IDENTIFICATION OF THE METABOLITES OF 3-NITROFLUORANTHENE AND 2-NITROFLUORANTHENE FORMED BY SALMONELLA TYPHIMURIUM STRAINS TA98, TA98 ND, and TA98/1,8-DNP6

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ABSTRACT

3-Nitrofluoranthene (3-NFA) and 2-nitrofluoranthene (2-NFA) are environmental pollutants produced during combustion processes, proven to be mutagenic in bacterial assays and potentially carcinogenic in mammalian species.

3-NFA was incubated with Salmonella typhimurium strain TA98 and its variants TA98 ND and TA98/1,8-DNP6, deficient in nitroreductase and transacetylase enzymes respectively. All three strains produced equal amounts of the reductive metabolite 3-aminofluoranthene (3-AFA) over six hours of incubation. The nitroreductase deficient strain TA98 ND produced the metabolite at a slower initial rate of conversion than the nitroreductase proficient TA98 and TA98/1,8-DNP6. 2-NFA incubated with TA98 and TA98 ND produced two identifiable metabolites 2-aminofluoranthene (2-AFA), and N-acetyl-2-aminofluoranthene (2-NAAFA) within 24 hours of incubation. The production of metabolites by TA98 ND was significantly slower than that of TA98. TA98/1,8-DNP6 produced only 2-AFA at slower rates than TA98.

The metabolism of 2-NFA by the Salmonella strains progressed at slower rates than that of 3-NFA. This reflects the difference observed in the mutagenicity of these compounds as measured by the Ames assay (3-NFA highly mutagenic, 2-NFA slightly to moderately mutagenic).

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ACKNOWLEDGEMENTS

I wish to thank my committee for all the time and effort they gave towards completion of this project. Special thanks goes to Dr. Louise M. Ball, my advisor, for taking time out of her hectic schedule to assist me in my quest to understand the finer points of metabolism and compound identification. Her humor, wit, and charm not only made the "rough" days bearable, but made the "smooth" days all the more enjoyable. Dr. Alvis Turner not only deserves thanks for reading, but also for helping me decide the path of my education in the School of Public Health. Dr. Avram Gold deserves recognition for his valuable input in the reporting of results as well as his reading of the final product.

Special thanks to my parents, Leverett Jr. and Linda, and my siblings, Leslie and Leverett III. Through their encouragement and affection, they gave me the strength I needed to complete this project.

Thanks also go to my friends and coworkers Heidi Bojes, David Cozzie, Donna Martin and Ravinder Singh. Without their personal support, completion and defense of this work would not have been possible. Sheila O'Grady magically supported me over 600 miles and will always be considered important in the completion of this work.

I. INTRODUCTION

3-NFA and 2-NFA are isomers of nitrated polycylic aromatic hydrocarbons commonly produced by combustion processes (Nielsen et al., 1984; Zielinska et al., 1986). Because of widespread environmental exposure and experimental evidence for mutagenicity and/or carcinogenicity associated with these compounds, 3-NFA and 2-NFA have the potential to affect human health (DiPaolo et al., 1983; Griebokk et al., 1985; Ball et al., 1986b.; Ball unpublished data).

A large part of the mutagenic response to nitroarenes seen in the Ames assay has been attributed to bacterial metabolism by the nitroreductive enzymes present in the cell system (Rosenkranz et al., 1982; Rosenkranz and Mermelstein, 1983). During the conversion of 3-NFA and 2-NFA to 3-AFA and 2-AFA respectively, a reactive intermediate, N-hydroxy-aminofluoranthene is produced. This reactive intermediate can form highly elctrophilic arylnitrenium ions which can either directly interact with cellular DNA or rearrange to a carbonium ion, also a potentially reactive electrophile (Karpinsky et al., 1984; Howard et al., 1983b.; Patton et al., 1986). This interaction with DNA causes the formation of adducts (Dietrich, 1987) which can interfere with normal cellular function.

The Salmonella typhimurium strains chosen for this study are commonly used in Ames assays to screen for a variety of possible mutagenic compounds. TA98 is a nitroreductase

competent strain of Salmonella expressing both the "classical" and "non-classical" nitroreductive enzyme systems responsible for the metabolism of most nitro-PAHs (Rosenkranz et al., 1980; McCoy et al., 1981). TA98 ND, a variant of TA98, is deficient in the "classical" nitroreductases (Rosenkranz et al., 1982; Orr et al., 1985). TA98/1,8-DNP6, also a variant of TA98, is "classical" and "non-classical" nitroreductase competent but lacks acetyl CoA dependent transferase activity which blocks esterification of arylhydroxylamines to hydroxamic esters (McCoy et al., 1983; Orr et al., 1985).

The purpose of this study was to analyze the reductive metabolites of 2-NFA and 3-NFA formed by three variant strains of Salmonella typhimurium (TA98, TA98 ND, and TA98/1,8-DNP6). A possible metabolic basis for differences in mutagenic potency among the variants of Salmonella typhimurium and isomers of nitrofluoranthene would be investigated. Enough metabolic products had to be collected to allow for characterization of the structure of the metabolite by analytical chemistry techniques.

II. LITERATURE REVIEW

A. 3-Nitrofluoranthene and 2-Nitrofluoranthene in the Environment

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are common combustion products widely dispersed throughout the environment (Nishioka et al., 1982; Gibson, 1982: Schuetzle et al., 1982; Wise et al., 1985; Brorstrom-Lunden and Lindskog, 1985). 2-Nitrofluoranthene (2-NFA), a member of the nitro-PAH family, is detected in extracts of ambient air particulate matter from urban and rural areas (Nielsen et al., 1984; Pitts et al., 1985). 3-Nitrofluoranthene (3-NFA), another family member, has been detected on diesel emission particulate matter in addition to other particulate matter from other sources of combustion (Gibson et al., 1982; Later et al., 1982; Nishioka et al., 1982; Shuetzle et al., 1982). 1-Nitropyrene (1-NP), a well characterized isomer of 2-NFA and 3-NFA (Figure 1), has also been detected in emissions from combustion sources.

Most nitro-PAHs are considered to be biologically inert, requiring metabolism to a reactive species before exerting biological activity. Many nitro-PAHs have proven to be highly mutagenic (Rosekranz et al., 1980; Mermelstein et al., 1981; DiPaolo et al., 1983; King et al., 1984; Wong, 1986; Berry, 1985; Vance and Levin, 1984) and carcinogenic (Oghaki et al., 1982; Lewtas, 1983; Hirose et al., 1984; Wislocki et al., 1986).

Because of widespread environmental exposure as well as experimental evidence for mutagenicity and/or carcinogenicity associated with these compounds, 2-NFA and 3-NFA have the potential to affect human health. In vivo studies have shown that even though inhaled diesel or other combustion particulates are rapidly cleared from the lungs, a significant fraction of the particulate matter remains in the lung allowing for release and metabolism of organic components from the particulates under normal physiological conditions (Brooks et al., 1980; King et al, 1981; King et al., 1983a.). Furthermore, the particulate matter that is cleared from the lung by the mucociliary elevator often descends into the gastro-intestinal tract where the associated organic matter is susceptible to further cellular and bacterial metabolism and absorption into the body.

B. Formation of Nitro-PAHs

The accepted theory of nitro-PAH formation revolves around the nitration of PAHs present in combustion gases and on particulate matter either during combustion or while circulating in ambient air (Nielsen et al., 1984; Zielinska et al., 1986). Many parameters influence the formation of nitro-PAHs including temperature, sunlight, reactant concentration, chemical structure of the parent PAH, ozone concentration, and surface characteristics of the material on which the PAH is adsorbed (Nielsen, 1983; Nielsen, 1984; Kamens et al., 1985; Zielinska et al., 1986).

Figure 2 outlines how nitro-fluoranthenes are thought to be produced during combustion and while circulating in ambient air (Kamens et al., 1985; Zielinska et al., 1986). Nitrogen oxide species (NO_x, dinitrogen pentoxide [N_2O_5], nitric acid [HNO_3]) produced during and after combustion can readily react with fluoranthene molecules, also produced during combustion, via free radical or ionic mechanisms. The most reactive site for electrophilic substitution on the fluoranthene molecule is believed to be the C3 position, the site of highest electron density (Zielinska et al., 1986). 3-NFA has been shown to be the dominant isomer formed in the liquid and solid phases arising from direct nitration of the fluoranthene molecule by NO_2/N_2O_4 or NO_x (Schuetzle et al., 1982; Zielinska et al., 1986). 2-NFA is believed to be the major isomer formed in the vapor phase or ambient air when volatile organic fluorathene reacts with N_2O_5 after free radical formation on the C3 position (Pitts et al., 1985; Zielinska et al., 1986; Zielinska et al., 1987).

C. Stucture Activity Relationship (SAR)

Structural features of nitro-PAHs have been investigated to determine which factors may affect the biological activity of the compounds (McCoy et al., 1983; Rosenkranz and Mermelstein, 1983; Klopman and Rosenkranz, 1984; Vance and Levin, 1984). Compounds that are good substrates for nitroreductase activity by bacterial strains often have high mutagenicity since it is theorized that nitro reduction is

necessary, but not sufficient, for mutagenicity (Vance and Levin, 1984). Vance and Levin (1984) have determined that four factors favor nitro-reduction of the compounds by Salmonella typhimurium and ultimately enhance the electrophilic attraction of metabolites to DNA. The physical dimensions of the aromatic rings plays a role in the determination of the mutagenicity of a nitro-PAH. Compounds with a length of three benzene nuclei are generally highly mutagenic. The isomeric position of the nitro group is important. If the nitro group is situated along the long axis of the molecule, mutagenicity is increased. When the nitro group and ring system of the compound are coplanar, mutagenicity is increased. The ability of the nitro-PAH to stabilize the final electrophilic ion by resonance affects the mutagenicity, an increase in resonance stabilization would be expected to increase mutagenicity. Klopman and Rosenkranz (1984) have linked the presence of certain molecular fragments seen with nitroarenes to an increase in mutagenicity.

According to the SAR requirements set forth by Vance and Levin, 3-NFA should be and is a highly mutagenic compound. It has the optimum length of 3 aromatic rings with the nitro group oriented along the long axis and coplanar to the rest of the molecule. 3-NFA also meets the requirements of the presence of molecular fragments and even though the stability of the arylnitrenium ion formed from the metabolism of 3-NFA is unknown, theoretical resonance

structures have suggested that a highly stabilized electrophile is possible. At this time, preliminary investigation is being done concerning the mutagenicity of 2-NFA, a compound that does meet many of the requirements necessary for mutagenicity.

D. Metabolism of Nitro-PAHs

Since the metabolic transformations of 3-NFA and 2-NFA have not been thoroughly investigated, the well characterized metabolic pathway of 1-NP is believed representative of the metabolic pathway involved with 2-NFA and 3-NFA biotransformation. At this time it is known that 1-NP is activated by both bacterial and mammalian enzymes (Howard and Beland, 1982; Lehninger, 1975; Andrews et al., 1986).

D.1. Bacterial metabolism

The principle metabolites produced from 1-NP biotransformation by Salmonella typhimurium strains and gut flora are those derived from the reduction of the nitro group with addition of six electrons (Howard et al., 1983a.; Jackson et al., 1985; Andrews et al., 1986; Stanton et al., 1986). Nitro-reductases present in the bacterial cell most likely convert 1-NP to N-hydroxy-1-aminopyrene (via reduction from 1-nitrosopyrene) which is considered the reactive intermediate responsible for the mutagenic effects associated with 1-NP. At this time it is difficult to

isolate this metabolic intermediate due to its high degree of instability at either ambient or physiological temperatures (Westra, 1981; Martin et al., 1982; Howard et al., 1983b.).

Under acidic conditions, the N-hydroxy-1-aminopyrene yields arylnitrenium ions which can either directly interact with cellular nucleophiles (including DNA) or rearrange to a carbonium ion, another highly reactive electrophile capable of nucleophile reaction with DNA (Karpinsky et al., 1982; Howard et al, 1983b.; Vance and Levin, 1984; Patton et al., 1986). When neutral conditions at room or physiological temperatures prevail, N-hydroxy-1-aminopyrene is further reduced to 1-aminopyrene, the fully reduced metabolite of 1-NP (Messier et al., 1981).

The 1-aminopyrene formed can undergo acetylation to form N-acetyl-1-aminopyrene via acetyl CoA dependent acetyl tranferase activity (Orr et al., 1985). The acetylated amine product can undergo further activation to form acetoxyamino groups which are also considered highly reactive electrophiles (Andrews et al., 1986).

The proposed metabolic pathway of 3-NFA is outlined in Figure 3 (adapted from Patton et al., 1986). 3-NFA is reduced to 3-nitrosofluoranthene which is further reduced to N-hydroxy-3-aminofluoranthene with a subsequent reduction producing 3-aminofluoranthene (3-AFA), the fully reduced metabolite. Under acidic conditions the N-hydroxy-3-aminofluoranthene can be converted to an arylnitrenium ion,

the reactive electrophile. The proposed metabolism of 2-NFA is similar to the pathway of 3-NFA with the exception of the nitro group being positioned on C2 rather than C3 (Figure 4). 3-AFA and 2-aminofluoranthene (2-AFA) are susceptible to acetylation to form N-acetyl-3-aminofluoranthene (3-NAAFA) and N-acetyl-2-aminofluoranthene (2-NAAFA).

D.2. Mammalian Metabolism

D.2.a. Intestinal Gut Flora Metabolism

In many mamalian systems, intestinal gut flora is considered the major site of in vivo reduction of nitro-PAHs (Ball and King, 1985). Bacterial suspensions from rat intestines have produced 1-aminopyrene from 1-NP (Howard et al., 1983a.). Rats with intact gut flora have been shown to produce a mutagenic, reduced and acetylated urinary tract metabolite of 1-NP, 6-hydroxy-N-acetyl-1-aminopyrene (Ball and King, 1985), while germ free rats were unable to produce large quantities of the metabolites, 8- and 6-hydroxy-N-acetyl-1-aminopyrene, indicating intestinal flora is involved in the metabolism of 1-NP (Ball et al., 1984a.). During the same experiments it was observed that phenols were the first metabolites to be excreted followed by conjugated metabolites some time later, suggesting enterohepatic circulation may be an important step in the bioactivation of 1-NP (Ball and King, 1985).

D.2.b. Reductive Metabolism

It is well known that eukaryotes have very low levels of nitroreductase enzymes (Karpinsky et al., 1982). Nitroreductase activity has been reported in rat hepatic microsomes (Peterson et al., 1979; Ball et al., 1984a.; Djuric et al., 1986) and small amounts of reduction of 1-NP to 1-aminopyrene has been attributed to liver tranformation in germ free rats (Ball and King, 1985). In perfused rat liver, Bond et al. (1984) have also observed reduction followed by N-acetylation of 1-NP resulting in the formation of acetylaminopyrine.

Reductive metabolism can indirectly occur in mammalian species by means of the xanthine oxidase system which is also able to reduce nitro-PAHs (Howard and Beland, 1982). Xanthine oxidase is a cytosolic enzyme normally used to metabolize hypoxanthine (a product of purine base deamination) to xanthine and then uric acid which in turn is excreted (Lehninger, 1975). Each step in the process of hypoxanthine metabolism involves two electron oxidation normally requiring the cofactor flavin adenine dinucleotide (FAD) which acts as the electron acceptor. Nitro-PAHs can be reduced by acting as a substitute for FAD, becoming the electron acceptor. The nitro-PAH enzymatic properties of xanthine oxidase have been supported by Djuric et al. (1986) who have determined that xanthine oxidase is able to catalyze the binding of 3-hydroxy-1-nitropyrene to DNA. Howard and Beland (1983b.) have also shown that 1-NP binds

to DNA in the presence of xanthine oxidase.

D.2.c. Oxidative Metabolism

Nitro-PAHs can undergo oxidative metabolism resulting in the epoxidation and hydroxylation of the aromatic ring. It is believed that mixed function oxidases found in microsomal preparations of both rat liver and rabbit lung tissue are responsible (King et al., 1987; El Bayoumy and Hecht, 1983; Howard et al., 1985). When 1-NP is incubated with liver and lung microsomes, a variety of metabolites are produced including 1-, 3-, 6-, 8- and 10-hydroxy-1-nitropyrene, 1-aminopyrene, N-acetyl-1-aminopyrene, 1-nitropyrene-4,5- or -9,10-dihydrodiol, 1-nitropyrene-4,5- or -9,10-oxide, and 4-, 5-, 6-, 8- or 9-monohydroxy-1-nitropyrene (Ball et al., 1984; Bond and Mauderly, 1984; King et al., 1984; Djuric et al., 1986).

1-NP, mutagenic to rabbit tracheal epithelial cells because of its ability to form 1-NP-DNA adducts, is believed to be metabolized by both oxidative and reductive pathways (King et al., 1987). The reductive metabolites, 1-aminopyrene and N-acetyl-1-aminopyrene, were produced to a lesser extent than the oxidative metabolites, 1-nitro-4,5-dihydro-4,5-dihydroxypyrene and several hydroxy-1-nitropyrenes (1-, 3-, 6-, 8- and 10-). It is important to note that oxidative metabolites are thought to be more readily reduced to DNA binding derivatives than the parent 1-NP suggesting their importance in the role of in vivo production of DNA adducts (Djuric et al., 1986).

3-NFA has been incubated with Aroclor 1254 treated rat liver S9 fraction to produce hydroxylated metabolites on at least 6 of the nine possible positions of the fluoranthene ring system (Ball et al., 1985; Ball et al., 1987). The major metabolites produced appeared to be 8- or 9-hydroxy-3-nitrofluoranthene and possibly an epoxide whose presence was suggested upon elution of a phenol, post acid hydrolysis. 3-NFA incubated with rat, mouse, guinea pig or goat microsomal fractions produced 1-, 6-, 7-, 8-, 9- or 10-hydroxy-3-nitrofluoranthene (Howard et al., 1988). The major metabolites produced in the rat liver were 8- or 9-hydroxy-3-nitrofluoranthene while the guinea pig fraction produced mainly 6-hydroxy-3-nitrofluoranthene. In all microsomal preparations, minor concentrations of 1-hydroxy-3-nitrofluoranthene were observed.

The oxidative metabolism of 2-NFA is currently being studied in this laboratory (unpublished data). Mammalian enzyme systems oxidatively metabolize 2-NFA on the benzo ring distal to the nitro group causing the formation of 2-nitrofluoranthen-8- or -9-ol. Initial epoxidation may occur across the C8-C9 bond and is followed by symmetric rearrangement to the 8- and 9- phenols. An alternative hypothesis considers the formation of phenols via direct hydroxylation at the distal end of the molecule. 2-NFA incubated with S9 liver fraction from Aroclor 1254 treated rat liver has produced 4 major metabolites; 2-nitrofluoranthen-8,9-dione,

2-nitrofluoranthen-5,6-dihydro-5,6-diol, and 2-nitrofluoranthen-8- and -9-ol (Ball in press, 1986a.). Zielenska et al. (1987) have also been able to identify 8-or 9-hydroxy-2-nitrofluoranthene in S9 incubations of 2-NFA.

E. Mutagenicity of Nitro-PAHs

When screened by the Ames assay (Ames et al., 1975), the mutagenicity of ambient air samples, diesel exhaust, fly ash, photocopier fluid and cigarette smoke has been attributed to the presence of direct acting nitro-PAHs (Nielsen et al., 1984b.; Lofroth, 1980; Lofroth 1980; Pitts, 1983; Lewtas, 1983; Ball et al., 1985; Schuetzle, 1983). Mutagenicity is frequently reported with the Ames reverse mutation assay test using a variety of Salmonella typhimurium tester strains including TA1538, TA98, TA98 ND, and TA98/1,8-DNP6 (Ames et al, 1975; Ball et al., 1986b.).

Most nitro-PAHs are considered direct acting mutagens because they do not require exogenous metabolic activation (provided by the addition of S9 liver fractions) during the Ames assay (Ball et al., 1986b.). Current theory suggests that nitro-PAHs must be biologically activated by target cells to induce mutagenicity. They can be converted by bacterial nitroreductases to arylhydroxylamines followed by non-enzymatic conversion to aryl nitrenium ions, highly mutagenic electrophiles that bind directly to DNA to form the N-(deoxyguanosin-8-yl)amino-PAH adduct under acidic conditions (Rosenkranz et al., 1982; Rosenkranz and

Mermelstein, 1983; Dietrich, 1987).

Some indirect acting nitro-PAHs do require exogenous oxidative activation by liver S9 preparations containing mammalian cytochrome P-450 metabolic enzymes (McCoy et al., 1983). Nitro-PAHs can be conjugated to form hydroxamic acid esters that can then be non-enzymatically converted to arylnitrenium ions. However, it has been noted that certain nitro-PAHs are less mutagenic in the Ames assay when S9 liver fraction is added as is the case of 3-NFA and 2-NFA (Ball et al., 1986b.; Ball, unpublished data). With some compounds both reductive and oxidative metabolism are required for significant mutagenicity, as suggested by an increase observed in the mutagenic potency of 9-hydroxy-3-NFA in Ames assays (Kirshner, unpublished data).

E.1. Bacterial Mutagenicity

1-NP has been shown to be moderately mutagenic in Salmonella typhimurium strains TA98 (360 - 450 revertants/nmole) with a decrease in mutagenicity seen with the nitroreductase deficient TA98 ND strain (Wang et al., 1975). When metabolized by rabbit lung S9 or Salmonella bacteria, 1-NP produces a number of reduced and oxidized metabolites most of which produce reversions in the TA98 tester strain with or without the addition of S9 (King et al., 1984; Andrews et al., 1986). It is believed that the 1-nitrosopyrene metabolite of 1-NP is the probable intermediate in the mutagenicity of the compound which upon

further reduction with ascorbic acid (a reducing agent) produces N-hydroxy-1-aminopyrene, a highly reactive electrophile (Howard et al., 1983a.; Heflich et al., 1985). Acetylated metabolites of 1-NP have been shown not to bind to DNA thereby decreasing its potential to induce mutagenicity (Djuric et al., 1986).

3-NFA is considered highly mutagenic, producing 4,000 - 5,500 revertants/nmole in nitro-reductase capable strains of Salmonella typhimurium (Griebrokk et al., 1985; Ball et al., 1986b.). In nitro-reductase deficient strains such as TA98 ND, 3-NFA is considered slightly mutagenic (Ball et al., 1985). Goldring (1986) has shown, however, that with the addition of zinc dust (a reducing agent) to the incubation, mutagenic activity was doubled further supporting the need for nitroreduction before mutagenicity. The reductive metabolites of 3-NFA, 3-aminofluoranthene (3-AFA) and N-acetyl-3-aminofluoranthene (3-NAAFA), were found to be at least 50 times less mutagenic than the parent 3-NFA with 3-NAAFA requiring exogenous activation to produce any mutagenicity at all (Ball et al, 1985; Ball et al., 1986b.). This further supports the theory that the formation of the arylhydroxylamine is an important intermediate in the mutagenicity of 3-NFA.

