THOMAS CLEMMER HOLLOWAY. Determination of the Mutagenicity of
Nitrocarbazole and 2-Hydroxynitrocarbazole Compounds Postulated to Occur
in Ambient Air. (Under the direction of Dr. L. M. Ball.)

ABSTRACT

Nitro derivatives of the heterocyclic compounds carbazole and 2-
hydroxycarbazole were synthesized and tested in the Ames plate-
incorporation assay in order to evaluate the contribution of these
compounds to the mutagenicity of hazardous pollutants in ambient air and
cigarette smoke. The results obtained in Salmonella typhimurium strain
TA 98 found 3,6-dinitrocarbazole to be the most mutagenic derivative,
followed by 2-hydroxy-1,3,6-trinitrocarbazole and 2-hydroxy-3-nitro-
carbazole. 2-hydroxy-1,3-dinitrocarbazole was moderately active, and 1-
nitrocarbazole weakly active, with 1,6-dinitrocarbazole and 3-nitro-
carbazole ranging from weakly to moderately active. Carbazole, 2-
hydroxycarbazole and 2-hydroxy-1-nitrocarbazole were completely non-
mutagenic. These varying activities appear to derive from the different
structures of the compounds, mainly the orientation of the nitro group,
the presence of the hydroxy group, and the extent of nitro-substitution,
as well as from the degree of nitroreduction by the "classical"
nitroreductase in Salmonella and by the nitroreductases in the S9 mix,
with the oxidases in S9 also possibly playing a role.

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I. INTRODUCTION

Concern over the presence of polycyclic aromatic hydrocarbons, or
PAH, in the environment, from industrial and auto emissions and the
danger they pose to public health has heightened in recent years. Of
particular concern are the nitro-PAH, which have been detected and
identified in extracts from ambient air pollution (Talcott and Harger,
1981; Pitts et al., 1983; Nielsen et al., 1984) and diesel exhaust
(Schuetzle et al., 1982; Xu et al., 1982) and found to contribute
significantly to the non-S9 or direct-acting mutagenicity of those
extracts (Wang et al., 1980; Talcott and Harger, 1981; Pitts et al.,
1982b).

As a result, studies have been undertaken to understand the
mechanism of formation of nitro-PAH in the environment during combustion
(Pitts et al., 1982a) or atmospheric (Pitts et al., 1985) processes and
their mutagenic (McCoy et al., 1981; Mermelstein et al., 1981) and
carcinogenic (Hirose et al., 1984; Moller et al., 1989) potential.

While the nitro- and dinitropyrenes have been recognized as
significant contributors to this direct-acting mutagenicity (Salmeen et
al., 1984), the rest of the mutagenicity in these extracts may be spread
among other nitro-PAH compounds, such as nitrocarbazoles, which have
been detected in the environment (Bayona et al., 1988) and found to be
mutagenic (Lavoie et al., 1981).

The objective of this research on nitrocarbazoles and their
oxidized form, the 2-hydroxynitrocarbazoles, was to assess their

contribution to the mutagenicity associated with hazardous pollutants in ambient air and cigarette smoke and to the potential health risk arising from exposure.

This objective was approached through the following tasks:

(1) Nitration of the carbazole and 2-hydroxycarbazole compounds using dinitrogen tetraoxide and nitric acid.

(2) Purification of the resulting nitrated compounds or isomers using silica gel column chromatography.

(3) Identification of the individual isomers using electron impact mass spectrometry, proton nuclear magnetic resonance, and melting point determination.

(4) Characterization of their bacterial mutagenicity, with and without S9 metabolic activation, using the Ames Salmonella typhimurium histidine-reversion assay.

II. BACKGROUND

2.1. Nitro-PAH in the Environment

2.1(a). Occurrence in the Atmosphere

While much of the attention in past years has focused on the PAH as the primary genotoxic agents in ambient air pollution, research in recent years has suggested that nitro-PAH also play an important role.

The presence of nitro-PAH in ambient air particulate matter has been well established (Talcott and Harger, 1981; Pitts et al., 1982b; Nielsen et al., 1983a, 1984). Nitro-PAH have been detected in a number of sources of air pollution--diesel and gasoline engine exhaust particulate matter, cigarette smoke condensates, fly ash particles, and emissions from incinerators, residential home heaters and wood-burning stoves (Rosenkranz and Mermelstein, 1983).

Diesel emissions have become a particular source of concern because economical diesel-powered vehicles produce significantly more particulate emissions than gasoline-powered vehicles of comparable performance. Furthermore, diesel exhaust particles are submicron in size and can deposit in the deepest regions of the respiratory system (Chan, et al., 1981). Many nitro-PAH have been identified in diesel particulate extracts (Pitts et al., 1982a; Schuetzle et al., 1982; Xu et al., 1982; Paputa-Peck et al., 1983; Campbell and Lee, 1984), including nitrocarbazoles (Wolfbeis et al., 1983; Bayona et al., 1988).

Carbazole, the parent compound of the nitrocarbazoles, has itself been detected in atmospheric particulate matter (Sawicki et al., 1961;

Bender et al., 1964b; Lane et al., 1973; Thomas et al., 1978; Moriske and Ruden, 1988), diesel fuel (Williams et al., 1986), petroleum (Helm et al., 1960), tobacco smoke (Hoffman et al., 1968; Hoffman and Wynder, 1971; Lee et al., 1976; Dong et al., 1978; Snook et al., 1978), combustion products of domestic fuel oil (Leary et al., 1983), tarring fumes (Sawicki et al., 1962; Bender et al., 1964; Hittle and Slukel, 1976), coke oven emissions (Buonicore, 1979), and waste gas from aluminum manufacturing (Hung and Bernier, 1983). Carbazole is especially prevalent, however, in coal tar and coal liquids (Later et al., 1982; Amateis and Taylor, 1983; Wood et al., 1984; Wright et al., 1984), which when combusted could release carbazole and any of its derivatives into the atmosphere as particulate matter.

2-hydroxycarbazole, a possible minor metabolite of carbazole and possible air pollutant, has also been detected in coal liquids (Bender et al., 1964a; Nishioka et al., 1985; Allen et al., 1987). Hydroxy-nitrocarbazoles have yet to be detected, but other hydroxynitro-PAH have been observed in ambient air (Nishioka et al., 1988) and diesel particulate matter (Schuetzle, 1983).

2.1(b). Formation in the Atmosphere

Nitro-PAH are readily formed when products of incomplete combustion or PAH, oxides of nitrogen, and traces of acid are present simultaneously (Rosenkranz and Mermelstein, 1983). This can occur during emissions from combustion sources (Pitts et al., 1982a; Schuetzle et al., 1982), during sample collection (Pitts et al., 1978), or during transport through the atmosphere by the reaction of PAH adsorbed to particulate matter with the gaseous pollutants NO_2 and HNO_3 (Nielsen et al., 1983b; Pitts et al., 1985).

Nitration during transport was tested with the PAH benzo(a)pyrene and perylene in simulated atmospheres containing 1 ppm NO_2 and traces of HNO_3. Directly mutagenic nitro derivatives were formed from both compounds (Pitts et al., 1978). Carbazole has also been shown to be susceptible to nitration by NO_2, forming two unidentified, directly mutagenic mononitro isomers, molecular weight 212 (Tokiwa et al., 1981).

Gaseous pollutants such as NO_2 and HNO_3 are expected to react as electrophiles with the carbon atoms of highest electron density on the PAH compound (van Cauwenberghe and van Vaeck, 1983). In the case of carbazole, the high electron density of the pyrrole ring predisposes it to nitration, with the resulting 9-nitro compound undergoing rearrangement to the 1- and 3-nitro isomers (Kyziol and Daszkiewicz, 1984). These isomers were most likely the ones formed by Tokiwa et al. after exposure of carbazole to NO_2.

Oxidized and nitrated compounds, such as the hydroxynitro-carbazoles, could also be expected to occur in ambient air as a result of such gas phase reactions, with ozone and peroxyacetylnitrate, or PAN, as possible photooxidants (Pitts, 1983).

Once the nitro-PAH and hydroxynitro-PAH have been formed, it is possible that they may photodegrade. Such a reaction has been observed on soot and airborne particulate matter for some nitro-PAH, such as 6-nitrobenzo(a)pyrene. The photodegradation involves rearrangement of the nitro-PAH to the corresponding aryl nitrite and subsequent dissociation into NO and a phenoxy-type radical. Ultimately, a quinone is formed (Nielsen et al., 1983b).

2.1(c). Direct-Acting Mutagenicity of Extracts

It has been demonstrated in a number of studies that nitro-PAH are responsible for a significant portion of the direct mutagenicity in particulate organic matter extracts (Wang et al., 1980; Talcott and Harger, 1981; Pitts et al., 1982b). The PAH themselves require metabolic activation in the Ames mutagenicity assay by the addition of liver microsomes to produce mutagenicity (Pitts et al., 1982b; Pitts, 1983; van Cauwenberghe and van Vaeck, 1983). The PAH have been found in the nonpolar fraction of diesel exhaust extract, which contains less than 5% of the direct-acting mutagenicity. Most of the direct-acting mutagenic activity was found in chemical fractions containing compounds of moderate polarity, such as 1-nitropyrene, the major nitro-PAH in diesel particulate matter (Schuetzle et al., 1982). Salmeen et al. (1984) found that 30-40% of the total direct-acting mutagenicity of the extracts could be attributed to those fractions containing compounds with retention times similar to those of 1-nitropyrene, 1,3-, 1,6-, and 1,8-dinitropyrene, and 3- and 8-nitrofluoranthene. However, Gibson (1983) found that only 8-12% of the direct-acting mutagenicity could be attributed to nitro- and dinitropyrenes.

That nitro-PAH could be significant in the direct-acting mutagenicity has been indirectly proven by the decrease in mutagenicity of the moderately polar diesel extracts containing nitro-PAH in Salmonella typhimurium strains TA 98ND and TA 98/1,8-DNP$_6$. The TA 98ND strain lacks an enzyme necessary for activating most nitro-PAH, like 1-nitropyrene, to an active form that can interact with the DNA. TA 98/1,8-DNP$_6$ lacks another enzyme necessary for activating 1,8-dinitropyrene (Salmeen et al., 1984).

Talcott and Harger (1981) found that the direct-acting mutagenicity of extracts from city air disappeared when the extracts were treated with a reducing agent. Since the aromatic amines derived from nitroaromatics by reduction reactions are usually nonmutagenic without microsomal activation, it could be reasoned that the nitro-PAH were the agents responsible for the direct-acting mutagenicity.

Furthermore, the organic matter extracted from city air was preferentially mutagenic to Salmonella typhimurium frameshift tester strain TA 98, as are many nitroaromatic compounds (Talcott and Harger, 1981).

2.1(d). Deposition in the Respiratory Tract

Direct-acting frameshift mutagens, detectable by TA 98, have been detected in city air on pollutant particles small enough (less than 2 microns) to penetrate into the parenchyma of human lungs (Talcott and Harger, 1980). Diesel particles are also readily deposited in the deepest regions of the respiratory system (Chan et al., 1981).

Particles in the bronchial airways can be removed by mucociliary action to the oral cavity and then deposited in the gastrointestinal tract (Chan et al., 1981), where nitroreduction to activated species could be performed by gut flora (Ball et al., 1984b). Particles deposited in the alveolar region are effectively removed by the phagocytic action of pulmonary alveolar macrophages to the ciliated airways or to the lymphatic system (Chan et al., 1981). It has been demonstrated in vitro that mutagens such as 1-nitropyrene can be released from diesel particles in the presence of lung macrophages, and that lung macrophages may have the capability to metabolize those mutagenic nitroaromatics (King et al., 1983).

2.2. Structure-Activity Relationships

Vance and Levin (1984) defined four structural determinants of mutagenic activity for nitro-PAH:

(1)   Degree of aromaticity of the PAH.

(2)   Resonance stabilization of the reactive electrophile, i.e., the nitrenium ion.

(3)   Orientation of the nitro group.

(4)   Conformation of the nitro group with respect to the plane of the aromatic rings.

The number and arrangement of aromatic rings in nitro-PAH has been found to affect the frameshift mutagenicity of the DNA adduct formed from nitro-PAH, possibly by influencing the adduct's position relative to the DNA helix. By observing the change in mutagenic activity with the progressive addition of five- and six-membered rings to a nitro-benzene ring, Vance and Levin demonstrated that mutagenic activity was a direct function of size. The results suggested an optimal length of three aromatic rings, as seen in 3-nitrofluoranthene, for mutagenic potency (Vance and Levin, 1984).

Increasing the width of the aromatic nucleus to that of two aromatic rings, as seen also in 3-nitrofluoranthene, has similarly been found to increase mutagenic activity. The additional ring may help to stabilize insertion of the aromatic nuclei by increasing the base-stacking forces (Vance and Levin, 1984).

However, any addition of rings beyond this number, as seen in 3-nitrobenzo(k)fluoranthene, may prevent the adduct from being inserted into the DNA due to the extensive strand separation necessary to accommodate the increased length of the molecule into the helix. As a result, it reduces the mutagenic activity. This has been confirmed by

the determination that 3-nitrofluoranthene is a more potent mutagen than its benzo derivative (Vance and Levin, 1984).

Further corroboration of the Vance and Levin results is provided by Rosenkranz and Mermelstein (1983). They found that activity increases from the bicyclic to the tetracyclic ring system, with tetracyclic nitro-PAH the most mutagenic mononitro-PAH reported. Pentacyclic nitro-PAH show an abrupt decrease in direct-acting mutagenicity from tetracyclic nitro-PAH (Rosenkranz and Mermelstein, 1983), as Vance and Levin found for 3-nitrobenzo(k)fluoranthene.

The same trend has been discovered in N-heterocyclics as well. Most of the N-heterocyclic compounds with one to four rings produce no mutagenicity, report Ho et al. (1981), while five-ring compounds have been found to produce significant activities.

Based on these findings, carbazole and its nitro derivatives would not be expected to be as mutagenic as their benzo and dibenzo derivatives.

The differences in mutagenic activity observed for nitro-PAH with homologous structures can be explained by differences in the second structural determinant of mutagenic activity outlined by Vance and Levin, which is resonance stabilization. Protonation of the hydroxylamine metabolite of a nitro-PAH compound can result in dehydration, with formation of an unstable nitrenium ion. Resonance of the positive charge from the nitrenium ion around the aromatic ring produces carbonium ions at the ortho and para positions. Factors that contribute to the stabilization of this aromatic carbonium ion would tend to increase the lifetime of the charged electrophile, thereby increasing its chances of binding with the DNA and of causing a

frameshift mutation. As a result, the mutagenic potency of the compound could be increased. An example of this is the greater mutagenic potency of 6-nitroindene over 5-nitroindene due to its ability to further resonate the positive charge from the benzene ring into the five-membered ring, resulting in a phenylene-like structure (Vance and Levin, 1984).

The presence of electron-donating groups, such as hydroxy groups, on ring carbons involved in resonance stabilization may effectively prevent resonance stabilization or reaction of the carbonium ion with a nucleophile such as DNA (Vance and Levin, 1984). The hydroxy group can contribute a free pair of electrons to resonance stabilize the positive charge and can then readily lose a hydrogen nucleus to restore electrical neutrality. As a result, the mutagenic potency of nitro-PAH possessing this group is reduced (Vance et al., 1985, 1987). Ball et al. (1984a), for example, observed a marked decrease in the direct-acting mutagenicity of 6-hydroxy-1-nitropyrene from that of 1-nitro-pyrene in Salmonella strain TA 98. 6-Hydroxy-1-nitropyrene required S9 metabolic activation to demonstrate its mutagenic activity in TA 98.

Electron-withdrawing groups, such as nitro groups, on the other hand, can increase the mutagenic potency of nitro-PAH, but not because of resonance stabilization effects. The unmetabolized nitro group exerts a strong electron-withdrawing effect on the aromatic structure, facilitating the initial reductions of another nitro group on the compound and increasing the stability of the hydroxylamine metabolic intermediate, both of which may increase the potential for genetic damage (Vance et al., 1985, 1987).

Further confirmation of this is found in the increase in mutagenicity which accompanies an increase in the nitro-substitution of a PAH compound. Dinitro-PAH, such as 1,8-dinitropyrene and 2,7-dinitrofluorene, have been observed to be much more mutagenic than their mononitro analogs. Maximal activity has been observed with the trinitro-substituted compounds, with mutagenic activity falling off with the addition of a fourth nitro group (McCoy et al., 1981; Mermelstein et al., 1981; Rosenkranz and Mermelstein, 1983). According to Vance et al. (1985, 1987), the reduction in activity may be due to an enzymatic inability to reduce the tetranitro-PAH to its active metabolic intermediate, the very thing that addition of a second nitro group had facilitated. Another possibility besides steric hindrance in enzymatic nitroreduction is steric hindrance in DNA adduct formation due to the excessive number of nitro groups (Rosenkranz and Mermelstein, 1983).

Additional evidence has been found in mammalian cells. While the addition of a single nitro group caused little change in sister chromatid exchanges in Chinese hamster ovary (CHO) cells, the number of mutations produced in the CHO/HGPRT assay did increase with an increasing number of nitro groups (Kitchin et al., 1988).

Based on these results, it would be expected that multinitro derivatives of carbazole would be more mutagenic than mononitro derivatives, and that the presence of a hydroxy group at the C2 position may reduce the mutagenic potency relative to the nonhydroxylated nitrocarbazole compounds.

The relative mutagenicities of positional isomers of nitro-PAH may reflect differences based on the third structural determinant of Vance and Levin, the orientation of the nitro group with respect to the long

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or short axis of the molecule. Nitro-PAH with a nitro group oriented along the long axis were discovered to be more potent mutagens than those with the nitro group oriented along the short axis (Vance and Levin, 1984).

A good example of this is the dramatic positional effects of the nitro group in the carbazole series. The 2-nitro isomer, which has the nitro group positioned along the long axis, was relatively potent, whereas the 1-nitro isomer, which has the nitro group positioned along the short axis, was completely inactive, according to mutagenicity data from Lavoie et al. (1981). These positional effects are even more dramatic when dinitro-PAH are examined (Rosenkranz and Mermelstein, 1983).

The differences in mutagenic activity between these positional isomers may be due to the orientation of the presumptive guanine C8 adduct in the major groove of the DNA helix. Adducts oriented end-on and perpendicular to the central axis of the helix will facilitate intercalation, whereas adducts which lie broadside will not. The orientation of the nitro group along the long axis allows the end-on, perpendicular orientation, whereas orientation along the short axis allows the broadside orientation (Vance and Levin, 1984).

Conformation of the nitro group with respect to the aromatic plane is related to its position on the PAH compound and, therefore, is also important in the determination of mutagenic activity of nitro-PAH. Nitro-PAH with nitro groups sterically forced out of the plane of the aromatic rings by structural features of the PAH, such as location between two peri protons, have been found to be either weakly mutagenic or nonmutagenic. This may be because of their inability to fit into the

active site of nitroreductase enzymes due to steric interactions. As a result, they fail to form stable N-hydroxylamine metabolic intermediates (Fu et al., 1985).

Coplanarity of the nitro groups can be detected by the characteristic deshielding effect of such groups on neighboring protons in their NMR spectra. (Vance and Levin, 1984; Fu et al., 1985). Nitro groups forced out of the plane, however, will create a slight upfield shift of neighboring protons (Fu et al., 1985). Since no carbon-substituted nitro groups in the nitrocarbazole series are located between two peri protons, no such upfield shift should be expected.

Another possible determinant of mutagenicity may be the presence of a nitrogen in the aromatic ring. In general, however, the mutagenic potential of basic type N-heterocyclic compounds, which include the weak base carbazole, are similar to their aromatic hydrocarbon analogs, according to Ho, et al. (1981). The presence of a nitrogen may increase the mutagenicity slightly. For example, 3,4- and 5,6-benzoquinoline were found to be more mutagenic than their analog phenanthrene in a mutant fraction of Salmonella typhimurium resistant to 8-azaguanine (Kaden et al., 1979). However, this effect is not as important as the number of fused rings, for example, in the determination of mutagenic activity (Ho et al., 1981).

Klopman and Rosenkranz (1984) reported another method for determining the relationship between the structure and mutagenic activity of nitro-PAH by the use of a newly developed "Computer-Automated Structure Evolution" program, or CASE, using a database of nitro-PAH compounds. They discovered two activating and two

deactivating fragments in nitro-PAH. To be mutagenic, the nitro-PAH must contain an activating subunit, while nonmutagenic molecules contain either no activating subunit or both an activating and a deactivating subunit. The activities of forty-seven of fifty-three nitro-PAH compounds, or 88.7%, were correctly predicted by this procedure. The activating and deactivating fragments described by Klopman and Rosenkranz and their relation to the nitrocarbazoles are shown in Figure 1 on pages 37 and 38.

Substitution at the carbon atom para to the nitro group in Structure I of Figure 1 is required for mutagenic activity, while the presence of a hydrogen atom at that position in Structure III prevents activity (Klopman and Rosenkranz, 1984).

This approach is supported by the mutagenicity results of the nitrocarbazoles. Of all the nitrocarbazoles, only 2- and 3-nitro-carbazole were found to contain an activating fragment (Klopman and Rosenkranz, 1984), and only those isomers possessed any substantial amount of mutagenicity (Lavoie et al., 1981).

Klopman and Rosenkranz suggested that substitution para to the nitro group may be necessary for activation of the compound by the nitro group, for substrate recognition by activating nitroreductase enzymes, or for DNA adduct formation.

The findings of Vance and Levin may support the last explanation. Since the substitution on the carbon occurs para to the nitro group, this suggests that the nitro group may be oriented along the long axis, thereby allowing the adduct to be better oriented with regard to the DNA helix for covalent binding.

Therefore, it should be expected from these structure-activity relationships that 2- and 3-nitro-carbazole will be the most mutagenic of the mononitro isomers, with any di- or trinitro isomers being even more mutagenic. If the hydroxy group in any of the 2-hydroxynitro-carbazoles interferes in the resonance stabilization of the resultant nitrenium ions, those compounds will have reduced mutagenic activity.

2.3. Genotoxic Activity

2.3(a). Salmonella Mutagenicity

Nitro-PAH are believed to contribute significantly to the direct-acting mutagenicity of air pollution. 1-Nitropyrene has been found to be responsible for a substantial portion of that mutagenicity (Pederson and Siak, 1981a; Schuetzle et al., 1981; Salmeen et al., 1984). Another major nitro-PAH in air pollution is 2-nitrofluorene, the aromatic hydrocarbon analog of 2-nitrocarbazole. Its mutagenic potency ranges from 2.8 to 88 revertants per nanomole without S9 metabolic activation (Rosenkranz and Mermelstein, 1983). The mutagenic potency of nitrated pyrenes (Mermelstein et al., 1981) and nitrated fluorenes (McCoy et al., 1981) has been found to increase with successive addition of nitro groups up to the third substitution. Activity then decreases, in accordance with the structure-activity relationships discussed above.

Carbazole, the parent compound of the nitrocarbazoles, has shown no mutagenic activity of its own in a number of Ames Salmonella mutagenicity studies (Anderson and Styles, 1978; Kaden et al., 1979; Ho et al., 1981; Tokiwa et al., 1981; Lavoie et al., 1982; Bechtold et al., 1985) using frameshift tester strains TA 98 and TA 1538 and base-pair tester strains TA 100 and TA 1535, with and without S9 metabolic activation. The same cannot be said for the nitrocarbazoles, however.

