THE METABOLISM OF 1,8-DINITROPYRENE BY *SALMONELLA typhimurium*

TITLE: The Metabolism of 1,8-Dinitropyrene by *Salmonella typhimurium*

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ABSTRACT

S. typhimurium strain TA98/1,8-DNP$_6$ is resistant to mutagenesis by 1,8-dinitropyrene and is deficient in an acetyl-CoA dependent acetyltransferase activity. Strains TA98 and TA98NR which are acetyltransferase competent are sensitive to 1,8-dinitropyrene mutagenesis. The coincidence of acetyltransferase deficiency and 1,8-dinitropyrene resistance in strain TA98/1,8-DNP$_6$ implicates acetylation as an important process in the metabolic activation of 1,8-dinitropyrene to a mutagenic intermediate. The acetyltransferase activity can be assayed by observing the formation of 1-N-acetylamino-8-aminopyrene and 1,8-N,N'-diacetyldiaminopyrene from 1,8-diaminopyrene. Reduction of the nitro-function is also an important enzymatic step involved in the activation of 1,8-dinitropyrene. Evidence is presented which suggests that a nitroreductase-acetyltransferase enzyme complex may exist. Further investigations of nitroreducatase activity have yielded results which indicate that three distinct nitroreductases exist, one specific for 1,8-dinitropyrene, one specific for 1-nitropyrene and one specific for nitrofurazone. The implications of these findings are discussed and an explanatory model is proposed.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

DNP$_6$: *Salmonella typhimurium* strain TA98/1,8-DNP$_6$
NR$_6$: *Salmonella typhimurium* strain TA98NR
NR/DNP$_6$: *Salmonella typhimurium* strain TA98NR/1,8-DNP$_6$

ANP	:	1-amino-8-nitropyrene
AP	:	1-aminopyrene
DAP	:	1,8-diaminopyrene
DNP	:	1,8-dinitropyrene
diAc	:	1,8-N,N'-diacetyldiaminopyrene
monoAc	:	1-N-acetylamino-8-aminopyrene
NP	:	1-nitropyrene
Ac CoA	:	acetyl coenzme A
DEAE	:	Diethylaminoethyl
DMSO	:	dimethylsulfoxide
DNA	:	deoxyribonucleic acid
FMN	:	flavin mononucleotide
HPLC	:	high performance liquid chromatography
N-OH-Glu-P-1	:	2-hydroxyamino-6-methyldipyrido-[1,2-9:5,2'-d] imidazole
NAD	:	nicotinamide adenine dinucleotide
NADH	:	reduced form of NAD
NADP	:	nictinamide adenine dinucleotide phosphate
NADPH	:	reduced form of NADP
nd	:	not done
ss	:	single strand
TLC	:	thin layer chromatography
UDS	:	unscheduled DNA synthesis

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1. INTRODUCTION

N-substituted polycyclic aromatic hydrocarbons have been recognized as carcinogens since as early as the 1890s (Miller and Miller, 1981). During the early 1900s, many dye industry workers were exposed to such aromatic amines as 1-naphthylamine, benzidine, 4-methylaminoazobenzene and 4-aminobiphenyl. The incidence of urinary bladder cancer among these workers was extremely high and since that time many studies have shown that this was due to exposure to some of the aromatic amines (Lower, 1982). A second group of N-aryl compounds has more recently become the focus of intense scientific study. These are the nitroarenes, a group of highly mutagenic compounds which are formed as incomplete combustion products and as such are widely distributed environmental pollutants (Mermelstein et al., 1981). Concern about the genotoxicity and mode of bioactivation of the nitroarenes is warranted since they have been detected in fly ash from incinerators, diesel emissions and even ambient air (Rosenkranz and Mermelstein, 1983). These compounds are much more potent mutagens than are the corresponding aromatic amines as measured by the Ames mutagenicity assay (ibid). Among the nitroarenes 1,8-dinitropyrene stands out because of its extreme mutagenic potency in Salmonella tymphimurium (McCoy et al., 1983). Recently a large number of studies have been reported which explore the biological effects of 1,8-dinitropyrene and the related compound 1-nitropyrene in other systems. These are summarized in Table 1. The reason for the inconsistent

TABLE 1: BIOLOGICAL EFFECTS OF NITROPYRENES

A. 1-Nitropyrene

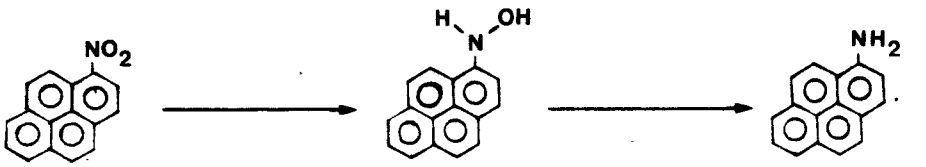
System	Biological Effect	References
E. Coli	mutagenesis	McCoy et al., 1985
Syrian hamster cells	transformation	DiPaolo et al., 1983
Chinese hamster cells	DNA ss breaks	Saito et al., 1984
rabbit lung	DNA adduct formation	Jackson et al., 1984
Wistar rats	DNA adduct formation	Stanton et al., 1985
F344	no tumors	Ohgati et al., 1985
new born rats	tumors at injection site	King, unpublished
BALB/c mice	no tumors	Tokiwa et al., 1985
Sencar mice	no tumors	Nesnow et al., 1984
A/J mice	lung tumors	El-Bayoumy et al., 1984

B. Dinitropyrenes

Isomer	System	Biological Effect	References
1,3-;1,6-;1,8-	E. coli	mutagenesis	McCoy et al., 1985
1,3-;1,6-;1,8-	Chinese hamster cells	mutagenesis	Katoh et al, 1984
1,6-;1,8-	Chinese hamster cells	DNA ss breaks	Saito et al, 1984
1,8-	Syrian hamster cells	transformation	DiPaolo et al, 1983
1,6-;1,8-	rat liver cells	clastogenesis	Danford et al, 1982
1,6-	rat hepatocytes	UDS	Butterworth et al, 1983
1,6-	human hepatocytes	UDS	Butterworth et al, 1983
1,6-;1,8-	F344 rats	tumors at injection site	Ohgaki et al, 1985
1,6-;1,8-	Sencar mice	skin tumors	Nesnow et al, 1984
1,6-	BALB/c mice	tumors at injection sites	Tokiwa et al, 1985

results regarding the carcinogenicity of 1-nitropyrene is not clear. One group of workers who previously reported on the carcinogenicity of 1-nitropyrene have now shown that it is not in fact carcinogenic to rats, but in their previous study the 1-nitropyrene was contaminated with trace amounts of highly carcinogenic dinitropyrenes (Ohgaki et al., 1985). This calls into question the purity of 1-nitropyrene being used in studies showing positive results and may explain why there are inconsistent reports regarding the carcinogenicity of 1-nitropyrene.

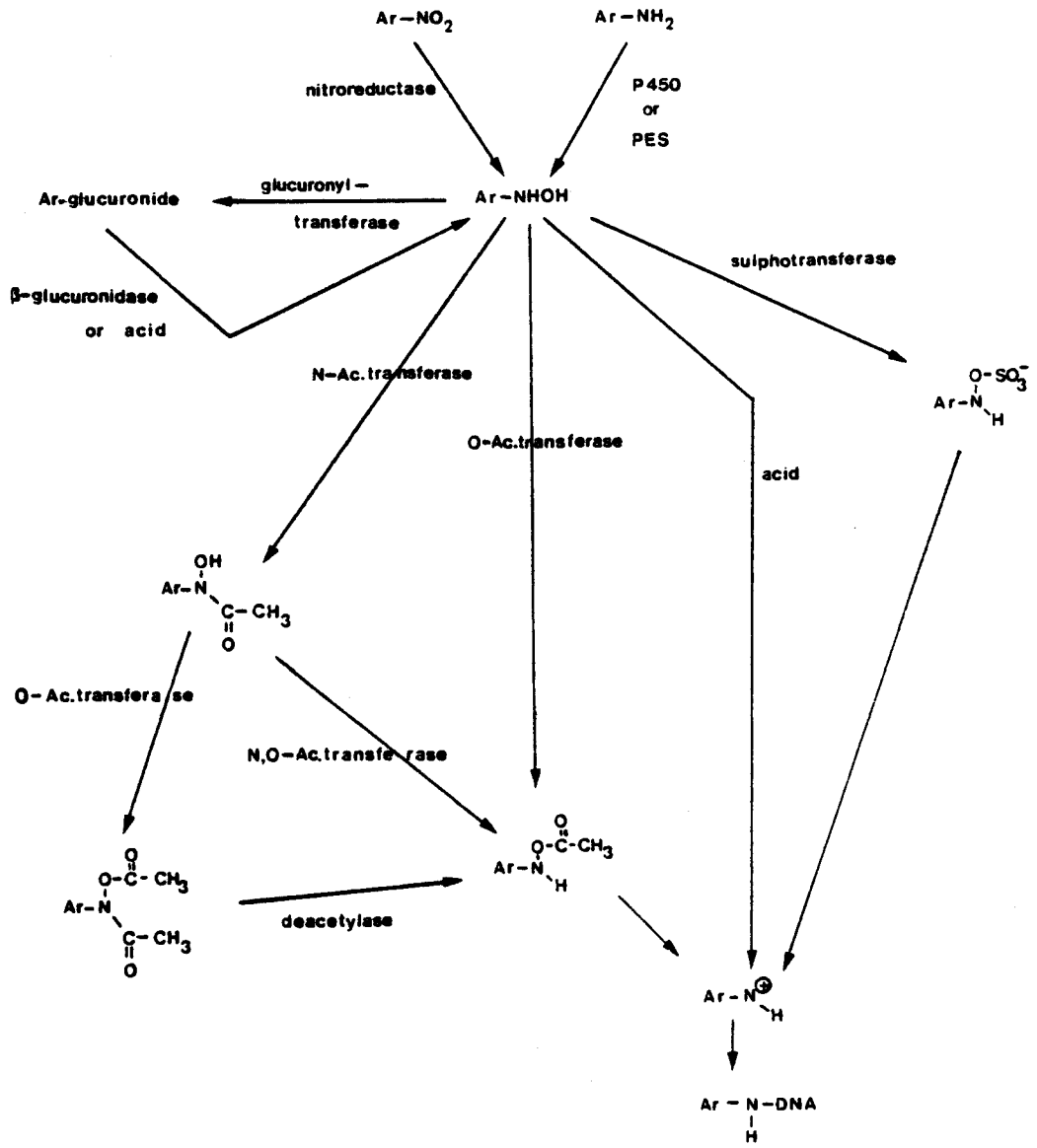
Since the nitropyrenes themselves do not react spontaneously with DNA, it is presumed that the carcinogen is metabolized in vivo to form a reactive intermediate which subsequently interacts with DNA leading to a loss of growth control by the cell (Miller and Miller, 1981). An early step in the metabolism of N-aryl compounds is probably the formation of an N-hydroxy derivative (see Figure 1). These are formed from nitroarenes by reduction of the nitro group (nitroreduction) (Rosenkranz and Mermelstein, 1983). These intermediates are identical to those formed by N-oxidation of the corresponding aminoarene. For example, it is generally accepted that 1-nitropyrene is reduced to 1-aminopyrene via the N-hydroxyarylamine derivative (ibid.) as shown in scheme 1 below:



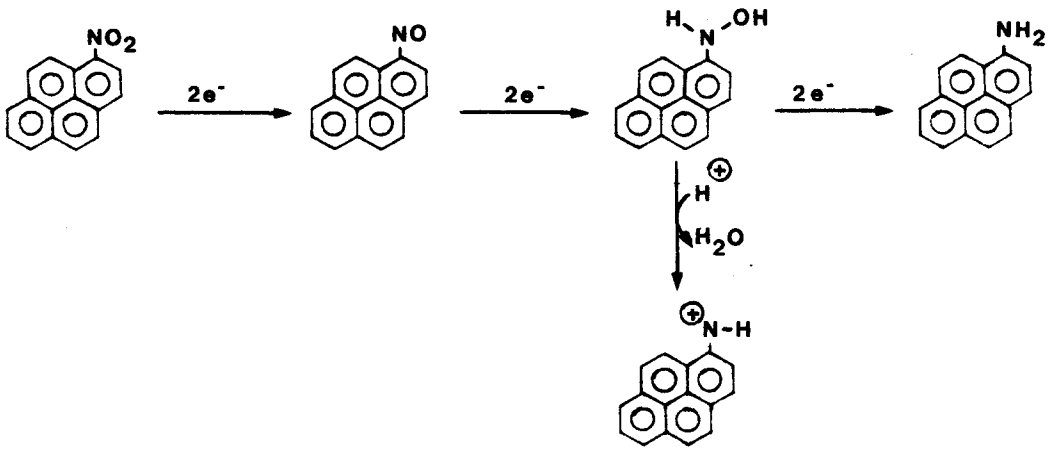
[1]

It is evident that N-oxidation of 1-aminopyrene would lead to the same N-hydroxyarylamine as would the nitroreduction of 1-nitropyrene. This type of relationship also exists for dinitro- and diaminopyrene (Ashby et al., 1983) and 2-nitro- and 2-aminofluorene (Boyd et al., 1983) suggesting that nitro- and aminoarenes cause mutation through a common active intermediate. For some compounds the hydroxyarylamine may be the ultimate DNA binding species, but for others further metabolism is required. Such metabolic processes include acetyltransfer (Bartsch et al., 1972), sulfotransfer (King and Phillips, 1968) and glucuronyl transfer (Irving et al., 1981 and Oglesby et al., 1981). The formation of glucuronides takes place in the liver and may be a means to allow transport of the N-hydroxyarylamines to the bladder for excretion or cleavage by the action of β-glucuronidase (Mitchell et al., 1984) or by acid catalysis (Oglesby et al., 1981). Cleavage of the glucuronide in the bladder may result in DNA damage to epithetial cells by the free hydroxylamine (ibid.). A summary of the various metabolic pathways followed by nitro- and aminoarenes is shown in Figure 1. Once a reactive intermediate has been formed (eg: N-hydroxyarylamine, N-acetoxyarylamine or N-sulfoxyarylamine) binding to nucleophiles such as DNA can occur. The mechanism of such binding may be through a nitrenium ion (Miller and Miller, 1981; Heller et al., 1951; Oglesby et al., 1981 and Beland et al., 1980) or a free radical (Floyd et al, 1981; Vasdev et al., 1982 and Wise et al., 1983).

FIGURE I: Summary of Metabolic Activation of N-Aryl Carcinogens



1,8-Dinitropyrene is a direct acting mutagen, therefore, the Salmonella used in the Ames assay must possess the enzymes necessary for metabolic activation of this compound. Indeed, it appears that bacterial nitroreductases are involved in the activation of nitroarenes (McCoy et al., 1981; Rosenkranz et al., 1982). 1,8-Dinitropyrene is readily reduced by S. tymphimurium strain TA98 to 1-amino-8-nitropyrene and further to 1,8-diaminopyrene (Bryant et al., 1984). S. typhimurium is also capable of metabolizing the related chemical 1-nitropyrene to 1-aminopyrene, and during the course of this reduction a DNA binding species is formed (Messier et al., 1981) and Howard et al., 1983). It has also been demonstrated that the first reduction product formed from 1-nitropyrene is 1-nitrosopyrene, and the proposed reduction pathway leading to the activation of 1-nitropyrene is shown in scheme 2 below (Heflich et al., 1985).



