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Title: Carcinogenicity and Acute Toxicity of Dimethylnitrosamine in Rainbow Trout (Salmo gairdneri)

Approved by: _____
Dr. Richard A. Scanlan

A dose related carcinogenic response was established for dimethylnitrosamine administered in the diet of rainbow trout (Salmo gairdneri). An equation was derived for the relationship between dose and hepatocellular carcinoma incidence. From a published dose response study using Porton rats as a test animal, a second equation was derived for comparison. The rat and the trout were approximately equivalent in their sensitivity to dimethylnitrosamine carcinogenesis. The histological nature of the carcinogenic response in trout was similar to to that of mammalian species. Apart from carcinogenesis, no indications of chronic toxicity were observed after a one year feeding experiment.

The median lethal dose after intraperitoneal injection of dimethylnitrosamine was 1,770 mg/kg body weight in rainbow trout. Relative to the range of 15 to 50 mg/kg body weight reported for several mammalian species, trout were resistant to the acutely toxic effects of dimethylnitrosamine.

# CARCINOGENICITY AND ACUTE TOXICITY OF DIMETHYLNITROSAMINE IN RAINBOW TROUT (*Salmo gairdneri*)

by

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# CARCINOGENICITY AND ACUTE TOXICITY OF DIMETHYLNITROSAMINE IN RAINBOW TROUT (SALMO GAIRDNERI)

## INTRODUCTION

Environmental factors in the origin of cancer have commanded increasing attention in recent years. Among the chemical carcinogens, the N-nitroso compounds were recognized as a possible hazard to humans. Well established as important carcinogens in test animals, these compounds have also demonstrated a potential for ubiquitous contamination of the environment, hence the possible significance in human carcinogenesis.

One of the most powerful toxins and carcinogens in the class of N-nitroso compounds, dimethylnitrosamine (DMN), has been extensively studied in experimental animal systems. DMN has been detected as an inadvertent contaminant of food and air, and used in various industrial applications.

Comparison of dose response studies for different species has been suggested as an aid in the estimation of human sensitivity to carcinogens. There have been few dose response studies for N-nitroso compounds, and the existing data often lacks the uniformity in experimental design needed for valid comparisons.

The purpose of this study was to quantitatively evaluate the toxicity of DMN in rainbow trout (Salmo gairdneri). The carcinogenicity of DMN in trout has been reported at relatively high dietary dose levels. The apparent need for high levels of DMN was attributed to the low capacity of trout liver to metabolize DMN. An experiment was initiated to investigate existing reports and to establish a dose

response curve for DMN carcinogenesis in trout. This data permitted comparison with the response of the rat to DMN carcinogenesis.

DMN has been noted for the acutely toxic reaction it produces in many species including man. As a second parameter of DMN toxicity in trout, the ten day median lethal dose was determined.

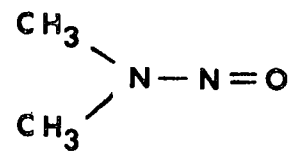
# LITERATURE REVIEW

## N-nitroso Compounds

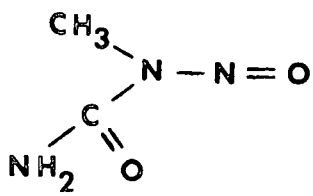
Carcinogenic N-nitroso compounds include dialkyl, alkyl aryl and cyclic N-nitrosamines as well as the acyl group containing N-nitrosoamides. Examples of these compounds are presented in Figure 1. Due to their greater chemical stability the N-nitrosamines are generally considered as a greater environmental hazard than the N-nitrosamides (Magee et al., 1972; Barnes, 1974). N-nitroso compounds encompass a wide structural array with the N-nitroso group as the only common feature. This variety poses an interesting problem for study in carcinogenesis. It also provides the basis for widespread environmental occurrence through the relatively mild nitrosation reaction between nitrite and many naturally occurring amines.

## General Significance

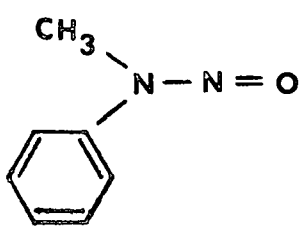
Although the carcinogenicity of N-nitroso compounds was established as early as 1956 by Magee and Barnes, the unintentional presence of N-nitroso compounds in the environment was not suspected until several years later. In 1960 there was an outbreak of liver disease in Norwegian sheep. These sheep had been fed nitrite preserved fish meal and the formation of N-nitrosamines in this fish meal was suggested as a possible explanation for this outbreak. Later, Ender et al. (1964) demonstrated the presence of DMN in fish meal at levels as high as 100 ppm. Concurrently, a similar situation was observed in mink fed nitrite preserved fish meal (Sebranek and Cassens, 1973; Koppang and



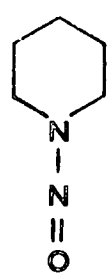
Dimethylnitrosamine



N-nitrosomethylurea



N-nitrosomethylaniline



N-nitrosopiperidine

Figure 1. Structure of N-nitroso compounds.

Rimeslatten, 1975).

After the fish meal incident in Norway, many foods were examined for the presence of N-nitrosamines. There have been a variety of confirmed reports of their occurrence in foods in the ppb range. Scanlan (1975) has reviewed this area. Although the demonstrated occurrence of N-nitroso compounds has not been at high levels, other evidence has produced concern as to their role in human carcinogenesis. Fine et al. (1976) have demonstrated ppb levels of DMN in the air of different urban areas. It has been suggested that the statistical correlation between the high levels of nitrogen dioxide and cancer incidence in many urban areas is due to N-nitrosamine formation (Shapley, 1976).

The nitrosation reaction is a common one which occurs under relatively mild conditions. Given the proper conditions, N-nitrosamines can be formed from amines which are found in foods, drugs and pesticides (Lijinsky, 1970; Mirvish, 1975; Scanlan, 1975).

The formation of N-nitroso compounds under conditions existing in the stomach has been demonstrated both in vivo and in vitro and in many species including man. Nitrite persisted long enough in the rat stomach for nitrosation of amines to occur (Mirvish et al., 1975). Tannenbaum (1972) reported the presence of nitrite in human saliva in quantities sufficient to produce N-nitroso compounds in the stomach. Hawksworth and Hill (1971) have demonstrated the formation of N-nitroso compounds by human intestinal bacteria. Lijinsky (1974) demonstrated the formation of N-nitroso compounds from the reaction of nitrous acid with several tertiary amine containing drugs.

The lack of adequate methods of analysis of N-nitroso compounds has been an obstacle to defining the carcinogenic risk from N-nitroso

compounds. A major concern in this area has been the possibility for occurrence of nonvolatile N-nitroso compounds which most current methods of analysis will not detect. This problem has been extensively reviewed by Foreman and Goodhead (1975).

Due to the nature of the nitrosation reaction, the occurrence of precursors and difficulties in analysis, N-nitroso compounds have produced widespread concern as to their potential role in human carcinogenesis.

## Toxicity and Carcinogenicity

N-nitroso compounds have been shown to be toxic, mutagenic and teratogenic as well as carcinogenic. The structure and chemical stability of each compound were crucial in determining the type and mode of action. Dialkyl and cyclic N-nitrosamines were not toxic or carcinogenic *per se*, but were first altered by metabolism to active metabolites. Unlike the N-nitrosamides, the N-nitrosamines did not produce a toxic reaction at the site of application, but had to be transported to a specific site of action. (Magee and Barnes, 1967; Magee, 1971; Montesano and Magee, 1974; Shank, 1975; Swann, 1975)

In acute toxicity testing, oral median lethal doses of N-nitrosamines for the rat ranged from a low of 18 mg/kg body weight for nitrosobenzylmethylamine to over 7,500 mg/kg body weight for nitrosoethyl-2-hydroxyethylamine (Magee and Barnes, 1967). Druckrey *et al.* (1967) reported a range from 4 mg/kg body weight for N-methyl-N-nitrosourethane to 1,200 mg/kg body weight for butylnitrosourea in the rat. In Druckrey's study, acute toxicity of the nitrosamines was expressed as liver necrosis and lung edema, whereas the nitrosamides

damaged the bone marrow and lymphatic tissue with some damage at the point of application.

Although the relationship between structure and toxicity has not been well defined, acute toxicity seemed to decrease with the increase in chain length of dialkylnitrosamines. Relative to other N-nitroso compounds, cyclic N-nitrosamines were generally more acutely toxic and N-nitrosamides had more moderate median lethal doses (Shank, 1975).

Mutagenicity was an outstanding characteristic of many of the N-nitroso compounds, especially the N-nitrosamides, and has been reviewed by Magee and Barnes (1967). The N-nitrosamide, N-methyl-N'-nitro-N-nitrosoguanidine is among the most powerful of chemical mutagens routinely used to induce mutations in bacteria (Magee et al., 1973). Some of the dialkyl N-nitrosamines were found to be mutagenic to Drosophilia melanogaster, but none of the dialkyl N-nitrosamines were mutagenic when tested in yeast, bacteria and Neurospora (Ong and Malling, 1975). The N-nitrosamines required metabolic activation by mammalian enzyme systems before they expressed mutagenicity. This has been established repeatedly in various host-mediated and microsomal-activated bacterial assays (Glettan et al., 1975; Czygan et al., 1973; Weekes, 1975; Baldwin et al., 1976; Braun and Schoneich, 1975).

Napalkov and Alexandrov (1968) were unable to demonstate a teratogenic effect after testing dialkyl N-nitrosamines in the rat. The teratogenic effects of N-nitrosamides have been reviewed (Magee and Barnes, 1967). Druckrey et al. (1969) did not find a close relationship between teratogenicity and carcinogenicity. In contrast to trans-placental carcinogenicity, they found that teratogenicity was absolutely dependent upon the total dosage administered at a specific

stage of pregnancy. As might be expected from their mutagenic action, N-nitrosamides were also the primary teratogens observed after testing N-nitroso compounds (Magee, 1973).

The primary toxic reaction to N-nitroso compounds has been carcinogenesis. Of the 100 plus N-nitroso compounds tested to date, more than 80 of them were carcinogenic to experimental animals. However, exposure to N-nitroso compounds has never been linked to specific instances of cancer in humans (Preussmann, 1973; Heidelberger, 1975; Montesano and Magee, 1974; Barnes, 1974). N-nitroso compounds produced an assortment of carcinogenic responses in a wide range of species. Single doses have proven carcinogenic (Magee and Barnes, 1959; Druckrey et al., 1964a), and dietary levels of approximately 1 ppm have induced tumors in animals (Druckrey et al., 1963).

Among the species sensitive to the carcinogenicity of N-nitroso compounds are the rat, mouse, Syrian golden hamster, Chinese hamster, European hamster, rabbit, guinea-pig, mastomys, dog, pig, mink, monkey, grass parakeet, rainbow trout, aquarium fish, Brachydanio rerio, Lebistes reticulatus, and Oryzias latipes, and newt, Triturus helveticus (Montesano and Magee, 1974; Khudolei, 1971; Ishikawa et al., 1975; Stanton, 1965). Diethylnitrosamine has proven to be carcinogenic in all of the eleven animal species tested. After treatment with uniformly low dosages of diethylnitrosamine, all of the species tested reacted in a similar manner and produced liver carcinomas. (Schmahl and Osswald, 1967).

Transplacental carcinogenesis has been demonstrated with both N-nitrosamines and N-nitrosamides (Shank, 1975). Low doses of

ethylnitrosourea produced malignant tumors of the nervous system in all offspring of rats treated on the fifteenth day of prengancy (Druckrey et al.,1969). Esophogeal tumors were produced in half of the descendants of Syrian golden hamster treated with diethylnitrosamine during the second week of pregnancy (Mohr et al., 1966).

A carcinogenic response has been elicited by simultaneously feeding the precursors of N-nitroso compounds to test animals. In most studies an amine was fed in the diet, and nitrite was administered in the daily drinking water. Greenblatt and Mirvish (1972) found a linear dose response after tabulating the incidence of lung tumors in mice given concurrent oral doses of piperazine and sodium nitrite. The dose response correlated well with the kinetics of piperizine nitrosation. Malignant tumors of the lymphatic system were induced in swiss mice after the in vivo formation of N-nitroso compounds from sodium nitrite and the pesticide, methyl-2-benzimidazole (Borzsonyi and Csik,1975).

The significance of N-nitroso compounds as chemical carcinogens was established in part by studies such as that of Druckrey et al. (1969). They treated BD rats over a lifespan with approximately 65 N-nitroso compounds. Their findings confirmed the potent carcinogenicity of N-nitroso compounds and contrasted the organ specificity of the systematically active N-nitrosamines to the direct activity of the N-nitrosamides. Druckrey et al. (1967) also provided the background necessary to use the N-nitroso compounds as a unique system for studying the mechanism of carcinogenesis. Barnes (1974) has suggested that N-nitroso compounds may be more important as a model system for studying carcinogenesis than as actual environmental carcinogens.

## Metabolism

Since N-nitrosamines produced an indirect carcinogenic activity only in specific organs, it has been theorized that only certain organs can metabolize the N-nitrosoamines from an inactive, stable precursor to the active carcinogen. Chemically unstable at physiological pH, the N-nitrosamides were rapidly hydrolyzed to active carcinogens in the tissue at the point of administration. The more stable N-nitrosamines required enzymatic intervention before forming active carcinogens which may be identical to those formed spontaneously from the N-nitrosamide decomposition. Factors which affect this specific metabolic process ultimately produced changes in the carcinogenic process. Therefore, the metabolism of N-nitrosamines has received considerable study which has been reviewed by several workers in the field (Heidleberger, 1975; Preussmann, 1973; Montesano and Magee, 1974; Swann, 1975).

Most of the metabolism studies to date were designed to either support or refute the alkylation theory (Druckrey et al., 1961) as the mode of action in N-nitroso compound carcinogenesis. The essence of this concept was that a mutation or other slight change in the genetic material of a cell was probably responsible for the transformation of a normal cell into a malignant cell. Such changes were explained by the alkylation of nucleotides, intact DNA, RNA, associated proteins, or other vital cellular constituants to produce either a toxic or a carcinogenic effect (Shank, 1975; Magee and Barnes, 1967). The mutagenicity of N-nitrosamides was explained by their alkylating capacity, and the enzymatic conversion of N-nitrosamines to similar

compounds has been suggested to explain their carcinogenic activity.

The early work which provided the basis for the alkylation theory was reviewed at length by Magee and Barnes (1967). More recent work used [$^{14}C$] dialkyl N-nitrosamines to measure both the $^{14}CO_2$ production and the occurrence of label in cellular constituants. Results were consistent with the alkylation theory (Montesano and Magee, 1974). A series of experiments with metabolites predicted from β or ω oxidation of dibutyl and dipropyl N-nitrosamines has also provided evidence for the alkylation theory of carcinogenesis (Druckrey et al., 1964b; Kruger, 1973; Kruger and Bertram, 1973; Althoff et al., 1973; Pour et al., 1974; Blattmann and Preussmann, 1975; Pour et al., 1975, Reznik et al., 1975).

This and other research with the dialkyl N-nitrosamines indicated that hydroxylation at either the α, β, or ω position of one or both alkyl groups initiated the breakdown of the compound. Metabolic action was generally attributed to the cytochrome P-450 microsomal mixed function oxidase system (McLean and Day, 1974). In the case of DMN, approximately one mole of formaldehyde was produced for each mole of DMN which was metabolized (Brouwers and Emmelott, 1960; Frantz and Malling, 1975; Arcos et al., 1976). Further oxidation of the hydroxyl group led to a shortened chain length in the larger alkyl groups (Heidleberger, 1975). Eventually, the transfer of an alkyl carbonium ion to a nucleophilic receptor site, such as RNA or DNA, occurred within the cell. This was thought to initiate the toxic and carcinogenic reactions (Shank, 1975; Lijinsky et al., 1968; Lijinsky et al., 1972; Czygan et al., 1973).

Baldwin et al. (1976) found that an α-acetoxy derivative of N-nitrosopyrrolidine did not require microsomal activation prior to producing bacterial mutations. A cyclic N-nitrosamine, N-nitrosopyrrolidine, did need activation by mammalian microsomes in order to mutate the same bacteria. The authors concluded that a transportable α-hydroxylated form may be involved in transformation of cyclic N-nitrosamines into active carcinogens.

