STUDIES ON SULFHEMOGLOBIN FORMATION BY VARIOUS DRUGS (5) THE MECHANISM OF SULFHEMOGLOBIN FORMATION AND SPECIES DIFFERENCES IN METHEMOGLOBIN AND SULFHEMOGLOBIN FORMATION

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Accepted March 15, 1981

Abstract—The mechanism of sulfhemoglobin (SHb) formation and species differences in the formation of SHb and methemoglobin (MHb) were investigated. Erythrocytes or hemolysates were incubated with phenylhydroxylamine (PHA), aniline or its monochloro derivatives in the presence or absence of liver microsomes from various species (mouse, rat, guinea pig, rabbit, cat, dog and monkey). It was confirmed that SHb was produced by the N-hydroxy metabolites of aniline and its derivatives and was not induced *via* MHb. There was a relationship in SHb or MHb formation between the chemical structure of aniline derivatives and their N-hydroxy metabolite production activities in the animals (*in vivo* and *in vitro*). Species differences in the production rates of N-hydroxy metabolites from aniline and its derivatives by liver microsomes (as well as in the susceptibility of erythrocytes to N-hydroxy compounds) *in vitro* were not reflected in species differences in MHb and SHb formation *in vivo*.

The observation that a N-(4-chlorophenyl) ethylurethane derivative caused typical sulfhemoglobinemia in animals led us to study the forming activities of methemoglobin (MHb) and sulfhemoglobin (SHb) by various compounds such as aniline, N-phenylanthranilic acid and N-(1-naphthyl) anthranilic acid derivatives, and reported the relationship between their MHb or SHb forming activity and chemical structures (1–3). In those studies, it was suggested that not only MHb but also SHb was produced by N-hydroxy metabolites of the aniline derivatives described above. Further it was suggested that SHb might be produced *via* MHb because SHb formation was remarkably

later than that of MHb. On the other hand, there is considerable evidence that SHb formation is not *via* MHb, for example, only SHb was produced by phenylurethane derivative (1) and the SHb formation was prevented by the pretreatment with ascorbic acid, which did not inhibit MHb formation (4).

The present study was designed to clarify MHb and SHb formation using mainly aniline and phenylhydroxylamine. We also investigated species differences in the formation of SHb and MHb.

MATERIALS AND METHODS

Animals used were as following: male ddY-Slc mice (25–34 g), male SD-Slc rats

(120–280 g) and male Hartley-STD guinea pigs (350–650 g) [Shizuoka Agric. Co-op. Assoc. for Laboratory Animals]; male JW-NIBS/RABITON rabbits (2.1–2.8 kg) [RABITON Farm. Inc.]; male and female cats (1.6–3.7 kg) [Nakajima Laboratory Animals Co.]; male beagle dogs (9–14 kg) [Laboratory Research Enterprises, Inc.] and male rhesus monkeys (5.3–6.1 kg) [Research Primates Japan Co.].

The chemicals used were as following: aniline hydrochloride (aniline), magnesium chloride, nicotinamide, phenylhydroxylamine (PHA), potassium ferricyanide, powder of sulfur and sodium sulfide [Wako Pure Chemical Industries]; 2-chloroaniline hydrochloride (2-Cl-A), 3-chloroaniline hydrochloride (3-Cl-A) and 4-chloroaniline hydrochloride (4-Cl-A) [Tokyo Chemical Industry Co.]; bovine serum albumin (Fraction V), glucose-6-phosphate disodium salt and glucose-6-phosphate dehydrogenase [Sigma Chemical Co.] and nicotinamide-adenine dinucleotide phosphate sodium salt (NADP) [Oriental Yeast Co.].

The amounts of MHb and SHb were measured by the modified method (1) of Evelyn and Malloy (5).

1) Experiments in MHb and SHb formation with intact erythrocytes and hemolysates *in vitro*

Fresh blood was drawn from healthy human beings and animals of various species described above. Erythrocytes were spun down and the plasma was discarded. Erythrocytes or hemolysates were incubated in M/15 phosphate buffer solution (pH 7.3–7.4) at 37°C for 30 minutes. Hemolysate solution was obtained by hemolysis of erythrocytes which was produced by adding hypotonic phosphate buffer solution (M/60), and then the final concentration of phosphates was adjusted to M/15 (isotonic). The final concentration of hemoglobin was adjusted to 10 g/dl both in erythrocyte suspension

and hemolysate solution.

