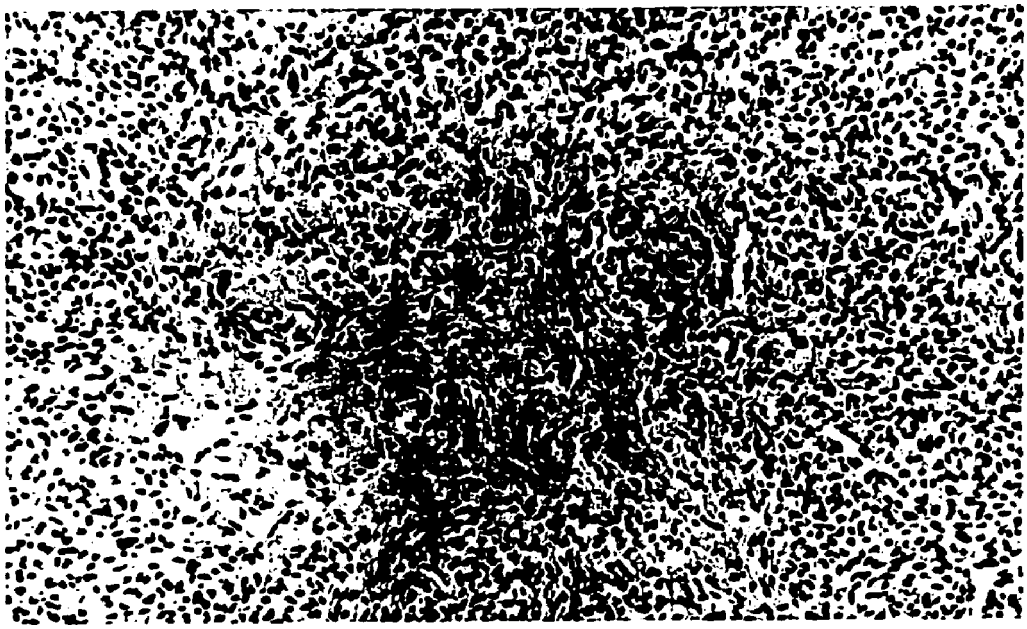


Figure 30

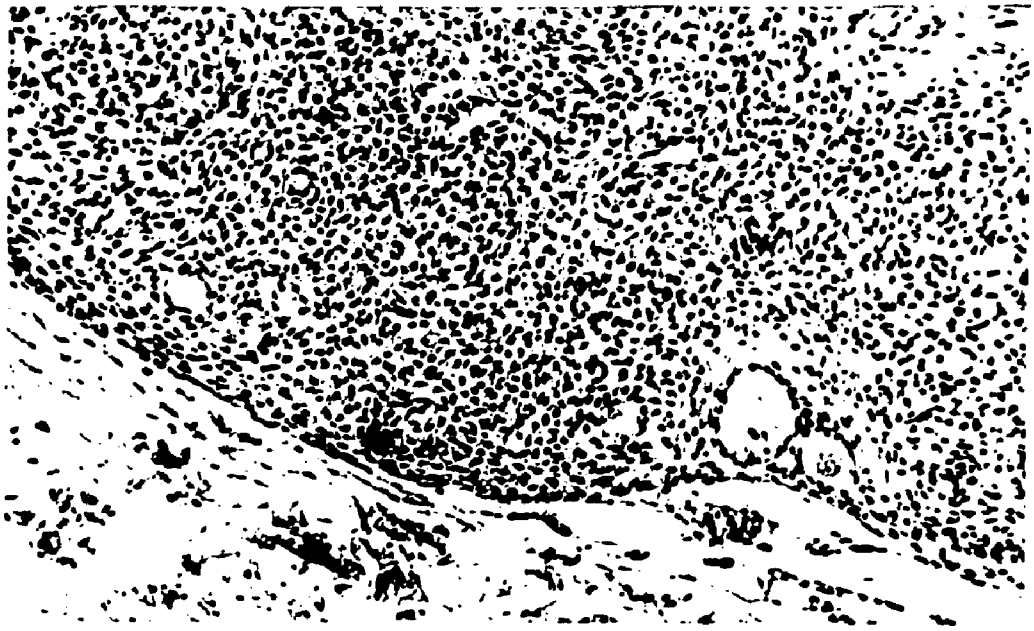


Figure 31

accompanied by a dense collagenous stroma (Figure 31). It was diagnosed as a sclerosing hemangioma.

Two rats (#DJ-1-NN16 and DJ-1-NN17) had small hard nodules in the abdominal wall at previous injection sites. These were both epidermal inclusion cysts. Four rats (#DJ-1-NN4, DJ-1-NN5, DJ-1-NN8 and DJ-1-NN9) had firm, white mesenteric nodules closely associated with the outer intestinal wall. Figure 32 illustrates on of these from rat #DJ-1-NN5. The appearance is that of a granulomatous reaction in relation to crystalline and oily material. One of these four rats (#DJ-1-NN4) and two other animals (#DJ-1-NN15 and DJ-1-NN16) also had clear cystic mesenteric or peritoneal nodules (Figure 33). They appeared as hyalinized fibrous walls circumscribing material which was washed away during the histological processing. The etiology of these cysts and the granulomatous nodules is almost certainly related to the chemical in the injected material since none appeared in the control group injected with vehicle alone.

Three rats (#DJ-1-NN14, DJ-1-NN15 and DJ-1-NN19) had small white lesions in the lungs. Histologically, these appeared to be nodules of lipid pneumonia in which there were numerous lipid laden macrophages, cholesterol clefts and histiocytic inflammation.

Figure 32

Nodule in mesenteric adipose tissue from rat #DJ-1-NN5. H&E, 143X.
The spaces represent pools of material, presumably oily, dissolved during processing. Macrophages and fibroblasts appear reactive to this material as well as interstitial crystalline material.

Figure 33

Clear cystic nodule in mesenteric adipose tissue of rat from 1-NN treated group.
1X.

