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Metabolic activation of the genotoxic environmental contaminants 2- and 3-nitrofluoranthene in variants of *Salmonella typhimurium* TA98

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The mutagenic environmental pollutants 2-nitrofluoranthene (2-NFA) and 3-nitrofluoranthene (3-NFA), labelled with ^3H and ^{14}C respectively, were incubated with *Salmonella typhimurium* strain TA98, its nitroreductase-deficient variant TA98NR and its *O*-acetytransferase-deficient variant TA98/1,8-DNP$_6$, to investigate the activity of these metabolic pathways under conditions approximating those of the Ames assay, hence their contribution to mutagenic potency. 2-Aminofluoranthene (2-AFA) was the major metabolite of 2-NFA (4 μM) in all three TA98 variants, isolated by reverse-phase HPLC and identified by UV-vis and NMR spectroscopy and mass spectrometry. 2-AFA was formed more slowly in TA98NR (65 pmol/h/ml resting phase bacterial broth, 1 to 2×10^9 bacteria/ml) than in TA98 (295 pmol/h/ml) or TA98/1,8-DNP$_6$ (82 pmol/h/ml). 2-Acetamidofluoranthene (2-AAFA) was also identified in incubations with TA98 (80 pmol/h/ml), TA98NR (21 pmol/h/ml), and TA98/1,8-DNP$_6$ (8 pmol/h/ml). 3-Aminofluoranthene (3-AFA, confirmed by UV-vis and NMR spectroscopy and mass spectrometry) was formed by all three variants from 3-NFA (4 μM): TA98, 1.76 nmol/h/ml; TA98NR, 0.55 nmol/h/ml; TA98/1,8-DNP$_6$, 2.93 nmol/h/ml. 3-Acetamidofluoranthene (3-AAFA) was not detected in any of the variants. 3-AFA and 3-AAFA were less mutagenic than 3-NFA, and required S9 for activation. Mutagenicity of 3-NFA relative to initial nitroreduction rate was similar in TA98 and in TA98NR, but almost 10-fold lower in TA98/1,8-DNP$_6$; hence *O*-acetylation considerably enhances the mutagenicity of reduction products of 3-NFA. Mutagenicity of 2-NFA relative to initial nitroreduction rate was similar in TA98 and in TA98/1,8-DNP$_6$; the bacterial genotoxicity of 2-NFA is therefore largely independent of *O*-acetyltransferase activity. Ratios of mutagenicity to nitroreduction rate were similar in TA98 for 2-NFA and 3-NFA; differences in the potency of these isomers arise primarily from their respective suitabilities as substrates for nitroreductase enzymes.

Introduction

Nitrated polycyclic aromatic hydrocarbons (nitroPAH) are widespread environmental contaminants; many compounds of this class exhibit remarkable mutagenicity in the Salmonella plate incorporation assay. 3-Nitrofluoranthene (3NFA; Figure 1) is found in diesel emission particulate matter and other combustion emissions (Gibson, 1982; Schuetzle *et al.*, 1982), where it is formed from direct nitration of fluoranthene (Streitweiser and Fahey, 1962; Zielinska *et al.*, 1987). 2-Nitrofluoranthene (2NFA; Figure 1) occurs on ambient air particulate matter rather than in direct combustion emissions; this isomer arises from attack of fluoranthene by hydroxyl radicals followed by atmospheric nitration (Pitts *et al.*, 1985; Zielinska *et al.*, 1986).

3-NFA is a highly potent bacterial mutagen (Greibrokk *et al.*, 1985; Zielinska *et al.*, 1988; Shane *et al.*, 1991; van Haeringen *et al.*, 1993) and also an animal carcinogen (Ohgaki *et al.*, 1982). 2-NFA is less active than 3-NFA in the Ames assay (Zielinska *et al.*, 1987), but preliminary results suggest tumorigenic activity in the newborn mouse (Herreno-Saenz *et al.*, 1994). 2-NFA can account for up to 5% of the direct-acting bacterial mutagenicity of ambient particulate matter (Arey *et al.*, 1988). The presence of these compounds in the environment is therefore a matter for concern, though the health implications have not been clearly defined as yet. Better information on the metabolic pathways followed by these compounds will permit evaluation of potential routes of activation in target organs and species, hence a more rational assessment of their genotoxicity and health impact.

Nitroreduction constitutes a primary route of genotoxic activation for nitrated aromatic compounds. This process is catalyzed by a variety of enzymes, both bacterial and mammalian, which differ in their mechanisms of action and their sensitivity to oxygen (Peterson *et al.*, 1979). Other enzymes such as acetyltransferases may also participate in the activation of nitro- and polynitroPAH (McCoy *et al.*, 1983; Orr *et al.*, 1985; Djuric *et al.*, 1985). In this study we attempt to define more closely the bacterial enzymes involved in the activation of nitrofluoranthenes, by making use of *Salmonella typhimurium* strains deficient in defined enzymes, in order to assess the contribution made by these different pathways to the mutagenicity of the two NFA isomers.

Salmonella typhimurium strain TA98 is historically the most responsive of the Ames tester strains towards nitroPAH. The variant TA98NR is deficient in the so-called 'classical' nitroreductase enzyme (Rosenkranz and Speck, 1975; McCoy *et al.*, 1981), and the variant TA98/1,8-DNP$_6$ is deficient in

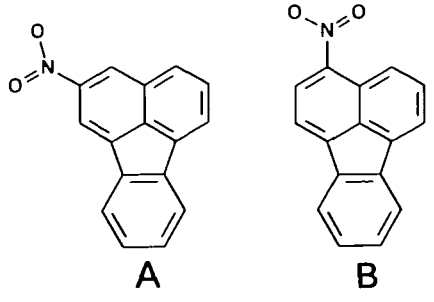

Fig. 1. Structure of 2-nitrofluoranthene (**A**) and 3-nitrofluoranthene (**B**).

one or more of the enzymes involved in transfer of acetyl groups (McCoy *et al.*, 1981, 1983; Orr *et al.*, 1985; Saito *et al.*, 1985). The absence of acetyl-CoA:*N*-hydroxyarylamine *O*-acetyltransferase activity in TA98/1,8-DNP_6, and its presence in TA98, have been explicitly documented by partial purification of the enzyme (Saito *et al.*, 1985). Sequencing of the *O*-acetyltransferase gene in a TA1538/1,8-DNP_6 strain derived by elimination of plasmid pKM101 from TA98/1,8-DNP_6 (Watanabe *et al.*, 1990) demonstrated the presence of a –1 frameshift deletion, resulting in a new termination codon (Watanabe *et al.*, 1992). We report identification and quantitation of the major metabolites formed in these TA98 variants and relate the extent of metabolism to the mutagenicity observed in the variants. We also use the TA98 variants to examine the mutagenicity of the reduced and acetylated derivatives of 3-NFA; since acetylation represents a major route of metabolism for nitroPAH *in vivo* (Ball *et al.*, 1984a; Upadhyaya *et al.*, 1992, 1994), information on the activity of these derivatives may be helpful in assessing the genotoxic potential of the nitroPAH in the intact animal.

