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# ENZYMIC REDUCTION OF CARCINOGENIC AROMATIC NITRO COMPOUNDS BY RAT AND MOUSE LIVER FRACTIONS*

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**Abstract**—The enzymatic reduction of 2-nitronaphthalene and similar aromatic nitro compounds by rat liver extracts was investigated. The incubation of 2-nitronaphthalene with the post-mitochondrial fraction of rat and mouse liver led to the slow formation of 2-naphthylamine and to the disappearance of 2-nitronaphthalene; the reaction also occurred with the cytoplasmic and microsomal fractions of rat liver and was generally accelerated by NADPH and FMN. No 2-naphthylhydroxylamine could be observed at any time during the reduction, even using a gas-liquid chromatographic system capable of detecting 5 μg of 2-naphthylhydroxylamine added to the incubation mixture. The similar incubation of 4-nitrobiphenyl and 1-nitronaphthalene also led to the slow formation of the corresponding arylamine, with no evidence of hydroxylamine accumulation. 2-Naphthylamine was also produced both chemically and enzymatically by incubating 2-naphthylhydroxylamine with rat liver post-mitochondrial supernatant. In contrast to 2-nitronaphthalene, 4-nitroquinoline-*N*-oxide was rapidly reduced by rat liver post-mitochondrial fraction to yield high levels of the 4-hydroxylamine derivative, as well as small but significant quantities of the corresponding amine.

THE ENZYMATIC reduction of aromatic nitro compounds has long been recognized as a common metabolic pathway both *in vivo* and *in vitro*.[1–9] The enzymatic reduction of aromatic nitro compounds has led to the detection of metabolites at each of the intermediate oxidative states: i.e. the nitroso, the hydroxylamino and the amine derivatives.[1–6] The biological formation of aromatic hydroxylamines and the hydroxamic acids often results in the formation of compounds with considerable toxic, carcinogenic and mutagenic activities.[10–12] The reduction of aromatic nitro compounds to their corresponding hydroxylamino derivatives may thus explain the carcinogenic action of such agents as 2-nitrofluorene,[13,14] 4-nitroquinoline-*N*-oxide,[15] certain heterocyclic nitro compounds,[16] 4-nitrobiphenyl[17] and 4,4′-dinitrobiphenyl. The present studies constitute an attempt to obtain information on the possible mode of action of aryl nitro compounds in rodents by investigating the enzymatic reduction of 2-nitronaphthalene by rat and mouse liver extracts.

## EXPERIMENTAL

*Compounds.* The following compounds were commercially obtained and used without further purification: 2-naphthylamine and 4-nitrobiphenyl (Aldrich Chemical Co., Milwaukee, Wis.), 2-nitronaphthalene (Fundamental Research Co., Berkeley,

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Calif.), 1-nitronaphthalene and 1-naphthylamine (Eastman Organic, Rochester, N.Y.). 4-Biphenylamine (m.p. 51–52°) was prepared by catalytic reduction of 4-nitrobiphenyl, and was generously supplied by Dr. E. K. Weisburger of NCI. 2-Naphthylhydroxylamine (m.p. 121–122°) was synthesized by the reduction of 2-nitronaphthalene with ammonium hydrogen sulfide according to the procedure of Willstätter and Kubli.[18] 4-Nitroquinoline-*N*-oxide (m.p. 152–153°) was kindly supplied by Dr. Y. Shirasu, and 4-aminoquinoline-*N*-oxide hydrochloride (m.p. 285–287°) was a gift of Dr. Y. Kawazoe, National Cancer Center, Tokyo, Japan. 4-Hydroxylaminoquinoline-*N*-oxide (m.p. 196 198°) was synthesized by the method of Ochiai and Mitarashi.[19]

*Animals and diets.* Male SPF/CD NIH Fischer rats weighing 75–100 g and female NIH Swiss mice (18–21 g) were used throughout the experiments. They were maintained on Wayne Lab Blox and water *ad lib.* until required for the enzyme assays.

