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STUDIES ON THE MECHANISM OF METHEMOGLOBIN FORMATION INDUCED BY AMINOAZO COMPOUNDS

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Abstract—Methemoglobinemia is induced by aminoazo dyes in rats of the Sprague–Dawley strain. 4-Aminoazobenzene (AB) induces about 66·6% of methemoglobin (MHb) by i.p. injection of a dose of $3{\cdot}23 \times 10^{-4}$ moles/kg, while in the same dose, *N*-methyl- and *N*,*N*-dimethyl-4-aminoazobenzene cause 51·7 and 22·3% maximum levels of MHb respectively. The MHb-forming activity of the metabolites of AB in rats and in isolated rat erythrocytes has been investigated. *N*-OH-AB seems to be the most active metabolite of AB in causing methemoglobinemia in rats and in inducing MHb in isolated erythrocytes. The reductive cleavage products of AB (aniline, *p*-aminophenol and *p*-phenylenediamine) are not the main MHb formers in the early stage, but they may contribute partially to the MHb concentration in the later period of methemoglobinemia induced by AB *in vivo*. The ring-hydroxylated metabolites of AB, such as 4′-OH-AB and 3-OH-AB, also have MHb-forming activity, but are not so active as *N*-OH-AB. *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene and *N*,*N*-dimethyl-4-aminoazobenzene-*N*-oxide are quite active in inducing MHb in the isolated rat erythrocytes.

WHEN AMINOAZO dyes were administered to rats of the Sprague–Dawley strain by intraperitoneal injection, the eyeballs, mucous membranes and toe-tips of the animals eventually developed typical symptoms of cyanosis. The cyanotic symptoms suggested that certain hematological changes in the animals were induced and prompted us to study the MHb-forming ability of these dyes.

In a previous communication[1] the authors have shown that aminoazo dyes are highly active in inducing MHb in rats and have proposed that the *N*-hydroxy derivatives of these dyes seem to be one of the major reactive metabolites in producing the blood pigment *in vivo*. In order to identify the real reactive metabolite, further study on the MHb-forming ability of these compounds in a well-defined *in vitro* system is required. In this study, the MHb-inducing ability of various aminoazo dye metabolites was tested in the isolated rat erythrocytes and in the intact animals. Attempts were made to correlate the MHb-inducing ability of aminoazo compounds *in vivo* and *in vitro* and to elucidate the mechanisms of MHb formation induced by these compounds.

EXPERIMENTAL

A. *Chemicals.* 4-Aminoazobenzene (AB),[2] *N*-methyl-4-aminoazobenzene (MAB),[3] *N*,*N*-dimethyl-4-aminoazobenzene (DAB),[4] *N*-benzoyloxy-*N*-methyl-4-aminoazobenzene (*N*-benzoyloxy-MAB),[5] *N*,*N*-dimethyl-4-aminoazobenzene-*N*-oxide (DAB-*N*-oxide),[6] *p*-aminophenol,[7] *N*-phenylhydroxylamine,[8] 3-hydroxy-4-aminoazobenzene

(3-OH-AB),[9] and 4'-hydroxy-4-aminoazobenzene (4'-OH-AB)[10] were prepared as described in the respective literature cited.

N-hydroxy-4-aminoazobenzene (*N*-OH-AB) was synthesized by the method of Sato *et al.*[11] except that 4-nitroazobenzene was prepared by direct nitration of azobenzene. Azobenzene (0·8 g) in 5 ml of glacial acetic acid was kept at 60° with magnetic stirring, and 1·6 ml of nitrating agent [nitric acid (s.g. 1·42):conc. H_2SO_4 (1:1, v/v)] was added dropwise. The reaction mixture was kept at 60° for an additional 30 min and then red–orange products were collected by filtration and washed with 1% ammonia water (10 ml). The crude product was further purified by Norit absorption and then Alumina column chromatography with hexane–toluene (8:2) as eluting solvent. The expected product which appeared in the first fraction was collected, evaporated to a small volume *in vacuo*, and cooled in a refrigerator for 2 hr. The orange crystals of 4-nitroazobenzene were obtained; m.p. 131–2°; (*lit.*[12]); 133–4°; yield, 80–85 per cent of theory.

