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COMPARISON OF *IN VIVO* BINDING OF AROMATIC NITRO AND AMINO COMPOUNDS TO RAT HEMOGLOBIN

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Abstract—The hemoglobin (Hb) binding of five nitroarenes, i.e. nitrobenzene (NB), 4-nitrobiphenyl (4-NBP), 1-nitropyrene (1-NP), 2-nitronaphthalene (2-NN) and 2-nitrofluorene (2-NF), and the corresponding amines, administered p.o. to male S.D. rats, was determined by HPLC, to evaluate the extent of *in vivo* reductive and oxidative activations of these compounds to *N*-hydroxylamines, which covalently bind to Hb to form acid-labile sulfinamides. Hb binding of the nitroarenes, except for NB, was significantly lower than that of the corresponding amines. Among the aromatic amines, 4-aminobiphenyl exhibited extremely high Hb binding. Hb binding of NB and 4-NBP decreased markedly after pretreatment with a mixture of antibiotics, but the binding of the others did not decrease appreciably. 1-Aminopyrene and 1-NP bound abundantly to plasma proteins, although the Hb binding was slight. Based on the Hb binding and the *in vitro* metabolism by liver microsomes and intestinal bacteria, the extent of *in vivo* reductive activation of nitroarenes is discussed.

Nitroarenes occur in various environments: gasoline and diesel emissions [1–3], fly ash particles [4, 5], cigarette smoke [6], grilled meat [7], urban atmosphere [8] and river water [9, 10]. Many of them are known to be potent mutagens to some bacterial strains that lack metabolic enzyme systems found in mammals [11–13]. This has been proven to be due to reduction to an active proximate form, *N*-hydroxylamine, by bacterial nitroreductases [13, 14]. Such metabolic activation via *N*-hydroxylamine is analogous to the activation by aromatic amines. As represented by 2-naphthylamine and 4-aminobiphenyl, aromatic amines have been shown by many studies to generate carcinogenicity via oxidative N-hydroxylation with hepatic microsomal enzymes [15, 19]. From these events, nitroarenes are considered currently to be important environmental carcinogens.

Some nitroarenes are activated reductively *in vitro* by various enzymes in mammalian tissues [13, 20–25], and a few compounds even show carcinogenicity [26–30]. It is unlikely, however, that the extent of reductive activation of nitroarenes is the same as that of oxidative N-hydroxylation of aromatic amines *in vivo* in mammalia. To evaluate the significance of nitroarenes as environmental carcinogens, it is necessary to know the extent of *in vivo* reduction of each nitroarene in mammalia.

Recently, it was found by Dolle *et al.* [31], Eyer [32] and Albrecht and Neumann [33] that aromatic nitroso compounds, which are active intermediates in the metabolism of aromatic nitro, amino, and azo compounds, bind covalently to the thiol residue of components of body fluids to give sulfinamide. *N*-Hydroxylamine, produced in liver and intestine, is also presumed to bind to the thiol residue of hemoglobin (Hb), because *N*-hydroxylamine had been known to be oxidized to a nitroso compound with oxy-hemoglobin in blood flow [34]. Since this adduct is stable throughout the life-span of the erythrocytes and is hydrolyzed to release the corresponding amine under mild acidic conditions, Neumann [35] stated that the adduct must be a suitable indicator for monitoring the exposure with their compounds. Actually, Bryant *et al.* [36, 37] attempted to estimate the exposure level of smokers to 4-aminobiphenyl and other aromatic amines by determining acid-labile sulfinamide adducts in blood with a GC–MS method.

We, therefore, estimated comparatively the extent of *in vivo* activation of some aromatic nitro and amino compounds in rats by monitoring their adducts with SH-groups of Hb. In the present paper, we demonstrate that the Hb binding of five nitroarenes, i.e. nitrobenzene (NB), 2-nitronaphthalene (2-NN), 4-nitrobiphenyl (4-NBP), 2-nitrofluorene (2-NF) and 1-nitropyrene (1-NP), administered orally to rats, was significantly less than in those of the corresponding amines under the same conditions, except for the case of NB. In addition, the relation between the extent of Hb binding and the activities of oxidative and reductive metabolism of hepatic microsomes and intestinal microflora is discussed.