[2]

Since 1-nitrosopyrene is one step closer to the ultimate mutagenic intermediate than is 1-nitropyrene, it is expected that 1-nitrosopyrene be more mutagenic. This is indeed the case (Heflich et al., 1985; L.M. Davidson and D.R. McCalla unpublished), with 1-nitrosopyrene causing four-times as much as mutation as 1-nitropyrene in strain TA98 (see Table 2). Table 2 also shows the comparative mutagenicities of 1-nitropyrene, 1,8-dinitropyrene and their derivatives in four related S. typhimurium strains (L.M. Davidson and D.R. McCalla, unpublished). If similar modes of activation were required to generate mutagenic intermediates from these two compounds, one would anticipate that 1-nitrosopyrene and 1-nitroso-8-nitropyrene show relatively similar increase in activity over their parent compounds. This is not the case. 1-Nitroso-8-nitropyrene is actually twenty times less mutagenic than 1,8-dinitropyrene. It may be that much of the 1-nitroso-8-nitropyrene is nonenzymatically reduced to 1-amino-8-nitropyrene (a much less mutagenic species) before even entering the bacterial cells, or before encountering the genetic material. Half-life studies with nitronitroso compounds support this idea (J. Fulton, personal communication). Examination of Table 2 reveals that 1,8-dinitropyrene is extremely mutagenic in both the wild type strain (TA98) and in strain TA98NR. TA98NR is a mutant of strain TA98 selected for resistance to niridazole (1-(5-nitro-2-thiazolyl)-2-imidazolidinone) (Speck et al., 1981) and showing cross-resistance to 1-nitropyrene (see Table 2).

TABLE 2: MUTAGENICITY OF DINITROPYRENE AND DERIVATIVES IN FOUR RELATED S. TYPHIMURIUM STRAINS[a]

Potential Mutagen Tested	TA98	TA98NR	DNP_6	TA98NR/DNP_6
1,8-dinitropyrene	171,210[b](5542)[c]	105,619(6944)	2434(123)	1251(114)
1-nitroso-8-nitropyrene	5919(563)	3131(287)	119(6)	60(5)
1-amino-8-nitropyrene	122(5)	102(5)	1.73(0.11)	
1,8-diaminopyrene	.01(0)	nd	nd	nd
1-N-acetylamino-8-nitropyrene	.04(0.01)	nd	nd	nd
1-N-acetylamino-8-aminopyrene	.16(0.01)	nd	nd	nd
1,8-N,N'-diacetyldiaminopyrene	.01(0)	nd	nd	nd
1-nitropyrene	157(6)	16(1.0)	129(8.0)	3(0.5)
1-nitrosopyrene	6827(314)	6152(161)	2892(228)	1794(70)

[a]Data were provided by L.M. Davison (personal communication).

[b]The units used to describe mutagenicity are revertants/nmol.

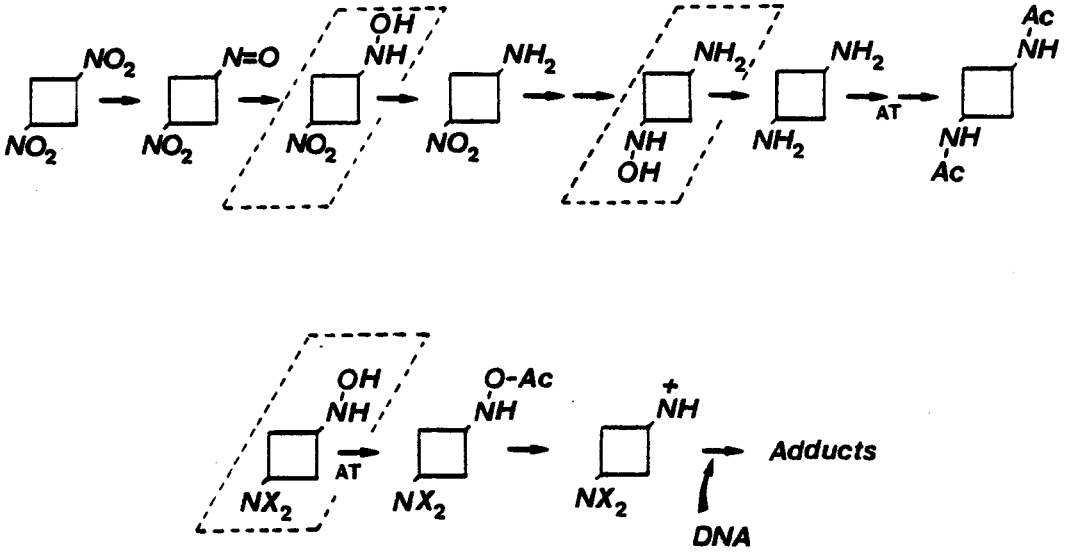
[c]Numbers in brackets are standard deviations.

When cell free extracts of TA98 and TA98NR are fractionated by ion exchange chromatography, nitroreductase activity (as measured by the rate of reduction of nitrofurazone, 5-nitro-2-furaldehyde semicarbazone), is much decreased in strain TA98NR compared with the major peak of nitroreductase activity found in the wild type. Only a small fraction of nitrofurazone-reducing activity remains in the mutant strain. It has been proposed that this small amount of residual activity is all that is required for the reduction of 1,8-dinitropyrene, but that this is not sufficient for the activation of 1-nitropyrene. This may explain the difference between the mutation patterns of 1-nitropyrene and 1,8-dinitropyrene across these two strains (Bryant et al., 1984). Comparison of the genotoxicity of 1-nitropyrene and 1,8-dinitropyrene in strain DNP_6 shows the opposite pattern. Strain DNP_6 is a derivative of strain TA98 which was selected for 1,8-dinitropyrene resistance (McCoy et al., 1982). This strain retains wild type nitroreductase activity, is unaltered in sensitivity to 1-nitropyrene mutagenesis but is resistant to 1,8-dinitropyrene mutagenesis. This difference between the mutagenicities of the two chemicals indicates that they are metabolically activated by different mechanisms. There has been some circumstantial evidence that strain DNP_6 lacks an acetyltransferase enzyme responsible for the activation of the hydroxyarylamine derivative of 1,8-dinitropyrene to a reactive acetoxyarylamine (see Figure 1) (McCoy et al., 1983). As noted above, acetyltransferase mediated activation of hydroxyarylamines is recognized as an

important mechanism (Weeks et al., 1985; Beland et al., 1980; King and Glowinski, 1983; Flammang et al., 1985; Saito et al., 1985; Shinohara et al., 1985) and it is reasonable to assume that it may play a role in the mutagenic action of 1,8-dinitropyrene, since this chemical is not mutagenic to a bacterial strain which evidently lacks this enzyme (see Table 2). Based on this assumption and the probability that the reductive pathway followed by 1,8-dinitropyrene is similar to that followed by 1-nitropyrene (see Page 5), Figure 2 shows the hypothetical route of metabolic activation of 1,8-dinitropyrene. Recent DNA adduct studies have shown that the adduct formed from 1,8-dinitropyrene by S. typhimurium strain TA98 in vivo retains one nitro group. This observation supports the notion that it is the N-acetoxy derivative of 1-N-hydroxylamino-8-nitropyrene which is the ultimate mutagenic species (P.A. Andrews et al., 1985).

The actual mechanism of adduct formation is most likely through a nitrenium ion intermediate intermediate (Miller and Miller , 1981). Nitrenium ion formation as first suggested in 1951 by Heller et al. occurs with the spontaneous dismutation of hydroxy, acetoxy or sulfoxy derivatives (Miller and Miller, 1981). In cases such as that of 1,8-dinitropyrene where a hydroxylamine derivative is implicated as an intermediate along the reaction pathway, the corresponding nitrenium ion is most likely to be the electrophile responsible for attacking DNA (Heller et al., 1951).

FIGURE 2: Postulated Pathway of Dinitropyrene Activation



Squares represent the 4-ring compound pyrene. Dotted boxes surround hydroxylamine derivatives which may be substrates for acetyltransferase enzymes (denoted AT). The pyrene derivates shown in the top row are 1,8-dinitro-,1-nitroso-8-nitro, 1-hydroxylamino-8-nitro, 1-amino-8-nitro, 1-amino-8-hydroxylamino, 1,8-diamino and 1,8-diacetyldiaminopyrene. The bottom row shows the hydroxylamino, acetoxyamino and nitremium ion derivatives.

The DNA adduct produced from 1,8-dinitropyrene does not contain an acetyl moiety (Andrews et al., 1985) and this is consistent with nitrenium ion formation (see Figure 2).

1.1. Research Goals

The mechanism of bioactivation of the potent mutagen 1,8-dinitropyrene appears to be complicated and there are several questions which remain unanswered and inconsistencies which remain unexplained. For instance, why does 1-nitropyrene require only nitroreduction to produce a reactive intermediate, while 1,8-dinitropyrene requires an additional metabolic step before it is activated by S. typhimurium? Why is 1-nitroso-8-nitropyrene twenty times less mutagenic than 1,8-dinitropyrene while 1-nitrosopyrene is four times more mutagenic than 1-nitropyrene? Why is 1,8-dinitropyrene one thousand times more mutagenic than 1-nitropyrene? Why does the loss of the majority of the nitrofurazone-reducing ability of strain TA98NR have very little effect on the mutagenicity of 1,8-dinitropyrene for this strain, when nitroreduction is required for activation? As well as the need for answers to these questions there also exists a need to characterize the enzymatic deficiencies in strain DNP_6. The goals of this research were to investigate the putative lack of acetyltransferase activity in strain DNP_6 and to try and shed additional light on the roles played by this enzyme and by nitroreductase in the metabolic activation of 1,8-dinitropyrene by S. typhimurium.

2. MATERIALS & METHODS

2.1. Chemicals and Strains

Salmonella typhimurium strains TA98NR, TA98/1,8-DNP_6 and TA98NR/1,8-DNP_6 were supplied by Dr. H. S. Rosenkranz, Case Western Reserve University, Cleveland, OH. NAD , NADH, glucose-6-phosphate, FMN, acetyl CoA, acetylphosphate, dithiothreitol (Cleland's reagent), N-ethyl maleimide, DEAE cellulose and DEAE sephacel were obtained from Sigma, St. Louis, MO. Affi gel blue (100-200 mesh) and the protein assay reagent were obtained from Biorad, Richmond, CA. NADP , NADPH and Glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides) were supplied by Boehringer-Mannheim, Montreal, P.Q. 1-Nitropyrene (purchased from Aldrich) was purified by HPLC as described by Bryant et al., 1984. 1,8-Dinitropyrene was a gift from LC Services, Woburn, MA. Sytheses of the dinitropyrene derivatives 1-amino-8-nitropyrene, 1,8-diaminopyrene and 1-N-acetylamino-8-nitropyrene have been previously described (Bryant et al., 1984). These compounds were provided by Paul Andrews and Dr. B. E. McCarry. 1-N-Acetylamino-8-aminopyrene and 1,8-N,N'-diacetyldiaminopyrene were prepared by acetylation of 1,8-diaminopyrene with acetic anhydride in methylene chloride. Nitrofurazone, (5-nitro-2-furaldehyde semicarbazone) was a gift from Norwich Pharmical, Norwich, NY. All other chemicals used were of reagent grade, except solvents, all of which were HPLC grade.

2.2. Preparation of Crude S. typhimurium Extracts

Bacteria were grown overnight in 1.5 L of Oxoid nutrient broth at 37°C to an absorbance at 600 nm of 1.1 - 1.3. Cells were harvested by centrifugation, washed with 50 mM tris-HCl buffer at pH 7.4 containing 1 mM dithiothreitol and 6% glycerol, resuspended in 15 ml of buffer and sonicated (Fisher Dismembrator 300) in ice. The resulting sonicate was centrifuged for 1 hour at 100,000 g at 4°C and the supernatant stored in 500 µl aliquots at -70°C.

2.3 Chromatography of Crude Extracts - DEAE Sephacel

For batch chromatography, crude extract containing 37 mg of protein (as determined by Biorad protein assay using BSA as the standard) was loaded onto a 1 x 6 cm gel bed of DEAE cellulose or Sephacel which had previously been equilibrated with 50 mM Tris-HCl (pH 7.4), washed with 40 ml of buffer and eluted with buffer containing 0.5M KCl. One ml fractions were collected and those five fractions which were a dark yellow colour were pooled and used for metabolism studies.

Chromatographic fractionation of crude extract was done by loading extract containing 37 mg of protein onto a 0.75 by 30 cm column of DEAE cellulose or Sephacel which had previously been equilibrated with 50 mM Tris-HCl (pH 7.4), washing with 50 ml of buffer and eluting with an 80 ml linear gradient of 0.1 to 0.5 M KCl in buffer. One ml fractions

were collected at a flow rate of approximately 0.75 ml per min. The column was washed with buffer containing one mole per litre KCl and then regenerated.

2.4. Affi-gel Blue Chromatography

The column used was 0.5 cm by 20 cm. Crude extract containing 74 mg of protein was loaded and washed with 50 mM Tris-HCl buffer, pH 7.4. Ten 2 ml fractions were collected. The eluting buffer was changed to 50 mM phosphate buffer, pH 6.0 containing 1 M KCl, 1 mM NADH and 1 mM NADPH and ten 2 ml fractions were collected.

2.5. Metabolism of 1-Nitropyrene, 1,8-Dinitropyrene and 1-Amino-8-nitropyrene

The chemical dissolved in methylene chloride was placed in an Eppendorf centrifuge tube and dried under a stream of argon. The amounts used were; 1-nitropyrene, 25 nmoles; 1,8-dinitropyrene, 25 nmoles; 1-amino-8-nitropyrene, 10 nmoles. The dried chemical was then dissolved in 20 µl DMSO, 200 µl enzyme was added followed by 20 µl of reaction mix containing 1.2 mg glucose 6-phosphate, 25 ng NAD 0.6 µg FMN, 0.8 mg acetyl CoA (or 0.4 mg acetyl phosphate), 0.1 units glucose-6-phosphate dehydrogenase and 50 mM Tris HCl pH 7.4. After mixing and incubation in the dark at room temperature, the mixture was extracted once with 1 ml methylene chloride and

the organic phase dried under nitrogen. The dried extracts were stored at -20°C for up to 48 hours and then analyzed by HPLC.

2.6. Metabolism of 1,8-Diaminopyrene

Ten nmoles of diaminopyrene were dried under argon, taken up in 20 µl DMSO and incubated with 200 µl enzyme preparation and 20 µl buffer, the entire mixture having a final concentration of 1 mM acetyl CoA or acetyl phosphate. Incubation and extraction procedures were as described in 2.5. Dried extracts were never stored longer than 24 hours.

2.7. Assay for Nitroreductase Using Nitrofurazone

Aliquots from chromatographic fractions were assayed for the ability to reduce nitrofurazone. The assay conditions have been described previously (McCalla et al., 1975).