B. *Estimation of methemoglobin induced* in vivo. All tested compounds were suspended in peanut oil by homogenization and were injected intraperitoneally into rats of the Sprague–Dawley strain weighing 200–300 g. After the tested compounds were injected, about 0·5 ml of blood was collected from the rats at a given time interval by heart puncture under ether anesthesia. The collected blood was immediately transferred to a tube containing EDTA (0·1 mg/ml of blood) as anticoagulant and was mixed thoroughly by gentle swirling. The concentration of MHb in the blood sample was determined as soon as possible according to the spectrophotometric method of Evelyn and Malloy[13] with slight modification. The following procedure was employed. Fresh whole blood (0·1 ml) was diluted to 10 ml with $1{\cdot}7 \times 10^{-2}$ M phosphate buffer (pH 6·6) in a test tube. After standing at room temperature (25°) for 5 min, the optical density of the resulting solution was read at 635 nm (D_1) with the same buffer as a blank. Then a drop of freshly prepared neutralized sodium cyanide (prepared by mixing an equal volume of 10% sodium cyanide and 12% acetic acid) was added to convert the MHb in the blood sample to cyanomethemoglobin. After 2 min, a second reading was taken at 635 nm (D_2). To the treated solution a drop of concentrated ammonium hydroxide was added for clarification. Two ml of the resulting solution was pipetted into a solution of 8 ml of $6{\cdot}8 \times 10^{-2}$ M phosphate buffer (pH 6·6) and one drop of 20% potassium ferricyanide. After standing for 2 min, all of the hemoglobin (Hb) was converted to MHb. One drop of 10% sodium cyanide was added to convert the MHb to cyanomethemoglobin. After standing for 2 min, the optical density was read at 540 nm (D_3) with $6{\cdot}8 \times 10^{-2}$ M phosphate buffer containing one drop each of 20% potassium ferricyanide and 10% sodium cyanide as a blank. The concentration of MHb and total Hb in blood samples were calculated according to the following equations:

$$\text{MHb (g/100 ml of blood)} = (D_1 - D_2) \times \text{Fm}$$

$$\text{Hb (g/100 ml of blood)} = D_3 \times \text{Ft.}$$

Fm and Ft represent the calibration factors which were estimated from 12 normal rat blood samples according to the procedure described by Hawk *et al.*[14] and were found to have average values of 52·0 and 72·3 respectively.

C. *Estimation of MHb induced* in vitro. Blood freshly drawn from rats by heart puncture under ether anesthesia was centrifuged at 500 *g* for 10 min and then the plasma and buffy coat were removed. The erythrocytes were then washed twice with isotonic Krebs–Ringer phosphate buffer (pH 7·4) containing 0·2% glucose.[15] After washing, the erythrocytes were suspended in an equal volume of the same buffer. Aliquots (0·9 ml) of the suspension were taken and incubated at 37° under air with 0·1 ml of various tested compounds dissolved in dimethyl sulfoxide at a concentration of 10^{-2} M or 10^{-3} M. At each given time interval (1, 5, 10, 20, 30, 60, 90 and 120 min), 0·1 ml of the incubation mixture was drawn out and the concentration of MHb in the blood sample was determined according to the procedure described in Section B.