MATERIALS AND METHODS

Chemicals. NB and aniline (AN) were obtained from the Kanto Chemical Co.; 1-NP, 1-aminopyrene (1-AP) and 2-NF were from the Tokyo Kasei Co.; and 2-NN and 2-aminofluorene (2-AF) were from the Aldrich Chemical Co. 4-NBP was prepared from biphenyl according to the method described by Maki and Ohbayashi [38] and purified by silica gel column chromatography [eluent, chloroform/cyclohexane (1/1)]. 4-Aminobiphenyl (4-ABP) and 2-aminonaphthalene (2-AN) were prepared by Pd/C-NH_2NH_2 reduction of 4-NBP and 2-NN, respectively, according to the method described by Bavin [39]. *N*-Hydroxyl-2-aminonaphthalene, *N*-hydroxyl-

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2-aminofluorene and *N*-hydroxyl-4-aminobiphenyl, which were used as standards in HPLC analysis of *in vitro* metabolites, were prepared by Pd/C-NH_2NH_2 reduction of the corresponding nitro compounds according to the method of Westra [40], and phenylhydroxylamine by Zn-NH_4Cl reduction of NB according to the method of Kamm [41]. The purities of these synthesized chemicals were more than 95% as determined by HPLC. Antibiotics, neomycin sulfate, bacitracin and tetracycline were purchased from the Sigma Chemical Co. Other chemicals were of the best grade commercially available.

Administration of chemicals to rats and antibiotics treatment. Male S.D. rats (6- to 7-weeks old, 150–200 g) were obtained from the Sankyo Labo Service Co. (Tokyo). In the standard experiments, NB, 2-NN, 4-NBP, 2-NF, 1-NP and their corresponding amines were each administered orally at a dose of 0.5 mmol/kg (as corn oil solution) to three rats. After the administration, blood was collected from the jugular vein with a heparinized syringe at the prescribed time intervals. In addition, in the experiments to evaluate the Hb binding in the rats without metabolism by intestinal microflora, the rats were treated five times at 12-hr intervals with an antibiotic mixture in 1% carboxymethylcellulose prior to the administration of nitro compounds according to the method described by Levin and Dent [42]. The mixture of antibiotics consisted of 100 mg of neomycin sulfate, 50 mg of tetracycline, and 50 mg of bacitracin in 0.75 ml of 1% carboxymethylcellulose solution.

Isolation of Hb and plasma proteins. The heparinized blood was separated into plasma and erythrocytes by centrifugation (5 min, 3000 rpm). The erythrocytes were rinsed with equal volumes of 0.9% NaCl solution, lysed with 4 vol. EDTA solution (10^{-4} M, pH 7.5), and then the cellular debris was removed by centrifugation. Hb and plasma proteins were obtained according to the following procedure described by Albrecht and Neumann [33]. Ethanol (4 vol.) was added under stirring to the hemolysate or plasma, and the precipitates were sedimented by centrifugation. The sediments were further washed, successively, with ethanol/water (80/20), ethanol, ethanol/ether (25/75), and ether. By this washing, free amines contained in the erythrocytes or plasma were removed completely. The preparations were dried *in vacuo*.

Acid hydrolysis and determination of released amine. The dried Hb or plasma proteins (10–50 mg, accurate weighing) were suspended in 5 ml of sodium dodecyl sulfate (SDS) solution (0.5%), 1 ml HCl (1 M) was added, and the suspension was stirred for 2 hr at room temperature. The pH was then adjusted to 9 by adding NaOH (10 M), and the solution was extracted twice with 15 ml ether. The ether phase was evaporated to dryness under reduced pressure in a dark room. For evaporating the ether containing volatile AN as released amine, 50 μl HCl (1 M) was added prior to the evaporation. The residue was dissolved in methanol, and the total volume was adjusted to 0.5 ml.

To follow the time–courses of Hb and plasma protein bindings, another simplified method was adopted. Erythrocytes and plasma were hydrolyzed by adding 5 ml SDS solution and 1 ml HCl after washing with ether (5 ml × 3) without the purification as mentioned above, and the released amine was extracted as mentioned above.

The free amine in the methanol solution was determined by HPLC using a Shimadzu LC-4A equipped with an SSC ODS-262 column (0.6ϕ × 10 cm), a Shimadzu SPS-2AS UV detector, and a Shimadzu RF-530 fluorescence detector. The solvents and the means of detecting amines in the HPLC analysis were as follows: MeOH/H_2O (40/60), UV at 235 nm for AN; MeOH/H_2O (65/35), fluorescence at emission (Em) 405 and excitation (Ex) 245 nm for 2-AN; CH_3CN/H_2O (70/30), fluorescence at Em 370 and Ex 280 nm for 2-AF; MeOH/H_2O (70/30), fluorescence at Em 390 and Ex 280 nm for 4-ABP; MeOH/H_2O (80/20), fluorescence at Em 430 and Ex 285 nm for 1-AP.