*Preparation of tissue extracts.* The animals were decapitated, and the livers were quickly removed and homogenized with a motor-driven glass-Teflon homogenizer in 4 vol. of ice-cold 1·15% KCl containing 0·02 M Tris-HCl buffer, pH 7·4. To prepare the post-mitochondrial supernatant, the homogenate was centrifuged for 20 min at 9000 *g* in a Sorvall centrifuge and the supernatant was carefully decanted. The cytosol and microsomes were obtained by recentrifugation for 1 hr at 105,000 *g* in a Spinco model L preparative ultracentrifuge. The cytosol was carefully decanted and the microsomal pellet was washed with a volume of KCl-Tris-HCl buffer equal to that of the decanted cytosol, recentrifuged at 105,000 *g* for 1 hr, and resuspended in $\frac{1}{3}$ of the initial homogenate volume of KCl-Tris-HCl pH 7·4 buffer.[20]

*Assay methods*

*Nitroreductase activity.* With the exception of studies on the reduction of 4-nitroquinoline-*N*-oxide, the reductase system used was a modification of the method of Feller *et al.*[21] for the enzymatic reduction of niridazole. It consisted of 0·01 to 0·5 ml or its equivalent of tissue extract, 300 nmoles of substrate in 50 μl ethanol, 0·1 ml of an NADPH-regenerating system containing 1·0 μmole NADPH, 15 μmoles $MgCl_2$, 15 μmoles glucose 6-phosphate, and 2 E. U. glucose 6-phosphate dehydrogenase in a final volume of 3·0 ml of 0·02 M Tris-HCl buffer at pH 7·4, containing 1·18% KCl. In certain instances 1·0 μmole FMN was added to the incubation mixture as an additional cofactor. To exclude oxygen, the reaction mixtures were prepared in 12-ml glass-stoppered test tubes while flushed with nitrogen and set in ice; the Tris-HCl buffer had been flushed with nitrogen for 20 min immediately prior to use. The samples were quickly stoppered and were then set in a 37° water bath for 0–60 min. No attempts were made to exclude light during the incubation. The reaction was terminated by the addition of 0·1 ml of 10 N HCl; the samples were extracted with 5·0 ml cyclohexane to remove the unreacted nitro compound. The acidified reaction mixture was re-extracted with 5·0 ml cyclohexane and the organic layer was discarded. Finally, the aqueous solutions were made alkaline with 0·3 ml of 4·0 N NaOH and the aromatic amine enzyme reaction products were extracted into 5·0 ml cyclohexane. Ultraviolet spectra of the initial and final cyclohexane extracts were taken to determine the contents of each of the aromatic nitro substrates and of the enzyme reduction products. The cyclohexane extracts appeared to contain only the nitro substrates or the amine products; the ultraviolet spectra of all cyclohexane

extracts of the incubation mixtures were clearly recognizable as those of the standard nitro and amine compounds. Further, when 300 nmoles of 2-naphthylhydroxylamine was added to the nitroreductase incubation system and immediately extracted as above, only 3 and 14 per cent, respectively, of unidentified u.v.-absorbing products could be recovered in the cyclohexane layers following acid and basic extractions.

*Hydroxylamine reductase.* Two general methods were used to measure the enzymatic reduction of 2-naphthylhydroxylamine. The first was based upon the *N*-dehydroxylase assay described by Williams *et al.*[20] The incubation mixture consisted essentially of the nitroreductase system described above except that 600 nmoles of 2-naphthylhydroxylamine was used as substrate. Enzyme reactivity was determined by the spectrophotometric determination of the unreacted hydroxylamine at 540 nm following the addition of 1·0 ml of 0·25% trisodium pentacyanoaminoferrate (TPF). Since early experiments indicated a rapid non-enzymatic loss of 2-naphthylhydroxylamine incubated with tissue extracts, an alternate detection procedure was devised. The incubation system was that described above, and the incubations were similarly run for 0–30 min. However, the reaction was terminated by the addition of 0·1 ml of 4 N NaOH. The incubation mixture was extracted with 5·0 ml cyclohexane, and the aqueous layer was discarded. The cyclohexane was extracted with 2·5 ml of 0·5 N HCl, and the organic layer was discarded. Finally, the acidic solution of 2-naphthylamine was made basic with 0·5 ml of 5 N NaOH and extracted with 5·0 ml cyclohexane. The u.v. spectra of the cyclohexane solution was then taken and the amount of 2-naphthylamine present was determined.