a) MHb and SHb induced by PHA: In the experiments in MHb formation, erythrocyte suspension or hemolysate solution prepared from the blood of guinea pigs was incubated with various concentrations of PHA. For the experiments in SHb formation, erythrocyte suspension or hemolysate solution was incubated with various concentrations of PHA in the presence of sodium sulfide. An aliquot of each reaction mixture was taken for the measurement of MHb and SHb.

b) Incubation of MHb with PHA and sodium sulfide: MHb was prepared by treatment of the hemoglobin from guinea pigs with potassium ferricyanide. It was incubated with PHA (0.05 mM) in the presence of sodium sulfide (0.2 mM). The sample was taken from the incubation mixture for the measurement of absorption spectra.

c) MHb and SHb formation by PHA or aniline with hemoglobin of various animal species: Erythrocytes and hemolysates were prepared from fresh blood of various animal species by the methods described above and were incubated with PHA or aniline at a final concentration of 0.05 mM in the absence or presence of sodium sulfide (0.2 mM).

d) Hepatic microsomal N-oxidations of aniline, 2-Cl-A, 3-Cl-A and 4-Cl-A: Hepatic microsomal fractions from animals of various species were prepared by the method of Hogeboom (6). The content of microsomal protein was determined by the method of Gornall et al. (7), using bovine serum albumin as a standard.

According to the method of Hjelm and Verdier (8), in each case, 10 μ moles of aniline, 2-Cl-A, 3-Cl-A and 4-Cl-A were incubated with 5 ml of solution containing magnesium chloride (5.71 mg), nicotinamide (14.65 mg), NADP (2.01 mg), glucose-6-phosphate (16.32 mg), glucose-6-phosphate dehydrogenase (1.4 unit) and liver microsomes (0.125–0.5 mg of microsomal protein)

at 37°C for 60 minutes.

To each reaction mixture was added 5 ml of erythrocyte suspension from guinea pigs for another 30 minute incubation in the absence or presence of sodium sulfide (0.2 mM). The final concentration of hemoglobin in erythrocyte suspension was adjusted to 10 g/dl. A sample of each reaction mixture was taken for the determination of MHb and SHb.

2) Experiments in MHb and SHb formation and species differences therein *in vivo*

In order to determine MHb formation and species differences of MHb formation, animals of various species were given PHA, aniline, 2-Cl-A, 3-Cl-A and 4-Cl-A intraperitoneally. Blood was drawn at various intervals for the determination of MHb. In the case of SHb formation, animals were given PHA, aniline and 4-Cl-A intraperitoneally with powder of sulfur (750 mg/kg/day p.o.) for three consecutive days. The SHb levels were measured 48 hours after the final administration.

RESULTS

1. Experiments in MHb and SHb formation with intact erythrocytes and hemolysates *in vitro*

1)–1 MHb formation by PHA: The results are shown in Fig. 1. The formation of MHb increased, depending roughly on the final concentration of PHA in the range from 0.003 to 0.1 mM in erythrocyte suspension or hemolysate solution of guinea pigs. MHb formation reached a plateau over 0.1 mM PHA concentration. At the hemoglobin concentration used in this experiment, the maximum concentration of PHA was obtained as 0.1 mM for erythrocytes and hemolysates. MHb formation in erythrocyte suspension by PHA, at each concentration, was more potent than that in hemolysates, while SHb formation was not observed at all in the case of either erythrocytes or hemolysates.

1)–2 SHb formation by PHA: The results are shown in Fig. 2. When erythrocytes or hemolysates from guinea pigs were incubated

Fig. 1. Methemoglobin formation of guinea pig hemoglobin incubated with various concentrations of phenylhydroxylamine (PHA). Erythrocytes or hemolysates were incubated at 37°C for 30 min. The final concentration of hemoglobin was adjusted to 10 g/dl. Each value indicates the mean $\pm$ standard error of 3 experiments. *: Significantly different from levels obtained with hemolysate solution, $p < 0.01$ (Student's t-test).

Fig. 2. Methemoglobin and sulfhemoglobin formation of guinea pig hemoglobin incubated with various concentrations of phenylhydroxylamine (PHA) and sodium sulfide (Na₂S). Erythrocytes or hemolysates were incubated at 37°C for 30 min. The final concentration of hemoglobin was adjusted to 10 g/dl. Each value indicates the mean±standard error of 3 experiments. *: Significantly different from levels obtained with hemolysate solution, $P<0.01$ (Student's t-test).

with PHA in the presence of 0.1 mM sodium sulfide, SHb was induced in a dose dependent manner, increasing up to 0.1 mM and 0.05 mM PHA concentration for erythrocytes and hemolysates respectively. In erythrocytes, a sharp concentration-dependent decrease of SHb formation was observed at concentrations of over 0.2 mM PHA. In hemolysates, on the other hand, SHb formation at concentrations of over 0.1 mM was slightly decreased. A remarkable SHb formation was observed in erythrocytes compared to that in hemolysates.