Until recently, not much has been known about the mutagenicity of 2-NFA. Our laboratory is involved with ongoing research studying the mutagenicity of this compound. In unpublished results, it has been determined that the

mutagenicity of 2-NFA is of a similar order of magnitude of that of 1-NP but via a slightly altered pathway. 2-NFA is considered a slight to moderate mutagen, approximately 5 to 15 fold less mutagenic in the Ames assay than 3-NFA. 2-NFA induces reversions in the three variant strains of Salmonella typhimurium (TA98, TA98 ND, and TA98/1,8-DNP6) with lower activity noted in the nitroreductase deficient strains. Partial restoration of mutagenic activity was observed in the nitro-reductase deficient strain TA98 ND when exogenous activation occured via the presence of S9. When compared to TA98 ND, TA98/1,8-DNP6 showed an increase in direct mutagenic activity with an S9 dependent increase highly noticeable with doses greater than 10 ug/plate. Ring oxidation of 2-NFA is not believed to be the major activating step in Ames assays done in the presence of S9 due to the fact that 8- and 9-hydroxy-2-NFA further oxidized by S9 showed a decrease in mutagenicity (Zielinska et al, 1987). 2-NFA has also been found to be mutagenic towards strain TM677W in the forward mutation assay in the presence of S9 consistent with studies done on other nitro-PAH substances (Ball, 1986b.; Ball, unpublished data; Williams and Lewtas, unpublished data).

E.2. Mammalian Mutagenicity

Many nitro-PAHs exhibit mutagenic activity in several mammalian cell assays. 1-NP and 1-nitrosopyrene can be reduced to the reactive electrophile N-hydroxy-1-aminopyrene

which is a potentially mutagenic compound to Chinese hamster ovary cells (Heflich et al., 1986). The electrophile binds to DNA to form the N-(deoxyguanosin-8-yl)-1-aminopyrene adduct assaociated with mutagenicity in Salmonella typhimurium tester strains. 1-NP and 1-nitrosopyrene also exhibit cytotoxic and mutagenic effects in diploid human fibroblasts (Patton et al., 1986).

3-NFA, 1-NP, and 1,8-dinitropyrene have been found to cause a dose dependent transformation of Syrian hamster embryo cells prior to addition of S9 (Dipaolo et al., 1983; Patton et al., 1986). 2-NFA was inactive and considered not toxic in the C3H101/2 mammalian cell transformation assay which is known for its oxidative metabolism of some nitro-PAHs (Ball, unpublished data).

F. Carcinogenesis

Chemical carcinogenesis is a complex multistep process in which damage realized at the molecular level can progress to cause cancer at the cellular level. Malignant cells arise as the result of interactions of genotoxic chemicals with one or more of the informational molecules of the target cell (Miller and Miller, 1977; Lutz, 1979; Farber, 1981; Hemminki, 1983).

In many tissues, tumor formation most likely occurs in two stages. The first stage is denoted as initiation and is thought to be the direct assault of the genotoxic substance on the DNA molecule. The genotoxic compound can enter the

organism as the ultimate carcinogen (direct acting) or as a procarcinogen requiring metabolic activation to a reactive (electrophilic) species. These electrophilic species randomly and non-enzymatically attack cellular nucleophiles such as RNA, DNA, proteins, lipids, and other cellular constituents. When DNA is the target molecule, covalent attachment occurs forming a DNA adduct which may be repaired, misrepaired, or remain unchanged thereby causing DNA conformational alterations.

Promotion, the second step in tumor formation, can occur immediately after DNA alteration or may require a latency period of an unspecified length of time. Eventually, adducts that have been misrepaired or unrepaired can transform a cell which can be either recognized as foreign and removed from the body or proliferate at an abnormal rate causing malignant or benign tumor formation.

DiPaolo et al. (1983) believe that due to their ubiquitous distribution and ability to induce morphological and mutational transformation in both bacterial and mammalian cells, nitro-PAHs should be considered carcinogenic. Malignant and benign tumors of the lung and liver were observed when eight nitro-PAHs were injected intraperitoneally into newborn male and female mice (Wislocki et al., 1986). Lewtas (1983) reviewed data that showed diesel exhaust to be carcinogenic in short term bioassays done to detect oncogenic transformation, viral enhancement of transformation, and skin tumor initiation.

Subcutaneous injections of 1-NP given to newborn rats may have caused the development of malignant tumors both local and distal to the sight of injection as well as in the mammary glands of the female rats (Hirose, 1984). Oghaki et al. (1982) have determined that 3-NFA and 1-NP may increase lung carcinomas as well as cause malignant fibrous histiocytomas at the site of injection. At this time, very little data exists on the carcinogenicity of 2-NFA. However, it is believed that S9 mediated genotoxicity observed with this compound may be attributed to the formation of an epoxide (Ball, unpublished data).

G. Formation of DNA Adducts

The mutagenicity and carcinogenicity of nitro-PAHs have been attributed to the binding of the electrophilic intermediates and metabolites to DNA (Dietrich, 1987, Singer and Grunberger, 1983; van Houte et al., 1987). Binding of nitro-PAHs and DNA can cause local distortion of the DNA by inducing kinking of the helical structure of DNA thereby inducing enzymatic repair which can lead to mutations if done incorrectly (van Houte et al., 1987). Frame shift mutations also occur when the aromatic moeity of the nitro-PAH-DNA adduct is intercalated into the DNA helix (van Houte et al., 1987).

The major adduct formed between nitro- or amino-PAHs and DNA is the N-(deoxyguanosin-8-yl)-aminopolycyclicaromatic hydrocarbon adduct which results from the covalent binding

of the nitrogen atom of the PAH to the C8 atom of 2'-deoxyguanosine (Singer and Grunberger, 1983; Hemminki, 1983; Howard et al., 1983b., Andrews et al., 1986; Dietrich, 1987). The high electron density at the C8 position of the deoxyguanosine molecule makes it the most likely site for attack by electrophilic arylnitrenium ions (Singer and Grunberger, 1983; Hemminki, 1983). Several other minor DNA adducts have been determined by HPLC methodologies and ^{32}P-postlabeling but none have been fully characterized (Gallegher et al., 1987; Dietrich, 1987).

1-NP does not interact directly with DNA to produce DNA adduct formation but several of its metabolites have (Mermelstein et al., 1981; Howard and Beland, 1982; Howard et al., 1983b.; Djuric et al., 1986; Edwards et al., 1986; Heflich et al., 1986; King et al., 1987). When 1-NP was reduced with xanthine oxidase in the presence of hypoxanthine and DNA, N-(deoxyguanosin-8-yl)-1-aminopyrene was produced along with two other minor DNA adducts which could not be characterized (Howard and Beland, 1982; Howard et al., 1983b.). 1-nitropyrene-9,10- and -4,5-oxides have also been observed to bind directly to DNA (Djuric et al., 1986). Chinese hamster ovary cells incubated with 1-NP and 1-nitrosopyrene have been shown to produce a single DNA adduct (N-(deoxyguanosin-8-yl)-aminopyrene) with the 1-nitrosopyrene being more potent than the 1-NP in adduct formation (Edwards et al., 1986; Heflich et al., 1986). 1-NP metabolites have also been determined to bind to DNA in

rabbit tracheal epithelial cells (King et al., 1987).

The arylnitrenium ion of the 3-NFA metabolic pathway has been shown to bind to DNA to form the N-(deoxyguanosin-8-yl)-3-aminofluoranthene adduct (Dietrich, 1987). It is believed that the adduct can then intercalate in the DNA helical structure to produce frameshift mutations, held partially responsible for the mutagenicity of 3-NFA (Blake & Peacock, 1968; Dietrich, 1987).

H. Analytical Techniques

H.1. Ames Test

The Ames Salmonella typhimurium assay is commonly used to screen for mutagenicity (Ames et al., 1975; Maron et al., 1983; Lewtas, 1983). Because of the lack of repair mechanisms in the strains of Salmonella typhimurium TA98 used, the reverse mutation assay responds to frameshift mutations resulting from base substitutions and frameshifts caused by xenobiotic assault. The Salmonella typhimurium strains normally used have distinct nitroreductase capabilities unless otherwise stated (Rosenkranz et al., 1982). The test is usually done with and without the addition of an S9 fraction to determine if oxidative transformation is a necessary step in a compound's mutagenicity. The added S9 fraction contains mammalian mixed function oxidases and requires the coadministration of a NADPH generating cofactor mix.

In this study, the Salmonella typhimurium strains that had been used for a variety of mutagenic screening tests of 3-NFA and 2-NFA were used to determine the metabolic products formed by each strain. TA98 is a nitroreductase competent strain of Salmonella that expresses both "classical" and "non-classical" nitroreductase activity (Rosenkranz et al., 1980; McCoy et al., 1981; Rosenkranz et al., 1982). The "classical" nitroreductases are responsible for the metabolism of most nitro-PAHs including nitrofurans, nitronaphthalenes and nitrofluorenes. The "non-classical" nitroreductases may be responsible for the metabolism of other nitro-PAHs including dinitropyrenes and nitroacridines.

TA98 ND is a variant of TA98 and is deficient in the "classical" nitroreductases yet still possesses "non-classical" nitroreductases and acetyl CoA dependent acetyl transferases (Rosenkranz et al., 1982; Orr et al., 1985). TA98/1,8-DNP6 is another variant of TA98 originally developed for its resistance to 1,8-dinitropyrene (McCoy et al., 1981). At first it was thought to be deficient in the "classical" nitroreductase activity but was later found to be deficient in acetyl CoA dependent transferase activity blocking the esterification of arylhydroxylamines to hydroxamic acid esters (McCoy et al., 1981; McCoy et al., 1983; Orr et al., 1985).

H.2. High Pressure Liquid Chromatography (HPLC)

Reverse phase chromatography is used to separate the highly polar nitro-PAHs and their metabolites. A hydrophobic rather than a hydrophilic column is used for the stationary phase of the elution process. HPLC can be used to qualitatively analyze and separate unidentified compounds through the comparison of retention times obtained by using certain columns under specified operating conditions. HPLC is most useful when purified standards are available so that retention times of unknowns can be matched to those of standards. If standards are coinjected with unknowns and coelution of both standard and unknown is observed, it is indicative of structural similarity.

H.3. Electron Impact Mass Spectrometry (EIMS)

EIMS provides valuable data as to the molecular weight and molecular structure of an unknown compound. In EIMS, an electron beam impinges on a vaporized molecule displacing an electron (ionizing) and imparting kinetic energy causing fragmentation. A fragmentation pattern is created by the ionized molecules and fragments as they accelerate through a magnetic field. Directed onto a detector, this creates a mass spectrum which is a chemical fingerprint of the sample (Watson, 1985). EIMS requires only a few ug of a sample which must be ionized and vaporized prior to mass analysis.

H.4. Proton Nuclear Magnetic Resonance (^{1}H-NMR)

^{1}H-NMR aids in the determination of the structure of an unknown molecule and helps to distinguish between isomers by determining the number and chemical environment of the protons of the unknown. It does this by determining the resonance frequency of the protons, the frequency at which a proton absorbs a quantum of energy causing it to change its orientation with respect to an applied magnetic field. Protons in different magnetic environments will resonate at different frequencies while protons in comparable environments will resonate at similar frequencies. ^{1}H-NMR requires at least 100 ug of an unknown sample to generate acceptable spectrum.

I. Summary

3-NFA and 2-NFA are wide spread environmental pollutants that have been determined to be mutagenic and possibly carcinogenic in both mammalian and bacterial assays. Because of the potential human exposure to high concentrations of these substances, investigations need to be conducted to elucidate mechanisms of metabolism. The purpose of this study is to analyze the reductive metabolites of these compounds formed by three variant strains of Salmonella typhimurium (TA98, TA98 ND and TA98/1,8-DNP6) and to investigate a possible metabolic basis for differences in mutagenic potency between the variants of Salmonella typhimurium and isomers of nitrofluoranthene.

III. MATERIALS AND METHODS

A. Materials

3-nitrofluoranthene was synthesized and characterized by Midwest Research Institute (Kansas City, MO). Radiolabeled 3-nitro-[^{14}C]-fluoranthene of 13.8 mCi/mMole specific activity and +99% purity was custom synthesized by Midwest Research Institute. 3-aminofluoranthene standard was purchased from Aldrich Chemical Company (Milwaukee, WI).

2-nitrofluoranthene was synthesized by Dr. Louise Ball from 3-aminofluoranthene (Aldrich Chemical Company) as described (Kloetzel et al., 1955). The purity of the compound was verified by HPLC and thin-layer chromatography. 2-nitro-[^{3}H]-fluoranthene was purchased from Chemsyn Science Laboratories (Lenexa, KS). 2-aminofluoranthene was produced from the chemical reduction of 2-nitrofluoranthene (Dr. Louise Ball). N-acetyl-2-aminofluoranthene was obtained from the acetlyation of synthetic 2-aminofluoranthene and N-acetyl-3-aminofluoranthene was obtained from the acetylation of 3-aminofluoranthene (Dr. Louise Ball).

HPLC grade methanol, ethyl acetate, and acetone were purchased from Fisher Scientific (Fairlawn, NJ). Scintiverse E liquid scintillation cocktail was purchased from Fisher Scientific. Baker analyzed grade dimethyl sulfoxide was purchased from Baker Chemical Company (Phillipsburg, NJ). Deuterated acetone (isotropic purity 100 atom %) was purchased from Aldrich Chemical Company (Milwaukee, WI).

Double distilled water was from the central water distribution system at Lineberger Cancer Research Center. Distilled, deionized, carbon-filtered distilled water from an all glass and teflon still was used for chromatography and obtained at the Lineberger Cancer Research Center.

Oxoid Nutrient Broth #2 was obtained from Oxoid Limited (Basingstoke, Hants., England or Columbia, MD). Salmonella typhimurium strain TA98 was obtained from Dr. Bruce Ames (University of California, Berkeley). Salmonella typhimurium strains TA98 ND and TA98/1,8-DNP6 were obtained from Dr. H. S. Rosenkranz (Case Western Reserve University, Cleveland, OH).

B. Methods

B.1. In Vitro Metabolism of 3-NFA by Salmonella typhimurium

Five 20 ml cultures per strain of Salmonella typhimurium TA98, TA98 ND, and TA98/1,8-DNP6 in nutrient broth (25g Oxoid Nutrient Broth #2 in 1 liter double distilled water, pH 7.5) were incubated for 16 hours at 37°C in a dry air shaker to reach a cell density of approximately 1.4×10^9 to 1.6×10^9 cells/ml (Dr. Ball, preliminary data).

To each 20 ml culture, 8 ul of 10 mM 3-nitro-[^{14}C]-fluoranthene in DMSO was added for a final incubation concentration of 4.0 uM with approximately 2.5×10^6 dpm per culture. All five treated cultures were then incubated at 37°C in a dry air shaker in sealed vials with minimal head space. At 0.5, 1, 2, 3, and 6 hour intervals, one culture

was removed and immediately extracted with 20 ml ethyl acetate/acetone (2:1, v/v) three times for a total of 60 ml. All experiments were carried out in duplicate.

Controls were done by incubating 8 ul of 10 mM 3-nitro-[^{14}C]-fluoranthene in 20 ml of nutrient broth for 6 hours at 37°C (dry air shaker) prior to ethyl acetate/acetone extraction.

B.2. In Vitro Metabolism of 2-NFA by Salmonella typhimurium

Five 20 ml cultures per strain of Salmonella typhimurium TA98, TA98 ND, and TA98/1,8-DNP6 (grown in the same manner as previously described) were incubated with 8 ul of 10 mM 2-nitro-[^{3}H]-fluoranthene in DMSO for a final concentration of 4.0 uM, 8.0 x 10^6 dpm per culture (37°C, dry air shaker). At 0.5, 1, 2, 3, and 6 hour intervals, one culture was removed and immediately extracted with ethyl acetate/acetone. All samples were done in duplicate.

Two additional 24 hour incubations (37°C, dry air shaker) were done for each strain of Salmonella typhimurium at concentrations of 4.0 uM (8.0 x 10^6 dpm per culture) 2-nitro-[^{3}H]-fluoranthene. At the end of incubation the cultures were immediately extracted with ethyl acetate/acetone.

Five 20 ml Salmonella typhimurium TA98 cultures were incubated with 40 uM 2-nitro-[^{3}H]-fluoranthene for 24 hours at 37°C (dry air shaker). They were immediately extracted with ethyl acetate/acetone upon removal.

Controls were done by incubating 8 ul of 10 mM 2-nitro-[^{3}H]-fluoranthene in 20 mls of nutrient broth for 24 hours at 37^{o}C (dry air shaker) prior to ethyl acetate/acetone extraction.

B.3. Extraction of 3-NFA and 2-NFA Salmonella typhimurium Incubations

The whole incubate was combined with 1 volume of ethyl acetate/acetone 2:1, v/v (HPLC grade) in a 50 ml capped centrifuge tube, inverted, and centrifuged at 1000 rpm for 5 minutes. The organic layer was removed and set aside. The extraction procedure was repeated twice to give a total organic pool of 3 x 1 volume of the original (60 ml). The extracts were evaporated to dryness on a rotary evaporator (Buchi Rotavapor TM, Flawil, Switzerland) and resuspended in 10 ml methanol (HPLC grade). Samples were then filtered through a C_{18} Sep Pak cartridge (Millipore Corp., Bedford, Ma) and evaporated to dryness under a stream of nitrogen prior to reconstitution with 1 ml methanol for a final concentration of approximately 1 uCi/ml for 3-NFA and 3 uCi/ml 2-NFA samples. When necessary, extracts were stored at -60^{o} C.

NOTE: Prior to each step in the extraction process, 2 x 0.1 ml of the original incubate and the resulting extraction fractions were aliquotted into scintillation vials for determination of radioactive content and percent recovery.

B.4. High Pressure Liquid Chromatography

Separation and purification of metabolites were done using a Varian LC-5000 (Palo Alto, CA) equipped with a six port Rheodyne injector using 20, 100 or 500 ul injection loops. Reverse phase Zorbax ODS columns (DuPont Instruments, Wilmington, DE) fitted with a 4.6 x 50 mm pellicular ODS precolumn were used for analytical separations (4.6 x 250 mm column) and preparative separations (9.4 x 500 mm column). Normal operating pressure for both columns was between 50 and 220 atm.

HPLC effluent was monitored at 254 nm using a Perkin-Elmer LC-85B Spectrophotometric Detector with an LC-85 autocontrol (Norwalk, CT), a dual beam variable wavelength detector with an 8 ul flow cell capable of detecting wavelengths between 190 and 600 nm as well as scanning spectra of selected chromatographic peaks. The instrument was operated at sensitivities of 0.16 to 0.64 absorbance unit maxima. For single wavelength monitoring, data were output to a Spectrophysics SP4270 Integrator (Piscataway, NJ). Stop flow scanning spectra between the wavelengths of 250 to 450 nm were recorded on a Perkin-Elmer 561 recorder.

Since samples were radiolabeled, qualitative and quantitative analysis was done using an ISCO Retriever III fraction collector (Lincoln, NE). Thirty second fractions of HPLC effluent were collected in 20 ml scintillation vials for determination of ^{14}C and ^{3}H content by liquid scintillation counting after the addition of 10 ml

Scinteverse E scintillation cocktail.

Two different HPLC gradient programs were used to separate the metabolites and parent compound present after incubation.

Program I: Separation of 3-NFA and its metabolites 3-AFA and 3-NAAFA

0 to 3 minutes:	30% water 70% methanol
3 to 18 minutes:	30% water 70% methanol to 0% water 100% methanol
18 to 23 minutes:	0% water 100% methanol
23 to 28 minutes:	0% water 100% methanol to 30% water 70% methanol

Flow: Analytical Column: 1 ml/minute
Preparative Column: 4 ml/minute

Program I: Separation of 2-NFA and its metabolites 2-AFA and 2-NAAFA

0 to 20 minutes:	30% water 70% methanol
20 to 25 minutes:	30% water 70% methanol to 0% water 100% methanol
25 to 30 minutes:	0% water 100% methanol
30 to 35 minutes:	0% water 100% methanol to 30% water 70% methanol

Flow: Analytical Column: 1 ml/minute
Preparative Column: 4 ml/minute

Chromatographic fractions were collected manually based on UV absorption peaks. The fractions were dried by evaporation under a stream of nitrogen and stored at -60° C.

B.5. Liquid Scintillation Counting

Samples were counted on an LKB-Wallace Betarack Liquid Scintillation Counter (Model 1217) equipped with a 1217-101 DPM package. 10 ml of Scintiverse E were added to the samples deposited in 20 ml standard glass scintillation vials. Vials were counted for five minutes on the preset

^{14}C channel (for ^{14}C-3-NFA) and ^{3}H channel (for ^{3}H-2-NFA).

The 1217-101 DPM package allows for the conversion of counts per minute (cpm) to disintegrations per minute (dpm). A calibration curve for ^{14}C was obtained by preparing eight replicates containing 10,000 dpm ^{14}C toluene in 10 ml Scinteverse E and then adding progressively greater amounts (by multiples of 10) of nitromethane, a quenching agent. The samples were then counted for five minutes and an automatic spline function was used to generate a "best fit" calibration curve. A calibration curve for ^{3}H was obtained in the same manner by substituting ^{3}H toluene for ^{14}C toluene. Unquenched ^{14}C samples were counted with approximately 90% efficiency while unquenched ^{3}H samples were counted with approximately 30% efficiency.

B.6. Mass Spectrometry

Mass spectrometry was done using the VG 70-250 SEQ Tandem Hybrid MS/MS (Vacuum Generator, Altrincham, Cheshire, England) operated at 70eV. The direct probe temperature was ramped manually from approximately 30°C to 300°C as quickly as possible. 1 to 3 ug of each metabolite produced by each Salmonella typhimurium strain was analyzed prior to pooling of metabolites.

Normally, the relative intensity of ions and ion fragments are assumed consistent within a single compound and are therefore often used as a diagnostic tool in MS. However, with many nitro-PAH, considerable variability in

ion fragment intensities have been attributed to varying sample concentrations and instrument conditions disallowing the use of this technique for identification purposes.

