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Methemoglobin—Induced by carcinogenic aminoazo dyes in rats

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WHEN a single dose ($3{\cdot}23 \times 10^{-4}$ moles/kg of body wt) of 4-aminoazobenzene (AB) was administered intraperitoneally to rats of the Sprague–Dawley strain, the animals eventually developed typical symptoms of cyanosis, as revealed by color changes in the eyeball, mucous membrane and tip of the toe, and occasionally by the presence of exertional dyspnea. Less severe symptoms were also observed in rats given the hepatocarcinogens, *N*-monomethyl-4-aminoazobenzene (MAB) or *N,N*-dimethyl-4-aminoazobenzene (DAB). The cyanotic symptoms suggest that certain hematological changes in the animals were induced by these dyes. Many arylamino and nitro compounds have been shown to be highly active in forming methemoglobin (MHb).[1,2] This prompted us to study the MHb-forming ability of aminoazo dyes.

The concentration of MHb in the blood of rats treated with AB, MAB or DAB was determined by the method of Evelyn and Malloy.[3] The results are given in Fig. 1. Aniline, *N*-monomethylaniline and *N,N*-dimethylaniline were used as controls for the purpose of comparison, since they have identical functional groups with AB, MAB and DAB respectively. With the same molar concentration ($3{\cdot}23 \times 10^{-4}$ moles/kg), the maximum levels of MHb induced by AB, MAB, DAB, aniline, *N*-mono-

methylaniline and *N,N*-dimethylaniline were 70, 46, 22, 22, 37 and 2·9 per cent of total hemoglobin (Hb) respectively. It is apparent that aminoazo compounds (Fig. 1, A, B and C) are more active and lasting in forming MHb as compared to the corresponding amino compounds (Fig. 1, D, E and F). The visible electronic absorption spectrum (from 400 to 700 nm) of MHb produced *in vitro* with potassium ferricyanide and that produced in the rat with an aminoazo compound are identical, indicating that the blood pigment formed in the blood of treated rats is indeed MHb.

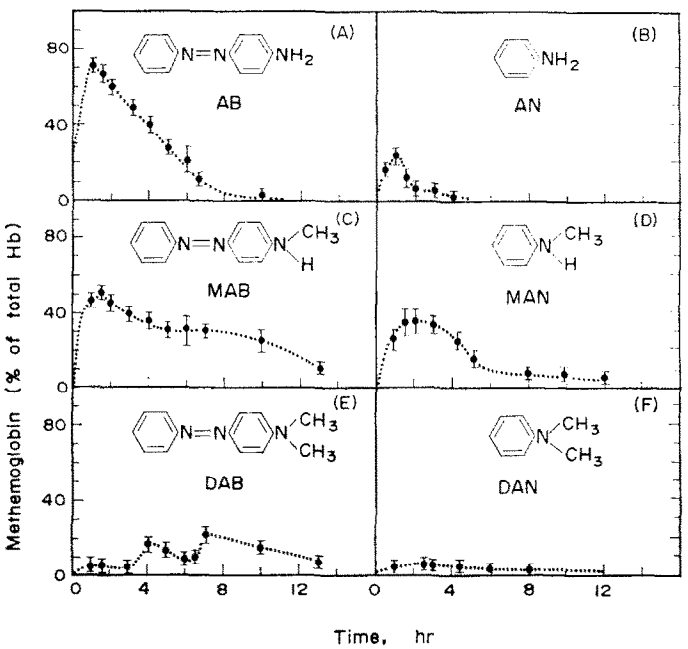

FIG. 1. Formation of methemoglobin by aminoazo compounds and amines in rats. The concentration of methemoglobin (as percentage of total hemoglobin) in the blood of rats injected i.p. with the following compounds ($3{\cdot}23 \times 10^{-4}$ moles/kg of body weight) in 0·5 ml peanut oil were determined: AB, 4-aminoazobenzene (A); MAB, *N*-monomethyl-4-aminoazobenzene (B); DAB, *N,N*-dimethyl-4-aminoazobenzene (C); AN, aniline (D); MAN, *N*-monomethylaniline (E); and DAN, *N,N*-dimethylaniline (F). Male albino rats (Sprague–Dawley strain) weighing 200–260 g were used. After the tested compounds were injected, the blood (0·5–0·7 ml) of rats was collected at a given time interval by heart puncture under ether anesthesia. The blood was transferred to an oxalated tube (potassium oxalate, 1·5 mg/ml of blood) and mixed thoroughly by gentle swirling. The concentration of methemoglobin in the collected sample was determined within 20 min, according to the spectrophotometric method of Evelyn and Malloy,[3] and calculated.[4] The symbols (●) indicate the means of three to five experiments; the vertical lines(I) show the standard error.