RESULTS

The induction of MHb by aminoazo dyes and their metabolites in rats is summarized in Table 1. Some of these data were described previously.[1] The primary aminoazo compound AB was more active in inducing MHb as compared to the secondary and tertiary aminoazo compounds like MAB and DAB, but the effects of the latter two dyes were more persistent. The ring-hydroxylated metabolite, 4′-OH-AB was moderately active, while its *N*-acetylated product, *N*-acetyl-4′-OH-AB was less active indicating that the *para*-hydroxy group was not important for the activity. This was further supported by the fact that *p*-aminophenol was less active than its parent compound aniline; furthermore, the amino group of the aminoazo dye was essential for its MHb-forming activity. Methylation or acetylation of the amino group reduces the activity as demonstrated in MAB, DAB or *N*-acetyl-4′-OH-AB (Table 1). *N*-hydroxylation of the amino group seems to be a necessary step for the metabolic activation of AB, since *N*-OH-AB induced nearly the same level of MHb as did AB. *N*-phenylhydroxylamine was more active than aniline, suggesting that the metabolic conversion of the latter in rat occurs through multiple pathways. *N*-hydroxylation may account for only a small fraction of aniline metabolism as judged from its MHb-forming ability. The patterns of MHb formation induced by AB, *N*-OH-AB, 4′-OH-AB and *N*-phenylhydroxylamine are given in Table 1. It is apparent that both the magnitude and induction peak are very similar in the aminoazo dyes AB and *N*-OH-AB.

For the investigation *in vitro* rat erythrocytes were isolated and incubated with various aminoazo compounds and the results are summarized in Tables 2 and 3. As shown in Table 2, aniline was inactive, whereas *p*-aminophenol was quite active. It is worthy to note that *p*-phenylenediamine alone had no significant effect on MHb induction but had a strong inhibitory effect when it was incubated together with *p*-aminophenol. *N*-phenylhydroxylamine (10^{-3}) induced the formation of 61·2% MHb in 1 min and reached a plateau of 85% within 20 min. The kinetics of MHb formation induced by *p*-aminophenol and *N*-phenylhydroxylamine are quite different. The former induces the formation of MHb slowly and reaches a plateau at 90 min, while the latter is a fast-acting compound.

DAB, MAB and AB did not produce MHb *in vitro* as shown in Table 3. 4′-OH-AB and 3-methoxy-AB were less active, whereas 3-OH-AB was moderately active in a short period and quite active after a 90-min incubation. *N*-OH-AB was highly active and its MHb-inducing ability was dose-dependent. The patterns of MHb formation

TABLE 1. INDUCTION OF METHEMOGLOBIN BY AMINOAZO DYES AND THEIR METABOLITES IN RATS

Compounds* (moles/kg)	Methemoglobin (% of total hemoglobin)†			
	1 hr	4 hr	7 hr	10 hr
AB ($3{\cdot}23 \times 10^{-4}$)	66·6 ± 5·2 (10)	44·5 ± 6·2 (5)	28·3 ± 5·2 (5)	7·2 ± 2·6 (5)
MAB ($3{\cdot}23 \times 10^{-4}$)	45·6 ± 4·2 (5)	38·7 ± 3·6 (5)	39·2 ± 4·2 (5)	30·2 ± 7·5 (5)
DAB ($3{\cdot}23 \times 10^{-4}$)	5·2 ± 2·1 (5)	21·0 ± 3·4 (5)	22·5 ± 3·6 (5)	18·3 ± 3·5 (5)
4′-OH-AB ($3{\cdot}23 \times 10^{-4}$)	27·9 ± 5·9 (7)	4·9 ± 0·8 (7)	4·6 ± 1·1 (7)	1·4 ± 1·4 (7)
N-Ac-4′-OH-AB ($3{\cdot}23 \times 10^{-4}$)	7·9 ± 2·9 (6)	3·6 ± 0·6 (6)	6·3 ± 0·11 (5)	2·5 ± 2·1 (5)
Aniline ($3{\cdot}23 \times 10^{-4}$)	22·3 ± 2·6 (6)	2·1 ± 0·6 (6)	1·2 ± 0·8 (5)	0·3 ± 0·2 (5)
p-Aminophenol ($3{\cdot}23 \times 10^{-4}$)	4·7 ± 3·0 (10)	6·2 ± 1·8 (6)	5·7 ± 3·5 (6)	7·8 ± 1·9 (6)
p-Phenylenediamine ($3{\cdot}23 \times 10^{-4}$)	3·7 ± 1·0 (9)	1·4 ± 0·6 (9)	3·8 ± 1·4 (9)	3·6 ± 1·5 (6)
Aniline ($3{\cdot}23 \times 10^{-4}$) + *p*-phenylenediamine ($3{\cdot}23 \times 10^{-4}$)	18·5 ± 1·4 (4)	17·2 ± 3·5 (4)	3·5 ± 1·3 (4)	5·6 ± 3·8 (4)
p-Aminophenol ($3{\cdot}23 \times 10^{-4}$) + *p*-phenylenediamine ($3{\cdot}23 \times 10^{-4}$)	2·1 ± 0·9 (8)	4·0 ± 1·4 (6)	2·1 ± 1·8 (6)	2·3 ± 1·0 (5)
N-phenylhydroxylamine ($3{\cdot}23 \times 10^{-4}$)	73 ± 6·8 (5)	15 ± 4·2 (5)		
N-phenylhydroxylamine ($1{\cdot}6 \times 10^{-4}$)	63·8 ± 7·2 (6)	7·3 ± 3·1 (6)		
N-OH-AB ($3{\cdot}23 \times 10^{-4}$)	63·0 ± 3·2 (8)	12 ± 3·0 (8)	6·8 ± 2·2 (5)	3·6 ± 2·0 (5)