Preparation of cell-free extract (CFE) of intestinal microflora in rats. A flask containing GAM medium (Nissui Co. Ltd.) was inoculated with the contents of the intestine of a non-treated male rat, and incubated under aeration with oxygen-free nitrogen gas at 37° for 24 hr. After incubation, the bacterial cells were harvested by centrifugation and washed twice with 0.1 M sodium phosphate buffer (pH 7.4). The bacterial pellets were homogenized with 3-fold weight of the same buffer and sonicated for 10 min with an Ultrasonic Disruptor (model UR-200p, Tomy Seiko Co. Ltd.) in an ice bath. Cellular debris was removed by centrifugation. The protein content of the supernatant fraction, that is CFE, determined by a modification [43] of the Lowry method, was 24 mg/ml.

Preparation of rat liver S9 fraction. Liver S9 fraction was prepared from liver of the same rats used in the *in vivo* experiments, according to the method described by Maron and Ames [44]. The protein content of the S9 was 18.5 mg/ml.

In vitro *metabolism by liver S9 and CFE of intestinal bacteria.* A mixture of 0.1 ml of the dimethyl sulfoxide (DMSO) solution of test chemicals (50 mg/ml) and 5 ml of S9 mix or CFE mix was incubated under nitrogen atmosphere (anaerobic metabolism) or under air (aerobic metabolism) for 1 hr at 37°. The S9 mix and the CFE mix (each 5 ml) contained 20 μmol $NADP^+$, 25 μmol G6P, 40 μmol $MgCl_2$, liver S9 fraction or bacterial CFE containing 47 mg protein, and 0.1 M sodium phosphate buffer (pH 7.4). After incubation, the mixture was extracted three times with ethyl ether (10 ml). The ether phase was dried under a nitrogen stream and dissolved in 1 ml methanol. After passing through a membrane filter (0.45 μm), the methanol solution was subjected to HPLC analysis for *N*-hydroxylamine and amine determinations. The HPLC conditions were as mentioned above except for the use of a UV detector (at 240 nm for phenylhydroxylamine and *N*-hydroxyl-2-aminonaphthalene, 270 nm for *N*-hydroxyl-2-aminofluorene, and 280 nm for *N*-hydroxyl-4-aminobiphenyl).

RESULTS

The amounts of Hb binding and plasma protein binding, which were determined using the purified Hb and plasma protein at 48 hr after administration, are shown in Table 1. The Hb binding of the nitro-

Table 1. Amounts of *in vivo* Hb and plasma protein bindings of aromatic nitro and amino compounds at 48 hr after p.o. administration in rats

Compounds	Hb binding (nmol/g Hb)	Plasma protein binding (nmol/g protein)
Nitrobenzene	657.0 ± 36.7*	ND†
Aniline	119.3 ± 8.1	ND
4-Nitrobiphenyl	231.0 ± 3.0	9.6 ± 0.8
4-Aminobiphenyl	3144.0 ± 19.2	33.9 ± 6.9
2-Nitronaphthalene	1.1 ± 0.2	ND
2-Aminonaphthalene	5.0 ± 1.1	0.1 ± 0.03
2-Nitrofluorene	0.8 ± 0.3	2.5 ± 0.04
2-Aminofluorene	85.7 ± 15.7	36.0 ± 1.1
1-Nitropyrene	ND	ND
1-Aminopyrene	0.16 ± 0.01	0.22 ± 0.03

* Each value is the mean ± SE of three rats.
† Not detected.

arenes tested decreased in order of NB, 4-NBP, 2-NN, 2-NF and 1-NP. In particular, the Hb binding of NB and 4-NBP was much higher than that of the others. No binding was observed for 1-NP. On the other hand, the Hb binding of aromatic amines decreased in order of 4-ABP, AN, 2-AF, 2-AN and 1-AP. That of 4-ABP was extremely high, 3144 nmol/g Hb. It is noteworthy that the Hb bindings of nitroarenes except for NB were significantly lower than those of the corresponding amines. Compared with the Hb binding, the amount of plasma protein binding was far less, ranging from 0.0 to 36 (nmol/g).

To determine the time–course of binding of these chemicals to Hb and plasma protein, the changes in the amount of acid-releasable amines in erythrocyte and plasma were followed by a simplified method as described in Materials and Methods. The results are shown in terms of the amount (μg or ng) of amines released per ml of blood (Figs. 1–5). The values of acid-releasable amine from Hb at 48 hr in these figures agreed approximately with those estimated from the values in Table 1 and the Hb content in blood, 130 mg/ml (this value was lower than that of the non-treated rat because of the frequent collection of blood). Therefore, these figures seem to reflect well the changes in Hb binding.