Since most arylamino and nitro compounds do not readily oxidize Hb *in vitro*, it is apparent that the formation of MHb *in vivo* must be accomplished through some metabolic activations.[1] No MHb could be detected *in vitro* when whole blood was directly incubated with AB, MAB or DAB at 37° for 1 hr. This suggests that aminoazo compounds must also be metabolically activated before they can produce MHb *in vivo*.

Phenylhydroxylamine and nitrosobenzene have been considered to be the active metabolites of aniline and *N*-monomethylaniline in producing MHb *in vivo*.[5] The reaction of phenylhydroxylamine with Hb and oxygen in red blood cells is a coupled oxidation that yields MHb and nitrosobenzene; the latter compound can be enzymatically reduced to phenylhydroxylamine in the presence of NADPH to complete a cycle.[6] It seems that different microsomal enzymes are responsible for *N*-hydroxylation of aniline and *N*-alkylaniline.[5] This may explain the different patterns of MHb formation from aniline and *N*-monomethylaniline (Fig. 1, D and E).

The metabolism of carcinogenic aminoazo dyes has been reviewed by Miller and Miller.[7] DAB, MAB and AB were cleaved by azo-reductase to the common product, aniline, and respectively to

N,*N*-dimethyl-*p*-phenylenediamine, *N*-monomethyl-*p*-phenylenediamine and *p*-phenylenediamine. *p*-Phenylenediamine was not quite active in producing MHb at $3{\cdot}23 \times 10^{-4}$ moles/kg, whereas *N*-monomethyl- and *N*,*N*-dimethyl-*p*-phenylenediamine were so toxic that the tested animals could not tolerate them at the same molar dose. In a smaller dose of $1{\cdot}08 \times 10^{-4}$ moles/kg, the latter two diamines induced 4·3 and 2·2 percent of MHb respectively; with the same dose, MAB induced 28·4 per cent of MHb. It is apparent that the major MHb-forming activity of the aminoazo compounds is not due to their reductive cleavage products. The data given in Table 1 seem to support this point of view.

TABLE 1. INDUCTION OF METHEMOGLOBIN BY 4-AMINOAZOBENZENE AND ITS METABOLITES IN RATS

Compounds* (moles/kg)	Methemoglobin (% of total Hb)† 1 hr	4 hr
4-Aminoazobenzene ($3{\cdot}23 \times 10^{-4}$)	66·6 ± 5·2 (10)	44·5 ± 6·2 (5)
4-Aminoazobenzene ($1{\cdot}61 \times 10^{-4}$)	53·1 ± 3·1 (4)	39·6 ± 2·6 (4)
4-Aminoazobenzene ($8{\cdot}0 \times 10^{-5}$)	48·9 ± 4·8 (4)	31·4 ± 3·7 (4)
4-Aminoazobenzene ($4{\cdot}0 \times 10^{-5}$)	24·6 ± 3·0 (3)	6·2 ± 0·2 (3)
4-Aminoazobenzene ($2{\cdot}0 \times 10^{-5}$)	18·5 ± 1·1 (3)	2·2 ± 0·2 (3)
Aniline ($3{\cdot}23 \times 10^{-4}$)	22·3 ± 2·6 (6)	2·1 ± 0·6 (6)
p-Aminophenol ($3{\cdot}23 \times 10^{-4}$)	4·0 ± 1·1 (9)	2·5 ± 1·4 (6)
p-Phenylenediamine ($3{\cdot}23 \times 10^{-4}$)	3·7 ± 1·0 (9)	1·4 ± 0·6 (6)
Aniline ($3{\cdot}23 \times 10^{-4}$) + *p*-phenylenediamine ($3{\cdot}23 \times 10^{-4}$)	18·5 ± 2·1 (4)	17·2 ± 1·7 (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)
4′-Hydroxy-4-aminoazobenzene ($3{\cdot}23 \times 10^{-4}$)	25·4 ± 2·6 (6)	4·9 ± 0·8 (5)
N-acetyl-4′-hydroxy-4-amino-azobenzene ($3{\cdot}23 \times 10^{-4}$)	9·0 ± 2·9 (4)	3·5 ± 0·6 (4)
N-hydroxy-4-aminoazobenzene ($3{\cdot}23 \times 10^{-4}$)	56·5 ± 2·1 (5)	16·6 ± 3·5 (4)