* All compounds were suspended by homogenization in 0·5 ml of peanut oil and injected intraperitoneally into male rats of the Sprague–Dawley strain weighing 250–300 g. AB, 4-aminoazobenzene; MAB, *N*-methyl-4-aminoazobenzene; DAB, *N*,*N*-dimethyl-4-aminoazobenzene; 4′-OH-AB, 4′-hydroxy-4-aminoazobenzene; *N*-AC-4′-OH-AB, *N*-acetyl-4′-OH-AB; and *N*-OH-AB, *N*-hydroxy-4-aminoazobenzene.

† The methemoglobin induced was measured by the method described in the Experimental section. The values given are means ± the standard error of the mean; the numbers in parentheses indicate the number of rats used.

TABLE 2. INDUCTION OF METHEMOGLOBIN BY AMINOAZO DYE METABOLITES IN ISOLATED RAT ERYTHROCYTES

Compounds* (M)	No. of expts.	Methemoglobin (% of total hemoglobin)† 1 min	5 min	10 min	20 min	30 min	60 min	90 min	120 min
Aniline (10^{-3})	4	0	0	0	0	0	0	0	0
p-Phenylenediamine (10^{-3})	4	2·0 ± 1·8	1·2 ± 0·5	1·8 ± 0·1	1·8 ± 0·1	2·4 ± 0·7	0·5 ± 0·5	3·9 ± 0·9	3·9 ± 0·9
p-Aminophenol (10^{-3})	5	7·5 ± 1·1	20·4 ± 3·6	25·5 ± 1·4	33·6 ± 1·4	44·1 ± 3·5	60·9 ± 3·0	64·0 ± 0·4	64·3 ± 4·4
Aniline (10^{-3}) + *p*-phenylenediamine (10^{-3})	4	1·6 ± 0·8	0·6 ± 0·9	1·1 ± 0·4	1·9 ± 1·2	4·8 ± 0·6	6·6 ± 0·4	10·4 ± 0·1	6·6 ± 2·4
p-Aminophenol (10^{-3}) + *p*-phenylenediamine (10^{-2})	4	4·0 ± 0·7	2·3 ± 0·7	5·7 ± 0·7	11·1 ± 1·0	13·5 ± 2·3	13·4 ± 0·6	14·7 ± 1·4	15·2 ± 1·9
N-phenylhydroxylamine (10^{-3})	6	61·2 ± 10·2	70·1 ± 11·2	76·2 ± 12·6	85·0 ± 9·2	83·1 ± 10·17	80·0 ± 9·6	78·5 ± 10·5	75·6 ± 7·6
N-phenylhydroxylamine (10^{-4})	3	23·1 ± 4·2	37·1 ± 3·8	45·2 ± 2·6	48·3 ± 5·1	46·3 ± 2·5	40·3 ± 2·5	35·2 ± 0·8	30·0 ± 4·6

* All compounds were dissolved in dimethyl sulfoxide (10^{-2} or 10^{-3} M) and 0·1-ml aliquots of the solutions were taken and incubated with 0·9 ml of isolated rat erythrocytes suspended in Krebs–Ringer phosphate–glucose buffer (pH 7·4) at 37°. For control, 0·1 ml of dimethyl sulfoxide was incubated with 0·9 ml of the erythrocyte suspension, no MHb formation could be detected throughout the whole incubation period in the control experiment.