Of the four possible mononitro isomers of carbazole, 2- and 3-nitrocarbazole were active mutagens in TA 98, according to Lavoie, et al. (1981). The activity of 2-nitrocarbazole was not unlike that of the known carcinogen 2-nitrofluorene, which was used as a positive control in the assay (Lavoie et al., 1981).

A summary of the results of Lavoie et al., in revertants per nanomole, is found in Rosenkranz and Mermelstein (1983) and is presented in Table 1 on page 39. According to those results, incorporation of rat-liver homogenate S9 reduced the mutagenic activity of 2-nitro-carbazole by more than 50%, while increasing the mutagenic activity of 3- and 4-nitrocarbazole only slightly. 1-Nitrocarbazole remained inactive when assayed in the presence of S9 (Lavoie et al., 1981).

In line with most other nitro-PAH, the nitrocarbazoles were stronger frameshift mutagens than base-pair mutagens. The mutagenic responses of the nitrocarbazoles in strains TA 1535 and TA 100 were either weak or nonexistent. No mutagenic activity was observed for any of the nitrocarbazoles in TA 1535 with or without S9. Both 1- and 3-nitrocarbazole were inactive as mutagens in TA 100 in the absence of S9, and 3-nitrocarbazole was only weakly active with the addition of S9, with the 1-nitro isomer remaining inactive. 2-nitrocarbazole was somewhat active in the absence of S9, but became inactive with the addition of S9. The 4-nitro isomer was the most active mutagen in TA 100 in either the presence or absence of S9, but was still only slightly mutagenic (Lavoie et al., 1981).

No information has been found in the literature on the bacterial mutagenicity of dinitrocarbazoles, but as previously mentioned, the

mutagenic potency of nitro-PAH increases with increasing number of nitro substitutions, up to the third substitution.

There is also no information on the mutagenicity of 2-hydroxy-carbazole or any of its nitro derivatives, but the hydroxy group is expected to reduce mutagenic potency relative to comparable nitro-carbazole compounds if it interferes with resonance stabilization. Also, its multinitro derivatives are expected to be more mutagenic than their mononitro analogs.

According to Rosenkranz and Mermelstein (1983), the mutagenicity of nitro-PAH is maximal in Salmonella strains containing the pKM101 plasmid, which codes for an error-prone DNA repair system, and missing the uvrB gene, which codes for the DNA excision repair system. Mutagenic activity for most nitro-PAH in Salmonella also requires the presence of the "classical" nitroreductase to reduce the nitro compound to its reactive metabolic intermediate (Rosenkranz and Mermelstein, 1983).

Nitro-PAH have give a variety of responses with the addition of S9 to the assay mixture, ranging from reduction or abolition of mutagenicity, to lack of any effect, to an absolute requirement for mutagenic activation. Incubation of nitro-PAH such as 2-nitrofluorene or 1-nitropyrene with S9 overcomes the block in the nitroreductase-deficient strain TA 98ND. This either suggests that rat microsomes contain nitroreductases capable of reducing the nitro compounds to active intermediates or that they activate the compound by ring oxidation to an epoxide, which then reacts with the DNA, or a combination of the two (Pederson and Siak, 1981b; Rosenkranz and Mermelstein, 1983).

2.3(b). Mammalian Cell Genotoxicity

No information has been found for any of the carbazole compounds with regard to their in vitro mammalian cell genotoxicity. However, other nitro-PAH have shown activity in mammalian cells. This activity includes the induction of unscheduled DNA synthesis, sister-chromatid exchanges (SCE), chromosomal aberrations, gene mutations, and cell transformations (Rosenkranz and Mermelstein, 1983).

In some cases, but not in others, biological activity is dependent on exogenous activation systems such as S9. For example, 2-nitro-fluorene requires S9 for transformation of the hamster BHK-21 cells but not for the transformation of normal human fibroblasts (Rosenkranz and Mermelstein, 1985).

Furthermore, some nitro-PAH are devoid of activity in some systems yet active in others. For example, 1-nitropyrene is nonmutagenic in Chinese hamster lung fibroblasts and demonstrates little or no activity in CHO cells, but induces significant unscheduled DNA synthesis and DNA damage as well as SCE's in other cells (Rosenkranz and Mermelstein, 1985). Also, several nitrofluorenes significantly increased the SCE rate in CHO cells in the presence or absence of S9, but did not produce any mutations in the CHO/HGPRT assay. All this suggests that there may be a sequence of nitroreductions needed for the formation of the ultimate mutagens. These nitroreductions cannot be carried out by many mammalian cells but are readily carried out in bacteria, in which nitro-PAH such as 1-nitropyrene are consistently active (Kitchin et al., 1988).

With regard to cell transformations, the same inconsistency seems to occur from one assay system to another. While nitro-PAH, such as

1-nitropyrene, 1,8-dinitropyrene, 2-nitrofluorene, and 3-nitrofluoranthene all demonstrated cell transformation activity in Syrian hamster embryos (SHE) and normal human fibroblasts as well, no activity at all was seen in the Mouse Balb 3T3 assay. It appears that nitroreduction mediates the transformation of normal human fibroblasts as well, since this enzymatic event was enhanced in the absence of oxygen (Rosenkranz and Mermelstein, 1985). Positive results in this assay system would be very significant for nitro-PAH such as these. Transformation of cultured cells into cells which have most of the properties of cancer cells and induce malignant tumors when injected into susceptible animals is considered highly predictive of the carcinogenic potential of a compound (Rosenkranz and Mermelstein, 1985).

2.4. Carcinogenic Activity

Nitro-PAH detected in ambient air pollution have been shown to be mutagenic (McCoy et al., 1931; Mermelstein et al., 1981; Tokiwa et al., 1981; Rosenkranz and Mermelstein, 1983), and some have been shown to be carcinogenic (El-Bayoumy et al., 1982; Ohgaki et al., 1982, 1984; Hirose et al., 1984). Although no results have been found in the literature on the carcinogenicity of nitrocarbazoles, there are results for 2-nitrofluorene, the aromatic hydrocarbon analog of 2-nitrocarbazole, and the parent compound carbazole. At a level of 1.62 millimoles of 2-nitrofluorene per kilogram of diet in rats, or 342 ppm, 2-nitrofluorene formed tumors in the forestomach, liver, small intestine, mammary gland and ear duct (Miller et al., 1955). 2-Nitrofluorene has also been found in a recent study to be a moderate initiator and a weak promoter of carcinogenesis (Moller et al., 1989).

Although earlier tests, summarized by World Health Organization (1983), gave inconclusive results, the parent compound carbazole has been demonstrated to be an animal carcinogen (Tsuda et al., 1982; Ito et al., 1988). In the study by Tsuda et al. there were significant increases after 96 weeks in the induction of neoplastic lesions in the livers and forestomachs of male and female mice given 0.6% carbazole in the basal diet, or 6000 ppm, as compared to control groups.

The incidence of preneoplastic enzyme-altered focal lesions, widely accepted as early indicators of neoplastic development, was statistically increased, compared to the control group, in male rats given 200 ppm of carbazole in the basal diet for 6 weeks (Ito et al., 1988). The carbazole was fed to the rats two weeks after the administration of a 200 mg/kg, or 200 ppm, intraperitoneal dose of diethylnitrosamine (DEN) to initiate carcinogenesis. Carcinogenic potential was scored by comparing the number and area per square centimeter of positive foci in the liver with those of the corresponding control group given DEN alone (Ito et al., 1988).

No results have been reported for 2-hydroxycarbazole or any of its nitro derivatives with regard to their carcinogenic activity.

2.5. Metabolism and DNA Adduct Formation

Like most mutagens and carcinogens, nitro-PAH are believed to be metabolized to reactive electrophiles in order to exert their effects on the DNA. Based on a comparison with those mutagens and carcinogens, three metabolic pathways are believed possible for nitro-PAH (Howard et al., 1983; Rosenkranz and Mermelstein, 1983):

(1) Reduction of the nitro function to N-hydroxyarylamines.

(2) Oxidation of the hydrocarbon ring to epoxides (the nature of which is influenced by the nitro group).

(3) Ring oxidation followed by nitroreduction.

Bacterial systems such as **Salmonella typhimurium** appear to rely solely on nitroreduction in the metabolic activation of nitro-PAH such as 1-nitropyrene, since mutation assays conducted with nitroreductase-deficient strains of Salmonella gave lower histidine reversion frequencies than those using nitroreductase-proficient strains (Howard et al., 1983). So far, three nitroreductases have been identified in Salmonella - A, B, and C, with B, the "classical" nitroreductase, accounting for 94% of the activity in TA 98 of 1-nitropyrene (Bryant et al., 1983).

The reactive electrophiles formed in bacterial systems as a result of this nitroreduction have been identified as either hydroxyarylamines (Howard et al., 1983) or arylnitrenium ions. Arylnitrenium ions form following non-enzymatic dehydration of the protonated form of the hydroxyarylamines (Vance and Levin, 1984). These electrophiles are produced as intermediates in the metabolic pathway of the nitro-PAH and covalently bind to the DNA at the C-8 position of guanine (Howard et al., 1983). The resulting bulky adducts displace the guanine to which they are covalently linked and swivel into the plane of the helix, resulting in a frameshift mutation (McCoy et al., 1981). These adducts have been detected as N-(deoxyguanosin-8-yl)-1-aminopyrene in the DNA of Salmonella frameshift tester strain TA 1538 following incubation with 1-nitropyrene (Howard et al., 1983).

A strong correlation has been found between the extent of DNA binding of the 1-nitropyrene adducts in TA 1538 and the frequency of induced histidine reversions, or mutagenicity (Howard et al., 1983).

This is supported by the results of Beranek et al. (1982), in which a linear relationship was discovered between the mutagenicity of N-hydroxy-2-aminofluorene compounds, the reactive intermediates of 2-nitrofluorene, and total DNA binding.

Reduction of the nitro function to N-hydroxyarylamines can also occur in eukaryotic cells. Mammalian enzymes which exhibit nitroreductase activity include xanthine oxidase, aldehyde oxidase, DT-diaphorase, cytochrome P-450, and NADPH-cytochrome P-450 reductase (Howard et al., 1983).

Xanthine oxidase catalyzes the 2-electron oxidations of hypoxanthine to xanthine and xanthine to uric acid. The reduced form of this enzyme can, in turn, catalyze the sequential reduction of a nitro compound to nitroso-, N-hydroxy-, and amine derivatives (Howard et al., 1983).

The N-(deoxyguanosin-8-yl)-1-aminopyrene adducts have been detected as major adducts in in vitro incubations of 1-nitropyrene with calf-thymus DNA using xanthine oxidase (Howard et al., 1983) and rat liver microsomal enzymes (Djuric et al., 1986) and in vivo in rats (Stanton et al., 1985). The adducts were even detected, although in small quantities, in cultures of respiratory tract tissues, where oxidation would be considered the major metabolic pathway (Jackson et al., 1985).

The major pathway of metabolism for PAH in eukaryotic cells, however, involves ring oxidation reactions, catalyzed mainly by the cytochrome P-450 mixed-function oxidase system (Ball and Lewtas, 1985).

In vitro incubations of 1-nitropyrene with hepatic microsomes resulted primarily in a series of hydroxylated metabolites, with

different DNA-binding strengths observed after nitroreduction by rat liver cytosol or xanthine oxidase (Djuric et al., 1986), and with different mutagenicities (Ball et al., 1984a). K-region epoxides were also formed as minor products which bind directly to DNA to give adducts. The ultimate reduced form of 1-nitropyrene, 1-aminopyrene, was also formed as a minor metabolite (Djuric et al., 1986).

In vivo metabolism of 1-nitropyrene in the rat gave a series of hydroxy-N-acetyl-aminopyrene urinary metabolites, which had undergone enterohepatic circulation, involving ring oxidation in the liver, nitroreduction in the gastrointestinal tract, and N-acetylation in the liver. The time course of excretion suggested that enterohepatic recirculation was necessary for, and gut flora were involved in, the formation of these metabolites. As mentioned previously, the gastrointestinal tract constitutes the major site for in vivo reduction of 1-nitropyrene and other nitro-PAH (Ball et al., 1984b).

Ring-oxidized nitro-PAH could be detoxified in the liver by conjugation with glucuronic acid at the hydroxy group, as in the case of the hydroxynitrofluorenes. While these conjugates have been verified as nonmutagenic, they could be hydrolyzed by intestinal B-glucuronidase and eventually enter the enterohepatic circulation, exposing both the intestines and the liver to potent mutagenic metabolites (Moller et al., 1989).

While no information is available on the metabolism of any nitrocarbazoles or hydroxynitrocarbazoles, the parent compound carbazole has been shown to be metabolized by ring oxidation to a hydroxy compound. 3-Hydroxycarbazole, conjugated with glucuronic acid, was detected as the major urinary metabolite in rats and rabbits after

administration of carbazole. Position-3 hydroxylation is in accordance with an attack by oxidizing enzymes at the most reactive position of the carbazole nucleus. Minor metabolites included 3-methoxycarbazole and an unidentified dihydroxycarbazole (Johns and Wright, 1964).

An unidentified hydroxycarbazole metabolite, presumably 3-hydroxycarbazole, was also detected as the major metabolite when female rats were orally administered carbazole. No mutagenicity was detected by the spore rec assay or the Ames Salmonella assay with strains TA 98 and TA 100 (Hasegawa et al., 1985), but no mention was made whether S9 had been included.

Nitrocarbazoles may be similarly metabolized. Nitrated 2-hydroxycarbazoles are being used as models for any putative 2-hydroxy-X-nitrocarbazole metabolites.

2.6. Analytical Techniques

2.6(a). Nitration

PAH compounds like carbazole and 2-hydroxycarbazole can be nitrated by a variety of methods, including dinitrogen tetraoxide (N_2O_4) in methylene chloride and nitric acid (HNO_3) in acetic acid or acetic anhydride.

Nitration of PAH with N_2O_4 by the method of Radner (1983) is very clean and rapid and provides under mild conditions almost quantitative yields of the most electronically favored mononitro-PAH after a simple work-up procedure that minimizes the handling of those hazardous PAH compounds (Radner, 1983).

The dissolved N_2O_4 dissociates into NO^+ and NO_3^- and is in equilibrium with its monomer NO_2. It is impossible at present to decide

whether the catalytic effect of N_2O_4 or NO_2 is caused by N_2O_4, NO_2, or NO^+, but recent experimental data indicate perhaps that dinitrogen tetraoxide is the real catalytic species (Nielsen, 1984).

No reports have been found in the literature on the nitration of carbazole or 2-hydroxycarbazole with N_2O_4.

To form nitro products, PAH are generally nitrated with HNO_3 in acetic acid at high temperatures or in acetic anhydride at low temperatures. Yields from this method are normally good, with a wider range of isomers possible than with N_2O_4, especially with acetic anhydride (Ruehle et al., 1985). However, work-up procedures can be tedious, and polynitration is sometimes encountered (Radner, 1983). The nitrating species is believed to be the protonated form of acetyl nitrate (Ruehle et al., 1985).

There have been a number of studies of the nitration of carbazole with HNO_3. The isomers formed from the nitration of carbazole with 60% HNO_3 in acetic acid (Ziersch, 1909; Lindemann, 1924; Preston et al., 1942) and in water (Morgan and Mitchell, 1931) at 80°C include mainly 3- and 1-nitrocarbazole. These compounds were later found to occur in an approximately 7:3 ratio in acetic acid, contaminated with carbazole and its dinitro derivatives (Kyziol and Daszkiewicz, 1984).

The formation of 3- and 1-nitrocarbazole has been hypothesized by Kyziol and Daszkiewicz (1984) to be a two-step process involving formation and intramolecular rearrangement of 9-nitrocarbazole. The high electron density of the pyrrole ring predisposes the compound to nitration at the 9-position on the pyrrole nitrogen. 3- and 1-nitro-carbazole isomers result from 9-nitrocarbazole in a nitramine rearrangement of that compound, following a "cartwheel" mechanism. This

reaction is illustrated by Kyziol and Daszkiewicz (1985) in Figure 2 on page 40.

C-nitrosooxy compounds are formed in the solution rapidly but in a minute amount and remain in the equilibrium state with the N-nitro form. Transformation of the nitrites (structures 1a and 1c in Figure 2) into the stable compounds (structures 2 and 3) occurs in the last, rate-limiting step, and is accompanied by proton expulsion (Kyziol and Daszkiewicz, 1985).

3- and 1-nitrocarbazole were also the major isomers formed from the nitration of carbazole with HNO_3 in acetic anhydride at 10-15^oC (Preston et al., 1942; Dewar and Urch, 1958). Carbazole reacts 222,000 times faster than benzene with HNO_3-acetic anhydride, having partial rate factors of 32,100 at the C1 position; 1,100 at the C2 position; 77,600 at the C3 position; and a rate factor too small to measure at the C4 position (Dewar and Urch, 1958). Thus, C3 is the most reactive position, followed by C1, C2, and C4 in that order.

Because of their low rate factors, 2- and 4-nitrocarbazole are not formed to any great extent by the simple nitration of carbazole with HNO_3. The presence of a more electron-withdrawing group, such as an acyl group, on the pyrrole nitrogen, to reduce the aromatic character of the central ring, is required for the formation of 2-nitrocarbazole (Joule, 1984).

According to Joule, the presence of a C3 substituent, whether electron-donating, such as a hydroxy group, or electron-withdrawing, such as a nitro group, usually leads to further substitution at the C6 position, which is the symmetrical equivalent of the C3 position. Grotta et al. (1964) discovered that carbazole could undergo dinitration

to a mixture of 3,6- and 1,6-dinitro derivatives. The 3,6-dinitro isomer comprised 50-50% of mixture, and the 1,6-dinitro isomer comprised 30-35% of the mixture.

Furthermore, carbazole, on treatment with 93% HNO_3, gave 3,6-dinitrocarbazole at 70-80°C and 1,3,6,8-tetranitrocarbazole at 100°C (Amemiya et al., 1952). 3,6-disubstituted carbazoles normally undergo electrophilic attack at C1 or C8 (symmetrically equivalent positions) and can form 1,3,6,8-tetra-substituted carbazoles under forcing conditions, such as high HNO_3 concentration and very high temperatures (Joule, 1984).

Carbazole undergoes tetranitration to form 1,3,6,8- and 1,2,6,8-tetranitrocarbazole by sequential treatment of carbazole with hot, fuming H_2SO_4 and then hot 93% HNO_3. The isomer formed in greatest quantity was the 1,3,6,8- isomer, with 1,2,6,8-tetranitrocarbazole comprising only 10% of the crude product (Murphy et al., 1953).

Much less is known of the substitution of 1-, 2-, or 4-substituted carbazoles, but an activating group at C2 has been found to lead to substitution at C1 and C3, the latter predominating (Joule, 1984), and, as demonstrated above, the presence of a nitro group at C1 could lead to further substitution at C6, to form the 1,6-dinitro isomer during carbazole nitration.

No data is available on the nitration of 2-hydroxycarbazole with HNO_3, but a similar route is probably followed. 2-hydroxycarbazole was chosen as a convenient route to specific synthesis of 2-hydroxy-X-nitrocarbazoles, possible hydroxylated metabolites and photooxidized products of the nitrocarbazoles.

2.6(b). Adsorption Chromatography

Among the methods of adsorption chromatography used for resolving individual isomers present in a reaction mixture, column chromatography and thin layer chromatography, or TLC, were chosen. The theory behind both methods is the same. In column chromatography a glass column is packed with adsorbent material, such as alumina or silica gel, whereas in TLC a glass or aluminum support is evenly coated with adsorbent material, such as silica gel. The adsorbent material contains hydroxy terminals which can interact with solute molecules from the reaction mixture by hydrogen bonding and London forces (Bailar et al., 1978; Braithwaite and Smith, 1985). A solvent is used to dissolve and carry the applied mixture down the column by gravitation for column chromatography or up the TLC plate by capillary action for thin layer chromatography. As a result, an equilibrium is established between the solute in the stationary phase (adsorbed to the adsorbent material) and the solute in the mobile phase (bound to and moving with the solvent). As the mixture proceeds down the column or up the plate, new and different equilibria are established as the material encounters fresh absorbent. Less polar molecules will displace more polar molecules since they are not as tightly adsorbed to the adsorbent material (Kirchner, 1978). As a result of these different adsorption affinities, a series of bands are formed on the column, and a series of spots on the TLC plate, each one corresponding to a particular isomer with a different polarity. The more polar an isomer is, the farther up the column, or the farther down the TLC plate, it will be.

At the end of the run, each band can be collected as fractions at the bottom of the column and dried. Each spot on the TLC plate can be

located visually or by fluorescence, and assigned an R_f value based on the distance the spot moved from the origin relative to the distance the solvent front moved from the origin. Although TLC plates can be used to obtain quantitative yields of individual isomers, in this case it was used to resolve the isomers present in the reaction mixture and also to analyze the purity of the fractions obtained from column chromatography.

2.6(c). Electron Impact Mass Spectrometry

Mass spectrometry can be used to determine the molecular weight and elemental composition of the isomer compound and to provide structural information from fragmentation patterns of the compound. Electron impact mass spectrometry works by bombarding a vaporized sample of the compound with an electron beam, fragmenting the compound, and thereby producing a beam of positive ions. After collimation, the beam is accelerated by an electrical potential of variable strength. The ions then pass through a magnetic field of variable strength perpendicular to their path, the force of which deflects the ions into curved paths, in which the radius of curvature depends on the mass and charge of the ions. By varying the accelerating electrical potential and the strength of the magnetic field, ions are directed onto a detector in the order of their mass-to-charge, or m/z, ratios, forming a distinctive mass spectrum that is a chemical "fingerprint" of the sample (Bailar et al., 1978).

Mass spectra "fingerprints" were made of 1- and 3-nitrocarbazole and 1,6- and 3,6-dinitrocarbazole by Kyziol et al. (1987). The main fragmentation routes of the nitrocarbazoles were found to be typical of other nitro-PAH, involving expulsion of the NO_2 and NO species as the primary processes, and were strongly dependent on the position of the

nitro group relative to the pyrrole nitrogen. For example, nitro-nitrite rearrangement, followed by expulsion of NO molecules played a more important role in the fragmentation of 3-nitro- and 3,6-dinitrocarbazole than of 1-nitro- and 1,6-dinitrocarbazole, suggesting a quinonoid structure for the intermediate, as illustrated by Kyziol et al. in Figure 3 on page 41.

No mass spectra have been found on other nitrocarbazoles or any 2-hydroxynitrocarbazoles in the literature.

2.6(d). Proton Nuclear Magnetic Resonance

Proton nuclear magnetic resonance, or proton NMR, can indicate the positions of substituents, such as nitro groups, on a compound based on the resonance frequencies of the protons remaining on the compound.

Protons produce a small magnetic moment as a result of their spinning about their axes, so when they are placed in an external magnetic field, they align, like magnets, parallel or antiparallel to that field. This alignment can be changed if the protons absorb energy in the radio frequency range of the electromagnetic spectrum (Bailar et al., 1978).