2.8. Acetyltransferase Assay Using TLC

Metabolism of 1,8-diaminopyrene was allowed to proceed as previously described, using 77 nmoles of chemical. After methylene chloride extraction and drying under argon the dried extract was redissolved in 10 µl methylene chloride. Samples (2-3 µl) were applied to $MKC_{18}F$ reverse phase TLC plates (Whatman Chem. Co., Clifton, NJ) and the spots dried with a stream of argon. The plates were developed in 4:3 V/V

acetonitrile/0.2 M phosphate buffer pH 7.6, for 5-10 minutes and examined under a long wave UV lamp before drying (spots disappear upon drying). Acetyltransferase activity is indicated by the presence of one or two fluorescent spots which migrate faster than the original diaminopyrene. These are the mono and diacetylated derivatives. The monoacetylated derivative has been previously characterized (Bryant et al., 1984). The characterization of the diacetylated derivative is described in the Results section which follows.

2.9. HPLC Analysis

Dried methylene chloride extracts of reaction mixtures were dissolved in 50 µl acetonitrile. A 20 µl sample was analyzed by reverse phase HPLC on an Altex ODS column (0.46 x 25 cm) using a linear gradient of phosphate buffered acetonitrile as previously described (Bryant et al., 1984). UV absorbance (254 nm) and fluorescence (excitation maximum 360 nm; emission greater than 430 nm) were monitored. Chromatograms were acquired by an Apple II + microcomputer equipped with a 12-bit analogue-digital converter and amplifier (Interactive Microware, Inc., State College, PA). The concentrations of each compound were calculated from peak areas determined with in-house software and using extinction coefficients determined from synthetic standards.

2.10. Ames Assays:

Mutagenicity assays were carried out according to the method of Ames *et al.* (1975), with bacterial extract and reaction mixture containing cofactors required for nitro-reduction and acetylation as the activation system instead of S9 mix. Preincubations were carried out by allowing the activation mix to metabolize the chemical in the presence of the bacteria for varying lengths of time before the addition of top agar and subsequent plating. Each concentration of chemical was tested in triplicate on at least two separate occasions.

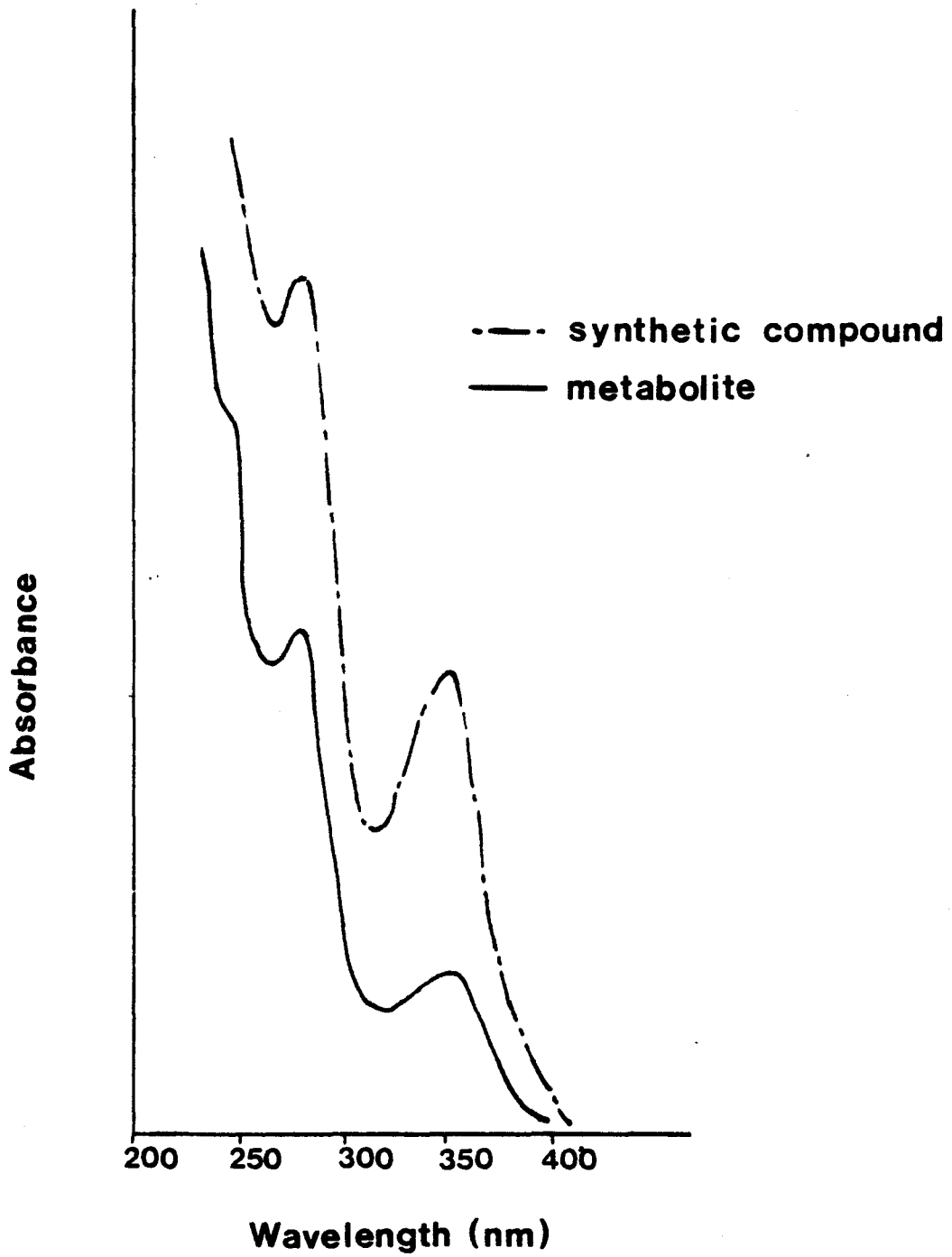
3. RESULTS

3.1 Identification of 1,8-N,N'-diacteyldiaminopyrene

The metabolism of 1,8-dinitropyrene by S. typhimurium strain TA98NR has been previously shown to produce the two reduction products 1-amino-8-nitro- and 1-8-diaminopyrene as well as 1-N-acetylamino-8-aminopyrene (Bryant et al., 1984). An additional metabolite has now been detected. This compound can be separated from the other metabolites by reverse phase HPLC or TLC. It migrates through the reverse phase chromatographic medium faster than the other compounds, indicating that it is more polar than they are. It seems logical to suppose that a further metabolite of 1-acetylamino-8-aminopyrene could be the more polar compound, 1,8-N,N'-diacetyldiaminopyrene. To determine whether or not the unknown metabolite was the diacetylated compound, a comparison was made between chemically synthesized 1,8-N,N'-diacetyldiaminopyrene and the unknown metabolite isolated from reaction mixtures. Methylene chloride extracts of reaction mixtures were subjected to TLC and the unknown was scraped from TLC plates and subsequently purified by HPLC. Figure 3 shows a comparison of the UV-visible spectra of the two compounds which are identical. Figures 4 and 5 show the fluorescence emission and excitation spectra respectively. Both of these show the same peaks for each of the compounds. The HPLC retention time for both the chemical standard and

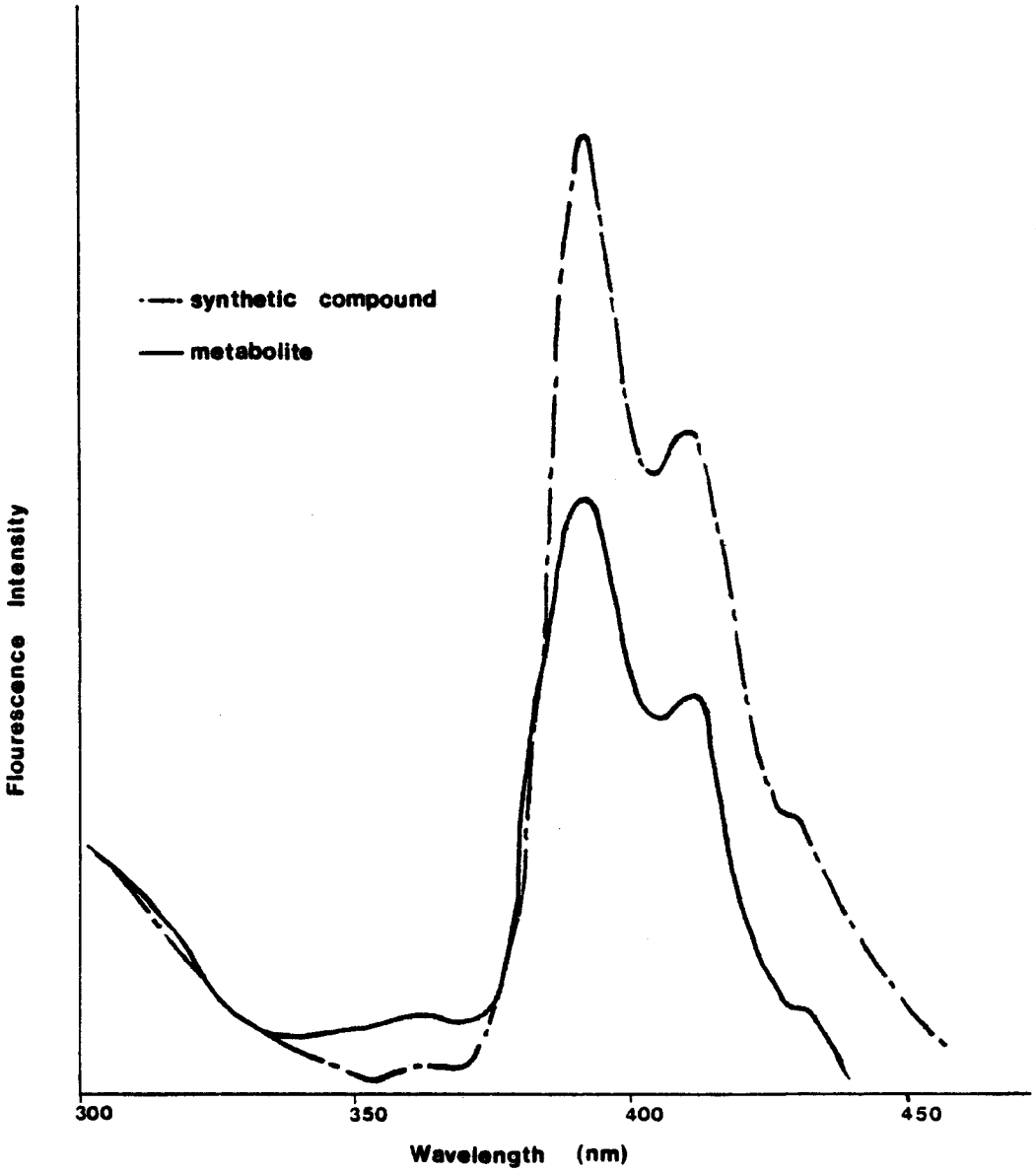
the metabolite is 17.9 minutes. Final confirmation that the unknown is indeed the diacetylated diamino derivative comes from mass spectral data provided by Dr. M. A. Quilliam and described as showing a molecular ion at m/z 274 (30%), 232 (60%) and 231 (100%) due to losses of CH_2CO, $2C_2CO$ and CH_2CO plus CH_3CO respectively.

FIGURE 3: UV-Vis Absorbance Scans of Unknown Metabolite and Synthetic Standard



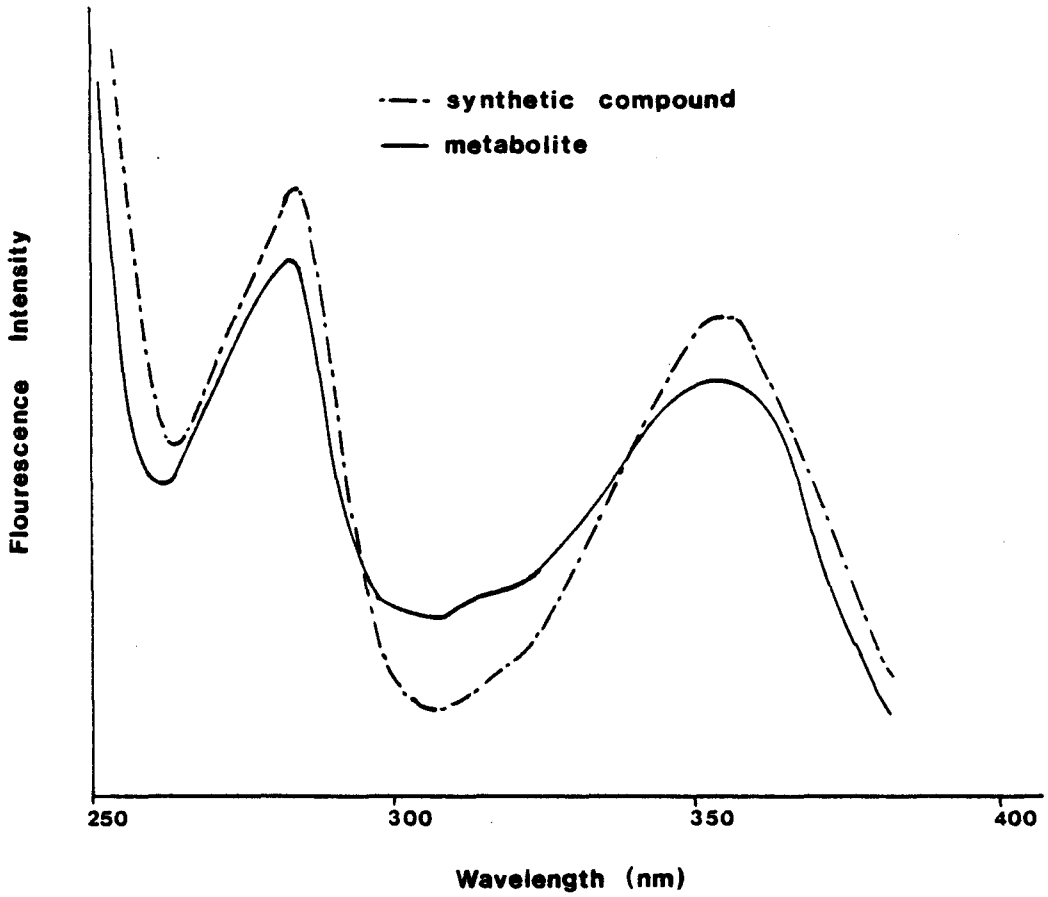
The absorbance profiles of authentic 1,8-N,N'-diacetyldiaminopyrene (-·—) and the unknown metabolite of 1,8-diaminopyrene purified from reaction mixtures (——) are shown.

FIGURE 4: Fluorescence Emission Spectra with Excitation at 280 nm of Unknown Metabolite and Synthetic Standard



The emission spectra of authentic 1,8-N,N'N-diacetyldiaminopyrene (-·—·-) and the unknown 1,8-diaminopyrene metabolite purified from reaction mixtures (——) are shown.

FIGURE 5: Fluorescence Excitation Spectra with Emission at 395 nm of Unknown Metabolite and Synthetic Standard



The excitation spectra of authentic 1,8-N,N' -diacetyldiaminopyrene (-——-) and the unknown 1,8-diaminopyrene metabolite purified from extraction mixtures (——) are shown.

All the results which follow are from single experiments, unless otherwise stated. Experiments were repeated at least twice, and some as many as five times to confirm trends which were observed. Mean results are not presented due to the variability of activity levels among bacterial extract preparations. The extracts used for experiments had the ability to metabolize between 20 and 23.7 nmoles of 1,8-dinitropyrene over a period of 2 hours. Extract containing 17 mg of protein was partially purified by DEAE cellulose chromatography prior to testing of activity.