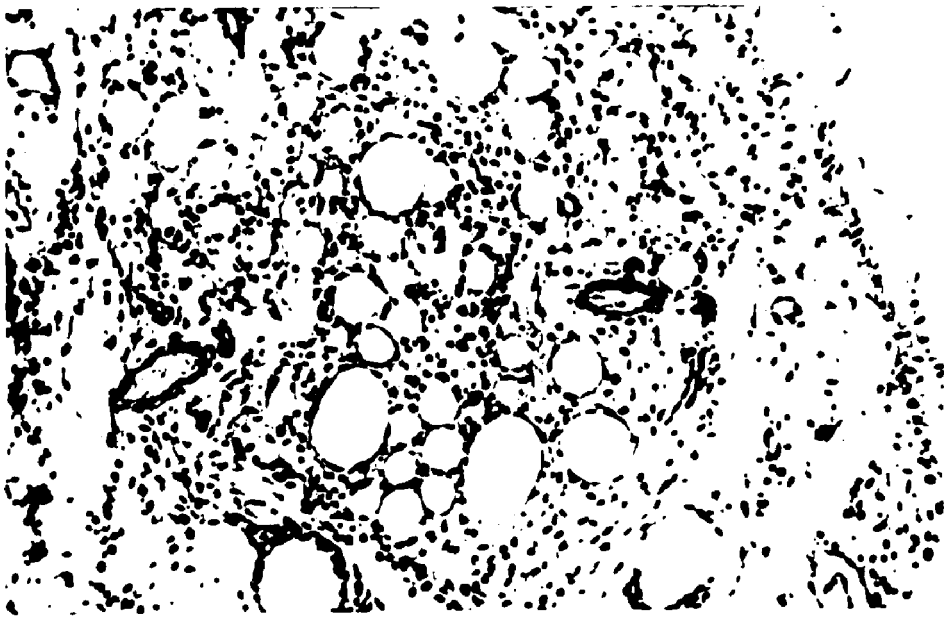


Figure 32



Figure 33

d. 2-NN Group

Two rats (#DJ-2-NN1 and DJ-2-NN3) had severe bronchiopneumonia involving one or more entire lobes of the lungs. Five rats (#DJ-2-NN1, DJ-2-NN12, DJ-2-NN17, DJ-2-NN19 and DJ-2-NN20) had enlarged bulbourethral glands with evidence of acute and subacute inflammation. Two of these glands, in rats #DJ-2-NN17 and DJ-2-NN19, were enlarged to such an extent by subacute abscesses that they were palpable and visible from the exterior abdominal surface of the animals.

The outstanding feature of the 2-NN group was the great number of rats bearing the firm, clear mesenteric or peritoneal cysts. Sixteen out of 20 rats had one or more of these nodules. They were identical to those seen in 1-NN group (See Figure 33). Again, it must be assumed that the presence of the chemical (or metabolites) in the peritoneal cavity is directly related to the formation of these cysts.

In addition, rat #DJ-2-NN11 had a ruptured epidermal cyst at the injection site and rat #DJ-2-NN20 had a fibrous adhesion to the outer surface of the liver.

F. Effect of Long-term Administration of 1-NN on Male Fertility

The three month treatment period (25 mg 1-NN/kg ip twice weekly for 12 weeks) apparently had no effect on male fertility as 9 out of 10 male rats mated successfully one month after the last injection. The data in Table 12 indicate that 1-NN did not cause dominant lethal mutations in males that were detectable with this assay. It should be pointed out, however, that several dosage levels with groups of 20 animals each (a minimum number for use with Fisher's exact test) should be used. Therefore, the question of whether or not 1-NN is a potential mutagen remains open. It is a critical question that must be answered at some point in the future since N-hydroxy-1-naphthylamine, a possible intermediate in the metabolism of 1-NN, is a very potent mutagen. The proper test system must allow for metabolism (nitroreduction) of 1-NN, such as dominant lethal or host mediated assays.

G. Teratogenicity and Embryotoxicity Assay

The importance of including teratogenicity testing in the overall assessment of the toxicity of a particular chemical compound must be viewed from two aspects. First, it is essential to find out if any danger exists in exposing pregnant women to the chemical either in the form

TABLE 12

Effect of Long Term 1-Nitronaphthalene Treatment[a] on Male Fertility[b]

Group	N	Conceptions (%)	Litters with resorbed fetuses	Litters with extra corpora lutea vs. viable fetuses	litters with extra corpora lutea vs. implantations
1-NN	10	90	4/9	7/9	5/9
Control	10	100	3/10	5/10	2/10

Above Results not significant using Fisher's Exact Test

Group	N	Litter size[c]	Resorption sites[c]	Corpora lutea[c]	Implantations[c]
1-NN	9	12.6 ± 0.4	0.7 ± 0.4	14.5 ± 0.6*	13.2 ± 0.3
Control	10	12.5 ± 0.4	0.5 ± 0.3	13.2 ± 0.4	13.0 ± 0.4

[a] Male rats administered 25 mg 1-NN/kg ip twice weekly for 12 weeks. Controls received vehicle injections for same schedule

[b] Male rats were mated with females (for one week) 16 weeks after initiation of injections. Dams were examined at midterm

[c] Mean values ± SE

* Difference from control group statistically significant only at ($p < 0.1$), Student's t test

of drugs, additives to consumer products or pollutants of the environment. Secondly and more pertinent in the study of the nitronaphthalenes, is whether certain metabolic pathways may be dangerous to the fetus. Nitroreduction or ring hydroxylation of nitro compounds appear to be bioactivating pathways (See Section V) and may pose a threat to man if the trend of increased use of nitro compounds as drugs continues (Kaplan, 1974).

Dosage selection and timing of administration are essential in teratogenicity testing, and the goal here was to chemically stress the maternal animal during the critical period of organogenesis without inducing abortion. Two methods of assessment were used; measurement of weight gain depression during and after the treatment period and visual observations of skeletal variations in cleared and stained fetal specimens. This latter method is extremely valuable but difficult to interpret in rats (Kimmel and Wilson, 1973). The difficulty arises when skeletal abnormalities are used to determine whether true embryotoxic effects were caused by a chemical or if the skeletal abnormalities are simply variations caused by maternal stress. Skeletal variations are well known in rats, particularly in teratogenic studies where appreciable manipulation of the pregnant animal is necessary (Kimmel and Wilson, 1973). The appearance of supernumerary 14th ribs, designated as 'rudimentary' (less than half the length of the 13th rib) or 'extra' (half or greater than the length of the 13th

rib) (Kimmel and Wilson, 1973), is the prime example. Green (1962) has stated that when an increase in supernumerary ribs occurs, it may be assumed that the maternal animal is being stressed sufficiently to express the developmental instability inherent in the species. Kimmel and Wilson (1973) have concluded that the presence of 'rudimentary' ribs should not be regarded as evidence of a specific teratogenic effect. However, the presence of 'extra' ribs may be taken as an indicator of possible teratogenic potential of the chemical compound under study.