B.7. Nuclear Magnetic Resonance (NMR)

The Varian XL400 Instrument housed in the UNC Chemistry Department was the source of all proton NMR spectra. Similar metabolite samples were pooled to obtain approximately 120 ug of each metabolite for NMR analysis. Purified metabolites were dissolved in 1 ml deuterated acetone and scanned from 0 to 10 ppm. Chemical shifts were reported in ppm, downfield from tetramethyl silane (TMS) with coupling constants (J) reported in Hz.

A. Separation and Identification of 3-NFA and Its Metabolites

A.1. Separation

Extraction methods employed returned 85.5% of the ^{14}C-3NFA incubated with TA98 while 87.4% and 91.9% of the radiolabeled material was recovered from TA98 ND and TA98/1,8-DNP6, respectively. The remainder of the radioactivity was accounted for in the aqueous phase of the extraction.

HPLC fractionation was accomplished by injecting 20 ul of each of the incubation extracts onto a Zorbax ODS analytical column using Program I (1 ml/minute). TA98, TA98 ND and TA98/1,8-DNP6 produced two compounds eluting at 11.2 and 21 minutes. These will be referred to as fraction 1 and fraction 2 respectively. A representative chromatogram (absorption 254 nm) is presented in Figure 5. Parallel controls produced one peak eluting at 21 minutes.

To ensure that fraction 1 was not composed of two or more coeluting compounds under Program I gradient conditions, 20 ul of the TA98 6hr incubation was fractionated under the conditions of Program II. Only one peak eluted, at 12.2 minutes (Figure 6).

A.2. Primary Identification

The retention time of fraction 1 was similar to that of the unlabeled 3-AFA standard. When 20 ul of the extracted

incubations from TA98, TA98 ND and TA98/1,8-DNP6 were coinjected with unlabeled 3-AFA standard, fraction 1 coeluted with the standard suggesting its identity. Fraction 1 produced a U.V. spectrum (when scanned between 250 and 450 nm) with maximum absorption at 300 nm with an additional broad peak at 370 nm. This resembled the spectrum of the 3-AFA standard under similar conditions (Figure 7). Fraction 1 also fluoresced yellow under long wave ultraviolet light (365 nm).

The retention time of fraction 2 was similar to that of the unlabeled 3-NFA standard. When extracted incubations were coinjected with unlabeled 3-NFA, fraction 2 coeluted with the standard. Fraction 2 had a U.V. spectrum with absorption peaks at 254 and 265 nm a broad peak between 370 and 390 nm. The spectrum was similar to that of the 3-NFA standard (Figure 8).

A.3. Quantitation

Quantitative analysis of the production of fraction 1 was made possible through the use of ^{14}C-3NFA and collection followed by counting of 30 second aliquots of eluant over a thirty minute period. The radioactivity profiles obtained from the injection 20 ul of the extracted incubations (approximately 40,000 DPM) were similar and corresponded to the chromatograms produced by absorption at 254 nm (Figure 9).

The curve representing TA98's production of fraction 1 (as percent radiolabeled metabolite) is presented in Figure 10 (Table 1). Metabolite production appears to follow first oder kinetics. Within the first half hour, 30.1% of the parent compound had been converted to fraction 1 followed by 48.9%, 68.6% and 77.9% conversion at 1, 2, and 3 hours respectively. By 6 hours, 97.2% of the radiolabeled material was present in fraction 1.

Figure 11 (Table 2) represents the metabolic curve produced by TA98 ND over the 6 hour incubation period. The metabolism of the parent compound appears to be slower than that in TA98 yet still appears linear with respect to time. At 1 hour, only 16.6% of the original radiolabeled compound had been converted to fraction 1 with 34.9% of the radioactivity present in the fraction at 2 hours and 47.3% present at 3 hours. By 6 hours, 92.9% of the original radiolabeled compound had been converted to fraction 1.

The metabolic curve produced by TA98/1,8-DNP6 is presented in Figure 12 (Table 3) and is similar to that of TA98. Once again, metabolite production appears linear with respect to time for the first two hours of incubation converting to first order kinetics some time after the that. At 0.5 hours, 39.6% of the original radiolabeled material was converted to fraction 1. At 1, 2 and 3 hours, 51.9%, 73.7% and 85.4% of the radioactivity eluted in fraction 1. By 6 hours, 95.6% of the radioactivity had been converted into fraction 1.

A.4. Secondary Identification

Upon separation of fractions 1 and 2 using HPLC methodologies employing the Zorbax ODS preparative column and Program I (4 ml/minute), respective fractions from all incubations were pooled for mass spectrometry and NMR evaluation. 159 ug of fraction 1 was available for analysis while 150 ug of fraction 2 was collected.

A.4.a. Mass Spectrometry (MS)

The mass spectrum produced by 2 ug of fraction 1 is presented in Figure 13. The signal at M/z 217 is the mass expected for the molecular ion (M+) of 3-aminofluoranthene. M/z 202 is believed to be a fragmentation product of M+, M-NH. M/z 189 may also be the fragmentation product M-CNH_2. M/z 229 and M/z 243 are contaminants (shown by examination of single ion chromatograms), possibly hydrocarbons due to column breakdown or the presence of plasticizers.

The mass spectrum produced by 2 ug of fraction 2 is presented in Figure 14. The signal at M/z 247 is consistent with the mass for the M+ of 3-nitrofluoranthene. M-O (M/z 231), M-NO (M/z 217), M-NO_2 (M/z 203) and M-CNO_2 (M/z 189) are common fragmentation peaks seen with many nitro-PAHs (Henderson et al., 1983; Schuetzle and Jensen, 1985). M/z 313 and M/z 385 are believed to be contaminants of hydrocarbon chains (shown by examination of single ion chromatograms). Based upon this information, as well as the U.V. spectrum and elution factors obtained on this fraction, fraction 2 is the parent compound, 3-nitrofluoranthene.

A.4.b. NMR of Fraction 1

The NMR sprectrum of fraction 1 is presented in Figure 15. Chemical shifts, reported in parts per million (ppm) downfield from the standard tetramethyl silane (TMS), and coupling constants (J), reported in Hz, are summarized in Table 4.

8.15 ppm

Doublet, integrated as one proton with $J_{1,2}$ = 8.0 Hz. H-1, a pseudo-bay region proton expected to be downfield in the same approximate region as H-6, H-7 and H-10.

8.05 ppm

Doublet, integrated as single proton with $J_{5,6}$ = 6.8 Hz. H-6, a pseudo-bay region proton expected to be downfield with H-1, H-7 and H-10 and coupled to H-5.

7.95 ppm

Split doublet, integrated as one proton with $J_{7,8}$ = 7.4 Hz and $J_{7,10}$ = 0.9 Hz. H-7, a pseudo-bay region proton expected to be downfield with H-1, H-6 and H-10 and coupled to H-8. The J observed is also similar to H-10, located para the proton and responsible for the fine splitting observed.

7.80 ppm

Doublet, integrated as one proton with $J_{4,5}$ = 8.3 Hz. H-4, located peri to the amino group and coupled to H-5. Located downfield.

7.78 ppm

Split doublet, integrated as single proton with $J_{9,10}$ = 7.5 Hz and $J_{7,10}$ =0.9 Hz. H-10, a pseudo-bay region

proton expected to be downfield with H-1, H-6 and H-7 and coupled to H-9. The J observed is also similar to that of its para proton, responsible for the fine splitting observed.

7.58 ppm

Triplet, integrated as one proton with $J_{5,6}$ = 6.9Hz and $J_{4,5}$ = 8.3 Hz. H-5, coupled with H-4 and H-6.

7.31 ppm

Split triplet, integrated as single proton with $J_{7,8}$ = 7.5 Hz, $J_{8,9}$ = 7.5 Hz and $J_{8,10}$ = 1.1 Hz. H-8, coupled with H-9 and H-7. Split peaks observed are due not only to the neighboring effects of H-9 and H-7 but also from H-10 located across the aromatic ring.

7.22 ppm

Split triplet, integrated as one proton with $J_{8,9}$ = 7.5 Hz, $J_{9,10}$ = 7.5 Hz and $J_{7,9}$ = 1.1 Hz. H-9, coupled with H-8 and H-10. Split peaks observed are due not only to the neighboring effects of H-8 and H-10, but also from H-7 located across the aromatic ring.

6.80 ppm

Doublet, integrated as one proton with $J_{1,2}$ = 7.6 Hz. H-2, normally located downfield of all other protons due to the electron-withdrawing capacity of the nitro group on the parent 3-NFA, but now shifted upfield due to the reduction of the nitro group to an amino group.

The amino protons can occur anywhere in the spectrum and are often difficult to locate because of exchange broadening. Amino groups can exchange with the solvent used for NMR

analysis, but to a much lesser extent than has been observed with hydroxyl groups.

The NMR spectrum, MS spectrum, U.V. spectrum and elution factors of fraction 1 identify it as 3-aminofluoranthene.

B. Separation and Identification of 2-NFA and Its Metabolites

B.1. Separation

Extraction methods recovered 85.1% of the radiolabeled material incubated with TA98, 84.5% incubated with TA98 ND and 92.5% incubated with TA98/1,8-DNP6.

HPLC fractionation used the Zorbax ODS analytical column and Program II (1 ml/minute). TA98 and TA98 ND produced three compounds eluting at approximately 12.2, 13.6 and 25.3 minutes and referred to as fraction A, B, and C respectively. A representative chromatogram (absorption 254 nm) is presented in Figure 16. The fractionation of TA98/1,8-DNP6 produced only fractions A and C (Figure 17). Parallel controls produced one peak eluting at 25.3 minutes.

B.2. Primary Identification

The retention time of fraction A was similar to that of the 2-AFA standard. When 20 ul of the extracted incubations of TA98, TA98 ND and TA98/1,8-DNP6 were coinjected with unlabeled 2-AFA standard, fraction A coeluted with the standard suggesting its identity as 2-AFA. Fraction A had a

U.V. spectrum with maxima at 250 nm and an additional peak at 280 nm. A broader, multi-band peak was also observed between 330 and 380 nm. The spectrum was similar to that of the 2-AFA standard (Figure 18). Fraction A was also fluorescent producing a blue-green fluorescence when placed under long wave ultra violet light (365 nm).

The retention time of fraction B was similar to that of the unlabeled 2-NAAFA standard. When extracted TA98 and TA98 ND samples were coinjected with unlabeled 2-NAAFA, fraction B coeluted with the standard. A well resolved U.V. spectrum of fraction B was difficult to produce due to its low concentration in the samples. However, the crude spectrum produced resembled the spectrum obtained from the standard (Figure 19). A maximum was oberved at 254 nm with two lesser peaks at 273 and 294 nm. A broad multi-band peak was located between 320 and 380 nm.

The retention time of fraction C was similar to that of the unlabeled 2-NFA standard. Upon coinjection with unlabeled 2-NFA standard, fraction C of TA98, TA98 ND and TA98/1,8-DNP6 coeluted with the standard. U.V. scanning fraction C gave a spectrum comparable to the standard with maximum absorption seen at 260 nm (Figure 20). An additional peak was observed at 290 nm with a broad multi-band peak located between 330 and 380 nm.

B.3. Quantitation

Quantitative analysis of the production of fractions A and B from fraction C was made possible through the use of ^{3}H-2NFA. The radioactivity profiles were obtained for 20 ul of each of the extracted incubations (approximately 120,000 DPM). Figure 21 represents the profile obtained for TA98 and TA98 ND, compared to the chromatogram produced by absorption at 254 nm. Figure 22 compares the radioactivity profile of TA98/1,8-DNP6 to the chromatogram produced at 254 nm.

The apparent rate of metabolism of fraction C by all three strains of Salmonella typhimurium was greatly reduced when compared to that of fraction 2 (3-NFA). The metabolite profile obtained with TA98 is presented in Figure 23 (Table 5) and appears to be linear with respect to time through the first six hours of incubation converting to first order kinetics some time after that. Through the first 6 hours, 41.6% of the radioactivity eluted as fraction A. When incubation was carried through for 24 hours, 78.7% of the radioactivity was eluting as fraction A. The kinetics of the reaction from 6 to 24 hours can not be determined withiut additional time points taken between 6 and 24 hours. The enzyme activity responsible for the production of fraction B from fraction A appears to be linear with respect to time with saturation of the enzyme occurring between 6 and 24 hours. By the end of the sixth hour of incubation, 10.9% of the radioactivity eluted as fraction B. 15.9% of the

radioactivity eluted as fraction B 24 hours after the initiation of incubation.

The metabolic profile of TA98 ND presented in Figure 24 (Table 6) reveals that only 6.2% of the radiolabeled material was converted to fraction A (2.7%) and fraction B (3.5%) by the sixth hour of incubation. After 24 hours of incubation, 80.1% of the radioactivity eluted in fraction A with 14.6% eluting in fraction B. Minimal production of both fractions is observed through the first six hours of incubation with a sharp increase seen in the production of fraction A between 6 and 24 hours. The production of fraction B also increased during this period. The kinetics of the enzyme activity are difficult to determine without additional time points between 6 and 24 hours.

TA98/1,8-DNP6 produced a metabolic profile presented in Figure 25 (Table 7). It was able to convert 13.3% of the radiolabeled material to fraction A within the first 6 hours of incubation. After 24 hours of incubation, 91.7% of the radioactivity was eluting in fraction A. The kinetics of the enzyme activity of TA98/1,8-DNP6 appear to be linear with respect to time through the first three hours of incubation, but additional concentrations of subtstrate and time points between 6 and 24 hours are needed to determine its exact kinetics.

In order to increase the amounts of fractions A and B, the concentration of ^{3}H-2NF incubated with TA98 was increased 10-fold. At the end of the incubation period, 22.5% (8.82

umoles) of the radiolabeled material had been converted to fraction A and 1.7% (0.68 umoles) eluted in fraction B. The previous 24 hour incubations of 4.0 umoles of ^{3}H-2NF produced 3.15 umoles of fraction A and 0.64 umoles of fraction B. These numbers suggest the saturation of the nitroreductase enzymes and trans-acetylase enzymes found in TA98 as previously hypothesized.

B.4. Secondary Identification

Upon separation of fractions A, B and C by HPLC using the Zorbax ODS preparative column and Program II (4 ml/minute), respective sample fractions from all strains and time points were pooled for MS and NMR analysis. 146 ug of fraction A was collected as well as 6.5 ug of fraction B. 213 ug of fraction C was also available for analysis.

B.4.a. Mass Spectrometry

MS of 2 ug of fraction A gave the spectrum presented in figure 26. The peak at M/z 217 represents the mass expected for M+ of 2-aminofluoranthene. The fragmentation product at M/z 200 represents M-NH_2. M-CNH_2 (M/z 189) is also a possible fragmentation product. M/z 229 and M/z 243 are possible hydrocarbon contaminants.

Two ug of fraction B gave the mass spectrum presented in figure 27. The signal at M/z 259 is consistent with the mass expected for M+ of N-acetyl-2-aminofluoranthene. M/z 217 is a fragmentation product of M-C_2H_2O with M/z 200 representing

M-NC_2H_4O. M-NC_3H_4O (M/z 189) may also be a fragmentation product. Contaminants of M/z 229 and M/z 243 are also present. This information along with the coelution factor and U.V. spectrum data favors the identification of fraction B as N-acetyl-2-aminofluoranthene. 2-NAAFA was isolated in quantities too low to permit definitive identification by NMR analysis.

MS of 2 ug of fraction C gave the spectrum presented in figure 28. The signal at M/z 247 is consistent with the mass expected for the M+ of 2-nitrofluoranthene. M/z 231, M/z 217, M/z 201, and M/z 189 represent the fragmentation products M-O, M-NO, M-NO_2, M-CNO_2, respectively. These are common fragmentation products of nitro-PAHs. This information as well as the U.V. spectrum and the elution factors identifies fraction C as 2-nitrofluoranthene.

B.4.b. NMR of Fraction A

The NMR spectrum of fraction A is presented in figure 29. The spectrum was not as strong as that of 3-AFA because of the smaller amount of fraction A available for NMR analysis. Chemical shifts and coupling constants that were identifiable are summarized in Table 8.

<u>7.93 ppm</u>

Multiplet, integrated as one proton with $J_{7,8}$ = 7.7 Hz and $J_{7,10}$ = 1.4 Hz. H-7, a pseudo-bay region proton coupled with H-8 and located downfield with H-1, H-6 and H-10. The J observed is similar to that of H-10, located para to the proton and contributing to the splitting observed in H-7.

7.90 ppm

Multiplet, integrated as one proton with $J_{9,10}$ = 7.5 Hz and $J_{7,10}$ = 1.4 Hz. H-10, a psuedo-bay region proton coupled with H-9 and located downfield with H-1, H-6 and H-7. The J observed is similar to that of its para proton, H-7.

7.72 ppm

Doublet, integrated as single proton with $J_{5,6}$ = 6.7 Hz. H-6, a pseudo-bay region proton coupled to H-5. Located downfield in the same approximate region as H-1, H-7 and H-10.

7.59 ppm

Meta-coupled singlet, integrated as single proton with J = 1.7 Hz. H-1, pseudo-bay region proton ortho to the amino group and located downfield with H-6, H-7 and H-10. The fine splitting seen is due to its proximity to the amino group.

7.58 ppm

Doublet, integrated as single proton with J = 6.5 Hz. H-4, coupled with H-5 (J = 6.8, 6.6 Hz).

7.50 ppm

Triplet, integrated as a single proton with J = 6.8, 6.6 Hz. H-5, coupled with H-4 (J = 6.5 Hz) and H-6 (J = 6.7 Hz).

7.36 ppm

Super-imposed triplets, integrated as two protons with J = 7.5, 1.7 Hz. H-8 and H-9, coupled to H-7 (J = 7.7, 1.4 Hz) and H-10 (J = 7.5, 1.4 Hz), respectively.

6.99 ppm

Meta-coupled singlet, integrated as a single proton with J = 1.6 Hz. H-3, a proton located ortho to the amino group and upfield from the remaining protons of the molecule.

This information as well as the information gathered from MS, U.V. spectra and coelution factors, identifies fraction A as 2-aminofluoranthene.

V. DISCUSSION

The bacterial metabolism of 1-NP, isomeric with nitrofluoranthene, has been extensively characterized. Salmonella typhimurium strain TA98 produced two major metabolites of 1-NP, 1-aminopyrene and N-acetyl-1-aminopyrene, and six minor metabolites (Messier et al., 1981). Howard et al. (1983) identified 1-aminopyrene as the major metabolite produced by incubating 1-NP with suspensions of bacteria from rat intestines.

Little information has been published on the bacterial metabolism of 3-NFA and 2-NFA. In this study, when 3-NFA was incubated with Samonella typhimurium strain TA98 and its variants TA98 ND and TA98/1,8-DNP6, one metabolic product was obsereved, 3-AFA. When 2-NFA was incubated with the same strains of Salmonella typhimurium, two metabolic products were produced, 2-AFA and 2-NAAFA.

A. Separation and Identification of 3-NFA and Its Metabolites

HPLC and radioactivity profiles of extracted medium from incubations of 3-NFA and Salmonella typhimurium strains TA98, TA98 ND, and TA98/1,8-DNP6 indicated the production of one metabolite which was identified as 3-AFA (Figure 5). All three strains of Salmonella typhimurium were able to metabolize over 90% of the parent compound present within six hours of the initiation of incubation. The production of 3-NAAFA was anticipated but not observed in any of the

incubations.

TA98 possesses both "classical" and "non-classical" nitroreductive enzymes as well as transacetylase enzymes (Rosenkranz et al., 1980; McCoy et al., 1981; Rosenkranz et al., 1982). The strain is able to accomplish the conversion of 3-NFA to 3-AFA in a relatively rapid manner. The metabolic profile of TA98 appears to approach saturation at six hours (Figure 10, Table 1). Additional experiments with varying concentrations of substrate are needed to determine the exact enzyme kinetics of the reaction.

The conversion of 3-NFA to 3-AFA by TA98 ND proceeds at a slower, more linear rate than that observed with TA98 (Figure 11, Table 2). TA98 ND is deficient in the "classical" nitroreductases associated with rapid conversion of many nitro-PAHs to their metabolic products and is resistant to the mutagenic activity of those nitro-PAHs (Rosenkranz et al., 1982; Orr et al., 1985). The reduction of 3-NFA to 3-AFA in this strain is therefore dependent upon the enzyme activity of the "non-classical" nitro-reductive enzymes. These "non-classical" nitroreductases may react at a slower rate than the "classical" enzymes or may work in harmony with the "classical" reductases in an additive or synergistic manner.

TA98/1,8-DNP6, possessing both "classical" and "non-classical" reductive capacity (McCoy et al., 1981; McCoy et al., 1983; Orr et al., 1985), was able to convert 3-NFA to 3-AFA at a rate similar to TA98 (Figure 12, Table 3). Once

again, the profile appears to be approaching saturation.

3-AFA was identified as the principle metabolite by HPLC, MS, and proton NMR analysis. Relative retention times and coelution with unlabeled 3-AFA standard suggested the identity of the metabolite early on. Further proof was obtained upon comparison of U.V. spectra of the compound and standard (Figure 7). MS of the pooled metabolite gave a molecular ion with the weight of 217, expected for 3-AFA (Figure 13). Identification of fragmentation products of M-NH (M/z 202) and M-CNH_2 (M/z 189) was also possible. Fragmentation products of this type have also been seen with nitro-PAH (Schuetzle, 1985). NMR spectrum of the fraction verified the presence of the nine aromatic protons expected for 3-AFA. Present were three triplets (H-5, H-8 and H-9) and six doublets (H-1, H-2, H-3, H-4, H-6, H-7 and H-10) with coupling constants between 7 to 8 Hz, indicative of aromatic protons (Silverstein et al., 1981).

B. Separation and Identification of 2-NFA and Its Metabolites

Salmonella typhimurium strains TA98, TA98 ND and TA98/1,8-DNP6 were unable to metabolize 2-NFA as quickly as 3-NFA. Six hour incubations produced insufficient metabolite for identification. An additional time point of 24 hours was needed to produce the minimum amount of metabolite required for analysis.

HPLC and radioactivity profiles of extracted medium from incubations of 2-NFA with Salmonella typhimurium TA98 and TA98 ND indicated the production of two metabolites identified as 2-AFA and 2-NAAFA (Figure 16). 2-NFA incubated with TA98/1,8-DNP6 produced only one metabolite, 2-AFA (Figure 17).

Approximately 63% of the parent compound had been converted into 2-AFA and 2-NAAFA by TA98 within the first six hours of incubation (Figure 23, Table 5). However, by 24 hours, 95% of the 2-NFA had been converted into the metabolites (79% 2-AFA and 16% 2-NAAFA). The production of 2-AFA by TA98 confirms the presence of reductive pathways in the strain. The 2-NAAFA produced by the strain was a product of the acetylation of the 2-AFA by acetyl CoA dependent acetyl transferases found in TA98 and TA98 ND (Orr et al., 1985). Throughout the first 6 hours of incubation, enzyme activity appears linear with respect to time for both enzyme systems with kinetics after the sixth hour of incubation difficult to determine. Saturation of the acetyl transferases observed in the presence of excessive amounts of 2-AFA.