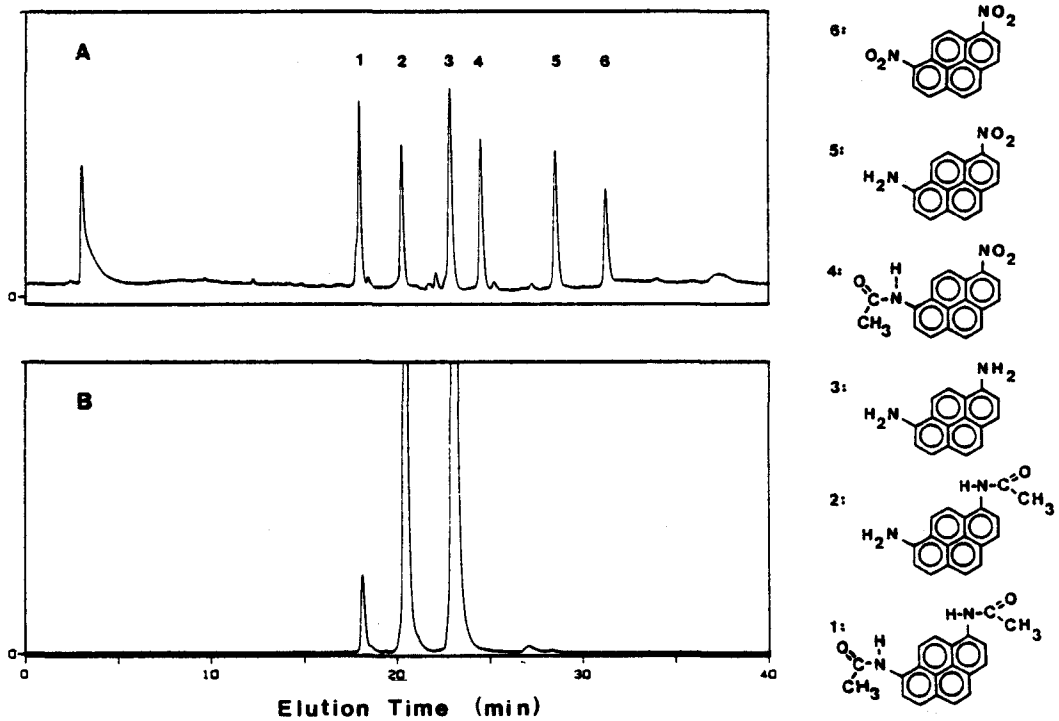
3.2. Metabolism of Dinitropyrene and Diaminopyrene

The HPLC profile of a mixture of chemically synthesized derivatives of 1,8-dinitropyrene is shown in Figure 6. The fluorescence trace is shown in panel B and indicates that only diaminopyrene and its acetylated derivatives are fluorescent. Figure 7 shows two sample HPLC traces obtained by chromatrography of methylene chloride extracts of reaction mixtures containing bacterial extract, cofactors and chemical. The top panel demonstrates the ability of TA98NR extract to acetylate diaminopyrene to both a mono- and a diacetylated compound. The bottom panel shows the pattern of metabolites formed from 1,8-dinitropyrene. Note that the monoacetylated compound is the major product when 1,8-dinitropyrene is the starting substrate. It is also interesting to note that one of the possible derivates of

1,8-dinitropyrene, 1-N-acetylamino-8-nitropyrene (compound #4 on Figure 6) is not produced in detectable amounts by bacterial extracts.

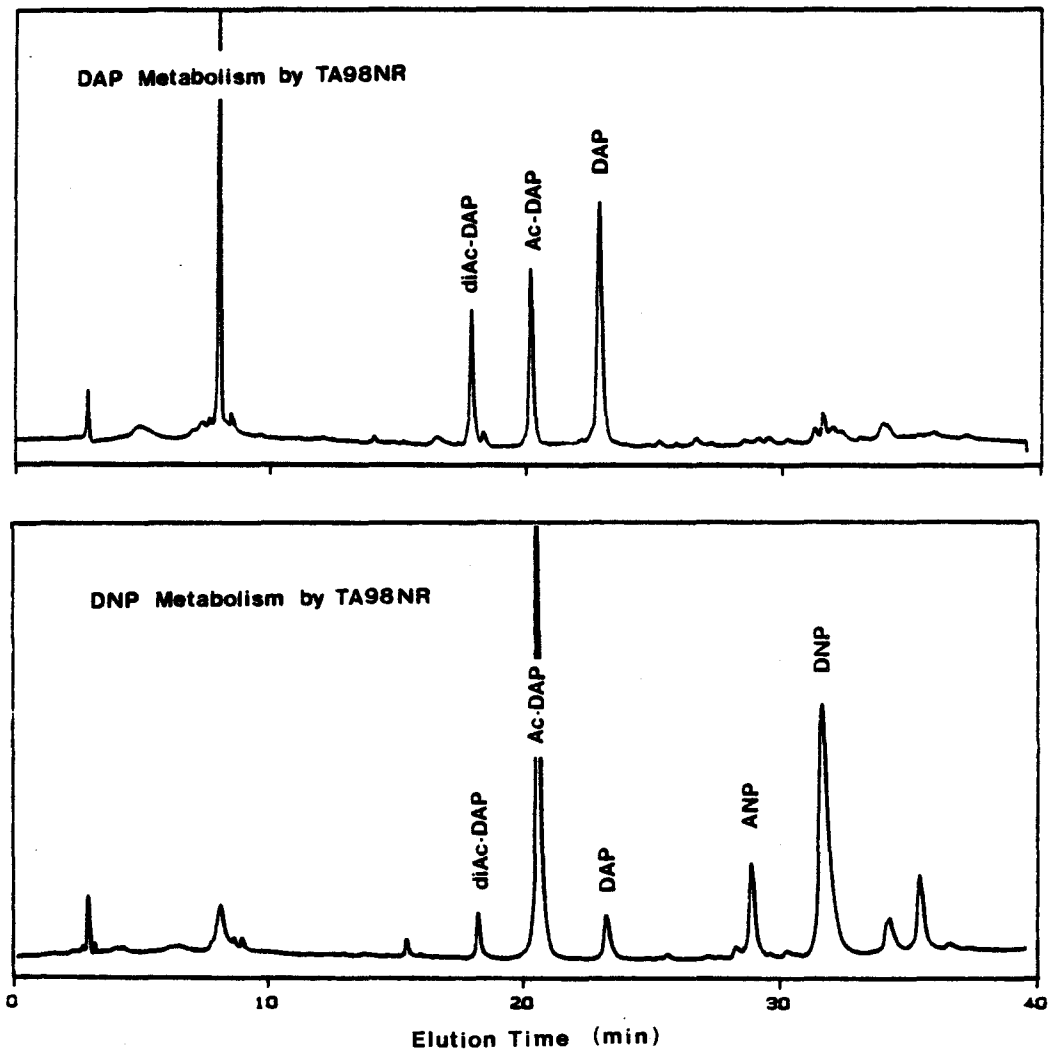
This pattern of metabolites has been confirmed by using [^{3}H]-1,8-dinitropyrene as the substrate for metabolism by TA98NR extract. The radioactive peaks coincide only with those peaks labelled in Figure 7 as metabolites (data not shown). The extraction efficiency was determined on two seperate occasions to be 90% and 95% respectively (data not shown).

FIGURE 6: HPLC Separation of Mixture of Chemically Synthesized Derivatives of 1,8-Dinitropyrene



Panel A shows the UV trace while Panel B shows the corresponding fluorescence trace. The standards shown are (1) 1,8-N,N'-diacetyldiaminopyrene; (2) 1-N-acetylamino-8-aminopyrene; (3) 1,8-diaminopyrene; (4) 1-N-acetylamino-8-nitropyrene; (5) 1-amino-8-nitropyrene; (6) 1,8-dinitropyrene.

FIGURE 7: HPLC Analyses of Reaction Mixtures Containing 1,8-Diaminopyrene and 1,8-Dinitropyrene

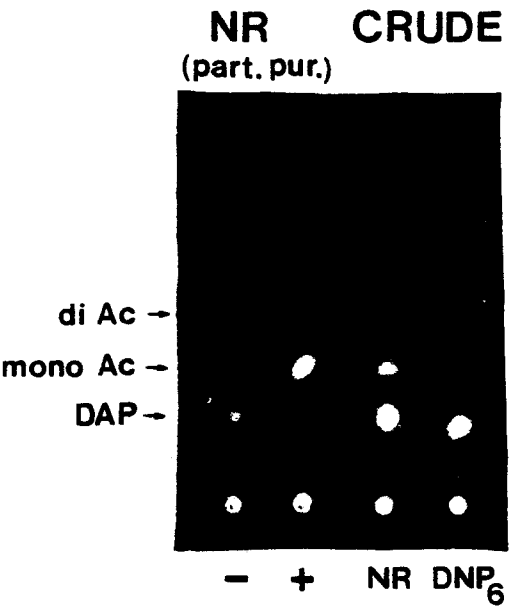


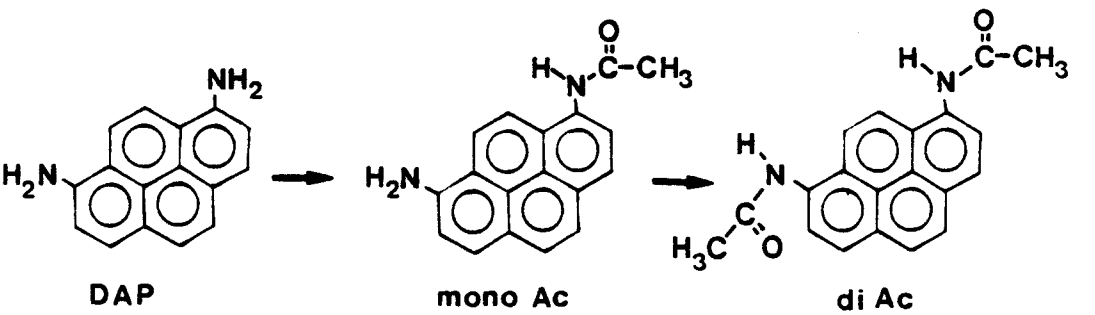
Shown are UV profiles of $MeCl_2$ extracts of reaction mixtures incubated for 2 hours with 1,8-diaminopyrene (top) or 1,8-dinitropyrene (bottom) and containing bacterial extract from S. typhimurium strain TA98NR which had been chromatographed by the batch method on DEAE cellulose.

3.2.1 Analysis of Reaction Mixtures by TLC

The fluorescent nature of 1,8-diaminopyrene and its acetylated derivatives provided the basis for a TLC assay for detection of acetyltransferase activity in bacterial extracts. Analysis of methylene chloride extracts of reaction mixtures containing TA98NR extract and with 1,8-diaminopyrene as the substrate results in the chromatogram seen in left portion of Figure 8. Partially purified TA98NR extract in the absence of acetyl CoA failed to metabolize the diaminopyrene while in the presence of acetyl CoA two fluorescent spots appeared which migrated beyond the starting compound. Typical R_f values are 0.24, 0.40 and 0.54 for 1,8-diaminopyrene, 1-N-acetylamino-8-aminopyrene and 1,8-N,N'-diacetyldiaminopyrene respectively. The requirement for acetyl CoA in the acetylation process is clearly indicated here. The right portion of Figure 8 compares the 1,8-diaminopyrene metabolizing ability of crude extracts of TA98NR and DNP_6. The DNP_6 extract was unable to produce any acetylated compound while TA98NR crude extract produced some monoacetylated compound. It is apparent that DNP_6 crude extract lacks the acetyltransferase activity present in TA98NR crude extract and that this activity was enhanced in the TA98NR extract by partial purification followed by supplmentation with acetyl CoA. Although clear differences can be seen using TLC analysis, quantitation is impossible. In order to obtain more informative quantitative data HPLC analysis is required.

FIGURE 8: TLC Analysis of Reaction Mixtures Containing 1,8-Diaminopyrene





The top portion shows a photograph taken under UV light of two TLC plates lying side by side. On the left is shown the pattern of metabolites formed by batch DEAE chromatographed TA98NR extract without (left) and with (right) acetyl CoA present. A dark purple fluorescent spot which is the diacetylated derivative is observed only in the presence of acetyl CoA - it does not show up in the photographs because of its very low intensity. On the right is shown a comparison of the metabolites formed by crude extracts of TA98NR (left) and DNP_6 (right). The bright spots at the origin are due to overloading of the plates.

3.2.2 Analysis of Reaction Mixtures by HPLC

The results of an HPLC analysis are shown in Table 3. Here a comparison is made between the 1,8-diaminopyrene metabolizing abilities of partially purified extracts of strains TA98NR and DNP_6. It is clear that acetyl CoA significantly enhanced the acetyltransferase activity of partially purified TA98NR extract while having no effect on that of DNP_6. As seen in the TLC assay, DNP_6 appeared to have much less acetyltransferase activity than did TA98NR. Table 4 shows the results of HPLC analyses of TA98NR and DNP_6 reaction mixtures which contained 1,8-dinitropyrene as the starting substrate. Again, the decreased effect of acetyl CoA on the production of an acetylated derivative by DNP_6 as compared to that of TA98NR is seen as well as the decreased acetyltransferase activity in DNP_6. A comparison of the data in Table 3 with that in Table 4 leads to an interesting observation. The pattern of metabolites formed from 1,8-diaminopyrene was dependent on the source of the diaminopyrene, ie., the extent of diaminopyrene metabolism was lower if diaminopyrene itself was provided as the original substrate than if it was produced in situ from its precursor 1-amino-8-nitropyrene. Further, the ratios of monoacetylated to diacetylated compound produced were quite different depending on the source of the diaminopyrene.

TABLE 3: METABOLISM OF DIAMINIOPYRENE BY DEAE CELLULOSE TREATED *S. typhimurium* CELL FREE EXTRACTS

Metabolite	Percent of Total Diaminopyrene Metabolites									
	TA98NR						DNP_6			
	+AcCoA			-AcCoA			+AcCoA			-AcCoA
	Trial Number									
	1	2	3	1	2	3	1	2	3	1
DAP	22	23	14	76	80	74	95	85	90	96
monoAc	71	77	82	24	20	26	5	15	10	4
diAc	7	0	4	0	0	0	0	0	0	0

Results shown are from analysis of methylene chloride extracted from reaction mixtures.