Materials and methods

Chemicals

3-Nitro[3-^{14}C]fluoranthene (sp. act. 13.8 mCi/mmol, radiochemical purity >99.9%) and unlabelled 3-NFA were synthesized and characterized by the Midwest Research Institute, Kansas City, MO, USA. 3-Aminofluoranthene (3-AFA) was purchased from Aldrich Chemical Co., Milwaukee, WI,USA, and recrystallized from aqueous ethanol to yield yellow flakes, melting point 107–108°C (lit. 113–115°C, Campbell *et al.*, 1951). 3-Acetamidofluoranthene (3-AAFA) was prepared by acetylation of 3-AFA with acetic anhydride and recrystallized from aqueous ethanol to give orange–yellow needles, melting point 243.5–245°C (lit. 244–245°C, Campbell *et al.*, 1951). The last two compounds were judged to be >99.5% pure by reverse-phase HPLC analysis, as described below. Mass spectrometry (MS) and proton nuclear magnetic resonance (NMR) spectra were consistent with the assigned structures.

2-Nitro[G-^{3}H]fluoranthene (sp. act. 439 mCi/mmole, radiochemical purity >99%) was synthesized by Chemsyn Science Laboratories (Lenexa, KS, USA) and diluted 1:10 or 1:100 with unlabelled 2-NFA (see below) as required. 2-Nitrofluoranthene (2-NFA; CAS no. 13177-29-2) was synthesized from 3-aminofluoranthene (Aldrich Chemical Co., Milwaukee, WI, USA) according to a procedure from the literature (Kloetzel *et al.*, 1955). The product was chromatographed on open-bed silica gel (eluted with benzene) and recrystallized repeatedly from glacial acetic acid until yellow needles were obtained with melting point 149.5–150°C (lit. 153–153.5°C; Kloetzel *et al.*, 1955). Exact mass was 247.0636 (nominal mass 247.0633, Δ 0.3 p.p.m.). Purity was verified by HPLC (>99%) as described below.

2-Aminofluoranthene (2-AFA) was obtained from 2-NFA (1 mg) by reduction with Zn dust/NH_4Cl (1 and 4 mg, respectively), and fluoresced yellow under long-wave (365 nm) UV light. Identity was confirmed by MS [*m/z* (relative abundance): 218 (19%) $[^{13}C\text{-}M]^+$; 217 (100%) $[M]^+$; 200 (6%) $[M\text{-}NH_3]^+$; 189 (23%) $[M\text{-}CNH_2]^+$.

Acetylation of 2-AFA with acetic anhydride yielded 2-acetamidofluoranthene (2-AAFA), which fluoresced blue under long-wave UV light. Proton NMR and MS data were consistent with the assigned structure. [^{1}H-NMR at 250 MHz, δ p.p.m. (multiplicity, assignment): 8.33 (d, 1H, $\underline{H}_1$, $J_{1,3}$ = 1.0); 8.23 (d, 1H, $\underline{H}_3$, $J_{3,1}$ = 1.0); 8.01–7.92(m, 2H, $\underline{H}_{10}$ and $\underline{H}_7$); 7.97 (d, 1H, $\underline{H}_6$, $J_{6,5}$ = 7.0); 7.83 (d, 1H, $\underline{H}_4$, $J_{4,5}$ = 8.2); 7.64 (t, 1H, $\underline{H}_5$); 7.42–7.38 (m, 2H, $\underline{H}_8$ and $\underline{H}_9$); MS, *m/z* (relative abundance): 259 (43%) $[M]^+$; 218 (20%) $[M\text{-}COCH_2]^+$; 217 (100%) $[M\text{-}COCH_3]^+$; 189 (28%) $[M\text{-}CNHCOCH_3]^+$].

HPLC-grade solvents were purchased from Burdick and Jackson, Muskegon, MI, USA and Fisher Scientific, Raleigh, NC, USA. All other chemicals and solvents were purchased from commercial sources, Sigma Chemical Co., St Louis, MO, USA Aldrich Chemical Co. and Fisher Scientific, at the highest degree of purity available.

Mutagenicity assays

The *S.typhimurium* plate incorporation assay was carried out as described (Ames *et al.*, 1975), with the following minor modifications: minimal histidine was added to the base agar rather than to the soft agar overlay, and the number of histidine-prototrophic revertants was counted (Model 800 Colony Counter, Artek Inc., Farmingdale, NY, USA) after 72 rather than 48 h (Claxton *et al.*, 1982). *Salmonella typhimurium* strain TA98 and its variants were from the stock of the Genetic Toxicology Branch, HERL, US Environmental Protection Agency, Research Triangle Park, NC, USA. Variants TA98NR and TA98/1,8-DNP_6 were originally obtained from Dr H.S.Rosenkranz, Case Western Reserve University, Cleveland, OH, USA. Bacteria were maintained on master-plates and tested for genetic markers (Claxton *et al.*, 1982); 0.1 ml of overnight bacterial broth was used per assay plate. The S9 fraction (9000 *g* supernatant) used to provide metabolic activation was prepared from the livers of Aroclor-1254-treated male CD-1 rats (Charles River, Wilmington, MA, USA), stored at –70°C until used, and supplemented with an NADPH-generating co-factor mix as previously described (Ames *et al.*, 1975). Protein content was assayed with bovine serum albumin as reference (Lowry *et al.*, 1951). The amount of S9 protein added (1.2–1.5 mg per plate) was determined by optimization with benzo[*a*]pyrene and 2-aminoanthracene for every batch of S9 prepared. Each compound was assayed on at least three separate occasions, at doses ranging from 0.01 to 10 or 20 μg per plate, both with and without exogenous metabolic activation (S9), up to the dose level at which toxicity (as manifested by decrease in revertant counts) was evident. Compounds were freshly dissolved in DMSO immediately before each assay. Positive controls were included in each experiment. Specific mutagenicity was calculated by least-squares linear regression from the linear portion of the dose–response curve.