When 0.05 mM PHA was added to the erythrocyte suspension under various concentrations of sodium sulfide, SHb formation was increased, depending on the amounts of

sodium sulfide. In other words, when less sodium sulfide was added, more MHb was produced and when large amounts of sodium sulfide were added, more SHb was produced. In the case of hemolysates, the increase of SHb was dependent on the sodium sulfide concentration up to 0.1 mM and SHb was decreased at over 0.2 mM sodium sulfide. Also, SHb formation was greater in erythrocytes than in hemolysates. In a separate experiment, unexpectedly, the amounts of MHb which had already been formed by PHA in the absence of sodium sulfide were not affected by post-treatment of sodium sulfide, namely, SHb was not produced unless the coexistence of PHA and sodium sulfide.

2) The incubation of MHb with PHA and

Fig. 3. Effects of phenylhydroxylamine (PHA) in the presence of sodium sulfide (Na_2S) on methemoglobin prepared from hemoglobin of guinea pigs. The absorption spectra of methemoglobin incubated with PHA in the presence of Na_2S . Figure insert—the absorption spectra of the mixture of sulfhemoglobin with methemoglobin or cyanmethemoglobin. Table insert—the incubation of methemoglobin with PHA in the presence of Na_2S did not lead to the sulfhemoglobin formation.

sodium sulfide: When sodium cyanide was added to a mixture of MHb and SHb, SHb remained unchanged with the characteristic absorption peak of 622 nm, as shown in the inset figure of Fig. 3, while the MHb absorption at 635 nm was eliminated due to the conversion of MHb to cyanmethemoglobin (Fig. 3). When MHb prepared from guinea pig hemoglobin was incubated with 0.05 mM PHA in the presence of 0.2 mM sodium sulfide, no SHb formation occurred from MHb, as shown in Fig. 3 (inset).

3) MHb and SHb formation by PHA or aniline with hemoglobin of various animal species: Erythrocyte suspension and

hemolysate solution were prepared from fresh blood of mice, rats, guinea pigs, rabbits, cats, dogs, monkeys and human beings, by the method described above. Each was incubated with 0.05 mM PHA or aniline in the absence or presence of sodium sulfide (0.2 mM). The results are shown in Fig. 4. Aniline, unlike PHA, showed no definite forming activity with regard to MHb and SHb in any animal species. PHA was more potent in the formation of MHb and SHb in erythrocytes than in hemolysates in all animal species, and the tendency was more remarkable in SHb formation compared with MHb formation.

In the cases of erythrocytes of dogs and cats, the level of MHb was slightly higher, and the level of SHb was lower, than that in other species.

4) N-Oxidation of aniline, 2-Cl-A, 3-Cl-A and 4-Cl-A by liver microsomes from various animal species: As mentioned above, PHA caused formation of both MHb and SHb, while aniline did not, thereby suggesting that SHb as well as MHb was produced through N-hydroxy metabolites of aniline, namely PHA. Accordingly, the levels of MHb and SHb were measured as an index of the production of N-hydroxy metabolites in a reaction mixture of aniline and its derivatives with liver microsomes. The results are shown in Figs. 5 and 6. The production of N-hydroxy metabolites from aniline was much lower in cats and monkeys and was markedly higher in guinea pigs. The same rank order was also observed in the production rate of N-hydroxy metabolites in the various species of animals, in the case of 4-Cl-A.

Making a comparison among aniline, 2-Cl-A, 3-Cl-A and 4-Cl-A in the case of mouse microsomes, the production rate of N-hydroxy metabolites of 4-Cl-A was highest, and was followed by 3-Cl-A, 2-Cl-A and aniline. In the case of cat microsomes, the rank order or production rate of N-hydroxy metabolites

Fig. 4. Species differences of methemoglobin and sulfhemoglobin formation induced by phenylhydroxylamine (PHA) or aniline (A) in the presence and absence of sodium sulfide (Na₂S). Erythrocytes or hemolysates were incubated at 37°C for 30 min. The final concentration of hemoglobin, PHA and Na₂S was adjusted to 10 g/dl, 0.05 mM and 0.2 mM, respectively. Each value indicates the mean±standard error of 3 experiments. *: Significantly different from levels obtained with hemolysate solution, p<0.01 (Student's t-test).

was 4-Cl-A>2-Cl-A>3-Cl-A>aniline.