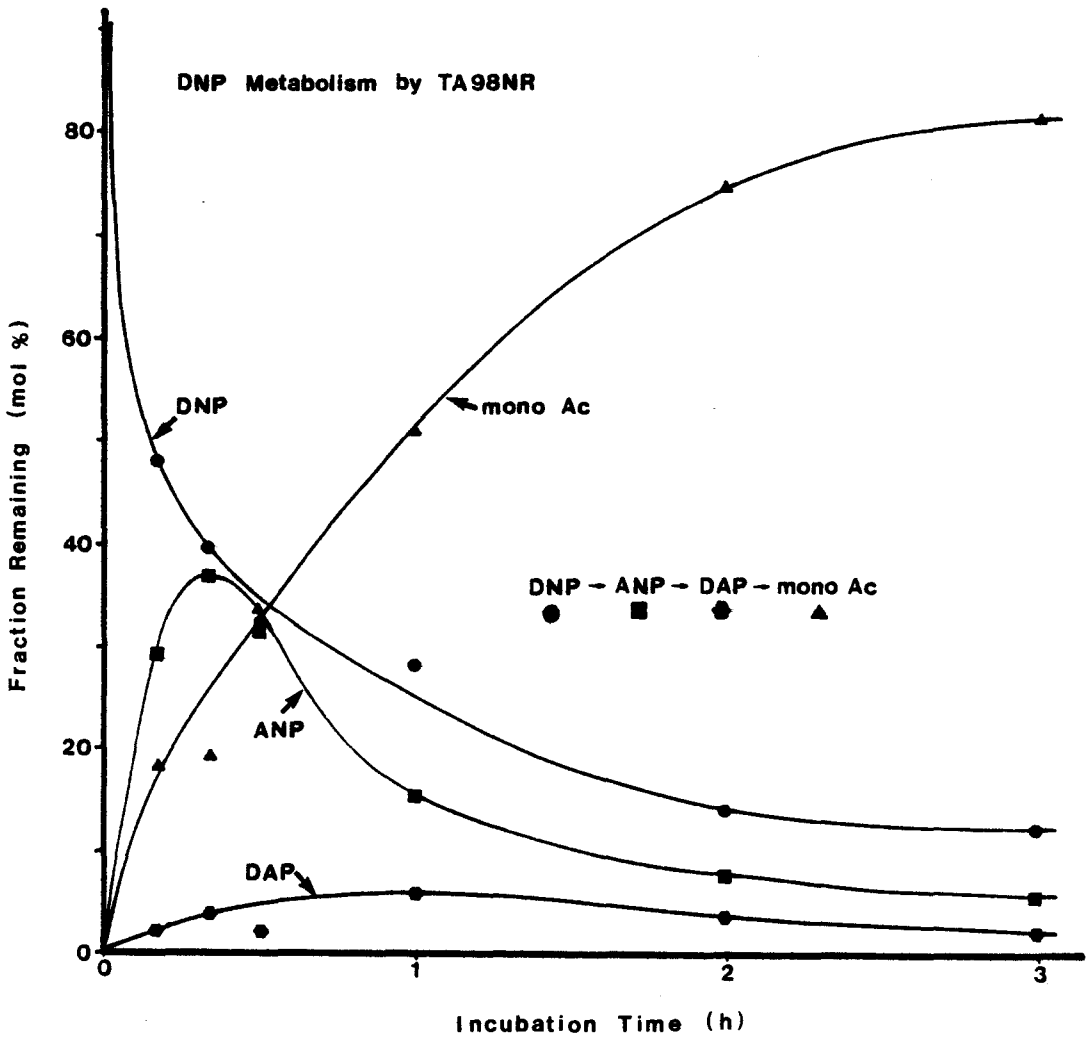
TABLE 4: METABOLISM OF DINITROPYRENE BY DEAE CELLULOSE TREATED *S. typhimurium* CELL FREE EXTRACTS

Metabolite	Percent of Total Dinitropyrene Metabolites											
	TA98NR						DNP_6					
	+Ac CoA			-Ac CoA			+Ac CoA			-Ac CoA		
	1	2	3	1	2	3	1	2	3			
DNP	10	11	5	25	19	15	33	30	20		5	
ANP	5	15	31	64	60	61	59	24	42		52	
DAP	1	26	1	5	3	8	6	41	15		28	
Mono Ac	81	72	63	3	18	16	6	4	23		14	
di Ac	1	1	1	0	0	0	0	0	0		0	

Results shown are from analysis of methylene chloride extracted reaction mixtures.

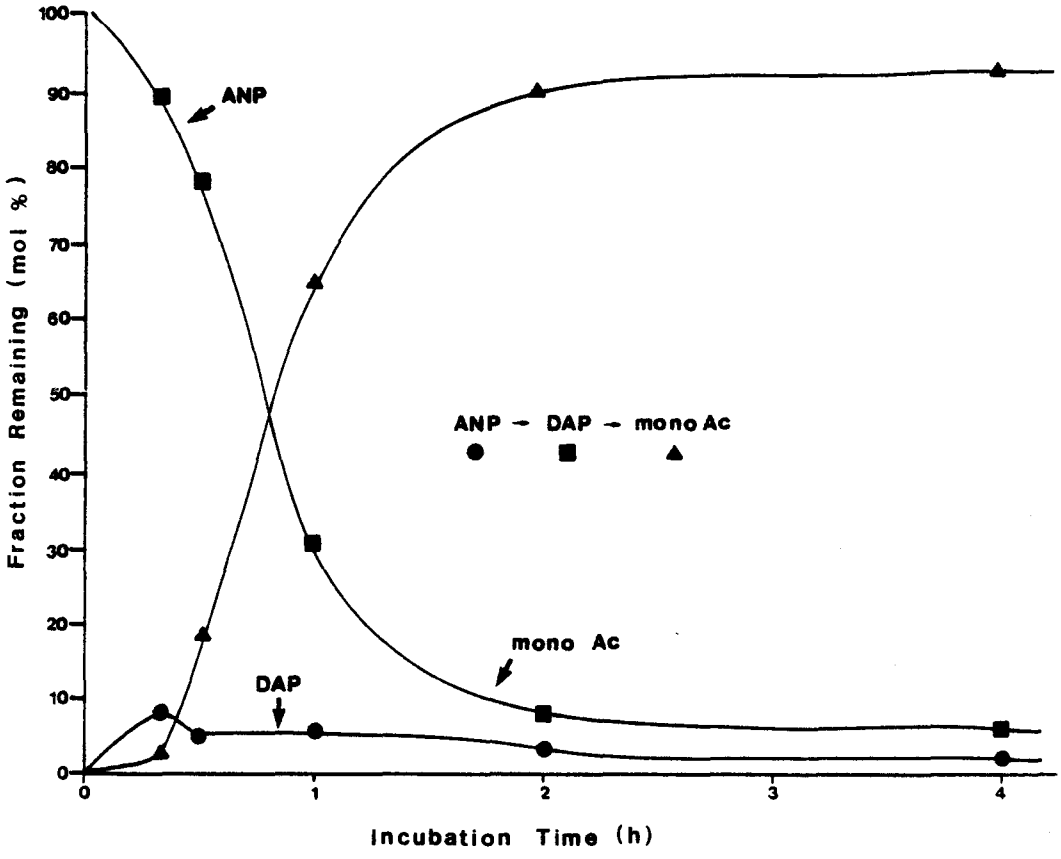
The data plotted in Figures 9 and 10 show the time courses of the metabolism of 1,8-dinitropyrene and 1-amino-8-nitropyrene respectively by TA98NR DEAE cellulose treated extract. The most noteworthy feature of these plots is that the diaminopyrene did not accumulate and thus must have been acetylated immediately upon its production from 1-amino-8-nitropyrene.

FIGURE 9: Time Course of 1,8-Dinitropyrene Metabolism by TA98NR



The time course of 1,8-dinitropyrene metabolism by TA98NR batch DEAE chromatographed extract. Percentages of metabolites at each time point were calculated from HPLC peak areas. Each time point represents a separate reaction mixture.

FIGURE 10: Time Course of 1-Amino-8-nitropyrene Metabolism by TA98NR



The time course of 1-amino-8-nitropyrene metabolism by TA98NR batch DEAE chromatographed extract. Percentages of metabolites at each time point were calculated from HPLC peak areas. Each time point represents a separate reaction mixture.

3.3. Acetyl Phosphate as Acetyl Donor:

It has been established that acetyl CoA may be used as the acetyl donor under the conditions of these studies, but being unstable and very expensive, it is not ideal for routine use. Another potential acetyl donor is the cheap and stable compound acetyl phosphate. Acetyl phosphate when substituted for acetyl CoA at the same concentration leads to the pattern of metabolites shown in Table 5 when 1-amino-8-nitropyrene is the substrate.

TABLE 5: ACETYL PHOSPHATE AS THE ACETYL DONOR IN THE METABOLISM OF 1-AMINO-8-NITROPYRENE BY TA98NR

Metabolite	% Total Aminonitropyrene Metabolites
ANP	25.0
DAP	6.6
mono Ac	68.0[a]
di Ac	0.4

[a]These results indicate that acetyl phosphate is a satisfactory acetyl donor and may be used to replace acetyl CoA in these experiments. There was no non-enzymatic acetylation of 1,8-diaminopyrene by acetylphosphate and this is demonstrated in figure 13 fractions 1 to 44 and 60 to 68.

3.4 Metabolism of 1-Nitropyrene:

It is evident that strain TA98NR is capable of reducing first one nitro group of 1,8-dinitropyrene and then the other. In the first instance, the substrate was a dinitro compound and in the second the substrate was a mononitro compound. 1-Nitropyrene, another mononitro compound was also reduced to its amino derivative by TA98NR. Figure 11 shows the HPLC separation of chemically synthesized 1-nitropyrene and 1-aminopyrene standards. The fluorescence trace (lower panel) indicates that 1-aminopyrene is fluorescent. Figure 12 shows the HPLC analysis of a methylene chloride extract of a TA98NR reaction mixture containing 1-nitropyrene and all cofactors required for both nitroreduction and acetylation. The only metabolite detected was 1-aminopyrene.

FIGURE 11: HPLC Separation of 1-Nitropyrene and 1-Aminopyrene Standards

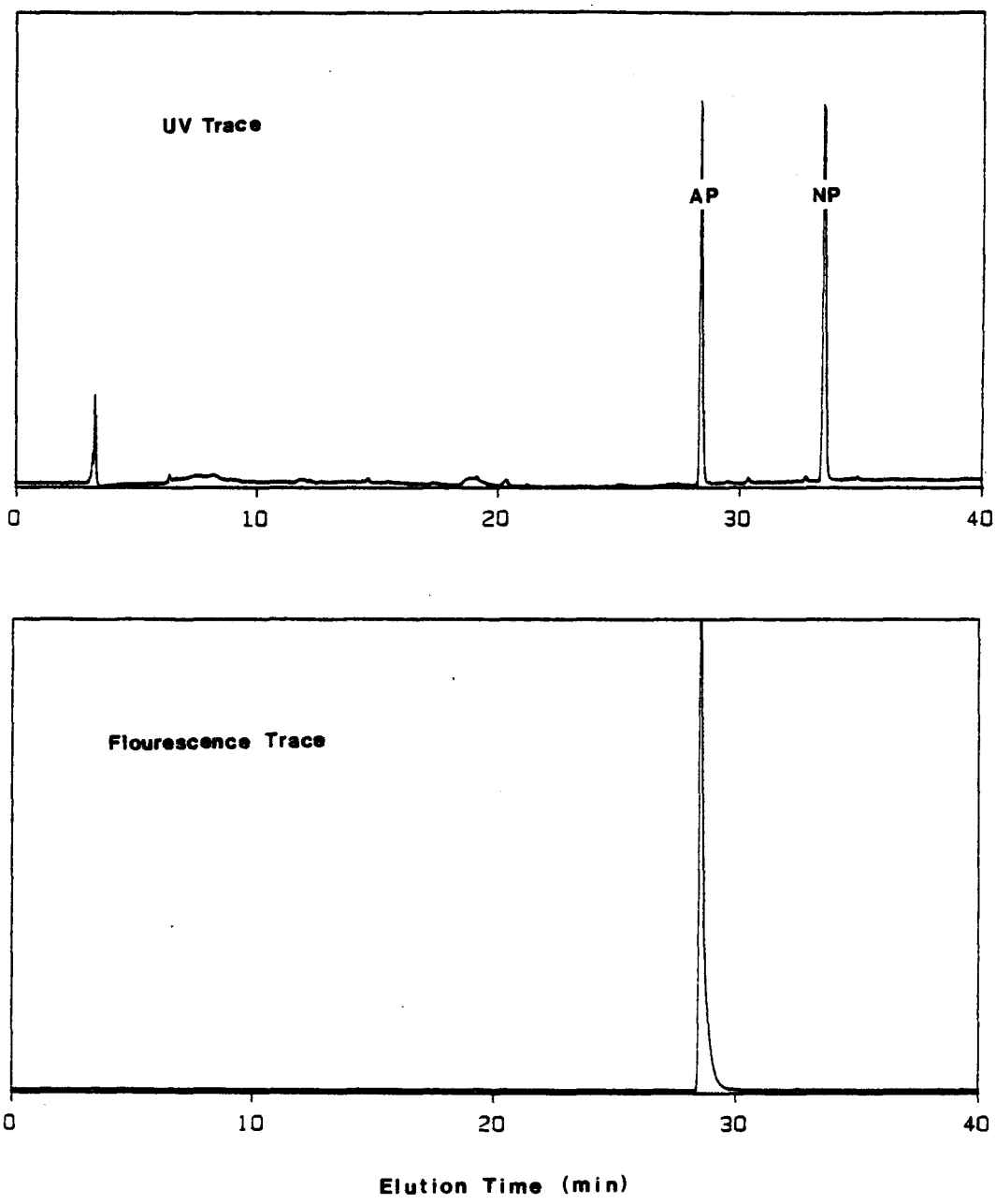
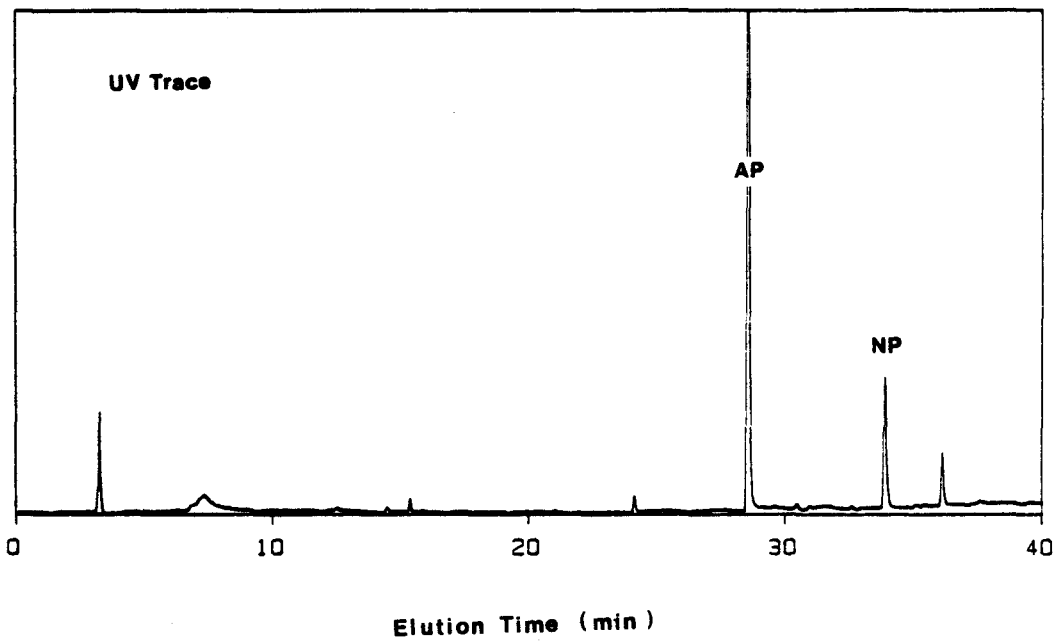


FIGURE 12: HPLC Analysis of Reaction Mixture Containing 1-Nitropyrene



UV trace of Methylene chloride extract of reaction mixture containing TA98NR batch DEAE chromatographed extract incubated for 2 hours with 1-nitropyrene. The only metabolite detected is 1-aminopyrene.