Determination of metabolite profiles and kinetics

Overnight (16 h) cultures of bacteria from each of *S.typhimurium* variants TA98, TA98NR and TA98/1,8-DNP_6 ($1–2\times10^9$ organisms/ml, 20 ml) in Oxoid no. 2 broth (25 g/l, pH 7.5, Oxoid Inc., Red Road, MD,USA) were incubated with 3-nitro[3-^{14}C]fluoranthene or 2-nitro[G-^{3}H]fluoranthene diluted 1:10 with unlabelled 2-NFA (8 μl of 10 mM nitroPAH in DMSO; ^{14}C, 2.4×10^6 dpm per culture, ^{3}H, 7.8×10^6 d.p.m. per culture) in sealed glass screw-capped vials with minimal head space (~1 ml) at 37°C in a dry-air orbital shaker. Separate reactions were stopped, at 0.5, 1, 2, 3 and 6 h for both nitroPAH, and at 24 h for 2-NFA only, by addition of 1 vol. of ethyl acetate/acetone 2:1 v/v, and extracted with a further 2×1 vol. of the same solvent, for a total of 60 ml. Five additional 20 ml cultures of TA98 were incubated with 40 μM 2-NFA (2-nitro[G-^{3}H]fluoranthene diluted 1:100 with unlabelled 2-NFA) for 24 h, in order to generate sufficient mass of metabolites for structural analysis. Control incubations consisted of 20 ml of nutrient broth with 4 μM 3-nitro[3-^{14}C]fluoranthene for 6 h or 4 μM 2-nitro[G-^{3}H]fluoranthene for 24 h, similarly extracted. All experiments were carried out in duplicate. The extracts were evaporated to dryness under reduced pressure (Rotavapor rotary evaporator, Büchi, Flawil, Switzerland).

Dried extracts were resuspended in MeOH (10 ml), filtered to remove insoluble material and evaporated to dryness under a stream of nitrogen gas. Recovery was monitored at each step by liquid scintillation counting (LSC; Wallac Rack-Beta Model 1217 liquid scintillation counter, LKB Instruments, Bromma, Sweden) of 2×0.1 ml samples of each extract in Scintiverse E (10 ml; Fisher Scientific, Fairlawn, NJ, USA). Where necessary, samples were stored at –60°C. The dried extracts were redissolved in MeOH (1 ml) and analysed by HPLC on a 4.6×250 mm Zorbax ODS column (Dupont Instrument Co., Wilmington, DE, USA). 3-NFA and its metabolites were eluted at 1 ml/min for 3 min at 70% MeOH in H_2O then with a linear gradient from 70% MeOH in H_2O to 100% MeOH over 15 min. For analysis of 2-NFA, the solvent (1 ml/min) was held initially at 70% MeOH in H_2O for 20 min, followed by a gradient to 100% MeOH over 5 min. The eluate was monitored for UV absorbance at 254 nm and collected in 30-s fractions (Model Retriever III fraction collector, ISCO, Lincoln, NE, USA) for determination of its ^{14}C or ^{3}H content by LSC. For large-scale isolation of metabolites, the same gradients were used with a semi-preparative Zorbax ODS column (9.4×250 mm) eluted at 4 ml/min. Metabolite fractions were collected manually based on UV absorbance peaks. The fractions were dried by evaporation under a stream of nitrogen gas and stored at –60°C.

Instrumentation

Proton NMR spectra were recorded in acetone-d_6 (99.5 atom%, Aldrich Chemical Co., Milwaukee, WI, USA) at 250 MHz (Model WM-250, Brucker Instrument Co., Billerica, MA, USA) or 400 MHz (Model XL-400, Varian Instrument Co, Palo Alto, CA, USA). Mass spectra were obtained in the direct probe insertion–electron impact ionization mode (70 eV) on a VG7070H or a VG70-250SEQ (Vacuum Generators, Altrincham, Cheshire, UK) mass spectrometer. The probe temperature was ramped ballistically from ~30 to 300°C. Melting points were determined on a Hoover capillary melting point apparatus (A.H. Thomas Co., Philadelphia, PA, USA) and are uncorrected. HPLC was performed on a Varian Model 5000 instrument (Varian Instrument Co., Palo Alto, CA, USA) equipped with a Zorbax ODS 4.6×250 mm (analytical) or 9.4×250 mm (semi-preparative) column (Dupont Instrument Co., Wilmington, DE, USA), and a Model LC-85B spectrophotometric detector (Perkin-Elmer, Norwalk, CT, USA), which also served to acquire UV spectra (from 250 to 450 nm) in the stopped flow mode.

Results

Mutagenicity of 3-nitrofluoranthene and derivatives

Table I summarizes the mutagenicity of 3-NFA, its reduction product 3-AFA, and the acetylated derivative of the latter, 3-AAFA, in *S.typhimurium* strain TA98 and in its variants TA98NR and TA98/1,8-DNP_6. Overall, little toxicity was seen except for 3-NFA without S9 at 0.5 μg/plate and above. As previously reported (Greibrokk *et al.*, 1985) 3-NFA is a highly potent direct-acting mutagen for strain TA98, producing about 2500 revertants/nmole. Its activity was decreased 10-fold to 244 revertants/nmole by the presence of S9. These results are well within the range of other reports: 7700 revertants/nmole (Zielinska *et al.*, 1988); 5982 revertants/nmole without S9, 590 revertants/nmole with S9 (Shane *et al.*, 1991); 1420 revertants/nmole (van Haeringen *et al.*, 1993). Activity in TA98NR was one-third of this level, and even less in TA98/1,8-DNP_6. The presence of S9 further decreased mutagenicity in both these variants.