Table 13 and Figure 34 reveal that treated dams did experience a decrease in their expected weight gain as compared to the control group. This indicates that the 1-NN dosage schedule (50 mg/kg sc on days 6, 8, 10, 12, and 14) was affecting the maternal animals but was not too high a level to cause abortion of the litter. Other toxic signs such as respiratory distress were not observed in any of the animals. Table 14 indicates that there were no statistically significant variations in the classic parameters which assess embryotoxicity, including a decrease in litter size, relative weight or number of implantations or an increase in resorptions or corpora lutea vs. implantations. Careful examination of the data, however, reveals a slight trend towards embryotoxicity which might become more significant at a higher dosage level.

After clearing the soft tissues and staining the skeletons, each fetal specimen was examined under a dissecting microscope at approximately 2x. There were no skeletal abnormalities except in the sternebrae and ribs (See Table 15). Using the Sign test, these were not shown to be statistically significant. However, the most notable deviations were in two fetuses in the 1-NN treated group which developed extra 14th ribs (Figure 35). According to the criteria of Kimmel and Wilson (1973), this indicates that 1-NN may have teratogenic potential and must be studied at a higher dosage level on specified days during the period of organogenesis.

H. Metabolism Studies (1-NN and 2-NN)

The procedures for the extraction of urine specimens and isolation of metabolites outlined in Section II.D.3. were the result of an intensive trial and error approach to extract and qualitatively detect trace amounts of metabolites of 1-NN. Demonstration of *in vivo* nitroreduction of 1-NN and 2-NN was considered to be of utmost importance to this study considering the toxic nature of expected intermediates, the N-hydroxynaphthylamines and/or the nitrosonaphthalenes.

TABLE 13

Body Weight Gain Depression[a] in Dams Treated with 1-Nitronaphthalene During Period of Organogenesis

Group	N	Day of Pregnancy 1	6	8	10	12
1-NN[b]	10	227.9±2.8	239.9±9.0	243.1±9.0	259.5±9.1**	263.3±11.4*
Control[c]	9	226.8±4.0	246.4±4.4	259.6±5.4	279.6±6.2	280.6±5.0

Group	N	Day of Pregnancy 14	16	18	20
1-NN	10	274.8±12.6*	290.9±12.1	321.2±15.9	352.2±17.2*
Control	9	295.6±6.9	308.6±8.3	345.1±9.1	384.3±10.0

[a] Expressed as mean body weight ± SE

[b] 50 mg 1-NN/kg subcutaneous injections on days 6, 8, 10, 12 and 14

[c] Vehicle injections on days 6, 8, 10, 12 and 14

* Difference from control group statistically significant ($p<0.1$), Student's t test

** Difference from control group statistically significant ($p<0.05$), Student's t test

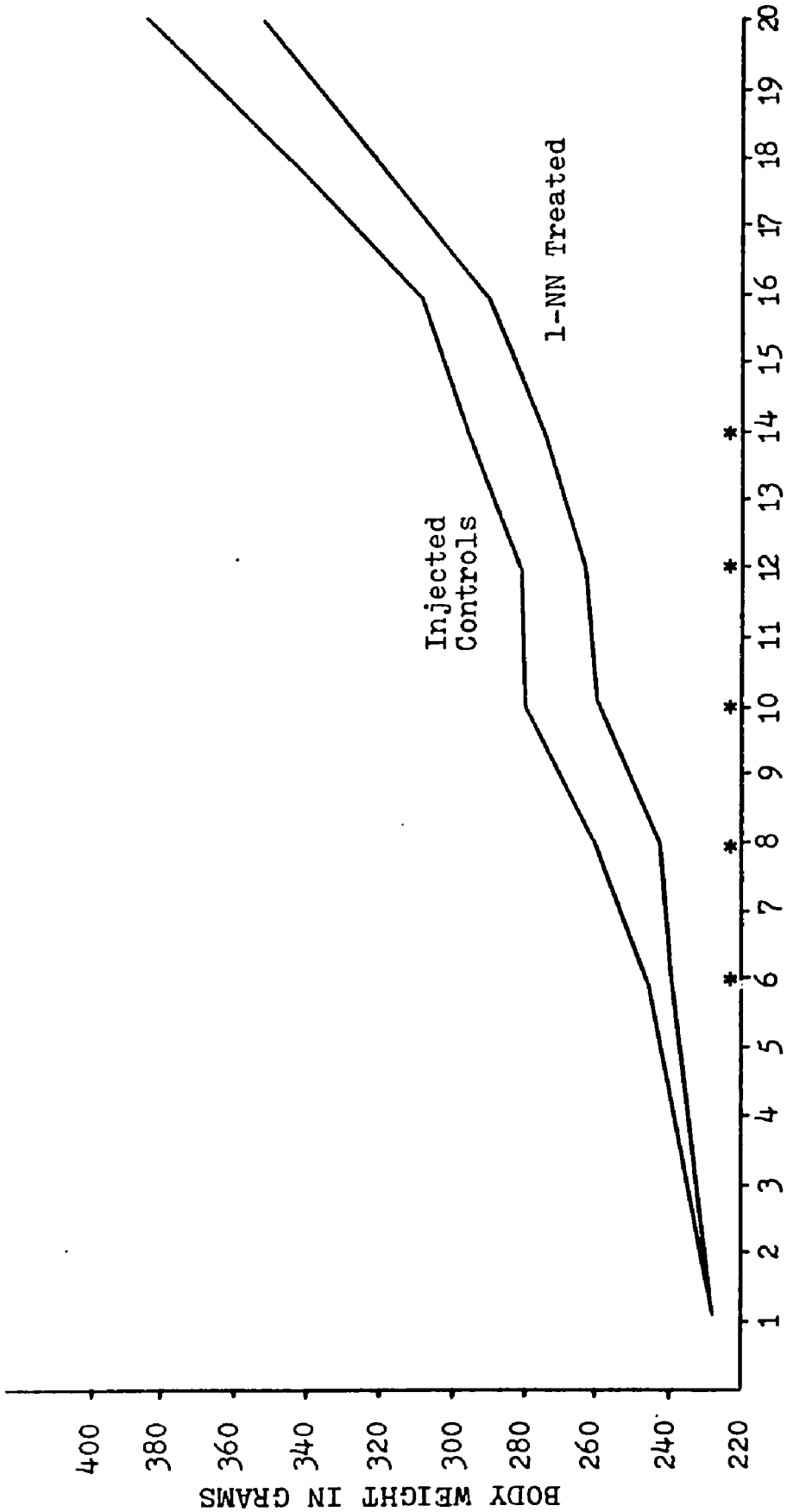


Figure 34. Dam Growth Curves During Gestation: 1-NN vs. Control

TABLE 14

Effect[a] of 1-Nitronaphthalene Treatment[b] During Period of Organogenesis on Embryotoxicity[c]