Saturation of the nitroreductase enzyme system found within TA98 was determined by increasing the amount of 2-NFA incubated with TA98 by a factor of 10. The TA98 was only able to convert approximately 23% (8.82 umoles) of the 2-NFA to 2-AFA and 2% (0.68 umoles) to 2-NAAFA. More 2-AFA and 2-NAAFA would have been produced in the excess of 2-AFA and

2-NFA available for conversion if the nitroreductases and acetyl tranferases responsible for their production had not become saturated.

TA98 ND produced minimal quantities of 2-AFA and 2-NAAFA through the first six hours of incubation (less than 10% of the 2-NFA present had been converted). However, by 24 hours approximately 95% of the parent compound had been converted to 2-AFA (80%) and 2-NAAFA (15%). Determination of the metabolic kinetics of the reactions requires additional incubation times between 6 and 24 hours. Saturation of the acetyl transferase is suggested in the presence of excessive amounts of 2-AFA (Figure 24, Table 6). At some point between 6 and 24 hours, either another nitroreductase is induced in the bacteria, or the culture has become sufficiently anaerobic that a previously silent oxygen-sensitive reductase becomes active.

The metabolism of 2-NFA by TA98/1,8-DNP6 produced minimal amounts of 2-AFA (13% of the original material) and no 2-NAAFA within the first 6 hours of incubation (Figure 25, Table 7). After 24 hours of incubation, approximately 92% of the 2-NFA had been converted to 2-AFA. Once again, the presence of another nitroreductase or silent nitroreductase should be considered. The lack of 2-NAAFA produced in these incubations confirms the absence of acetyl tranferase enzymes systems in TA98/1,8-DNP6 (Orr et al., 1985).

2-AFA was identified as the principle metabolite of 2-NFA by HPLC, MS, and proton NMR analysis. Relative retention times and coelution with unlabeled 2-AFA suggested the identity of the compound in the early stages of analysis. Comparison of the U.V. spectrum of the metabolite with that of the standard also was consistent with the structural assignment(Figure 18). Mass spectrum of the metabolite indicated a molecular ion expected for 2-AFA (molecular weight 217). Identification of fragmentation products M-NH_2 (M/z 200) and M-CNH (M/z 189) was also possible. A NMR spectrum obtained on the fraction showed two singlets (H-1 and H-3) and one triplet (H-5) coupled to two doublets (H-4 and H-6), all indicative of a naphthalene ring structure (Figure 19). The remainder of the protons appeared as multiplets with comparable coupling constants as would be expected for H-7, H-8, H-9 and H-10.

2-NAAFA was identified as the minor metabolic product by HPLC and MS analysis. When compared to the unlabeled standard of 2-NAAFA, the fraction in question coeluted with the standard and had a similar U.V. spectrum (Figure 20). The MS showed a molecular ion with the weight expected for 2-NAAFA (M/z 259). Fragmentation products of M-C_2H_2O (M/z 217) and M-NC_2H_4O (M/z 200) were also observed (Figure 27). Unfortunately, TA98 and TA98 ND did not produce enough of the metabolite to confirm the structural assignment of the metabolite by proton NMR analysis.

C. Relevance to Mutagenicity

Most nitro-PAH are regarded as direct acting mutagens in the Ames assay since they do not require exogenous activation with S9 fractions (Rosenkranz et al., 1980; Rosenkranz and Mermelstein, 1980). It is thought that nitro-PAH are biologically activated by the nitroreductase and acetylase enzymes present in Salmonella typhimurium strain TA98 and its variants TA98 ND and TA98/1,8-DNP6. The nitroreductases present convert the nitro-PAHs to amino-PAHs forming the arylhydroxylamine as an intermediate. This intermediate can be non-enzymatically converted to a highly electrophilic arylnitrenium ion which can bind directly to DNA (Rosenkranz et al., 1982; Rosenkranz and Mermelstein, 1983; Dietrich, 1987).

1-NP has been shown to be moderately mutagenic in strain TA98 with a decrease in mutagenicity observed in the nitroreductase deficient strain TA98 ND (Wang et al., 1975). It is thought that the metabolic intermediate 1-nitrosopyrene is the probable mutagenic intermediate which is further reduced to N-hydroxy-1-aminopyrene which can then form a highly reactive electrophile (Howard et al., 1983a., Heflich et al., 1985).

The hypothesized route of mutagenicity of nitro-PAH is further supported by 3-NFA's behavior in the Ames assay. The parent compound is highly mutagenic in strain TA98 while it is less mutagenic in strain TA98 ND ("classical" nitroreductase deficient) and TA98/1,8-DNP6 (acetyl

transferase deficient) (Griebokk et al., 1985; Ball et al., 1985b; Ball et al., 1986; Ball, unpublished data). The final proposed metabolic products of 3-NFA, 3-AFA and 3-NAAFA, are also significantly less mutagenic than the parent compound (Ball et al., 1985; Ball et al., 1986). This implies that the metabolism of 3-NFA to 3-AFA via "classical" nitroreductase and acetyl tranferase pathways is an important step in the mutagenic activation of the compound and further metabolism may only detoxify the compound.

This study confirms that 3-NFA is quickly and efficiently metabolized to 3-AFA by Salmonella typhimurium strain TA98. TA98 thus has the ability to produce arylhydroxylamine intermediates thought responsible for mutagenicity. The compound is also metabolized to 3-AFA by TA98 ND but at a slower rate, and/or by a different nitroreductive enzyme system. This alternative pathway may not produce the same electrophilic intermediates necessary for high levels of mutagenicity.

TA98/1,8-DNP6 is also able to produce 3-AFA from 3-NFA yet still has a lower degree of mutagenicity. This suggests that even though the "classical" nitroreductive compounds thought responsible for elevated levels of mutagenicity are present, an additional step of transacetylation or an alternative metabolic pathway may be required for mutagenicity. Since TA98/1,8-DNP6 is deficient in acetyl transferase and possibly other as yet unidentified metabolic

pathways, the electrophilic intermediates needed for mutagenicity may not be present.

Recently, other studies in this laboratory have shown that 2-NFA can be considered a slight to moderate mutagenic compound proving to be 5 to 15 fold less mutagenic than 3-NFA with the metabolic products 2-AFA and 2-NAAFA producing very little mutagenicity (Ball, unpublished data).

The mutagenicity of 2-NFA observed is attributed to the "classical" nitroreductive capacity of the Salmonella typhimurium strains. TA98 had a higher mutagenic response than the TA98 ND strain deficient suggesting once again that "classical" nitroreduction is an important pathway for mutagenicity. TA98/1,8-DNP6 also showed a lower level of mutagenicity when compared to TA98. This suggests once again that acetyl transferases or some other metabolic pathway is important in the mutagenic response of these compounds.

The lower mutagenicity of 2-NFA compared to 3-NFA is consistent with the slower rate of metabolism of 2-NFA in all strains of Salmonella typhimurium. The slower rate of metabolism could be indicative of the strains' decreased ability to metabolize this isomer of nitrofluoranthene. Without metabolism, mutagenicity can not occur.

The positional isomerism of the nitro group on the fluoranthene molecule may affect the metabolism of the isomers. The nitro group of 3-NFA is situated along the long axis of the molecule (Figure 2), a factor Vance and

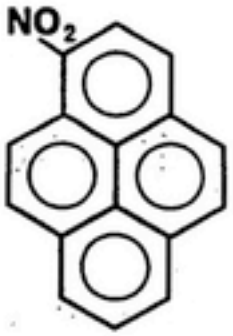
Levin (1984) consider important for efficient nitroreductive metabolism of the compound. The nitro group of 2-NFA is positioned off the long axis (Figure 1) this change in geometry may be enough to decrease the rate of metabolism of 2-NFA by interferring with the access to the active site of the nitroreductase thereby decreasing mutagenicity.

D. Future Work

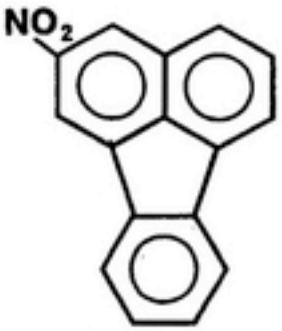
Definitive confirmation by NMR analysis of the 2-NAAFA produced by TA98 and TA98 ND incubated with 2-NFA should be considered. Unfortunately, the Salmonella typhimurium strains produce small quatities of the metabolite as the acetyl tranferase enzyme responsible appears to become staurated in the presence of even small amounts of 2-AFA, the precursor of 2-NAAFA.

Additional incubations of varying concentrations of substrate and increased time of incubation should be considered to determine the kinetics of the metabolism of 3-NFA and 2-NFA.

The mutagenicity of the metabolites produced directly from the bacterial incubations should be evaluated by the Ames assay to determine whether these compounds yield the same results as synthetic preparations of 3-AFA, 2-AFA, and 2-NAAFA.



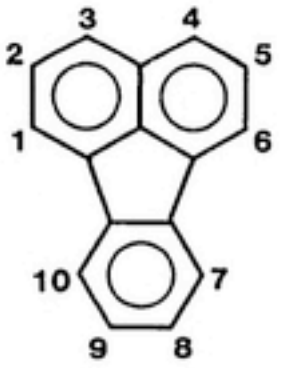
1-NP



2-NFA

3-NFA

Figure 1



3-NFA
(in combustion)

* on diesel exhaust particles
* HNO3 [NO2+] in CH3COOH
* NO2/N2O4 in CH2Cl2
* N2O5 (adsorbed onto filters)

2-NFA
(atmospheric)

* in ambient air
* N2O5 in CCl4 (22°C)
* N2O5 (in gas phase)

Fig. 2 Formation of2-NFA and 3-NFA

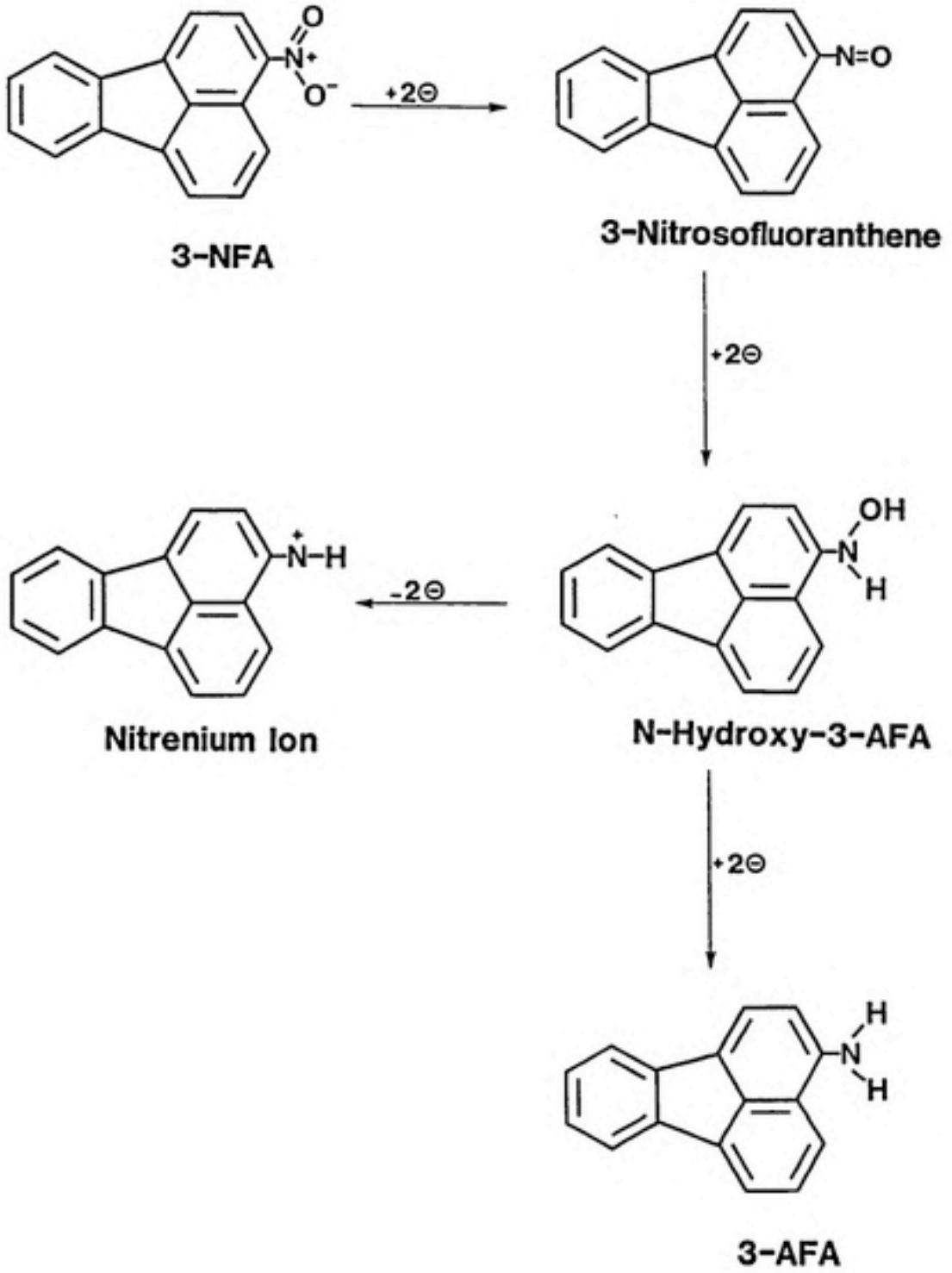


Fig. 3 Proposed Mechanism for Metabolism of 3-NFA

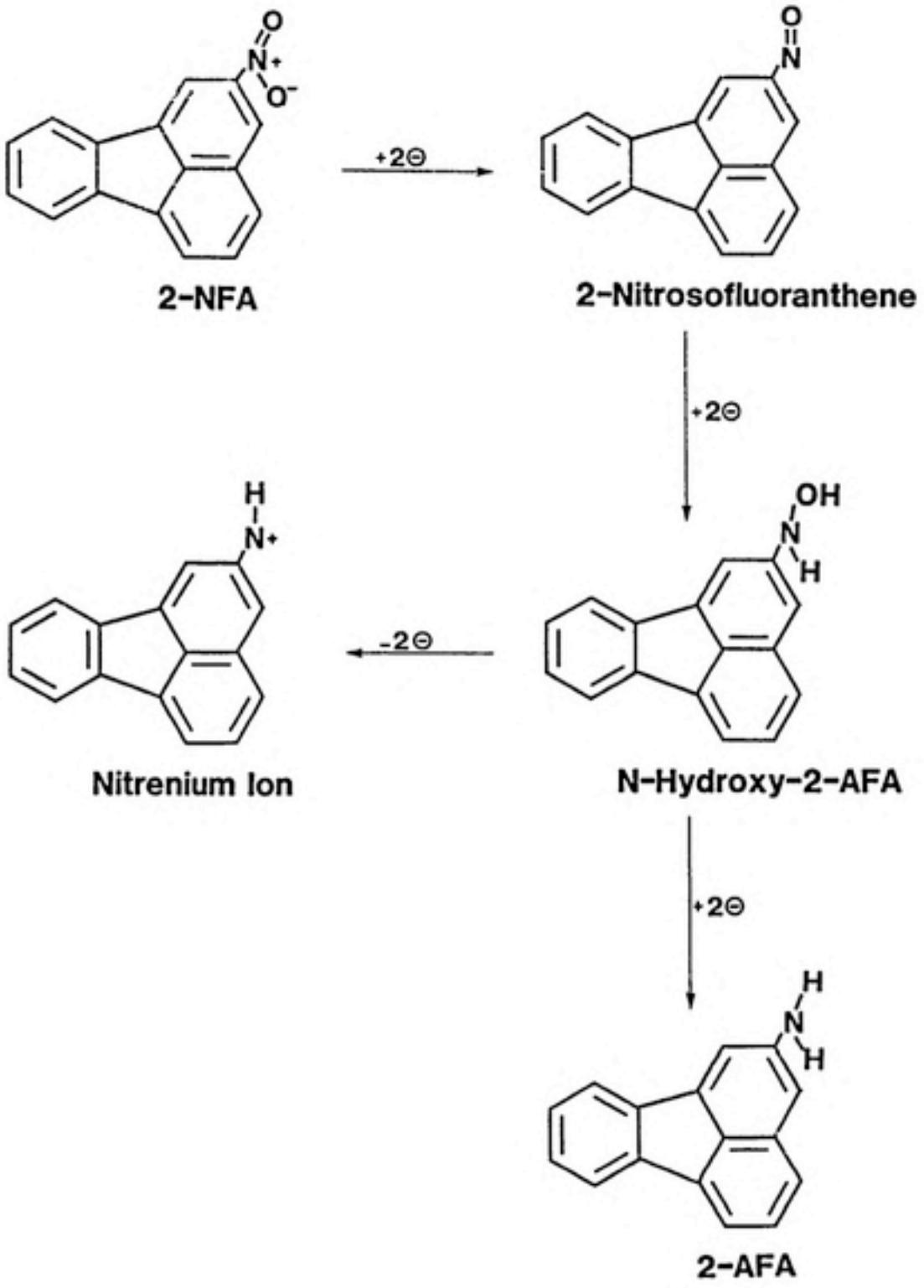


Fig. 4 Proposed Mechanism for Metabolism of 2-NFA

Fig. 5 Representative HPLC Chromatogram (254 nm) of Extracted 3-NFA and Salmonella typhimurium Incubations (Program I)

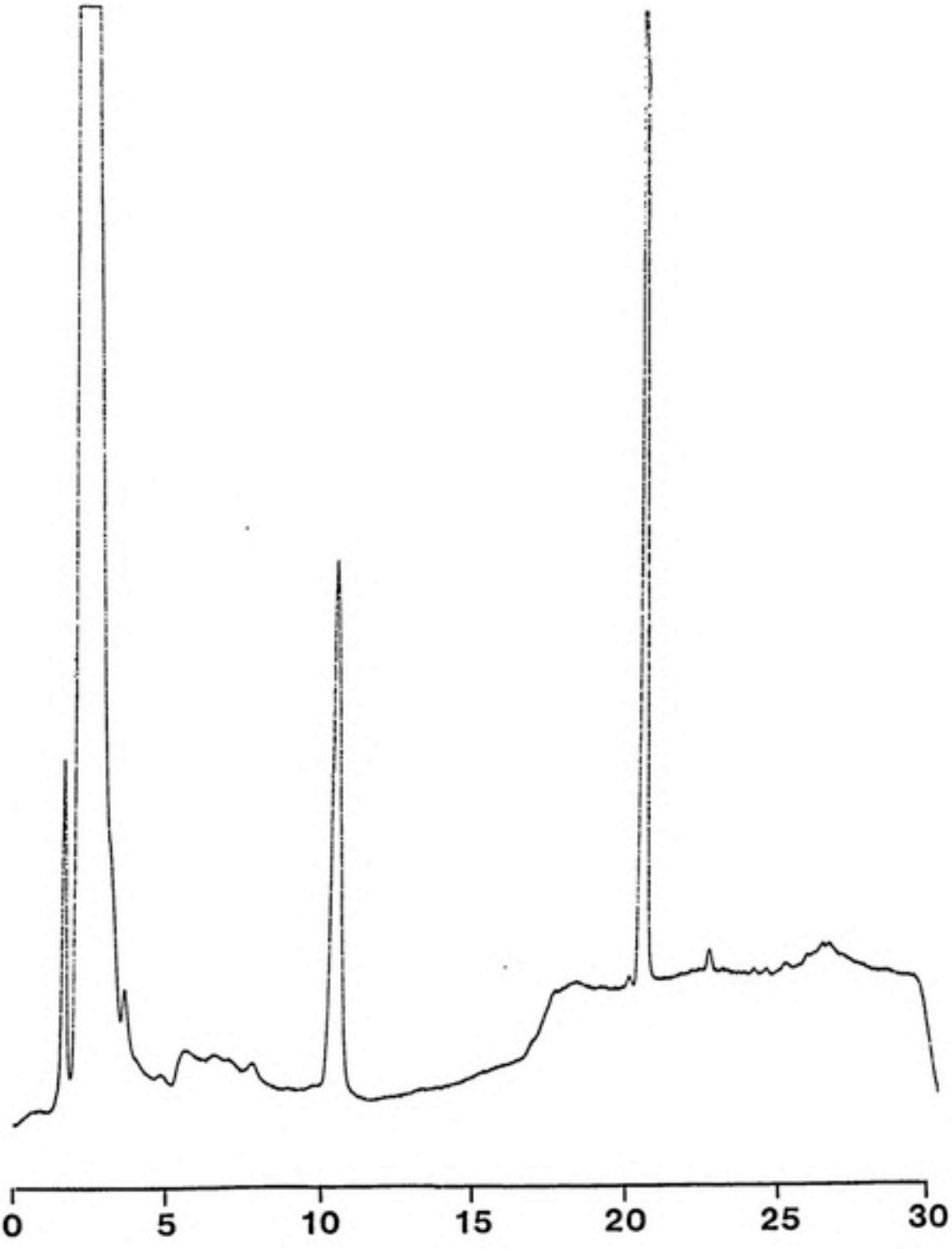


Fig. 6 Representative HPLC Chromatogram of Fraction I Eluted with Program II Parameters