3.5 Fractionation of Enzymatic Activities:

Upon reviewing the results of over eighty experiments, all of which were designed to study the metabolism of three or four of the substrates, 1,8-dinitropyrene, 1-amino-8-nitropyrene, 1,8-diaminopyrene and 1-nitropyrene by one or more of the S. typhimurium strains TA98NR, DNP_6 or TA98NR/1,8-DNP_6 (double mutant), it became apparent that nine of these experiments yielded unexpected results. For reasons which remain unknown, the bacterial extracts used in these experiments exhibited an inability to metabolize one or more substrate while retaining the ability to metabolize another. The results of these are tabulated in Table 6. Plus signs indicate that more than seventy percent of the substrate at the head of the column was metabolized. Minus signs indicate less than twenty percent metabolism. In all cases except one (Experiment #46), the metabolism of dinitropyrene occurred to a different extent from that of the other compounds tested. This finding suggests that perhaps there is more than one nitroreductase enzyme and that separation of these from each other and/or inactivation of one and not the other may be possible.

TABLE 6: EXPERIMENTS WHICH DEMONSTRATE LOSS OF ONE TYPE OF METABOLIZING ACTIVITY, BUT NOT ANOTHER

Experiment #	Strain	Substrate			
		DNP	ANP	DAP	NP
12	DNP_6	+[a]	-	-	
14	DNP_6	+	-	-	
28	NR	+	-	-	
31	NR	-	+	+	
46	NR	-	+	-	
50	NR	+	-	-	
	DNP_6	+	-	-	
53	NR/DNP_6	+	-	-	
57	NR/DNP_6	+	-	-	-
	NR	+	-		-
70	NR	+	-		-

[a]Extracts demonstrated the ability to metabolize greater than 70% (+) or less than 20% (-) of the substrate provided. Extracts were prepared by DEAE cellulose gel filtration and metabolism of the various substrates was carried out under standard assay conditions.

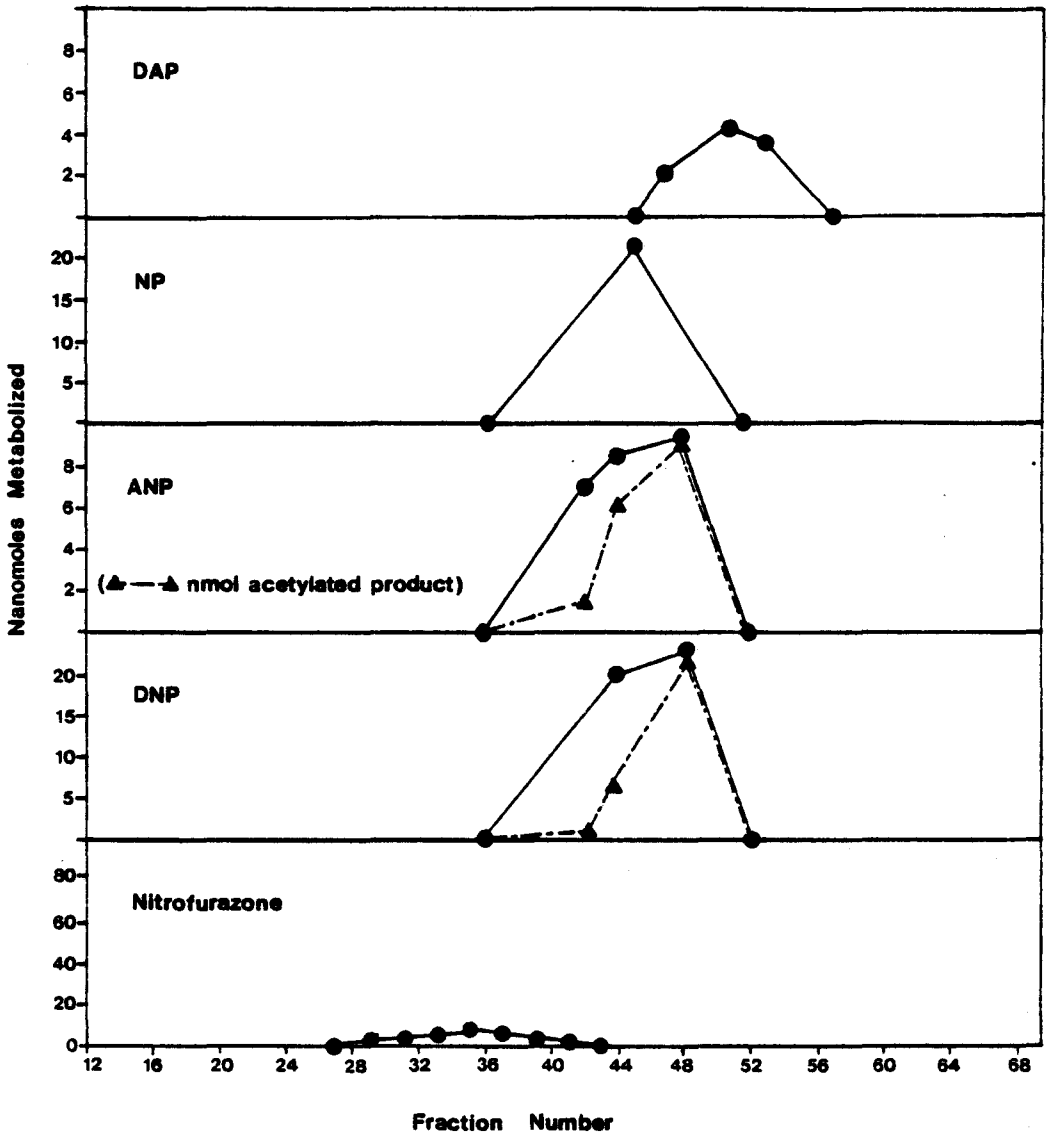
3.5.1 DEAE Sephacel Chromatoraphy

In an attempt to fractionate the different activities, crude extracts from strains TA98NR, DNP_6 and TA98NR/DNP_6 were fractionated by DEAE cellulose chromatography and the fractions tested for the ability to metabolize 1,8-diaminopyrene, 1-nitropyrene, 1-amino-8-nitropyrene and 1,8-diaminopyrene as well as nitrofurazone, the substrate used to measure "classical" nitroreductase activity (McCalla *et al.*, 1975).

The chromatographic profiles are shown in Figure 13 (TA98NR), Figure 14 (DNP_6) and figure 15 (TA98NR/DNP_6). These were from experiments in which NADH was present. Alternate fractions were tested concurrently with NADPH present and results were very similar. TA98NR extract (Figure 13) exhibited acetyltransferase activity in fractions 48 to 56 (top panel). 1-Nitropyrene, 1-amino-8-nitropyrene and 1,8-dinitropyrene metabolizing activity existed in fractions 42 to 50 (centre three panels). Interestingly, acetylated derivatives were detected in fractions 42 and 44 when 1-amino-8-nitro- or 1,8-dinitropyrene were the original substrates. These fractions showed no acetyltransfrase activity when 1,8-diaminopyrene was the original substrate. Again a difference between the acetylation of externally provided diaminopyrene versus that which was produced *in situ* was observed (see also results in Tables 3 and 4). The bottom panel indicates that the nitrofurazone-nitroreductase activity resided in fractions 29 to 41. Although there are

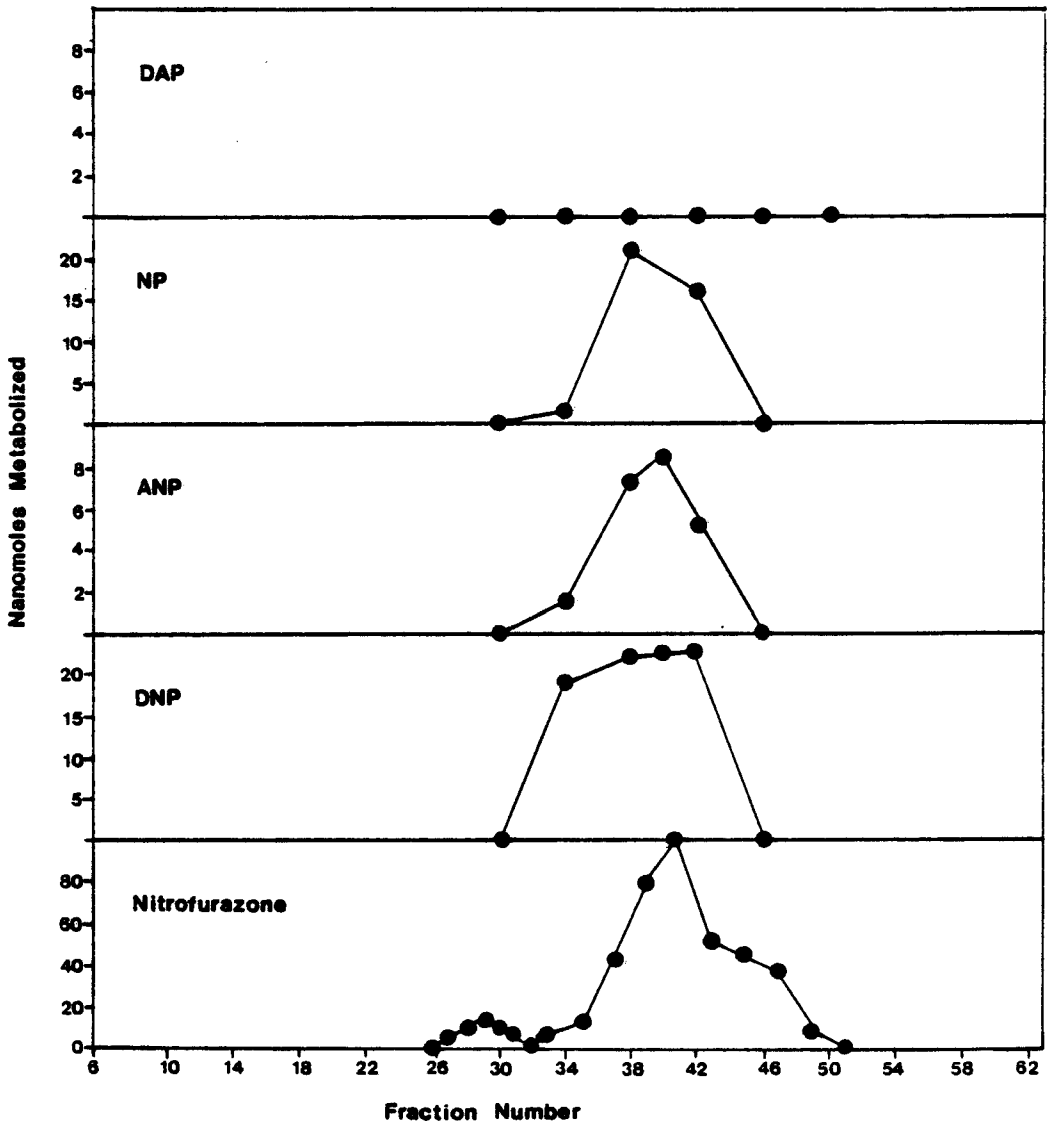
slight overlaps, it appears that at least three activities exist in TA98NR, those of acetyltransferase, nitropyrene nitroreductase and nitrofurazone nitroreductase. There are two obvious differences between the chromatographic profiles of TA98NR extract (Figure 13) and those of DNP_6 (figure 14). DNP_6 showed no acetyltransferase activity (top panel) as is expected in light of results previously presented. It also exhibited two peaks of nitrofurazone metabolizing activity, a minor peak which was also seen with TA98NR (Figure 13, bottom panel) and a major peak (Figure 14, bottom panel) which coincided with the nitropyrene metabolizing activities (Figure 14, centre three panels). Figure 15 shows a similar fractionation of extract from the double mutant (TA98NR/DNP_6) extract. As expected, neither acetyltransferase activity (top panel) nor the major nitrofurazone nitroreductase activity (bottom panel) was present. Although obviously different in some respects, the three mutant strains TA98NR, DNP_6 and TA98NR/DNP_6, all appeared to have identical abilities to metabolize 1-nitropyrene, 1-amino-8-nitropyrene and 1,8-dinitropyrene. The lack of the major nitroreductase in the two NR mutants (TA98NR and TA98NR/DNP_6) did not affect the nitropyrene nitroreductase activity indicating that these are two separate activities which happen to be eluted coincidentally from DEAE-sephacel under the conditions used in these experiments.

FIGURE 13: Fractionation of TA98NR Crude Extract by DEAE-Sephacel Chromatography



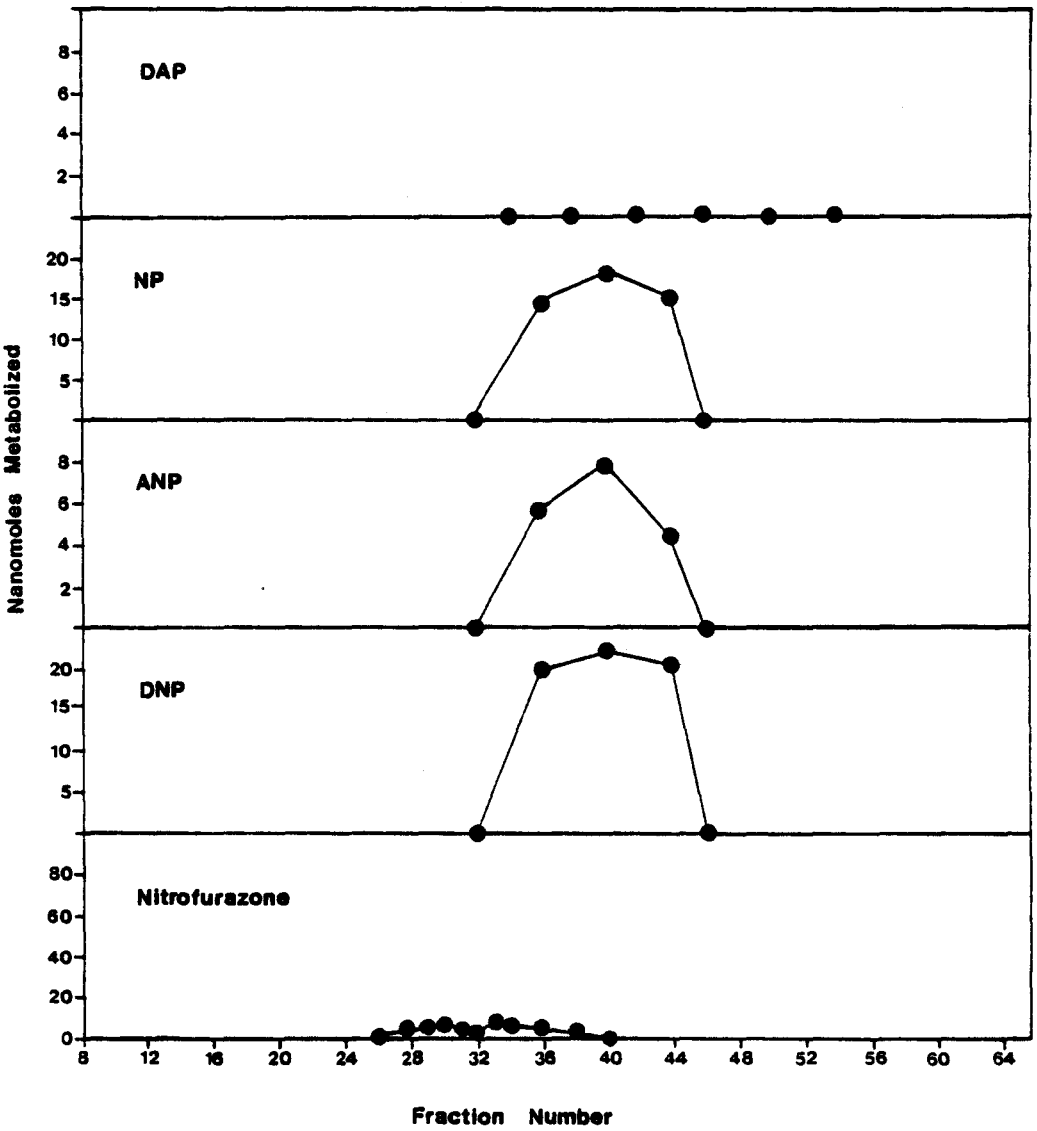
The ability of 1 ml fractions to metabolize a variety of substrates is shown. Extent of formation of acetylated products from 1-amino-8-nitropyrene (ANP) and 1,8-dinitropyrene (DNP) is indicated by broken lines (- —— -). Nanomoles of pyrene derivates metabolized were calculated from HPLC peak areas. Each point represents a separate overnight reaction mixture. Nanomoles of nitrofurazone metabolized were calculated based on results of a spectrophotometric assay for which the appearance of aminofurazone was monitored by observing the loss of absorbance at 375 nm.