Lijinsky et al. (1973) studied alkylation of nucleic acids, expressed as 7-methyl-guanine, after subjecting rats to five cyclic N-nitrosamines. Only one of the five N-nitrosamines produced alkylation of guanine. In the one case where alkylation did occur, there was no relationship between the site of tumor induction and the degree of in vivo alkylation. Although Lijinsky et al. (1973) speculated that some type of chemical interaction occurred between the cyclic N-nitrosamines and RNA, DNA and protein, they suggested that alkylation, as observed with dialkyl N-nitrosamines, was not important in the conversion of cyclic N-nitrosamines into carcinogens. However, their results did not preclude the possibility of important metabolites being produced from hydroxylation by the microsomal mixed function oxidase system. Lijinsky and Taylor (1976) fed rats a group of N-nitrosopiperdine derivatives which had been substituted with methyl groups in different positions on the ring. When four methyl groups were placed alpha to the N-nitroso group, carcinogenic activity was eliminated. The presence of as few as one methyl group adjacent to the N-nitroso group greatly reduced carcinogenicity, but no other

positions on the ring had a significant effect on activity. The key role of the alpha carbon could be related to the discovery of Baldwin et al (1976) that a transportable α-hydroxylated form may be important in carcinogenicity by cyclic N-nitrosamines.

An alternate mode of action which could explain the carcinogenicity of cyclic N-nitrosamines was outlined by Schoental (1973). This theory called for oxidation of N-nitrosamines to their respective aldehydes or acids which would be relatively reactive compounds within the cell. This modified but still intact N-nitrosamine could then form crosslinks or bridges between DNA, RNA or proteins causing a disruption of the normal functioning of the cell and subsequently a carcinogenic response.

Unfortunately, nearly all of the research on alkylation of nucleic acids by N-nitrosamines, including that of Lijinsky's outlined earlier, has used 7-methyl-guanine as the sole indicator of alkylation. Lijinsky et al. (1969) found a discrepancy between production of 7-methyl-guanine and tumor target organs after administration of tritium labeled alkylaryl N-nitrosamines. However, had other alkylation indicators been used for this study, such a discrepancy may not have been observed. After studying DNA repair systems in tumor tissue from mice, Ganatt et al. (1975) concluded that aberrant nucleic acid alkylation need not be hyperalkylation. The patterns established by the position and nature of the alkylating group were considered as important as the degree of alkylation. This was due to the specificity and relative rates of the DNA repair systems.

As noted by Shank (1975), Swann (1975), and others, alkylation of the $O^6$ position of guanine or of the 3 position of cytosine may be more important than the 7 position of guanine in the disruption of DNA base pairing and ultimately in carcinogenesis by N-nitroso compounds.

In an explanation for an apparent discrepancy with the alkylation theory, strongly alkylating, but non-carcinogenic, agents such as methyl iodide were proposed to attack nucleophilic sites through an $S_N2$ mechanism in contrast to the N-nitroso compounds which would react through an $S_N1$ mechanism. The different chemical mechanisms were thought to be responsible for the selection of a sensitive verus a non-sensitive site and ultimately for carcinogenic activity (Swann, 1975). Consequently, most reviewers of the metabolism of N-nitroso compounds concur that the observations reported in the literature to date support the alkylation theory of N-nitroso carcinogenicity (Swann, 1975; Shank, 1975; Montesano and Magee, 1974; Heidleberger, 1975; Barnes, 1974).

# Dimethylnitrosamine

## Chemical Characteristics

DMN is a non-viscous yellow liquid with a molecular weight of 74 and the structure indicated in Figure 1. This compound is extremely volatile, infinitely soluble in water and soluble in most lipids and organic solvents. Chemically, DMN is relatively stable, but it is sensitive to decomposition by ultraviolet light (IARC Monograph, 1972).

## Use and Occurrence

There are several patents available for the use of DMN as a solvent in plastics and chemical industries, but the demonstrated toxicity in humans exposed to DMN on an industrial scale has limited its use in these applications (Barnes and Magee, 1954). Specifically, it has been used as an antioxidant, an ingredient in certain lubricants, and to alter dieletric constants in condensers. An early report of toxicity in humans grew out of its use in the automobile industry as a paint component (Shank, 1975; IARC, 1972). Until April 1976, DMN was the sole source of 1,1-dimethylhydrazine which was manufactured on a large scale in the U.S. as rocket fuel (Shapley, 1976).

The toxicity of DMN to humans has been noted in several studies. In 1937, Freund observed that acute necrosis of the liver occurred after short term exposure and liver cirrhosis appeared in two chemists exposed for a period of two years. This initial observation was followed by those mentioned in the previous paragraph. Liver injury was indicated in all instances. Barnes and Magee (1954) calculated that 250 mg of DMN

per cubic meter of atmosphere would present a dangerous level for humans. A safe level was thought to be less than 25 mg per cubic meter. Their study of acute toxicity of DMN was prompted by two cases of liver cirrhosis in men working with the compound in an industrial research laboratory. This in turn led to the discovery of the carcinogenicity of DMN and subsequently to the study of the entire spectrum of N-nitroso compounds (Barnes, 1974).

The occurrence of DMN in foods at ppb levels has been confirmed by mass spectrometry in numerous studies. The presence of DMN has been confirmed in raw, smoked and cured salmon, sable and shad at 4 to 26 ppb. It was found at 1 to 4 ppb in fried and raw bacon, cheese, salami, luncheon meat and chopped pork. In addition, DMN was detected at 120 to 450 ppb in fish meal, 11 to 84 ppb in frankfurters, 5 ppb in ham and from 1 to 35 ppb in numerous other cured meat products (Scanlan, 1975). In general, the reports of the occurrence of DMN in foods have been sporatic and in relatively low amounts (Mirvish, 1975; Scanlan, 1975). Reports of the occurrence of DMN in cigarette smoke and urban air have been made (Hoffmann et al., 1973; Fine et al., 1976). The in vivo production of dimethylnitrosamine from sodium nitrite and the drug, aminopyrine has been documented (Lijinsky and Greenblatt, 1972). Low amounts of dimethylnitrosamine can be formed in vivo from dimethylamine and sodium nitrite (Mirvich, 1976).

## Carcinogenicity

Dimethylnitrosamine evoked a carcinogenic response in ten different species of test animals. Tumors were produced in one or more

of these species by different routes of administration including feeding in the diet or drinking water, stomach cannulation, inhalation, intraperitoneal, subcutaneous and intramuscular injections, the transplacental route and large single doses, usually in the form of an injection. The primary target organs were the liver, kidney, and lungs. To date, no epidemilogical evidence indicates that DMN has produced cancer in humans. The major observations on the carcinogenicity of DMN are presented by species.

Rat

Long term oral administration of DMN in low concentrations to the rat resulted in the development of malignant liver tumors (Magee and Barnes, 1956; Schmahl and Preussmann, 1959; Terracini and Magee, 1964; Terracini et al., 1967). Single doses or several large doses over a short time span produced renal tumors at levels as low as 20 mg/kg body weight (Magee and Barnes, 1959; Zak et al., 1960; Magee and Barnes, 1962; Hoch-Ligeti et al., 1968; Ripopelle and Jasmin, 1969). In one case oral doses totaling 10 to 20 mg per rat induced tumors (Shuemberger, 1966). A greater incidence of renal tumors was also observed when DMN was fed in a protein-deficient diet (McLean and Magee, 1970). Pretreatment with aminoacetonitrile inhibited DMN carcinogenesis in rat livers (Hadjiolov, 1971).

Tumors of the lung were induced by feeding DMN in two different studies (Zak et al., 1960; Argus and Hoch-Ligeti, 1961). One dose response study related the frequency of liver tumors to the level of DMN in the diet (Terracini et al., 1967). Renal tumors were produced

by a single intraperitoneal injection of DMN at 18 mg/kg body weight (Murphy et al.,1966). Single or repeated exposure to DMN through inhalation at 4 mg/kg body weight induced tumors of the kidney and nasal cavities (Druckrey et al., 1967).

DMN has also been implicated as a transplacental carcinogen in the rat. Alexandrov (1968) found that treating rats in the last week of pregnancy resulted in a low frequency of renal tumors in the offspring. Lung and liver tumors were induced after administration of 125 ug of DMN to 24 hour and one week old rats (Terracini et al., 1969). In contrast, liver tumors have never been induced in intact adult rats following large doses of DMN (Magee and Barnes, 1967).

Mouse

Doses as low as 1 mg/kg body weight were carcinogenic in newborn BALB/c mice after subcutaneous injection of DMN (Toth et al 1964). DMN induced tumors of the liver, lung, kidney and testes, and sporatic incidence of leukemia when fed in the diet of ddN, ICR and C3H/Ahe mice. At high doses, ICR mice developed lung tumors exclusively (Takayama and Oota, 1963; 1965). Other studies have also established the strain and organ specific carcinogenic effects of feeding DMN to mice (IARC Monograph,1972).

At 50 ppm in the drinking water, DMN produced tumors of the kidney and lung (Terracini et al,1966). Long-term administration of DMN in drinking water which corresponded to a total dose of 89 mg/kg body weight was the lowest carcinogenic dose reported for oral administration of DMN (Clapp and Toya, 1970).

Otsuka and Kuwahara (1971) gave weekly subcutaneous injections ranging from 0.15 to 3.75 mg/mouse which produced tumors of the liver, lung and other organs. Single intraperitoneal doses of 7 or 14 mg/kg body weight resulted in lung tumors in mice of the GR and CFW/D strains (Den Engelse et al,1970; and Frei,1970). Through transplacental dosage, liver and lung tumors were induced in the offspring of mice injected with DMN at 12.5 to 75 mg/kg body weight during the last days of pregnancy (Smetanin,1971).

DMN precursors, dimethylamine and sodium nitrite, failed to elicit a carcinogenic response when fed concurrently to mice (Greenblatt et al.,1971). The authors theorized that the low nitrosation rate of dimethylamine prevented the formation of DMN in the stomachs of mice.

Two dose response studies have been reported for DMN carcinogenesis in mice. Vesselinovitch (1969) gave young, (C57BLXC3H)F1 mice intraperitoneal injections six times at three day intervals. Dose levels tested were 1, 2 and 4 mg/kg body weight. After one year, a positive logrithmic relationship was obtained between dose and tumor incidence with the males showing a significantly greater tendency to develop liver tumors than did the females. Vesselinovitch suggested that sex hormones could have affected DMN metabolism in a manner similar to that observed with urethan, and therefore were responsible for the observed sex difference in tumor susceptability.

In the second dose response study, tumor incidence was found to vary with both the dose rate and the quantity of DMN used in long-term oral administration. The data suggested that damage was repaired before initiation of a tumor when DMN was fed at a slower rate. Tumor

incidence was not strictly dose dependent within a given dose rate treatment (Clapp and Toya, 1970). Unfortunately, comparison with the similar long-term oral dose response study in the rat was not possible due to the high incidence of spontaneous tumors observed in these mice. In general, the carcinogenicity of DMN was similar in both the rat and the mouse with the exception being the wider range of susceptable organs and the greater differences in strain susceptibility observed in the mouse.

Other Mammals

Tomatis and Cefis (1967) found that stomach cannulation of 1.0 to 1.6 mg of DMN per hamster induced liver tumors. Weekly subcutaneous injections of 6 to 14 mg/hamster produced tumors of the liver and of the nasal cavity in the Syrian golden hamster (Herrold, 1967). DMN at a dosage of 25 ppm in the drinking water for 11 weeks induced liver tumors in these hamsters (Tomatis et al., 1964).

No evidence of a carcinogenic response was observed by Magee and Barnes (1956) after feeding DMN to male rabbits at 20 to 50 ppm in the diet. However, by the eighth week of the experiment, all rabbits had died from the toxic effects of DMN. In a later study by LePage and Christie (1969a) tumors of the liver and kidneys with lung metastases were observed in rabbits fed DMN at 25 ppm in the diet.

Male guinea-pigs which were given DMN orally at 1 to 2 mg/kg body weight for one year developed liver tumors (LePage and Christie, 1969b). Liver tumors were observed in Mastomys after twice-weekly injections

of DMN at 0.1 mg/rodent for 10 to 44 weeks (Fujii and Sato, 1970). The mink has been primarily noted for its role in the first observation of unintentional environmental poisoning traced to a N-nitroso compound. Recently, the mink has also been reported to be susceptable to the carcinogenic as well as toxic effects of DMN and may be the most sensitive species tested to date. Twice-weekly exposure of as little as 0.05 mg/kg body weight in the diet, or a total dose as low as 25 mg/kg body weight over the life span, produced liver tumors in all of the animals tested (Koppang and Rimeslatten, 1975).

Aquarium Fish

In 1971 Khudolei reported liver tumors in Lebistes reticulatus, the guppy, after dissolving DMN in the aquarium water at levels from 3 to 100 ppm for eight weeks. DMN fed in the diet at 5 ppm to guppies produced a questionable carcinogenic response in 2 out of 20 fish examined after 13 months of feeding (Sato et al., 1973). The only other N-nitroso compound tested in fish, diethylnitrosamine, produced a carcinogenic response in the guppy, the medaka (Oryzias latipes) and the Brachydanio rerio (Khudolei, 1971; Ishikawa et al., 1975; Stanton, 1965).

Rainbow Trout

Preliminary reports of the induction of liver tumors in rainbow trout with DMN feeding were made by Halver (1965b). DMN fed at 480 and 1920 ppm for 15 months produced an incidence of tumor-bearing animals of 41% and 100% respectively (Halver,

1967). In a separate screening experiment, DMN at 150 ppm in the diet produced a 3% incidence of tumor-bearing animals after one year (Halver, 1967).

A complete description of these early feeding trials and the results of a second confirmatory feeding trial were reported by Ashley and Halver (1968). Replicate groups of 100 trout at each dose level were fed 75, 300, 1,200, 4,800 and 19,200 ppm DMN in the diet for 20 months. A second experiment provided a confirmatory test of the 300 and 1,200 ppm dose levels, and was conducted in the same manner.

Table 1 presents the observed tumor incidence reported from these experiments. These observations were made during surgical inspection with termination at the 20 month observation period. Tumors occurred exclusively in the liver. Although the results of the first experiment were not conclusive, a time dependent dose response was indicated in the second confirmatory experiment. The histology report included observations of basophilic preneoplastic nodules, eosinophilic nodules, trabecular carcinomas and mixed cholangiolar and trabecular carcinomas in a pattern similar to that observed in DMN carcinogenesis in mammals. No metastases were observed, nor were there any indications of hemorrhagic fluid accumulation in the peritoneal cavity.

The authors concluded that the latent period for tumorogenesis in DMN fed trout appeared to be longer than that of DMN fed rats. This was attributed to the lower temperatures at which trout exist. There was also a casual mention of the lower quantities of feed consumed by the trout.

TABLE 1. Hepatocellular carcinoma incidence in trout fed DMN.[a]

| Dose of DMN (mg/kg diet) | Percent Tumor Bearing Trout | | | | | | |
|---|---|---|---|---|---|---|---|
| | 6 months Exp. 1 | 9 months Exp. 1 | 12 months Exp. 1 | 16 months Exp. 1 | 16 months Exp. 2 | 20 months Exp. 1 | 20 months Exp. 2 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 75 | 0 | 0 | 0 | 0 | - | 0 | - |
| 300 | 0 | - | 42 | 41 | 48 | 76 | 72 |
| 1200 | 0 | 2 | 58 | 54 | 59 | 89 | 91 |
| 4800 | 0 | - | - | 41 | - | 76[b] | - |
| 19200 | 0 | - | - | 100 | - | -[c] | - |

a) Data was tabulated from Ashley and Halver (1968).

b) DMN was fed for only 14 months at this dose.

c) All trout were dead at 20 months.

Rainbow trout have proven to be a useful animal model for the study of hepatocellular carcinoma (Ashley, 1973). Use of trout in this capacity was an outgrowth of an epizootic of hepatocellular carcinomas in fish hatcheries. The cause of this epizootic was traced to the presence of alflatoxin in oilseed meals which were fed in standard hatchery diets (Halver, 1965a; 1967).

Other chemical carcinogens were also capable of inducing hepatocellular carcinomas in trout (Halver, 1967). The histology of this condition has been well defined and showed little variation regardless of the carcinogenic agent (Wales, 1970; Wales and Sinnhuber, 1973). A semi-synthetic experimental diet which does not produce hepatocellular carcinomas has been designed for trout (Castell et al., 1972; Lee and Putnam, 1973; Lee and Wales, 1973).