3-AFA and 3-AAFA were both much less active than 3-NFA and, in contrast, more potent in the presence than the absence of S9. 3-AFA exhibited minimal direct activity (relative to the potency of 3-NFA), consistent with the results of Van Haeringen *et al.* (1993), but 3-AAFA (overall half as active as 3-AFA) was entirely S9-dependent. For both these compounds, activity in the presence of S9 was similar in TA98NR and in TA98, as would be expected since nitroreduction would play no role here. Mutagenicity was much lower in TA98/1,8-DNP_6, also pointing to involvement of acetyltransferase in activation of S9-generated intermediates.

Bacterial metabolism of 3-nitrofluoranthene

When incubated at a dose (4 μM, 1 μg/ml of overnight culture) selected to represent a substrate-to-bacteria ratio (0.1 μg/plate

Table I. Mutagenicity of 3-nitrofluoranthene and its reduced metabolites in the plate incorporation assay

Dose (μg/plate)	Activity in *S typhimurium* strain[a]					
	TA98		TA98NR		TA98/1,8-DNP_6	
	−S9	+S9	−S9	+S9	−S9	+S9
Positive control	320 ± 24[b]	536 ± 24[c]	53 ± 10[b]	578 ± 64[c]	74 ± 21[b]	648 ± 182[d]
DMSO alone	41 ± 4	59 ± 3	18 ± 2	44 ± 4	24 ± 4	33 ± 5
3-Nitrofluoranthene						
0.005	112 ± 8	58 ± 8	35 ± 4	46 ± 6	24 ± 6	35 ± 5
0.01	186 ± 11	58 ± 6	49 ± 36	44 ± 3	33 ± 4	30 ± 4
0.05	810 ± 55	87 ± 7	191 ± 23	61 ± 6	84 ± 8	33 ± 6
0.1	1,671 ± 63	118 ± 7	339 ± 27	69 ± 6	150 ± 12	37 ± 7
0.3	3,030 ± 98	255 ± 23	929 ± 63	137 ± 14	526 ± 56	49 ± 8
0.5	3,265 ± 191	460 ± 29	1,620 ± 74	247 ± 31	976 ± 84	57 ± 11
1.0	3,055 ± 10	1,067 ± 21	–	–	–	–
rev/nmole[e]	2,469 ± 271	244 ± 13	781 ± 11	97 ± 6	467 ± 17	13 ± 1
r^2	⩾0.95 (0.3)	⩾0.98 (1.0)	⩾0.99 (0.5)	⩾0.98 (0.5)	⩾0.99 (0.5)	⩾0.97 (0.5)
3-Aminofluoranthene						
0.05	40 ± 4	52 ± 5	–	–	–	–
0.1	70 ± 19	57 ± 4	54 ± 5	58 ± 5	25 ± 7	36 ± 7
0.3	109 ± 13	70 ± 5	–	–	–	–
0.5	224 ± 99	163 ± 16	146 ± 13	155 ± 18	36 ± 7	55 ± 9
1.0	256 ± 91	359 ± 56	389 ± 60	515 ± 94	38 ± 6	109 ± 21
3.0	450 ± 239	2,060 ± 240	441 ± 51	2,094 ± 241	56 ± 11	512 ± 101
5.0	535 ± 282	2,774 ± 138	675 ± 110	2,881 ± 84	62 ± 6	1,031 ± 177
10.0	421 ± 270	3,377 ± 138	683 ± 129	3,125 ± 90	67 ± 9	2,043 ± 273
rev/nmole[e]	30 ± 4	127 ± 7	26 ± 5	132 ± 9	2 ± 0.2	45 ± 1
r^2	⩾0.91 (5)	⩾0.98 (5)	⩾0.88 (5)	⩾0.98 (5)	⩾0.92 (1)	⩾0.99 (1)
3-Acetamidofluoranthene						
0.1	52 ± 15	67 ± 7	28 ± 4	48 ± 5	26 ± 6	34 ± 7
0.3	39 ± 5	81 ± 3	–	–	–	–
0.5	34 ± 7	122 ± 17	26 ± 3	98 ± 7	24 ± 6	42 ± 5
1.0	36 ± 6	183 ± 25	30 ± 4	162 ± 16	22 ± 4	58 ± 24
3.0	40 ± 10	722 ± 26	24 ± 6	602 ± 63	19 ± 5	128 ± 33
5.0	40 ± 6	1,377 ± 48	25 ± 3	1,639 ± 374	21 ± 4	263 ± 45
10.0	38 ± 3	2,611 ± 106	21 ± 3	2,522 ± 202	23 ± 5	655 ± 76
rev/nmole[e]	0	67 ± 1	0	68 ± 5	0	16 ± 1
r^2	–	⩾0.99 (1)	–	⩾0.97 (5)	–	⩾0.97 (10)

[a]Activity is expressed as His^+ revertants/plate (mean ± S.E.M.) determined with the Salmonella plate incorporation assay (Ames *et al.*, 1975) in triplicate on three separate occasions.
[b]2-Nitrofluorene, 3 μg/plate.
[c]2-Anthramine, 0.5 μg/plate.
[d]2-Anthramine, 3.0 μg/plate.
[e]Specific mutagenicity, expressed as His^+ revertants/nmole, was calculated by least squares linear regression from the linear portion of the dose–response curve. Correlation coefficients r^2 and the limits of linearity (μg/plate) in parentheses are also known.

to 0.1 ml of overnight culture/plate) well within the linear portion of the mutagenicity dose–response curve so as to minimize toxicity, with oxygen limited but not excluded, to approximate the conditions under which the Ames plate incorporation assay is conducted, 3-NFA was rapidly and extensively metabolized by strains TA98 and TA98/1,8-DNP_6, less rapidly by TA98NR (Figure 2). At all time-points >95 % of the total radioactivity could be extracted into the organic phase (TA98, 94.6 ± 4.0 %; TA98NR, 103.1 ± 9.0% and TA98/1,8-DNP_6, 100.0 ± 3.6%). No time-related trend in extractibility was observed in any of the three variants. No transformation or decomposition was noted in the control incubations.