*Hydroxylamine formation.* Several attempts were made to detect 2-naphthylhydroxylamine during the enzymatic reduction of 2-nitronaphthalene. The incubation system was that described above for nitroreductase studies except that 600 nmoles of 2-nitronaphthalene was used as substrate, and the reactions were generally run for only 5–10 min to maximize the accumulation of the presumed hydroxylamine intermediate.[1,4]

Three methods were employed to detect the 2-naphthylhydroxylamine metabolites: colorimetry following the TPF treatment[20] described above, thin-layer chromatography (TLC)[1] and gas-liquid chromatography (GLC). In both chromatographic procedures, the enzyme incubation mixtures were extracted with 2·5 ml ether or 5·0 ml $CHCl_3$, and the organic layers were taken to dryness under $N_2$ prior to chromatographic analysis. The samples were taken up in a minimal volume of ethanol for TLC or $CCl_4$ for GLC analyses. The TLC was performed on Eastman Silica gel sheets with a solvent system of petroleum ether–acetone (4:1, v/v). The mobilities of the standard compounds were: 2-nitronaphthalene, 0·70; 2-nitrosonaphthalene, 0·79; 2-naphthylhydroxylamine, 0·31; and 2-naphthylamine, 0·50. The compounds were detected by examination under a u.v. lamp and by spraying with an aqueous solution of 0·25% TPF.

The gas-liquid chromatography was performed using a Hewlett–Packard model 7620 Research Chromatograph containing 6 ft columns of 3% OV-1 in 80–100 mesh Chromasorb W, operated at a temperature of 120–180°. All of the naphthalene derivatives tested could be identified when present in quantities as low as 0·3 μg. 2-Naphthylamine, 2-nitroso- and 2-nitronaphthalene all had clearly discernible peaks. 2-Naphthylhydroxylamine appeared to decompose on GLC to 2-naphthylamine, 2-nitrosonaphthalene and 2-azoxynaphthalene.

Microgram quantities of 2-nitrosonaphthalene were synthesized from 2-naphthylhydroxylamine by oxidation with potassium ferricyanide.[1] The u.v. spectrum of the 2-nitrosonaphthalene thus obtained was identical to that previously described.[1] Prior to extraction with organic solvent, the incubation mixtures were occasionally treated with 0·5 ml potassium ferricyanide to form the presumed 2-nitrosonaphthalene intermediate, and the samples were then treated as described above for chromatographic analysis. Under such conditions, when 2-naphthylhydroxylamine was added directly to the chromatographic system, as little as 3 μg and 1 μg could be detected with TLC and GLC respectively. When 2-naphthylhydroxylamine was added to the standard incubation systems and immediately extracted into the appropriate organic solvent, the minimum amount of 2-naphthylhydroxylamine that could be detected by either GLC or TLC was 5 μg.

The same chromatographic procedures were used to seek the hydroxylamine intermediate during the enzymatic reduction of 1-nitronaphthalene and 4-nitrobiphenyl. The enzymatic reduction of 4-nitroquinoline-*N*-oxide and 4-hydroxylamino-quinoline-*N*-oxide was studied by the method of Kato *et al.*[3]

## RESULTS

The enzymatic reduction of 2-nitronaphthalene by the post-mitochondrial fraction was studied by both the disappearance of 2-nitronaphthalene and by the appearance of 2-naphthylamine. The rates of 2-nitronaphthalene disappearance and of 2-naphthylamine production were very similar, and were both virtually linear for the first 30 min of the reaction (Fig. 1). The nitroreductase activity in the subfractions of the post-mitochondrial supernatant as well as the cofactors required for such reductase activity are listed in Table 1. In the absence of exogenous cofactors, virtually all of the nitroreductase activity of the post-mitochondrial supernatant is found in the