Metabolism in Rainbow Trout

There have been two reports concerning the metabolic activation of DMN by rainbow trout (Kruger et al., 1970; Montesano et al., 1973). Kruger et al. injected [$C^{14}$] DMN intraperitoneally into trout which weighed from 300 to 400 grams. Examination of the RNA, DNA and protein of the livers revealed no [$C^{14}$] in any of these components. Increased dosages of DMN, changes in injection site and changes in incubation time all failed to produce any indication of methylation in cellular constituants. Enzymatic oxidation of DMN was thought to be necessary for nucleic acid methylation. The authors argued that since fish lacked the necessary oxidative enzymes, the trout would not be expected to produce nucleic acid methylation. The demonstrated carcinogenicity of DMN in trout coupled with their findings resulted in the conclusion that the alkylation theory did not apply to DMN carcinogenesis in the trout.

In response to their conclusion, Montesano et al. (1973) investigated the capacity of rainbow trout to metabolize DMN. They detected low levels of $^{14}CO_2$ and low levels of [$C^{14}$] in the 7-methylguanine of liver nucleic acids after incubation of [$C^{14}$] DMN with trout liver slices. The authors stated that DMN metabolism was occurring at a lower level than observed in a rat study and that this was in agreement with the higher dietary levels of DMN required for carcinogenesis in the trout. Rapid elimination of unchanged DMN, as observed in goldfish and newts, was given as a possible explanation for the absence of in vivo methylation in the report of Kruger et al. (1970).

Montesano et al. (1973), further agreed that Kruger et al. (1970), were in error about the oxidative drug metabolism capacity of fish, and concluded that rapid elimination of DMN coupled with a low capacity for enzymatic oxidation of DMN in trout livers was responsible for the negative results obtained by Kruger.

The capacity of rainbow trout microsomal enzymes to oxidatively metabolize various zenobiotics has been confirmed (Buhler and Rasmusson, 1968a; Dewaide, 1971; Schoenhard et al., 1974). Specifically, the oxygen and NADPH dependent N-demethylation of aminopyrine by rainbow trout liver microsomes has been demonstrated (Buhler and Rasmusson, 1968b; Dewaide, 1971). In addition to microsomal enzyme activity, trout liver microsomes were found to contain relatively high concentrations of cytochrome P-450 and NADPH-cytochrome-c reductase (Chan et al., 1967). Therefore, in contrast to the implications in the study of Kruger et al. (1970), it is probable that rainbow trout possess a liver microsomal N-demethylase capable of activating DMN.

Acute Toxicity

Centrilobular necrosis with hemorrhages into the liver and lungs characterized the acute toxicity of DMN in most species (Magee and Barnes, 1967). In the rat, there was associated hemorrhagic ascites and blood in the lumen of the gut. When the rat succumbed to the dose, death was within two to four days. There was a sharp demarcation between the liver cells which were either totally necrotic or survived to begin division within 48 hours of the DMN treatment (Barnes and Magee, 1954). The kidneys of the dog and rabbit also showed some damage after DMN dosage including altered urine flow and loss of tubular function.

In the rat, biochemical changes during DMN induced toxicity included an increase in liver lipid content, a decrease in liver glycogen content, a decrease in amino acid incorporation into microsomal proteins, loss of RNA and phospholipids and the leakage of liver enzymes into the blood serum (Magee and Barnes, 1967). Pretreatment of rats with acetoaminonitrile, cysteine, ethanol 2-diethylaminoethyl-2 2-diphenyl-valerate (SKF 525 A), 3-methyl cholanthrene, pregnenolone-16α-carbonitrile or a protein free diet resulted in a reduction in the acute toxicity of DMN (Magee and Barnes, 1967; Maling et al., 1975; Gravela, 1974; Somogyi et al., 1972; Venkatesan et al., 1970b). The most characteristic sign of acute DMN poisoning was inhibition of protein synthesis (Magee and Barnes, 1967).

The median lethal doses after oral or intraperitoneal dosage were between 15 and 50 mg/kg body weight for the rabbit, mouse, guinea-pig,

rat and dog (Barnes and Magee, 1954; LePage and Christie, 1969a). By the inhalation route, the median lethal dose in BD rats was 37 mg/kg body weight (Druckrey et al., 1967). In sheep daily doses less than 0.1 mg/kg body weight did not produce any toxic symptoms. Larger dosages in amounts from 17 to 40 mg/kg body weight were lethal to most sheep tested (Koppang, 1974a). Liver damage and hemorrhage into the peritoneal cavity and certain organs were the major features of the toxic reaction.

Koppang recorded a similar toxic reaction in cows and pigs fed DMN (Koppang, 1974b; 1974c). In milk cattle significant toxic effects in the liver were observed after a dose of 20 mg/kg body weight, with a no-effect toxicity threshold of approximately 0.1 mg/kg body weight daily. Koppang did not report median lethal doses, but stated that pigs and duckling required ten times the DMN dosage needed by cattle, sheep and mink in order to produce a similar toxic reaction (Koppang, 1974c). By subcutaneous injection the median lethal dose of DMN in the mink was 7 mg/kg body weight (Koppang and Rimeslatten, 1975).

Dose Response in Carcinogenesis

In a review of environmental factors in carcinogenesis, Jones and Grendon (1975) discussed the stages of cancer development observed in experimental systems. They reported a valid relationship between the time of appearance of tumors and the inverse cube root of the dose of several carcinogens. This led to their statement,

> If the cube-root of the dose applies to the estimation of the time of appearance of cancers, low-dosage exposure at some levels is virtually without risk because the expected lifespan of those exposed is exceeded by the time necessary for low concentrations of altered cells to develop into cancers.

A 'zero tolerance' level for chemical carcinogens in the human environment has generally been accepted in principle, but the practical feasibility and analytical limitations have modified this concept towards positions such as the one described above. This has been described as the 'no effect' level philosophy of chemical carcinogenesis (Kroes et al.,1973). For this concept to function properly, estimations of 'no effect' doses from animal experiments are essential (Preussmann, 1973; Kroes et al.,1973).

In extrapolation of a 'safe' level for humans from animal studies it should be remembered that single doses may induce tumors. Synergistic and additive effects from a group of chemicals must also be considered in evaluation of dose response experiments. As exemplified by the study of Nixon et al. (1974), the absolute amount of a chemical required for carcinogenesis, rather than the daily dosage, might be more useful in calculating a 'no effect' level. The use of high levels of chemicals in animal studies relative to probable

human exposure levels becomes less important if one considers the short lifespan of a test animal relative to the human lifespan. Finally, humans may be more or less sensitive to a chemical as a function of species to species variations (Kroes et al., 1973; Preussmann,1973; Lijinsky,1976).

There were several dose response experiments reported in the literature on N-nitroso compounds. Many studies of N-nitroso carcinogenesis used this relationship as inherent proof of causation, especially when high incidences of spontaneous tumors occurred in the test animals. A classic dose study of the carcinogenicity of N-nitroso compounds was reported by Druckrey et al.(1967). As mentioned earlier, they treated BD rats over a lifespan with approximately 65 N-nitroso compounds, and calculated daily dose, mean induction time for tumor emergence, mean carcinogenic dose and median lethal dose for each compound tested. For DMN the daily oral dose was 4 mg/kg diet; the mean tumor induction time was 270 days; the mean carcinogenic dose was 400 mg/kg body weight; and the median lethal dose was 40 mg/kg body weight.

Quantative analysis resulted in a normal distribution for both total dose and induction time of each compound tested. A linear function was obtained for the relationship between the log of the dose and the log of the induction time. A plot of this relationship resulted in the formula: $dt^n$ = a constant.[1] Since the values of the exponent

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[1] d is the dose, t is the induction time for tumor emergence. The exponent, n, and the constant were drived from the plot.

were between two and four in most cases, N-nitroso compound carcinogenesis was considered an accelerated process which could result from a single dose (Druckrey et al., 1967).

Wishnok and Archer (1976) took advantage of Druckrey's large, internally consistent set of data to define some quantitative relationships in N-nitroso carcinogenesis. A daily dose of carcinogen corresponding to one to three percent of the median lethal dose consistantly produced the mean carcinogenic dose when administered over a lifespan. Linear regression analysis revealed a highly significant correlation between mean carcinogenic dose and the number of carbon atoms in the N-nitroso compound. The mean carcinogenic dose was approximately three and one half times the amount of the median lethal dose. Wishnok and Archer concluded that these relationships can be used to estimate carcinogenic doses for N-nitroso compounds. These doses would be useful both in experimental design and in definition of the potential carcinogenic risk. N-nitrosamines with more than 14 carbon atoms were thought to be unlikely carcinogenic agents, and the importance of the number of carbon atoms in defining the mean carcinogenic dose was probably a reflection of the ability of a given compound to reach the site of action. However, the authors warned that the data may not be directly applicable to other species and other classes of carcinogens, or to modes of application other than the long term daily exposure. In addition, no corrections were made for organ specificity which varies with different N-nitroso compounds (Wishnok and Archer, 1976).

Dose response curves varied in slope and intercept depending on the type of animal, type of tumor under consideration, distribution of

the chemical over time and route of administration (Kroes et al., 1973). Due to the presence of low levels of DMN in human foods, dose response data from chronic animal feeding experiments could provide information valuable to risk evaluation. The response of different species to equivalent treatment with DMN may be identical or vary widely, and the results of this type of comparison strengthen a hypothesis that humans will or will not be sensitive to a given dose of DMN. Therefore, the carcinogenic dose response in rainbow trout fed DMN provides data which could aid in determining the human risk resulting from environmental exposure to DMN.

# MATERIALS AND METHODS

## Dimethylnitrosamine

The DMN used in the feeding trial was purchased in a single lot from Eastman Kodak Company, Rochester, New York. The same lot of DMN was routinely used as a gas chromatography and mass spectrometry standard in our lab,[2] and was found to be over 99% pure. The DMN used in the median lethal dose determination was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, and specified to be 99+% pure by infrared spectrum and gas chromatographic analysis.

All work with DMN was done under a fume hood and gas masks with appropriate filters were used when necessary. Safety procedures designed by the National Institute of Health for handling N-nitrosamines were employed at all times. As indicated by the circumstances, destruction of DMN contaminated waste materials was by the addition of sodium hydroxide and alumina to solutions and by UV irradiation or incineration of solid materials.

## Diet Preparation

The diet was prepared by the method of Castell et al. (1972), which was modified only to permit variation in the size of individual diet batches. Experimental diets were prepared by the addition of weighed amounts of DMN to batches of diet and were otherwise identical to the control diet. The DMN was initially prepared for use by weighing a

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[2] Department of Food Science and Technology, Oregon State University, Corvallis, Oregon.

quantity sufficient for incorporation into ten kg of dry diet. This DMN was then distributed among ten vials fitted with teflon lined screw caps, diluted to ten mls with distilled water and stored in the dark at 0°C for periods averaging two months prior to use. During diet preparation, the contents of the appropriate vial were thawed and thoroughly mixed with a one kg batch of diet. As the feed consumption increased, diet was prepared more frequently and in larger amounts ranging up to 3 kg batches. The content of the DMN solutions was adjusted accordingly. The use of single weighings followed by dilutions was to minimize exposure to DMN during diet preparation.

## Feeding Experiment

Mt. Shasta strain rainbow trout were spawned and hatched in our laboratory.[3] The trout were fed the semi-synthetic diet described in Table 2 for thirty days prior to initiation of the experimental diets. The levels of DMN used in experimental diets have been listed in Table 3.

Fingerling trout in groups of 100 were randomly selected and placed in 100 gallon fiberglass tanks with a water flow rate of four gallons per minute 12°C. Duplicate tanks of trout were designated for each of the dose levels. In the first month of the trial trout were fed ad libitum several times daily. As the trout matured, feeding was reduced to twice daily ad libitum for the rest of the trial. Tanks of trout were weighed monthly and feed consumption was recorded weekly. Calculations of the total amount of DMN ingested during the feeding trial were made from these feed consumption records.

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[3]Food Toxicology and Nutrition Laboratory, Department of Food Science and Technology, Oregon State University, Corvallis, Oregon.

TABLE 2. Composition of semi-synthetic diet used in the DMN feeding experiment.

| Ingredient | Percent in Diet |
|---|---|
| Casein | 49.5 |
| Gelatin | 8.7 |
| Dextrin | 15.6 |
| Mineral mix[a] | 1.0 |
| Carboxymethylcellulose | 1.0 |
| α-cellulose (celufil)[b] | 8.2 |
| Vitamin mix[c] | 2.0 |
| Choline chloride (70%) | 1.0 |
| Salmon oil | 10.0 |

[a]Modified Barnhart-Tomarelli (1966) salt mix (0.002% NaF and 0.02% $CoCl_2$ were added).

[b]United States Biochemicals Corporation, Cleveland, Ohio.

[c]Supplies vitamins at the following levels (mg/kg diet): thiamin 64, riboflavin 144, niacinamide 512, biotin 1.6, Ca D-pantothenate 288, pyridoxine (HCl) 48, folic acid 19.2, menadione 16, cobalamine ($B_{12}$) 0.159, i-insitol (meso) 2500, ascorbic acid 1,200, para-aminobenzoic acid 400, Vitamin A 25,000 I.U./kg, Vitamin $D_2$ 4,000 I.U./kg, Vitamin E 660 00 I.U./kg.

After 6 and 12 months, batches of 30 trout were randomly removed from each tank and killed with tricaine methanesulfonate. Individual body and corresponding liver weights were recorded, and visera were examined for gross abnormalities. Livers were examined under a disecting microscope for the presence of tumors or other toxic effects.

After 6 months of feeding DMN, samples of five different livers and kidneys were removed from each tank and preserved in Bouin's fixative. Sections of this tissue were imbedded in paraffin, and stained with hematoxylin and eosin as described by Humason (1962). Slides prepared in this manner were read by a fisheries pathologist.

After 12 months of feeding DMN, a similar procedure was followed. However, during this sample all liver tissue was fixed in Bouin's solution and after one week was removed and hand sliced into 1 mm sections to check for additional internal tumors. Samples of tumor tissue were taken from each dosage to confirm the presence of carcinomas, and define the type of damage.

## Dose Response Calcualtions

Enumeration of tumor-bearing animals was determined on the basis of histological classification of lesions as either preneoplastic nodules or actual carcinomas. The definition of hepatocellular carcinomas given by Canton *et al* (1975) for trout was used in this classification. The total intake of DMN over a 12 month period was related to the percentage of tumor bearing animals by maximum likelihood analysis (Cox, 1970).

As listed in Table 6, the data from a similar dimethylnitrosamine feeding trial was used for the calculation of the dose response curve in Porton rats. Daily dietary doses were converted to total amounts of dimethylnitrosamine fed by using the feed consumption factors given by Terracini et al.(1967). The percentage of tumor bearing animals was calculated from hepatocellular carcinomas incidence after histological confirmation. Maximum likelihood analysis was again used to define the dose response relationship.

## Median Lethal Dose Determination

Yearling rainbow trout weighing an average of 300 gms. were subjected to three replicate trials to determine the ten day median lethal dose for dimethylnitrosamine. At each dose level, ten trout were given a single intraperitoneal injection. The dimethylnitrosamine dose levels tested were 0, 200, 400, 800, 1600 and 3,200 mg/kg body weight. Due to the large amounts needed, dimethylnitrosamine was administered without a carrier solvent except when distilled water was needed to equalize the injection volume. Controls were dosed with distilled water.

The handling procedures described by Bauer et al. (1969) were used with the following minor modifications. The diet described in Table 2 was fed prior to and during the ten day trial. Representative liver tissue samples were taken after the ten day period for histology. Statistical determination of the median lethal dose was by maximum likelihood analysis (Cox,1970).

Oral administration of acutely toxic doses of dimethylnitrosamine was also attempted, but was unsuccessful due to regurgitation of the stomach contents by the treated trout. This occurred even after direct injection of DMN into the stomach through the body wall. This problem has been discussed and verified by Bauer et al. (1969), Doster (1972), and Doster et al. (1972).

# RESULTS AND DISCUSSION

## Chronic Toxicity of Dimethylnitrosamine in Rainbow Trout

The 12 month feeding experiment was a vehicle for determining the chronic toxicity of DMN in trout. Initially, the dose levels were 0, 3, 50, 200, 400, and 800 mg/kg body weight. After 3 months of feeding, both tanks at the 50 mg/kg level were accidently mixed with both tanks at the 200 mg/kg level. Since there was no way of identifying the fish from either dose level, all four tanks were fed the 200 mg/kg level diet for the rest of the experiment. The choice of the higher dose insured that the dose response would not be skewed towards excessive sensitivity.