† At each time interval, the formation of MHb was measured by the method described in the text. The values given are means ± the standard error of the mean.

TABLE 3. INDUCTION OF METHEMOGLOBIN BY AMINOAZO DYES AND THEIR METABOLITES IN ISOLATED RAT ERYTHROCYTES

Compounds* (M)	No. of expts.	Methemoglobin (% of total hemoglobin)† 1 min	5 min	10 min	20 min	30 min	60 min	90 min	120 min
DAB (10^{-3})	3	0	0	0	0	0	0	0	0
MAB (10^{-3})	3	0	0	0	0	0	0	0	0
AB (10^{-3})	6	0	0	0	0	0	0	0	0
4′-OH-AB (10^{-4})	3	2·1 ± 0·1	5·0 ± 0·2	8·5 ± 2·5	9·0 ± 2·4	9·1 ± 2·5	9·5 ± 2·5	9·6 ± 2·7	10·0 ± 2·0
3-OH-AB (10^{-4})	4	9·8 ± 2·1	13·5 ± 3·8	17·1 ± 3·1	22·2 ± 4·1	29·5 ± 4·0	36·2 ± 4·6	40·0 ± 5·0	42·1 ± 4·8
3-Methoxy-AB (10^{-4})	4	0	1·3 ± 0·7	0·3 ± 0·2	2·2 ± 0·3	1·3 ± 0·6	2·1 ± 0·7	0	1·6 ± 0·4
N-OH-AB (10^{-4})	8	22·1 ± 3·1	30·3 ± 3·4	34·5 ± 4·0	35·0 ± 3·6	32·5 ± 2·8	27·5 ± 3·1	23·1 ± 5·2	22·0 ± 5·4
N-OH-AB (10^{-3})	9	38·6 ± 2·6	57·3 ± 4·2	66·7 ± 4·6	64·2 ± 5·2	60·1 ± 6·3	48·5 ± 4·3	38·6 ± 5·3	27·5 ± 5·8

* DAB, *N*,*N*-dimethyl-4-aminoazobenzene; MAB, *N*-methyl-4-aminoazobenzene; AB, 4-aminoazobenzene; 3-OH-AB, 3-hydroxy-4-aminoazobenzene; 4′-OH-AB, 4′-hydroxy-4-aminoazobenzene; 3-methoxy-AB, 3-methoxy-4-aminoazobenzene; and *N*-OH-AB, *N*-hydroxy-4-aminoazobenzene. The experimental conditions are as described in Tab.e 2.

† As described in Table 2.

induced by 4′-OH-AB, 3-OH-AB and *N*-OH-AB are given in Table 3. The pattern of MHb formation induced by *N*-phenylhydroxylamine (Table 2) is also given for comparison. A very rapid induction of MHb formation *in vitro* was noted in the case of *N*-hydroxy derivatives, whereas a rather slow induction was observed for ring-hydroxy derivatives. A decline in MHb formation was indicated after incubation of *N*-OH-AB and *N*-phenylhydroxylamine with isolated rat erythrocytes for 30 min. The same phenomenon was absent in the system of 4′-OH-AB and 3-OH-AB. This difference may be due to the different stability of these compounds under the experimental conditions, since it has been shown that *N*-OH-AB is extremely thermolabile.[11]