Group	N	Litter size	Litter weight (gm.)	Implantations	corpora lutea	Resorptions	Body wt.[d] ratios
1-NN	10	9.8±1.6	43.9±7.2	11.6±1.4	13.9±0.6	1.8±0.8	11.7±2.0
Control[e]	9	12.1±0.9	55.1±3.5*	12.9±0.7	13.4±0.4	0.8±0.5	14.2±0.6

[a] Expressed as mean value ± SE

[b] 50 mg 1-NN/kg subcutaneous injections on days 6, 8, 10, 12 and 14 of pregnancy

[c] Litters removed by caesarian section one day prior to term

[d] Expressed as fetus weight/Dam weight x 100

[e] Vehicle injections on days 6, 8, 10, 12 and 14 of pregnancy

* Difference from control group statistically significant only at ($p < 0.1$), Student's t test

TABLE 15

Teratogenesis Assay: Fetal Skeltal Variations

Sternebral Variations

Group	N	One or More Split Centra		Three or More Cleft or Malaligned Centra		One or More Sternebral Variations (Including poor Ossification)	
		N	Per Cent	N	Per Cent	N	Per Cent
Control	91	20	22.0	7	7.7	45	49.5
1-NN	78	24	30.8	2	2.6	51	65.4

Vertebral Variations

Group	N	One or More Supernumerary Ribs		Two Super-numerary Ribs		One Extra Rib	
		N	Per Cent	N	Per Cent	N	Per Cent
Control	91	28	30.8	9	9.9	0	0.0
1-NN	78	25	32.1	14	17.9	2	2.6

Vertebral Variations: 1-NN vs. Control



Figure 35

Fetal rat skeleton stained with Alizarin Red S. Maternal animal treated with 1-NN during period of organogenesis. 2X.

Note extra 14th rib on left side.

Initially, several non-polar solvents were used in an attempt to extract non-conjugated metabolites of 1-NN for comparison with standards using TLC analyses. Several interesting points were noted. (1) There seemed to be no qualitative difference in urinary metabolites excreted by rats in respiratory distress compared to rats which appeared normal (See TLC results in Table 16). (2) There appeared to be several metabolites that reacted with Ehrlich's reagent indicating the presence of an amine functional group. (3) 1-Naphthylamine (1-NA) possessed its own fluorescence appearing as light blue against the green background of the TLC plates with added fluorescent indicator . And, (4) trace amounts of 1-NA were thought to be present in the urine of rats injected with 1-NN. This last point was intriguing since it would prove the assumption that <u>in vivo</u> nitroreduction occurred and show that a compound appearing on the OSHA restricted list was excreted in the urine of animals exposed to a 'safe' chemical. First attempts to isolate 1-NA were not successful, resulting in the isolation of a compound with TLC characteristics similar to 1-NA but different GLC and mass spectral characteristics. The compound had a molecular weight of 173, was not 1-NN or 1-nitroso-2-naphthol and did not possess an amine functional group. It appeared that this compound was an oxidized form of 1-NA which could have been an artifact of the extraction and isolation procedure. This led to the

TABLE 16

TLC Analyses of Urinary Extracts, Isolated Metabolites and Standard Compounds

A. Urinary metabolite excretion pattern of 1-NN in rats with and without 1-NN induced respiratory distress. Merck TLC sheets were cut in strips and not activated prior to use. Detection was made using a UV lamp.

Compound or Extract	Major Rf Values in Solvent System A	B	C
1-NN	.37	.57	.62
24 hour urine, rats in resp. dist.[a]	.24*	.27;.41* .53;.57	.40;.55* .63
24 hour urine, rats not in resp. dist.[a]	.24*	.27;.41* .53;.57	.40;.55* .63
24 hour urine, rats pretreated with PB[a]	.24*	.27;.41* .53;.57	.40;.55* .63
control; vehicle injection	Nothing	Nothing	Nothing
control; vehicle injection, pretreated with PB	Nothing	.63	.64
1-NA	.26*	.41*	.53*

* Spot turned yellow-brown overnight

[a] Treatment of 100 mg 1-NN/kg ip

TABLE 16 (continued)

B. TLC analyses of standard compounds and 1-NA isolated as a urinary metabolite of 1-NN. Applied Sciences Prekotes (20x20cm) were activated prior to use. Compounds were spotted in order listed starting from left-hand side of plate. Detection with spray reagents, or UV if necessary.

Compound	Rf Values in Solvent System					Color Reaction to Spray Reagent		
	A	B	C	D	E	E	G	N
1	.27	.62	.72	.41	.64	NC	Y	NC
2	.12	.39	.56	.20	.65	NC	Y	NC
3	.25	.54	.70	.48	.79	YP	G	NC
4	.49	.73	.77	.69	.75	NC	NC	NC
5	.51	.75	.79	.71	.75	NC	NC	NC
6	.26	.52	.67	.41	.72	Y	TP	P
7	.34	.54	.61	.53	.76	NC	P	NC
8	.10	.15	.29	.11	.78	YF	LP	NC
9	.26	.52	.67	.41	.72	Y	TP	P

code: 1=2-hydroxy, 1-nitronaphthalene; 2=2-hydroxy, 1-nitrosonaphthalene; 3=2-hydroxy, 1-naphthylamine; 4=1-nitronaphthalene; 5=2-nitronaphthalene; 6=1-naphthylamine; 7=1-naphthol; 8=4-hydroxy, 1-naphthylamine; 9=1-naphthylamine isolated as metabolite of 1-nitronaphthalene.