NMR is measured, therefore, by placing the sample to be analyzed, in solution, in a constant radio frequency field, and varying the strengths of the applied magnetic field. At the appropriate combination of radio frequency and magnetic field, absorption occurs and is detected and recorded as a plot of magnetic field strength versus radio frequency absorption, which appears as a series of peaks (Bailar et al., 1978).

The frequency at which a proton absorbs varies with the chemical environment, and that variation is called a chemical shift. The chemical shift of a particular peak is the difference between its

absorption and that of the standard tetramethylsilane, or TMS, and is reported in parts per million and is called delta. The area under each peak is proportional to the number of protons in that particular environment (Bailar et al., 1978).

The presence of electronegative groups, such as nitro groups, on a molecule create a chemical shift downfield, to higher delta values, of the protons near them as a result of their deshielding of the molecule around them. This decreases the electron density near the nitro group, thereby increasing the magnetic field reaching the nucleus of a proton nearby. As a result, protons near the nitro group will be shifted downfield on the spectrum (Brescia et al., 1978). The nitrogen present in the pyrrole ring of carbazole and the pseudo bay region between C4 and C5 have a similar deshielding effect on ring protons.

Hydroxy groups, on the other hand, have the opposite effect. They shield the molecule and shift protons upfield to lower delta values on the spectrum. Specific chemical shifts at various positions on the molecule relative to the nitro or hydroxy group are given for benzene by Jackman and Sternhell in Table 2 on page 42.

Splitting of the absorption peak occurs for protons in different chemical environments as a result of the influence of the magnetic moment of protons on adjacent atoms (Bailar et al., 1978). If there are two adjacent protons, the proton's absorption peak will be split twice, thereby forming a triplet. If there is only one other adjacent proton, it will be split once, thereby forming a doublet. If no other protons are adjacent, it will not be split, forming only a singlet. There can also be partial splitting of those peaks by protons on atoms in meta positions, i.e. "meta splitting."

With this information on chemical shift, or resonance, and splitting patterns, it should be possible to determine the positions of substituents on compounds such as nitrocarbazoles and hydroxynitro-carbazoles. However, no information has been found in the literature on the NMR spectra of those compounds.

2.6(e). Melting Point Determination

Because each compound has its own unique melting point, melting point determination is a good tool for identifying a particular isomer by comparing its experimental melting point to those found in the literature. The melting point of a solid compound is defined as the temperature at which the solid and liquid phases of the compound are at equilibrium (Bailar et al., 1978). A number of melting point determinations have been done on carbazole and its hydroxy and nitro derivatives and are listed in Table 3 on page 43. No melting points have been found of hydroxynitrocarbazole compounds in the literature.

2.6(f). Mutagenicity Assay

The Ames Salmonella mutagenicity assay is considered a rapid, relatively inexpensive way to screen a wide range of chemicals for genotoxic activity. Initial validations of this assay (McCann et al., 1975; Purchase et al., 1978; Bartsch et al., 1980), using known carcinogens, gave correlations of about 90% between carcinogenicity and the mutagenicity determined in those tests. However, this correlation began dropping over time, first to 83% (Ames and McCann, 1981) and then to 62% (Tennant et al., 1987). This may be due to the inclusion of more compounds for which the carcinogenicities were not well established, or for which the Ames assay could not detect, such as polychlorinated

pesticides (Rinkus and Legator, 1979). Tennant et al. also found correlations around 60% or lower for mammalian cell short-term tests such as chromosomal aberrations, SCE's in CHO cells, and the mouse lymphoma L5178Y cell mutagenicity assay, with no significant complementarity observed between any of those tests, including the Ames assay, in detecting carcinogens.

For detecting mutagens, the Ames assay uses a set of histidine-requiring strains, each with a different type of mutation in the histidine operon, eliminating the strain's ability to synthesize its own histidine for growth (Maron and Ames, 1983). In strain TA 98 there is a -1 frameshift mutation in the histidinol dehydrogenase gene hisD3052 (Isono and Yourno, 1974). As a result, the reading frame of the gene necessary for histidine production is shifted, eliminating histidine synthesis by the strain. Frameshift mutagens such as nitro-PAH can restore the correct reading frame by inducing another frameshift mutation in the gene. As a result, the bacteria revert to the wild type and can grow again. They can form colonies on a minimal histidine agar plate, the number of which is proportional to the mutagenic potency of the frameshift mutagen. A dose-response relationship can be determined by using various doses of the compound, and the genotoxic potency can be calculated from the slope of the linear portion of that dose-response curve.

The standard tester strains contain two other mutations, in addition to the histidine mutation, that greatly increase their ability to detect mutagens. One mutation (rfa) causes a partial loss of the lipopolysaccharide barrier coating the bacterial surface. As a result,

the permeability of the cell wall to large molecules such as benzo(a)pyrene is increased (Maron and Ames, 1983). Another mutation (uvrB) deletes the gene coding for the DNA excision repair system, greatly increasing the sensitivity to detect mutagens. Because, for technical reasons, this deletion also extends through the bio gene, these bacteria also require biotin for growth (Maron and Ames, 1983).

The strains also contain the R-factor plasmid, pKM101, which enhances an error-prone DNA repair system, allowing the strains to detect a number of mutagens that are detected weakly or not at all with the non R-factor strains TA 1535 and TA 1538 (Maron and Ames, 1983).

Before using the strains to detect mutagens, the presence of the mutations and the plasmid must be verified. Histidine requirement can be tested by determining if bacterial growth occurs on histidine-free agar plates. Since the rfa mutation confers cell wall permeability, the presence of the mutation can be tested by observing if any toxic effect occurs from the intrusion of the large toxic molecules of crystal violet through the bacterial cell wall. Because one side effect of the uvrB mutation is the strain's sensitivity to ultraviolet, or uv, light, a good test of this mutation is determining whether any growth occurs on a plate after exposure to uv light. Finally, since the pKM101 plasmid confers ampicillin resistance, the presence of the plasmid can be confirmed by streaking bacteria on plates containing ampicillin and watching for growth (Maron and Ames, 1983).

Nitroreductase-deficient derivatives of TA 98 and other strains have been isolated by Rosenkranz and Speck (1975, 1976). These strains can be useful for studying the mutagenicity of nitro-PAH, since they are

metabolized by a family of bacterial nitroreductases to electrophilic intermediates that can bind to the DNA and cause a frameshift mutation.

For use in the Ames assay, tester strain cultures are obtained from master plates made from frozen stock and grown in nutrient broth overnight to a density of $1-2 \times 10^9$ cells per milliliter. Spontaneous reversion rates and positive mutagenesis controls are then measured for the strains. Each strain reverts spontaneously to histidine independence at a frequency that is characteristic of the strain (Maron and Ames, 1983). The spontaneous rates for TA 98 (-S9) have been given by Maron and Ames as 30-50 revertants per plate, and by Claxton et al. (1982) as 15-35 revertants per plate, with slightly higher rates on plates with S9 (Maron and Ames, 1983).

To qualitatively assess the sensitivity and specificity of the strains, positive mutagenesis controls are used. Strains respond more easily to some compounds as positive controls than to others (Maron and Ames, 1983). 2-Nitrofluorene, a potent frameshift mutagen, is used as the positive control for strain TA 98 without S9, and 2-anthramine as the positive control for TA 98 with S9 (Claxton et al., 1982). They commonly produce 254-356 and 361-809 revertants/plate respectively in TA 98 (Goldring et al., 1987).

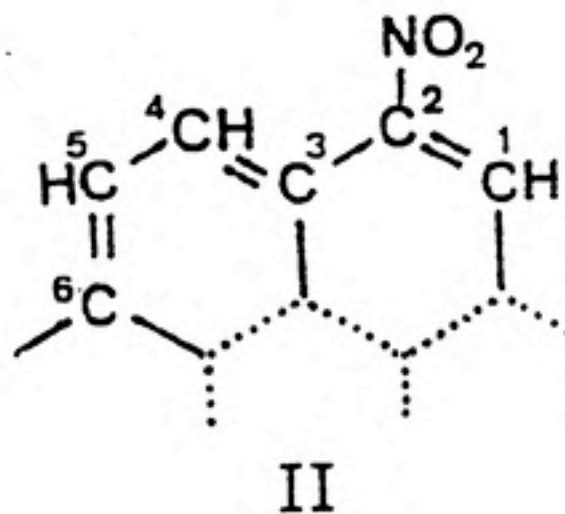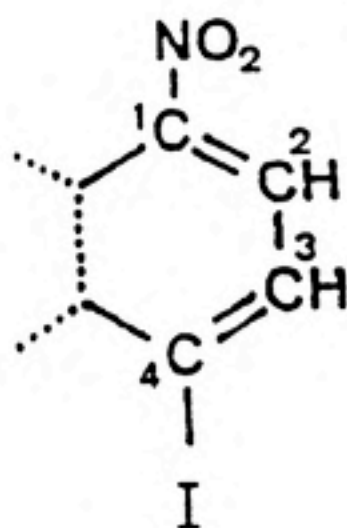S9 is the 9000 x g fraction obtained from the centrifugation of liver homogenate from rats induced with the polychlorinated biphenyl mixture Aroclor 1254. By inducing the animals in this way to produce cytochrome P-450 metabolicc enzymes, a wide variety of carcinogens requiring metabolic activation can be efficiently detected (Maron and Ames, 1983). S9 is incorporated in the Ames assay in order to determine

the effect of mammalian metabolic activation on the genotoxicity of a particular chemical.

While its correlation with carcinogenicity has declined with the addition of more compounds, the Ames mutagenicity assay remains a potent tool for the detection of environmental mutagens, if the limitations of the assay are known and taken into account.

FIGURE 1 Activating and Deactivating Fragments of NitroPAH Compounds

Activating Fragments

Deactivating Fragments

FIGURE 1 Activating and Deactivating Fragments of Nitro-PAH Compounds, continued

Containing Structure I Activating Fragment:

2-Nitrocarbazole   3-Nitrocarbazole

Containing Structure III Deactivating Fragment:

1-Nitrocarbazole   4-Nitrocarbazole

TABLE 1 Mutagenicity Results for Nitrocarbazole Compounds by Lavoie et al.(1981)

| Compound | Revertants/nanomole | | | |
| | TA 98 | | TA 100 | |
	-S9	+S9	-S9	+S9
1-nitrocarbazole	0.0	0.0	0.0	0.0
2-nitrocarbazole	10.3	4.7	0.5	0.1
3-nitrocarbazole	0.2	0.8	0.1	0.4
4-nitrocarbazole	0.01	0.06	0.5	0.8

FIGURE 2 Mechanism of
Carbazole Nitration

FIGURE 3 Fragmentation Pattern of
Nitrocarbazole Compounds

TABLE 2 Chemical Shifts of

Model PAH Benzene

Group	Chemical Shift (ppm) Ortho	Meta	Para
Nitro	+0.95	+0.17	+0.33
Hydroxy	-0.50	-0.14	-0.40

TABLE 3 Melting Points for
Carbazole Compounds

Compound	Melting Point (°C)	Physical Description	References
Carbazole	242-243		Kyziol and Daszkiewicz, 1984
	247-248	white crystals, plates, or leaves	WHO, 1983
	245-246		Aldrich MSDS
1-Nitrocarbazole	186-187	needles	Dewar and Urch, 1958
	189-190		Kyziol and Daszkiewicz, 1984
	187	yellow	Lindemann, 1924
	185-187		Campbell and Barclay, 1945
	188-189	yellow rods	Kyziol et al., 1987
2-Nitrocarbazole	174-175		Kyziol and Daszkiewicz, 1984
	173-174		Smith and Brown, 1951
3-Nitrocarbazole	215.5-216.5	yellow crystals	Dewar and Urch, 1958
	213-215		Kyziol and Daszkiewicz, 1984
	215-216	orange plates	Kyziol et al., 1987
	214		Lindemann, 1924

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TABLE 3 Melting Points for

Carbazole Compounds, continued

Compound	Melting Point (°C)	Physical Description	References
3-Nitrocarbazole	212-213		Smith and Brown, 1951
	203-206		Campbell and Barclay, 1945
4-Nitrocarbazole	182-183	orange	Campbell and Barclay, 1945
9-Nitrocarbazole	123-125	light yellow needles	Kyziol and Daszkiewicz, 1985
1,6-Dinitro-carbazole	344-346	glistening golden leaflets	Grotta et al., 1964
	343-344	golden yellow leaflets	Kyziol et al., 1987
3,6-Dinitro-carbazole	386-387	fine yellow needles	Grotta et al., 1964
	383-385	red needles	Kyziol et al., 1987
1,2,6,8-Tetra-nitrocarbazole	253-254	small, glistening, yellow plates	Murphy et al., 1953
1,3,6,8-Tetra-nitrocarbazole	295.6-296	pale yellow, transparent needles	Murphy et al., 1953
2-Hydroxy-carbazole	266.5-269.5		Smith et al., 1958
	273-275		Aldrich MSDS
	274-275		Teuber and Cornelius, 1964

III. EXPERIMENTAL

3.1. Materials

For the nitration reaction, the parent compounds carbazole and 2-hydroxycarbazole were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). The nitrating agents dinitrogen tetraoxide and nitric acid were purchased from Matheson Division, Searle Medical Products USA, Inc. (E. Rutherford, NJ) and Fisher Scientific (Fair Lawn, NJ) respectively.

For the thin layer chromatography of the nitro products, silica gel TLC plates were obtained from E. Merck (Darmstadt). Silica gel (230-400 mesh ASTM) for column chromatography was purchased from American Scientific Products, American Hospital Supply Corporation (McGaw Park, IL). Solvents that were employed in the chromatography included ACS grade toluene, methylene chloride, and acetone, all obtained from Fisher Scientific.

For identification of the fractions from the chromatography, electron impact mass spectrometry was performed using a VG7070F instrument, and proton nuclear magnetic resonance with a 400 MHz instrument. Deuterated acetone, 99.5 atom % D, and deuterated dimethylsulfoxide (DMSO), 99.9 atom % D, for use in NMR, were obtained from Sigma Chemical Co. (St. Louis, MO).

For the preparation of minimal agar assay plates, master plates, and genotype test plates, agar was purchased from United States Biochemical Corporation, or USBC (Cleveland, OH), dextrose from

Mallinkrodt, Inc. (Paris, KY), and L-histidine, biotin, and chemicals for the Vogel-Bonner medium E (50X), or VBME, from Fisher Scientific. VBME chemicals included magnesium sulfate ($MgSO_4 \cdot 7H_2O$), citric acid monohydrate, potassium phosphate dibasic (anhydrous) (K_2HPO_4), and sodium ammonium phosphate ($NaHNH_4PO_4 \cdot 4H_2O$). The ampicillin trihydrate used in making master plates for the bacterial strains and the crystal violet used in testing them were obtained from Aldrich Chemical Co., Inc.

For the Ames mutagenicity assay, Salmonella typhimurium strains TA 98 and TA 98ND were obtained from Dr. Bruce Ames, University of California at Berkeley and United States Environmental Protection Agency respectively. The genotypes of the two bacterial strains were the same--his D3052, rfa, uvrB, and pKM101, but with TA 98ND lacking nitroreductase enzyme B. Bacterial strains were incubated in Oxoid Nutrient Broth #2, purchased from Oxoid, Ltd. (Basingstoke, Hants., England).

For the preparation of the S9 mix in the assay, NADP and glucose-6-phosphate were purchased from Boehringer Mannheim (West Germany). Magnesium chloride ($MgCl_2$) and potassium chloride (KCL) for the $MgCl_2$-KCl salts solution in the S9 mix were obtained from Fisher Scientific. The phosphate-buffered saline, or PBS, solution employed in the S9 mix was supplied by the Tissue Culture Facility at the Lineberger Cancer Research Center. The S9 itself was from frozen stock of the 9000 x g supernatant of livers of Aroclor 1254-treated male Syrian hamsters, purchased from Moltox (Molecular Toxicology, Inc.) (College Park, MD).

For the assay, positive controls 2-nitrofluorene and 2-anthramine were obtained from Aldrich Chemical Co., Inc., and Sigma Chemical Co.

respectively. The molecular biology grade DMSO, used as a solvent for the assay compounds and positive controls and as a solvent control, was purchased from Fisher Scientific.

3.2. Methods

3.2(a). Synthesis

Carbazole and 2-hydroxycarbazole were nitrated by two methods, one using dinitrogen tetraoxide (N_2O_4) in methylene chloride (CH_2Cl_2) and the other using nitric acid (HNO_3) in acetic acid.

A modification of the N_2O_4 method of Radner (1983) utilized by Goldring et al. (1987) was employed. 250 mg of carbazole or 2-hydroxycarbazole were dissolved in 50 ml of CH_2Cl_2 to form a 5 mg/ml solution. 2.5 ml of N_2O_4 were dissolved in 100 ml of CH_2Cl_2, and 25 ml of the solution were added to the carbazole- or 2-hydroxycarbazole- CH_2Cl_2 solution (0.1 ml of N_2O_4-CH_2Cl_2 solution for each mg of carbazole or 2-hydroxycarbazole).

The reaction was carried out at room temperature (20^oC), and its course was monitored using thin layer chromatography. When the carbazole or 2-hydroxycarbazole disappeared from the TLC plate and no further products were seen to be created, usually within 5 to 10 minutes, the reaction was terminated by evaporating the solvent. This was done under a stream of nitrogen or with a rotary evaporator.

A modification of this method was used for 2-hydroxycarbazole in order to produce a wider isomer profile. Reaction at 20^oC with 25 ml of N_2O_4-CH_2Cl_2 produced mostly a trinitro isomer based on mass spectrometry results. The reaction was slowed somewhat by adding 12.5 ml of N_2O_4-CH_2Cl_2 in a 0^oC water ice bath and produced mostly a dinitro product. The reaction was slowed down even further by adding 2.5 ml of the

N_2O_4-CH_2Cl_2 solution in a -70°C dry ice bath and produced mostly two mononitro products.

The nitrocarbazole isomer profile was essentially unchanged when the N_2O_4 concentration was increased or decreased and when the reaction temperature was reduced using a 0°C water ice bath, unlike the 2-hydroxynitrocarbazole isomer profile.

A modification of the widely used method of HNO_3 nitration of Ziersch (1909) was also employed. A volume of glacial acetic acid sufficient to dissolve the parent compound under heat (30 ml for carbazole and 40 ml for 2-hydroxycarbazole, both at 65°C) was added to 500 mg of the compound. 1 ml of concentrated HNO_3 was added to 10 ml of glacial acetic acid. 2 ml of the HNO_3-acetic acid solution was then added to the parent compound-acetic acid solution and allowed to react for 30 minutes in a 65°C water bath. As with the N_2O_4 method, this reaction was monitored using thin layer chromatography. The reaction was terminated by either adding ice-cold distilled water and filtering off the product or by rotary evaporation.

3.2(b). Purification

Crude mixtures of the nitrocarbazoles or 2-hydroxynitrocarbazoles were applied to a silica gel column and chromatographed successively with toluene, methylene chloride, and acetone as needed. The fractions collected from each of the distinct bands on the column were then dried under a stream of nitrogen or with a rotary evaporator. The degree of purification of the fractions relative to the crude mixture was checked using thin layer chromatography.

3.2(c). Identification

Following the separation, the identity of each isomer was determined using electron impact mass spectrometry, or EIMS, proton nuclear magnetic resonance, and melting points. EIMS determined the molecule weight of each isomer; proton NMR determined the positions of the nitro substituents; and melting points provided a reference for comparison to nitrocarbazole compounds in the literature. The melting point of each compound was obtained by placing a sample of each in a capillary tube and immersing in an oil bath. The temperature of the bath was then slowly increased until the melting point was reached.

3.2(d). Mutagenicity Assay

Mutagenicity of the compounds was assayed by the standard histidine-reversion plate-incorporation method as described by Maron and Ames (1983), using the constructed strains TA 98 and TA 98ND described earlier.

The strains were stored at $-70^{\circ}C$ in a low-temperature freezer, and from these stocks, master plates were prepared in the following manner. After overnight incubation of the strains to a density of $1-2 \times 10^9$ cells/ml, the bacteria were streaked onto the master plates and incubated for 48 hours at $37^{\circ}C$. The plates were then securely wrapped and stored at $4^{\circ}C$ in a refrigerator. By the method of Maron and Ames (1983), single colonies of the bacteria were picked from these master plates for individual experiments or for new master plates (prepared every 4 to 6 weeks).

The presence of the genetic markers described in the Background Section on pages 33 and 34 was verified for each master plate before its use in growing overnight cultures in nutrient broth for mutagenicity

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assays. The histidine requirement and ampicillin resistance of the strains were verified by streaking the bacteria across histidine and ampicillin agar plates respectively, incubating overnight, and checking for growth. Sensitivity to ultraviolet light was verified by streaking the bacteria across a nutrient agar plate, exposing half the plate to ultraviolet light, incubating overnight, and checking for any growth on the exposed side. The deep rough (rfa) character of the strains was checked by adding paper discs containing crystal violet to nutrient agar plates to which bacteria were added, incubating overnight, and checking for any growth around the discs.

The spontaneous and positive control responses were tested for each master plate as well as with each assay. The positive control chemicals and the amount added per plate for TA 98 and TA 98ND were as follows: -S9: 3.0 ug 2-nitrofluorene
          +S9: 0.5 ug 2-anthramine

When spontaneous and positive control responses were outside the historically acceptable range, an experiment was rejected.

The agar plates and the top agar for the assay and the genotype tests were prepared using the chemicals mentioned earlier, according to the methods suggested by Maron and Ames (1983).

The test compounds and positive controls were dissolved in DMSO, with serial tenfold dilutions of 100, 10, 1, and 0.1 ug/plate of each test compound. DMSO also served as a solvent control. Duplicate plates were used for each dose, with and without S9 metabolic activation.

To prepare the 0.3 mg/plate S9 solution, 92.4 mg of NADP and 42.3 mg of glucose-6-phosphate were dissolved in 10 ml of sterile phosphate-buffered saline solution and filter-sterilized into a sterile beaker on

ice. To this was added 600 ul of a sterile KCl/MgCl$_2$ solution, 18.2 ml of PBS solution, and 1.2 ml of S9, giving a total of 30 ml of S9 solution.

0.1 ml of bacteria, 0.1 ml of compound (for each dilution), and 0.5 ml of S9 (if S9 used) were then added to 2-3 ml of molten top agar, vortexed, and poured onto a minimal agar plate. Afterwards, the plates were inverted and incubated for 48 hours at 37°C. Each compound was assayed until consistent, replicative results were obtained.

The revertant colonies on each plate were counted and averaged for each dose of a compound, plus or minus two standard deviations for 95% confidence. This was done for each strain, with and without S9. Spontaneous rates were not subtracted from these average values. From these data a dose-response curve was drawn. Any linear dose-response above the background rate was seen as evidence of positive mutagenicity. The mutagenic potency, or specific activity, of a compound, in revertants/microgram, was determined from the slope of the dose-response curve in the linear range. The curve was fitted by linear regression to the data points and a correlation calculated. When the correlation factor r was equal to or greater than 0.9, the result was accepted.