The peak eluting at 20.5 min was confirmed as 3-NFA by UV-vis spectroscopy and MS identity with authentic compound [MS, *m/z*, relative intensity: 249 (8%) $[^{14}CM]^+$; 248 (5%) $[^{13}C]M^+$; 247 (26%) M^+; 219 (44%) $[^{14}CM\text{-}NO]^+$; 218 (35%) $[^{13}CM\text{-}NO]^+$; 217 (100%) $[M\text{-}NO]^+$; 189 (49%) $[M\text{-}CNO_2]^+$. The major metabolic product was identified as

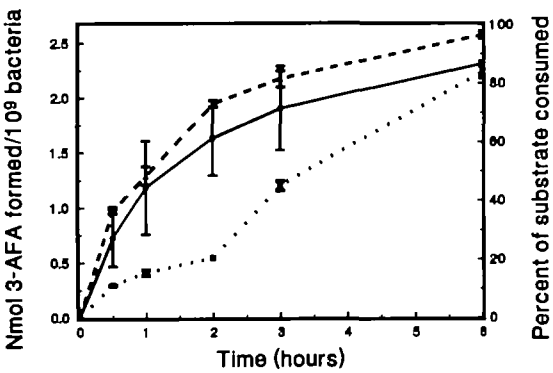

Fig. 2. Time-course of transformation of 3-NFA by *Salmonella typhimurium* TA98 (—●—) and its variants TA98NR (··■··) and TA98/1,8-DNP_6 (–▲–). The values shown are the means ± SEM of duplicate experiments.

3-AFA by comparison of its HPLC retention time (R_T, 12 min) with that of the standard synthetic compound, by UV spectroscopy, by ^{1}H-NMR at 400 MHz (δ p.p.m., multiplicity, assignment: 8.16, d, 1H, $\underline{H}_4$, $J_{4.5}$ = 8.1 Hz; 8.06, d, 1H, $\underline{H}_6$, $J_{6.5}$ = 6.8 Hz; 7.95, broad d, 1H, $\underline{H}_7$, $J_{7.8}$ = 7.4 Hz; 7.81, broad d, 1H, $\underline{H}_{10}$, $J_{10.9}$ = 7.4 Hz; 7.78, d, 1H, $\underline{H}_1$, $J_{1.2}$ = 7.5 Hz; 7.58, t, 1H, $\underline{H}_5$, $J_{5.4}$ = 8.2 Hz, $J_{5.6}$ = 6.9 Hz; 7.31, dt, 1H, $\underline{H}_9$, $J_{9.8}$ = 7.5 Hz, $J_{9.10}$ = 7.5 Hz, $J_{9.7}$ = 1.1 Hz; 7.22, dt, 1H, $\underline{H}_8$, $J_{8.9}$ = 7.5 Hz, $J_{8.7}$ = 7.4 Hz, $J_{8.10}$ = 1.0 Hz; 6.80, d, 1H, $\underline{H}_2$, $J_{2.1}$ = 7.6 Hz) and by MS [*m/z*, relative intensity: 219 (40%, $[^{14}CM]^+$); 218 (28%, $[^{13}CM]^+$); 217 (100%, $[M]^+$); 189 (34%, $[M\text{-}CNH_2]^+$)]. No 3-AAFA (R_T 13.5 min, clearly separated from 3-AFA) was detected (<0.1 % conversion) in any of the strains of bacteria; 3-AFA is apparently a poor substrate for bacterial *N*-acetyltransferase activity, as was 1-amino-8-nitropyrene (Orr *et al.*, 1985).

Conversion in TA98NR was slower than in TA98, and remained linear with time to 6 h, but the extent of nitroreduction at 24 h was the same as in TA98 (~90%). Kinetics in TA98/1,8-DNP_6 were initially more rapid than observed in TA98, then slowed down as substrate was used up. Again, by 6 h conversion to 3-AFA was almost complete. Initial rates of reduction are shown in Table III, along with ratios of direct-acting mutagenicity to initial reduction rates. Mutagenic potency bears the same quantitative relationship to nitroreduction in TA98NR as in TA98, but is substantially lower relative to initial rate of reduction in the *O*-acetyltransferase-deficient variant, since TA98/1,8-DNP is as competent in nitroreduction with respect to 3-NFA as TA98 itself. This implies an essential role for *O*-acetyltransferase activity in potentiating mutagenicity of the reduction products of 3-NFA.

Mutagenicity of 2-nitrofluoranthene

2-NFA induced reversion to histidine prototrophy in all three TA98 variants (Table II). In accord with previous estimates (Pitts *et al.*, 1985), the overall level of activity of 2-NFA (around 350 revertants/nmole) was comparable to that reported

Table II. Mutagenicity of 2-nitrofluoranthene in the Ames plate incorporation assay

Dose (μg/plate)	Activity in *S.typhimurium* strain[a]					
	TA98		TA98NR		TA98/1,8-DNP_6	
	−S9	+S9	−S9	+S9	−S9	+S9
0	37 ± 14	52 ± 9	31 ± 9	44 ± 8	24 ± 9	35 ± 10
0.05	155 ± 27	54 ± 5	30 ± 3	36 ± 3	27 ± 3	22 ± 7
0.1	261 ± 41	58 ± 11	28 ± 1	43 ± 3	48 ± 9	22 ± 3
0.3	599 ± 50	95 ± 28	89 ± 41	58 ± 13	136 ± 36	39 ± 8
1.0	1,539 ± 107	422 ± 139	168 ± 108	130 ± 29	379 ± 100	46 ± 6
3.0	1,809 ± 204	1,671 ± 423	235 ± 129	730 ± 328	451 ± 134	99 ± 17
5.0	1,846 ± 133	2,239 ± 416	233 ± 119	1,230 ± 488	421 ± 91	170 ± 35
10.0	1,758 ± 82	1,837 ± 522	233 ± 118	1,885 ± 567	383 ± 89	448 ± 89
13.0	–	–	293 ± 117	2,178 ± 295	464 ± 86	650 ± 76
15.0	–	–	295 ± 103	2,221 ± 387	382 ± 41	818 ± 75
20.0	–	–	281 ± 109	2,152 ± 542	346 ± 40	1,050 ± 58
Positive control	363 ± 43[b]	488 ± 171[c]	50 ± 13[b]	436 ± 34[c]	66 ± 14[b]	598 ± 77[d]
rev/nmole[e]	362 ± 24	116 ± 23	43 ± 25	45 ± 15	87 ± 22	11 ± 2
r^2	≥0.99 (1)	≥0.96 (5)	≥0.99 (1)	≥0.97 (15)	≥0.99 (1)	≥0.97 (20)

[a]Mutagenicity is expressed as His^+ revertants per plate (mean ± SD), determined by the Ames plate incorporation assay [(Ames *et al.*, 1975) and text] in duplicate on four separate occasions.
[b]2-Nitrofluorene, 3 μg/plate.
[c]2-Anthramine, 0.5 μg/plate.
[d]2-Anthramine, 3.0 μg/plate.
[e]Calculated by least-squares linear regression from the linear portion of the dose–response curve, with limits of linearity (in μg/plate) in parentheses.