In all chemicals except for 1-NP and 1-AP, the amount of amines released from Hb reached maximum at 12–24 hr after administration and then gradually decreased at different rates. In addition, 10–50% of the maximal values remained even after 5 days. The order of the amount of amine released from Hb at the maximal point was the same as that obtained for the Hb binding at 48 hr after administration, as shown in Table 1.

On the other hand, the amount of amines released from plasma reached a maximum at 6 hr after administration and then decreased rapidly. We cannot discuss on the same level the values in Table 1 and these figures, because the determination of the content of plasma protein in blood was impossible, owing to the loss during purification of the protein. However, taking into account the rapid decrease of the acid-releasable amine in plasma, the values shown in Table 1 should not necessarily reflect the binding of test chemicals to plasma protein. In the case of NB and AN, the amine released from plasma was undetectable (detection sensitivity was very low, 100 ng/ml blood, because of UV detection). In the case of 4-NBP, 4-ABP, 2-NN, 2-AN, 2-NF, 2-AF, 1-NP and 1-AP, the amounts of amines released from plasma at the maximal points were 52, 910, 11, 20, 25, 320, 110 and 9100 (ng/ml) respectively. These values were far less than those from Hb in the test chemicals except for 2-NF, 1-NP and 1-AP. On the

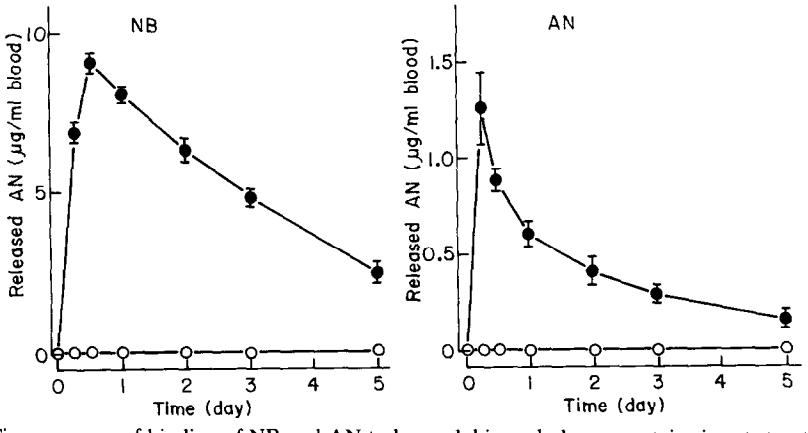

Fig. 1. Time–courses of binding of NB and AN to hemoglobin and plasma proteins in rats treated p.o. Results are shown in terms of the amounts of AN (μg/ml blood) released from erythrocytes (●) and plasma (○) by acid hydrolysis. Values are means ± SE, N = 3.

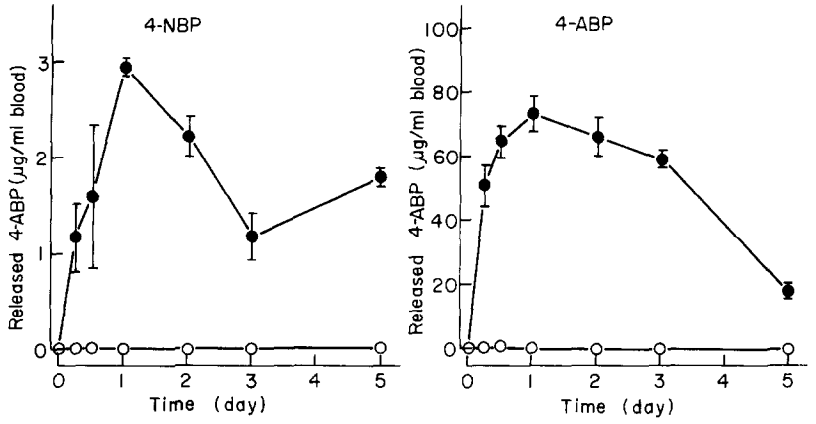

Fig. 2. Time–courses of binding of 4-NBP and 4-ABP to hemoglobin and plasma proteins in rats treated p.o. Results are shown in terms of the amounts of 4-ABP (μg/ml blood) released from erythrocytes (●) and plasma (○) by acid hydrolysis. Values are means ± SE, N = 3.