IV. RESULTS

The results are presented by compound. Figures and tables of those results are shown at the end of this chapter. Mass spectra are presented in Figures 4a-13a, NMR spectra in Figures 4b-13b, and physicochemical results, such as melting points, R_f values, molecular weights, and proton assignments, in Tables 4a-13a. Graphs and tables of mutagenicity results for each compound can be found in Figures 4c-13c, 7d-8d, and 11d-13d, and Tables 4b-13b, 7c-8c, and 11c-13c respectively. A final summary of mutagenicity results for all the compounds in revertants per nanomole is given in Table 14.

4.1. Carbazole

4.1(a). Physicochemical Results

As a means of comparison with its nitrated isomers, a mass spectrum and NMR spectrum were done of the parent compound, carbazole. As expected, the $[M]^+$ peak on the mass spectrum had a m/z ratio of 167, the molecular weight of carbazole. The NMR spectrum exhibited a pattern of two triplet peaks and two doublet peaks. Because carbazole is a symmetrical molecule, corresponding pairs of protons on the molecule are in the same chemical environment and, therefore, have identical chemical shifts, with peaks at the same delta values. As a result, each peak represents two protons. In the NMR spectrum of carbazole, protons H3 and H6 form one triplet peak, since they are adjacent to two carbon atoms with one proton each. Protons H2 and H7 also form a triplet, but

further downfield due to their closer proximity to the deshielding electronegative pyrrole nitrogen. Protons H1 and H8 form one doublet peak, each proton being adjacent to only one other proton, further downfield than H2 and H7 due to their even closer proximity to the pyrrole nitrogen. Protons H4 and H5 also form a doublet, but even further downfield due to their proximity to the pseudo bay region. These proton assignments are confirmed by assignments in the literature for carbazole (The Sadtler Handbook of Proton NMR Spectra, 1978).

A melting point of 245-246°C has been reported for carbazole in the material safety data sheet, or MSDS, from Aldrich Chemical Co., Inc. An R_f value of 0.67 has been found for carbazole on a silica gel TLC plate using toluene as the solvent. As with the spectra, both of these values were useful in comparing the parent compound with its nitrated forms.

4.1(b). Mutagenicity

In agreement with previous tests using Salmonella tester strain TA 98 (Anderson and Styles, 1978; Kaden et al., 1979; Ho et al., 1981; Tokiwa et al., 1981; Lavoie et al., 1982; Bechtold et al., 1985), carbazole was found to be nonmutagenic in our laboratory. Specific activities of only 0.08 revertants per microgram, or 0.01 revertants per nanomole, were obtained without S9 and 0.07 revertants per microgram, or 0.01 revertants per nanomole, with the addition of S9. As in the other tests, S9 did not appear to have any effect on the mutagenicity of carbazole, either positive or negative.

4.2. 1-Nitrocarbazole

4.2(a). Physicochemical Results

The nitration of carbazole with either N_2O_4 or HNO_3 yielded four nitro products. The first product to elute in silica gel column chromatography, with an R_f value of 0.535 in TLC, using toluene as solvent, was a yellow substance labelled A. Product A formed bright yellow needles upon drying. Mass spectrometry of this fraction gave a molecular weight of 212, that of a mononitrocarbazole compound. Because there were three triplets and no singlets present in the NMR spectrum of product A, nitro-substitution must have occurred at either the C1 or C4 position to enable a third triplet to form, but no singlet. Since the C1 position is much more reactive than the C4 position to electrophilic attack, based on the results of Dewar and Urch (1958), substitution at the C1 position is more likely, thereby identifying the compound as 1-nitrocarbazole. The compound's melting point of 184-186°C and its physical description as yellow needles provided further confirmation of the compound's identity. Both are similar to literature results for 1-nitrocarbazole listed in Table 3 on page 43.

4.2(b). Mutagenicity

The 1-nitrocarbazole compound was found to be a weak mutagen in TA 98. It produced only 1.2 revertants per microgram, or 0.3 revertants per nanomole, without S9. Addition of S9 reduced the activity of the isomer slightly to 0.9 revertants per microgram, or 0.2 revertants per nanomole. These results agree somewhat with those of Lavoie et al. (1981), found in Table 1 on page 39. Lavoie et al. found 1-nitrocarbazole to be completely nonmutagenic, producing 0.0 revertants per nanomole, both with and without S9.

However, a previous preparation of 1-nitrocarbazole gave quite different activities. It produced 46.0 revertants per microgram, or 9.7 revertants per nanomole, without S9, and 37.8 revertants per microgram, or 8.0 revertants per nanomole, with S9. These results are similar to those for 2-nitrocarbazole by Lavoie et al.--10.3 and 4.7 revertants per nanomole, without and with S9. Furthermore, Kyziol and Daszkiewicz (1984) found 2-nitrocarbazole to follow 1-nitrocarbazole in TLC.

An assay of this preparation with TA 98ND, the nitroreductase-deficient strain of TA 98, produced 31.7 revertants per microgram (6.7 revertants per nanomole) without S9 and 25.1 revertants per microgram (5.3 revertants per nanomole) with S9. S9 appeared to reduce activity slightly in both strains. Because of the lack of any distinct reduction in activity in TA 98ND and any subsequent increase with the addition of S9, this contaminant is either a nonnitro compound, or a nitro compound which does not depend on the missing nitroreductase in TA 98ND for its activity. The presence of a contaminant was confirmed by peaks on the NMR spectrum of this preparation besides those of 1-nitrocarbazole, but its identity is not known.

4.3 3-Nitrocarbazole

4.3(a). Physicochemical Results

Following product A on the silica gel column was product B, with a R_f value of 0.256 in toluene. Product B formed yellow crystals with N_2O_4 and orange crystals with HNO_3. Both forms generated identical NMR and mass spectra. The mass spectrum identified B as another mononitro-carbazole with a molecular weight of 212. The presence of a singlet and absence of a third triplet in the NMR spectrum of compound B suggested that nitro-substitution occurred at the C2 or C3 position. Nitro-

substitution at either position would prevent the formation of a third triplet and isolate one proton, thereby causing a singlet to be generated. Due to the higher reactivity at the C3 position, 3-nitro-carbazole is the more likely identity of the compound. Furthermore, its melting points of 209-211°C (orange crystals) and 211-213°C (yellow crystals) and its physical description are similar to those in the literature for 3-nitrocarbazole (Table 3 on page 43).

4.3(b). Mutagenicity

Unlike the 1-nitro isomer, addition of S9 appears to have distinctly increased the activity of 3-nitrocarbazole in TA 98. For the orange crystals, activity increased from 0.3 revertants per microgram (0.06 revertants per nanomole) without S9 to 1.0 revertants per microgram (0.2 revertants per nanomole) with S9. For the yellow crystals, activity was higher, increasing from 6.9 revertants per microgram (1.5 revertants per nanomole) without S9 to 10.7 revertants per microgram (2.3 revertants per nanomole) with S9. Lavoie et al. (1981) found a similar increase, from 0.2 to 0.8 revertants per nanomole with the addition of S9.

4.4. 1,6-Dinitrocarbazole

4.4(a). Physicochemical Results

Product C was the third fraction to elute from the silica gel column and gave an R_f value of 0.186 in toluene. It dried as intensely fluorescent yellow needles, with a decomposition melting point of 253-255°C. A similar melting point was not found in the literature. Mass spectrometry identified the compound as a dinitrocarbazole isomer, molecular weight 257. Nitro-substitution must have occurred on both of

the benzene rings of carbazole in order for there to be only one triplet in the NMR spectrum of C. Furthermore, one substitution must have been at either the C1 or C4 position, to enable that one triplet to be generated. Substitution on the other ring must, therefore, have occurred at either the C6 or C7 position, to prevent the generation of a triplet on that ring. That left possibilities of 1,6-, 1,7-, 3,5- (4,6-), and 2,5-(4,7-)dinitrocarbazole. Since the reactivities at C2 (equivalent to C7) and C4 (equivalent to C5) are relatively low compared to those at C1 and C3 (equivalent to C6), based on the results of Dewar and Urch, the 1,6-dinitro isomer appears the most likely identity for the compound. That compound was also detected as a major dinitro isomer in the nitration of carbazole by Grotta et al. (1964).

4.4(b). Mutagenicity

The 1,6-dinitro compound was somewhat more mutagenic than the 1-nitro compound and the orange 3-nitro compound. It produced 2.1 revertants per microgram, or 0.5 revertants per nanomole, without S9, and 6.6 revertants per microgram, or 1.7 revertants per nanomole, with S9. The addition of S9 appears to have distinctly increased the mutagenicity of the compound. Assaying the compound with TA 98ND distinctly decreased activity. Only 0.4 revertants per microgram (0.1 revertants per nanomole) were produced as residual mutagenicity. Addition of S9 completely restored activity to TA 98 (+S9) levels, producing 6.7 revertants per microgram (1.7 revertants per nanomole). Mutagenicity results for this compound and all succeeding compounds have not been found in the literature.

4.5. 3,6-Dinitrocarbazole

4.5(a). Physicochemical Results

The last nitro product of carbazole to elute was product D, with an R_f value of 0.056 on TLC, using toluene as solvent. It formed golden-brown crystals, with a decomposition melting point of 232-234°C, which was not found in the literature.

The mass spectrum of Compound D identified it as a dinitro-carbazole compound, molecular weight 257. The 'H NMR spectrum of the compound, with only three sets of peaks for six protons, suggested a symmetrical dinitro-substitution pattern, with nitro groups at opposite ends to each other on the molecule. Since there were no triplets, the molecule could not be 1,8- or 4,5-dinitrocarbazole, which would generate two triplets, but only 3,6- or 2,7-dinitrocarbazole, which would prevent the generation of triplets by the positions of their nitro groups on the molecule. The C3 and C6 positions are the most reactive positions of carbazole to electrophilic attack, based on the results of Dewar and Urch. Therefore, 3,6-dinitrocarbazole is the more likely identity of the compound. Furthermore, the 3,6-dinitro isomer was the major dinitro product in a nitration of carbazole by Grotta et al. (1964).

4.5(b). Mutagenicity

As with the 1,6-dinitro isomer, addition of S9 appears to have increased the mutagenic potency of 3,6-dinitrocarbazole. Without S9 activity was 101.9 revertants per microgram (26.2 revertants per nanomole). With S9 activity increased to 134.4 revertants per microgram (34.5 revertants per nanomole). These high activities make 3,6-dinitrocarbazole the most mutagenic compound of the nitrocarbazoles tested. When assayed with TA 98ND, 3,6-dinitrocarbazole gave a much

lower activity, 34.7 revertants per microgram (8.9 revertants per nanomole) residual mutagenicity. Also as with the 1,6-dinitro isomer, addition of S9 completely restored activity to previous levels. It produced 113.5 revertants per microgram, or 29.2 revertants per nanomole.

4.6. 2-Hydroxycarbazole

4.6(a). Physicochemical Results

As with carbazole, a mass spectrum and an NMR spectrum were generated of the parent compound 2-hydroxycarbazole to provide a means of comparing it with its nitro products. A m/z ratio of 183 for the $[M]^+$ peak in the mass spectrum of the compound confirmed its identity as a hydroxycarbazole, and the lack of a third triplet and presence of a singlet narrowed that identity to 2- or 3-hydroxycarbazole. Its melting point of 273-275°C from the Aldrich MSDS and TLC R_f value of 0.676 in toluene and 0.76 in methylene chloride were also used as points of reference for its nitro derivatives.

4.6(b). Mutagenicity

Like carbazole, 2-hydroxycarbazole was found to be nonmutagenic in both the presence and absence of S9. It produced only -0.02 revertants per microgram (-0.004 revertants per nanomole) without S9 and -0.1 revertants per microgram (-0.02 revertants per nanomole) with S9, with no toxic effects detected. Although addition of S9 increased activity somewhat, the value was still in the inactive range, even lower than that for carbazole.

4.7. 2-Hydroxy-1-Nitrocarbazole

4.7(a). Physicochemical Results

Three nitro compounds were formed by the nitration of 2-hydroxy-carbazole with 10% of the standard N_2O_4 concentration in a $-70^\circ C$ dry ice bath. The first of those three to elute was a product labelled A', which had R_f values of 0.44 in toluene and 0.875 in methylene chloride. Upon drying product A formed red needles. Mass spectral analysis of this compound identified it as a mononitro isomer of 2-hydroxycarbazole, molecular weight 228. The absence of a singlet in the 'H NMR spectrum of A' strongly suggested that this compound was the 1-nitro isomer. Because the C2 position is occupied by the hydroxy group, the proton at C1 is isolated from other protons. As a result, if that proton were unsubstituted, it would generate a singlet. The absence of that singlet, therefore, indicated substitution occurred at the C1 position. This identity could not be confirmed by comparing the melting point of A', $203-205^\circ C$, with literature results since no results could be found for this or any other 2-hydroxynitrocarbazoles.

4.7(b). Mutagenicity

The 1-nitro isomer was also determined to be nonmutagenic in TA 98. Without S9, it had a specific activity of 0.07 revertants per microgram, or 0.02 revertants per nanomole. Addition of S9 increased activity slightly, to 0.2 revertants per microgram, or 0.04 revertants per nanomole, but still in the inactive range and less active than 1-nitrocarbazole.

4.8. 2-Hydroxy-3-Nitrocarbazole

4.8(a). Physicochemical Results

The second product to elute from the column after the nitration of 2-hydroxycarbazole was a yellow-orange product labelled B', which had R_f values of 0.227 in toluene and 0.625 in methylene chloride. It also was a mononitro isomer, with a molecular weight of 228, based on mass spectrometry results. Since there were two triplets in the ^1H NMR spectrum of B', substitution must have occurred on the same ring as the hydroxy group to allow those triplets to be generated. The presence of two singlets on the spectrum negated the possibility of B' being the 1-nitro isomer, even though its melting point of 205-207°C was close to that of the 1-nitro isomer. That left the 3- and 4-nitro forms as the only remaining possibilities. The more likely identity of B' is 2-hydroxy-3-nitrocarbazole for the following reasons. First, Joule (1984) found that C2 substitution encourages further substitution at C1 and C3, with substitution at C3 predominating. Furthermore, the C4 position was determined to be practically nonreactive to electrophilic attack according to Dewar and Urch. Finally, only C3 substitution would create singlets like those in the NMR spectrum that are not meta split. Meta splitting would occur with C4 substitution.

4.8(b). Mutagenicity

Unlike its nonhydroxylated counterpart, 2-hydroxy-3-nitro-carbazole demonstrated quite high activity in the Ames test. It produced 27.0 revertants per microgram (6.2 revertants per nanomole) in the absence of S9, while addition of S9 reduced activity by about 50%, to 13.6 revertants per microgram (3.1 revertants per nanomole). Assaying the compound with TA 98ND also reduced activity substantially,

to 2.1 revertants per microgram (0.5 revertants per nanomole) residual activity without S9. Instead of restoring activity, addition of S9 actually reduced activity even further, to 1.5 revertants per microgram (0.3 revertants per nanomole), an almost 50% decrease.

4.9. 2-Hydroxy-1,3-Dinitrocarbazole

4.9(a). Physicochemical Results

A small amount of another product, labelled C', was also formed in the nitration of 2-hydroxycarbazole in a $-70^{\circ}C$ dry ice bath with 10% N_2O_4 concentration. This compound was the major product, however, when nitration was carried out in a $0^{\circ}C$ ice water bath with 50% N_2O_4 concentration, as well as in a $65^{\circ}C$ water bath with standard HNO_3 concentration. It was present on the silica gel column as a deep red band and eluted and dried as orange needles, with R_f values of 0.033 in toluene, 0.068 in methylene chloride and 0.800 in acetone. Its decomposition melting point of $203-205^{\circ}C$ was similar to the melting points of the 1-nitro and 3-nitro isomers of 2-hydroxycarbazole, but its mass spectrum and NMR spectrum identified it as a different compound. An $[M]^+$ peak with a m/z ratio of 273 identified product C' as a dinitro isomer. Like products A' and B', product C' had two triplets in its NMR spectrum, indicating nitro-substitution occurred on the hydroxylated, but not the nonhydroxylated, benzene ring of carbazole. This left three possibilities for nitro-substitution--1,3-, 1,4-, and 3,4-dinitro-substitution. The latter two are unlikely due to the lack of reactivity at the C4 position. Steric interactions may also tend to discourage 3,4-dinitro-substitution. Furthermore, only substitution at C1 and C3 could shift the remaining proton on the hydroxylated ring closest to the delta value of 9.19 ppm on the spectrum. This is because the nitro

groups would be in ortho and para positions relative to the proton, thereby creating the greatest downfield shifts, based on data from Jackman and Sternhell (1969) shown in Table 2 on page 42. Finally, as previously mentioned, the presence of an activating group at the C2 position encourages substitution at the C1 and C3 positions. Thus, the identity of C' must be 2-hydroxy-1,3-dinitrocarbazole.

4.9(b). Mutagenicity

The 1,3-dinitro isomer was also mutagenically active in TA 98, although not quite as much so as the 3-nitro isomer. In the absence of S9, it produced 7.2 revertants per microgram, or 2.0 revertants per nanomole. Unlike the 3-nitro isomer, addition of S9 increased activity somewhat, to 8.7 revertants per microgram, or 2.4 revertants per nanomole. Assaying C' with the nitroreductase-deficient strain TA 98ND decreased activity dramatically, with a residual mutagenicity of 0.9 revertants per microgram, or 0.2 revertants per nanomole. Also unlike the 3-nitro isomer, addition of S9 recovered a small amount of the mutagenicity, increasing activity to 1.3 revertants per microgram, or 0.4 revertants per nanomole.

4.10. 2-Hydroxy-1,3,6-Trinitrocarbazole
4.10(a). Physicochemical Results

Product D' was formed as the major product in the nitration of 2-hydroxycarbazole with the standard amount of N_2O_4 at room temperature. Present as a golden-brown band on the silica gel column, D' produced a reddish-orange product after recrystallization with acetone. Its R_f values in TLC were 0.0 in toluene, 0.023 in methylene chloride, and 0.810 in acetone. Its decomposition melting point was 188-190°C. Mass

spectral analysis identified D' as a trinitro isomer of 2-hydroxy-carbazole, molecular weight 318. Because a small amount of the 1,3-dinitro isomer was produced along with the trinitro isomer, based on TLC's of the reaction mixture, it is likely that the trinitro compound would have nitro groups at C1 and C3 positions. Further substitution at the C6 position is likely since, according to Joule, substitution at the C3 position leads to further substitution at the C6 position. This was supported by the 'H NMR spectrum of the trinitro compound. Because there were no triplets in the spectrum, substitution must have occurred on both rings. Only seven possibilities for isomer assignment remained that could create the two doublets and two singlets seen in the spectrum and not have steric interactions resulting from two or more nitro groups adjacent to one another on a ring. Those possibilities were the 1,3,6-, 1,6,8-, 1,3,7-, 1,4,6-, 1,5,7-, 3,5,8-, and 4,5,8-trinitro isomers of 2-hydroxycarbazole. Of those, only the 1,3,6- and 1,6,8-trinitro isomers had all nitro groups at reactive positions. Of the two, only 1,3,6-trinitro-substitution followed the pattern of substitution set by the 2-hydroxy-1,3-dinitro isomer. Only 1,3,6-trinitro-substitution would lack the upfield shifts of the C1 and C3 protons ortho to the 2-hydroxy group due to nitro-substitution at those positions. Furthermore, only the 1,3,6-trinitro isomer would produce the meta split singlet and doublet and non-meta split singlet and doublet seen in an NMR spectrum of unpurified D'. 1,6,8-trinitro-substitution would generate two non-meta split doublets and two meta split singlets not seen in the spectrum. Thus, the likely identity of compound D' is 2-hydroxy-1,3,6-trinitrocarbazole.

4.10(b). Mutagenicity

Similar to the 3-nitro isomer, the 1,3,6-trinitro isomer produced 23.7 revertants per microgram (7.5 revertants per nanomole) without S9 and 10.2 revertants per microgram (3.3 revertants per nanomole) with S9, showing a 50% decrease in activity with the addition of S9. Activity also decreased dramatically when the compound was assayed with TA 98ND. The number of revertants per microgram declined to 5.6 without S9 (1.8 revertants per nanomole), and again declined by 50% with the addition of S9 to 2.7 revertants per microgram (0.9 revertants per nanomole).