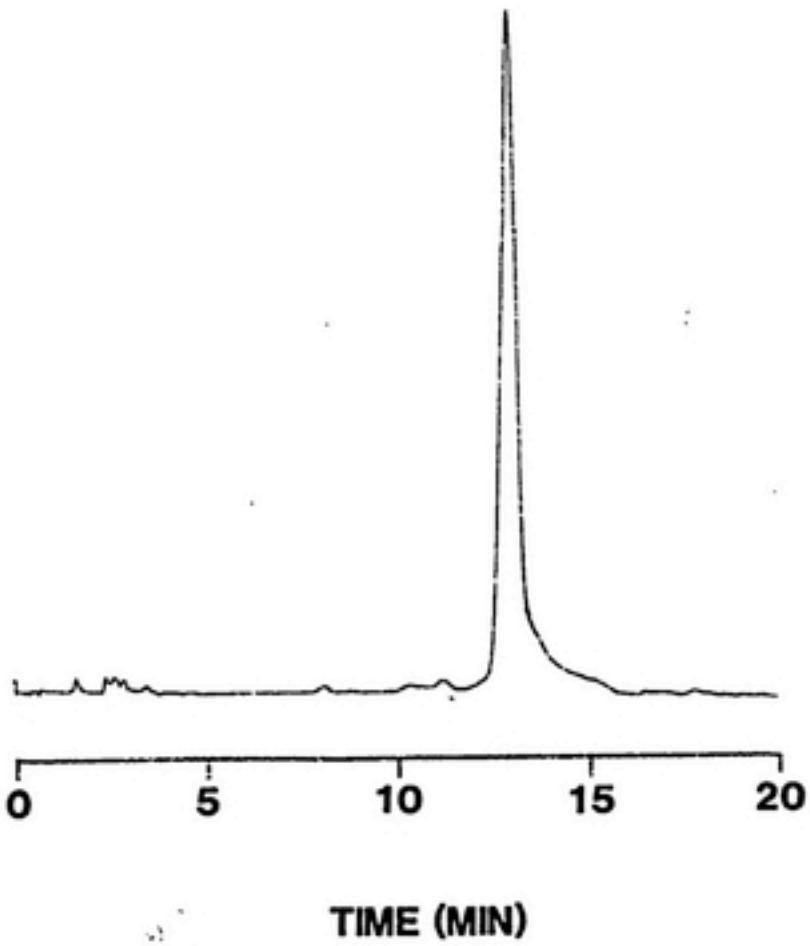


Fig. 7 UV-VIS Spectrum of Fraction 1

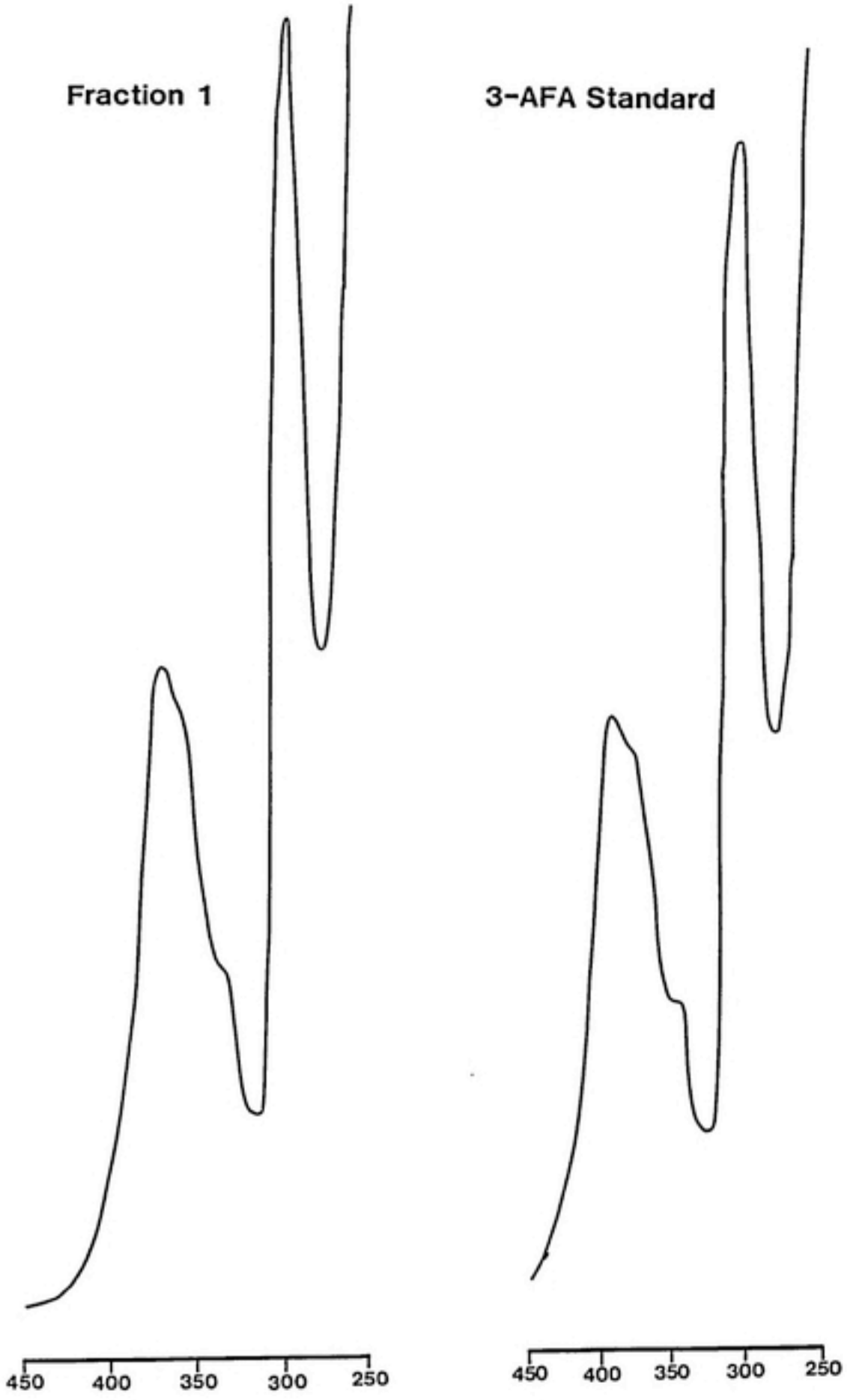


Fig. 8 UV-VIS Spectrum of Fraction 2

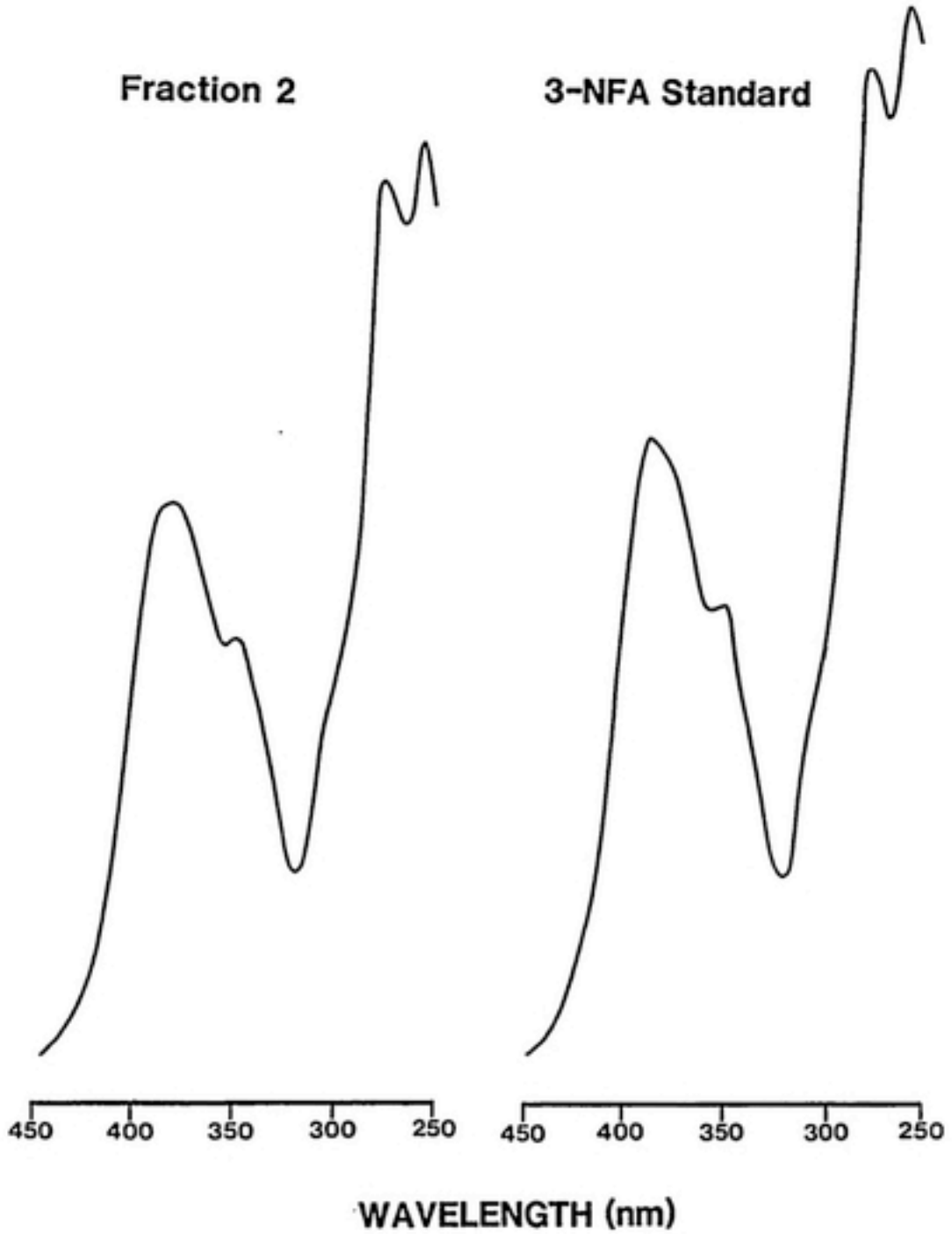


Fig. 9 Representative Radioactivity Profile of Extracted 14C-3NFA Incubations vs. Chromatogram at 254 nm (Program I)

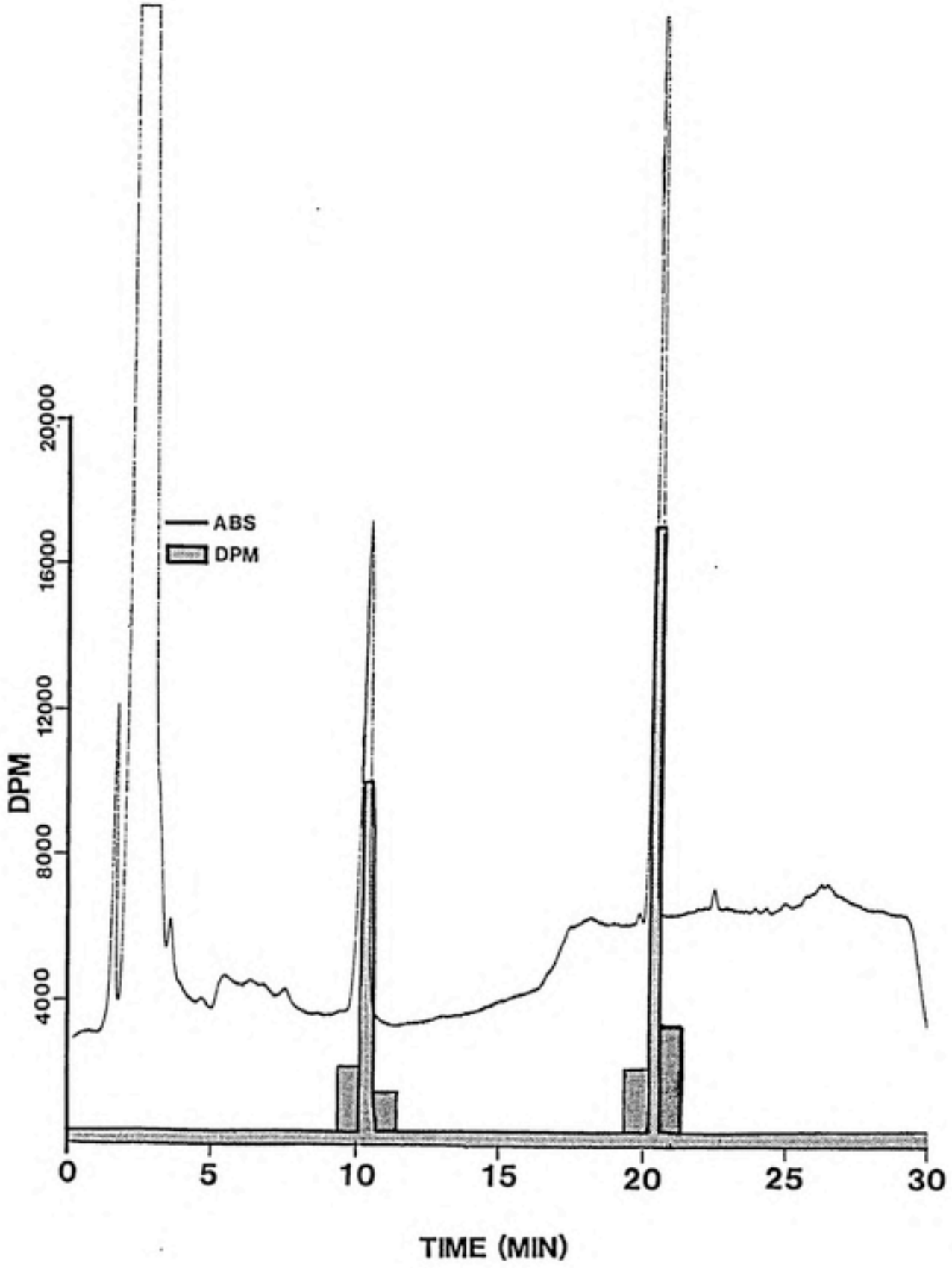


Fig. 10

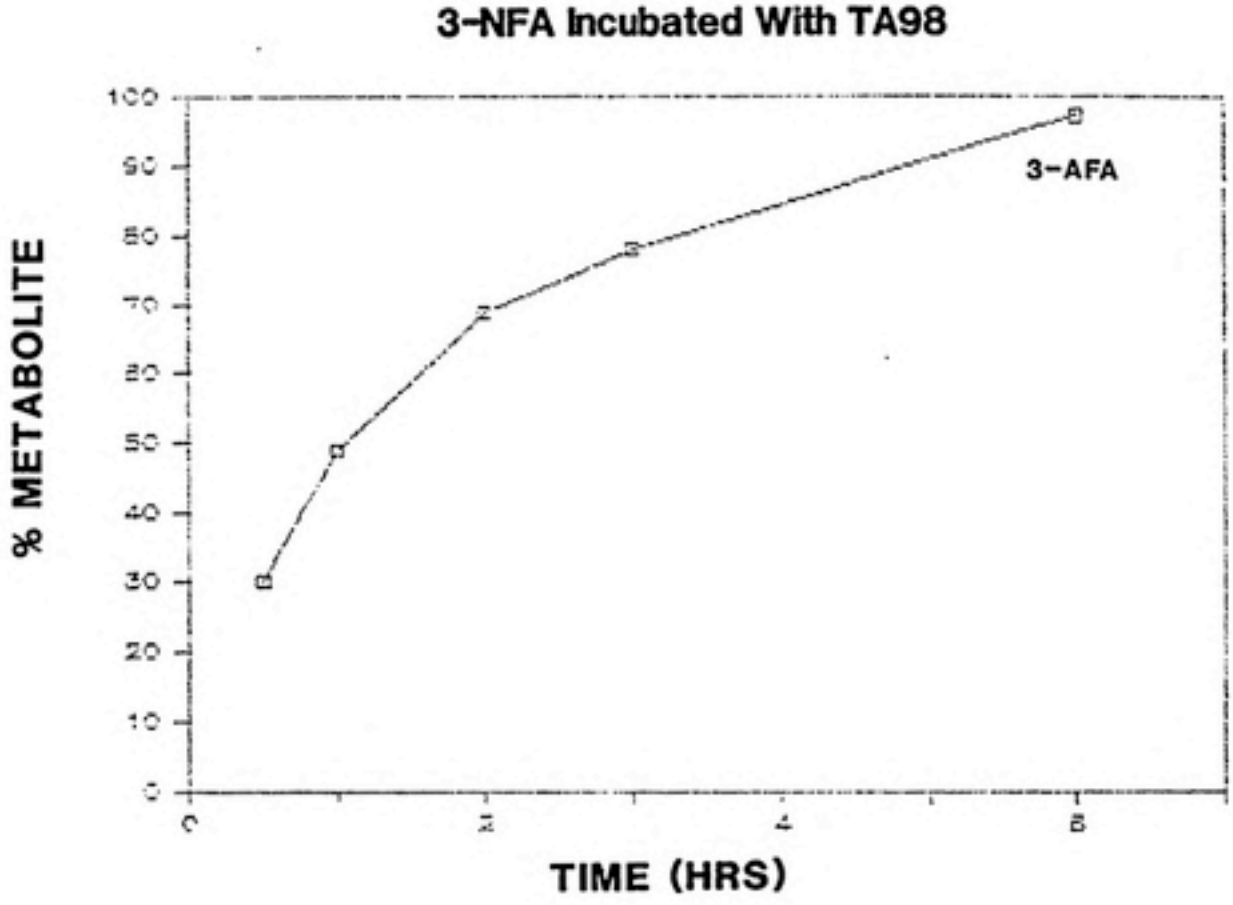


TABLE 1

RADIOACTIVE METABOLITES OF
14C-3NFA INCUBATED WITH TA98

Time (hour)	% 3-AFA[a.b.c.]
0.5	30.1 ± 4.2
1.0	48.8 ± 2.8
2.0	68.6 ± 5.7
3.0	77.9 ± 1.4
6.0	97.2 ± 2.1

a. % radioactivity present as fraction 1
b. n = 2
c. ± standard deviation

Fig. 11

3-NFA Incubated With TA98 ND

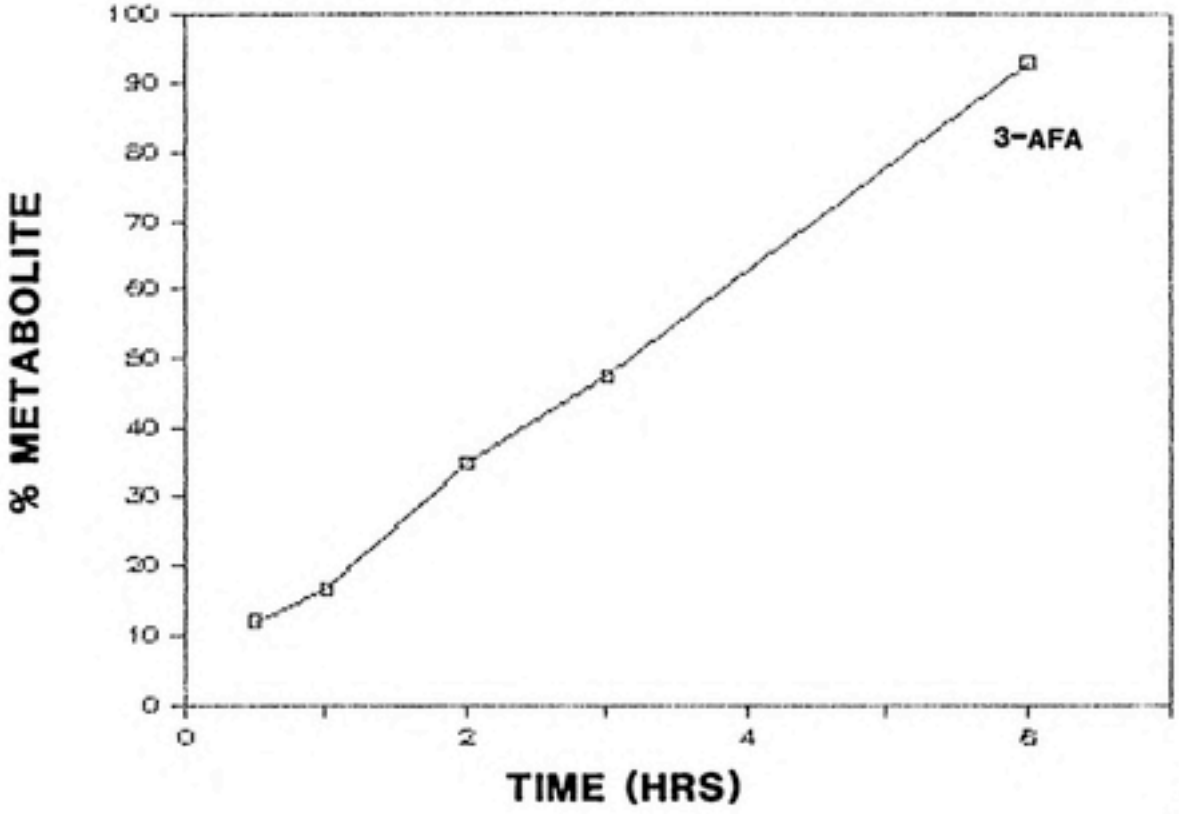


TABLE 2

RADIOACTIVE METABOLITES OF
14C-3NFA INCUBATED WITH TA98 ND

Time (hours)	% 3-AFA[a.b.c.]
0.5	12.1 ± 0.3
1.0	16.7 ± 2.5
2.0	34.9 ± 2.8
3.0	47.3 ± 2.8
6.0	92.9 ± 3.9

a. % radioactvity present as fraction 1
b. n = 2
c. ± standard deviation

Fig. 12

3-NFA Incubated With TA98/1,8-DNP6

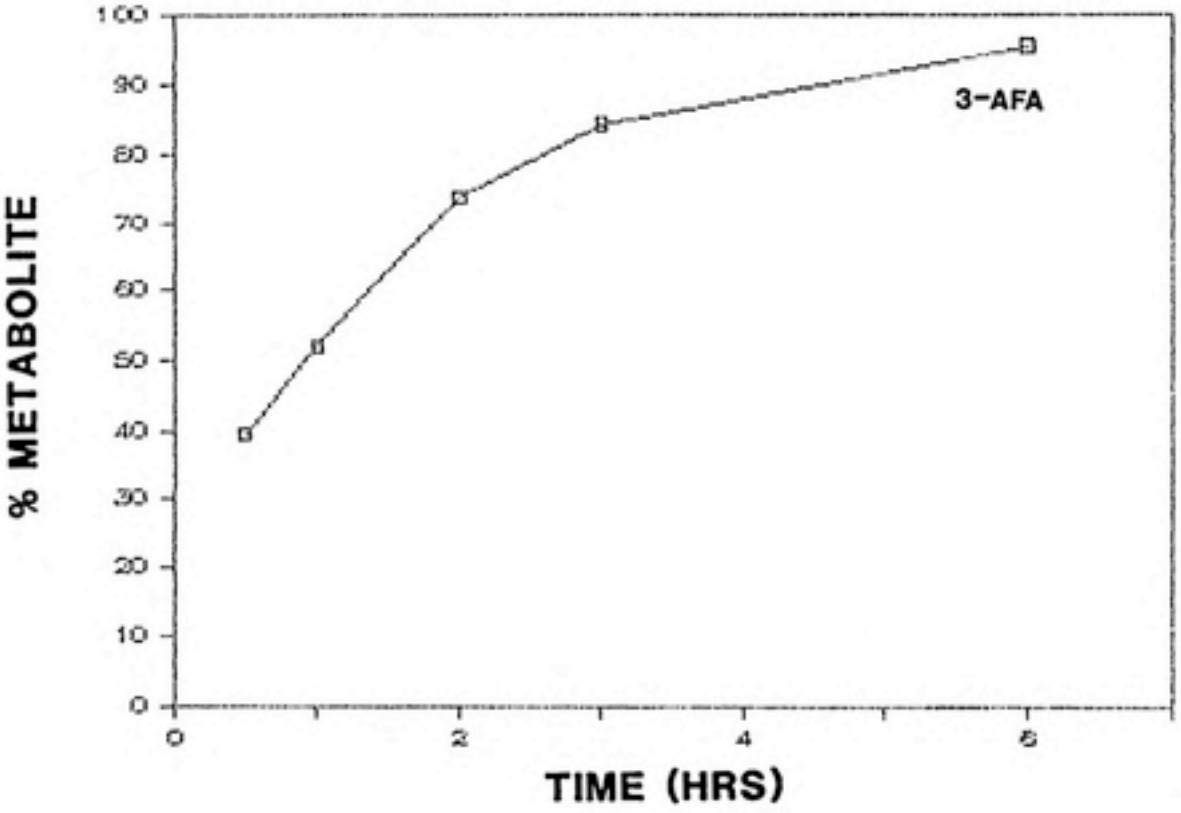


TABLE 3

RADIOACTIVE METABOLITES OF 14C-3NFA
INCUBATED WITH TA98/1,8-DNP6

Time (hour)	% 3-AFA[a.b.c.]
0.5	39.6 ± 1.3
1.0	51.9 ± 3.3
2.0	73.7 ± 1.8
3.0	85.4 ± 2.2
6.0	95.6 ± 1.4