color code: NC=No Color; Y=Yellow; YP=Yellow changing to Purple; G=No Color turning Grey; TP=Turquoise turning Purple; P=Purple, YF=Yellow fading to No Color; LP=Light Purple.

spray reagent code: E=Ehrlich's reagent; G=Gibb's reagent; N=Ninhydrin

final refinement of the procedure to include collecting the urine on a freezing unit as outlined earlier (Section II.D.3.b.). To test the effectiveness of the procedure, two critical assays were performed. A standard methanol solution of 1-NA was applied to a TLC plate and appropriate 1-NA band isolated. A subsequent GLC-mass spectral analysis indicated that this 'isolated' standard was identical to a freshly prepared 1-NA standard in methanol. Next, a trace amount of 1-NA was added to a control urine sample. The spiked urine was incubated at refrigerator temperature overnight, extracted and the 1-NA isolated, proving again to be identical to a freshly prepared 1-NA standard.

1-NA was then successfully isolated as a urinary metabolite from the pooled urines of 14 rats injected with 1-NN 100 mg/kg. The isolated metabolite had the same Rf values as standard 1-NA in five TLC solvent systems and gave identical color reactions with three spray reagents. Retention times in GLC were identical as were the mass spectra. The data confirming its identity appear in Table 17. Critical to the isolation was the fact that 1-NA had its own fluorescence. In trace amounts this could best be viewed under short-wave UV light while the TLC plate was still somewhat wet with solvent system B.

The procedure used to isolate 2-NA as a metabolite of 2-NN was identical to that used for 1-NN treated animals. Standard 2-NA TLC analyses were not run due to

TABLE 17

GLC-Mass Spectral
Identification of 1-NA as a Urinary Metabolite of
1-NN in the Rat

Sample	GLC Conditions for Mass Spectral Analysis	Retention Time
Isolated Metabolite	3% ov17, 180°isothermal	3.3 min.
Standard 1-NA	SAME	3.3 min.

Mass Spectral Quantitative Report
(computer generated)
Conditions: 24 ev, 50 mv

m/e	Relative Abundance Isolated Metabolite	Relative Abundance Standard 1-NA
144	9.9	9.7
143	100.0	100.0
142	7.6	10.9
141	.0	.7
140	.9	.7
127	.0	.4
117	1.0	.9
116	6.5	11.5
115	21.9	22.1
114	.8	3.2
91	.0	.4
89	.9	1.4
77	.0	.5
71.5	.9	1.2

the restrictions and safeguards required for using 2-NA in the laboratory. The only 2-NA used was a freshly prepared standard solution for use in the GLC-mass spectral analysis. The isolation of 2-NA from the pooled 24 hour urines of 9 rats injected with 100 mg 2-NN/kg was successful and the data which characterize 2-NA as a urinary metabolite of 2-NN appear in Table 18.

The importance and implications of these findings will be explored in detail in the Discussion Section.

As was mentioned earlier, the mass spectral analyses were run at 24 ev, a condition which tends to maximize the detection of the molecular ion of a compound. In many cases, this could result in a less than complete fragmentation pattern in comparison to analyses run at 70 ev. However, in this particular case it appears that the fragmentation pattern for 2-NA was not compromised. In the Registry of Mass Spectral Data, Volume I, 1974, page 331, the mass spectrum of 2-NA, run at 70 ev, gives essentially the same pattern, except for fragments m/e 72 and 71. The results, obtained by measuring a line representation, were: m/e 144 (relative abundance 14.0); 143 (100.0); 142 (4.0); 141 (1.0); 140 (1.0); 128 (2.8); 127 (3.6); 126 (2.0); 117 (2.0); 116 (13.0); 115 (38.0); 114 (2.0); 89 (4.0); 88 (1.0); 87 (1.0); 72 (10.0); and 71 (5.0). Mass spectral data for 1-NA were not found in the literature.

TABLE 18

GLC-Mass Spectral
Identification of 2-NA as a Urinary Metabolite of
2-NN in the Rat

Sample	GLC Conditions for Mass Spectral Analysis	Retention Time
Isolated Metabolite	3% ov17, 180°x5 min. then, 8°/min to 250°	3.1 min.
Standard 2-NA	SAME	3.1 min.

Mass Spectral Quantitative Report
(calculated)
Conditions: 24 ev, 50 mv

m/e	Relative Abundance Isolated Metabolite	Relative Abundance Standard 2-NA
144	19.1	14.6
143	100.0	100.0
142	20.2	12.4
127	3.2	2.2
126	3.2	3.4
117	5.3	5.6
116	24.5	19.1
115	40.4	25.8
114	4.3	5.6

V. Discussion

A. Acute Toxicity of the Nitronaphthalenes

Treon and Cleaveland (1960) studied the toxicity of 2-NN after various routes of administration including oral, application to the skin and respiratory exposure to sublimed vapor and dust. The oral LD_{50} values (2-NN suspension in aqueous Methocel) were 2.65 g/kg for rabbits and 4.4 g/kg for rats. Fatally poisoned animals of both species exhibited severe and diffuse degeneration and necrosis of the hepatic cells, involving primarily the centrilobular region. Severe degeneration and necrosis also occurred in the distal and proximal segments of the convoluted tubules of the kidneys. There were no significant alterations of other viscera except for slight degeneration of neurons in the brain. Additionally, oral administration of 2-NN to rabbits (above the LD_{50} value) resulted in the formation of methemoglobin (15 to 20 per cent of hemoglobin) in 2 out of 4 rabbits. Other findings included large numbers of Heinz bodies in erythrocytes and an increase in clotting time in the peripheral blood.

Interesting comparisons can be made from the results of the present investigation with 1-NN and those of Treon and Cleaveland (1960) with 2-NN.

Although the routes of administration were different in the two studies, the results indicate that both 1- and 2-NN have the ability to induce centrilobular liver necrosis. Findings from this study tend to indicate that 1-NN is the more toxic of the two naphthalene derivatives on an acute basis; 100 mg 1-NN/kg induced liver necrosis and death in a majority of rats tested while 100 mg 2-NN/kg, with or without phenobarbital pretreatment, failed to induce liver necrosis or death. Kidney toxicity was not seen in this study. The total amount of 2-NN given by Treon and Cleaveland (1960) to an individual animal was greater, thus possibly accounting for the difference in their results.