While the synthesis of *N*-hydroxy-*N*-methyl-4-aminoazobenzene (*N*-OH-MAB) has not been achieved, an ester of this compound, *N*-benzoyloxy-MAB, has been prepared.[5,16] Theoretically, hydrolysis of this ester under mild conditions should give some *N*-OH-MAB in the medium and initiate various types of reactions. When *N*-benzoyloxy-MAB was incubated with isolated rat erythrocytes, a remarkable MHb-forming activity was detected. At a concentration of 10^{-5} M, it caused 13·1 per cent of MHb formation, then gradually decreased to 7·1 per cent at the end of incubation (Fig. 1, D). But when the concentration was increased to 10^{-4} M, the formation of MHb quickly increased to 34·2 per cent, then gradually attained a maximum level (47·8 per cent) at the end of incubation (Fig. 1, B). The peak levels built up by *N*-benzoyloxy-MAB at 10^{-4} M or 10^{-5} M are very near to the peak level built up by MAB *in vivo* (Table 2). It is assumed that the early MHb induced by MAB *in vivo* may derive from the action of *N*-OH-MAB or its *N*-hydroxy ester, possibly MAB-*N*-sulfate.

DAB-*N*-oxide is considered by Terayama[17] to be an intermediate metabolite of DAB preceding *N*-demethylation and *ortho*-hydroxylation. However, there is still no

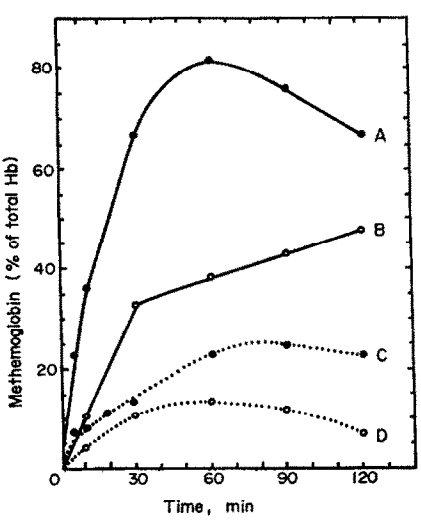

FIG. 1. Induction of methemoglobin by *N*-benzoyloxy-MAB and DAB-*N*-oxide in isolated rat erythrocytes. Methemoglobin was induced and determined as described in the Experimental section C. The data indicate the means of three to five experiments; the standard error of the mean was always less than 10 per cent of each mean value. Curve A: DAB-*N*-oxide, 10^{-3} M; curve B: *N*-benzoyloxy-MAB, 10^{-4} M; curve C: DAB-*N*-oxide, 10^{-4} M; and curve D: *N*-benzoyloxy-MAB, 10^{-5} M.

direct evidence that this compound is formed *in vivo*. When DAB-*N*-oxide was incubated with rat erythrocytes, a remarkably high level of MHb was induced. At a concentration of 10^{-3} M, DAB-*N*-oxide induced the formation of 36 per cent MHb in 10 min and reached a peak of 82 per cent in 60 min (Fig. 1, A); at the lower concentration (10^{-4} M), the compound induced far less MHb formation (Fig. 1, C). It is apparent that the reaction of DAB-*N*-oxide with Hb is quite slow, since it reaches its maximum level within 1 hr, while *N*-OH-AB and *N*-phenylhydroxylamine reach their maximum levels within 10–20 min (Tables 3 and 2 respectively).

DISCUSSION

The present and previous studies have demonstrated that the aminoazo dyes, DAB, MAB and AB, have MHb-forming activity *in vivo* and have no detectable activity *in vitro*. Thus, they all must be metabolically transformed to active derivatives to be capable of oxidizing Hb. Since most processes of metabolism of DAB and MAB are channeled to that of AB,[18] our efforts were centered on the methemoglobinemia induced by AB. The major metabolic pathways of AB in the rat are summarized in Fig. 2. According to Miller and Miller,[18] AB is metabolically cleaved to aniline and