4.11. Summary of Results

The activities of these ten compounds tested in the Ames Salmonella mutagenicity assay may be categorized in five ways--inactive, weak, moderately active, active, and very active. Based on the results obtained, carbazole, 2-hydroxycarbazole, and 2-hydroxy-1-nitrocarbazole were determined to be inactive as mutagens, with activities only as high as 0.04 revertants per nanomole, with and without S9. The 1-nitro and orange 3-nitro isomers were weak mutagens with and without S9, having activities only as high as 0.3 revertants per nanomole. The dinitro isomer 1,6-dinitro-carbazole was weak without S9 (0.5 revertants per nanomole) but moderately active with S9 (1.7 revertants per nanomole). The yellow 3-nitro and 2-hydroxy-1,3-dinitro isomers were moderately active mutagens, with 1.5 to 2.4 revertants per nanomole with and without S9. 2-Hydroxy-3-nitro- and 2-hydroxy-1,3,6-trinitrocarbazole gave somewhat higher results in TA 98 without S9, producing 6.2 and 7.5 revertants per nanomole respectively, thereby making them active mutagens. With S9, their activities dropped to 3.1 and 3.3 revertants

per nanomole, making them only moderately active mutagens. 3,6-Di-nitrocarbazole was a very active mutagen, producing 26.2 revertants per nanomole without S9 and 34.5 revertants per nanomole with S9. The 1-nitro contaminant, possibly 2-nitrocarbazole, was also active, with 6.7 and 5.3 revertants per nanomole without and with S9 respectively.
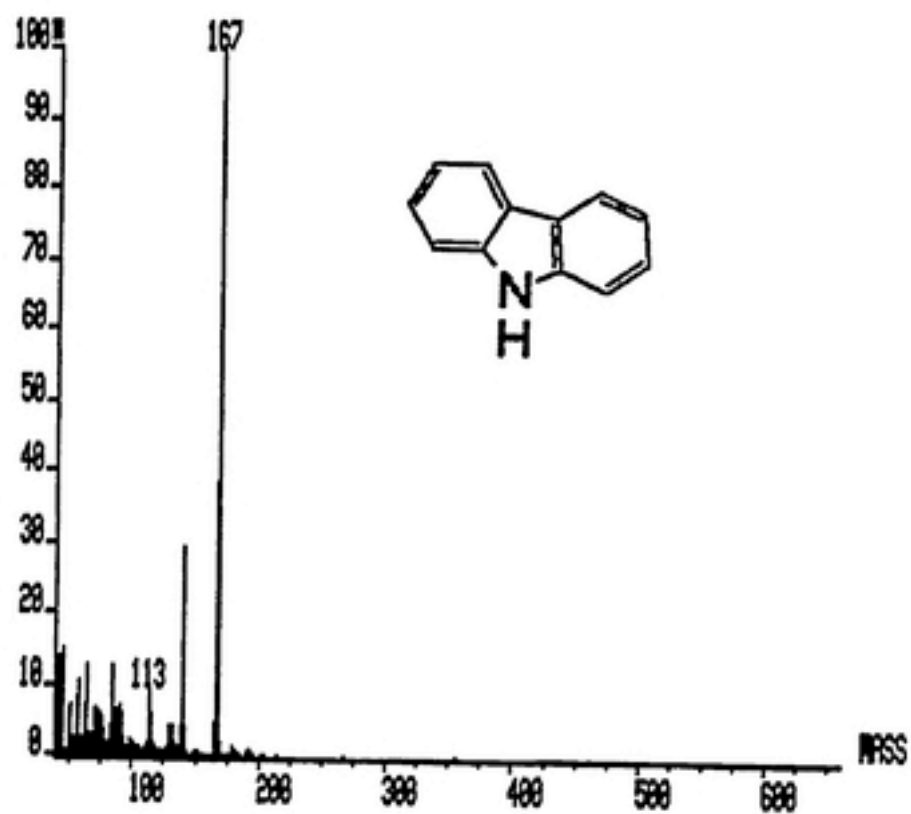
As seen with these results, S9 had varying effects on the compounds. S9 increased activity by over 200% for orange 3-nitro-carbazole and 1,6-dinitrocarbazole, and by 100%, 53%, and 32% for 2-hydroxy-1,3-dinitrocarbazole, yellow 3-nitrocarbazole, and 3,6-dinitrocarbazole respectively. While 2-hydroxycarbazole and its 1-nitro isomer were more active with the addition of S9, their activities still remained in the inactive range. Activity was decreased with the addition of S9, however, for 1-nitro-carbazole, the contaminated 1-nitrocarbazole preparation, 2-hydroxy-3-nitrocarbazole, and 2-hydroxy-1,3,6-trinitrocarbazole by 33%, 18%, 50%, and 56% respectively. Carbazole was relatively unaffected by S9, its activity remaining in the inactive range.
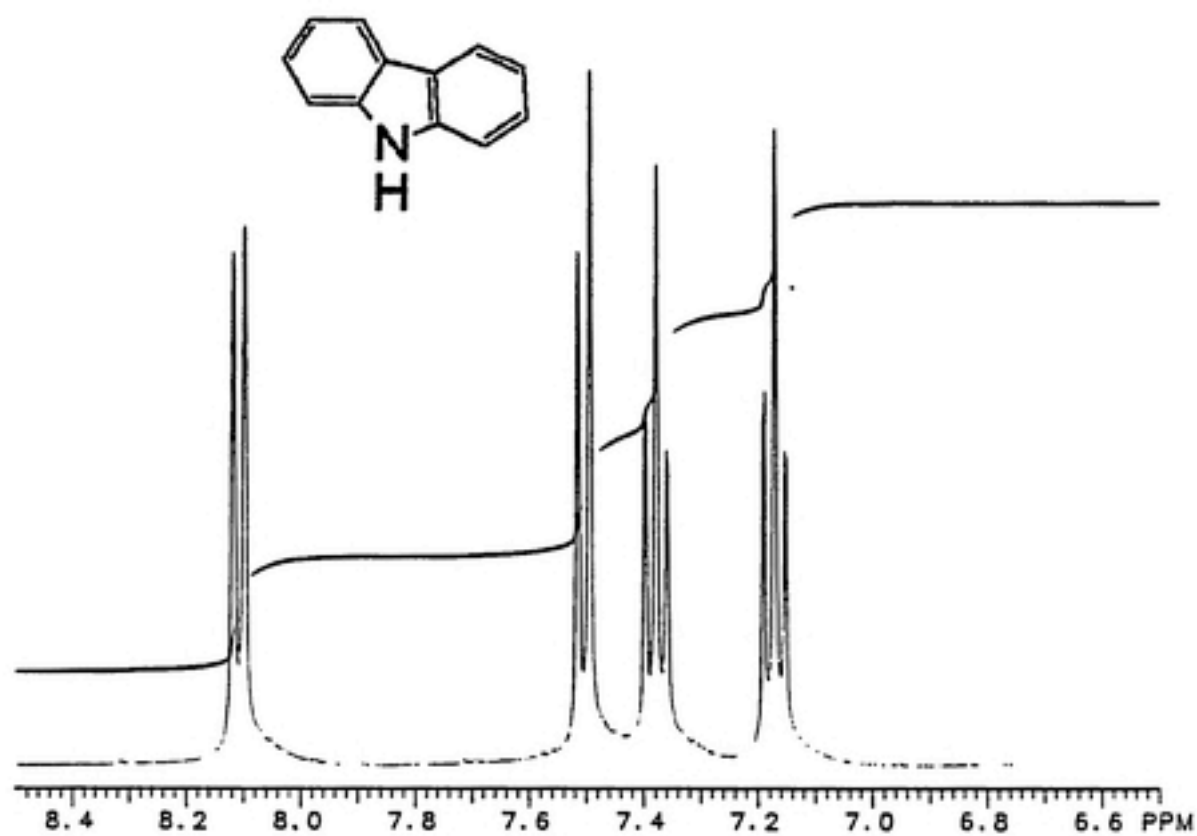
With the exception of the 1-nitro contaminant, all of the compounds which were assayed with the nitroreductase-deficient strain TA 98ND lost a substantial portion of their activity, 80% for 1,6-dinitro-carbazole, 60% for 3,6-dinitrocarbazole, 92% for 2-hydroxy-3-nitro-carbazole, 90% for 2-hydroxy-1,3-dinitrocarbazole, and 76% for 2-hydroxy-1,3,6-trinitrocarbazole. Some residual mutagenicity remained for all compounds tested, however, especially 3,6-dinitrocarbazole and 2-hydroxy-1,3,6-trinitrocarbazole. The 1-nitro contaminant lost no activity.

Those compounds assayed with TA 98ND which regained some or all of their mutagenic activity with the addition of S9 were also the ones which increased in activity with the addition of S9 in TA 98--1,6- and 3,6-dinitrocarbazole and 2-hydroxy-1,3-dinitrocarbazole. 1,6- and 3,6-dinitrocarbazole regained 100% or more of their activity, while the 1,3-dinitro isomer regained only 20% of its activity. As in TA 98, the 2-hydroxy-3-nitro and 2-hydroxy-1,3,6-trinitro isomers lost even more activity in TA 98ND with S9, 40% and 50% respectively. The contaminant of the 1-nitrocarbazole preparation lost 20% of its -S9 activity.

The different activities of all these compounds in TA 98 and TA 98ND, with and without S9, create a varied picture of the mutagenicity of nitrocarbazoles and hydroxynitrocarbazoles, which may be a result of the differences in their structures and metabolic activation pathways.

FIGURE 4(a) Mass Spectrum of Carbazole

FIGURE 4(b) NMR Spectrum of Carbazole

TABLE 4(a) Physicochemical Data for

Carbazole

---

m.p.: 245-246°C (Aldrich MSDS)
TLC: R_f 0.667 (toluene)
Mass spectrum: MW 167
^{1}H NMR spectrum (400 MHz, acetone-d_6):
    delta 7.51 (d, two protons, H1, H8)
    delta 7.38 (t, two protons, H2, H7)
    delta 7.18 (t, two protons, H3, H6)
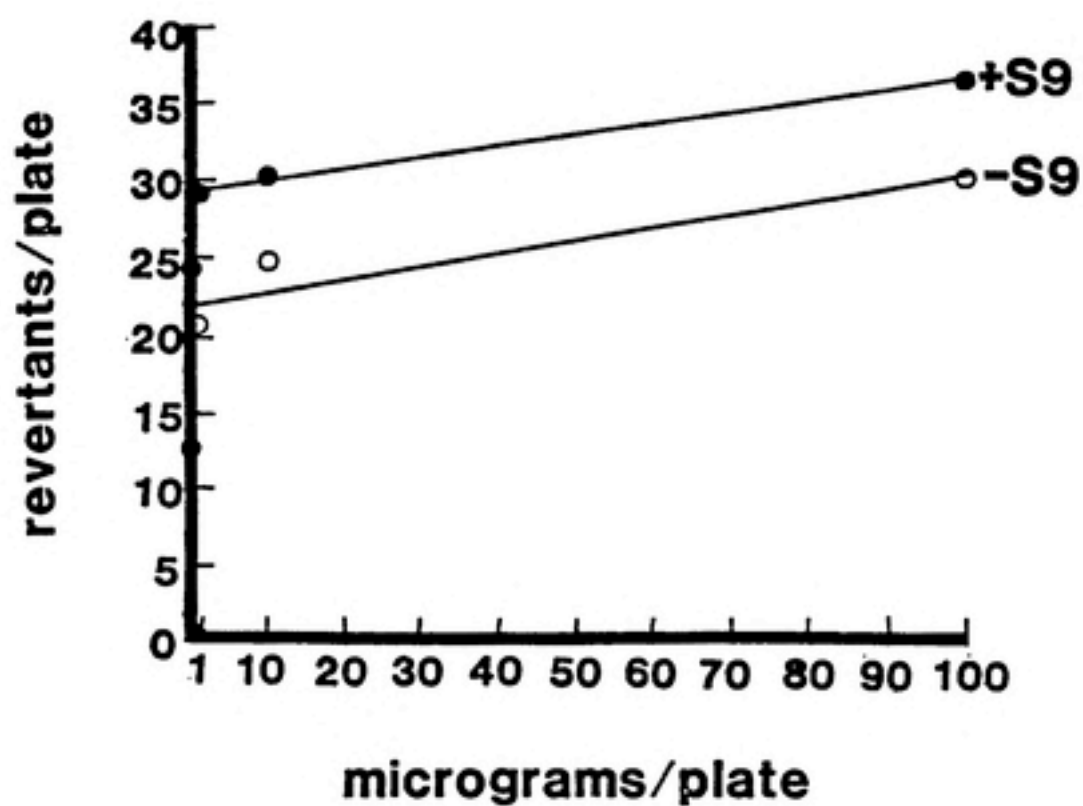   delta 8.11 (d, two protons, H4, H5)
    delta 10.32 (s, one proton, H9)

---

s: singlet peak
d: doublet peak
t: triplet peak

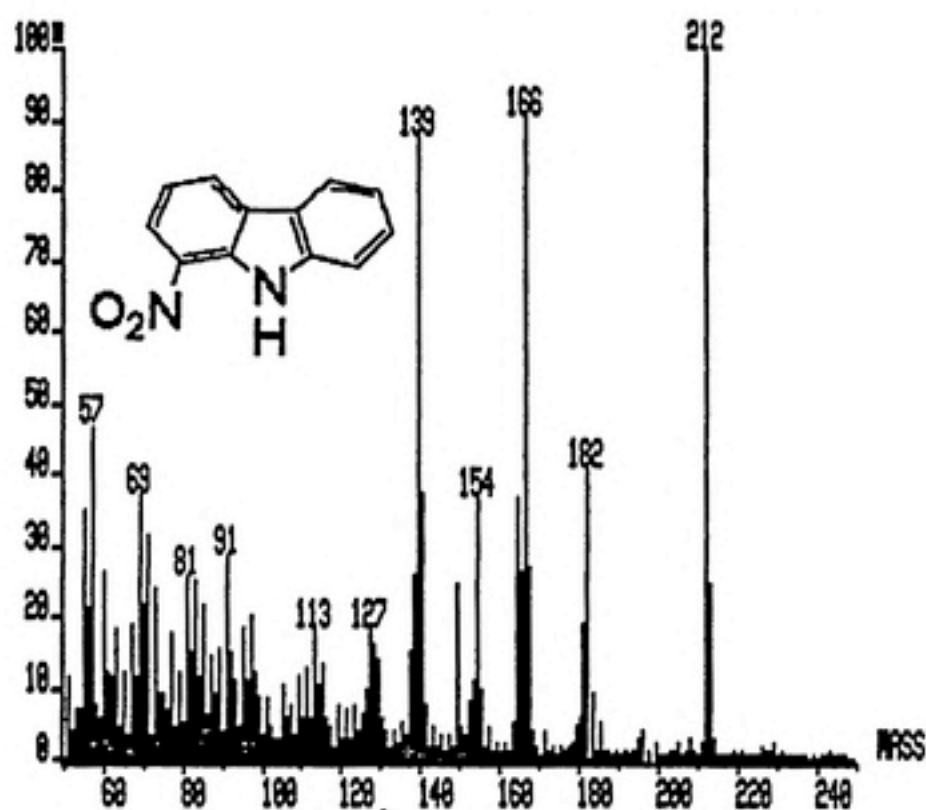# FIGURE 4(c) Mutagenicity of Carbazole in Salmonella Strain TA 98

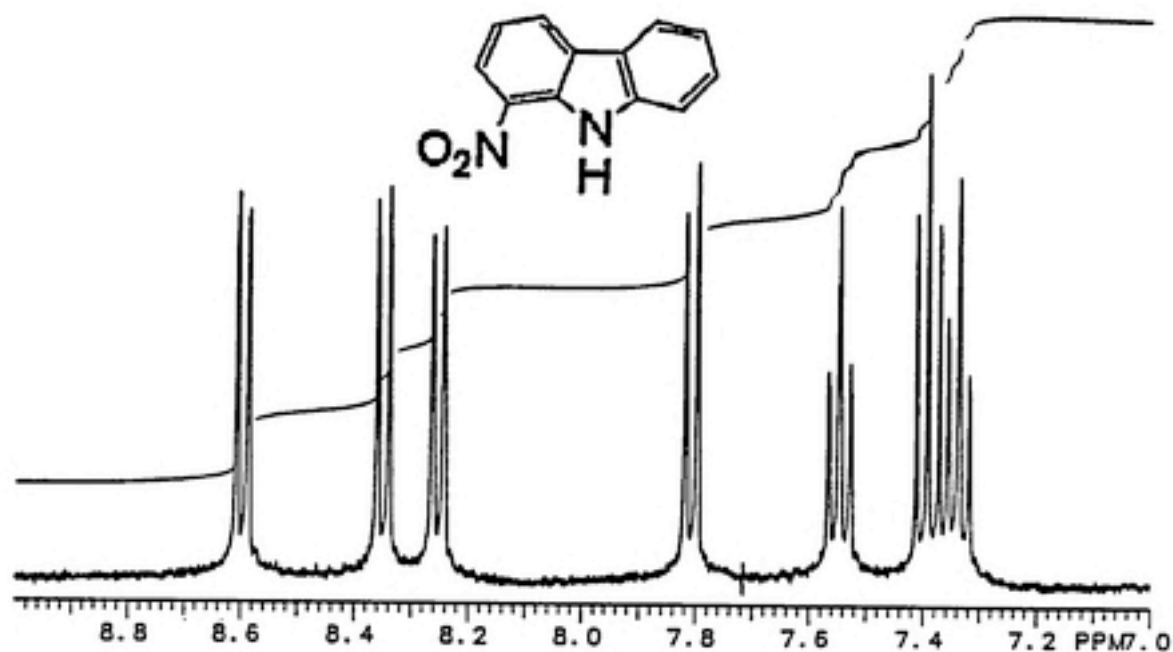# TABLE 4(b) Mutagenicity of Carbazole
in Salmonella Strain TA 98

Dose (ug/plate)	Salmonella Typhimurium Strain TA 98 -S9	+S9
Positive control	260.0±50.9	312.0±39.6
Spontaneous	20.0±11.3	30.5±7.1
0.1	12.5±1.4	24.0±2.8
1.0	20.5±12.7	29.0±8.5
10.0	24.5±1.4	30.0±33.9
100.0	29.5±7.1	36.0±0.0
Rev/ug	0.08	0.07
r	0.9	0.999

Duplicate plates were employed for each dose.

FIGURE 5(a) Mass Spectrum of
1-Nitrocarbazole

FIGURE 5(b) NMR Spectrum of

1-Nitrocarbazole

TABLE 5(a) Physicochemical Data for

1-Nitrocarbazole

---

m.p.: 184-186°C (yellow needles)
TLC: R_f 0.535 (toluene)
Mass spectrum: MW 212
^{1}H NMR spectrum (400 MHz, acetone-d_6):

      delta 8.60 (d, one proton, H2)
      delta 7.39 (t, one proton, H3)
      delta 8.35 (d, one proton, H4)
      delta 8.25 (d, one proton, H5)
      delta 7.34 (t, one proton, H6)
      delta 7.55 (t, one proton, H7)
      delta 7.81 (d, one proton, H8)
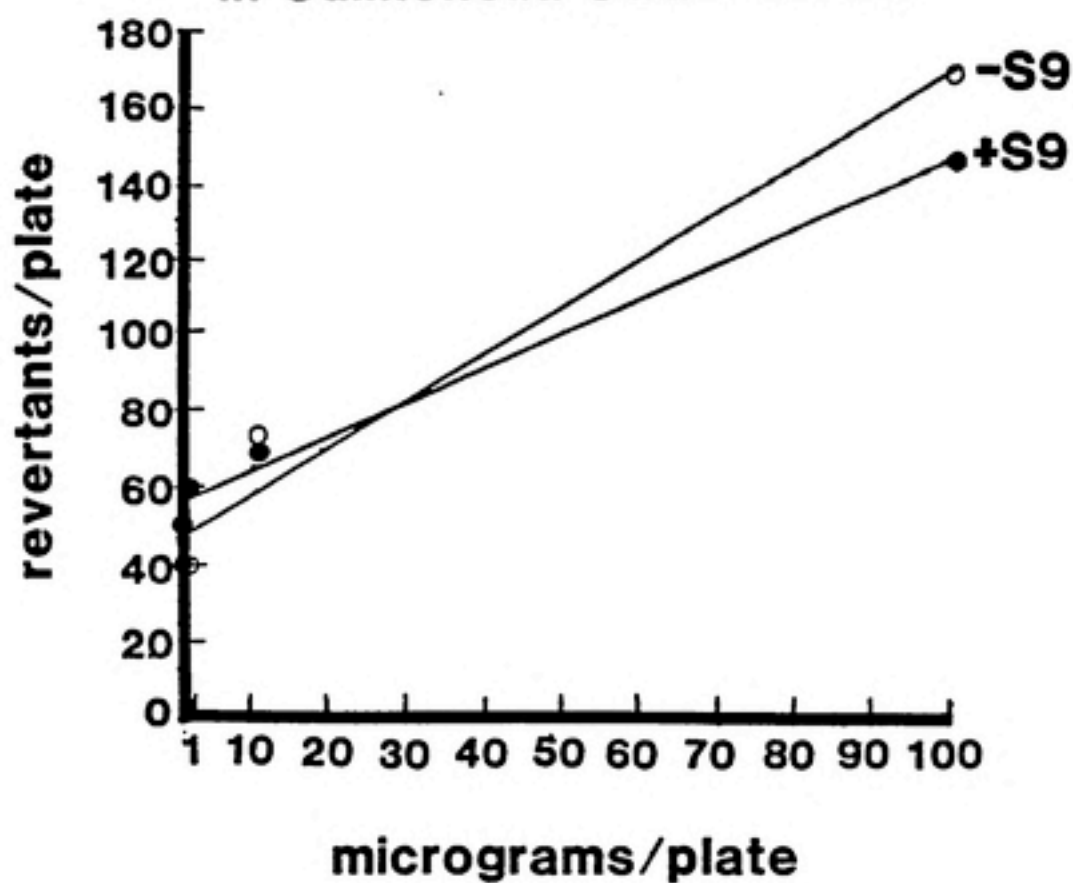   delta 11.49 (s, one proton, H9)

---

s: singlet peak
d: doublet peak
t: triplet peak

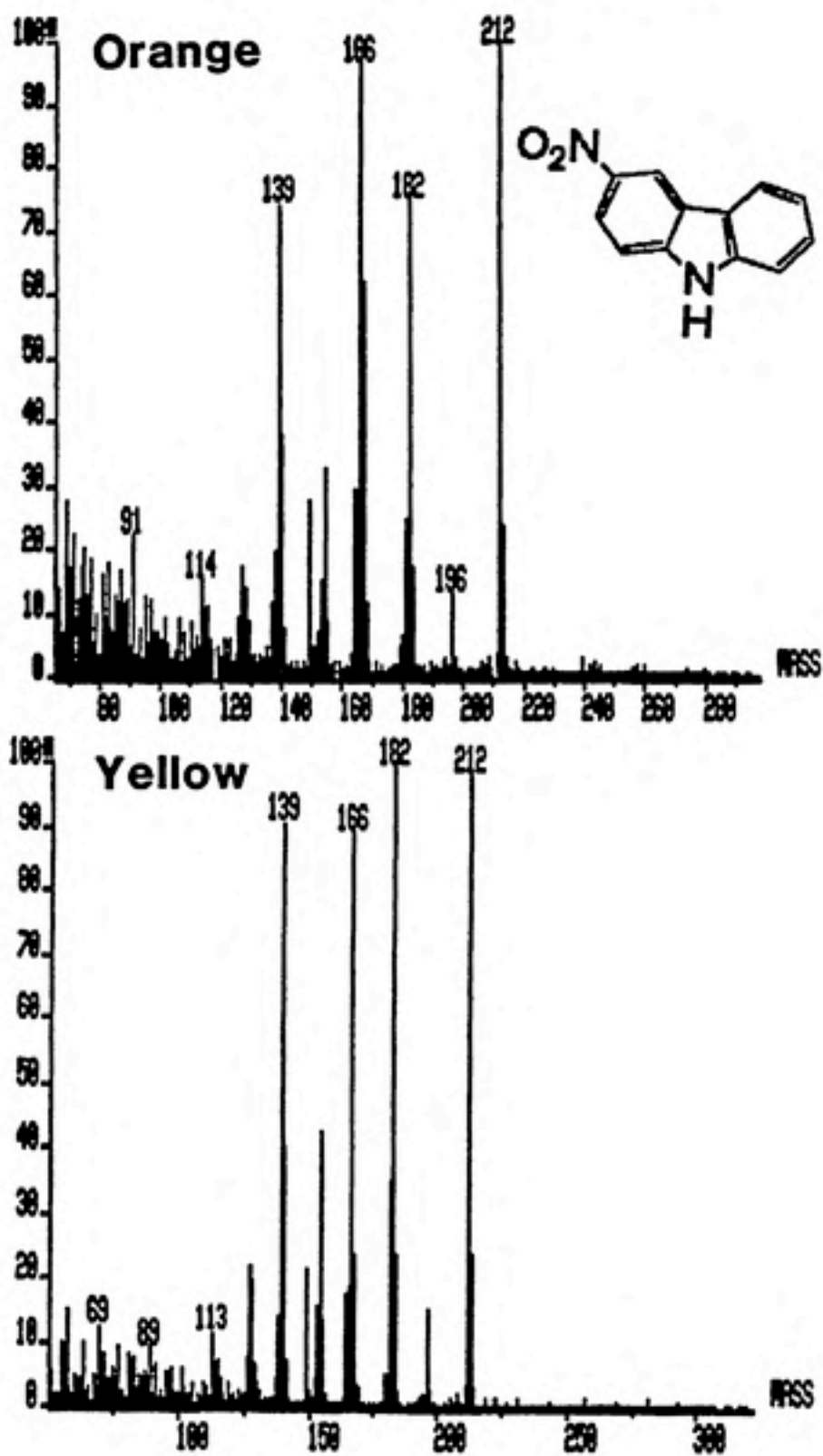FIGURE 5(c) Mutagenicity of 1-Nitrocarbazole in Salmonella Strain TA 98