One striking difference in the acute toxicities of 1- and 2-NN is the dose related respiratory distress induced by 1-NN. This response is apparently not elicited by 2-NN exposure and is another example of differences in the biological response to chemicals due to seemingly slight variations of chemical structure.

B. Bioactivation of 1-Nitronaphthalene

Although most foreign compounds are metabolized *in vivo* to less toxic polar derivatives, certain compounds are converted to chemically reactive intermediates capable of producing biochemical or structural damage to animal tissues (Reid, 1973). Overwhelming evidence indicates that many chemically unreactive chemicals induce

cancer after 'bioactivation' to electrophilic reactants capable of binding to nucleophilic centers. These targets include certain protein bound amino acids and bases of nucleic acids (Miller and Miller, 1969). Brodie and associates, when at the Laboratory of Chemical Pharmacology in the National Heart and Lung Institute, proposed that not only cancer, but other types of chemically-induced diseases including liver, bone-marrow, kidney or adrenal gland necrosis, red blood cell hemolysis, methemoglobinemia and porphyria are also mediated by activated derivatives formed *in vivo* (Brodie, 1973).

The evidence accumulated here on the effect of phenobarbital and SKF-525A pretreatment on 1-NN toxicity supports the notion that hepatic microsomal metabolism of 1-NN involves an activating step with respect to liver damage and potential bladder carcinogenicity. Similar results have also been shown with various aromatic hydrocarbons, especially halogenated ones of which bromobenzene is the classic example (Brodie, *et al.* 1971). The important similarity between 1-NN and the halogenated hydrocarbons, which include chloro, bromo, iodobenzene and the chloronaphthalenes, is their ability to induce centrilobular liver necrosis (Brodie, 1973). Several studies with bromobenzene have indicated that the liver necrosis is due to a metabolite and not to bromobenzene itself. The evidence supporting this view was summarized by Brodie (1973) and is

similar to the results of this study with 1-NN. Pretreatment of rats with phenobarbital, a compound that stimulates the activity of the cytochrome P-450 drug metabolizing enzymes, also stimulated the disappearance of bromobenzene from the body, the covalent binding of radiolabeled bromobenzene in liver and the severity of liver necrosis. On the other hand, pretreatment of rats with SKF-525A or piperonyl butoxide, compounds that decrease the activity of drug metabolizing enzymes, also decreased the disappearance of bromobenzene, the covalent binding of radiolabeled bromobenzene and the severity of the liver necrosis.

Since the results with inhibitors and stimulators of microsomal metabolism of bromobenzene parallel this work with 1-NN, it is attractive to hypothesize that the mechanisms might be similar. Brodie and co-workers (1971) have indicated that the activation step in bromobenzene metabolism involves the formation of bromobenzene epoxide with subsequent formation of a glutathione conjugate. In <u>in vitro</u> microsomal assays, it was found that the amount of this complex is increased with microsomes from rats pretreated with phenobarbital and decreased in the presence of SKF-525A. Additionally, carbon monoxide, which lowers the activity of cytochrome P-450 enzymes, decreased the formation of the bromobenzene-glutathione complex (Brodie, 1973). It is evident that a strong relationship exists

between the bioactivation of bromobenzene and the cytochrome P-450 enzymes.

The bioactivation of 1-NN might be viewed in a similar fashion. Ring hydroxylation more than likely proceeds through an epoxide intermediate. The relative stability of an epoxide in a fused ring series is apparent from the work done with 1,2-epoxy-1,2,3,4-tetrahydronaphthalene (Booth, et al.,1960). Also, compounds with a nitro group frequently form mercapturate metabolites (Mandel, 1971) either from an activated hydrogen (Clayson, 1964) or through an epoxide intermediate (Mandel, 1971). This type of reactivity could result in binding to liver macromolecules particularly with sulfhydryl residues and account for the liver necrosis. The possibility that nitroreductase activity might be linked to cytochrome P-450 has been studied by Gillette and co-workers (1968) and would offer another explanation for the liver necrosis induced by the bioactivation of 1-NN. In their studies, Gillette, et al. (1968) concluded that nitroreduction of p-nitrobenzoate was mediated by cytochrome P-450 and proposed an electron transport system which would explain both the oxidative and reductive function of the microsomal metabolizing system. The fact that nitroreductase is only active under anaerobic conditions might indicate that the hydroxylamine intermediate is sensitive to oxygen resulting in autooxidation. Clayson (1964) has proposed how an N-hydroxy

derivative of 2-naphthylamine might bind to macromolecules via a quinone-imine type intermediate.

The evidence accumulated here indicates that 1-NN is bioactivated to a metabolite, quite possibly with electrophilic properties, capable of binding to liver macromolecules. The activating step may involve cytochrome P-450 and could be linked to either ring hydroxylation or nitroreduction.

C. *In vivo* Nitroreduction of 1- and 2-Nitronaphthalene

The isolation of 1- and 2-naphthylamine as urinary metabolites of 1- and 2-nitronaphthalene is highly significant from several aspects. First, it proves that nitroreduction of the nitronaphthalenes occurs *in vivo* at least in one pathway separate from ring hydroxylation. This means that the identical intermediates of the N-oxidation of the naphthylamines, the N-hydroxy and nitroso derivatives, probably occur *in vivo* (at least in the rat) after exposure to the nitronaphthalenes. If the carcinogenic and mutagenic properties of these intermediates are considered, the implications of these findings are obvious. 1- and 2-Nitronaphthalene must now be considered to be potentially carcinogenic and/or mutagenic. Since no attempts were made to quantitate the excretion of the naphthylamines, it is not known to what extent this complete nitroreduction occurs. Also, it should be pointed

out that these data pertain only to a single dose of the chemical. It is not known what occurs metabolically after long-term exposure. Certainly, since these compounds may induce microsomal hydroxylase activity (See Section IV. D.1.), the possibility exists that long-term exposure might increase the percentage of nitronaphthalenes excreted as ring hydroxylated species or reduce the amount of N-hydroxy or nitroso intermediates occurring. Further, the naphthylamines produced may be largely excreted as conjugates which are then broken down in the urine by hydrolytic enzymes such as β-glucuronidase.