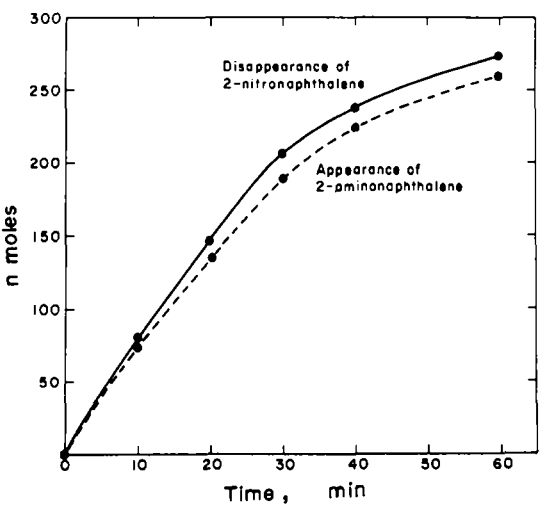


FIG. 1. Kinetics of 2-nitronaphthalene disappearance and 2-aminonaphthalene appearance in the presence of rat liver post-mitochondrial supernatant. The incubation mixture contained 0·05 ml ethanol, 0·3 μmole of substrate and 0·1 ml of an NADPH-regenerating system, in a final volume of 3·0 ml of 0·02 M Tris-HCl buffer at pH 7·4, containing 1·18% KCl.

cytosol and not in the microsomes (Table 1). FMN stimulated the enzymatic reduction of 2-nitronaphthalene in all fractions studied, while NADPH stimulated the enzymatic reduction of 2-nitronaphthalene by rat liver post-mitochondrial supernatant and microsomes, but not by the cytosol. Also, the rate of appearance of 2-naphthylamine closely paralleled the rate of 2-nitronaphthalene disappearance.

TABLE 1. NITROREDUCTASE ACTIVITY IN THE SUBCELLULAR FRACTIONS OF RAT LIVER*

| Fraction | Cofactors added | Activity (nmoles/min/50 mg liver) Nitro disappearance | Amine appearance |
|---|---|---|---|
| Post-mitochondrial supernatant | None | 1·15 | 1·33 |
| | NADPH | 1·94 | 1·94 |
| | NADPH + FMN | 3·95 | 3·37 |
| Cytosol | None | 0·96 | 1·10 |
| | NADPH | 0·86 | 0·76 |
| | NADPH + FMN | 4·42 | 3·46 |
| Microsomes | None | 0·07 | 0·01 |
| | NADPH | 0·24 | 0·15 |
| | NADPH + FMN | 0·64 | 0·60 |

* Anaerobic incubation mixture contained 300 nmoles of 2-nitronaphthalene in 50 μl ethanol, the post-mitochondrial supernatant and cytosol from 40 mg rat liver or the microsomes from 120 mg rat liver, 1·0 μmole of FMN and 0·1 ml of the NADPH-regenerating system described in Methods, in a final volume of 3·0 ml of 0·02 M Tris-HCl buffer at pH 7·4, containing 1·18% KCl.

The subcellular distribution of nitroreductase in mouse liver closely resembled that seen in the rat. Most of the activity noted in mouse liver post-mitochondrial supernatant was localized in the cytosol; in the absence of exogenous cofactors, very little nitroreductase activity could be found in the microsomal fraction (Table 2). As in the case of the rat, the enzymatic reduction of 2-nitronaphthalene was greatly accelerated by FMN in all fractions studied, but by NADPH only in the post-mitochondrial supernatant and microsomes; this effect may result from the presence of endogenous NADPH in the cytosol. Again there was a close correlation between the rate of 2-nitronaphthalene disappearance and the rate of 2-naphthylamine appearance.