Table III. Initial rates of nitroreduction of 2- and 3-nitrofluoranthene, compared to mutagenicity in variants of *S.typhimurium* TA98

	Salmonella strain:	TA98	TA98NR	TA98/1,8-DNP$_6$
3-NFA:	Formation of 3-AFA[a]	1,761 ± 915 (1)	551 ± 17 (6)	2,933 ± 125 (0.5)
	Ratio of mutagenicity/nitroreduction[b]	14.0	14.2	1.6
2-NFA:	Formation of 2-AFA[c]	295 ± 28 (3)	64.5 ± 34.5 (1)	82.3 ± 29.5 (2)
	Formation of 2-AAFA	80.3 ± 18.4 (3)	20.6 ± 13.4 (24)	8.4 1.0 (2)
	Ratio of mutagenicity/nitroreduction	12.3	6.6	10.5

[a]Nitroreduction is expressed in pmol/h/ml of bacterial broth ± S.E.M., with linearity in h in parentheses.
[b]Revertant yield determined in the absence of S9, expressed in rev/nmol nitroPAH for 0.1 ml of broth per pmol nitroPAH reduced/h/0.1 ml of broth.
[c]Includes material present as 2-AAFA.

for 1-nitropyrene (1-NP; Mermelstein *et al.*, 1981; Ball *et al.*, 1984b), and considerably lower than that of 3-NFA. Other published reports of the mutagenicity of 2-NFA without S9 range from 1030 revertants/nmole (Zielinska *et al.*, 1987) to 100 revertants/nmole (van Haeringen *et al.*, 1993) and 61 revertants/nmole (Shane *et al.*, 1991). Addition of S9 decreased this activity by about two-thirds. In comparison, activity in the presence of S9 has been variously reported as only one-third less than (Zielinska *et al.*, 1987), or approximately equivalent to (Shane *et al.*, 1991), the activity without S9.

Differences in pattern of activity were notable between the different TA98 variants. Mutagenicity was decreased by three-quarters in TA98/1,8-DNP$_6$, and by almost an order of magnitude in TA98NR. This is in general agreement with Zielinska *et al.* (1987), who reported one-fifth the activity of TA98 with variants TA98NR and TA98/1,8-DNP$_6$, while Shane *et al.* (1991) detected at most 5 revertants/nmole in these two variants. Contrary to prior observation with 1-NP (Ball *et al.*, 1984b), addition of S9 did not restore mutagenic activity in TA98NR. The presence of S9 generally decreased responsiveness at low doses, but prolonged linearity of the dose–response curve to higher dose levels. Accordingly, higher absolute plate counts were achieved at high doses with TA98NR and TA98/1,8-DNP$_6$ in the presence than in the absence of S9.

Bacterial metabolism of 2-nitrofluoranthene

Under the same incubation conditions, overall metabolism of 2-NFA was slower than that of 3-NFA, and complete consumption of substrate required a longer time, so that incubations were continued to 24 h. No transformation or decomposition was noted in the control incubations. Aqueous (non-extractable) radioactivity increased with time from <1 % at 0.5 and 1 h to ~20% at 6 h in all three variants, and decreased to <10% at 24 h. This material was not further analysed. Both 2-AFA (R_T 13 min; MS: 217 (100%, $[M]^+$); 200 (15%, $[M\text{-}NH_3]^+$; 189 (39%, $[M\text{-}CNH_2]^+$)]; and ^{1}H-NMR at 400 MHz [δ p.p.m., multiplicity, assignment: 7.94–7.88, m, 2H, $\underline{H}_8$ and $\underline{H}_9$; 7.72, d, 1H, $\underline{H}_6$, $J_{6,5}$ = 6.7 Hz; 7.59, d, 1H, $\underline{H}_1$, $J_{1,3}$ = 1.6 Hz; 7.58, d, 1H, $\underline{H}_4$, $J_{4,5}$ = 8 Hz; 7.49, t, 1H, $\underline{H}_5$, $J_{5,4}$ = 8.1 Hz, $J_{5,6}$ = 6.7 Hz; 7.38–7.34, m, 2H, $\underline{H}_8$ and $\underline{H}_9$; 6.99, d, 1H, $\underline{H}_3$, $J_{3,1}$ = 1.6 Hz]) and 2-AAFA (R_T 15 min; MS, *m/z* (relative intensity): 259 (44%) $[M]^+$; 218 (19%) $[M\text{-}COCH_2]^+$ and $[^{13}CM\text{-}COCH^3]^+$; 217 (100%) $[M\text{-}COCH_3]^+$; 189 (28%) $[M\text{-}CNHCOCH_3]^+$]) were formed. Unchanged 2-NFA (R_T 29 min) was identified by UV-vis spectroscopy and mass spectrometry [*m/z* (relative intensity): 248 (23%, $[^{13}CM]^+$); 247 (80%, $[M]^+$); 217 (28%, $[M\text{-}NO]^+$); 201 (100%, $[M\text{-}NO_2]^+$); 200 (70%, $[M\text{-}HNO_2]^+$); 189 (35%, $[M\text{-}CNO_2]^+$)].