After 6 months of receiving DMN in the diet, duplicate groups of thirty trout were sampled at each dose level. Visual examination of the internal organs revealed no dose related toxic reaction. Histological examination of liver and kidney sections also disclosed no apparent effects from the presence of DMN in the diet. Toxicity indicators including enlarged nuclei, intranuclear inclusions and occasional necrotic cells were sporatically seen in the liver sections. However, these abnormalities did not present a clear dose relationship, nor were they consistent within dose levels.

Liver to body weight ratios were calculated to detect enlargement of the livers due to toxic damage. The average liver weight was expressed as a percentage of total body weight and presented in Table 3. There was a significant difference ($P<0.01$) between the liver weight of the 200 mg/kg dose level and the liver weights of

TABLE 3. Liver to body weight ratios of trout fed DMN for 6 months.

| Dose of DMN (mg/kg dry diet) | Liver weight as a percentage of body weight[b] | |
|---|---|---|
| | Tank A | Tank B |
| 0 | 1.22 | 1.24 |
| 3 | 1.26 | 1.18 |
| 200[a] | 1.30 | 1.27 |
| 200[a] | 1.29 | 1.39 |
| 400 | 1.20 | 1.15 |
| 800 | 1.25 | 1.18 |

[a]Half of these trout were fed DMN at 50 mg/kg dry diet for the first 3 months of the feeding trial and were inadvertantly mixed with the remainder which had been fed DMN at 200 mg/kg dry diet for the first 3 months of the feeding trial. Both groups of trout were fed DMN at 200 mg/kg dry diet for the remainder of the trial.

[b]The 200 mg/kg dry diet dose levels were significantly ($P>0.01$ T test of the means) higher than other treatments.

all other treatments including controls. There were no other significant differences between the remainder of the treatments.

The difference observed in the liver weight of the 200 mg/kg dose level was the only indication of toxicity apparent at the 6 month sample. The liver enlargement reflected in these weight ratios could have been due to several factors. The absence of this reaction in the higher dose levels could mean that the enlargement of the livers in the 200 mg/kg dose level was due to some factor unrelated to the presence of DMN in the diet. Conversely, the liver enlargement could be a symptom of an early stage of toxicity from which the higher dose levels had advanced by the 6 month sample. If the latter were correct, some sign of a second stage of toxic damage would be expected from the higher dose, but no such evidence was observed.

Table 4 lists the average body weights for each tank used in the feeding experiment. Although the weights of the control trout were significantly different ($P<0.01$) from those of the DMN fed trout, there was no significant correlation found between dose level and weight of the trout. No signs of inappetence were observed in the DMN fed trout. The difference between the control and treatments was probably due to the availability of the diets and subsequently, the relative amount of diet given to the trout at each feeding.

Table 4 also lists the average body weights recorded at the 12 month sample. Again there was a significant difference ($P<0.01$) between the controls and the treatments with the same probable explanation still valid. There was no significant correlation between dose level and body weight of the trout at 12 months.

TABLE 4. Average body weight of trout fed DMN for 6 and 12 months.

| Dose of DMN (mg/kg dry diet) | Average Weight of Trout[b,c] 6 months Tank A | 6 months Tank B | 12 months Tank A | 12 months Tank B |
|---|---|---|---|---|
| 0 | 33 | 34 | 243 | 254 |
| 3 | 28 | 28 | 217 | 221 |
| 200[a] | 28 | 27 | 211 | 217 |
| 200[a] | 30 | 24 | 228 | 196 |
| 400 | 31 | 26 | 216 | 200 |
| 800 | 29 | 30 | 212 | 218 |

[a] Half of these trout were fed DMN at 50 mg/kg dry diet for the first 3 months of the feeding experiment and were then inadvertantly mixed with the remainder which had been fed DMN at 200 mg/kg body weight for the first 3 months of the feeding experiment. Both groups of trout were fed DMN at 200 mg/kg dry diet for the duration of the feeding experiment.

[b] There was a significant difference between the weights of the controls and the weights of the treatments of both 6 and 12 months ($P < 0.01$, T test of the means). There was no significant correlation between dose level and body weight.

[c] Weight is in grams.

After 12 months of receiving DMN in the diet, duplicate groups of thirty trout were sampled at each dose level. Visual examination of the viscera revealed tumors of the liver in many of the trout from the 200, 400 and 800 mg/kg dose levels. No other visual signs of chronic toxicity were observed at this time.

Hepatocellular carcinoma incidence

A carcinogenic response was observed in the trout fed DMN at 200, 400 and 800 mg/kg dry diet. Tumors were produced exclusively in the liver with no metastasis to other organs noted at the 12 month sample.

The hepatocellular carcinoma incidence listed in Table 5 occurred in trout after 12 months of receiving DMN in the diet. The average percentage of trout bearing hepatocellular carcinomas was 5, 34, and 73% for the 200, 400 and 800 mg/kg dose levels respectively.

Varying in size from a minimum of 0.5 mm to a maximum of 40.0 mm, these tumors were located both on the interior and the exterior of the livers. In contrast to the normal deep red color of the trout liver, the tumors appeared as light yellowish patches or nodules on the surface of the liver. After hand slicing of livers fixed in Bouin's solution the normal liver tissue was yellow and the tumors appeared as distinct spherical white areas on the yellow background.

In the 800 mg/kg dose level, several of the livers were entirely composed of a large internal tumor. The remaining normal liver tissue was covered by numerous small tumors in a 'shotgun' effect. These small tumors were both preneoplastic nodules and actual carcinomas depending on the individual tumor under consideration. This 'shotgun' effect

TABLE 5. Hepatocellular carcinoma incidence in trout fed DMN for 12 months.

| Dose of DMN (mg/kg dry diet) | Cummulative Dose of DMN (mg/trout)[b] | Number of Tumor Bearing Trout at 12 months | |
|---|---|---|---|
| | | Tank A | Tank B |
| 0 | 0 | 0/30 | 0/30 |
| 3 | 0.72 | 0/30 | 0/30 |
| 200[a] | 47.8 | 0/30 | 2/30 |
| 200[a] | 45.6 | 2/30 | 2/30 |
| 400 | 96.3 | 9/30 | 11/30 |
| 800 | 195.9 | 22/30 | 22/30 |

[a] Half of these trout were fed DMN at 50 mg/kg dry diet for the first 3 months of the feeding experiment and were inadvertantly mixed with the remainder which had been fed DMN at 200 mg/kg dry diet for the first 3 months of the feeding experiment. Both groups of trout were fed DMN at 200 mg/kg dry diet for the remainder of the experiment.

[b] This expression of the dose levels was used in all calculations.

seen in Figure 2, prevented accurate enumeration of the number of tumors per trout. Therefore, dose response calculations employed the number of tumor bearing trout rather than the number of tumors per trout.

Histology

The normal trout liver as described by Simon et al (1967) possessed several salient features when examined by light microscopy. The liver cords were two cells wide and were separated by sinusoids possessing a distinct endothelium. The nuclei tended to be displaced towards the sinusoids and were spherical with a sharply defined membrane and a prominant central nucleolus. In contrast to mammalian liver tissue, the trout liver had no distinct lobular structure and rarely displayed the complete portal triad. Trout also had fewer central veins and bile canaliculi were located in a branched web-like fashion between the parenchymal cells.

Frequently in hatchery reared trout, hepatocytes were highly vacuolated. Histochemical evidence indicated that this vacuolation was due to deposits of glycogen in the cytoplasm. After fasting these trout, the vacuoles disappeared, which again indicated glycogen deposition.

The liver sectioned from the control trout displayed the normal histology described in the preceding paragraphs. A typical liver section as illustrated in Figure 3 was taken from a control trout. The light angular areas within the cells were glycogen vacuoles.

The majority of the tumor bearing livers from DMN fed trout exhibited classical trabecular hepatocellular carcinomas. The tumor tissue contained a greater number of smaller cells, an abundance of

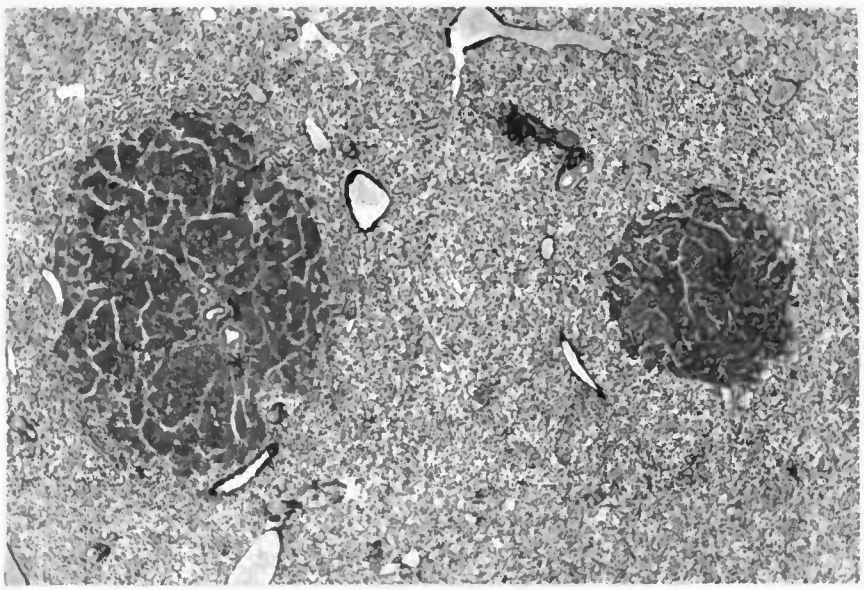

Figure 2. The 'shotgun' effect in DMN carcinogenesis. Section of liver taken from a trout fed DMN at 800 mg/kg diet for 12 months showing two small tumors. Hematoxylin and eosin X70.

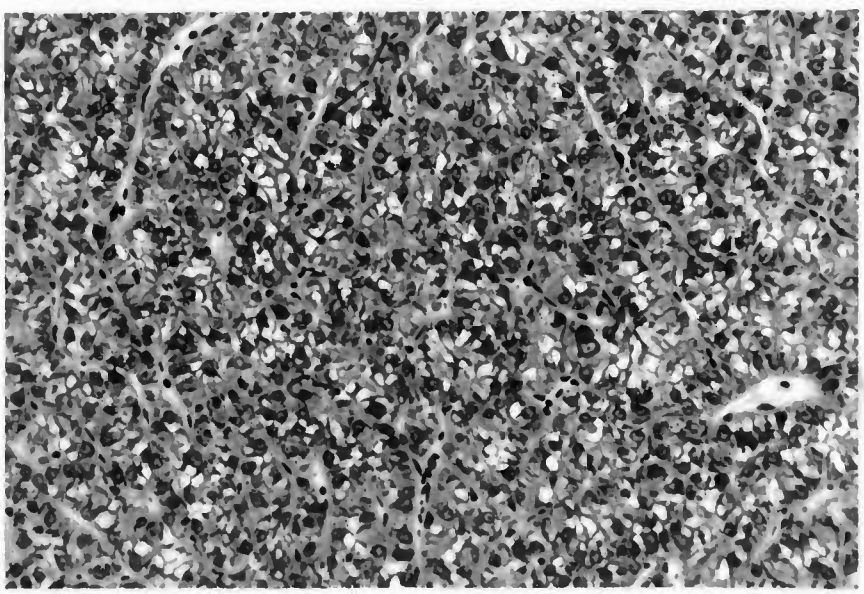

Figure 3. Liver section from trout fed control diet. Note the two cell wide cord structure (A), polarization of the nuclei towards the sinusoids (B), light areas of glycogen deposition (C), and light, eosinophilic staining character of cytoplasm. Hematoxylin and eosin X450.

bizzare nuclei, frequent mitoses and an increased nuclear/cytoplasm ratio. In addition, this tissue was deeply basophilic and had a widened cord structure accompanied by compression of surrounding normal tissue. Figure 4, the edge of a hepatocellular carcinoma from a trout fed DMN at 200 mg/kg dry diet, was typical of these features. An entire small trabecular tumor was seen in the section in Figure 5.

Figures 6 and 7 illustrate extensive foci of necrosis which was observed in the center of a large trabecular tumor from the 200 mg/kg dry diet dose level. The necrosis seen here was typical of all of the larger tumors. It was probably due to an insufficient blood supply in the abnormally widened cords which resulted in anoxia.

A second type of tumor was observed in the trout fed DMN. These tumors were classified as mixed lesions resulting from the proliferation of bile ducts in the center of trabecular carcinomas. As illustrated in Figure 9, a trabecular carcinoma with cholangiolar components was observed in the 200 mg/kg dry diet dose level. As seen in Figure 8, hyperplastic connective tissue accompanies the bile duct proliferation. The increase in connective tissue was not attributed to sarcomatous transformation, but was considered to be bile duct induced hyperplasia.

A type of lesion which was not considered to be a hepatocellular carcinoma was observed in many cases in the livers from trout fed DMN. These lesions were basophilic nodules showing much of the cellular

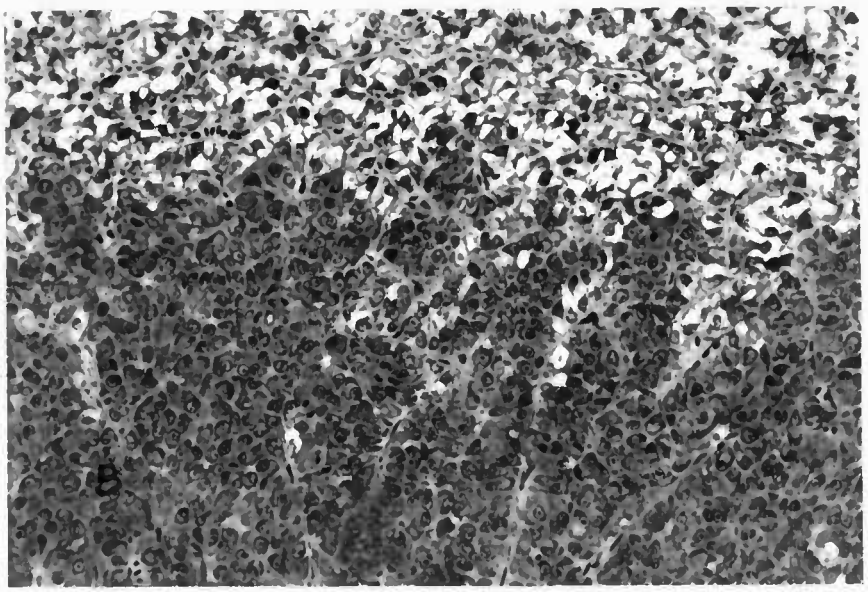


Figure 4. Edge of DMN induced trabecular tumor. Sectioned from a trout fed DMN at 200 mg/kg diet for 12 months, this was the most common type of hepatocellular carcinoma. Both normal tissue (A) and the edge of the tumor (B) are apparent. Hematoxylin and eosin X450.

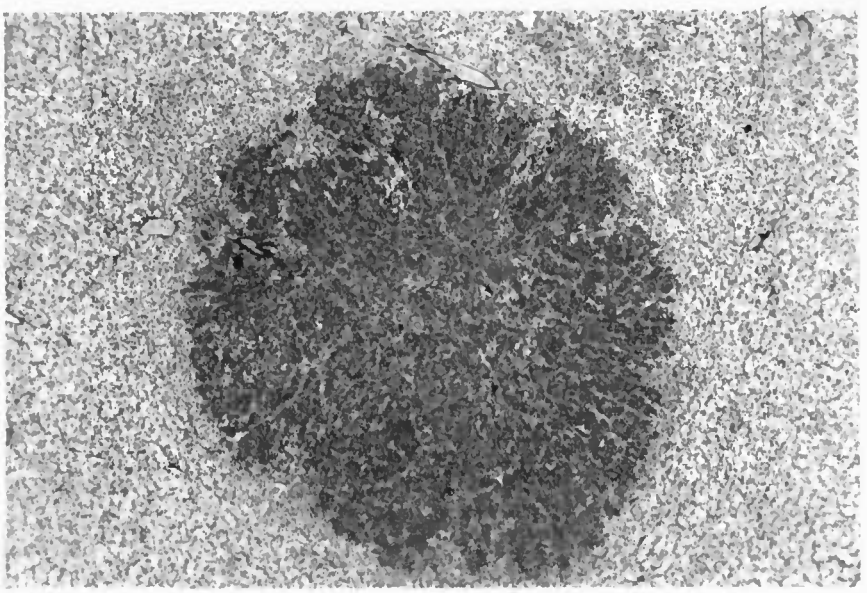

Figure 5. Edge of DMN induced trabecular tumor illustrating compression of surrounding tissue. Liver section was from trout fed DMN at 200 mg/kg diet for 12 months. Hematoxylin and eosin X70.