FIGURE 14: Fractionation of DNP_6 Crude Extract by DEAE Sephacel Chromatography



The ability of 1 ml fractions to metabolize a variety of substrates is shown. Experimental details can be found in materials and methods and in the legend to Figure 13.

FIGURE 15: Fractionation of TA98NR/1,8-DNP$_6$ Crude Extract by DEAE Sephacel Chromatography



The ability of 1 ml fractions to metabolize a variety of substrates is shown. Experimental details can be found in materials and methods and in the legend to Figure 13.

3.5.2 Affi-Gel Blue Chromatography

A second chromatographic gel was employed in an attempt to separate acetyltransferase activity from nitroreductase activity. The medium used was Affi-gel blue, a derivative of blue dextran which specifically binds many pyridine nucleotide dependent enzymes (Thompson *et al.*, 1975), including nitrofurazone nitroreductase present in *E. coli* crude extract (Trus and McCalla, unpublished). This gel was used under the assumption that acetyltransferase (which is not a pyridine nucleotide dependent enzyme) would not be retained by the Affi-gel blue, but that nitroreductase would be reversibly bound thus allowing separation of these two activities. Initial experiments demonstrated a clear separation of activities when acetyltransferase activity was assayed by the TLC method using 1,8-diaminopyrene as the substrate and nitroreductase was assayed using the nitrofurazone standard assay (McCalla *et al.*, 1975). The results are shown in Table 7. Activity in the wash fraction represents that which was not retained by the Affi-gel blue. Activity in the eluent fraction represents that which was retained and subsequently eluted. The total nitroreductase eluted was much less than in the originally loaded crude extract. The reason for this is unknown and is irrelevant in light of the purpose of the experiment, *ie.* isolation of acetyltransferase from nitroreductase. Although there was a clear separation of activities, the relevance of this data is questionable in light of the evidence presented in Figures 13 to 15 which indicate

that the nitrofurazone assay does not test for the nitroreductase activity of interest, ie. nitropyrene nitroreductase. Results shown in Figure 13 also call into question the practice of using diaminopyrene as the starting substrate for the acetyltransferase assay since two fractions which were clearly able to produce acetylated products when 1,8-dinitropyrene was the original substrate that failed to do so when provided with diaminopyrene. On the basis of these findings, the Affi-gel blue experiments were repeated and the enzyme activites were assayed differently. 1-Nitropyrene, 1-amino-8-nitropyrene and 1,8-diaminopyrene were used as original substrates, instead of using the nitrofurazone assay (McCalla et al., 1975) to measure nitroreductase activity.

TABLE 7: AFFI-GEL BLUE CHROMATOGRAPHY OF TA98NR CRUDE EXTRACT

Experiment	Enzyme Activity	Activity in Crude	Activity in Wash[c]	Activity in Eluent[d]
18	nitroreductase[a]	6.8	-	0.16
20	"	6.8	-	0.31
26	"	6.6	-	0.24
18	acetyltransferase[b]	+	+	-
20	"	+	+	-
26	"	+	+	-

[a] Nitroreductase activity is expressed as nmol nitrofurazone reduced per minute per ml of extract.

[b] Acetyltransferase activity was assayed by TLC and described as present (+) or absent (-).

[c] The wash is that material which was not retained by the Affi-gel.

[d] The eluent is that material which was retained and subsequently eluted.

Metabolism of the various substrates was qualitatively determined using TLC. The results are shown in Table 8. These results differ from previous Affi-gel results, in that two acetyltransferase fractions were observed. One of these was separable from nitroreductase activity, and one was not. The acetyltransferase activity found in the eluent appears to be the same as that which was evident in fractions 42 and 44 (Figure 13) from the DEAE fractionation, since in both cases the activity was only observed when 1,8-dinitropyrene was the original substrate and not when 1,8-diaminopyrene itself was provided directly.

TABLE 8: AFFI-GEL BLUE CHROMATOGRAPHY OF TA98NR CRUDE EXTRACTS

Fraction #		Metabolites Detected[cd]			
Wash[a]	Eluent[b]	mono Ac (from DAP)	mono Ac (from DNP)	DAP (from DNP)	AP (from NP)
1		+	-	-	-
2		+	-	-	-
3		+	-	-	-
4		+	-	-	-
5		+	-	-	-
6		+	-	-	-
7		+	-	-	-
8		+	-	-	-
9		+	-	-	-
10		+	-	-	-
	1	-	-	-	-
	2	-	-	-	-
	3	-	+	+	+
	4	-	+	+	+
	5	-	+	+	+

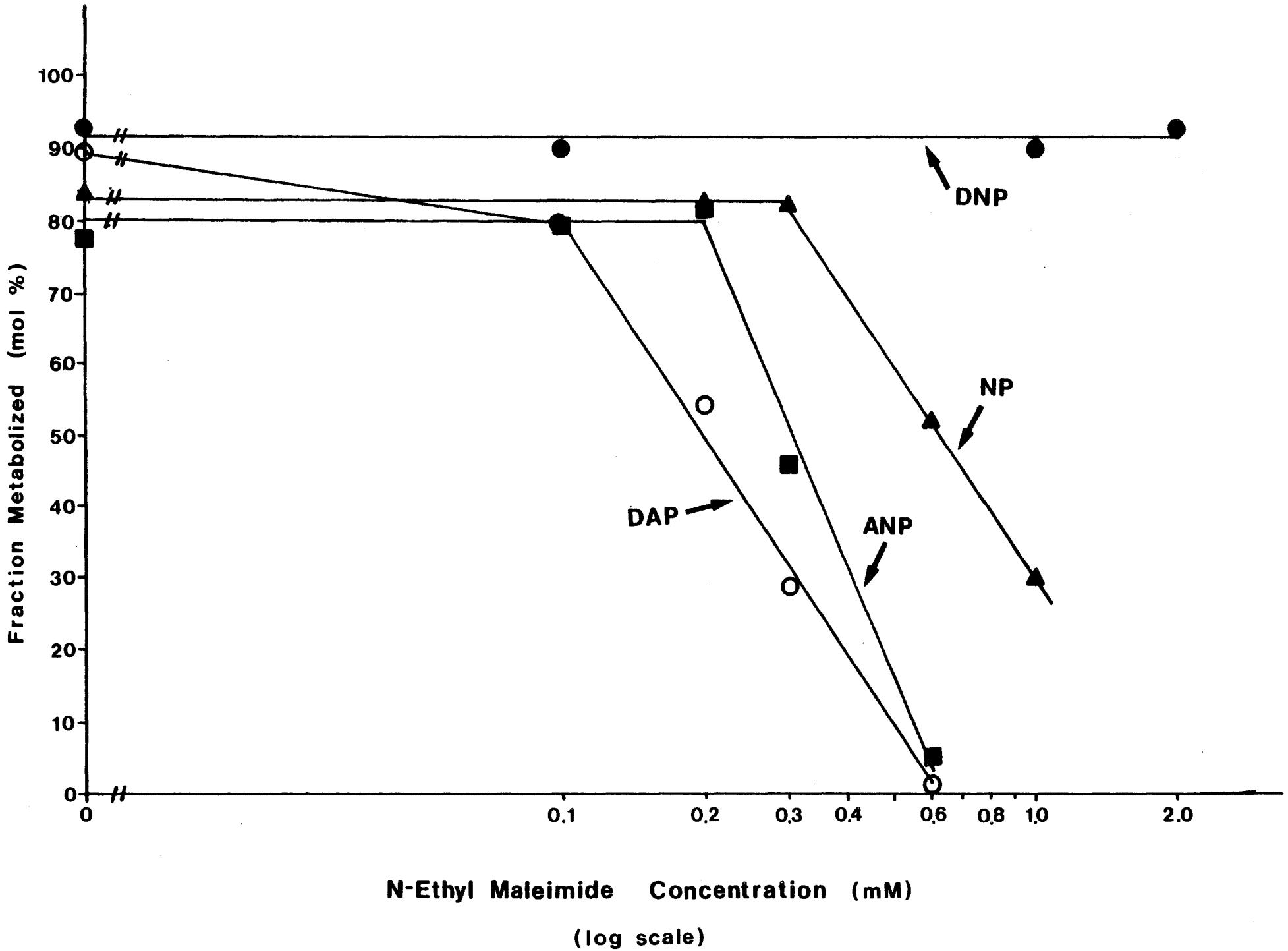
[a]The wash is that material which is not retained by the Affi-gel.
[b]The Eluent is that which is retained and subsequently eluted.
[c]The original substrate provided is indicated in brackets below the metabolites detected.
[d]Metabolites were detected by inspection of TLC plates and designated present (+) or absent (-).

3.6 Inhibition of Enzymatic Activity by N-Ethyl Maleimide

Both the gel chromatography methods demonsrated the ability to separate at least part of the acetyltransferase activity from the nitroreductase. The data shown in Table 6 provide some evidence of distinct nitroreductase components, one which metabolizes mononitro-, and one which metabolizes dinitro-compounds. Previously the results of several experiments in which bacterial extracts had somehow lost one or the other of these activities, but not both were presented (see Table 6 and accompanying description). Evidence to support the existence of two nitroreductases has been obtained using the sulfhydryl blocking agent N-ethyl maleimide. Figure 16 displays the effect of the increasing N-ethyl maleimide concentration on the metabolism of 1,8-diaminopyrene, 1-amino-8-nitropyrene, 1,8-dinitropyrene and 1-nitropyrene by DEAE treated TA98NR extract. The acetyltransferase activity as well as the mononitropyrene nitroreduction activities were clearly inhibited by the N-ethyl maleimide. The dinitropyrene nitroreductase activity on the other hand was not inhibited, even at an inhibitor concentration of 2 mM.

FIGURE 16: The Effect of N-Ethyl Maleimide on Metabolism of Various Substrates by TA98NR

The effect of increasing concentrations of N-ethyl maleimide on the ability of TA98NR batch DEAE chromatographed extract to metabolize 1,8-dinitropyrene (●), 1-nitropyrene (▲), 1-amino-8-nitropyrene (■) and 1,8-diaminopyrene (○). Percentages were calculated from peak areas from individual HPLC runs. The incubation time for all reactions was 1.5 hours.


100
90
80
70
60
50
40
30
20
10
0
Fraction Metabolized (mol %)
DNP
NP
ANP
DAP
0
0.1
0.2
0.3
0.4
0.6
0.8
1.0
2.0
N-Ethyl Maleimide Concentration (mM)
(log scale)

3.7. Ames Assays:

The lack of acetyltransferase activity in strain DNP_6 coincides with a significantly reduced sensitivity to mutagenesis by 1,8-dinitropyrene (see Table 2). Strain TA98NR possesses acetyltransferase activity, and is extremely sensitive to 1,8-dinitropyrene mutagenesis (see Table 2). Based on this, a series of Ames assays were performed using DNP_6 as the tester strain, 1,8-dinitropyrene as the mutagen and crude extract or partially purified crude extract from TA98NR as the external activitation system (instead of S9). It was predicted that a reactive intermediate could be generated outside the DNP_6 cells by the TA98NR extract and that this species would diffuse into the cells causing some increase in mutagenicity. The presence of the TA98NR extract did not enhance the mutagenicity of 1,8-dinitropyrene for DNP_6 in fact, it significantly reduced it (data not shown).

4. DISCUSSION

4.1. Acetyltransferase

It has been clearly demonstrated that S. typhimurium strain DNP_6 is lacking in the ability to N-acetylate 1,8-diaminopyrene. The inability of this strain to produce significant amounts of 1-N-acetylamino-8-aminopyrene and 1,8-N,N'-diacetyldiaminopyrene does not in itself explain the resistance to mutagenicity by the parent compund, 1,8-dinitropyrene since neither of these two N-acetylated metabolites is mutagenic (see Table 2). If the acetyltransferase plays a role in the activation of 1,8-dinitropyrene, it does not do so by producing N-acetylated derivatives. A more likely role for this enzyme is the production of a reactive acetoxy derivative through O-acetylation of a hydroxylamine (King and Glowinski, 1983). There is evidence to suggest that O-acetylation plays a part in the activation of the hydroxylamines 2-N-hydroxylaminofluorene (McCoy et al., 1982) and N-OH-Glu-P-1 (Saito et al., 1983; Saito et al., 1985; Shinohara et al., 1985). The latter studies are very convincing since N-OH-Glu-P-1 is a stable hydroxylamine and an assay has been developed to measure the rate of its O-acetylation (Saito et al., 1983). It has not been possible to develop such an assay to measure the acetylation of the 1-N-hydroxylamino-8-nitro derivative of 1,8-dinitropyrene since this compound is very unstable in aqueous solution (J. Fulton, personal communication). Speculation that 1-N-

acetyoxyamino-8-nitropyrene is the ultimate mutagenic derivative of 1,8-dinitropyrene is based largely on two observations: (1) that the hydroxylamino derivative generated *in vitro* from 1-amino-8-nitropyrene does not react spontaneously with DNA at neutral pH (P. Andrews *et al.*, 1985). which suggests the need for further activation, and (2) that the acetylase-deficient strain, DNP_6, is resistant to 1,8-dinitropyrene mutagenesis (McCoy *et al.*, 1983). Data presented here support the second of these, as far as proving that DNP_6 is indeed acetyltransferase deficient. This leads to the conclusion that the further activation of 1-N-hydroxylamino-8-nitropyrene may occur via acetyltransferase activity.