Formation of 2-AFA in TA98 was linear with respect to time for the first 3 h, then fell away (Figure 3). Ultimate conversion was >90%, including the fraction present as 2-AAFA. Production of 2-AAFA in TA98 followed a similar time-course. Nitroreduction started out slowly in variant TA98NR, but at some point after 6 h accelerated so that total conversion at 24 h was ~85%. In this variant, production of 2-AAFA was approximately linear with respect to time throughout. The initial rate of nitroreduction in TA98/1,8-DNP$_6$ was intermediate between that of TA98 and TA98NR, then accelerated after 4 h. Percentage conversion at 24 h was also ~85%. This amount includes <5% as 2-AAFA, whereas 2-AAFA represented 8 and 12% of the radioactivity in the other two variants. Detection of even low levels of an acetylated product in the DNP$_6$ strain is unexpected, since here the *O*-acetyltransferase enzyme (also responsible for *N*-acetyltrans-

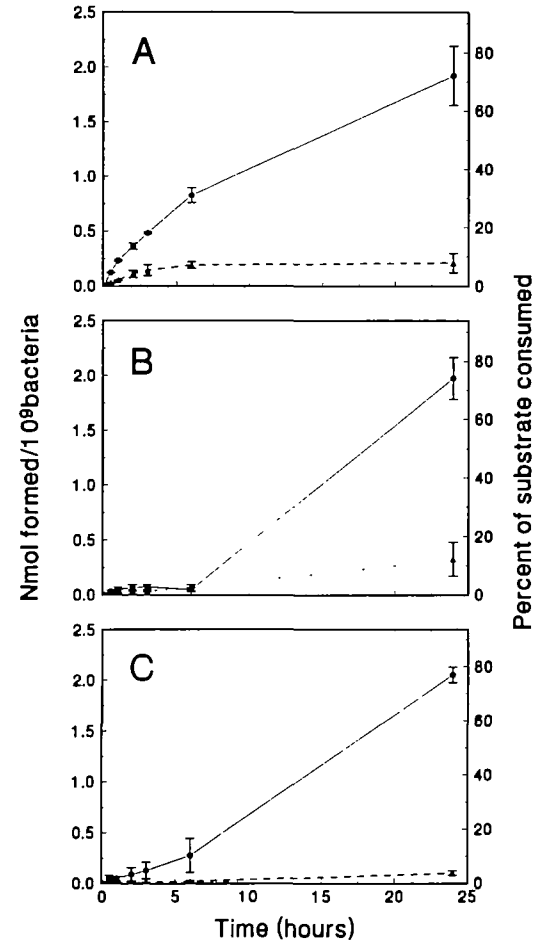

Fig. 3. Time-course of transformation of 2-NFA by *S.typhimurium* TA98 (**A**) and its variants TA98NR (**B**) and TA98/1,8-DNP$_6$ (**C**). Solid lines indicate material present as 2-AFA (—●—) and dashes indicate 2-AAFA (--▲--). The values shown are the means ± SEM of duplicate experiments.

ferase activity) is rendered non-functional by the presence of a termination codon in its coding gene (Watanabe *et al.*, 1992).

Initial rates of conversion to 2-AFA are shown in Table III, along with ratios of direct-acting mutagenicity to reduction rates. In contrast to 3-NFA, the mutagenic yield of nitroreduction was similar in TA98 and in TA98/1,8-DNP_6, indicating that *O*-acetyltransferase is not a strong requirement for activation of 2-NFA. The decrease in mutagenic yield in TA98NR may not be meaningful considering the variability in the low mutagenicity and very low reaction rate measurements.

Discussion

Reduction of 3-NFA either chemically or *in vitro* by the mammalian nitroreductase enzyme xanthine oxidase in the presence of calf thymus DNA gives rise to *N*-(deoxyguanosin-8-yl)-3-aminofluoranthene as the major nucleoside adduct (Dietrich *et al.*, 1988). An *N*-(deoxyguanosin-8-yl)-2-aminofluoranthene adduct has been characterized in xanthene oxidase- and rat-liver-microsome-mediated reductions of 2-NFA in the presence of calf-thymus DNA (Herreno-Saenz *et al.*, 1992), and also in *S.typhimurium* TA98 and neonatal $B6C3F_1$ mice (Herreno-Saenz *et al.*, 1994), although structural identification has not yet been confirmed by independent chemical synthesis. Though deamination of cytosine in conjunction with ascorbic acid-catalyzed reduction of another nitroPAH, 1-NP has been reported *in vitro* (Malia and Basu, 1994), the implications for bacterial mutagenesis are not as yet clear. For both NFA isomers the *N*-(deoxyguanosin-8-yl) adduct ostensibly formed by nitroreduction would thus appear to be the major DNA lesion involved in genotoxic activity. The results of the present studies indicate that the two NFA isomers follow different pathways to formation of analogous adducts.

In Figure 4, generic activation routes for nitroPAH and their metabolites are outlined, illustrated by 3-NFA. Steps *6–4* and *8–9* require exogenous S9. All others except for steps *9–7* (*N,O*-transacetylation; Kato and Yamazoe, 1988) are believed to be active in TA98. In addition, steps *1– 4* are defective in TA98NR, and steps *4–7* are absent in TA98/1,8-DNP_6 (Saito *et al.*, 1985). Our observation that initial rate of formation of 2-AAFA is lower in TA98/1,8-DNP_6 than in TA98 or TA98NR indicates that steps *6–8*, *N*-acetylation, are impaired but not inactive in this variant. It is also apparent that 3-AFA does not readily undergo *N*-acetylation, in contrast to 2-AFA (this study) and 1-aminopyrene (Messier *et al.*, 1981). Diminished mutagenicity in variant TA98/1,8-DNP_6 implies that activation of 3-NFA proceeds more efficiently through intermediate *7*, *N*-acetoxy-3-amino-fluoranthene, than by the direct route *4–5*, consistent with the greater leaving efficiency of the acetoxy group. The action of S9 in each variant is to detoxify the 3-NFA, whether by metabolism, protein binding or other sequestration mechanism. The low direct-acting potency of 3-AFA, enhanced by the presence of S9 in all three variants and higher in the acetyltransferase-competent strains than in TA98/1,8-DNP_6, is consistent with a requirement for S9-mediated formation of hydroxylamine, *4*, which is then activated via *7* more readily than by direct loss of hydroxide.