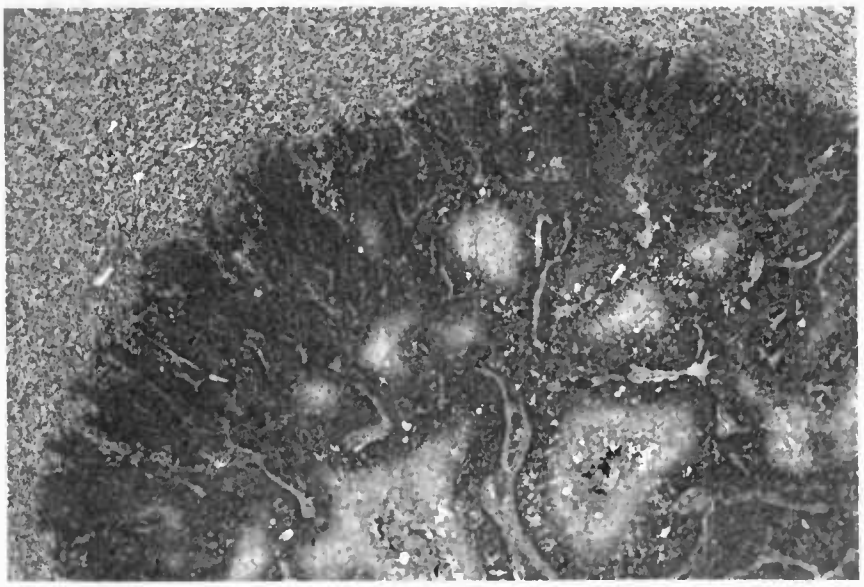

Figure 6. Large hepatocellular carcinoma from trout fed DMN at 200 mg/kg diet. Note the greatly widened cord structure and extensive necrotic foci of this trabecular tumor. Hematoxylin and eosin X70.

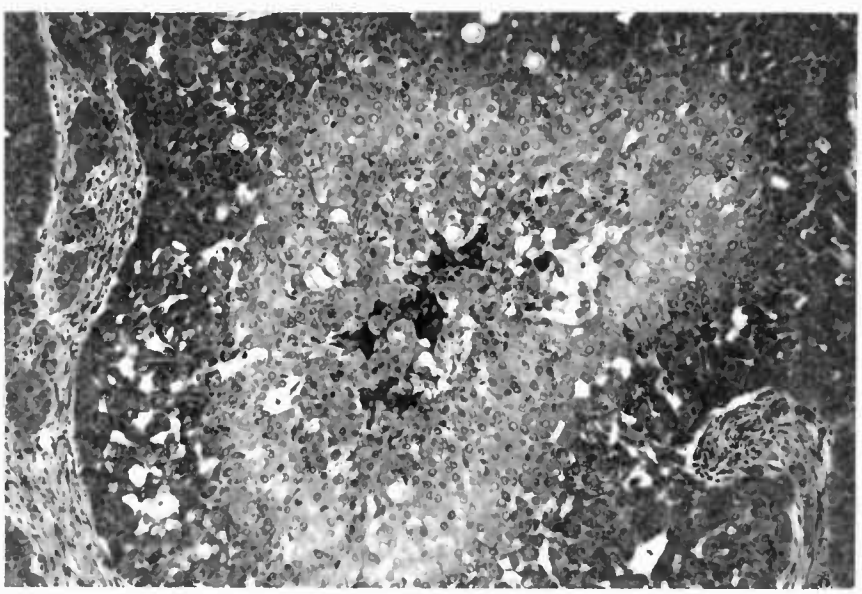

Figure 7. Enlargement of necrotic foci. This damage was typical of the centers of large carcinomas. Hematoxylin and eosin X280.

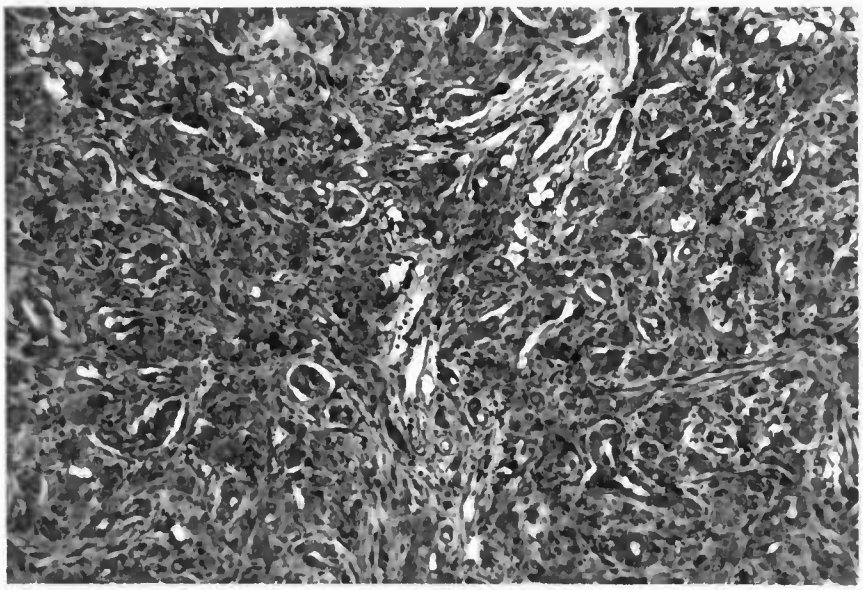

Figure 8. Cholangiolar tissue from trout fed DMN at 200 mg/kg diet. This tissue was observed in the center of a mixed tumor and consists of hyperplastic bile ducts and supportive connective tissue. Hematoxylin and eosin X280.

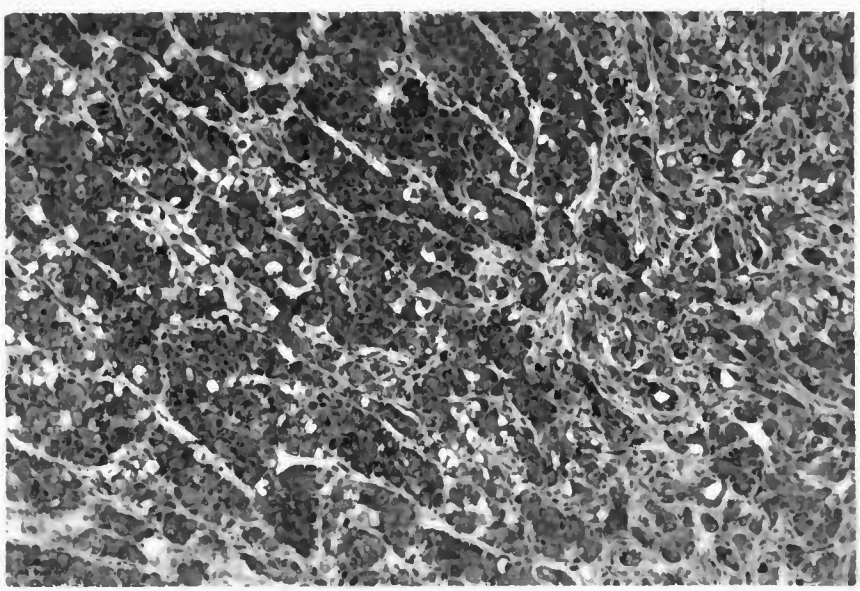

Figure 9. Mixed trabecular and cholangiolar tumor. Section of a liver from trout fed DMN at 200 mg/kg diet for 12 months. The right side of the photograph shows the cholangiolar components from the center of the tumor; the left side shows the surrounding trabecular tumor tissue. Hematoxylin and eosin X280.

atypia of the trabecular tumor. However, these nodules were smaller than the trabecular tumor, no central necrosis was present, and there was no compression of adjacent tissue. Many of the tumors forming the 'shotgun' effect were actually preneoplastic basophilic nodules (Figure 2). Differentiation between hepatocellular carcinomas and basophilic nodules was made primarily on the basis of secondary tumor characteristics.

A second preneoplastic condition was observed in DMN treated livers. This type of lesion occurred as a focus of large eosinophilic cells which had been invaded by lymphocytes. Livers from the 200, 400, and 800 mg/kg dry diet dose levels contained several of these areas. An eosinophilic nodule from the 400 mg/kg dry diet dose level was observed in the liver section in Figures 10 and 11.

These eosinophilic nodules apparently stimulated an immune reaction resulting in lymphocyte invasion. These nodules were not thought to be important in the ultimate formation of a tumor since these eosinophilic areas were presumably destroyed. Conversely, the small basophilic nodules which probably developed into hepatocellular carcinomas were rarely invaded by lymphocytes.

Non-tumerous tissue from trout fed DMN displayed signs of low level toxicity after 12 months. However, there were no effects observed in the liver tissue from the 3 mg/kg dry diet dose level at the 12 month sample. Among the toxic effects seen in the remainder of the dosages were enlargement, pleomorphism and inclusions of the nuclei. Scattered focal necrosis and destruction of individual cells were also present in a dose related manner in the trout fed

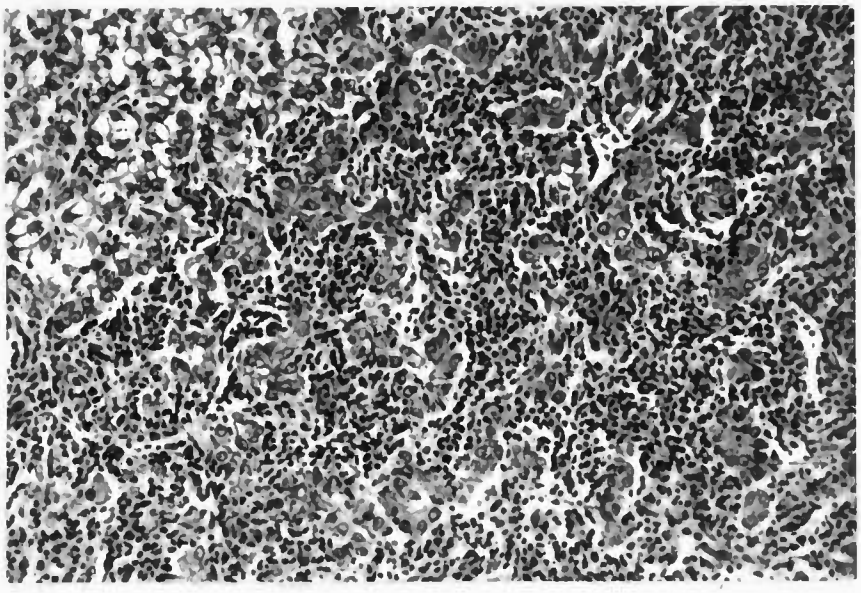

Figure 10. Eosinophilic nodule from DMN treated trout liver. This trout was fed DMN at 400 mg/kg diet for 12 months. Note the nuclear and cytoplasmic atypia, with reference to the normal tissue in the upper left hand corner, and the masses of invading lymphocytes. Hematoxylin and eosin X450.

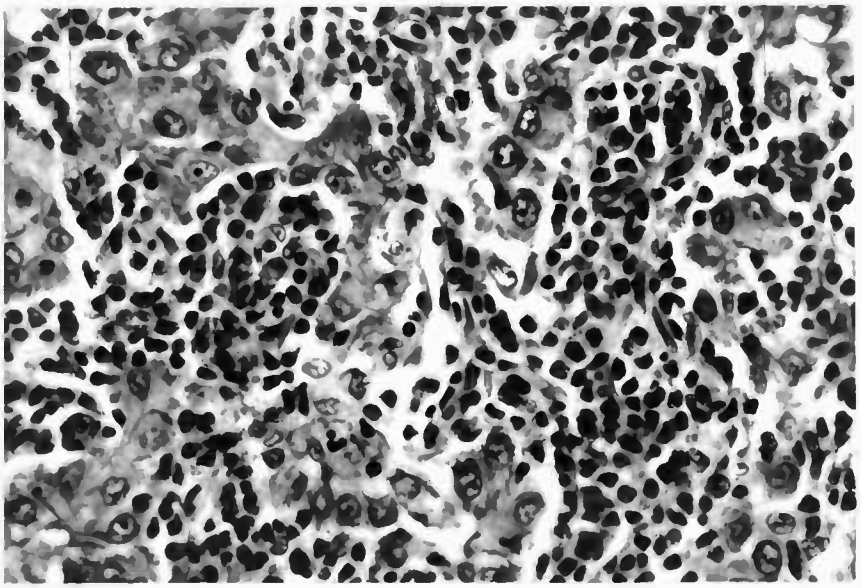

Figure 11. Enlargement of eosinophilic cells. Note the extremely large cells and the evidence of their destruction by lymphocytes. Hematoxylin and eosin X1,120.

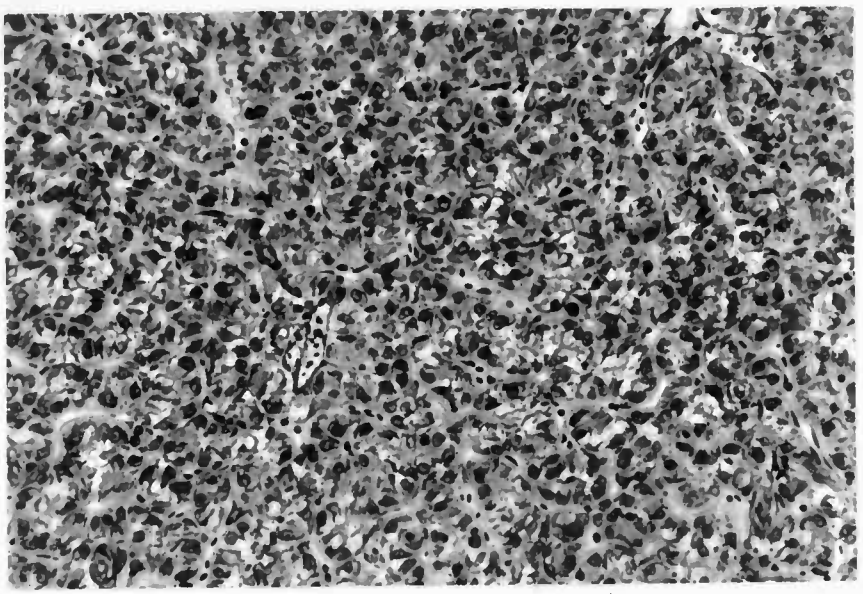

Figure 12. Low level toxic effects in livers of trout fed DMN at 200 mg/kg diet. Atypical nuclei and frequent necrotic, sloughed cells are evident in this section. Hematoxylin and eosin X450.

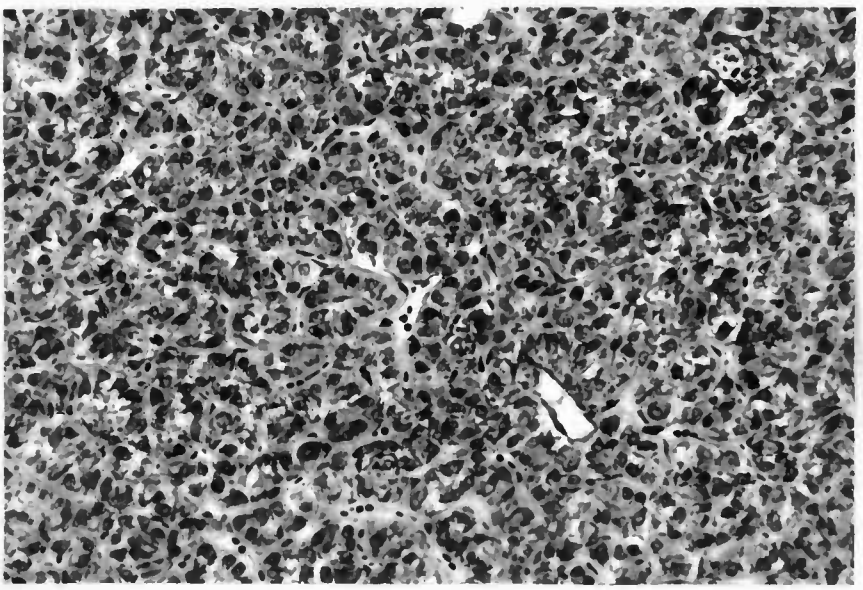

Figure 13. Dose related toxic effects in trout fed DMN at 400 mg/kg diet. The toxic effects seen at 200 mg/kg diet are present in a greater amount in this dose level. Hematoxylin and eosin X450.