TABLE 2. NITROREDUCTASE ACTIVITY IN THE SUBCELLULAR FRACTIONS OF MOUSE LIVER*

| Fraction | Cofactors added | Activity (nmoles/min/50 mg liver) Nitro disappearance | Amine appearance |
|---|---|---|---|
| Post-mitochondrial supernatant | None | 0·81 | 0·75 |
| | NADPH | 1·59 | 1·28 |
| | NADPH + FMN | 4·09 | 3·77 |
| Cytosol | None | 0·91 | 0·76 |
| | NADPH | 0·72 | 0·25 |
| | NADPH + FMN | 3·26 | 2·60 |
| Microsomes | None | 0·11 | 0·01 |
| | NADPH | 0·30 | 0·12 |
| | NADPH + FMN | 0·42 | 0·41 |

* Anaerobic incubation mixture contained 300 nmoles of 2-nitronaphthalene in 50 μl ethanol, the post-mitochondrial supernatant and cytosol from 40 mg mouse liver or the microsomes from 120 mg mouse liver, 1·0 μmole of FMN and 0·1 ml of the NADPH-regenerating system described in Methods, in a final volume of 3·0 ml of 0·02 M Tris-HCl buffer at pH 7·4, containing 1·18% KCl.

TABLE 3. REDUCTION OF AROMATIC NITRO COMPOUNDS BY THE POST-MITOCHONDRIAL SUPERNATANT FRACTION OF RAT LIVER*

| Compound | Enzyme activity (nmoles/min/50 mg liver) | | |
|---|---|---|---|
| | Nitro disappearance | Hydroxylamine appearance | Amine appearance |
| 2-Nitronaphthalene | 1·84 | 0 | 2·07 |
| 1-Nitronaphthalene | 1·20 | 0 | 1·41 |
| 4-Nitrobiphenyl | 1·06 | 0 | 1·58 |
| 4-Nitroquinoline-*N*-oxide | 107·9 | 84·6 | 1·53 |

* Anaerobic NADPH incubation system for the reduction of 1- and 2-nitronaphthalene and 4-nitrobiphenyl is described in the footnote to Table 1. The anaerobic-reducing system for 4-nitroquinoline-*N*-oxide contained: the post-mitochondrial supernatant from 250 mg rat liver, 50 μmoles nicotinamide, 50 μmoles $MgCl_2$, 1·4 ml of 0·2 M sodium phosphate buffer (pH 7·0), 1·5 ml of 1·15% KCl, 5 μmoles of substrate, and an NADPH-regenerating system in a final volume of 5·0 ml.

The reduction of other aromatic nitro compounds by the post-mitochondrial supernatant fraction of rat liver was also examined. As with 2-nitronaphthalene, the rates of reduction of 1-nitronaphthalene and 4-nitrobiphenyl were very slow, whether determined by the rate of disappearance of the nitro compound or by the appearance of the amine (Table 3). Ten to 15 min after the start of the reduction of 2-nitronaphthalene, 1-nitronaphthalene or 4-nitrobiphenyl aliquots were taken and examined for the presence of the corresponding hydroxylamine derivative using thin-layer chromatography on Silica gel.[1] No hydroxylamine derivative could be detected with the three nitro compounds studied. The reduction of 4-nitroquinoline-*N*-oxide proceeded quite differently, however (Table 3). Confirming previous results, the rate of amine appearance was much lower than the rate of nitro compound disappearance, although the reduction of 4-nitroquinoline-*N*-oxide was much faster than the reduction of the other nitro compounds tested. Also, as expected, the enzymatic reduction of 4-nitroquinoline-*N*-oxide led to a considerable accumulation of 4-hydroxylaminoquinoline-*N*-oxide (Table 3).