The acetylated product 3-AAFA is the weakest mutagen considered in this series, in contrast to the analogous derivative of 1-NP, 1-acetamidopyrene, which in the presence of S9 is more active than 1-NP (Ball *et al.*, 1984b). 3-AAFA requires S9 for *N*-hydroxylation to the hydroxamic acid *9*; deacetylation to 3-AFA may also occur. The decrease in activity of 3-AAFA

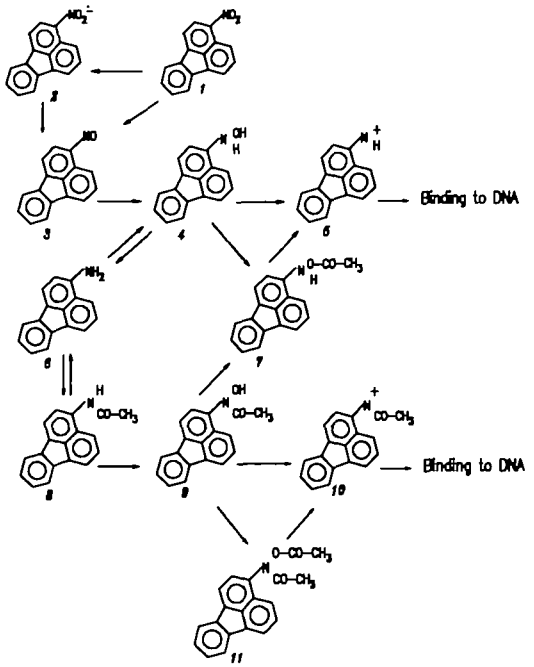

Fig. 4. Bacterial metabolic activation routes of nitrofluoranthenes, illustrated for 3-nitrofluoranthene (*1*). One-electron reduction forms the nitro anion radical (*2*), and a second single-electron reduction gives 3-nitrosofluoranthene (*3*), which is also produced by direct two-electron reduction catalyzed by oxygen-insensitive nitroreductase. A second two-electron reduction yields 3-hydroxylaminofluoranthene (*4*). The hydroxylamine can form an electrophilic arylnitrenium ion (*5*) that reacts with nucleophilic sites on macromolecules, including DNA, or can undergo activation via *O*-acetylation (*7*). The hydroxylamine can also be further reduced to 3-aminofluoranthene (*6*); this step may be reversed by oxidation catalyzed by cytochrome P450. *N*-acetylation converts *6* to 3-acetamidofluoranthene (*8*). Oxidation of *8* produces a hydroxamic acid, *N*-hydroxy-*N*-3-acetamidofluoranthene (*9*), that can form an electrophilic *N*-acetylarylnitrenium ion (*10*) or undergo *N,O*-acetyl transfer to an ester, *N*-acetoxy-3-aminofluoranthene (*7*), which itself readily yields the arylnitrenium ion *5*. *O*-acetylation of *9* is an alternative route to the *N*-acetylarylnitrenium ion (*10*) via the acetoxyacetamidofluoranthene *11*.

in TA98/1,8-DNP_6 relative to the other two variants is greater than that of 3-AFA. Possible explanations include more facile *N*-hydroxylation of 3-AFA than of 3-AAFA therefore simply greater flux through potentially activating pathways, a greater dependence on activation via *O*-acetyltransferase by the hydroxamic acid than by the hydroxylamine, that the arylnitrenium ion *4* is intrinsically more stable or a stronger electrophile than the *N*-acetylnitrenium ion *10*, or that activation of 3-AAFA in fact proceeds through deacetylation to 3-AFA. Identification of (hitherto unreported) acetamide-derived DNA adducts, and quantitation relative to amine-derived adducts following incubation with 3-AAFA would provide useful information on this point.

The mutagenicity of 2-NFA in the Ames assay is of a similar order of magnitude to that of 1-NP, but the overall pattern of activity is quite different from that of 1-NP (Ball *et al.*, 1984b). The presence of S9 does not decrease activity in TA98 as much as for 3-NFA, indicating either very little interference or binding by the S9, or the presence of alternative microsomal activation steps. The drop in activity in strain TA98NR relative to TA98, and the proportionality observed between mutagenicity and nitroreduction in all three variants, indicate that the majority of 2-NFA's activity is directly due

to nitroreduction by the bacterial reductase enzyme. The slight drop in activity relative to nitroreduction in TA98/1,8-DNP_6 may reflect a small contribution by *O*-acetyltransferase. Initial net nitroreduction rates were also decreased in TA98/1,8-DNP_6, whereas reduction of 3-NFA was enhanced in this variant. The substrate specificities of nitroreductases in the TA98 variants may therefore need further exploration. In variant TA98NR, and to a less marked extent in TA98/1,8-DNP_6, nitroreductase activity accelerated at some point between 6 and 24 h. While bacterial regrowth or induction of a latent form of nitroreductase in the presence of substrate cannot be excluded, enhanced product formation by an oxygen-sensitive nitroreductase, as the incubation medium becomes more anaerobic overnight, seems more plausible.

Howard *et al.* (1987) postulate a specific 2-electron nitroreductase pathway, active in TA98NR as well as TA98, which is tightly coupled to the *O*-acetylation step and which exhibits definite substrate preferences. If 3-NFA were a good substrate for this enzyme, such a pathway would be consistent with the elevated activity of 3-NFA in TA98, and the smaller decrease in mutagenicity in TA98NR, compared to 1-NP which appears not to be a good substrate. This hypothesis does not adequately explain why the numerical relationship between total nitroreduction and mutagenicity of 3-NFA in TA98NR, where virtually all nitroreduction would pass through the tighly-coupled pathway, is similar to that seen in TA98, where the majority of nitroreduction presumably goes through the reductase pathway not coupled to esterification.

The 'mutagenic yield' of 2-NFA nitroreduction is very similar to that of 3-NFA in TA98 and considerably higher than 3-NFA in TA98/1,8-DNP_6, so that even though *N*-hydroxy-2-AFA apparently does not go through the activity-enhancing *O*-acetylation step, it yields an arylnitrenium ion intrinsically as potent as that from *N*-acetoxy-3-AFA. This could be explained by enhanced ability of the 2-isomer to stabilize the arylnitrenium ion by *ortho* delocalization (Vance and Levin, 1984). Overall mutagenicity of 2-NFA would thus be limited by the rate of formation of hydroxylamine through nitroreduction rather than by the rate of *O*-acetylation of hydroxylamine as in the case of 3-NFA. Thus differences in mutagenic potency between these isomers must stem primarily from their respective abilities to serve as substrates for nitroreductase, consistent with an earlier correlation of mutagenicity with ease of electrochemical reduction (Klopman *et al.*, 1984), and secondarily from the reactivity of the arylnitrenium ion formed.

Whereas 3-NFA is activated via *O*-acetyltransferase and 3-AFA is not readily acetylated, 2-NFA and 1-NP do not require *O*-acetyltransferase for activity and their respective amines do undergo acetylation. It remains to be seen whether this emerging pattern holds true for other nitroPAH, and what its predictive value may be for mammalian genotoxicity.

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