DMN at 200, 400, and 800 mg/kg dry diet dose levels. The dose response nature of the toxic reactions was shown by the livers in Figures 12 and 13.

At the 6 month sampling time, no toxic or carcinogenic effects were observed in any of the liver tissue sectioned for histology. None of the kidneys examined at either 6 or 12 months contained any histological evidence of DMN induced damage.

Dose Response Curve

The carcinogenic response described in the previous section was present in the dose related manner seen in Figure 14. This relationship produced the following equation.

$$\ln \frac{P}{100-P} = -13.06 + 2.68 \ (\ln \text{ mg DMN fed})^4$$

The standard error for this set is $2.68 \pm 0.33$.

A similar curve was derived from the rat dose response experiment outlined in Table 6. DMN induced carcinogenicity in Porton rats produced the following equation.

$$\ln \frac{P}{100-P} = -11.10 + 2.20 \ (\ln \text{ mg DMN fed})^4$$

The standard error for this equation is $2.20 \pm 0.47$.

The dose response curves for both species have been plotted (Figure 15). The slopes of these curves suggested that while rats were sensitive to lower DMN doses, the sensitivity of trout increased with dosage at a faster rate. The total dose of DMN needed to induce

[4] P is the percentage of tumor bearing animals.

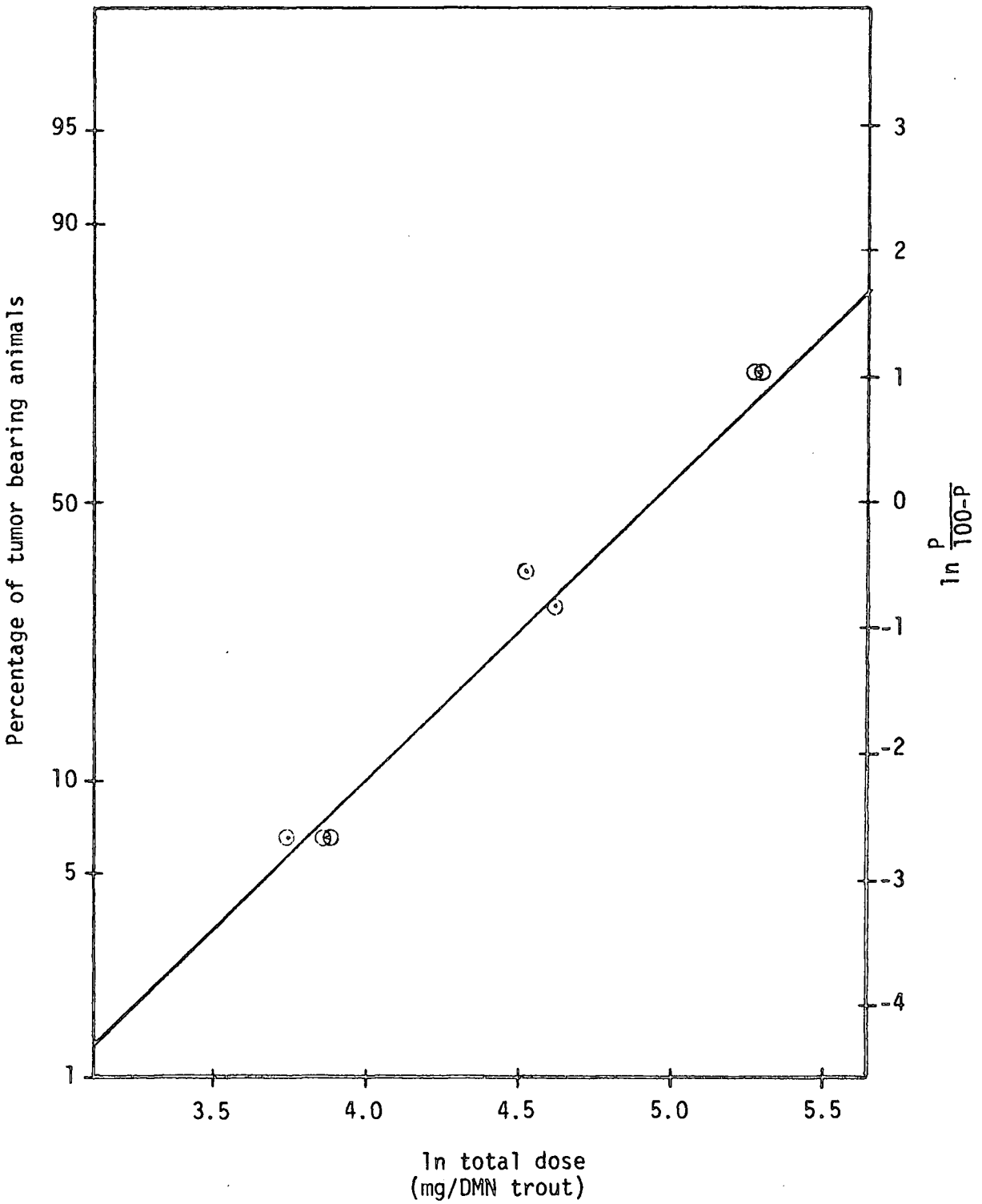


Figure 14. Dose response curve for DMN carcinogenicity in trout.

TABLE 6. Hepatocellular carcinoma incidence in Porton rats fed DMN.[a]

| Dose of DMN (mg/kg diet) | Cummulative Dose of DMN (mg/rat)[b] | Number of tumor-bearing rats at 24 months[c] |
|---|---|---|
| 0 | 0 | 0/36 |
| 2 | 25 | 1/26 |
| 5 | 63 | 8/74 |
| 10 | 126 | 2/5 |
| 20 | 252 | 10/31 |

a) Data tabulated from Terracini et al. (1967).

b) Doses were calculated from the average daily feed consumption of 15 gm/rat reported by Terracini et al. (1967).

c) All tumors were hepatocellular carcinomas.

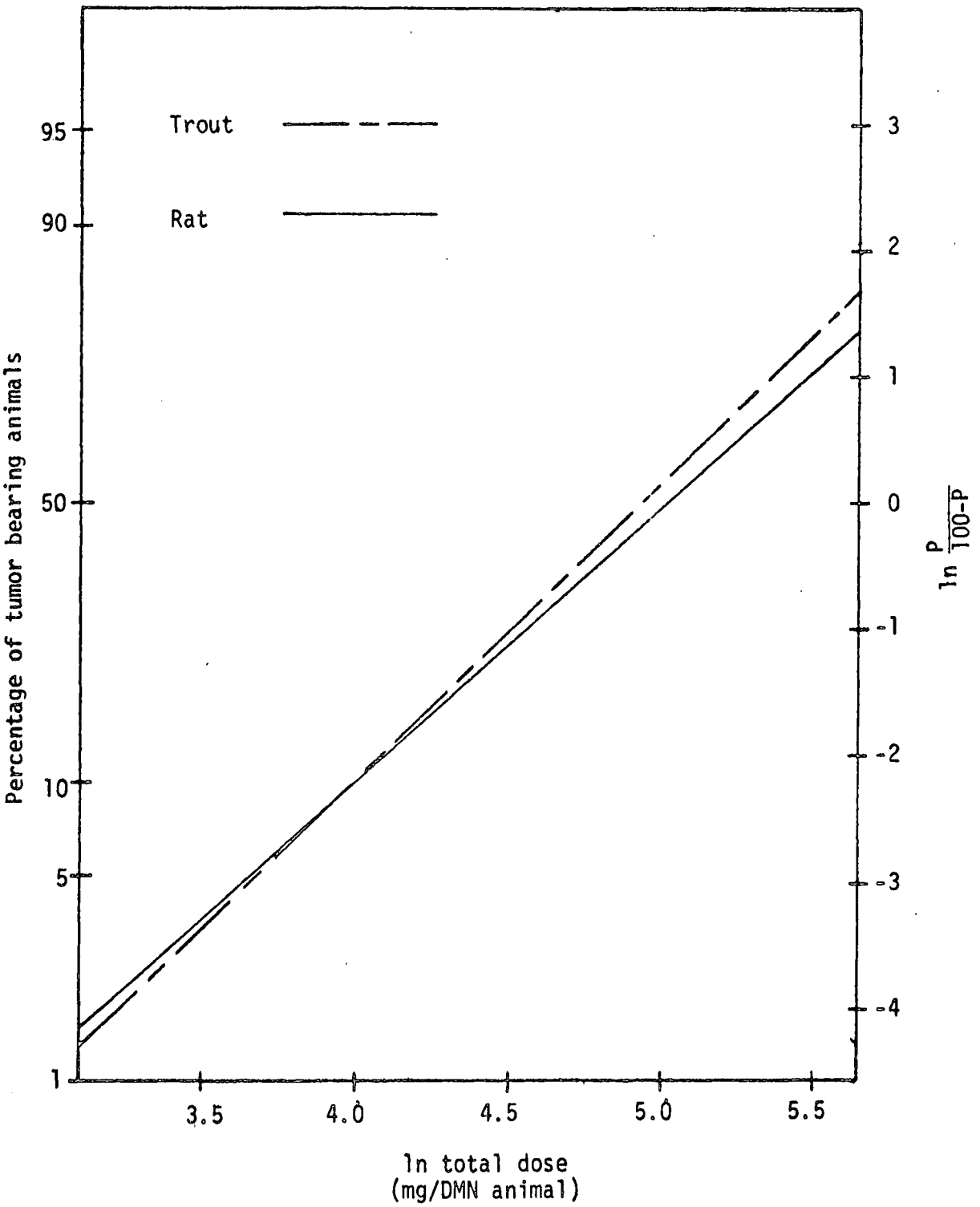


Figure 15. Comparative dose response curve for DMN carcinogenicity in trout and rats.

a 1% hepatocellular carcinoma incidence was 23.5 mg for the trout and 19.1 mg for the rat. Corresponding 95% confidence intervals were 12.2 to 45.5 mg for the trout and 7.5 to 49.1 mg for the rat. Therefore, in this evaluation of dose response, the sensitivity of the trout and the rat to DMN induced liver carcinoma were approximately equal.

Earlier work with hepatocellular carcinoma in trout assumed that trout were not acutely sensitive to DMN carcinogenesis. This conclusion resulted from a lack of attention towards relative feed intake in the trout and the rat, and the subsequent inaccuracies in the calculation of carcinogenic dose and tumor induction time. In the study of Montesano et al. (1973) a lower rate of DMN metabolism in trout compared to that of rats was attributed to their relative insensitivity to DMN carcinogenesis. However, since the sensitivity of the trout and the rat were not significantly different herein, the conclusions of Montesano et al. (1973) may bear further investigation.

A consideration of tumor induction time revealed the potential for an even greater sensitivity to DMN carcinogenesis in trout. The study with Porton rats was conducted over the entire lifespan of the rat, whereas the trout were only fed for the first year of life. Since trout have lifespans from two to four times as long as the rat, dietary levels of DMN lower than those used in this study would probably produce carcinomas in trout when fed over an entire lifespan.

## Acute Toxicity of DMN in Rainbow Trout

Table 7 presents the mortalities observed during the ten days following intraperitoneal injection of DMN to 15 month old Mt. Shasta strain rainbow trout. The addition of the 3,200 mg/kg body weight dose level and the omission of the 200 mg/kg body weight dose level from Trials 2 and 3 was to provide a range of data suited for statistical analysis.

Of the three consecutive trials only Trial 2 differed greatly from the others. A thorough examination of these experiments revealed that Trial 2 was conducted after a typical 48 hour fasting period, whereas Trials 1 and 3 were performed on trout which had been fed a few hours prior to injection of DMN. Food was supplied to the trout during the ten day observation periods of Trial 1 and 3, but it was inadvertantly withdrawn from the trout during Trial 2.

Since the experimental methods varied in the manner described, statistical analysis to determine the median lethal dose did not include data from Trial 2. Although the results of Trial 2 were reported, they are considered important only insofar as they suggest the role of diet in toxicity testing.

## Median Lethal Dose

Analysis of the data obtained in Trial 1 and 3 produced the following equation to describe the dose response curve.

$$\ln \frac{P}{100-P} = -17.57 + 2.35 \ (\ln \text{dose level})^{5}$$

[5] P is the percent mortality observed after ten days.

TABLE 7. Mortalities in trout ten days after intraperitoneal administration of DMN.

| Dose of DMN (mg/kg body weight) | Mortalities | | | |
|---|---|---|---|---|
| | Trial 1 | Trial[a] 2 | Trial 3 | Total of 1 and 3 |
| 0 | 0/10 | 0/10 | 0/10 | 0/20 |
| 200 | 0/10 | - | - | 0/10 |
| 400 | 1/10 | 0/10 | 0/10 | 1/20 |
| 800 | 0/10 | 0/10 | 1/10 | 1/20 |
| 1,600 | 5/10 | 0/10 | 6/10 | 11/20 |
| 3,200 | - | 8/10 | 7/10 | 7/10 |

[a] This trial was not used in calculations of the median lethal dose due to variation in the parameters under which the study was conducted.

The standard error was calculated to be $2.35 \pm 0.58$. The ten day median lethal dose after intraperitoneal injection of DMN was calculated to be 1,770 mg/kg body weight. The 95% confidence limits were 556 - 5,650 mg/kg body weight.

The median lethal dose for DMN was high in the rainbow trout compared to other species. Depending on whether administration was oral, intravenous, intraperitoneal or subcutaneous, the median lethal dose for the rat was between 25 and 41 mg/kg body weight. Other species were also more sensitive than trout to the acutely toxic effects of DMN.

One explanation for the relative lack of acute toxicity in trout was indicated by preliminary experiments. In these experiments, unchanged DMN was rapidly eliminated from the gills following intraperitoneal injection of DMN. Partitioning of the water soluble DMN into the constantly changing water of the tanks probably occurred before DMN could reach the liver and create the toxic reaction seen in other species.

## Physiological Changes

General reaction to administration of DMN included weakness and inactivity accompanied by a darkening in coloration and inappetence. Most of the mortalities occurred in the period from two to five days after injection of DMN. Autopsied trout showed hemorrhages in the pyloric caeca, stomach, adipose tissue, intestines and wall of the peritoneal cavity. Intestines were always blood filled and edema was present in a few cases. Grossly, the livers and kidneys did not appear

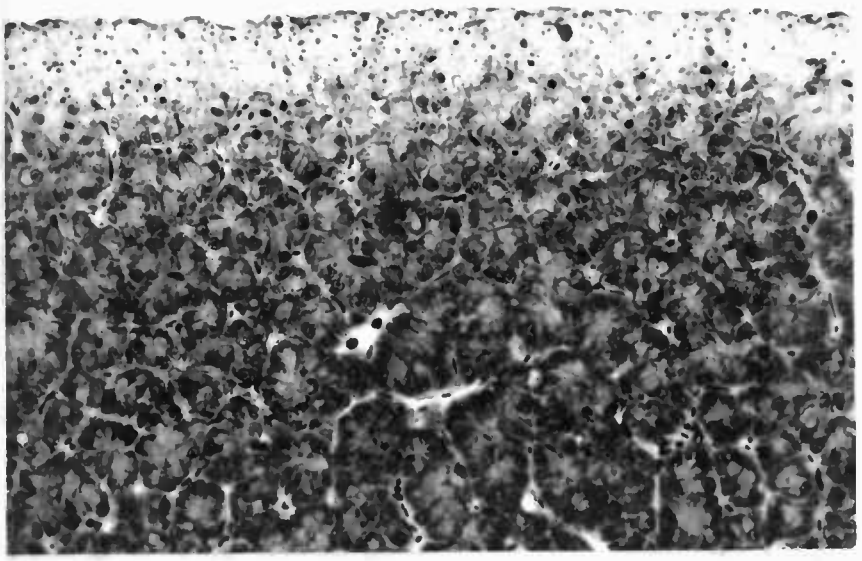

Figure 16. External liver necrosis from trout treated with DMN. Section of liver taken ten days after an intraperitoneal dose of 1,600 mg/kg body weight. Hematoxylin and eosin X450.