Another interesting implication of these findings is the fact that chemical compounds that appear on the OSHA restricted carcinogen list (listed as α- and β-naphthylamine) are excreted in the urine of animals exposed to chemicals (the nitronaphthalenes) considered relatively safe. Where does this place the nitronaphthalenes with respect to safety and compliance with OSHA regulations? Certainly at this point they cannot be listed as definite carcinogens, but it seems apparent that the nitronaphthalenes and biological samples from animals treated or exposed to the nitronaphthalenes must be handled in a manner consistent with the safety precautions and laboratory practice used for the handling of the naphthylamines. Also, it would seem prudent that laboratory and industrial personnel should minimize their exposure to the nitronaphthalenes in the course of research or manufacturing activities.

D. Carcinogenicity Assay

As mentioned in Section I.C.1.c., three studies on the potential carcinogenicity of 2-NN have appeared in the literature, whereas none have been published on 1-NN. Bryan et al. (1963) implanted various compounds compressed with cholesterol as pellets into mouse bladders and observed tumors in animals surviving at least 25 weeks. Seven out of 87 mice (8 per cent) developed carcinomas with cholesterol alone, whereas 15 out of 52 (29 per cent) ($p=0.002$) developed tumors when N-hydroxy-2-naphthylamine was incorporated into the pellet. The figure with 2-NN was 11 out of 78 (14 per cent) ($p=0.15$) and not considered significant. Bryan, et al. (1964) expanded on the above study and again showed that 2-NN was inactive when implanted into the bladders of mice. This might be expected, however, since neither bladder mucosa nor urine have been reported to have nitroreductase activity. Conzelman, Moulton and Flanders (1970) reported a single case in which a rhesus monkey receiving 2-NN orally for 54 months, developed multiple, irregular, papillary tumors of the urinary bladder transitional epithelium.

The results of this present investigation indicate that the nitronaphthalenes have the potential to induce proliferative changes in the urinary bladder in rats after ip administration. Since 7/40 rats in the two treated groups developed changes ranging from mild focal hyperplasia in five bladders to a pronounced diffuse hyperplasia in one bladder to a single animal with a transitional cell

carcinoma, the significance of these findings must be carefully evaluated. According to several literature sources (See Section IV.E.3.) spontaneously arising bladder tumors in rats are extremely rare. However, there appears to be a correlation between the presence of bladder stones and bladder tumors in chemically treated animals (Clayson, 1974) as shown in several studies with both artificially implanted and naturally occurring calculi. If the correlation actually exists, then chemicals which induce urinary calculus formation need careful evaluation before being accepted as being directly carcinogenic. In this study, rat #DJ-1-NN2 developed a low grade transitional cell carcinoma of the bladder and was the only animal in the 1-NN group which had bladder stones. On the other hand, three rats in the injected control group had bladder calculi but did not develop abnormalities. In the 2-NN group, four rats had bladder stones at autopsy. Two of these had normal bladders, while a third had a mild focal transitional cell hyperplasia. The fourth rat with bladder calculi had a diffuse marked hyperplasia of the bladder mucosa. Two additional rats developed a focal hyperplasia without evidence of bladder stones. While it appears that the nitronaphthalenes do have the potential to induce proliferative lesions in the bladder mucosa, the co-carcinogenicity of bladder stones must be considered, as the two most severely affected animals, rats #DJ-1-NN2 and DJ-2-NN6, both had bladder calculi. However, the number of animals without

abnormalities and with stones and the animals with abnormalities and without stones tends to make the correlation less tenable.

It will be imperative that these compounds be further evaluated under more extensive test procedures that will allow evaluation of the co-carcinogenicity of bladder stones.

Trichosomoides crassicauda, the rat bladder threadworm, has been implicated as a co-carcinogenic factor in several rat bladder carcinogenesis studies (Clayson, 1974). Infection apparently causes a slight inflammation in the bladder wall. Schreiber, et al. (1969) found that infection greatly stimulated the response of urothelial DNA synthesis to irradiation, while Chapman (1969) found that when 2-fluorenylacetamide was fed to rats, there were no bladder tumors in 78 noninfected animals, compared to 5 in 75 rats infected with the parasite. In this present study, it is not known if the animals were infected with T. crassicauda, but this must be considered a possibility since no precautionary measures were taken. Future studies should include the prophylactic treatment of rats with methyridine (Peardon, et al. 1966) or nitrofurantoin (Chapman, 1964) to avoid any complications which may arise.

It would be premature to draw any firm conclusions on the ability of the nitronaphthalenes to induce bladder cancer in rats other than to point out that a potential exists.

Certainly, these results, coupled with the report from Conzelman, et al. (1970) indicate that an intensive evaluation of the carcinogenic potential of the nitronaphthalenes should be undertaken.

VI. Summary

Several toxicological testing procedures were carried out in order to assess the toxic potential of 1- and 2-nitronaphthalene (1- and 2-NN) in Sprague-Dawley rats. Of particular concern was (1) correlating acute target organ toxicity with metabolism to show that an 'activating' step is involved in the metabolism of the nitronaphthalenes, (2) proving that nitroreduction of the nitronaphthalenes occurs *in vivo* indicating that metabolism may lead to the identical intermediates produced in the N-oxidation of the naphthylamines and (3) assessing the carcinogenic potential of the nitronaphthalenes.

Results from preliminary acute toxicity studies indicated that 1-NN induced liver necrosis in the centrilobular region and caused severe respiratory distress manifested by acute dyspnea and focal atelectasis in the lungs. The ip LD_{50} for 1-NN was calculated to be 86.3 mg/kg. Relative lung weights (organ/body weight ratios) increased markedly at 24, 48 and 72 hours after a 100 mg 1-NN/kg injection apparently due to pulmonary congestion. At 72 hours after the 1-NN injection, there was also an increase in the relative liver weight. An equimolar dose of 2-NN (100 mg/kg ip) failed to induce either the respiratory distress or liver necrosis.

The ED_{50} for 1-NN induced respiratory distress was found to be 59.8 mg/kg ip. A comparison of probit analyses (respiratory distress vs. mortality) indicated that this acute toxic response may be a factor but not the major cause of acute mortality in 1-NN treated rats.