The apparent lack of production of 2-naphthylhydroxylamine during the enzymatic reduction of 2-nitronaphthalene was studied in considerable detail. Neither TLC nor colorimetry with TPF gave evidence of 2-naphthylhydroxylamine during the enzymatic reduction of 2-nitronaphthalene by rat and mouse liver microsomes, cytosol or post-mitochondrial supernatant either in the presence or absence of NADPH. Similarly, after a 10-min incubation of 2-nitronaphthalene with rat liver post-mitochondrial supernatant, cytosol and microsomes in the presence of NADPH, no 2-naphthylhydroxylamine could be detected by GLC. The detector systems employed were sensitive to as little as 1–5 μg of pure 2-naphthylhydroxylamine. However, the addition of 2-naphthylhydroxylamine to the incubation mixtures led to the rapid disappearance of the 2-naphthylhydroxylamine (Table 4). Much of this loss was due to chemical and enzymatic reduction. Zero-time blanks and incubation mixtures containing boiled rat and mouse liver extracts led to 15 per cent recoveries of the substrate as 2-aminonaphthalene. Figure 2 illustrates the enzymatic reduction of 2-naphthylhydroxylamine by the post-mitochondrial supernatant fraction of rat liver; during the first 10 min of reaction, 2-naphthylhydroxylamine was reduced at a rate of 16·3 and 5·05 nmoles/min/50 mg tissue in the presence and absence, respectively, of

TABLE 4. RAPID DISAPPEARANCE OF 2-NAPHTHYLHYDROXYLAMINE WITH SUBCELLULAR FRACTIONS OF RAT AND MOUSE LIVER*

| Fraction | Rate of disappearance (nmoles/min/50 mg liver) | |
|---|---|---|
| | +NADPH | −NADPH |
| Rat liver | | |
| Post-mitochondrial supernatant | 68·9 | 38·5 |
| Cytosol | 65·0 | 37·2 |
| Microsomes | 0·2 | 0·6 |
| Mouse liver | | |
| Post-mitochondrial supernatant | 115 | 115 |
| Cytosol | 110 | 93 |
| Microsomes | 15 | 32 |

* Anaerobic incubation system contained: 600 nmoles of 2-naphthylhydroxylamine in 100 μl ethanol, the cytosol and post-mitochondrial supernatant obtained from 40 mg liver, or the microsomes obtained from 120 mg liver, 0·1 ml of the NADPH-regenerating system described in Methods, in a final volume of 3·0 ml of 0·02 M Tris-HCl buffer at pH 7·4, containing 1·18% KCl; the reactions were run for 10 min, and the unreacted 2-naphthylhydroxylamine was measured with TPF.[20]

NADPH. A comparison of these data with those in Table 1 indicates that the enzymatic reduction of 2-naphthylhydroxylamine to 2-aminonaphthalene by rat liver post-mitochondrial supernatant was four to eight times faster than the similar reduction of 2-nitronaphthalene. On the other hand, the production of 2-aminonaphthalene by both the chemical and enzymatic reduction of 2-naphthylhydroxylamine still

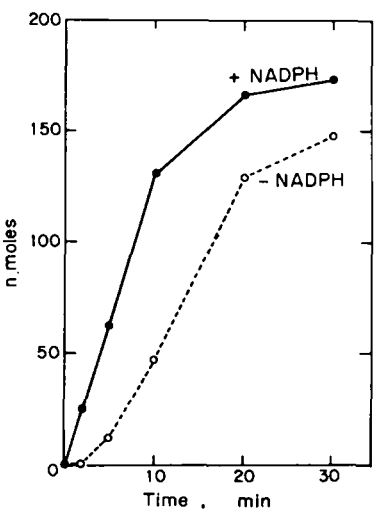


FIG. 2. Kinetics of the enzymatic formation of 2-aminonaphthalene from 2-naphthylhydroxylamine in the presence of rat liver post-mitochondrial supernatant. The incubation mixture contained 0·1 ml of a 20% post-mitochondrial supernatant, 1·2 μmoles of substrate, 0·1 ml ethanol and 0·1 ml of an NADPH-regenerating system, in a final volume of 30 ml of 0·02 M Tris-HCl buffer at pH 7·4, containing 1·18% KCl.

accounted for only 40 per cent of the loss of 2-naphthylhydroxylamine from the 10-min incubation mixture. Since the reduction of 2-nitronaphthalene proceeded almost quantitatively to 2-aminonaphthalene, it seems clear either that no free 2-naphthylhydroxylamine accumulated during the incubation or that the metabolism of 2-naphthylhydroxylamine varied according to whether the compound was a reaction intermediate or a substrate. In either case, these data may explain the lack of detectability of 2-naphthylhydroxylamine during the enzymatic reduction of 2-nitronaphthalene.