2. Experiments on MHb and SHb formation and species differences therein *in vivo*

The results are shown in Figs. 7 and 8. After intraperitoneal administration of PHA, aniline and 4-Cl-A to mice, rats, guinea pigs, rabbits, cats and dogs, MHb formation was measured at various intervals. MHb formation by PHA, aniline and 4-Cl-A in guinea pigs was less prominent than in cats and dogs. The rank order of MHb formation activity of animals was completely reversed to that of *in vitro* experiments. For SHb formation, the three compounds showed no activity in guinea pigs, some activity in rabbits and a somewhat higher in cats. These results also differed to the results *in vitro*.

To elucidate the correlation between MHb formation and the chemical structure, MHb forming activities of aniline and its mono-chloro derivatives were investigated in mice and cats, *in vivo*. The rank order of MHb forming activity was 4-Cl-A>3-Cl-A>2-Cl-A>aniline in mice and 4-Cl-A>2-Cl-A>3-Cl-A>aniline in cats, such corresponding to the results *in vitro*.

DISCUSSION

1) Mechanism of SHb formation: MHb formation by aromatic amines, aliphatic amines and nitrites is induced by their N-hydroxy metabolites (9-13). Usually, N-hydroxylation of those compounds in the body is less than the C-hydroxylation, with

Fig. 5. Species differences of methemoglobin formation by N-hydroxy metabolites of aniline derivatives in erythrocytes of guinea pigs. The substrates [aniline (A), 2-chloroaniline (2-Cl-A), 3-chloroaniline (3-Cl-A) and 4-chloroaniline (4-Cl-A)] and the liver microsomes from animals of various species were incubated at 37°C for 60 min, and then, the incubated mixture was added to erythrocyte suspension and the preparation was incubated for a further 30 min. The final concentration of hemoglobin in the erythrocyte suspension was adjusted to 10 g/dl. Each value indicates the mean $\pm$ standard error of 3 experiments.

phenacetin the N-hydroxy metabolites were detected at a rate of less than 0.52% of the administered dose (14). However N-hydroxy metabolites have toxicologically an important role in Mhb formation and in carcinogenicity (11, 15–17).

It was reported that the ferro-hydrogen peroxide complex ($\text{HbFe}^{II}\cdot\text{H}_2\text{O}_2$) was produced from oxyhemoglobin by PHA and it was changed to ferri-hemoglobin (HbFe^{III} , Mhb), with hydrogen peroxide decomposition (18). Nicholls (19) and Allen and Jandl (20) proposed that SHb was produced through the reaction of Mhb formation. Nichol and coworkers (18) suggested that ferro-SHb (SHbFe^{II}) was produced directly

from the complex ($\text{HbFe}^{II}\cdot\text{H}_2\text{O}_2$) in the presence of hydrosulfide. We have already reported that there was no correlation between Mhb and SHb formation in a treatment with nitrite, "inorganic" hydroxylamine and a phenylurethane derivative and also that the delayed SHb formation was prevented by ascorbic acid which did not inhibit Mhb formation (1, 4). Such evidence can hardly be explained by only the one mechanism proposed by Nicholls (19) and Allen and Jandl (20). Our results showed that Mhb was produced only by treatment with PHA or ferricyanide, in the absence of sodium sulfide, and that Mhb was never converted to SHb when sodium sulfide was

Fig. 6. Species differences of sulfhemoglobin formation by N-hydroxy metabolites of aniline derivatives in erythrocytes of guinea pigs. The substrates [aniline (A), 2-chloroaniline (2-Cl-A), 3-chloroaniline (3-Cl-A) and 4-chloroaniline (4-Cl-A)] and the liver microsomes from animals of various species were incubated at 37°C for 60 min, and the mixture was added to erythrocytes in the presence of sodium sulfide and the preparation was incubated for a further 30 min. The final concentrations of hemoglobin and sodium sulfide in the erythrocyte suspension were adjusted to 10 g/dl and 0.2 mM, respectively. Each value indicates the mean $\pm$ standard error of 3 experiments.

added. It was thus confirmed that SHb was not produced *via* MHb.

The incubation of aniline with erythrocytes resulted in little formation of either MHb or SHb, but formation of both MHb and SHb was apparent when liver microsomes were added to the incubated reaction mixture. Thus SHb was also formed by N-hydroxy metabolites.

Even though there was little MHb and SHb formation without microsomes following the treatment with aniline, these formation might be caused by the possible existence of a certain conversion mechanism in erythrocytes, because nitrobenzene was detected in the reaction mixture containing erythrocytes

and aniline (21).