TABLE 5(b) Mutagenicity of 1-Nitrocarbazole

in Salmonella Strain TA 98
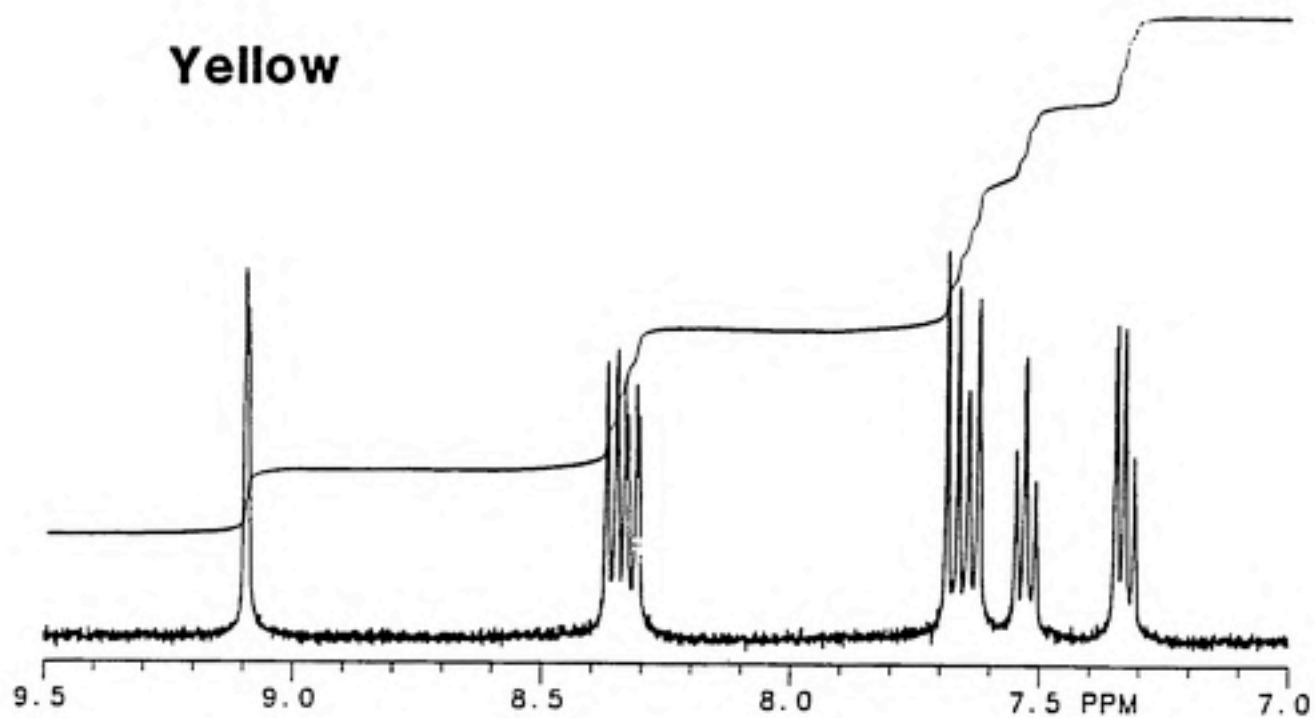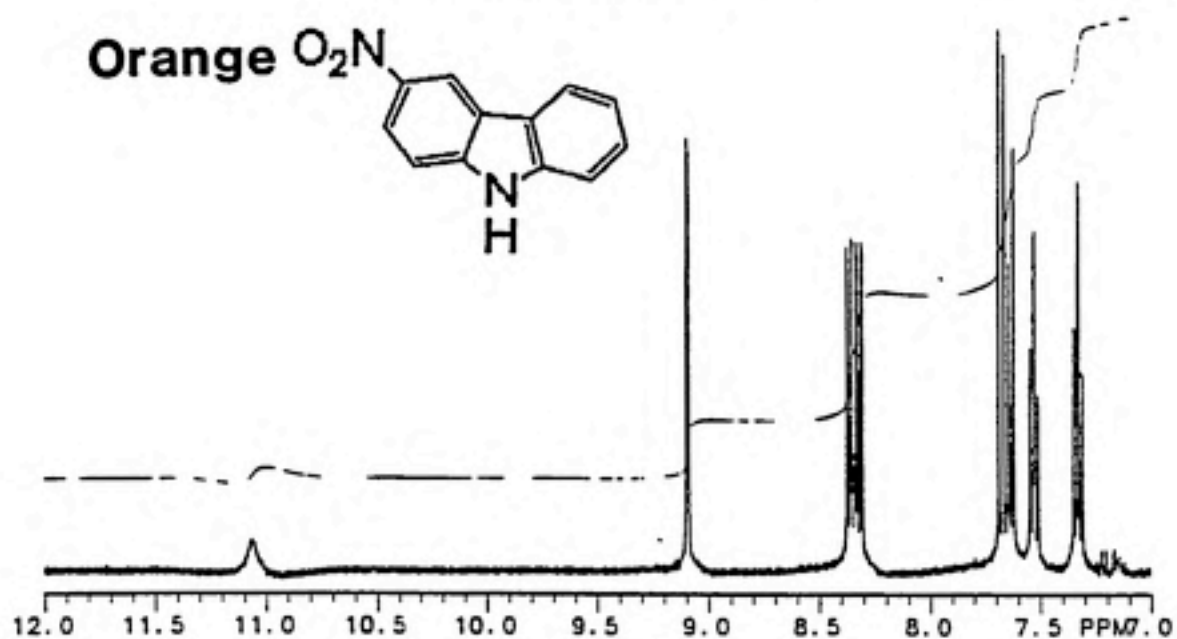
Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98	
	-S9	+S9
Positive control	285.5±80.6	449.0±28.3
Spontaneous	26.5±7.1	66.0±5.7
0.1	40.5±1.4	50.5±15.6
1.0	41.0±14.1	60.5±26.9
10.0	74.0±8.5	69.0±17.0
100.0	169.0±19.8	146.0±19.1
Rev/ug	1.2	0.9
r	0.99	0.995

Duplicate plates were employed for each dose.

FIGURE 6(a) Mass Spectrum of
3-Nitrocarbazole

FIGURE 6(b) NMR Spectrum of

3-Nitrocarbazole

TABLE 6(a) Physicochemical Data for

3-Nitrocarbazole

Orange and Yellow

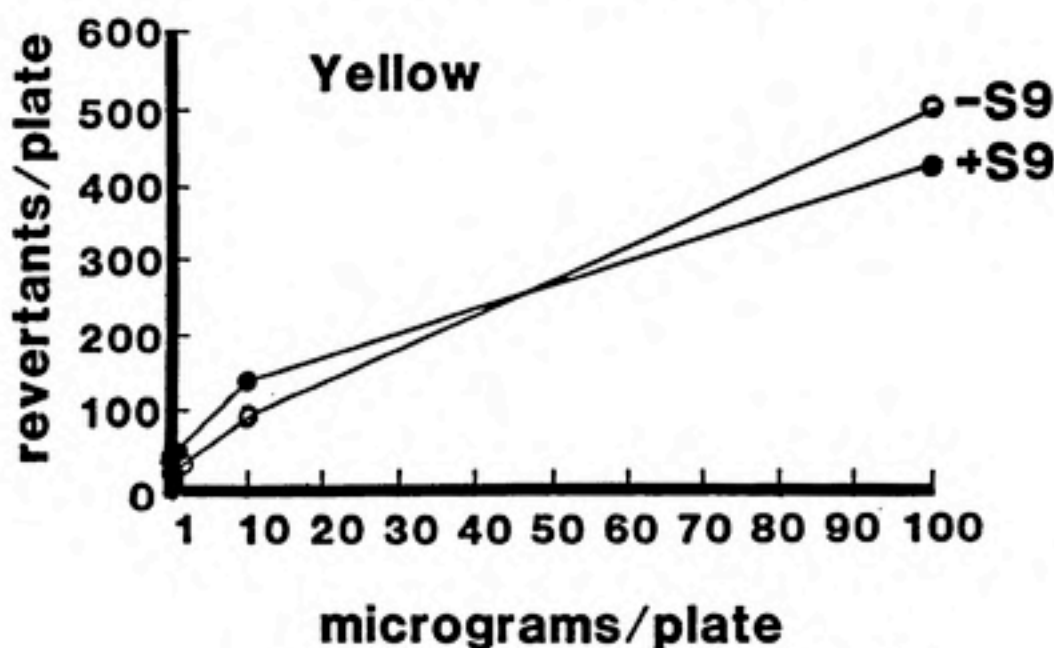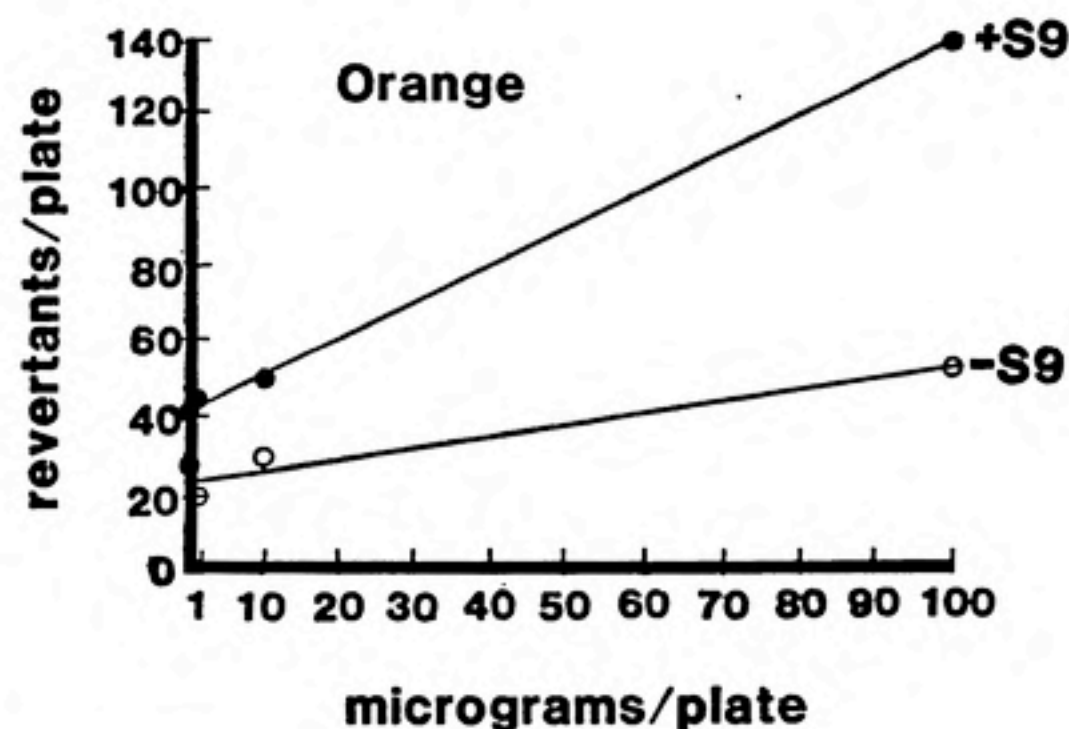---

m.p.: 209-211ºC (orange crystals)
211-213ºC (yellow crystals)
TLC: R_f 0.256 (toluene)
Mass spectrum: MW 212
^{1}H NMR spectrum (400 MHz, acetone-d_6):
    delta 7.68 (d, one proton, H1)
    delta 8.32 (d, one proton, H2)
    delta 9.09 (s, one proton, H4)
    delta 8.36 (d, one proton, H5)
    delta 7.33 (t, one proton, H6)
    delta 7.53 (t, one proton, H7)
    delta 7.63 (d, one proton, H8)
    delta 11.07 (s, one proton, H9)

---

s: singlet peak
d: doublet peak
t: triplet peak

FIGURE 6(c) Mutagenicity of 3-Nitrocarbazole in Salmonella Strain TA 98

TABLE 6(b) Mutagenicity of 3–Nitrocarbazole
in Salmonella Strain TA 98

Orange

Dose (ug/plate)	Salmonella Typhimurium Strain TA 98 -S9	+S9
Positive control	229.5±9.9	304.5±49.5
Spontaneous	29.0±2.8	29.0±14.1
0.1	27.0±14.1	41.5±7.1
1.0	19.0±8.5	44.5±9.9
10.0	29.0±8.5	49.5±1.4
100.0	53.0±2.8	139.0±96.2
Rev/ug	0.3	1.0
r	0.97	0.999

Duplicate plates were employed for each dose.

Yellow

Dose (ug/plate)	Salmonella Typhimurium Strain TA 98 -S9	+S9
Positive control	260.0±50.9	312.0±39.6
Spontaneous	20.0±11.3	30.5±7.1
0.1	18.5±1.4	36.0±11.3
1.0	31.0±5.7	44.0±5.7
10.0	89.5±4.2	141.0±17.0
100.0	502.0±76.4	419.0±353.6
Rev/ug	6.9	10.7
r	0.997	0.9999

Duplicate plates were employed for each dose.

FIGURE 7(a) Mass Spectrum of

1,6-Dinitrocarbazole

2

FIGURE 7(b) NMR Spectrum of
1,6-Dinitrocarbazole

TABLE 7(a) Physicochemical Data for

1,6-Dinitrocarbazole

m.p.: 253-255°C (decomposition) (fluorescent yellow needles)
TLC: R_f 0.186 (toluene)
Mass spectrum: MW 257
^{1}H NMR spectrum (400 MHz, DMSO-d_6):

      delta 8.91 (d, one proton, H2)
      delta 7.51 (t, one proton, H3)
      delta 8.43 (d, one proton, H4)
      delta 9.34 (s, one proton, H5)
      delta 8.42 (d, one proton, H7)
      delta 7.90 (d, one proton, H8)

s: singlet peak
d: doublet peak
t: triplet peak

FIGURE 7(c) Mutagenicity of

1,6-Dinitrocarbazole in Salmonella

Strain TA 98

TABLE 7(b) Mutagenicity of
1,6-Dinitrocarbazole in Salmonella
Strain TA 98

Dose (ug/plate)	Salmonella Typhimurium Strain TA 98 -S9	+S9
Positive control	229.5±9.9	304.5±49.5
Spontaneous	29.0±2.8	29.0±14.1
0.1	22.5±4.2	35.5±4.2
1.0	46.5±9.9	48.0±14.1
1Q.0	78.0±8.5	103.5±38.2
100.0	246.0±76.4	258.0±31.1
Rev/ug	2.1	6.6
r	0.99	0.996

Duplicate plates were employed for each dose.

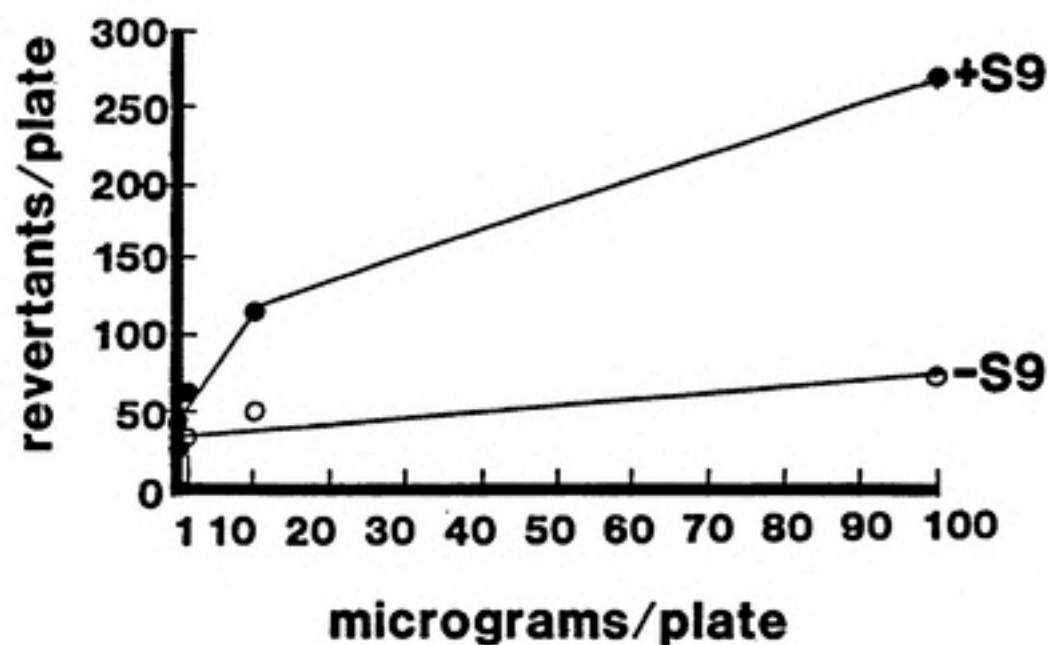# FIGURE 7(d) Mutagenicity of 1,6-Dinitrocarbazole in Salmonella Strain TA 98ND

TABLE 7(c) Mutagenicity of

1,6-Dinitrocarbazole in Salmonella

Strain TA 98ND

Dose (ug/plate)	Salmonella Typhimurium Strain TA 98ND -S9	+S9
Positive control	66.0±22.6	410.5±43.8
Spontaneous	30.5±12.7	43.5±7.1
0.1	26.0±8.5	43.0±8.5
1.0	33.0±5.7	62.5±4.2
10.0	50.0±17.0	116.0±0.0
100.0	73.5±9.9	273.5±32.5
Rev/ug	0.4	6.7
r	0.9	0.98

Duplicate plates were employed for each dose.

FIGURE 8(a) Mass Spectrum of
3,6-Dinitrocarbazole

FIGURE 8(b) NMR Spectrum of
3,6-Dinitrocarbazole

TABLE 8(a) Physicochemical Data for

3,6-Dinitrocarbazole

---

m.p.: 232-234°C (decomposition) (golden-brown crystals)
TLC: R_f 0.056 (toluene)
Mass spectrum: MW 257
^{1}H NMR spectrum (400 MHz, DMSO-d_6):
     delta 7.76 (d, two protons, H1, H8)
     delta 8.39 (d, two protons, H2, H7)
     delta 9.50 (s, two protons, H4, H5)

---

s: singlet peak
d: doublet peak

FIGURE 8(c) Mutagenicity of
3,6-Dinitrocarbazole in Salmonella
Strain TA 98

TABLE 8(b) Mutagenicity of
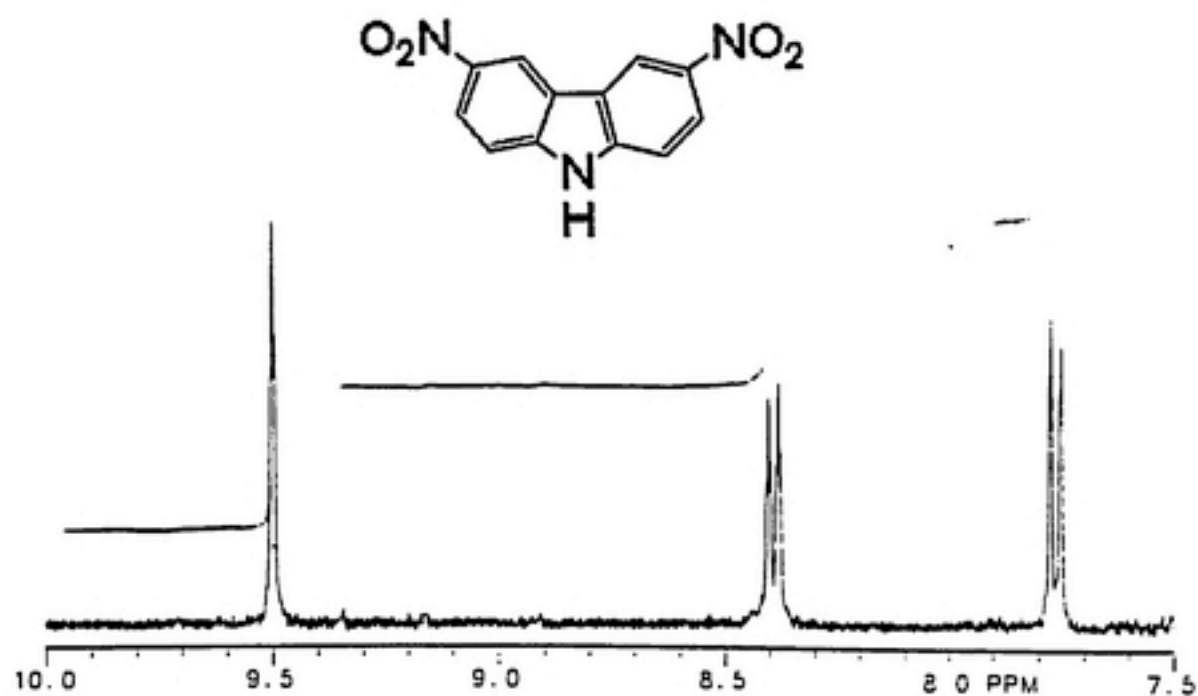
3,6-Dinitrocarbazole in Salmonella

Strain TA 98

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98 -S9	+S9
Positive control	267.0±8.5	372.5±77.8
Spontaneous	32.5±7.1	51.0±5.7
0.1	55.5±8.5	61.0±0.0
1.0	259.0±82.0	209.0±17.0
10.0	1112.5±63.6	1403.0±39.6
100.0	1029.5±154.1	1457.5±1.4
Rev/ug	101.9	134.4
r	0.995	0.9998

Duplicate plates were employed for each dose.

FIGURE 8(d) Mutagenicity of 3,6-Dinitrocarbazole in Salmonella Strain TA 98ND

TABLE 8(c) Mutagenicity of

3,6-Dinitrocarbazole in Salmonella

Strain TA 98ND

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98ND	
	-S9	+S9
Positive control	66.0±22.6	410.5±43.8
Spontaneous	30.5±12.7	43.5±7.1
0.1	43.0±2.8	62.0±11.3
1.0	216.5±1.4	228.0±25.5
10.0	447.5±106.1	1213.0±393.2
100.0	574.5±87.0	901.0±62.2
Rev/ug	34.7	113.5
r	0.9	0.999

Duplicate plates were employed for each dose.

FIGURE 9(a) Mass Spectrum of
2-Hydroxycarbazole

FIGURE 9(b) NMR Spectrum of

2-Hydroxycarbazole

TABLE 9(a) Physicochemical Data for

2-Hydroxycarbazole

---

m.p.: 273-275°C (Aldrich MSDS)
TLC: R_f 0.676 (toluene)
   R_f 0.76 (CH_2Cl_2)
Mass spectrum: MW 183
^1H NMR spectrum (400 MHz, acetone-d_6):
   delta 6.93 (s, one proton, H1)
   delta 6.74 (d, one proton, H3)
   delta 7.88 (d, one proton, H4)
   delta 7.94 (d, one proton, H5)
   delta 7.09 (t, one proton, H6)
   delta 7.25 (t, one proton, H7)
   delta 7.40 (d, one proton, H8)
   delta 10.07 (s, one proton, H9)
   delta 8.33 (s, one proton, OH)

---

s: singlet peak
d: doublet peak
t: triplet peak

FIGURE 9(c) Mutagenicity of 2-Hydroxycarbazole in Salmonella Strain TA 98

101

TABLE 9(b) Mutagenicity of

2-Hydroxycarbazole in Salmonella

Strain TA 98

Dose (ug/plate)	Salmonella Typhimurium Strain TA 98 -S9	+S9
Positive control	357.0±0.0*	174.0±0.0*
Spontaneous	43.5±3.5	66.5±13.4
0.1	42.5±7.1	69.0±19.8
1.0	41.0±8.5	67.5±38.2
10.0	56.5±1.4	66.0±17.0
100.0	39.5±35.4	57.5±15.6
Rev/ug	-0.02	-0.1
r	-0.9	-0.99

Duplicate plates were employed for each dose unless otherwise indicated.
*Only one plate employed.