## DISCUSSION

The present results provide further evidence to support the generalization that the carcinogenic activities of aromatic nitro compounds may be attributed to their conversion *in vivo* to reactive hydroxylamino intermediates. There are three major categories of aromatic nitro compounds that give rise to tumor production: (1) derivatives of 4-nitroquinoline-*N*-oxide,[15] (2) certain monocyclic heterocyclic nitro compounds[16] and (3) the nitro analogues of classical aromatic amine carcinogens.[13,14,17,22] Several studies, including our own, have demonstrated that the versatile carcinogen 4-nitroquinoline-*N*-oxide is readily reduced enzymatically to 4-hydroxylaminoquinoline-*N*-oxide, which is then only slowly reduced to the corresponding amine.[3] Further, the carcinogenic activities of various nitro analogues of 4-nitroquinoline-*N*-oxide has been correlated with their ease of enzymatic reduction.[23] Cohen *et al.*[16] have reported on the carcinogenic activity of a variety of heterocyclic nitro compounds. Recently it was demonstrated that the enzymatic reduction of structurally similar nitro compounds leads to the accumulation of the corresponding hydroxylamine.[21,24]

The nitro analogues of classical aromatic amine carcinogens showing significant carcinogenic activity include: 4-nitrobiphenyl,[17] 2-nitrofluorene,[13,14] 2,7-dinitrofluorene,[25] 4-nitrostilbene[26] and 4,4'-dinitrobiphenyl;[22] in one study, 2-nitronaphthalene did not show appreciable carcinogenic activity in mice.[27] Further, the available evidence tends to indicate that, in general, the nitro analogues of the aromatic amines are weaker carcinogens than their corresponding amino or acetamido derivatives.[11,13,14] Such data suggest a slow rate of formation *in vivo* of the carcinogenic hydroxylamine carcinogens. The present results support this concept. Thus, 2- and 1-nitronaphthalene as well as 4-nitrobiphenyl were only slowly reduced by hepatic nitroreductase, and their hydroxylamine intermediates could not be detected.

Previous aromatic nitro compounds reported to form hydroxylamine intermediates *in vitro* during enzymatic reduction include: *p*-nitrobenzoic acid,[4] nitrobenzene[28] and 4-nitrobiphenyl.[1] The reductive incubation of the *p*-nitrobenzoic acid and nitrobenzene with rat liver extracts led to the accumulation of relatively large amounts of the hydroxylamine analogue.[4,28] Uehleke and Nestel[1] reported the detection of 4-nitrosobiphenyl during the enzymatic reduction of 4-nitrobiphenyl by rat liver post-mitochondrial supernatant. The rate of accumulation of both 4-nitrosobiphenyl and 4-biphenylamine reported by the workers was approximately 5-fold greater than the rate of disappearance of 4-nitrobiphenyl seen in our own studies. The very high substrate and FMN concentrations used by these workers may have contributed to the differences observed in the results.

In general, the major characteristics of hepatic nitroreductase closely resembled those previously reported.[1-4] Hepatic nitroreductase, especially that localized in the microsomes, was stimulated by NADPH and FMN. The low proportion of the total nitroreductase activity found in the microsomes is reminiscent of the findings of Kato *et al.* for the enzymatic reduction of 4-nitroquinoline-*N*-oxide. The major difference noted between the present and earlier studies[1-4] on nitroreductase was the low activity of nitroreductase noted with the aromatic nitro compounds used as substrates in the present studies. On the other hand, the arylhydroxylamines were rapidly reduced to the amines by liver extracts. Thus, the failure to detect measurable amounts of the arylhydroxylamines may perhaps be accounted for. Nonetheless, it seems quite certain that the arylhydroxylamines are the key intermediates in the pathologic effects—methemoglobin formation, anemia, allergies and cancer—of nitroaryl compounds. Hence, it would seem that specific intracellular trapping reagents can interact with recently formed arylhydroxylamine prior to the further reduction of the latter.

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