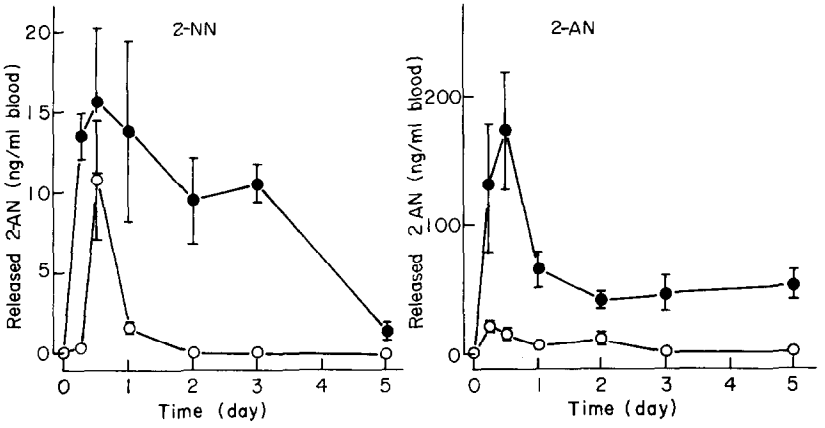

Fig. 3. Time–courses of binding of 2-NN and 2-AN to hemoglobin and plasma proteins in rats treated p.o. Results are shown in terms of the amounts of 2-AN (ng/ml blood) released from erythrocytes (●) and plasma (○) by acid hydrolysis. Values are means ± SE, N = 3.

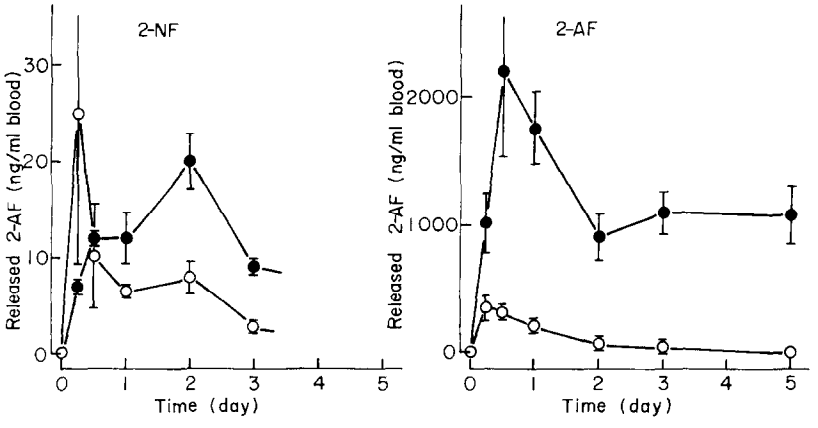

Fig. 4. Time–courses of binding of 2-NF and 2-AF to hemoglobin and plasma proteins in rats treated p.o. Results are shown in terms of the amounts of 2-AF (ng/ml blood) released from erythrocytes (●) and plasma (○) by acid hydrolysis. Values are means ± SE, N = 3.

contrary, the amounts of amine released from plasma in rats administered 1-NP and 1-AP were much larger than those from Hb.

To confirm whether the acid-releasable amine in the plasma observed during the short period after administration of 1-NP and 1-AP was an amine covalently bound to plasma proteins, the plasma at 3 hr after administration of 1-AP, after washing with ether, was fractionated by gel filtration chromatography (G-200). As shown in Fig. 6, no ether-extractable free amine was observed in any fractions, but acid-releasable amine was detected in the frac-

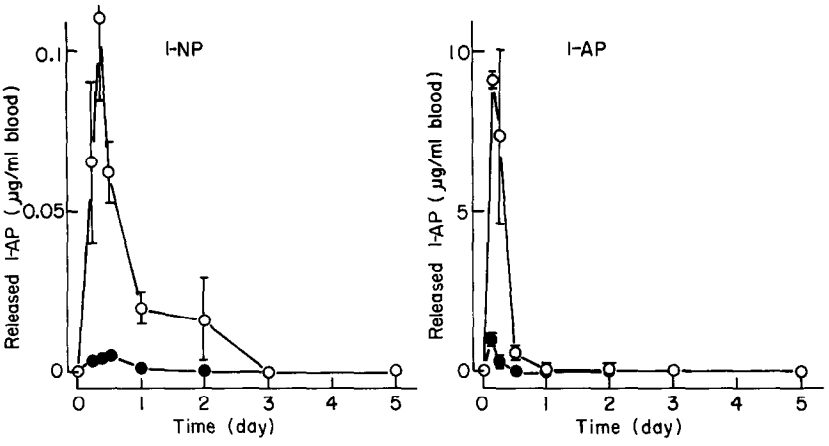

Fig. 5. Time–courses of binding of 1-NP and 1-AP to hemoglobin and plasma proteins in rats treated p.o. Results are shown in terms of the amounts of 1-AP (μg/ml blood) released from erythrocytes (●) and plasma (○) by acid hydrolysis. Values are means ± SE, N = 3.