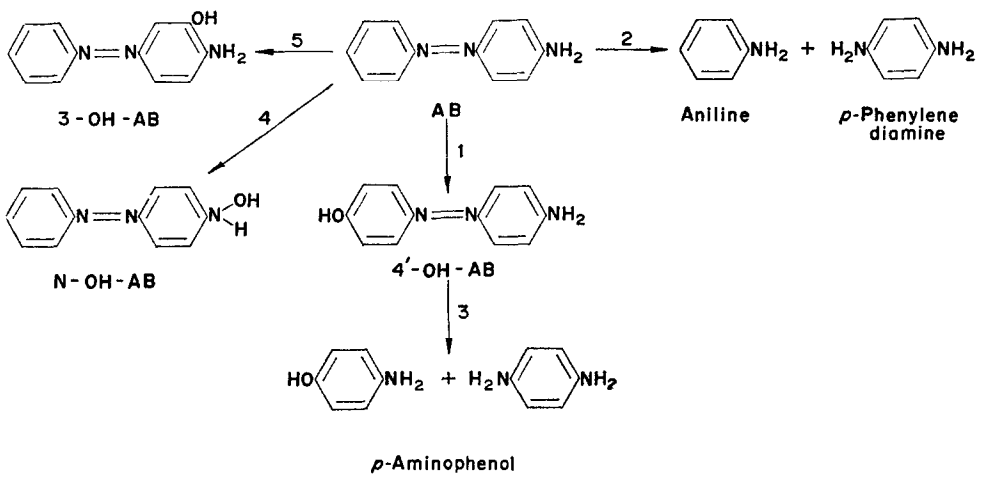

FIG. 2. Major metabolic pathways of 4-aminoazobenzene in rats. The metabolism of aminoazo compounds has been extensively discussed by Miller and Miller.[18] DAB was demethylated stepwise to MAB and AB; in addition to 4′-hydroxylation, 2-, 3-, 2′- and 3′-hydroxylation of AB were also detectable. *p*-Hydroxylation of aniline led to *p*-aminophenol.

p-phenylenediamine, and aniline is further metabolized to *p*-aminophenol and *o*-aminophenol with a molar ratio of approximately 6.[19] The major ring-hydroxylated metabolite of AB, 4′-OH-AB, can be reduced to *p*-aminophenol and *p*-phenylenediamine by azo reductase (Fig. 2, reaction 3). The above two pathways account for the presence of large amounts of conjugated forms of *p*-aminophenol and *p*-phenylenediamine in rat urine after the administration of AB. Although *p*-aminophenol and *p*-phenylenediamine are produced in large quantities, they cannot contribute to the major part of methemoglobin induced by AB at the early period (Table 1). The studies *in vitro* with isolated rat erythrocytes also led to a similar conclusion (Table 2).

The *N*-hydroxy derivatives of several aromatic amines are considered to be the major metabolites which are active in producing MHb after the absorption of aromatic amines.[20] In the present experiment, it seems that the initial step in the metabolism of AB after injection into the rat may be *N*-hydroxylation (Fig. 2, reaction 4), since the time course and quantity of MHb induced by *N*-OH-AB and AB in the early stage are quite similar. The *N*-hydroxylation may occur about 10–20 min after the injection of AB, since the study of MHb-forming activity of *N*-OH-AB *in vitro* showed that it attained 39·1, and 21·3% MHb after only a 1-min incubation with 10^{-3} M and 10^{-4} M of *N*-OH-AB respectively (Table 3). The data *in vivo* indicate that AB requires about 10–20 min to attain this range of MHb.

The studies *in vitro* demonstrate that 3-OH-AB is more active than 4'-OH-AB. However, the ring-hydroxylation of aminoazo dyes occurs mainly at the 4'-position[21] and the concentration of 3-OH-AB may be not significantly great *in vivo*. It has been proposed[22] that 4'-hydroxy compounds might form through the *p-p'* intramolecular rearrangement of their *N*-hydroxy derivatives. If this is true for AB metabolism, 4'-OH-AB has to be derived from *N*-OH-AB.

As reported by Meyer,[23] Sieburg,[24] Baader *et al.*[25] and Kiese,[26,27] *N*-hydroxy derivatives can be readily reduced to original amines or transformed to other inactive or less active metabolites in blood or other tissues and thus rapidly inactivate the MHb-forming activity of *N*-hydroxy derivatives. This fact may well explain the short-action in formation of MHb by *N*-OH-AB and *N*-phenylhydroxylamine *in vivo*. Thus it is clear that the intensity and the full extent of action of a MHb-forming agent *in vivo* is determined not only by the rate of its enzymatic production from the parent amine and its intrinsic activity, but also by its metabolic change to inactive or less active derivatives.

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