a. % radioactivity present as fraction 1
b. n = 2
c. ± standard deviation

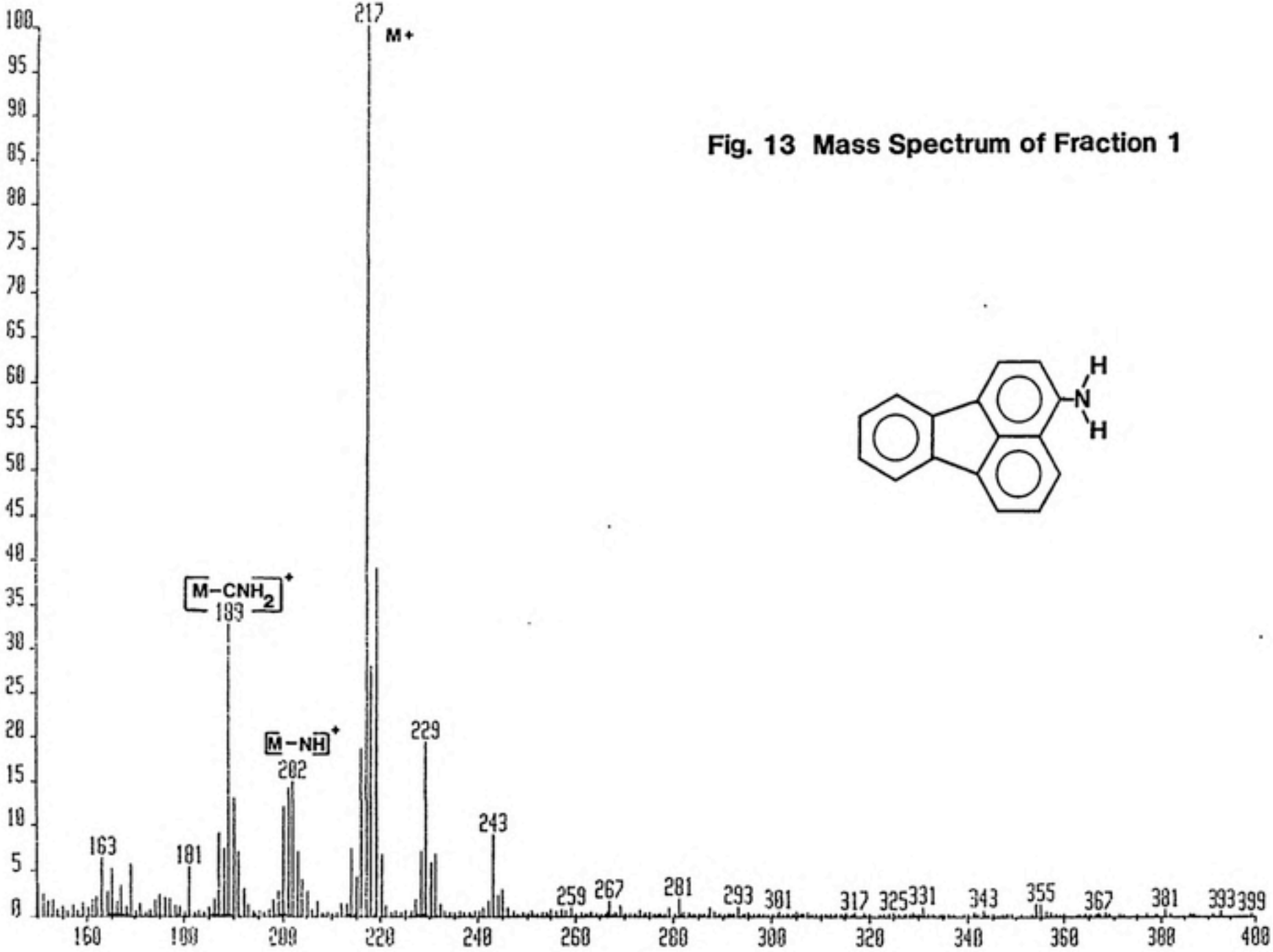


Fig. 13 Mass Spectrum of Fraction 1

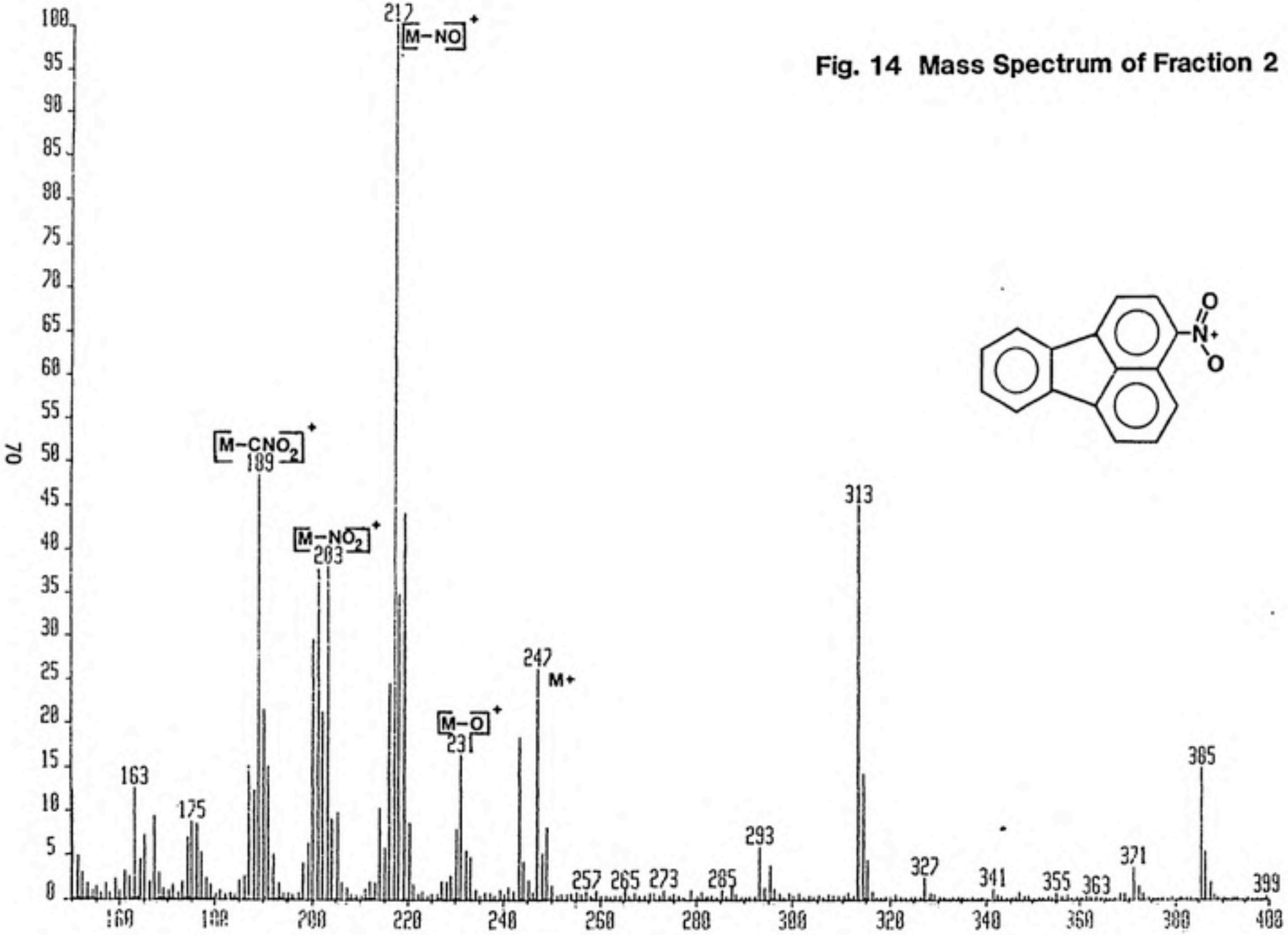


Fig. 14 Mass Spectrum of Fraction 2

Fig. 15 Proton NMR of Fraction 1

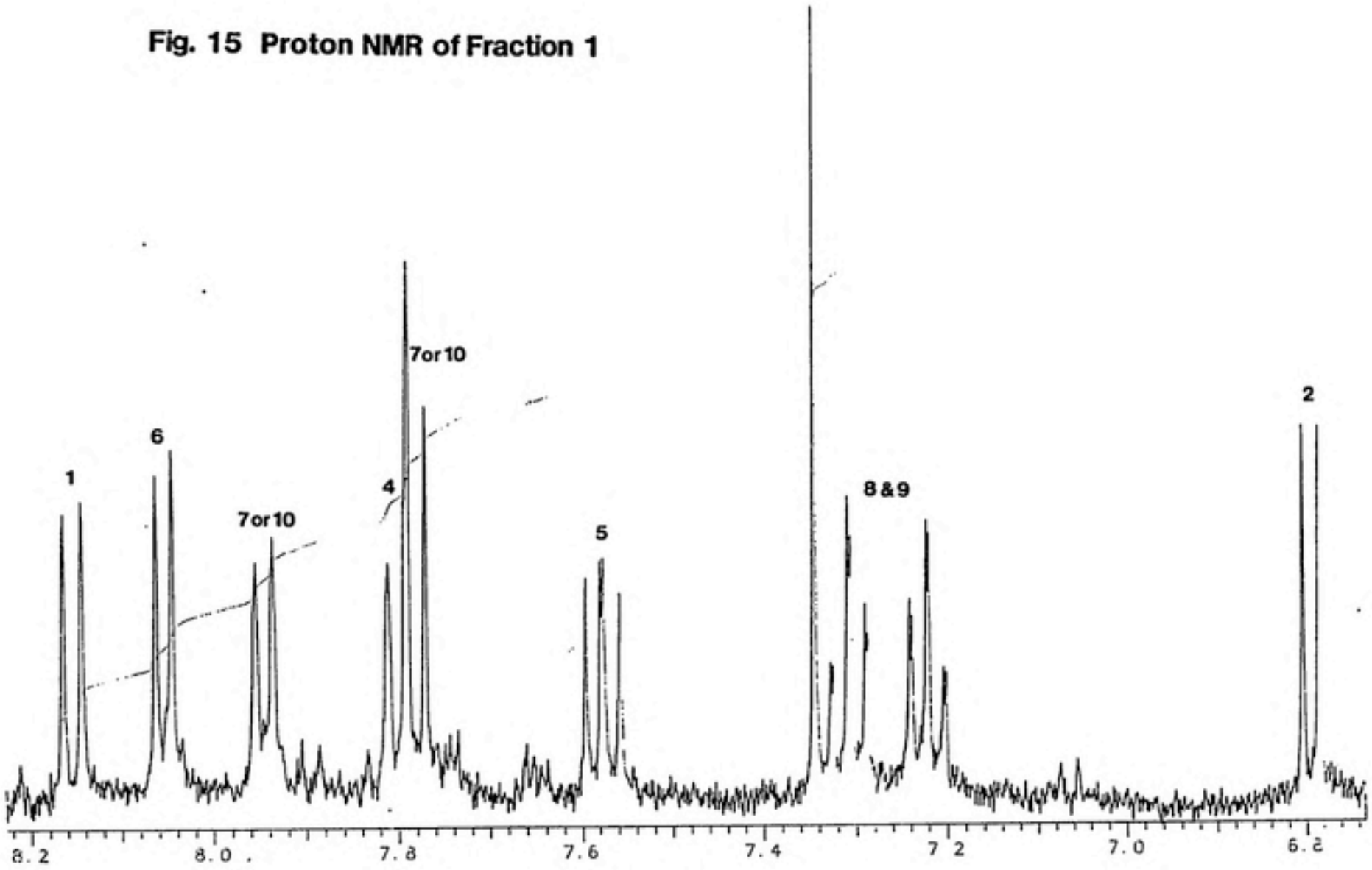


Table 4

PROTON NMR CHARACTERISTICS OF FRACTION 1 (3-AFA)

Chemical Shift[a] (ppm)	Splitting Pattern	J (Hz)	Proton
8.15	doublet	(8.0)	H-1
8.05	doublet	(6.8)	H-6
7.95	doublet	(7.4, 0.9)	H-7 or H-10
7.80	doublet	(8.3)	H-4
7.78	doublet	(7.5, 0.9)	H-7 or H-10
7.58	triplet	(6.9, 8.3)	H-5
7.31	triplet	(7.5, 7.5, 1.1)	H-8 or H-9
7.22	triplet	(7.5, 7.5, 1.0)	H-8 or H-9
6.80	doublet	(7.6)	H-2

a. Integral for resonance signals was 1 proton

Fig. 16 Representative HPLC Chromatogram (254 nm) of Extracted 2-NFA and Salmonella typhimurium TA98/TA98 ND Incubations (Program II)

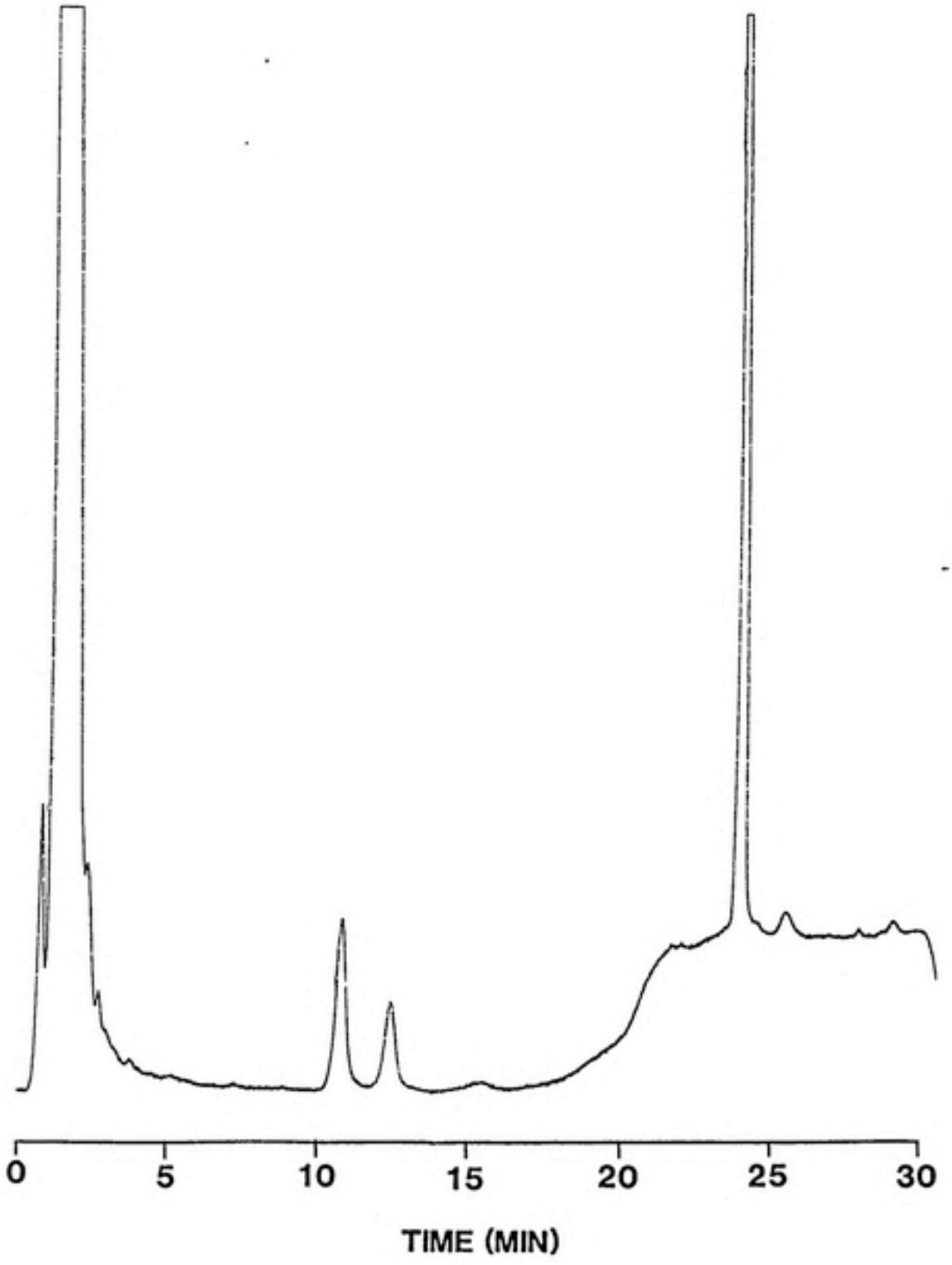


Fig. 17 Representative HPLC Chromatogram (254 nm) of Extracted 2-NFA and Salmonella typhimurium TA98/1,8-DNP6 Incubation (Program II)