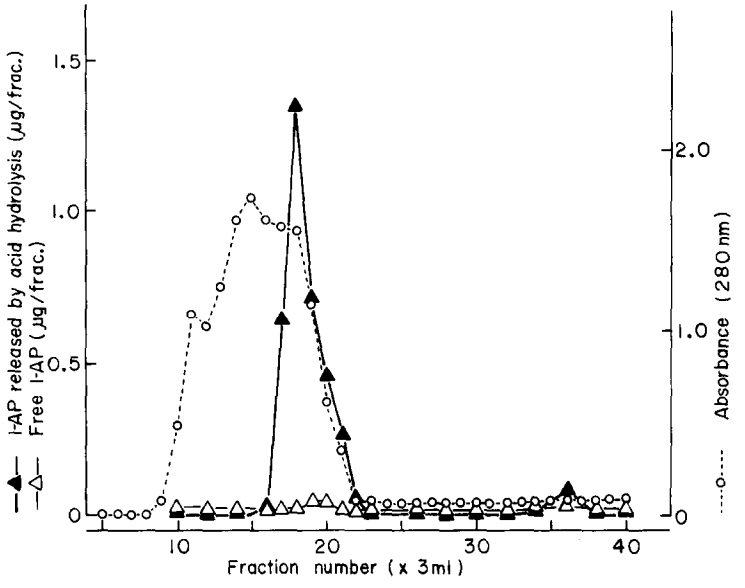

Fig. 6. Elution profile of the bound 1-AP in gel filtration chromatography of plasma obtained from the rat at 3 hr after p.o. administration of 1-AP. The column was packed with Sephadex G-200 (2φ × 30 cm); the solvent was 0.1 M Tris–HCl buffer (pH 8.0) with 0.2 M NaCl. Blue dextran (mol. wt 2,000,000), albumin (mol. wt 66,000) and NaCl were eluted in fraction No. 11, 18 and 28 respectively.

tions, No. 17–21, in which albumin (mol. wt 66,000) was eluted, indicating that the acid-releasable amine was covalently bound to plasma protein with high molecular weight.

'Of the above-mentioned results, at least the Hb bindings are attributable to the metabolic activation via *N*-hydroxylamines, as reported by Neumann [35]. Accordingly, the amounts of Hb binding of the chemicals tested in Table 1 should imply the extent of the *in vivo* metabolic activation via *N*-hydroxylamines in rats. A main organ for metabolism of amines is the liver, and also the intestine for nitroarenes [15, 42, 45].

Table 2 shows the rates of *in vitro* N-hydroxylation of the aromatic amines by rat liver S9 under aerobic conditions. The highest rate of *N*-hydroxylamine production was observed in 4-ABP, and the rate was much higher than in the other compounds. The *N*-hydroxylamine of 1-AP was so unstable that the isolation of standard chemical was impossible. Accordingly, the *N*-hydroxylaminopyrene must not be stably present in the incubation mixture of 1-AP

Table 2. N-Hydroxylation of aromatic amines by rat liver S9 under aerobic conditions

Compounds	*N*-Hydroxylamine production (nmol/hr/mg protein)
Aniline	0.45 ± 0.09*
4-Aminobiphenyl	5.83 ± 0.08
2-Aminonaphthalene	0.12 ± 0.03
2-Aminofluorene	0.33 ± 0.03
1-Aminopyrene	†

* Each value is the mean ± SE of three determinations.
† Standard *N*-hydroxylamine was not obtainable.

Table 3. Amounts of *in vivo* Hb binding of nitroarenes at 48 hr after p.o. administration in rats pretreated with antibiotics

Compounds	Hb binding (nmol/g Hb)
Nitrobenzene	88.2 ± 10.5*
4-Nitrobiphenyl	8.3 ± 0.2
2-Nitronaphthalene	0.6 ± 0.2
2-Nitrofluorene	0.6 ± 0.1
1-Nitropyrene	ND†

* Each value is the mean ± SE of three rats.
† Not detected.

with S9 mix. The rates of N-hydroxylation of the five aromatic amines in Table 2 correlated well with the amounts of Hb binding in Table 1 ($r = 0.9994$), indicating that the Hb binding is an excellent indicator of the extent of *in vivo* N-hydroxylation of test chemicals.