FIGURE 10(a) Mass Spectrum of
2-Hydroxy-1-Nitrocarbazole

FIGURE 10(b) NMR Spectrum of
2-Hydroxy-1-Nitrocarbazole

TABLE 10(a) Physicochemical Data for

2-Hydroxy-1-Nitrocarbazole

m.p.: 203-205°C (red needles)
TLC: R_f 0.44 (toluene)
R_f 0.875 (CH_2Cl_2)
Mass spectrum: MW 228
^1H NMR spectrum (400 MHz, acetone-d_6):
delta 6.94 (d, one proton, H3)
delta 8.44 (d, one proton, H4)
delta 8.12 (d, one proton, H5)
delta 7.30 (t, one proton, H6)
delta 7.44 (t, one proton, H7)
delta 7.76 (d, one proton, H8)
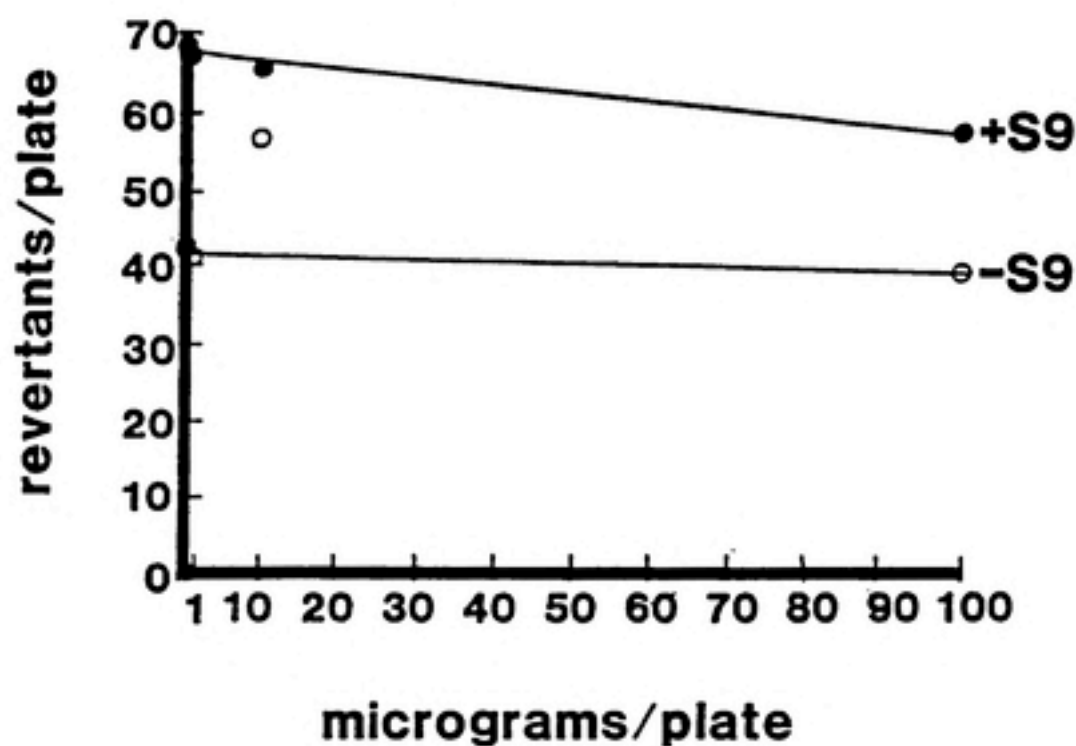delta 11.55 (s, one proton, H9)
delta 11.35 (s, one proton, OH)

s: singlet peak
d: doublet peak
t: triplet peak

FIGURE 10(c) Mutagenicity of
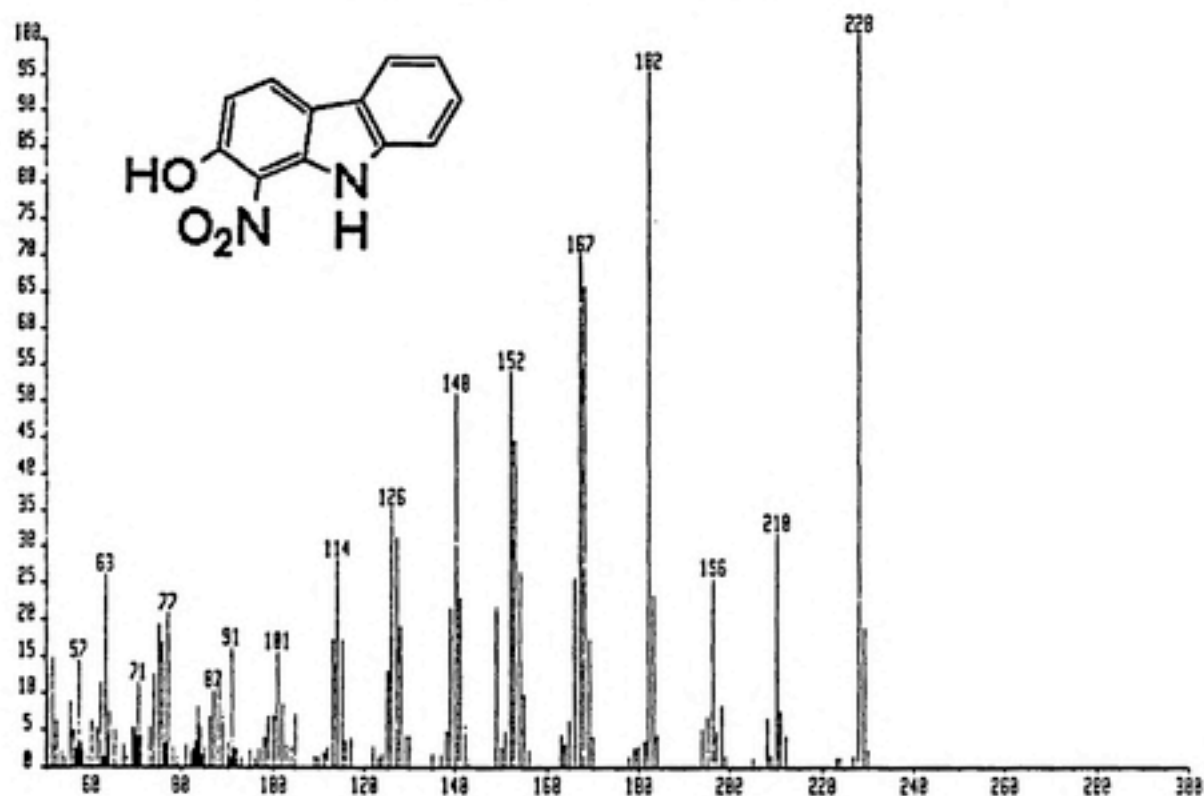
2-Hydroxy-1-Nitrocarbazole in Salmonella

Strain TA 98

TABLE 10(b) Mutagenicity of

2-Hydroxy-1-Nitrocarbazole in Salmonella

Strain TA 98

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98 -S9	+S9
Positive control	377.5±18.4	119.0±2.8
Spontaneous	19.5±4.2	53.5±7.1
0.1	26.0±25.5	43.0±5.7
1.0	28.5±4.2	48.5±7.1
10.0	34.0±2.8	49.0±11.3
100.0	35.5±9.9	64.5±1.4
Rev/ug	0.07	0.2
r	0.7	0.998

Duplicate plates were employed for each dose.

FIGURE 11(a) Mass Spectrum of
2-Hydroxy-3-Nitrocarbazole

FIGURE 11(b) NMR Spectrum of

2-Hydroxy-3-Nitrocarbazole

TABLE 11(a) Physicochemical Data for

2-Hydroxy-3-Nitrocarbazole

---

m.p.: 205-207°C (yellow-orange plates)
TLC: R_f 0.227 (toluene)
      R_f 0.625 (CH_2Cl_2)
Mass spectrum: MW 228
^1H NMR spectrum (400 MHz, acetone-d_6):
      delta 7.24 (s, one proton, H1)
      delta 9.11 (s, one proton, H4)
      delta 8.40 (d, one proton, H5)
      delta 7.45 (t, one proton, H6)
      delta 7.62 (t, one proton, H7)
      delta 7.70 (d, one proton, H8)
      delta 11.01 (s, one proton, H9)
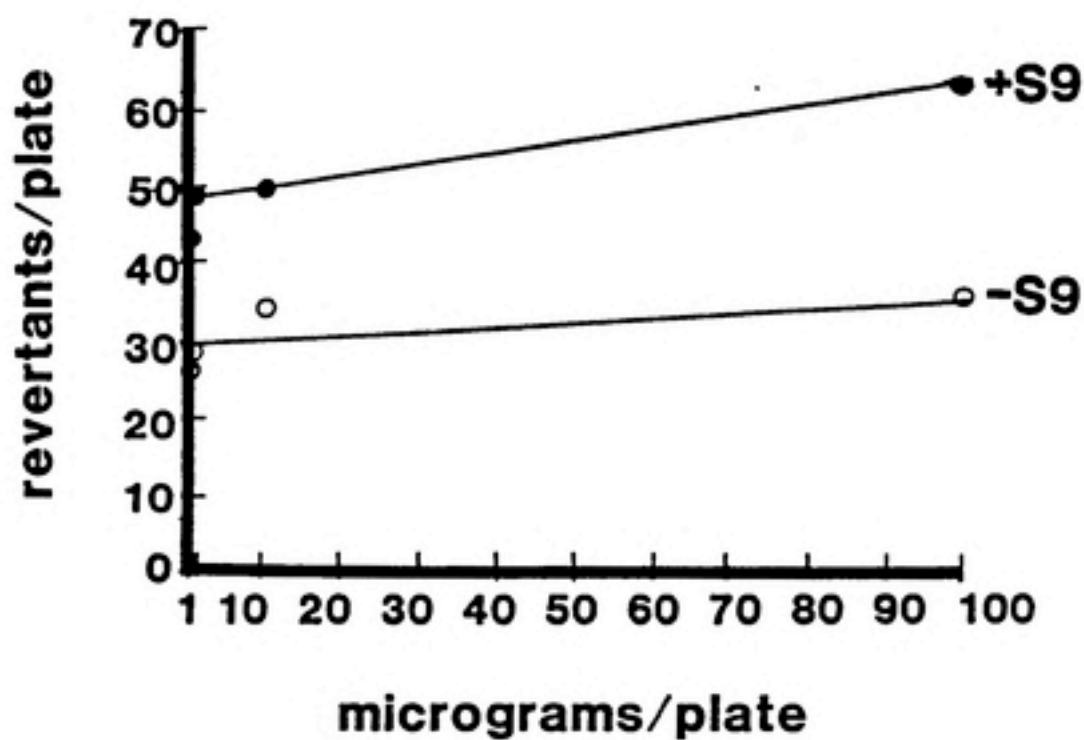   delta 11.12 (s, one proton, OH)

---

s: singlet peak
d: doublet peak
t: triplet peak

FIGURE 11(c) Mutagenicity of 2-Hydroxy-3-Nitrocarbazole in Salmonella Strain TA 98

TABLE 11(b) Mutagenicity of

2-Hydroxy-3-Nitrocarbazole in Salmonella

Strain TA 98

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98	
	-S9	+S9
Positive control	285.5±80.6	449.0±28.3
Spontaneous	26.5±7.1	66.0±5.7
0.1	36.5±15.6	51.5±9.9
1.0	65.0±11.3	62.0±2.8
10.0	306.0±124.5	185.0±28.3
100.0	712.0±5.7	936.0±172.5
Rev/ug	27.0	13.6
r	0.9999	0.9999

Duplicate plates were employed for each dose.

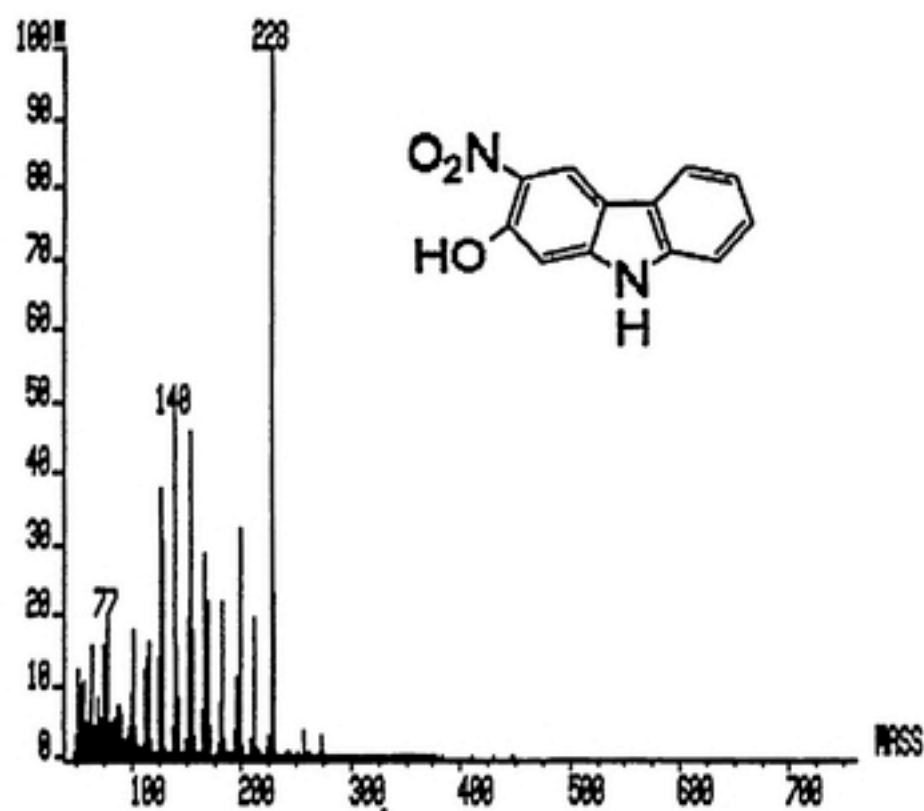FIGURE 11(d) Mutagenicity of 2-Hydroxy-3-Nitrocarbazole in Salmonella Strain TA 98ND

TABLE 11(c) Mutagenicity of

2-Hydroxy-3-Nitrocarbazole in Salmonella

Strain TA 98ND

Dose (ug/plate)	_Salmonella Typhimurium_ Strain TA 98ND -S9	+S9
Positive		
Control	60.5±9.9	333.0±62.2
Spontaneous	21.0±11.3	35.0±8.5
0.1	16.5±4.2	27.5±9.9
1.0	24.5±7.1	33.0±0.0*
10.0	39.5±7.1	48.5±15.6
100.0	90.5±35.4	182.5±83.4
Rev/ug	2.1	1.5
r	0.96	0.999

Duplicate plates were employed for each dose, unless otherwise in-
dicated.
*Only one plate employed.

FIGURE 12(a) Mass Spectrum of

2-Hydroxy-1,3-Dinitrocarbazole

FIGURE 12(b) NMR Spectrum of
2-Hydroxy-1,3-Dinitrocarbazole

TABLE 12(a) Physicochemical Data for

2-Hydroxy-1,3-Dinitrocarbazole

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m.p.: 203-205°C (decomposition) (orange needles)
TLC: R_f 0.033 (toluene)
   R_f 0.068 (CH_2Cl_2)
   R_f 0.800 (acetone)
Mass spectrum: MW 273
^1H NMR spectrum (400 MHz, acetone-d_6):
   delta 9.19 (s, one proton, H4)
   delta 8.29 (d, one proton, H5)
   delta 7.39 (t, one proton, H6)
   delta 7.53 (t, one proton, H7)
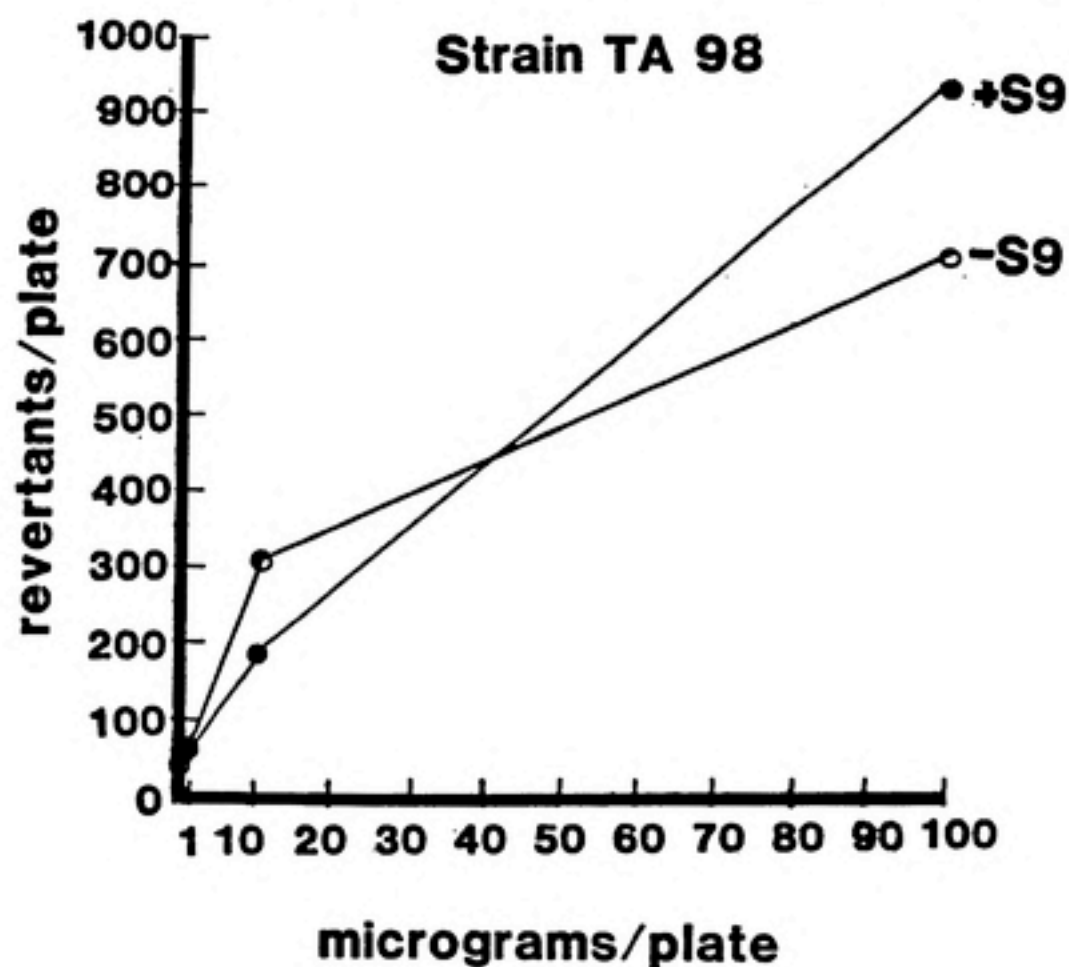   delta 7.78 (d, one proton, H8)
   delta 11.87 (s, one proton, H9)

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s: singlet peak
d: doublet peak
t: triplet peak

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FIGURE 12(c) Mutagenicity of
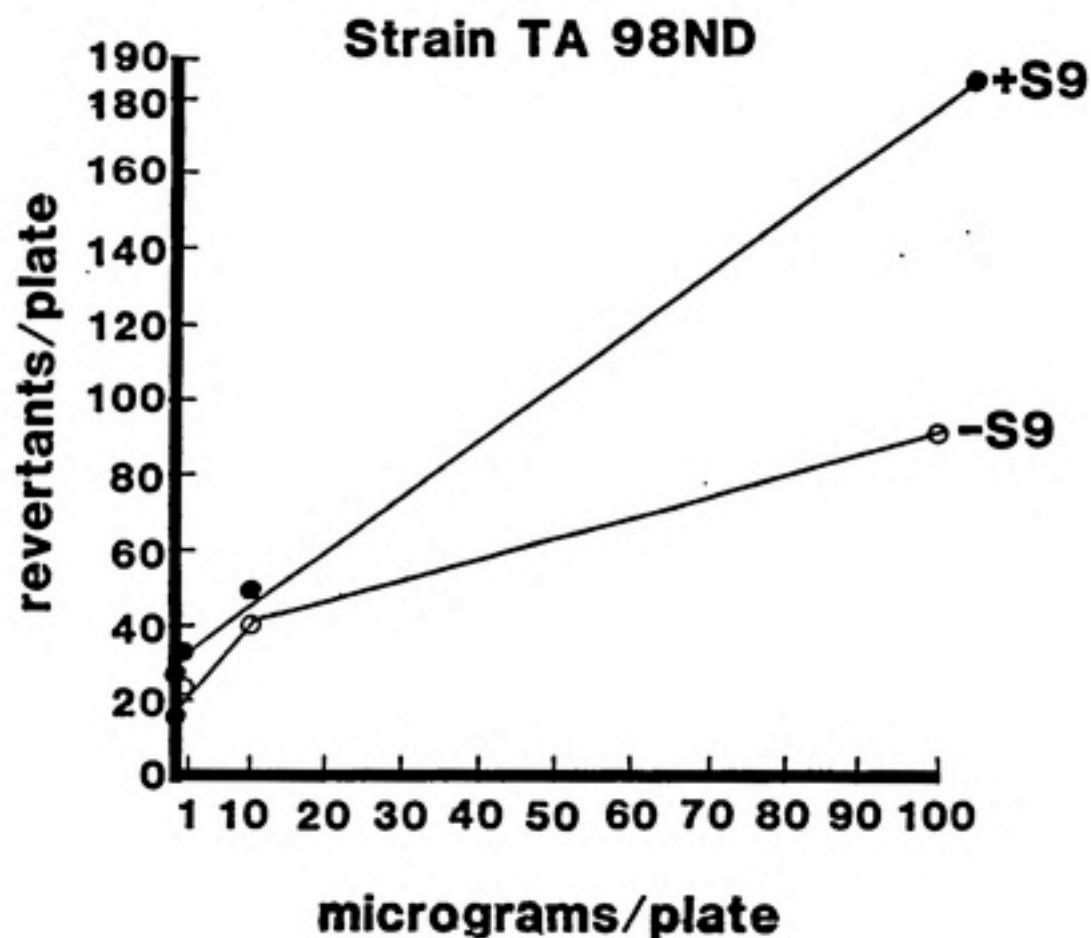
2-Hydroxy-1,3-Dinitrocarbazole in Salmonella

Strain TA 98

TABLE 12(b) Mutagenicity of

2-Hydroxy-1,3-Dinitrocarbazole in Salmonella

Strain TA 98

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98 -S9	+S9
Positive control	377.5±18.4	119.0±2.8
Spontaneous	19.5±4.2	53.5±7.1
0.1	35.0±11.3	36.5±9.9
1.0	34.0±5.7	52.5±4.2
10.0	103.0±11.3	126.0±14.1
100.0	320.0±31.1	286.5±21.2
Rev/ug	7.2	8.7
r	0.996	0.996

Duplicate plates were employed for each dose.