The major DNA adduct formed both *in vitro* and in intact bacteria retains one of the original nitro groups from 1,8-dinitropyrene (P. Andrews, 1985). Assuming that this adduct arose from reaction with an acetoxy derivative, this acetoxy derivative must have resulted from O-acetylation of 1-N-hydroxylamino-8-nitropyrene (see Figure 2). Such a reactive species would not be detectable by the HPLC analyses used in the studies reported here, but the analagous N-acetylated compound 1-N-acetylamino-8-nitropyrene is very stable and would be easily detected (see Figure 6, compound #4). This compound was never detected in reaction mixtures (see Figure 7) and therefore it was concluded that it was not produced. There are two possible explanations for the apparent

inability to produce 1-N-acetylamino-8-nitropyrene: (1) the presence of the second nitro group prevents 1-amino-8-nitropyrene from being a suitable substrate for the acetyltransferase or (2) the extreme electron withdrawing effect of the nitro group in this conjugated system effectively deactivates the amino group, preventing N-acetylation. If the first of these explanations is correct then it would be expected that 1-N-hydroxylamino-8-nitropyrene would be as unsuitable a substrate for O-acetylation as 1-amino-8-nitropyrene is for N-acetylation, thereby ruling out the participation of acetyltransferase activity in the activation of 1,8-dinitropyrene. If the second explanation is correct, then O-acetylation may be possible where N-acetylation is not, since the tenacity of the oxygen atom for its lone pair of electrons may somewhat override the deactivating effect of the electron withdrawing nitro group, permitting formation of the acetoxy derivative. Although insufficient evidence exists to prove or disprove either of these suggestions, the second explanation is favoured since it explains the presence of a nitro group on the major adduct and does not contradict evidence that implicates acetylation as an activating process.

It has been observed that the pattern of acetylated metabolites produced from 1,8-diaminopyrene by DEAE treated extracts of TA98NR was dependent on the source of the 1,8-diaminopyrene, ie, externally provided as the original substrate, or produced in situ from its precursor 1-amino-8-

nitropyrene. When 1,8-diaminopyrene was provided as the original substrate there was less total metabolism and more diacetylated product formed (Table 3) than when 1,8-dinitropyrene was the original substrate. Recent fluorimetric evidence has shown that 1,8-diaminopyrene molecules stack together probably forming dimers above a critical concentration of monomer (B.E. McCarry, personal communication). If the dimer was not a suitable substrate for the acetyltransferase then acetylation efficiency would be greater when only a small amount of 1,8-diaminopyrene was available at a time (as when it was being enzymatically produced from 1-amino-8-nitropyrene). It is evident from Figures 9 and 10 that indeed 1,8-diaminopyrene did not accumulate to high levels in reaction mixtures. While possibly explaining the difference in extent of total metabolism, the dimer theory does not explain why so very little diacetylated product is formed when 1,8-dinitropyrene is the original substrate, even when a large amount of monoacetylated product is formed (compare results in Tables 3 and 4). The phenomenon exhibited in Figure 13 is also unexplained by the dimer theory. Here the concentration of 1,8-diaminopyrene was sixteen times lower than that used to generate the data shown in Table 3, so the extent of dimer formation should be significantly lower. Fractions 42 and 44, both of which exhibited acetyltransferase activity when 1,8-dinitropyrene or 1-amino-8-nitropyrene were provided as the original substrates, showed no such activity when 1,8-diaminopyrene was provided. Frac-

tion 48 metabolized 100% of the 1,8-diaminopyrene produced from either 1,8-dinitro- or 1-amino-8-nitropyrene but this fraction (by interpolation) metabolized only 30% of the diaminopyrene provided as the substrate. A possible explanation may be that there are two different acetyltransferase enzymes, one which operates independently, and one which is complexed with a nitroreductase so that any diaminopyrene produced remains bound rather than being released into solution, and is thus shunted straight to the acetyltransferase. This idea is supported by the fact that diaminopyrene did not accumulate in reaction mixtures, but was rapidly and efficiently acetylated (Figures 9 and 10). It seems quite reasonable to presume that an organism such as Salmonella would have a number of acetyltransferases, of which more than one may be capable of the non-specific acetylation of arylamines. Further support comes from the Affi-gel blue experiments in which one acetyltransferase activity was found to separate from nitroreductase activity and another was inseparable from nitroreductase. The separable activity metabolized 1,8-diaminopyrene as provided, but the inseparable activity only produced acetylated diaminopyrene metabolites when 1,8-dinitropyrene was provided as the substrate (see Table 8). It is suspected that the nitroreductase and acetyltransferase activities are not separated because they exist as a complex. There is not enough evidence to allow speculation about whether the separable acetyltransferase has diffused from the complex, or whether it is an entirely different enzyme, but the lack of both separable and insepa-

rable activity in strain DNP_6 suggests that they may actually be the same enzyme.

4.2 Nitroreductase

Before evidence became available which indicated that DNP_6 may be lacking acetyltransferase activity, it had been suggested that its resistance to 1,8-dinitropyrene mutagenesis was due to lack of an essential nitroreductase (Rosenkranz et al., 1982). The postulation of multiple nitroreductases was one possible explanation for the pattern of mutagenesis observed with the various strains (ibid.) Support for such an idea comes from Figures 13 to 15. The "classical" nitroreductase present in strain DNP_6 (Figure 14) appears to have no effect on the metabolism of the nitropyrenes, since it is absent in strain TA98NR (Figure 13) and in the double mutant (Figure 15) and yet all three strains showed identical abilities to metabolize the nitropyrenes. It is concluded from these data that the nitrofurazone-nitroreductase is a distinct enzyme from nitropyrene nitroreductase and the fact that it elutes from the DEAE gel at the same place is coincidental. Evidence from studies with Bacteriodes fragilis also supports the idea that there are several different nitroreductases capable of nitroarene metabolism (Kinouchi and Ohnishi, 1983). Four distinct nitroreductases were discovered, and numbered I, II, III and IV. Nitroreductase I is highly specific for mononitropyrenes, III

for dinitropyrenes, IV for 4-nitroquinoline-1-oxide and II has broad specificity. Substrate specificities were determined by competition experiments. Separate nitroreductases responsible for the metabolism of 1-nitropyrene and 4-nitroquinoline-1-oxide have also been implicated by mutagenicity studies with a mutant yeast strain (McCoy et al., 1984). Table 6 shows results from a set of experiments in which the ability to metabolize one substrate but not another was demonstrated. Closer evaluation of these results reveals that in all but one case, an extract which metabolized 1,8-dinitropyrene did not metabolize the mononitropyrenes 1-nitropyrene and 1-amino-8-nitropyrene, or vice versa. These results suggest that there may be individual nitroreductases specific for mono- and dinitropyrenes. It was further shown that the reduction of mononitropyrenes can be inhibited with N-ethyl maleimide whereas the dinitropyrene reduction is not affected (Figure 16). Acetyltransferase activity was also sensitive to N-ethyl maleimide. These results strongly suggest that two distinct nitroreductases exist, one specific for mononitro- and one specific for dinitropyrene. This possibility is supported by the results of Kinouchi and Ohnishi (1983) which have been previously described. These workers also reported inhibition of nitropyrene nitroreductase (designated enzyme I) by *p*-chloromercuribenzoic acid which, like N-ethyl maleimide, is a sulfhydryl blocker. Inhibition of acetyltransferase activity in TA98 extract by N-ethyl maleimide has also been reported by other workers (Saito et al., 1985). A second line of evi-

dence supports the hypothesis that mono- and dinitropyrene specific nitroreductases exist. This comes from polarographic studies in which the number of electrons required to reduce 1-nitropyrene, 1,3-dinitropyrene, 1,6-dinitropyrene and 1,8-dinitropyrene was determined (Rozenkranz, unpublished). The following table summarizes the data:

TABLE 9: NUMBER OF ELECTRONS REQUIRED TO REDUCE VARIOUS NITROPYRENES

Compound	Number of Electrons		Relative Mutagenicity	
	1st reduction	2nd reduction	TA98	TA98NR
1-NP	1	0	high	low
1,3-DNP	1	1	high	low
1,6-DNP	2	1	high	high
1,8-DNP	2	1	high	high

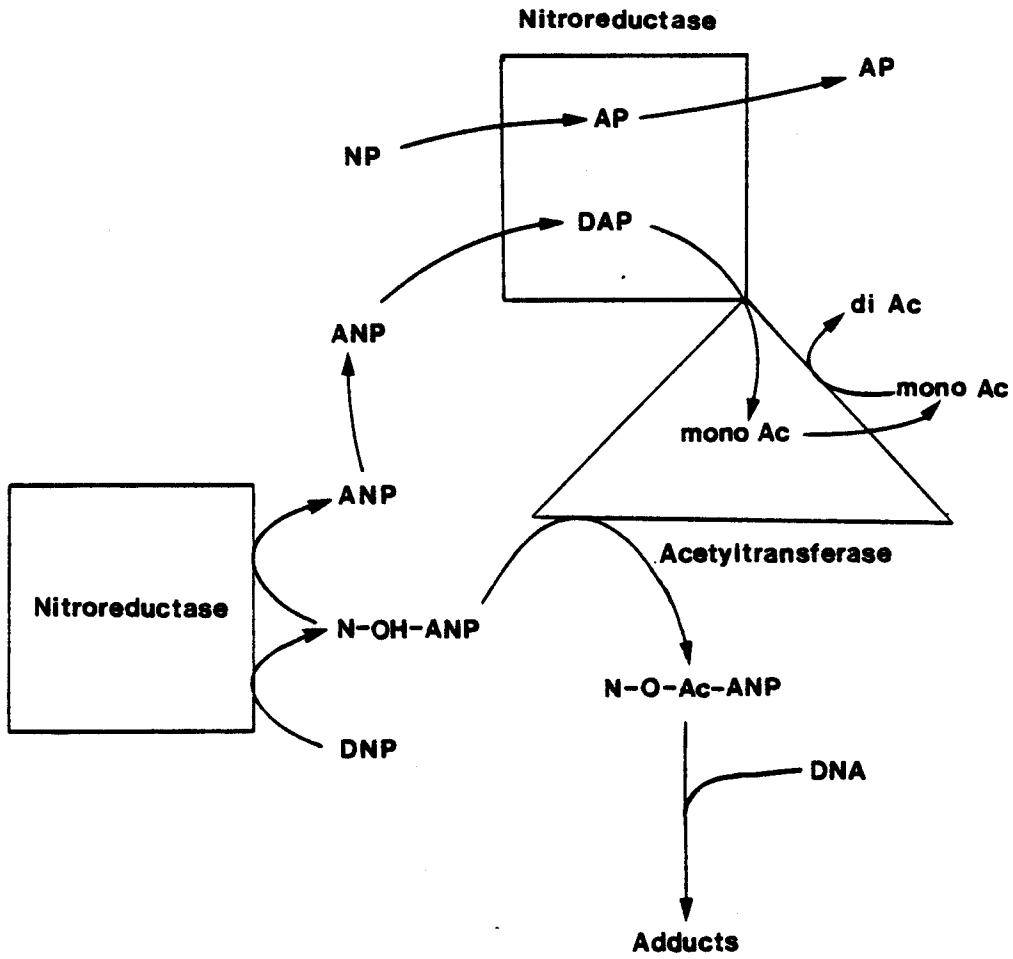
According to Rosenkranz's interpretation of the data, the 1 electron reductions require the "classical" nitroreductase (i.e., nitrofurazone nitroreductase) and the 2 electron reductions require some other nitroreductase. While data presented herein do not implicate the "classical" nitroreductase in the metabolism of 1-nitropyrene, they do support the idea that 1,8-dinitropyrene is metabolized by a distinct nitroreductase from that which metabolizes 1-nitropyrene.

When the extent of metabolism of 1-nitropyrene, 1-amino-8-nitropyrene and 1,8-dinitropyrene are compared across the three strains TA98NR, DNP_6 AND TA98NR/DNP_6 (Figures 13-15), it appears as though all are identical. No apparent difference in nitroreductase activity is observed although only strain DNP_6 possesses nitrofurazone nitroreductase (Fig. 14). This is surprising when the mutagenicity data is considered (Table 2). Strains TA98NR and TA98NR/DNP_6 are resistant to 1-nitropyrene mutagenesis, yet they metabolize this compound just as well as does the sensitive strain DNP_6. This observation leads to the conclusion that the metabolism being measured here is not directly relevant to the production of a mutagenic intermediate from 1-nitropyrene. It is possible that a nitroreducatase exists which reduces the nitro function directly to an amino group without the intermediate release of a hydroxylamine, thus eliminating the possibility of DNA binding. Perhaps the "classical" nitrofurazone nitroreductase which is missing in strain TA98NR is capable of metabolizing 1-nitropyrene to a mutagenic intermediate, but another nitroreductase is also present which reduces the nitro to an amino group without hydroxylamine release.

4.3. A Theoretical Model

Based on the data presented here, a model is proposed to explain the relationship between nitroreductase and transacetylase and their roles in the metabolism of 1-nitropyrene and 1,8-dinitropyrene (Figure 17). This model is based on

FIGURE 17: Model to Explain Metabolism of Pyrene Derivatives in TA98NR



Closed shapes represent the enzymes. Metabolites shown outside shapes are free in solution, those inside are enzyme-bound. Arrows touching or entering the shapes indicate reactions which occur on the enzyme. Arrows outside the shapes indicate diffusion, or non-enzymatic reactions.

strain TA98NR, in which little activating metabolism of 1-nitropyrene occurs. It takes into account the apparent presence of two nitroreductase enzymes (one specific for mono- and one for dinitropyrenes), and the link between nitroreductase and transacetylase. This model can be used to explain why 1,8-diaminopyrene never accumulates, and why dinitropyrene is activated to a hydroxylamine and 1-nitropyrene is not. The decreased mutagenicity of 1-nitroso-8-nitropyrene relative to 1,8-dinitropyrene can also be explained if the former is treated as a mononitro compound.

4.4. Summary

In summary, this work has resulted in (1) the development of a qualitative TLC assay for acetyltransferase activity; (2) the identification of 1,8-N,N'-diacetyldiaminopyrene as a metabolite of 1,8-diaminopyrene; (3) proof that Salmonella tymphimurium TA98/1,8-DNP_6 lacks acetyltransferase activity; (4) circumstantial evidence for a nitroreductase-acetyltransferase complex; (5) evidence for the existence of both a mononitropyrene-specific and a dinitropyrene-specific nitroreductase; and (6) the development of a model which attempts to explain the data presented.

4.5. Predictions And Further Work

Based on this model it is predicted that the dinitropyrene nitroreductase should be separable from the mononitropyrene nitroreductase-transacetylase complex. This might be accomplished through chromatography on an affinity gel which presents free nitropyrene groups to the enzyme. The dinitropyrene specific enzymes would be expected to pass through the gel, while the mononitropyrene specific nitroreductase is retained. Molecular exclusion gel chromatography may also separate these enzymes if their molecular weights are substantially different. If the nitroreductase-acetylase complex is stable enough to withstand such treatment, then a large molecular weight difference would be expected. If the complex is unstable, then isoelectric focussing under non-denaturing conditions should separate the mononitropyrene nitroreductase from the acetyltransferase. For example, applying this procedure to fractions 42 to 46 (Figure 13), which do not acetylate diaminopyrene unless it is produced *in situ*, may free the acetyltransferase, producing a band in the gel which is capable of acetylation of exogenous diaminopyrene and devoid of nitreductase activity.

4.6. Conclusions

It has been demonstrated that the metabolism of 1,8-dinitropyrene by *S. tymphimurium* is a complicated process, involving both nitroreduction and subsequent acetylation.

Some insight into the roles of these two processes in the activation of 1,8-dinitropyrene to an active intermediate has been gained through the studies presented here. Additional work of this type may eventually lead to a complete understanding of the biochemistry involved in chemical carcinogenesis of nitroaromatics.

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