In vivo Hb bindings of nitroarenes in rats pretreated with the antibiotic mixture were also determined to evaluate the extent of participation of intestinal microflora on the Hb bindings shown in Table 1. The results are shown in Table 3. A comparison of the amounts of Hb binding in Tables 1 and 3 indicated that the antibiotic treatment decreased drastically the Hb bindings of NB and 4-NBP but only slightly decreased those of 2-NF and 2-NN. The antibiotic treatment was confirmed to have little effect on the metabolism in organs other than the intestinal microflora by the fact that the amount of Hb binding of 2-AN in the rats treated with the antibiotics was about the same as that in non-treated rats (data not shown). These results suggest that the reductive activation of NB and 4-NBP is largely dependent on the metabolism by intestinal microflora, although that of the other nitroarenes is not so dependent.

Table 4 shows the reduction rates of the five nitroarenes to the corresponding amine and *N*-hydroxylamine under anaerobic conditions by the CFE of bacteria from the intestinal contents in a rat. The reduction rates to amines increased in the order of 1-NP, 2-NF, 4-NBP, 2-NN and NB. *N*-Hydroxylamine production was very high in NB, but only to a slight extent in the other nitroarenes. The same tendency for *N*-hydroxylamine production was also observed in the metabolism with whole cells of the intestinal bacteria (data not shown). This phenomenon seems to be attributable to the differences in stability of *N*-hydroxylamines. That is to say, the *N*-hydroxylamines of nitroarenes other than NB are presumed to be reduced rapidly into amines by the bacterial enzymes or to be bound with bacterial proteins.

Binding of *N*-hydroxylamine to Hb has been considered to occur via its oxidation to a nitroso derivative by oxy-hemoglobin in blood [33–35]. Therefore, the phenylhydroxylamine produced in the intestine is expected to enter directly into the blood flow to bind rapidly with Hb. Thus, the abnormally high Hb binding of NB *in vivo* in rats and its drastic lowering by treatment with antibiotics were attributable to the unusually high production rate of phenylhydroxylamine by microflora in intestine. On the other hand, the Hb binding of 4-NBP, exhibiting a drastic lowering by antibiotic treatment like NB, could not be accounted for by the *N*-hydroxylamine production by intestinal microflora. It is presumed to result from both the 4-ABP production by intestinal microflora and its ready oxidation to *N*-hydroxylamine by microsomal oxygenases in the liver. The reason why the Hb bindings of other nitroarenes were affected slightly by the antibiotic treatment seems to be that the N-hydroxylation activity of liver enzymes was very low, as shown in Table 2, even if the corresponding amines were produced by nitroreduction in the intestine.

On the other hand, the amounts of Hb binding of nitroarenes in the rats treated with antibiotics should reflect the *in vivo* nitroreduction activity excluding that of intestinal microflora in rats. Therefore, in order to examine whether the amounts of these Hb bindings are correlated to the nitro reduction activity of hepatic microsomes or not, the *in vitro* reduction activities of rat liver S9 on five nitroarenes were evaluated under anaerobic conditions. As shown in Table 5, a slight amount of *N*-hydroxylamine was observed only in NB and 2-NN, although the reductions to the corresponding amines in the same extent as those by intestinal bacteria were observed in all nitroarenes tested. Under aerobic conditions, *N*-hydroxylamines were not detected at all. Thus, the amounts of Hb binding of nitroarenes in the antibiotic-treated rats were not correlated to the reduction activity of liver S9 under the present experimental conditions. Since the liver S9 used in this experiment was fortified with only cofactors for a NADPH-generating system, this result may indicate that some nitroreductases, other than NADPH-dependent ones such as cytochrome P-450, for

Table 4. Nitroreduction of nitroarenes by CFE of rat intestinal bacteria under anaerobic conditions

Compounds	*N*-Hydroxylamine production (nmol/hr/mg protein	Amine production (nmol/hr/mg protein)
Nitrobenzene	2.27 ± 0.19*	150.3 ± 12.3
4-Nitrobiphenyl	0.06 ± 0.01	18.6 ± 1.5
2-Nitronaphthalene	0.03 ± 0.02	68.2 ± 1.3
2-Nitrofluorene	ND†	5.7 ± 0.3
1-Nitropyrene	‡	4.6 ± 0.8

* Each value is the mean ± SE of three determinations.
† Not detected.
‡ Standard *N*-hydroxylamine was not obtainable.

Table 5. Nitroreduction of nitroarenes by rat liver S9 under anaerobic conditions

Compounds	*N*-Hydroxylamine production (nmol/hr/mg protein)	Amine production (nmol/hr/mg protein)
Nitrobenzene	0.10	100.7
4-Nitrobiphenyl	ND*	25.1
2-Nitronaphthalene	0.04	69.4
2-Nitrofluorene	ND	10.4
1-Nitropyrene	†	10.6

* Not detected.
† Standard *N*-hydroxylamine was not obtainable.

example, xanthine oxidase, DT-diaphorase or aldehyde oxidase [21, 23, 25], play an important role in the reductive activation of nitroarenes *in vivo*. From the fact that the amount of Hb binding in the antibiotic-treated rats was very small, it is concluded that the reductive activation of nitroarenes *in vivo* was far less than the oxidative N-hydroxylation of aromatic amines.