The reason why SHb formation in intact cells was more efficient than that in hemolysates may be that PHA and sulfide were taken up effectively by the cells, and that hemoglobin was extremely concentrated more in the cells than that in hemolysate solution.

2) Correlation between the chemical structure and MHb and SHb formation: In previous work we referred to the correlation between the chemical structure (aniline and anthranilic acid derivatives) and the formation of MHb and SHb *in vivo* (1–3). In this study, the rates of MHb and SHb formation used as indices of production rates of N-hydroxy

Fig. 7. Species differences of methemoglobin formation, *in vivo* after a single administration of phenylhydroxylamine (PHA), aniline (A), 2-chloroaniline (2-Cl-A), 3-chloroaniline (3-Cl-A) and 4-chloroaniline (4-Cl-A) to animals of various species. Each value indicates the mean $\pm$ standard error of 10, 5, 5, 3, 3, and 3 experiments of mice, rats, guinea pigs, rabbits, cats and dogs, respectively.

metabolites from aniline derivatives by liver microsomes *in vitro* were compared with the formation rates of MHB and SHb *in vivo*. We found that the order of amounts of N-hydroxy metabolites was in parallel with the rates of MHB formation *in vivo*. This relationship was also observed in the cases of the SHb formation as described previously (1). Therefore, it would be possible to estimate the correlation between the chemical structure and MHB and SHb formation from the production rate of N-hydroxy metabolites *in vitro*.

3) Species differences in MHB and SHb formation: Species differences in MHB formation have been investigated *in vivo* (whole animals) (12, 22) as well as *in vitro* (using liver microsomes) (15, 23–28). When

aniline (as well as 4-Cl-A) was incubated with liver microsomes from various species, there was a production of N-hydroxy metabolites, as assessed by MHB and SHb formation. In cats, dogs and monkeys, the production rate was low, but it was extremely high in guinea pigs. The high N-hydroxylation activity of guinea pigs was also reported by Uehleke (15), Appel et al. (25) and Debackere and Uehleke (23).

We found that the formation of MHB and SHb *in vivo* was extremely high in cats and dogs, as compared with that in guinea pigs. These results were contrary to the production rates *in vitro*. The high level of MHB in both species may be due to: a) deficiency of the glucuronyl transferase system in cats causing a higher level in the blood of the induced

Fig. 8. Species differences of sulfhemoglobin formation, *in vivo* after the consecutive administration of phenylhydroxylamine (PHA), aniline (A) and 4-chloroaniline (4-Cl-A) to animals of various species for three days. The levels of sulfhemoglobin were measured 48 hr after the final administration of drugs. Each value indicates the mean $\pm$ standard error of 10, 5, 5, 3, 3, and 3 experiments of mice, rats, guinea pigs, rabbits, cats and dogs, respectively.

arylhydroxylamines for a comparatively long time (11), b) arylamines remained unchanged in a higher level in dogs because as such have a lower acetylation capability (11). In addition, it might be considered that MHb formation was less induced by the effect of high MHb reductase activity which was found in rabbits, rats and mice (11). However, the discrepancy between *in vivo* and *in vitro* results in guinea pigs cannot be fully explained at present. The discrepancy in the case of SHb formation can hardly be explained from the view of enzymatic activity such as MHb reductase, because of the irreversible property of SHb (29, 30).

As the levels of MHb induced by PHA in the erythrocyte suspension were slightly higher in dogs and cats than that in guinea pigs, it is assumed that this phenomenon reflected species differences for MHb for-

mation *in vivo*. In the case of SHb formation, the results *in vitro* did not follow the species differences *in vivo*; SHb formation in the erythrocyte suspension was less prominent in dogs and cats than in the other species. The following are significant for a proper understanding the unexpected results in species differences: a) There were differences in some properties of hemoglobin of individual animal species, b) There were differences in the reduction mechanism of MHb within erythrocytes through the pentose phosphate shunt, c) The formation of N-hydroxy metabolites in the body was induced not only in the liver but in other organs such as lung and kidney (11, 15, 31, 32), d) N-Hydroxy compounds were further metabolized through the glucuronic acid conjugation, the N-acetylation, the reduction of N-hydroxy moiety and so on in different manners in

different animal species.

Acknowledgements: We thank Mr. Y. Sasaki and Mrs. F. Iwaku for technical assistance. A part of this work was presented at the 53rd Regional Meeting of the Japanese Pharmacological Society (Wakayama, June 16, 1978).

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