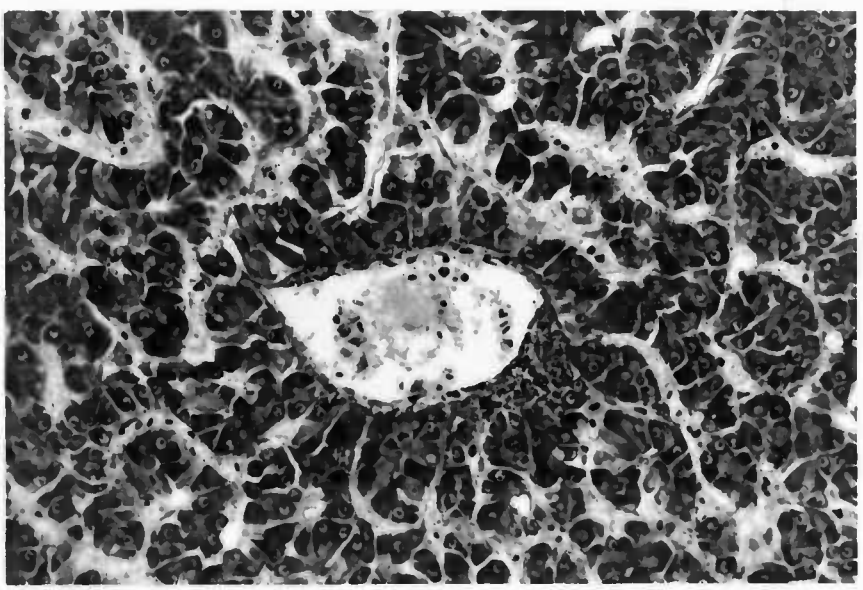

Figure 17. Central lobular necrosis in trout treated with a lethal dose of DMN. Section of liver taken ten days after an intraperitoneal dose of 1,600 mg/kg body weight. Hematoxylin and eosin X450.

to be affected by DMN.

At the conclusion of the ten day observation period, surviving trout were killed and examined for abnormalities. To a lesser extent and in a dose related manner, these trout displayed the same symptoms as the trout which succumbed to toxic doses. These symptoms and related mortalities were probably due to the presence of large quantities of DMN in the peritoneal cavity. This effect was seen in Figure 16, a section of the outer edge of a liver taken from a trout dosed with 1600 mg/kg body weight DMN.

The type of damage illustrated in Figure 16 also indicated that DMN produced acute necrosis when it was taken up by a cell. This possibility received support from the occurrence of early stages of necrosis around central veins as seen in the liver section in Figure 17. This type of liver necrosis was the primary toxic mechanism in other species. Presumably, if DMN had been retained longer and reached the liver in higher concentrations, more of this type of damage would have been observed in trout.

# SUMMARY AND CONCLUSIONS

This work was designed to provide a quantitative definition of the toxicity of DMN in rainbow trout. Both acute and chronic toxicity were examined.

Chronic toxicity was expressed solely as hepatocellular carcinomas. A dose response curve was obtained for the hepatocellular carcinoma incidence in trout fed DMN for one year. A similar relationship was derived from previously reported data in Porton rats. The two species were approximately equal in sensitivity to DMN carcinogenesis and in histology of DMN induced hepatocellular carcinoma.

Acute toxicity of DMN in trout was measured by a ten day median lethal dose determination. Following intraperitoneal injection, the median lethal dose for yearling trout was much higher than the doses reported for mammalian species.

In contrast to previous reports of DMN carcinogenesis in trout, the trout was as sensitive as the rat particularly when feed intake was considered. This contrast with previous investigators was significant in two respects. Montesano *et al.*, (1973) found that the trout liver was capable of only low levels of DMN metabolism when compared to the rat. They explained this on the basis of lower sensitivity to DMN carcinogenesis in the trout. The contrast presented herein suggests that further research be undertaken to investigate the metabolism of DMN in trout.

Secondly, this investigation demonstrated that low levels of DMN in the diet elicited a carcinogenic response in another species. All species tested to date with DMN have been sensitive to low levels. This observation suggests that humans may also be susceptable to carcinogenesis by low doses of DMN (Schmahl and Osswald, 1967).

# BIBLIOGRAPHY

Alexandrov, V. A. Blastomogenic effect of dimethylnitrosamine on pregnant rats and their offspring. Nature (London) 218:280-281. 1968.

Althoff, J., F. W. Kruger and U. Mohr. Carcinogenic effect of dipropylnitrosamine and compounds related by β-oxidation. Journal of the National Cancer Institute 51:287-288. 1973.

Arcos, J. C., R. T. Valle, G. M. Bryant, N. P. Buu-Hoi, and M. F. Argus. Dimethylnitrosamine-demethylase:molecular size-dependence of repression by polynuclear hydrocarbons. Nonhydrocarbon repressors. Journal of Toxicology and Environmental Health 1:395-408. 1976.

Argus, M. F. and C. Hoch-Ligeti. Comparative study of the carcinogenic activity of nitrosamines. Journal of the National Cancer Institute 27:695-709. 1961.

Ashley, L. M. Animal model of human disease; liver cell carcinoma, hepatocellular carcinoma, hepatoma. American Journal of Pathology 72:345-348. 1973.

Ashley, L. M. and J. E. Halver. Dimethylnitrosamine-induced hepatic cell carcinoma in rainbow trout. Journal of the National Cancer Institute 41:531-552. 1968.

Baldwin, J. E., S. E. Branz, R. F. Gomez, P. L. Kraft, A. J. Sinskey, and S. R. Tannenbaum. Chemical activation of nitrosamines into mutagenic agents. Tetrahedron Letters No. 5 pp. 333-336. 1976.

Barnes, J. M. Nitrosamines. Essays in Toxicology 5(1):1-15. 1974.

Barnes, J. M. and P. N. Magee. Some toxic properties of dimethylnitrosamine. British Journal of Industrial Medicine 11:167-174. 1954.

Barnhart, F. W. and R. M. Tomarelli. A salt mix supplying the national research council estimates of mineral requirements of the rat. The Journal of Nutrition 89:495-500. 1966.

Bauer, D., D. J. Lee and R. O. Sinnhuber. Acute toxicity of aflatoxins $B_1$ and $G_1$ in rainbow trout (Salmo gairdneri). Toxicology and Applied Pharmacology 15:415-419. 1966.

Blattmann, L. and R. Preussmann. Metaboliten von (2-hydroxybutyl)-n-butylnitrosamin im rattenurin. Zeitschrift fuer Krebsforschung und Klinische Onkologie 83(2)125-127. 1975.

Borzsonyi, M. and M. Csik. Induction of malignant lymphomas in Swiss mice by N-nitroso compounds in vivo. International Journal of Cancer 15:830-838. 1975.

Braun, R. and J. Schoneich. The mutagenicity of dimethylnitrosamine, diethylnitrosamine and nitrosomorpholine in the host-mediated assay with Salmonella typhimurium G46 and Salmonella typhimurium TA 1950. Biologisches Zentralblatt 94:661-669. 1975.

Brouwers, J. A. and P. Emmelot. Microsomal N-demethylation and the effect of the hepatic carcinogen dimethylnitrosamine on amino acid incorporation into the proteins of rat livers and hepatomas. Experimental Cell Research 19:467-474. 1960.

Buhler, D. R., and M. E. Rasmusson. Reduction of p-nitrobenzoic acid by fishes. Archives of Biochemistry and Biophysics 103:582-595. 1968a.

Buhler, D. R. and M. E. Rasmusson. The oxidation of drugs by fishes. Comparative Biochemistry and Physiology 24:223-239. 1968.

Canton, J. H., R. Kroes, M. J. vanLogten, M. vanSchothorst, J. F. C. Stavenuiter and C. A. H. Verhulsdonk. The carcinogenicity of aflatoxin $M_1$ in rainbow trout. Food and Cosmetics Toxicology 13:441-443. 1975.

Castell, J. D., R. O. Sinnhuber, J. H. Wales, and D. J. Lee. Essential fatty acids in the diet of rainbow trout (Salmo gairdneri). Growth, feed conversion, and some gross deficiency symptoms. The Journal of Nutrition 102:77-85. 1972.

Chan, T. M., J. W. Gillett and L. C. Terriere. Interaction between microsomal electron transport systems of trout and male rat in cyclodiene epoxidation. Comparative Biochemistry and Physiology 20:731-742. 1967.

Clapp, N. K. and R. E. Toya. Effect of cumulative dose and dose rate on dimethylnitrosamine oncogenesis in RF mice. The Journal of the National Cancer Institute 45:495-498. 1970.

Cox, David R. The Analysis of Binary Data. London, Methuen. 1970. 142 p.

Czygan, P., H. Greim, A. J. Garro, F. Hutterer, F. Schaffner, H. Popper, O. Rosenthal and D. Y. Cooper. Microsomal metabolism of DMN and the cytochrome P-340 dependency of its activation to a mutagen. Cancer Research 33:2983-2986. 1973.

Den Engelse, L., P. A. J. Bentvelzen, and P. Emmelot. Studies on lung tumors. I. Methylation of deoxyribonucleic acid and tumor formation following administration of dimethylnitrosamine to mice. Chemico-Biological Interactions 1:395-399. 1970.

Dewaide, J. H. Metabolism of Zenobiotics. Comparative and Kinetic Studies as a Basis for Environmental Pharmacology. Institute of Pharmacology, University of Nijmegen, Nijmegen, The Netherlands. 1971. 164 p.

Doster, R. C. Acute Toxicity and Carcinogenic Activity of Ochratoxin in Rainbow Trout (Salmo gairdneri). M.S. Thesis, Oregon State University, Corvallis. 1972. 90 numb. leaves.

Doster, R. C., R. O. Sinnhuber, and J. H. Wales. Acute intraperitoneal toxicity of ochratoxins A and B in rainbow trout (Salmo gairdneri). Food and Cosmetics Toxicology 10:85-92. 1972.

Druckrey, H. and R. Preussmann. Zur entstehung carcinogener nitrosamine am beispiel des tabakrauches. Naturwissenschafter 49:498-499. 1962.

Druckrey, H., R. Preussmann and S. Ivankovic. N-nitroso compounds in organotropic and transplacental carcinogenesis. Annals of the New York Academy of Sciences 163(2):676-696. 1969.

Druckrey, H., R. Preussmann, S. Ivankovic and D. Schmahl. Organotrope carcinogene wirkungen bei 65 verschiedenen N-nitroso-verbindungen an BD-ratten. Zeitschrift fuer Krebsforschung 69:103-201. 1967.

Druckrey, H., R. Preussmann, S. Ivankovic, C. H. Schmidt, H. D. Mennel and K. W. Stahl. The selective induction of bladder cancer in rats using di-butyl and n-butyl-n-butanol (4) nitrosamine. Zeitschrift fuer krebsforschung 66:280-290. 1964b.

Druckrey, H., R. Preussmann, D. Schmahl and M. Muller. Chemische konstitution and carcinogene wirkung bei nitrosaminen. Naturwissenschaften 48:134-135. 1961.

Druckrey, H., A. Schildbach, D. Schmahl, R. Preussmann and S. Ivankovic. Quantitative analyses der carcinogenen wirkung von diathylnitrosamin. Arzneimittelforschung 14:841-851. 1963.

Druckrey, H., D. Steinhoff, R. Preussmann, and S. Ivankovic. Carcinogenesis in rats by a single administration of methylnitrosourea and various dialkylnitroamines. Zeitschrift fuer Krebsforschung 66:1-10. 1964a.

Ender, F., G. Havre, A. Helgebostad, N. Koppang, R. Madsen and L. Ceh. Isolation and identification of a hepatotoxic factor in herring meal produced from sodium nitrite preserved herring. Naturwissenschaften 51:637-638. 1964.

Fine, D. H., D. P. Rounbehler, N. M. Belcher, and S. S. Epstein. N-nitroso compounds: Detection in ambient air. Science 192: 1328-1330. 1976.

Foreman, J. K. and K. Goodhead. The formation and analysis of N-nitrosamines. The Journal of the Science of Food and Agricutlure 26:1771-1783. 1975.

Frantz, C. N., and H. V. Malling. Factors affecting metabolism and mutagenicity of DMN and DEN. Cancer Research 35:2307-2314. 1975.

Frei, J. V. Toxicity, tissue changes, and tumor induction in inbred Swiss mice by methylnitrosamine and -amide compounds. Cancer Research 30:11-17. 1970.

Freund, H. A. Clinical manifestations and studies in parenchymatous hepatitus. Annals of Internal Medicine 10:1144-1155. 1937.

Fujii, K. and H. Sato. Response of adult mastomys (Praomys natalensis) to subcutaneous injection of N-nitrosodimethylnitrosamine. Gann 61:425-434. 1970.

Gantt, R. G., H. Smith and B. J. Julian. Viron associated and cellular RNA methylase activity in normal and neoplastic mammary tissue from mammary tumor virus-infected and -uninfected mice. Cancer Research 35:1847-1853. 1975.

Gletten, F., U. Weekes and D. Brusick. In vitro metabolic activation of chemical mutagens. I. Development of an in vitro mutagenicity assay using liver microsomal enzymes for the activation of DMN to a mutagen. Mutation Research 28:113-122. 1975.

Gravela, E., P. Pani and G. Bertone. Changes in the hepatotoxicity by DMN in relation to modifications of the drug metabolizing enzyme system. The Italian Journal of Biochemistry 23:402-411. 1974.

Greenblatt, M. and S. S. Mirvish. Dose response studies with concurrent administration of piperazine and sodium nitrite to strain A. mice. The Journal of the National Cancer Institute 49:119-124. 1972.

Greenblatt, M., S. S. Mirvish and B. T. So. Nitrosamine studies: Induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice. The Journal of the National Cancer Institute 46:1029-1034. 1971.

Hadjiolov, D. The inhibition of dimethylnitrosamine carcinogenesis in the rat liver by aminoacetonitrile. Zeitschrift fuer Krebsforschung 76:91-92. 1971.

Halver, J. E. Aflatoxicosis and rainbow trout hepatoma. In: Symposium on Mycotoxins and Foodstuffs, 1964 Proceedings. Cambridge, Mass., M.I.T. Press, 1965a. pp. 209-234.

Halver, J. E. Hepatoma in fish. In: Primary Hepatoma. ed. by W. J. Burdette, Salt Lake City, University of Utah Press. 1965b. pp. 103-112.

Halver, J. E. Crystalline aflatoxin and other vectors for trout hepatoma. In: Trout Hepatoma Research Conference Paper, ed. by J. E. Halver and I. A. Mitchell. Research Report 70. Washington, D.C., Bureau of Sport Fisheries and Wildlife, 1967. pp. 78-102.

Hawksworth, G. and M. J. Hill. The formation of nitrosamines by human intestinal bacteria. Biochemistry Journal 122:28-29. 1971.

Heidelberger, C. Chemical carcinogenesis. Annual Review of Biochemistry 44:79-121. 1975.

Herrold, K. M. Histogenesis of malignant liver tumors induced by dimethylnitrosamine. An experimental study in Syrian hamsters. The Journal of the National Cancer Institute 39:1099-1111. 1967.

Hoch-Ligeti, C., M. F. Argus and J. C. Arcos. Combined carcinogenic effects of dimethylnitrosamine and 3-methylcholanthrene in the rat. The Journal of the National Cancer Institute 40:535-549. 1968.

Hoffmann, D., G. Rathkamp and Y. Y. Liu. Chemical studies on tobacco smoke. XXVI. On the isolation and identification of volatile and nonvolatile N-nitrosamines and hydrazines in cigarette smoke. Proceedings of the Third Meeting on the Analysis and Formation of N-nitroso Compounds; International Agency for Research on Cancer. Lyon, France. October 17-20, 1973. pp. 159-165.

Humason, G. L. Animal Tissue Techniques. San Francisco, W. H. Freeman, 1962. 468 p.

International Agency for Research on Cancer. Monographs on the Elevation of Carcinogenic Risk of Chemicals to Man. Vol. 1, 1972. World Health Organization-IARC. Lyon, France. pp. 95-106.