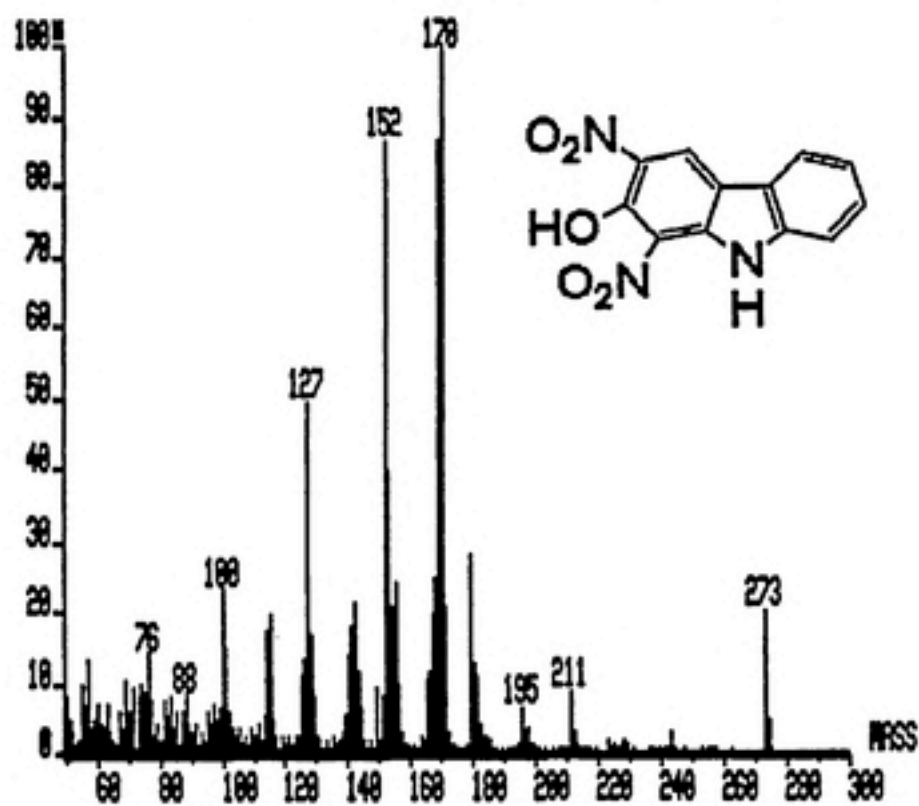# FIGURE 12(d) Mutagenicity of 2-Hydroxy-1,3-Dinitrocarbazole in Salmonella Strain TA 98ND

TABLE 12(c) Mutagenicity of

2-Hydroxy-1,3-Dinitrocarbazole in Salmonella

Strain TA 98ND

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98ND -S9	+S9
Positive control	66.0 ± 22.6	410.5 ± 43.8
Spontaneous	30.5 ± 12.7	43.5 ± 7.1
0.1	27.0 ± 2.8	54.0 ± 19.8
1.0	25.0 ± 14.1	50.5 ± 9.9
10.0	44.5 ± 7.1	63.5 ± 7.1
100.0	116.0 ± 22.6	180.5 ± 41.0
Rev/ug	0.9	1.3
r	0.99	0.9995

Duplicate plates were employed for each dose.

FIGURE 13(a) Mass Spectrum of 2-Hydroxy-1,3,6-Trinitrocarbazole

FIGURE 13(b) NMR Spectrum of

2-Hydroxy-1,3,6-Trinitrocarbazole

TABLE 13(a) Physicochemical Data for

2-Hydroxy-1,3,6-Trinitrocarbazole

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m.p.: 188-190°C (decomposition) (red-orange plates)
TLC: R_f 0.0 (toluene)
      R_f 0.023 (CH_2Cl_2)
      R_f 0.810 (acetone)
Mass spectrum: MW 318
^{1}H NMR spectrum (400 MHz, acetone-d_6):
      delta 9.07 (s, one proton, H4)
      delta 8.62 (s, one proton, H5)
      delta 8.19 (d, one proton, H7)
      delta 8.33 (d, one proton, H8)

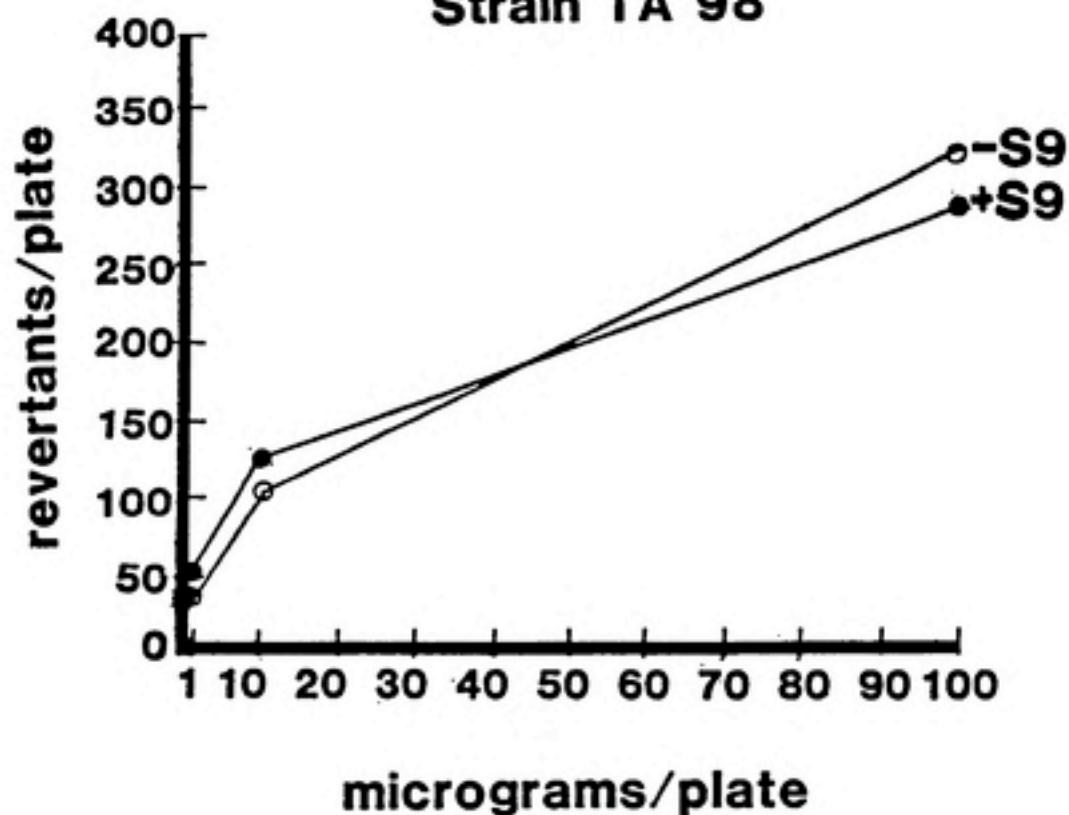
s: singlet peak
d: doublet peak

FIGURE 13(c) Mutagenicity of 2-Hydroxy-1,3,6-Trinitrocarbazole in Salmonella Strain TA 98

TABLE 13(b) Mutagenicity of
2-Hydroxy-1,3,6-Trinitrocarbazole in
Salmonella Strain TA 98

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98 -S9	+S9
Positive control	229.5±9.9	304.5±49.5
Spontaneous	29.0±7.1	29.0±14.1
0.1	19.5±4.2	30.5±7.1
1.0	36.0±11.3	40.0±17.0
10.0	252.0±39.6	132.0±2.8
100.0	992.0±181.0	662.0±65.1
Rev/ug	23.7	10.2
r	0.9998	0.999997

Duplicate plates were employed for each dose.

FIGURE 13(d) Mutagenicity of
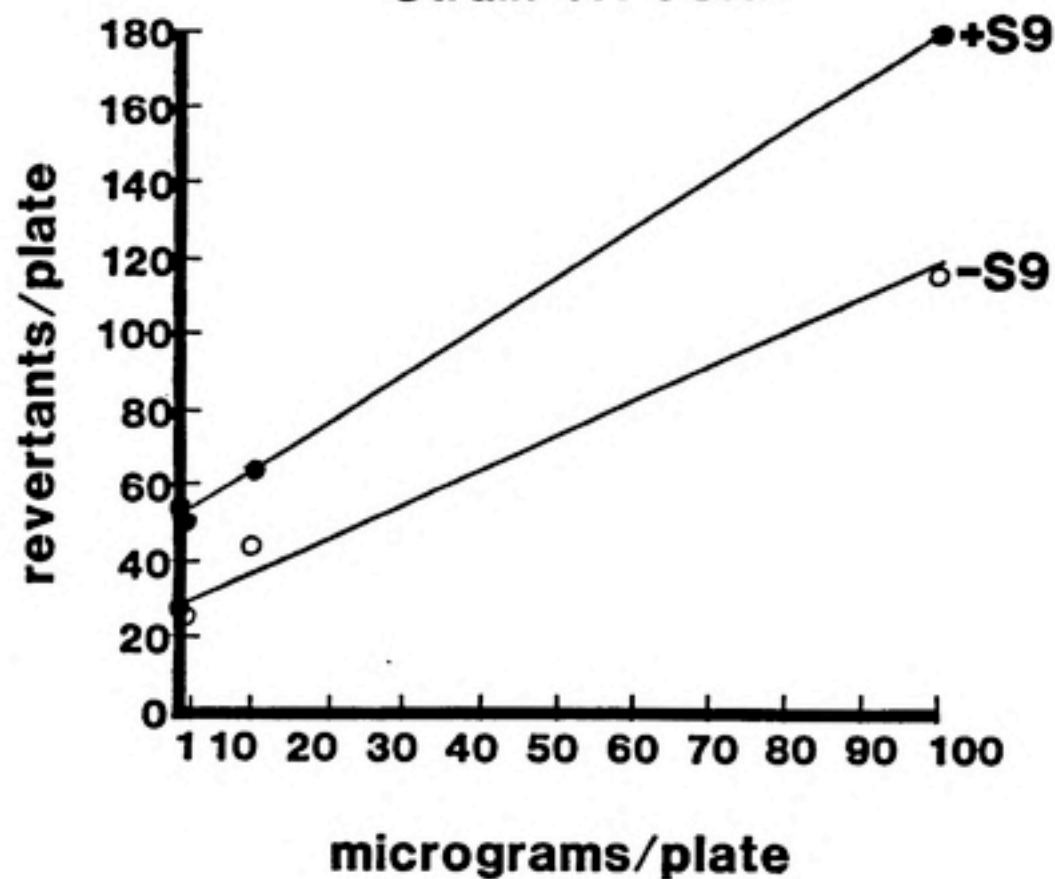# 2-Hydroxy-1,3,6-Trinitrocarbazole in
Salmonella Strain TA 98ND

TABLE 13(c) Mutagenicity of

2-Hydroxy-1,3,6-Trinitrocarbazole in

Salmonella Strain TA 98ND

Dose (ug/plate)	*Salmonella Typhimurium* Strain TA 98ND -S9	+S9
Positive control	66.0±22.6	410.5±43.8
Spontaneous	30.5±12.7	43.5±7.1
0.1	27.0±2.8	56.0±5.7
1.0	32.5±12.7	57.0±17.0
10.0	82.5±1.4	85.0±0.0*
100.0	256.0±39.6	327.0±2.8
Rev/ug	5.6	2.7
r	0.99997	0.9999

Duplicate plates were employed for each dose, unless otherwise indicated.

*Only one plate employed.

TABLE 14 Mutagenicity Results for Carbazole Compounds

Compound	Revertants/nanomole TA 98 -S9	+S9	TA 98ND -S9	+S9
Carbazole	0.01	0.01	-	-
1-Nitrocarbazole	0.3	0.2	-	-
3-Nitrocarbazole:				
Orange product	0.06	0.2	-	-
Yellow product	1.5	2.3	-	-
1,6-Dinitrocarbazole	0.5	1.7	0.1	1.7
3,6-Dinitrocarbazole	26.2	34.5	8.9	29.2
2-Hydroxycarbazole	-0.004	-0.02	-	-
2-Hydroxy-1-nitro-carbazole	0.02	0.04	-	-
2-Hydroxy-3-nitro-carbazole	6.2	3.1	0.5	0.3
2-Hydroxy-1,3-di-nitrocarbazole	2.0	2.4	0.2	0.4
2-Hydroxy-1,3,6-tri-nitrocarbazole	7.5	3.3	1.8	0.9

V. DISCUSSION

5.1. Discussion of Results

The reasons for the varying activities of the carbazole compounds may lie in some of the structure-activity relationships outlined in the Background section. Those relationships are the following:

(1) Degree of aromaticity of the PAH.

(2) Resonance stabilization of the reactive electrophile, i.e., the nitrenium ion.

(3) Orientation of the nitro group.

(4) Degree of nitro-substitution.

According to Ho et al. (1981), most heterocyclic compounds with one to four rings produce no mutagenicity, while five-ring compounds have been found to give significant activities. With its lack of activity, the tricyclic compound carbazole appears to follow this trend. The presence of five rings in the aromatic structure may allow for better insertion of the compound into the DNA to form a DNA adduct.

The same reason may be given for the inactivity of 2-hydroxy-carbazole, with an additional possible deactivating effect of the hydroxy group at the C2 position. The presence of such an electron-donating group, according to Vance and Levin (1984), may effectively prevent resonance stabilization or reaction of the carbonium ion with a nucleophile such as DNA by its presence on a ring carbon involved in resonance stabilization.

That may also explain why 2-hydroxy-1-nitrocarbazole is inactive as a mutagen, even less active than 1-nitrocarbazole. Unlike the 1-nitro isomer, addition of a hydroxy group at the C2 position distinctly increased activity for 3-nitrocarbazole, perhaps by assisting in resonance stabilization.

Another reason for the nonmutagenic behavior of 2-hydroxy-1-nitrocarbazole, as well as for the weak mutagenicity of 1-nitrocarbazole, may be the orientation of the nitro group along the short axis of the molecule. This, according to Vance and Levin, causes the adduct to orient broadside to the DNA helix, which does not facilitate intercalation and the subsequent frameshift mutation.

Somewhat analogous to this is the approach taken by Klopman and Rosenkranz (1984), who used the presence of activating and deactivating fragments to predict mutagenic activity. The presence of a substituted carbon para to the nitro group constitutes an activating fragment, whereas the presence of an unsubstituted carbon para to the nitro group constitutes a deactivating fragment. Figure 1 on page 38 illustrates these fragments. Both 1-nitrocarbazole and 2-hydroxy-1-nitrocarbazole possess deactivating fragments.

On the other hand, 3-nitrocarbazole, 2-hydroxy-3-nitrocarbazole, and 3,6-dinitrocarbazole all have nitro groups oriented along the long axis, creating activating fragments. Such orientations allow adducts to orient end-on and perpendicular to the DNA helix, facilitating intercalation and DNA frameshift mutation. 2-Hydroxy-3-nitrocarbazole and 3,6-dinitrocarbazole were both quite active, and the yellow 3-nitrocarbazole isomer possessed significant activity. However, the orange 3-nitrocarbazole was only weakly active. The yellow 3-nitro

product may have been more active than the orange 3-nitro product due to the very small amount of a dinitrocarbazole in the mass spectrum of the yellow product or to an orange contaminant co-eluting with 3-nitro-carbazole and diluting or deactivating the orange product. The contaminated preparation of 1-nitrocarbazole may be active due to the presence of 2-nitrocarbazole, which also has a nitro group along the long axis of symmetry, creating an activating fragment.

1,6-Dinitrocarbazole and 2-hydroxy-1,3-dinitrocarbazole have nitro groups oriented along both axes, creating both activating and deactivating fragments. Such orientations may be responsible for the compounds' weak to moderate activity. The activating and deactivating fragments may tend to cancel each other out.

2-Hydroxy-1,3,6-trinitrocarbazole has two nitro groups along the long axis, creating two activating fragments, and one nitro group along the short axis, creating a deactivating fragment. The C1 deactivating fragment and the C6 (or C3) activating fragment may cancel each other out, leaving the C3 (or C6) activating fragment as the remaining determinant of mutagenicity. This may explain why 2-hydroxy-3-nitro-carbazole and 2-hydroxy-1,3,6-trinitrocarbazole have similar activities.

In addition to the possession of two activating fragments, the high activities of 3,6-dinitrocarbazole and 2-hydroxy-1,3,6-trinitrocarbazole may be due to the greater extent of nitration of the compounds. Activity has been found to increase with the extent of nitration of other PAH compounds such as pyrene and fluorene. Maximal activity occurs with the trinitro-substituted compounds. Activity falls off with the addition of a fourth nitro group (McCoy et al., 1981; Mermelstein et al., 1981; Rosenkranz and Mermelstein, 1983). According

to Vance et al. (1985, 1987), the presence of additional electron-withdrawing groups such as nitro groups can increase the mutagenic potency of nitro-PAH. The unmetabolized nitro group serves to exert a strong electron-withdrawing effect on the aromatic structure, facilitating the initial reductions of the other nitro group and increasing the stability of the hydroxylamine intermediate, both of which may increase the potential for genetic damage.

This effect of the greater extent of nitration may also explain the relatively high activities of 1,6-dinitrocarbazole and 2-hydroxy-1,3-dinitrocarbazole compared to some of the mononitro isomers. 1,6-Dinitrocarbazole and 2-hydroxy-1,3-dinitrocarbazole were probably not as mutagenic as their respective 3-nitro isomers, however, because of their possession of both activating and deactivating fragments.

The dramatic decrease in mutagenic activity for all compounds tested in TA 98ND indicates the major role played in their reduction to active intermediates by the "classical" nitroreductase B, which is absent in TA 98ND. However, not all the activity of these compounds was due to nitroreduction by this enzyme, since some residual activity did remain, especially for 3,6-dinitrocarbazole and 2-hydroxy-1,3,6-trinitrocarbazole. Activity was not affected significantly in the contaminated 1-nitrocarbazole preparation. This may be due to the contaminant being either a nonnitro compound or a nitro compound, such as 2-nitrocarbazole, that is not reduced by the "classical" nitroreductase.

Addition of S9 to 1,6-dinitro, 3,6-dinitro-, and 2-hydroxy-1,3-dinitrocarbazole probably increased their activity in TA 98 and restored their activity in TA 98ND due to more effective nitroreduction of the

compounds by mammalian nitroreductases in the S9. Addition of S9 to 3-nitrocarbazole may have increased the compound's activity by both more effective nitroreduction and oxidation of the compound to 2-hydroxy-3-nitrocarbazole and other X-hydroxy-3-nitrocarbazole compounds by the cytochrome P-450 mixed function oxidase system in the S9. 2-Hydroxy-3-nitrocarbazole would not have been very abundant, however, since the C2 position is not very reactive. Hence, the increase in activity for 3-nitrocarbazole by this route may not have been very great.

Activity was decreased with the addition of S9 for 1-nitro-carbazole in TA 98 and for 2-hydroxy-3-nitrocarbazole and 2-hydroxy-1,3,6-trinitrocarbazole in TA 98 and TA 98ND. Oxidation of those compounds by the S9 to hydroxy or dihydroxy compounds perhaps prevented resonance stabilization from occurring, thereby lowering activity. Evidence of this effect may be found in the inactivity of 2-hydroxy-1-nitrocarbazole relative to 1-nitrocarbazole. The decrease in activity with S9 of the contaminated 1-nitrocarbazole preparation, possibly containing 2-nitrocarbazole, may also suggest a deactivating effect of hydroxylation. Other explanations may be that the compounds adsorbed to a certain protein or proteins present in the S9 or that they were converted to less active amino compounds through a hydroxylamino intermediate. 1-Nitropyrene has been found to readily absorb to bovine serum albumin and readily convert to 1-aminopyrene in the presence of rat microsome fraction (Tokiwa et al., 1981).

5.2. Conclusions

From these results, the following conclusions may be made:

(1) Nitro group orientation is important in determining the mutagenic activity of nitrocarbazole and 2-hydroxynitro-carbazole compounds. Activity was higher for compounds

possessing nitro groups at the C3 and/or C6 position and lower for compounds possessing nitro groups at the C1 position. Compounds possessing nitro groups at both positions were only weakly or moderately active.

(2) The extent of nitration was also an important consideration, with activity in general rising with increasing number of nitro groups on the compound.

(3) Based on the different results of 1- and 3-nitrocarbazole and their 2-hydroxy derivatives, the presence of a hydroxy group can greatly affect mutagenicity, perhaps through its effect on resonance stabilization of the arylnitrenium ion, increasing activity for the 3-nitro isomer, but decreasing it for the 1-nitro isomer.

(4) Activity was also dependent on nitroreduction by the "classical" nitroreductase B, based on results in TA 98ND. However, the presence of some residual mutagenicity indicated that other nitroreductases were also involved.

(5) S9 played an important role in the activity of most carbazole compounds, increasing activity distinctly for some, and decreasing it distinctly for others. Based on results in TA 98ND, nitroreduction by S9 mammalian nitroreductases may have played the major role in increasing activity, with the oxidases possibly playing a role as well. The oxidases and other proteins present in S9 may have played the major role in reducing activity.

5.3. Recommendations

Of the compounds studied in this project, three possess sufficient mutagenic activity to merit further study--3,6-dinitrocarbazole, 2-hydroxy-3-nitrocarbazole, and 2-hydroxy-1,3,6-trinitrocarbazole. To obtain a more accurate picture of their mutagenic activity, further purification of the compounds could be done with high-performance liquid chromatography before assaying. Metabolite and DNA adduct studies could also be conducted of all the compounds, or at least those most active, to understand the patterns of metabolism undergone by carbazole compounds and the effect of a heterocyclic nitrogen on the patterns of metabolism undergone by nitro-PAH compounds in general.

Since the C-1 and C-3 positions of carbazole are the most reactive positions, and since 3-hydroxycarbazole has been detected by Johns and Wright (1964) as the major metabolite of carbazole, synthesis of 1- and 3-hydroxycarbazole as additional parent compounds for nitration may provide additional information about the mutagenicity of hydroxynitro-carbazole metabolites and air pollutants. Other carbazole compounds which may be worthy of future study are 2-nitrocarbazole, based on the results of Lavoie et al. (1981) and structure-activity relationships dealing with nitro group orientation, and benzo-and dibenzocarbazoles and their nitro derivatives, based on structure-activity relationships dealing with the degree of aromaticity of heterocyclic compounds. In order to obtain a wider isomer profile of nitrocarbazoles and hydroxy-nitrocarbazoles, nitration with nitric acid in acetic anhydride may also be attempted. The method of Murphy, et al. (1953) in synthesizing 1,2,6,8- and 1,3,6,8-tetranitrocarbazole could be repeated as well. Attempts could be made to nitrate other heterocyclic compounds found in ambient air, such as acridine and benzoquinoline and their hydroxy derivatives.

Present results have shown the nitrocarbazoles and 2-hydroxy-nitrocarbazoles as a group to be relatively inactive. Therefore, they would have to be present in high concentrations to contribute significantly to the mutagenicity of hazardous pollutants in ambient air and cigarette smoke and to the potential resulting health risk arising from exposure.

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