Pretreatment of animals with phenobarbital, a stimulator of microsomal metabolism (1 mg/ml substituted for drinking water for 4 days), completely abolished the respiratory distress induced by 1-NN, whereas pretreatment with SKF-525A, an inhibitor of microsomal metabolism, did not decrease the respiratory distress. Respiratory distress of at least 24 hours duration was observed in 17/23 (73.9%) rats given 100 mg 1-NN/kg, in 0/16 (0.0%) rats given phenobarbital + 100 mg 1-NN/kg, and in 7/9 (77.7%) rats given SKF-525A + 100 mg 1-NN/kg. These data tend to indicate that 1-NN induced respiratory distress is probably due to the parent compound rather than a metabolite.

Pretreatment of animals with phenobarbital substantially increased the severity of centrilobular liver necrosis induced by 1-NN, while pretreatment with SKF-525A protected the rats from 1-NN induced liver necrosis. This indicates that the hepatotoxicity is caused by a metabolite of 1-NN. This theory of metabolism involving an 'activating' step which leads to tissue damage is similar to studies with other hepatotoxic agents including bromobenzene.

An analysis of 1-NN mortality data from phenobarbital pretreated rats indicates that a different mechanism of lethal toxicity may be involved in induced animals than in rats treated with 1-NN alone.

Contrary to results from other studies with monocyclic aromatic nitro compounds and amines, 1-NN, 2-NN or 1-naphthylamine did not elicit a methemoglobinemia significantly higher than controls in any group tested.

In a preliminary assessment of hepatic microsomal enzyme activity, 1-NN treatment (25 mg/kg ip for 3 days) was found to increase the activity of hydroxylase enzymes. This may indicate that 1-NN has the ability to stimulate its own metabolism.

The carcinogenic potential of 1- and 2-NN was studied in a 6 month protocol - 3 months of twice weekly injections followed by a 3 month latency period. One treatment group (20 rats) received 25 mg 1-NN/kg ip twice weekly while another 20 rats received 2-NN in the identical dosage schedule. One control group (20 rats) was given vehicle injections twice weekly for 3 months, and a second control group was not injected. One rat in the 1-NN group developed a low grade, noninvasive transitional cell carcinoma of the urinary bladder mucosa. Three other rats developed focal hyperplasia of the bladder mucosa. One rat in the 2-NN group developed a diffuse marked hyperplasia of the bladder mucosa, while two other rats developed focal hyperplasia.

There were no abnormalities noted in any of the control bladders. The possible co-carcinogenicity of bladder calculi was taken into account. Other tumor bearing animals included 3 in the non-injected control group; 2 nephroblastomas and 1 fibrosarcoma. The 1-NN group had 1 sclerosing hemangioma while the 2-NN group had no evidence of tumors. The vehicle injected control group was devoid of any tumors. Additionally, the treated groups both developed unusual peritoneal and/or mesenteric cysts which were not seen in the controls. The occurance of these structures was related to the presence of foreign chemical in the peritoneal cavity.

Long-term 1-NN treatment (25 mg/kg ip twice weekly for 12 weeks) apparently had no effect on male fertility as 9/10 (1-NN) vs. 10/10 (control) rats mated successfully with untreated females one month after the last injection. There were no significant findings when standard dominant lethal mutagenesis parameters were measured.

In a preliminary assessment of teratogenic potential, 1-NN at a dosage of 50 mg/kg sc on days 6, 8, 10, 12 and 14 of pregnancy did not cause a statistically significant increase in embryotoxic signs. However, two fetuses in the treated group were found to have an extra 14th rib indicating that 1-NN may have teratogenic potential at a higher dosage level.

The amine analogs of 1- and 2-NN, 1- and 2-naphthylamine, were isolated as urinary metabolites of the parent nitro compounds in male Sprague-Dawley rats. Urine samples were collected on a freezing unit for 24 hours after a 100 mg/kg ip injection of 1- or 2-NN, and pooled specimens were extracted with ether. The naphthylamines were isolated by preparative thin-layer chromatography (TLC). Structure identification for 1-NA was accomplished using TLC and gas liquid chromatography - mass spectral analysis (GLC-MS), whereas 2-NA was identified as a metabolite with GLC-MS alone. The isolated metabolites were identical to pure standards.

This investigation indicates that 1-NN is bioactivated to a reactive metabolite, probably electrophilic, which is capable of inducing centrilobular necrosis. 1-NN induced respiratory distress is prevented when metabolism of 1-NN is stimulated. In addition, 1- and 2-NN have been shown to be potentially carcinogenic to the bladder mucosa of rats, although further work is required to assess the actual biological hazard. Isolation of the amine analogs of 1- and 2-NN as urinary metabolites suggests the possibility that the same intermediates are formed during the nitroreduction of the nitronaphthalenes as in the oxidation of the corresponding naphthylamines. These metabolic

findings also suggest that the nitronaphthalenes and biological samples from animals treated with nitronaphthalenes should be handled in a manner consistent with laboratory practice for handling potentially dangerous compounds.

APPENDIX A

Carcinogenicity Studies with 2-Naphthylamine[a]

Species	No. of Studies	Total No. of Animals	No. of Studies showing tumors in treated animals	Total No. of animals with tumors	Overall Tumor incidence (per cent)
Dog	17	208	12	101	48.55
Rat	11	367	7	54	14.71
Mouse	8	830	7	186	22.40
Rabbit	13	288	8	63	21.87
Guinea Pig	1	63	1	13	20.63
Hamster	2	68	0	0	0.00
Monkey	1	24	1	12	50.00

[a] See statement at the end of Appendix B

APPENDIX B

Carcinogenicity Studies with 1-Naphthylamine[a]

Species	No. of Studies	Total No. of Animals	No. of Studies showing tumors in treated animals	Total No. of animals with tumors	Overall tumor incidence (per cent)
Dog	1	5	0	0	0.00
Rat	3	27	2	2	7.40
Mouse	4	251	3	32	12.74
Rabbit	3	29	0	0	0.00
Hamster	1	120	0	0	0.00

[a] The information for appendices A and B was complied from 5 volumes of Public Health Service Publication No. 149, Survey of Compounds Which Have Been Tested for Carcinogenic Activity, and represents all published studies through 1960 and from 1968 through 1972. The volume for the years 1961-1967, in which 21 studies on 2-NA and 3 on 1-NA appeared, is not currently available from the National Library of Medicine. The studies listed refer only to those which included the number of animals used, the number of animals with tumors and the tumor type. This resulted in the omission of a few of the earlier studies.

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