* All compounds were homogeneously suspended in 0·5 ml of peanut oil and injected intraperitoneally into male rats of the Sprague–Dawley strain weighing 250–300 g.

† The methemoglobin induced was measured by the method described in Fig. 1. The values given are means ± the standard error of the mean; the numbers in parentheses indicate the number of rats used.

The MHb-forming activity of AB is dose-dependent and is about ten times as active as that of aniline. Both reductive cleavage products of AB, *p*-phenylenediamine and *p*-aminophenol, are not quite active. Tests on the combination of aniline and *p*-phenylenediamine or of *p*-aminophenol and *p*-phenylenediamine did not show their additive effect in producing MHb; the reason for this is not clear. 4′-Hydroxy-4-aminoazobenzene is the major ring-hydroxylated metabolite of AB[7] and is moderately active, while its *N*-acetylated derivative is far less active. It is of interest to note that *N*-hydroxy-4-aminoazobenzene (N-OH-AB), the *N*-hydroxylated metabolite of AB, is highly active in producing MHb. As mentioned in Table 1, at the 1-hr period, the level of MHb produced by AB (66·6 per cent) is slightly higher than that produced by N-OH-AB (56·5 per cent). When the formation of MHb was measured at the 0·5-hr period, AB and N-OH-AB induced 50 and 64 per cent of MHb respectively. This indicates that N-OH-AB is a rather fast-acting inducer and may be the reactive metabolite of AB *in vivo*. In order to evaluate this conclusion, futher studies on the MHb-forming ability of N-OH-AB *in vitro* are now in progress.

Demethylation is another important metabolic reaction of *N*-alkyl aminoazo compounds. DAB was demethylated stepwise by liver demethylase to MAB and then to AB.[8] Both of the demethylated products are more active in producing MHb and may account for the appearance of the later two peaks in the MHb formation curve of DAB (Fig. 1, C). DAB and MAB are equally active in inducing liver cancer in rats,[9] whereas AB is inactive[9] or very weak.[10] It is obvious that MHb formation of DAB and MAB is more lasting than that of AB, suggesting that the metabolic change of AB is faster than that of DAB and MAB. The difference of these patterns might be due to the different enzymes responsible for the metabolic conversion of these aminoazo compounds or to other factors such as transport from the peritoneal cavity and efficiency as substrates for the enzymes involved. *N*-hydroxyl-

ation has been suggested to play an important role in aminoazo dye carcinogenesis,[11] and it seems that the same reaction may also be an essential metabolic reaction in producing MHb *in vivo*.

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Conversion of a substituted 3,4,5-trimethoxycinnamide to a 3,5-dihydroxy compound in the rat and inhibition of the conversion by neomycin

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IN OUR study of the metabolism of carbon-14 labeled *N*-(3,3-dimethylpropynyl)-3,4,5-trimethoxycinnamide* in rats, there was isolated from the urine a metabolite identified by nuclear magnetic resonance (n.m.r.) and mass spectrometry as α-(3,5-dihydroxycinnamido)isobutyric acid. This compound accounted for approximately 12 per cent of the radioactivity found in glucuronidase-sulfatase-treated rat urine, and could be extracted by diethyl ether at pH 7. It is not known that any mammalian system is capable of transforming a trimethoxyphenyl structure into a dihydroxyphenyl structure. Yet the dihydroxy compound was radioactive and therefore must have been derived from the radioactive parent drug, and some means had to exist for this conversion. We thus decided to investigate the source of this unique transformation.

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