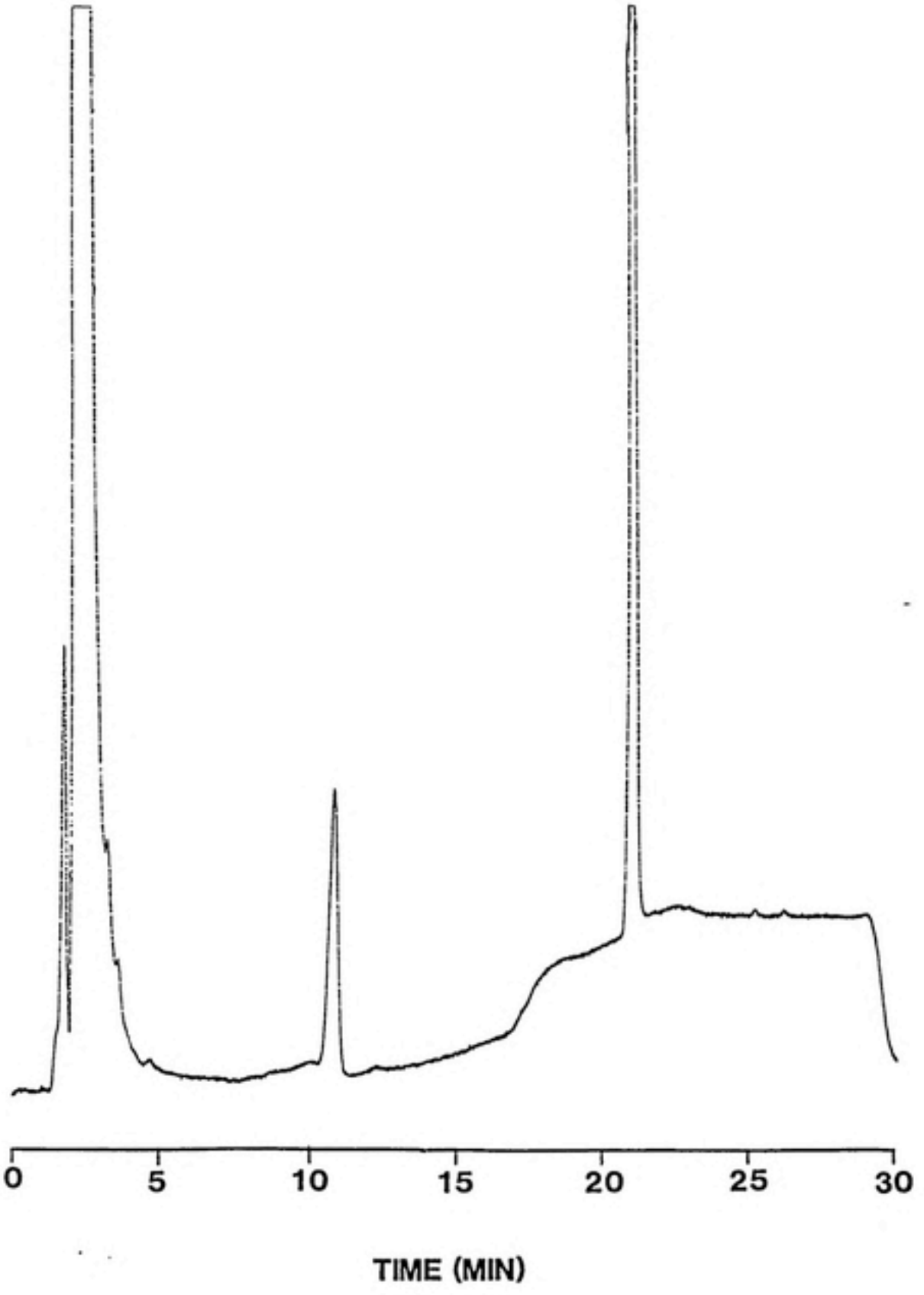


Fig. 18 UV-VIS Spectrum of Fraction A

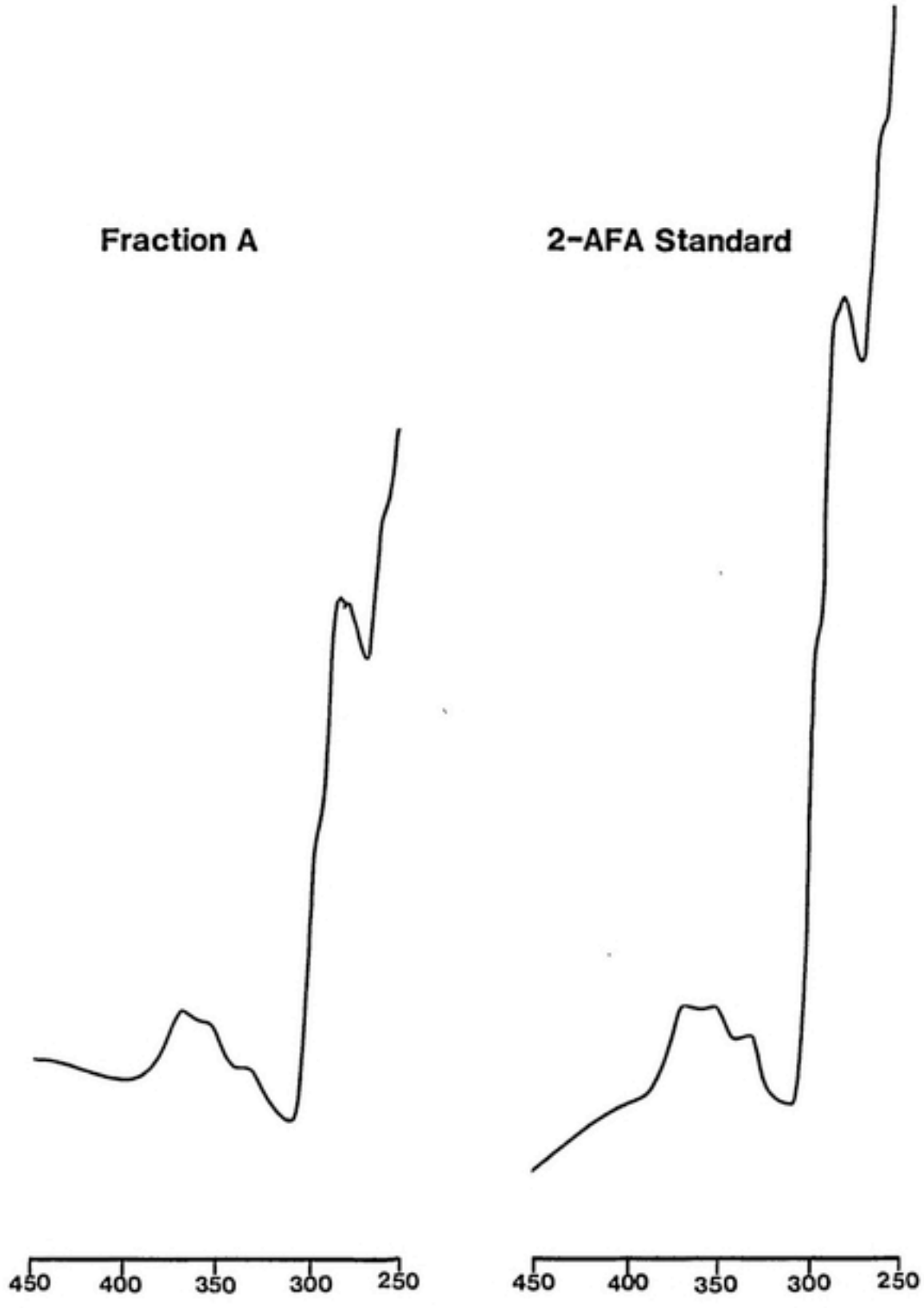


Fig. 19 UV-VIS Spectrum of 2-NAAFA Standard

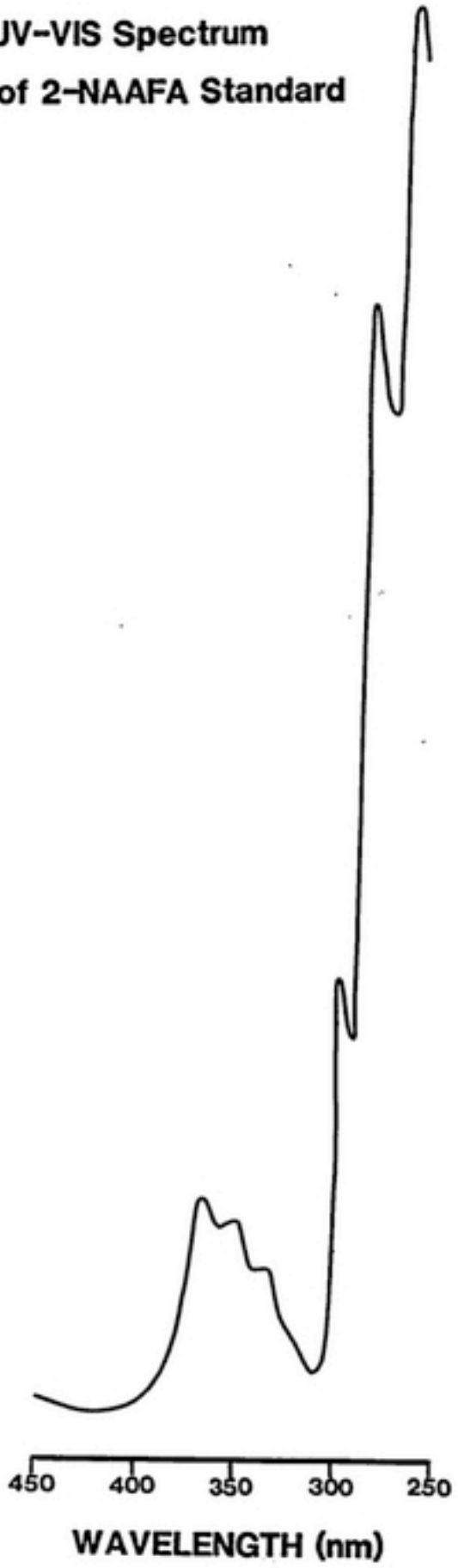


Fig. 20 UV-VIS Spectrum of Fraction C

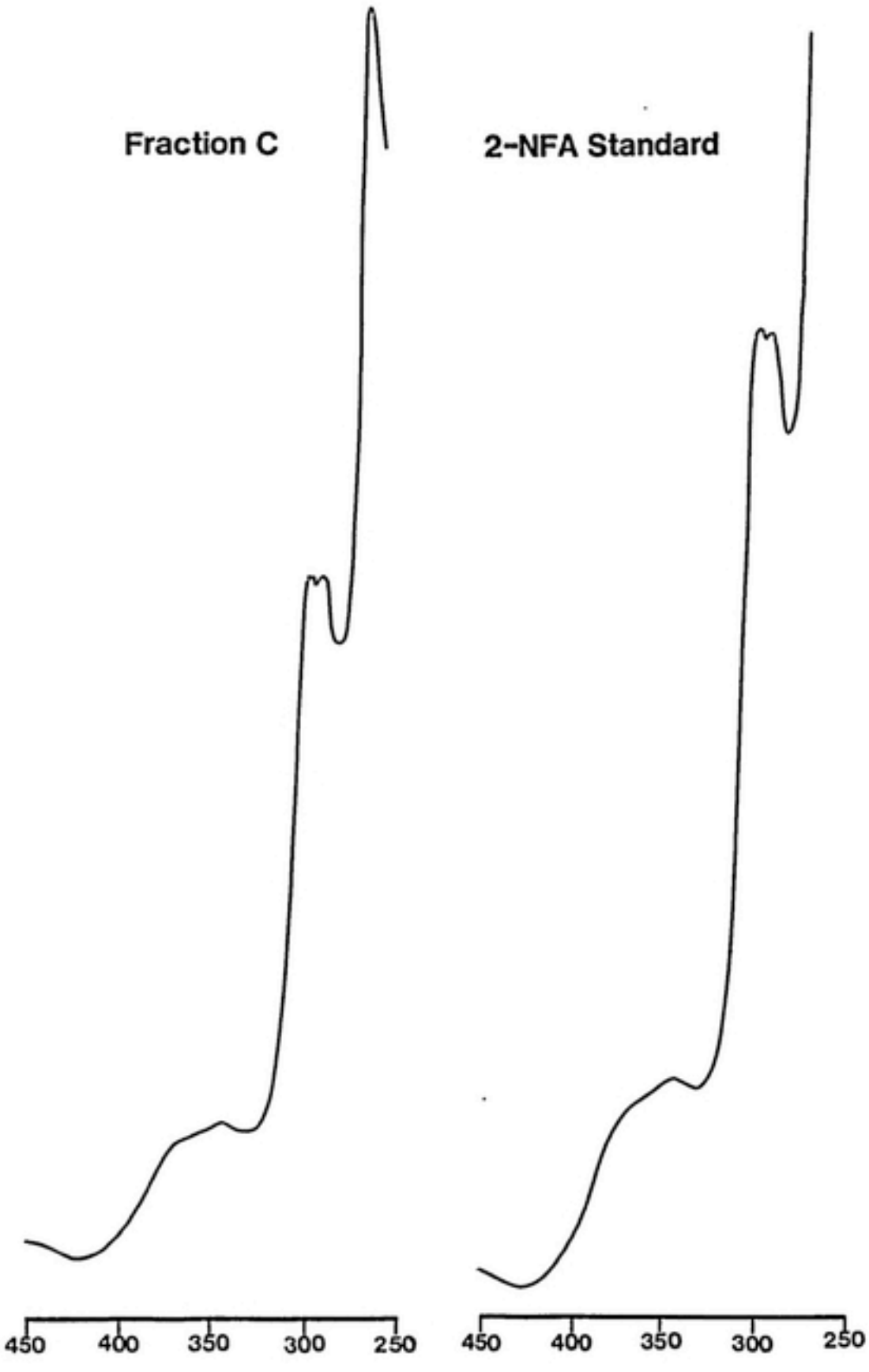


Fig. 21 Representative Radioactivity Profile of Extracted 3H-2NFA and TA98/TA98 ND Incubations vs. Chromatogram at 254 nm (Program II)

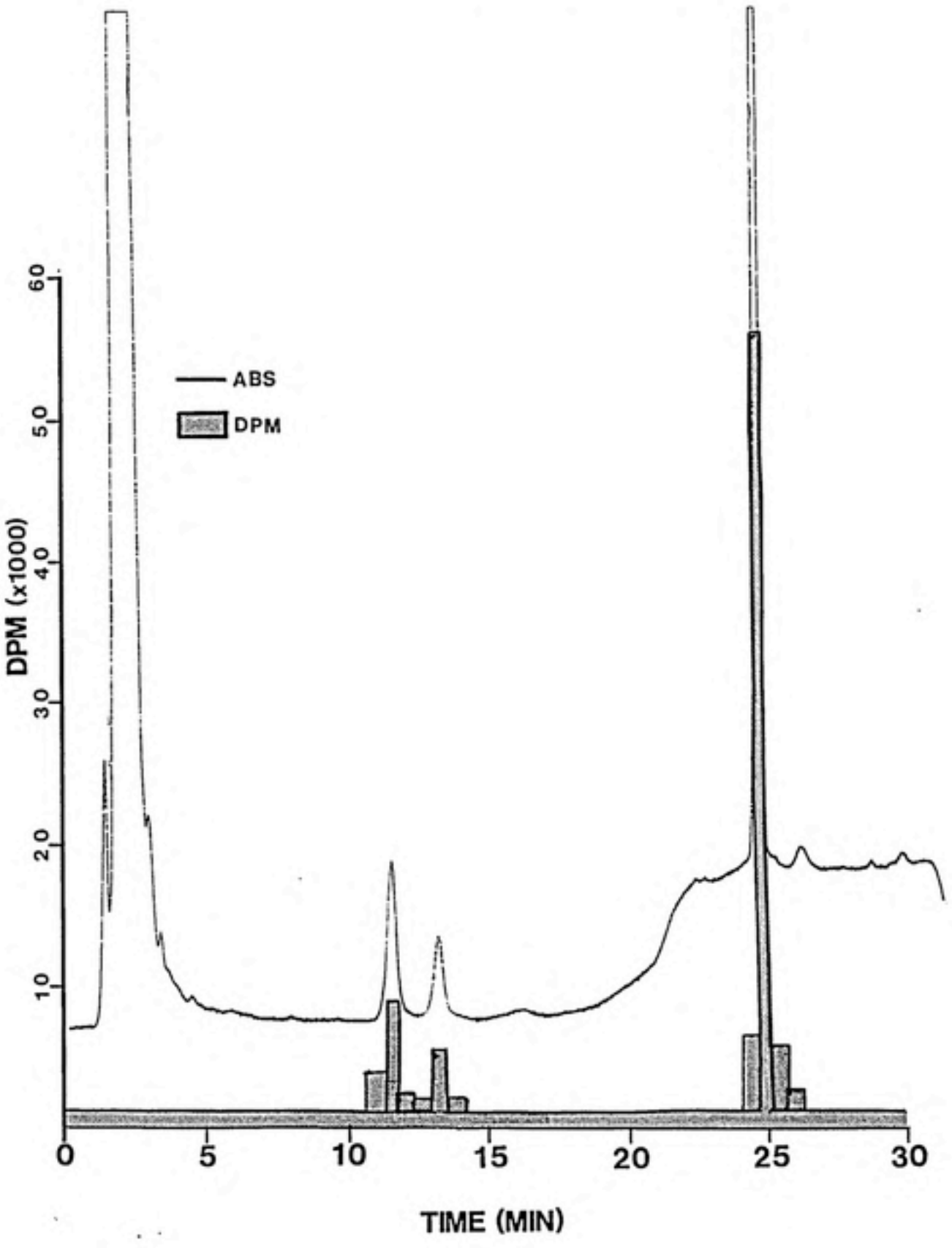


Fig. 22 Representative Radioactivity Profile of Extracted 3H-2NFA and TA98/1,8-DNP6 Incubations vs. Chromatogram at 254 nm (Program II)

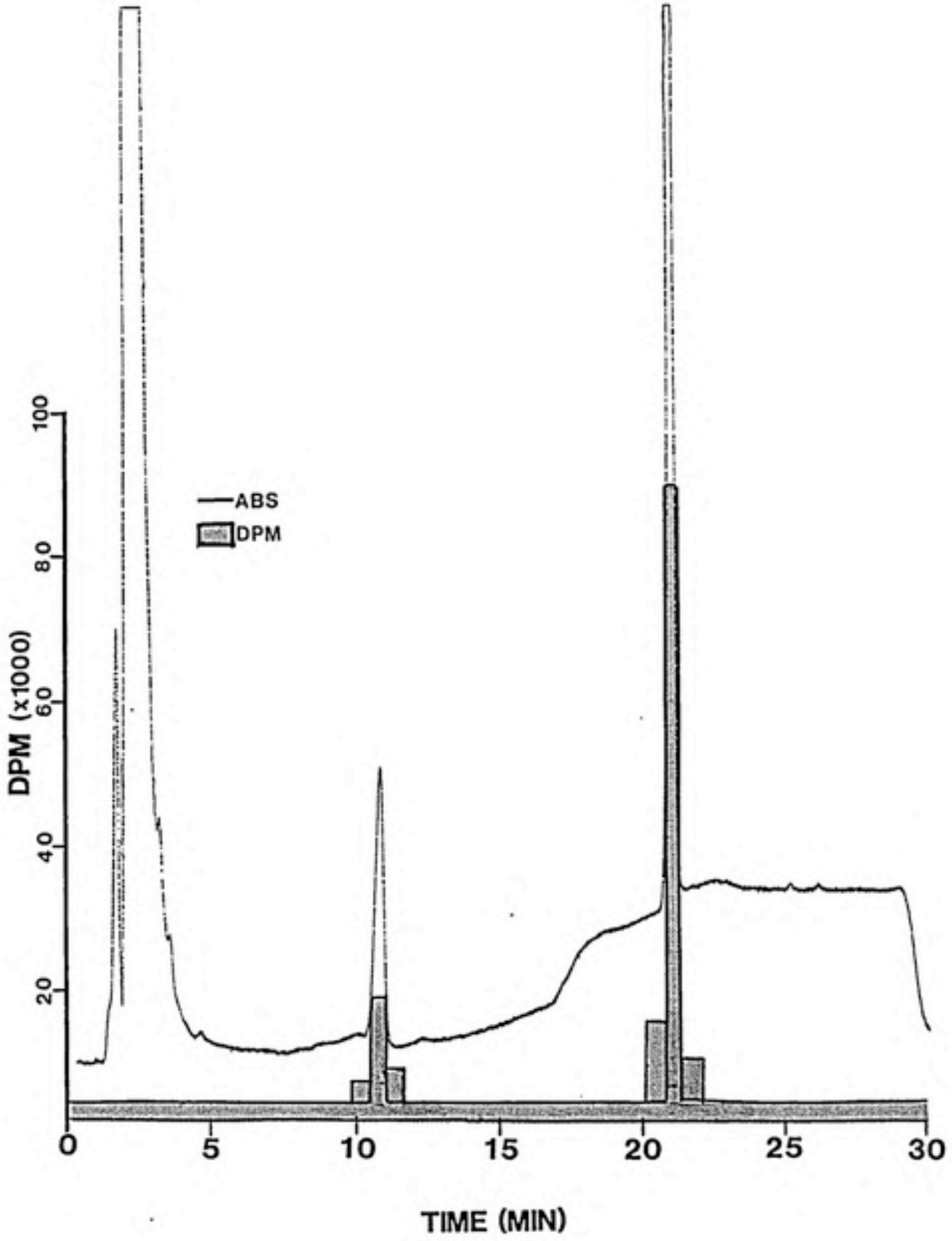


Fig. 23

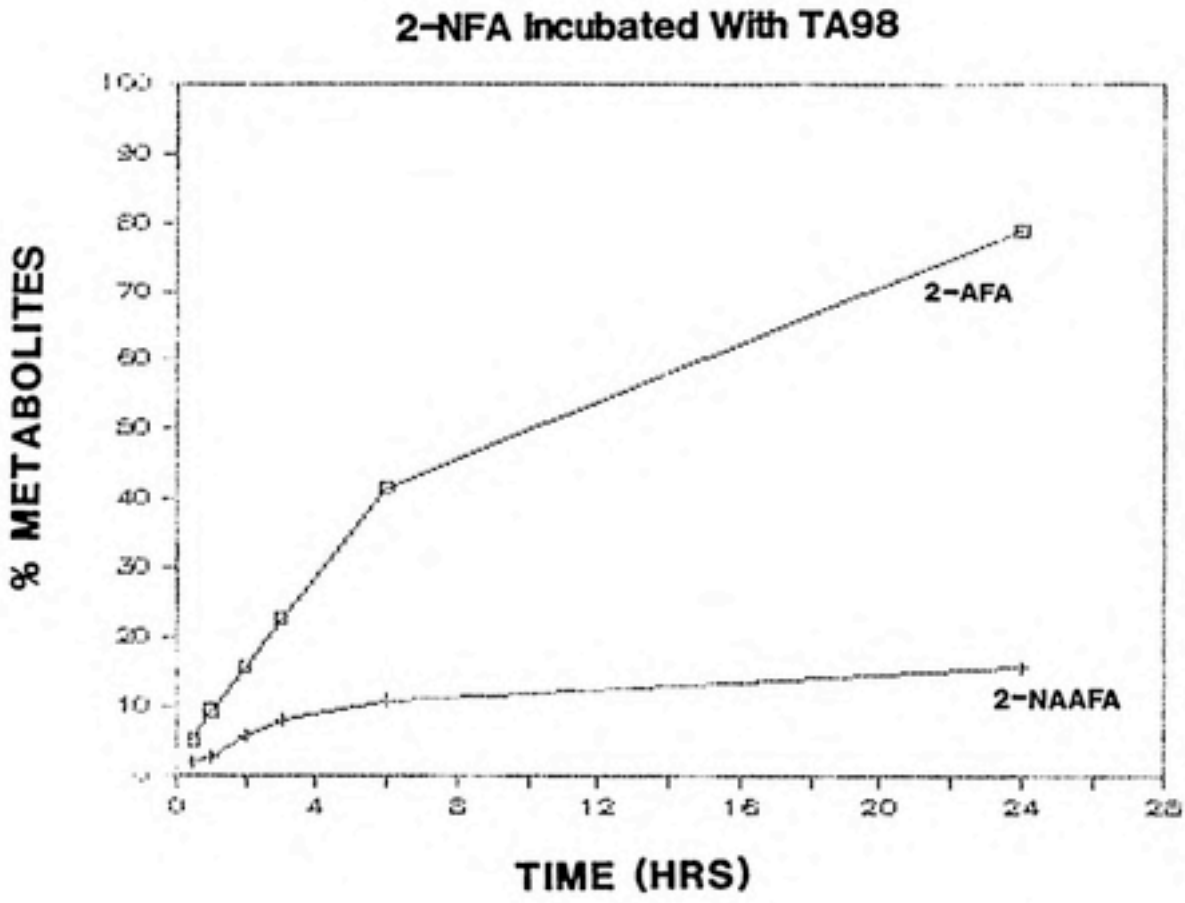


TABLE 5

RADIOACTIVE METABOLITES OF 3H-2NFA
INCUBATED WITH TA98

Time (hours)	% 2-AFA[a.b.c.]	% 2-NAAFA[d.]
0.5	5.1 ± 0.1	1.7 ± 0.1
1.0	9.3 ± 0.3	2.7 ± 0.5
2.0	15.9 ± 1.1	5.8 ± 1.7
3.0	22.9 ± 4.0	7.9 ± 2.8
6.0	41.6 ± 1.8	10.9 ± 1.9
24.0	78.7 ± 4.2	15.9 ± 0.9

a. % radioactivity present as fraction A
b. n = 2
c. ± standard deviation
d. % radioactivity present as fraction B