DISCUSSION

As mentioned above, the present *in vivo* experiments demonstrated that the amounts of Hb binding of five nitroarenes, i.e. NB, 2-NN, 4-NBP, 2-NF and 1-NP, were much less than those of the corresponding amines, except for the case of NB and AN. In addition, a good correlation between the amounts of HB binding and the oxidative N-hydroxylation activities of liver microsomes for aromatic amines tested was observed, indicating that the amount of Hb binding is a good indicator of the extent of *in vivo* activation via *N*-hydroxylamine. Accordingly, the data of the *in vivo* Hb binding describe that the extent of *in vivo* reductive activation of nitroarenes, except for NB in rats, is significantly lower than those of oxidative activation of aromatic amines even if intestinal microflora should take part in the metabolism.

The contribution of intestinal microflora to the *in vivo* reductive activation of nitroarenes was large in NB and 4-NBP, as judged by the change in the amounts of HB binding by antibiotic treatment. However, the causes of the contribution in both nitroarenes were found to differ from each other. In the case of NB, the relatively stable phenylhydroxylamine produced by intestinal microflora contributed directly to the HB binding in blood flow. Such direct contribution of intestinal microflora must be an unusual phenomenon observed in only stable *N*-hydroxylamine and scarcely occurs in most nitroarenes. The contribution of intestinal microflora to HB binding in most nitroarenes must be an indirect one which is attributable to the production of aromatic amines by nitroreduction and the N-hydroxylation of their amines by liver microsomes. Although the contribution in the case of 4-NBP was also indirect, it was considered to be large because of the high N-hydroxylation rate of 4-ABP by liver microsomes. In other words, these results indicate that the extent of *in vivo* reductive activation in organs other than intestine is so low that even the indirect contribution of intestinal microflora affects the HB binding of nitroarenes.

Most of the conventional findings on nitroreduction activity of mammalian enzymes are based on *in vitro* experiments under anaerobic conditions [20–25] and they do not always reflect the *in vivo* activity because the oxygen concentration in experiments *in vitro* usually differs from that in experiments *in vivo*. Actually, some investigators have reported that the *in vitro* metabolism of nitroarenes with liver microsomes under aerobic conditions is oxidative ring hydroxylation rather than nitroreduction [46–50]. El-Bayoumy and Hecht [51], Ball *et al.* [52] and Howard *et al.* [53] have also reported that the *in vivo* metabolites in rats administered 1-NP were hydroxy-1-nitropyrenes, hydroxy-1-aminopyrenes and their conjugates. Furthermore, the *in vivo* metabolites in germfree rats were only ring-hydroxylated 1-NPs, indicating that the nitroreduction in *in vivo* metabolism is due mainly to intestinal microflora [45].

Such low values of the *in vivo* reductive activation suggest that the risk of nitroarenes as environmental carcinogens is not so high relative to potent mutagenicities exhibited in bacterial test systems in which the mutagenicities are due to bacterial nitroreductases. Because a major route of exposure to nitroarenes is inhalation, the risk of exposure to nitroarenes should be far less than to aromatic amines.

The present *in vivo* experiments manifested another interesting phenomenon, that the amounts of plasma binding of 1-NP and 1-AP were much larger than those of HB binding, unlike other test compounds. The *N*-hydroxylamines of 1-AP and 1-NP were so unstable that the isolation of standard was impossible. The unique behavior of 1-NP and 1-AP in plasma and HB bindings is presumed to be attributable to the instability of its *N*-hydroxylamine. The adduct in plasma was confirmed to be a covalently bound 1-NP or 1-AP with a high molecular weight protein (presumably albumin) and to release 1-AP by acid hydrolysis, although it was unclear whether the adduct is a sulfinamide or not. The labile *N*-hydroxylamine produced in liver may have difficulty in reaching erythrocytes because it binds rapidly to cytosolic proteins in liver or plasma proteins in blood.

Although 1-NP is considered to be carcinogenic [29, 30, 54, 55], a negative response has been reported recently [56]. Such a discrepancy seems to suggest the weakness of carcinogenicity, and it may be closely related to the unique behavior of plasma binding as observed in this experiment. Accordingly, clarification of the mechanism of the plasma binding

of 1-NP and 1-AP is an interesting and important subject, which is currently under investigation.

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