Ishikawa, T., T. Shimamine and S. Takayama. Histologic and electron microscopy observations on DEN-induced hepatomas in small aquarium fish. The Journal of the National Cancer Institute 55:909-916. 1975.

Jones, H. B. and A. Grendon. Environmental factors in the origin of cancer and estimation of the possible hazard to man. Food and Cosmetics Toxicology 13:251-268. 1975.

Khudolei, V. V. The induction of hepatic tumors by nitrosamines in aquarium fish (Lebistes reticulatus). Voprosy Onkologii 17:67-72. 1971.

Koppang, N. The toxic effects of dimethylnitrosamine in sheep. Acta Veternaria Scandinavica 15:533-543. 1974a.

_____. Dimethylnitrosamine formation in fish meal and toxic effect in pigs. American Journal of Pathology 74:95-106. 1974b.

_____. Toxic effects of dimethylnitrosamine in cows. The Journal of the National Cancer Institute 52:523-531. 1974c.

Koppang, N. and H. Rimeslatten. The toxic and carcinogenic effect of dimethylnitrosamine (DMNA) in mink. International Agency for Research on Cancer. Fourth Meeting on the Analysis and Formation of N-nitroso Compounds. Tallinn, Estonian, SRR, 1-2 October, 1975. World Health Organization-IARC. In Press.

Kroes, R., G. J. VanEsch and J. W. Weiss. Philosophy of 'no effect level' for chemical carcinogens. Proceedings of the International Symposium on Nitrite in Meat Products, Zeist, Pudoc, Wageningen. 1973. pp. 227-241.

Kruger, F. W. Metabolism of nitrosamines in vivo. II. On the methylation of nucleic acids by aliphatic di-n-alkylnitrosamines in vivo, caused by β-oxidation. The increased formation of 7-methyl-guanine after application of β-hydroxypropyl-nitrosamine compared to that after application of di-n-propyl-nitrosamine. Zeitschrift fuer Krebsforschung 79:90-97. 1973.

Kruger, F. W. and B. Bertram. Metabolism of nitrosamines in vivo. III. On the methylation of nucleic acids by aliphatic di-n-alkyl-nitrosamines in vivo resulting from β-oxidation: The formation of 7-methyl-guanine after application of 2-oxo-propyl-propyl-nitrosamine and methyl-propyl-nitrosamine. Zeitschrift fuer Krebsforschung 80:189-196. 1973.

Kruger, F. W., and B. Bertram. Metabolism of nitrosamines in vivo. IV. Isolation of 3-hydroxyl-1-nitrosopyrrolidine from rat urine after application of 1-nitrosopyrrolidine. Zeitschrift fuer Krebsforschung 83:255-260. 1975.

Kruger, F. W., G. Walker and M. Wiessler. Carcinogenic action of DMN in trout not related to methylation of nucleic acids and protein in vivo. Experientia 26:520-522. 1970.

Lee, D. J. and G. B. Putnam. The response of rainbow trout to varying protein/energy ratios in a test diet. The Journal of Nutrition 103:916-922. 1973.

Lee, D. J. and J. H. Wales. Observed liver changes in rainbow trout (Salmo gairdneri) fed varying levels of a casein-gelatin mixture and herring oil in experimental diets. Journal of Fisheries Research Board of Canada 30:1017-1020. 1973.

LePage, R. N. and G. S. Christie. Induction of liver tumors in the rabbit by feeding dimethylnitrosamine. British Journal of Cancer 23:125-131. 1969a.

LePage, R. N. and G. S. Christie. Induction of liver tumors in the guinea pig by feeding dimethylnitrosamine. Pathology 1:49-54. 1969b.

Lijinsky, W. Carcinogen dimethylnitrosamine produced in vivo from nitrite and aminopyrine. Nature 236:177-178. 1972.

_____.Nitrites represent a problem, not a scare. Community Nutrition Institute Weekly Report 6:4-6. July 15, 1976.

_____. Reaction of drugs with nitrous acid as a source of carcinogenic nitrosamines. Cancer Research 34:255-258. 1974.

Lijinsky, W. and S. S. Epstein. Nitrosamines as environmental carcinogens. Nature 225:21-23. 1970.

Lijinsky, W., H. Garcia, L. Keefer, J. Loo and A. E. Ross. Carcinogenesis and alkylation of nucleic acids by nitrosomethylurea administered by intraportal injection. Cancer Research 33:893-897. 1972.

Lijinsky, W., L. Keefer, J. Loo, and A. E. Ross. Studies of alkylation of nucleic acids in rats by cyclic nitrosaimes. Cancer Research 33:1634-1641. 1973.

Lijinsky, W., J. Loo and A. E. Ross. Mechanism of alkylation of nucleic acids by nitroso dimethylamine. Nature 218:1174-1175. 1968.

Lijinsky, W., and A. E. Ross. Alkylation of rat liver nucleic acids not related to carcinogenesis by N-nitrosamines. The Journal of the National Cancer Institute 42:1095-1100. 1969.

Lijinsky, W., and H. W. Taylor. Carcinogenicity of methylated nitrosopiperidines. International Journal of Cancer 16:318-322. 1975.

Magee, P. N. Toxicity of Nitrosamines: Their possible human health hazards. Food and Cosmetics Toxicology 9:207-218. 1971.

Magee, P. N. and J. M. Barnes. Carcinogenic nitroso compounds. Advances in Cancer Research 10:163-246. 1967.

Magee, P. N. and J. M. Barnes. Induction of kidney tumors in the rat with dimethylnitrosamine (N-nitroso-dimethylamine). Journal of Pathology and Bacteriology 84:19-31. 1962.

Magee, P. N. and J. M. Barnes. The production of malignant primary hepatic tumors in the rat by feeding dimethylnitrosamine. British Journal of Cancer 10:114-122. 1956.

Magee, P. N. and J. M. Barnes. The experimental production of tumors in the rat by dimethylnitrosamine (N-nitroso-dimethylamine). Acta Unio Internationalis Contra Cancrum 15:187-190. 1959.

Magee, P. N., A. Hawks, B. W. Stewart, and P. F. Swann. Mechanism of carcinogenesis by alkylnitrosamine and related compounds. Pharmacology and the Future of Man. Proceedings of the 5th International Congress of Pharmacology, San Francisco, Vol. 2, 1972. pp. 140-149.

Maling, H. M., B. Stripp, I. G. Sipes, B. Highman, W. Saul and M. A. Williams. Enhanced hepatoxicity of $CCl_4$, thioacetamide and DMN by pretreatment of rats with EtOH and some comparisons with potentiation by isopropanol. Toxicology and Applied Pharmacology 33:291-308. 1975.

McLean, A. E. and P. A. Day. The use of new methods to measure the effect of diet and inducers of microsomal enzyme synthesis on cytochrome P-450 in liver homogenates and on metabolism of DMN. Biochemical Pharmacology 23:1173-1180. 1974.

McLean, A. E. and P. N. Magee. Increased renal carcinogenesis by dimethylnitrosamine in protein deficient rats. British Journal of Experimental Pathology 51:587-590. 1970.

Mirvish, S. S. Formation of N-nitroso compounds: Chemistry, kinetics and in vivo occurrence. Toxicology and Applied Pharmacology 31:325-351. 1975.

Mirvish, S. S. Kinetics of dimethylnitrosamine nitrosation in relation to nitrosamine carcinogenesis. The Journal of the National Cancer Institute 44:633-639. 1970.

Mirvish, S. S., K. Patil, P. Ghadirian and V. R. C. Kommineni. Disappearance of nitrite from the rat stomach. Contribution of emptying and other factors. The Journal of the National Cancer Institute 54:869-875. 1975.

Mohr, U., J. Althoff, and A. Authaler. Diaplacental effect of the carcinogen diethylnitrosamine in the Syrian golden hamster. Cancer Research 26:2349-2352. 1966.

Montesano, R., A. J. Ingram and P. N. Magee. Metabolism of dimethylnitrosamine by amphibians and fish in vitro. Experientia 29: 599-601. 1973.

Montesano, R. and P. N. Magee. Comparative metabolism in vitro of nitrosamines in various animal species including man. In: Chemical Carcinogenesis Essays. Proceedings of a Workshop on approaches to assess the significance of experimental chemical carcinogenesis data for man. International Agency for Research on Cancer, Scientific Publication No. 10, Lyon, France. 1974. pp. 39-56.

Murphy, G. P., E. A. Mirand, G. S. Johnston, J. D. Schmidt and W. W. Scott. Renal tumors induced by a single dose of dimethylnitrosamines: Morphologic, fuctional, enzymatic and hormonal characterizations. Investigative Urology 4:39-56. 1966.

Napalkov, N. P. and V. A. Alexandrov. On the effects of blastomogenic substances on the organism during embryogenesis. Zeitschrift fuer Krebsforschung 71:32-50. 1968.

Nixon, J. E., R. O. Sinnhuber, D. J. Lee, M. K. Landers, and J. R. Harr. Effect of cyclopropenoid compounds on the carcinogenic activity of diethylnitrosamine and aflatoxin $B_1$ in rats. The Journal of the National Cancer Institute 53:453-459. 1974.

Ong, T. and H. V. Malling. Microsomal activation of DMN to metabolites mutagenic in Neurospora crassa. Mutation Research 31:195-197. 1975.

Otsuka, H. and A. Kuwahara. Hemangiomatous lesions of mice treated with nitrosodimethylamine. Gann 62:147-156. 1971.

Pour, P., J. Althoff, A. Cardesa, F. W. Kruger and U. Mohr. Effect of β-oxidized nitrosamines on Syrian golden hamsters. II. 2-oxopropyl-n-propyl-nitrosamine. The Journal of the National Cancer Institute 52:1869-1874. 1974.

Pour, P., F. W. Kruger, J. Althoff, A. Cardesa and U. Mohr. Effect of β-oxidized nitrosamines on Syrian hamsters. III. 2,2'dihydroxydi-n-propylnitrosamine. The Journal of the National Cancer Institute 54:141-146. 1975.

Preussmann, R. Toxicity of nitrite and N-nitroso compounds. Proceedings of the International Symposium on Nitrite in Meat Products, Zeist, Pudoc, Wageningen. 1973. pp. 217-226.

Reznik, G., U. Mohr, and F. W. Kruger. Carcinogenic effects of di-n-propyl-nitrosamine, β-OH-propyl-n-propyl-nitrosamine and methyl-n-propyl-nitrosamine on Sprague-Dawley rats. The Journal of the National Cancer Institute 54:937-941. 1975.

Riopelle, J. L. and G. Jasmin. Nature, classification and nomenclature of kidney tumors induced in the rat by dimethylnitrosamine. The Journal of the National Cancer Institute 42:643-662. 1969.

Scanlan, R. A. N-nitrosamines in foods. CRC Critical Reviews in Food Technology 5:357-402. 1975.

Schmahl, D. and H. Osswald. Carcinogenesis in different animals species by diethylnitrosamine. Experientia 23:497-498. 1967.

Schmahl, D. and R. Preussmann. Cancerogene Wirkung von Nitroso-dimethylamin bei Ratten. Naturwissenschaften 46:175-181. 1959.

Schoenhard, G. L., D. J. Lee, S. E. Howell, N. E. Pawlowski, L. M. Libbey and R. O. Sinnhuber. Alfatoxin $B_1$ metabolism to alfatoxicol and derivatives lethal to Bacillus subtilis GSY 1057 by rainbow trout (Salmo gairdneri) liver. Cancer Research 36:2040-2045. 1976.

Schoental, R. The mechanism of action of the carcinogenic nitroso and related compounds. British Journal of Cancer 28:426-439. 1973.

Sebranek, J. G. and R. G. Cassens. Nitrosamines: A review. Journal of Milk and Food Technology 36:76-91. 1973.

Shank, R. C. Toxicology of N-nitroso compounds. Toxicology and Applied Pharmacology 31:361-368. 1975.

Shapley, D. Nitrosamines: Scientists on the trail of prime suspect in urban cancer. Science 191:268-270. 1976.

Simon, R. C., A. Dollar and E. A. Smuckler. Descriptive classification on normal and altered histology of trout livers. In: Trout Hepatoma Research Conference Papers, ed. by J. E. Halver and I. A. Mitchell. Research Report 70. Washington, D. C., Bureau of Sport Fisheries and Wildlife, 1967. 199 p.

Smetanin, E. E. On transplacental blastomogenic effect of dimethylnitrosamine and nitrosomethylurea. Voprosy Onkologii 17:75-81. 1971.

Somogyi, A., A. H. Conney, R. Kantzmant and B. Solymoss, Protection against dimethylnitrosamine toxicity by pregnenolone-16 carbonitrile. Nature 237:61-63. 1972.

Stanton, M. F. Diethylnitrosamine-induced hepatic degeneration and neoplasia in the aquarium fish, Branchydanio rerio. The Journal of the National Cancer Institute 34:117-130. 1965.

Swann, P. F. The toxicology of nitrate, nitrite and N-nitroso compounds. The Journal of the Science of Food and Agriculture 26:1761-1770. 1975.

Takayama, S. and K. Oota. Induction of malignant tumors in various strains of mice by oral administration of N-nitroso-dimethylamine and N-nitroso-diethylamine. Gann:46:189-199. 1965.

_____. Malignant tumors induced in mice fed with N-nitroso-dimethylamine. Gann 54:465-469. 1964.

Tannenbaum, S. R., A. J. Sindkey, M. Weismann and W. Bishop. Nitrite in human saliva. Its possible relationship to nitrosamine formation. The Journal of the National Cancer Institute 53: 79-84. 1974.

Terracini, B. and P. N. Magee. Renal tumors in rats following injection of dimethylnitrosamine at birth. Nature 202:502-503. 1964.

Terracini, B., P. N. Magee, and J. M. Barnes. Hepatic pathology in rats on low dietary levels of dimethylnitrosamine. British Journal of Cancer 21:559-565. 1967.

Terracini, B., G. Palestro, M. Ramella Gigliardi and R. Montesano. Carcinogenicity of dimethylnitrosamine in Swiss mice. British Journal of Cancer 20:871-876. 1966.

Terracini, B., G. Palestro, S. Rua and A. Trevisio. Studio sul ruolo dell'iperplasia compensatoria nella cancerogenesis renale da dimetilnitrosamina nel ratto. Tumori 55:357-359. 1969.

Tomatis, L. and F. Cefis. The effects of multiple and single administration of dimethylnitrosamine to hamsters. Tumori 53:447-451. 1967.

Tomatis, L., P. N. Magee and P. Shubik. Induction of liver tumors in Syrian golden hamsters by feeding dimethylnitrosamine. The Journal of the National Cancer Institute 33:341-345. 1964.

Toth, B., P. N. Magee and P. Shubik. Carcinogenesis study with dimethylnitrosamine administered orally to adult and subcutaneously to newborn BALB-c mice. Cancer Research 24:1712-1721. 1964.

Venkatesan, N., J. C. Arcos and M. F. Argus. Amino acid induction and carbohydrate repression of dimethylnitrosamine demethylase in rat liver. Cancer Research 30:2563-2567. 1970a.

Venkatesan, N., M. F. Argus and J. C. Arcos. Mechanism of 3-methylcholanthrene-induced inhibition of dimethylnitrosamine demethylase in rat liver. Cancer Research 30:2556-2562. 1970b.

Vesselinovitch, S. D. The sex dependent difference in the development of liver tumors in mice administered dimethylnitrosamine. Cancer Research 29:1024-1027. 1969.

Wales, J. H. Hepatoma in rainbow trout. In: A Symposium on Diseases of Fishes and Shellfishes. Sniesko, ed. Special Publication No. 5, American Fish Soc., Washington, D.C., pp. 351-365.

Wales, J. H. and R. O. Sinnhuber. Trout hepatoma: Fibrosis and lymphocytosis as supressive mechanisms in the rainbow trout (Salmo gairdneri). The Anatomical Record 175:97-106. 1973.

Weekes, U. Y. Metabolism of dimethylnitrosamine to mutagenic intermediates by kidney microsomal enzymes and correlation with reported host susceptibility to kidney tumors. The Journal of the National Cancer Institute 55:1199-1201. 1975.

Wishnok, J. S. and M. C. Archer. Structure-activity relationships in nitrosamine carcinogenesis. British Journal of Cancer 33:307-311. 1976.

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

Zak, F. G., J. H. Holzner, E. J. Singer and H. Popper. Renal and pulmonary tumors in rats fed dimethylnitrosamine. Cancer Research 20:96-99. 1960.