Fig. 24

2-NFA Incubated With TA98 ND

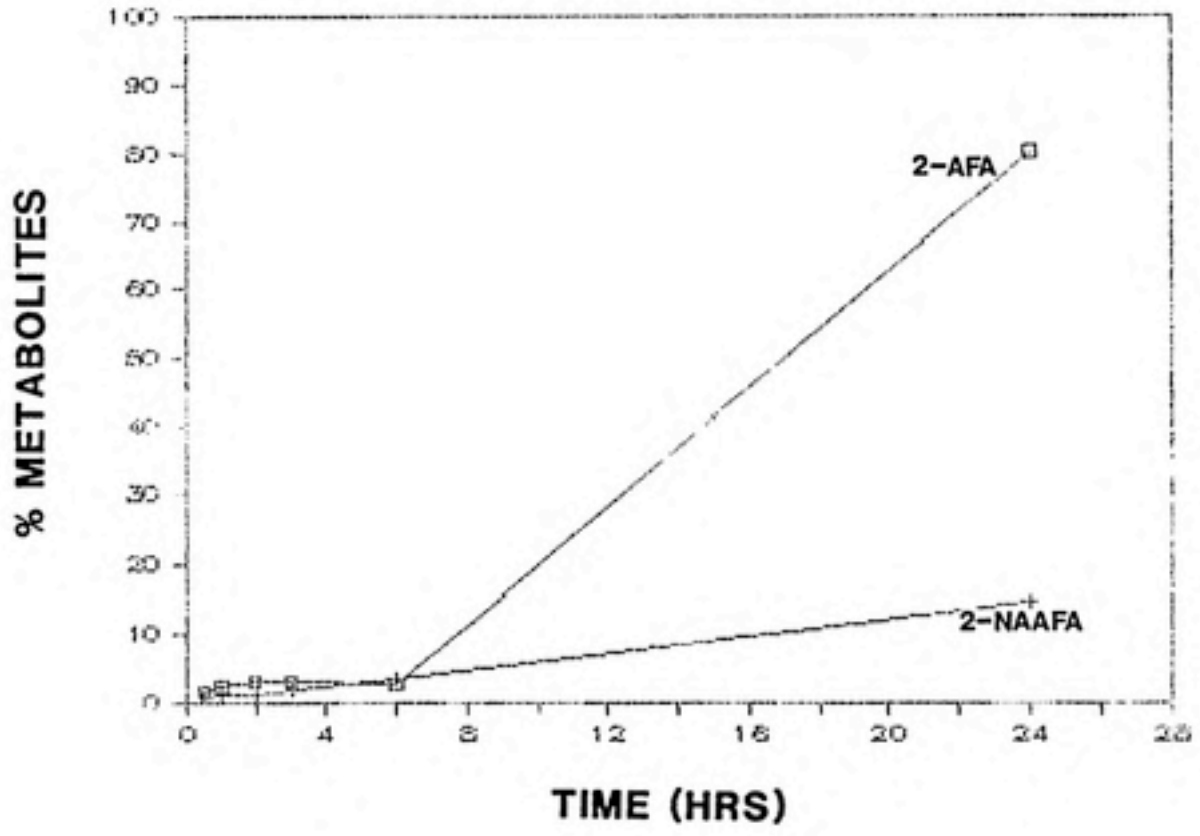


TABLE 6

RADIOACTIVE METABOLITES OF 3H-2NFA
INCUBATED WITH TA98 ND

Time (hour)	% 2-AFA[a.b.c.]	% 2-NAAFA[d.]
0.5	1.4 ± 0.7	0.6 ± 0.1
1.0	2.1 ± 1.0	1.0 ± 0.4
2.0	2.9 ± 1.6	1.2 ± 0.5
3.0	3.1 ± 1.5	1.9 ± 0.7
6.0	2.7 ± 1.1	3.4 ± 1.2
24.0	80.1 ± 2.8	14.7 ± 1.6

a. % radioactivity present as fraction A
b. n = 2
c. ± standard deviation
d. % radioactvity present as fraction B

Fig. 25

2-NFA Incubated With TA98/1,8-DNP6

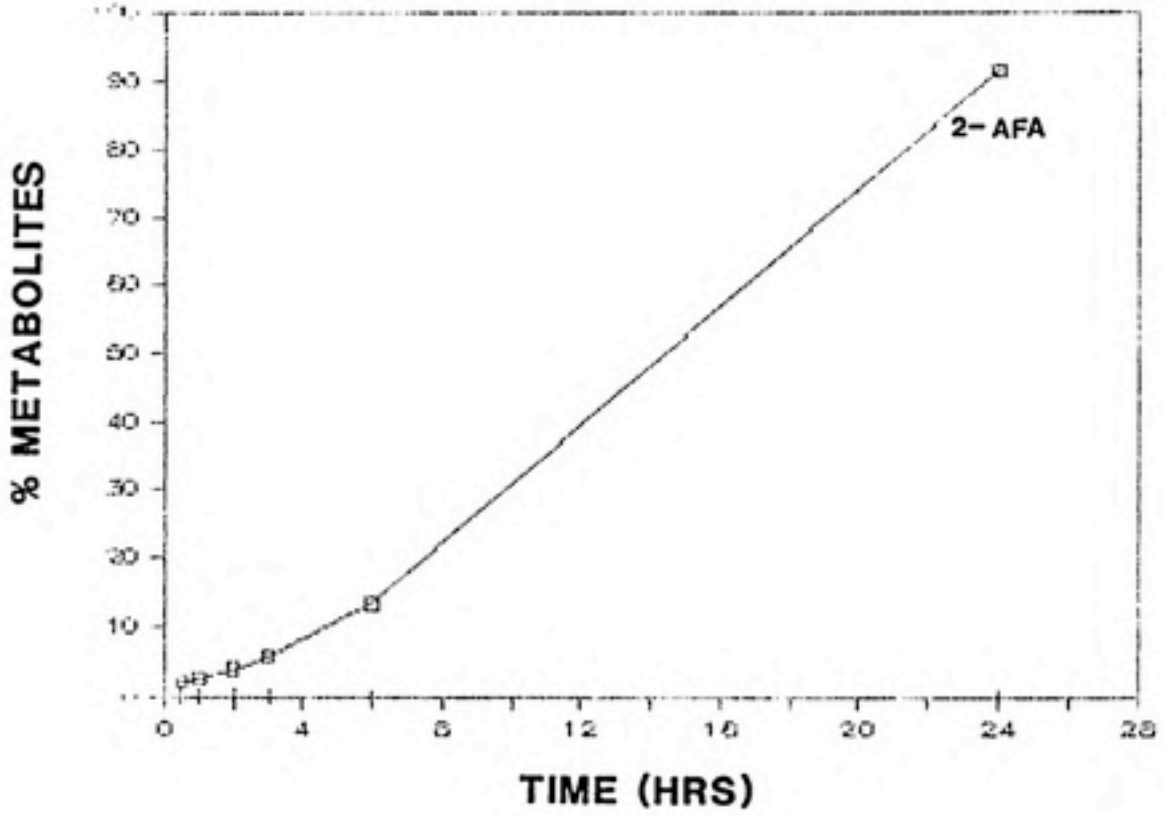


TABLE 7

RADOACTIVE METABOLITES OF 3H-2NFA
INCUBATED WITH TA98/1,8-DNP6

Time (hour)	% 2-AFA[a.b.c.]	% 2-NAAFA[d.]
0.5	2.3 ± 0.5	0
1.0	2.7 ± 1.0	0
2.0	3.9 ± 0.9	0
3.0	5.7 ± 1.1	0
6.0	13.3 ± 1.4	0
24.0	91.7 ± 4.2	0

a. % radioactivity present in fraction A
b. n = 2
c. ± standard deviation
d. % radioactivty present as fraction B

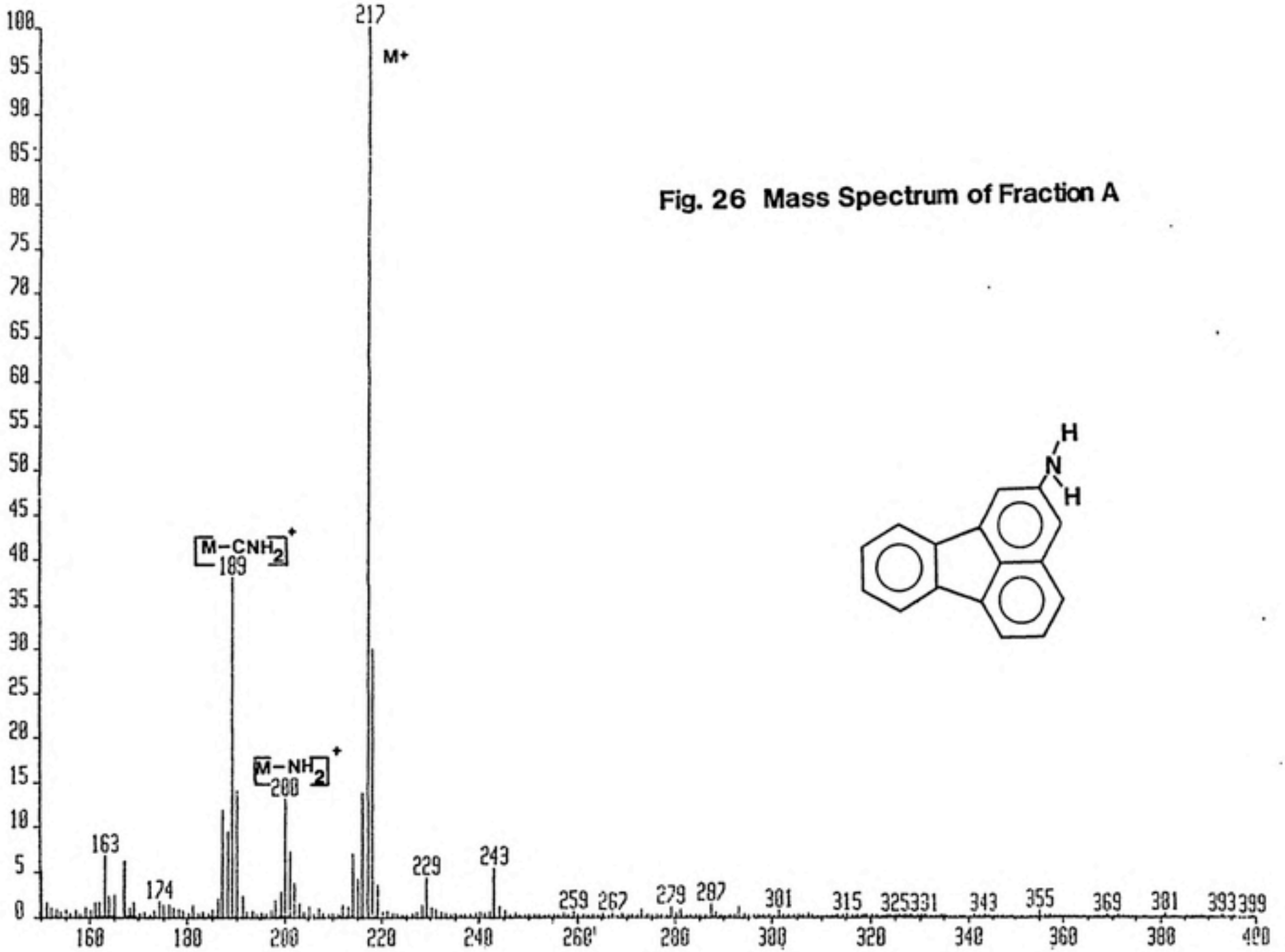


Fig. 26 Mass Spectrum of Fraction A

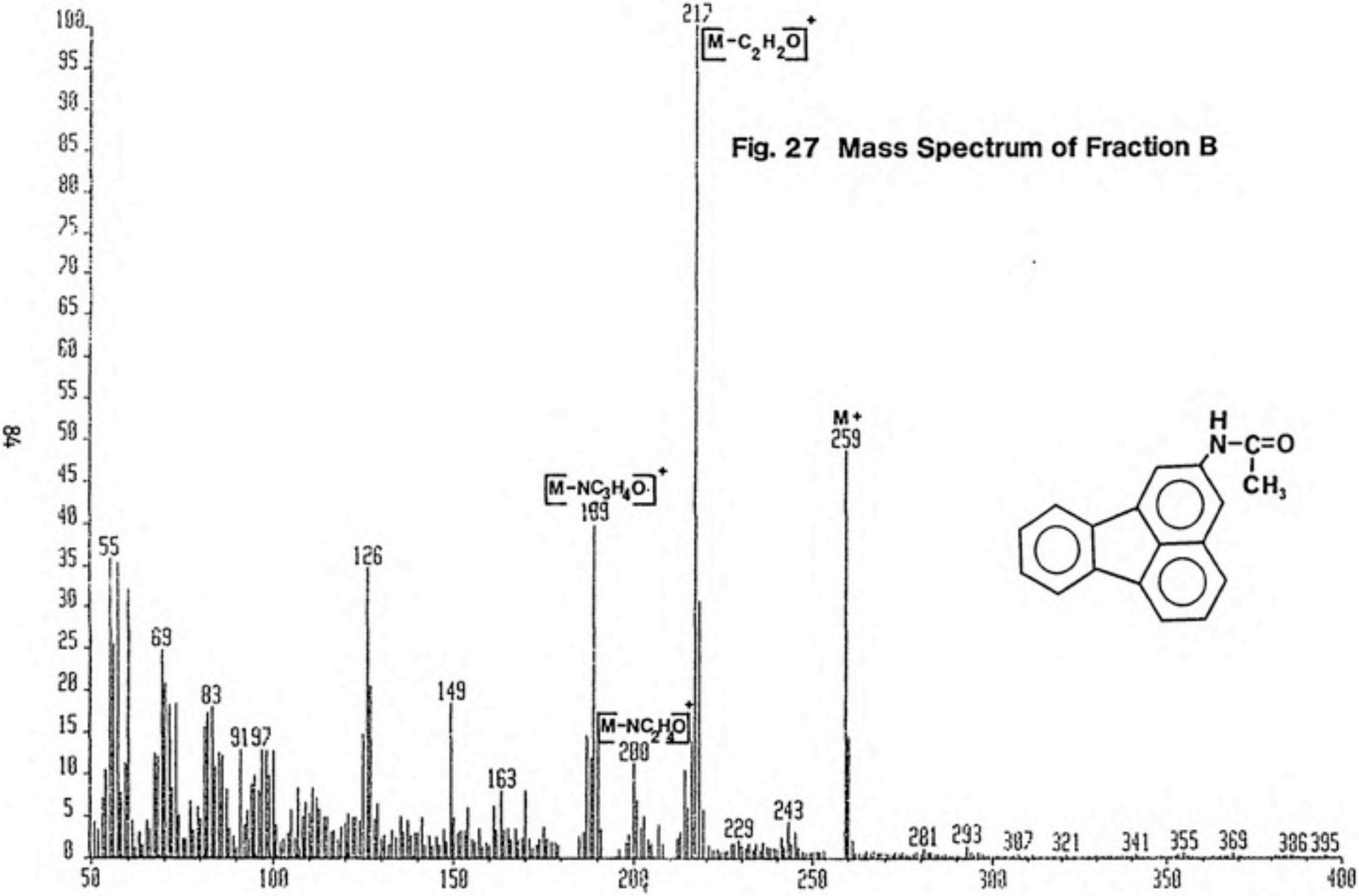


Fig. 27 Mass Spectrum of Fraction B

Fig. 28 Mass Spectrum of Fraction C

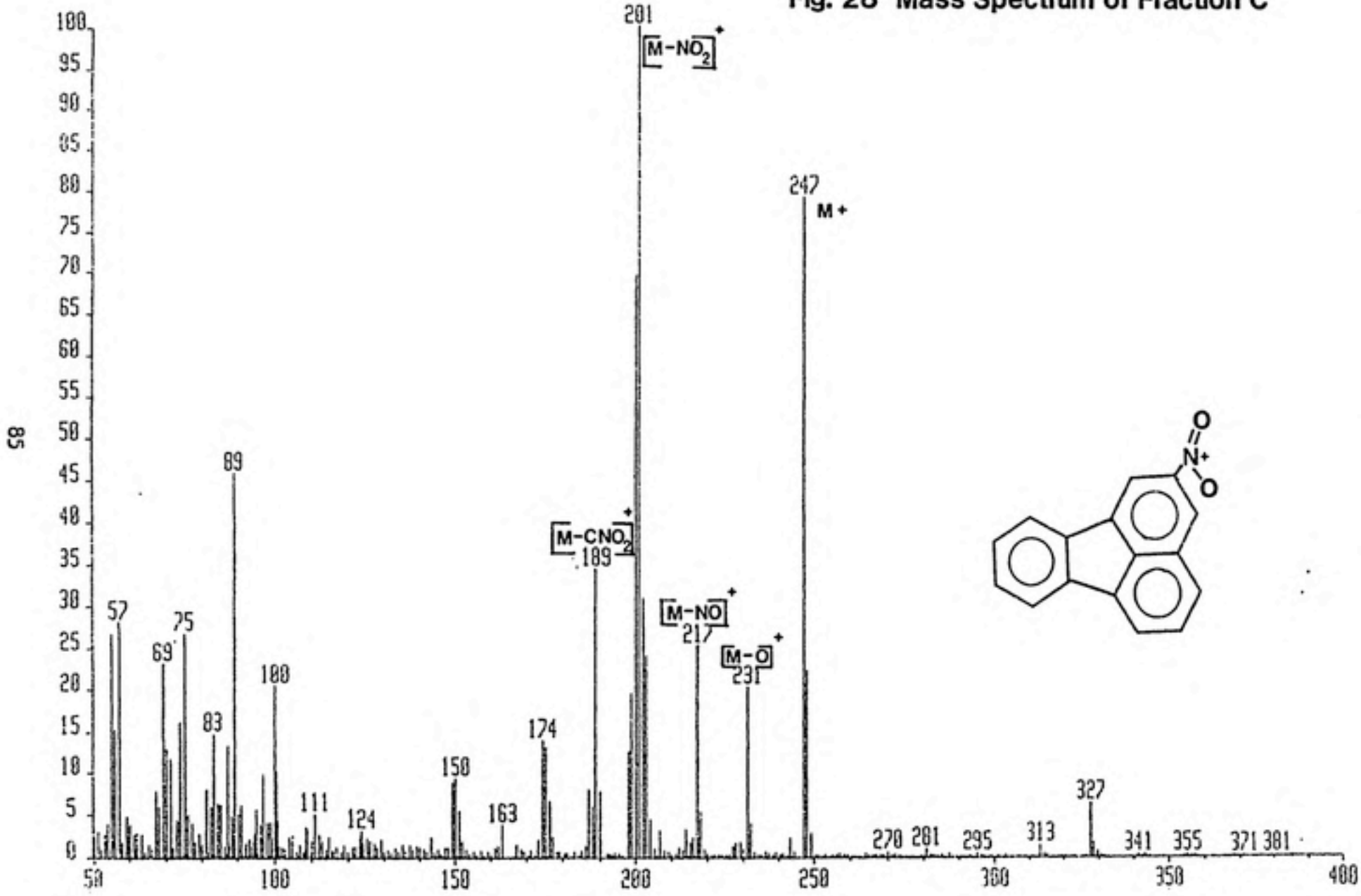


Fig. 29 Proton NMR of Fraction A

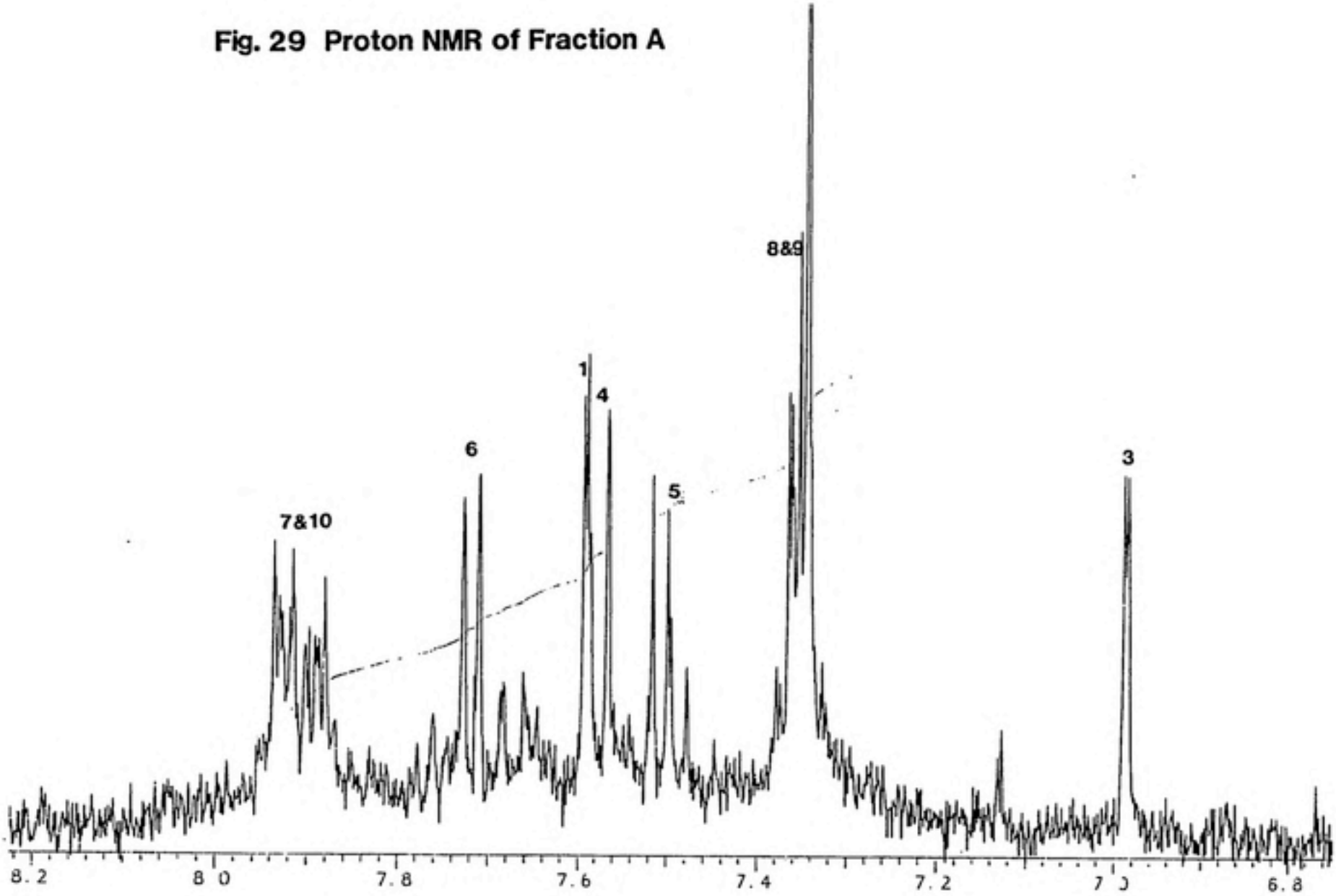


TABLE 8

PROTON NMR CHARACTERISTICS OF FRACTION A (2-AFA)

Chemical Shifts[a] (ppm)	Splitting Pattern	J (Hz)	Proton
7.93	multiplet	(7.7, 1.4)	H-7 or H-10
7.90	multiplet	(7.4, 1.4)	H-7 or H-10
7.72	doublet	(6.7)	H-6
7.59	meta-coupled singlet	(1.7)	H-1
7.58	doublet	(6.5)	H-4
7.50	triplet	(6.8, 7.9)	H-5
7.36	2 super-imposed multiplets	(7.5, 1.7)	H-8 & H-9
6.99	meta-coupled	(1.6)	H-3

a. Integral for resonance signal was 1 proton

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