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SPECIES DIFFERENCES IN METHEMOGLOBIN REDUCTASE ACTIVITY*

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Abstract—Sodium nitrite induced equivalent levels of methemoglobin in washed erythrocytes from cat, dog, and man, all suspended in Krebs-Ringer phosphate–glucose (pH 7·4). The same levels occurred in human cells with or without added substrate (glucose or lactate). In all these incubations, reduction of methemoglobin was minimal or absent over a 2-hr period. When 10^{-5}M methylene blue was added with glucose, equivalent increases in rates of methemoglobin reduction occurred in the cells of all three species. Similar rates were seen in rabbit and mouse red cells even without added methylene blue, as long as lactate or glucose was present. Methylene blue further enhanced reductase activity in mouse cells but only in the presence of glucose. Rabbit cells responded much less dramatically, if at all, to methylene blue. Lysates of human, rabbit, and mouse cells were equally sensitive to nitrite, and no spontaneous reduction occurred. These findings suggest that the high reductase activity of rabbit and mouse erythrocytes is NADH-dependent. The mouse but not the rabbit appears to possess also a NADPH-dependent reductase like man, dog and cat.

METHEMOGLOBIN reductase activity can be attributed to one or both of two mechanisms possessed by the erythrocytes of a particular species (literature reviewed by Jaffé[1]). The NADH or so-called diaphorase system is thought to proceed by a rapid transfer of electrons to an enzyme, or combination of enzymes, diaphorase, followed by the slower reduction of the final acceptor, the ferric iron of methemoglobin. NADH is generated by glycolysis, which may be the principal energy-producing pathway in the normal metabolizing erythrocyte. NADPH is generated by hexose monophosphate shunt metabolism, and NADPH reduction of methemoglobin requires the presence of either an unknown cofactor or an artificial electron carrier such as methylene blue. The NADPH-dependent system reduces methemoglobin rapidly, in contrast to the slower activity of the NADH system.

The pattern of the methemoglobinemic response induced when erythrocytes are exposed to an oxidant challenge is undoubtedly a balance between methemoglobin formation and its subsequent reduction back to hemoglobin. Some of the so-called species differences in sensitivity to methemoglobin-forming agents can probably be attributed to differences in reductase activity. In a frequently misquoted paper, Lester[2] points out some striking species differences in sensitivity to the methemoglobin-forming properties of acetanilid and acetophenetidin. However, interpretation of this work is complicated by probable species differences in the rates of metabolism of these drugs to active methemoglobin-forming agents.

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A better agent for elucidating species differences in reductase activity is sodium nitrite because it does not require biotransformation in order to be active. In one such study Kiese and Weis[3] report that of several species tested, the erythrocytes of rabbits exhibited the highest rate of reductase activity if either glucose or lactate was present; lactate was said to be somewhat more effective than glucose as a substrate. In the presence of glucose and nile blue the reductase activity was increased two to tenfold in all species tested, while with lactate and nile blue only the guinea pig, rat, and dog exhibited any increase over lactate alone; in all cases this increase was less than twofold. Malz,[4] however, reports conflicting data on rabbit erythrocytes. In the presence of methylene blue and glucose, the reductase activity of his rabbit cells was only slightly stimulated, about 1·5 times over that of glucose control values.

In the above studies[3, 4] reductase activity was estimated on erythrocytes in which high levels of methemoglobin were induced by sodium nitrite. However, the red cells of the various species tested had different reductase activities, and it was not possible to treat them all similarly with nitrite and arrive at the same concentration of intraerythrocytic methemoglobin from which to begin the assay. Our experiments follow the time course of the methemoglobinemia when erythrocytes are challenged *in vitro* with sodium nitrite. While these observations sum two phenomena, namely methemoglobin formation and methemoglobin reduction, the data obtained permit comparison with results of previously reported experiments *in vivo*.[5]

METHODS

Experiments on mice were performed as previously described, and the control response of mice to sodium nitrite shown in Table 2 has been reported elsewhere.[5] Methylene blue, 50 mg/kg in water, was given i.p. 20 min prior to sodium nitrite, 75 mg/kg. Methemoglobin analyses were performed on samples of blood removed from the tail, as previously described.[5]

Heparinized blood samples obtained from the various animal species and freshly drawn, pooled, human blood with Versene added were centrifuged and washed repeatedly with isotonic Krebs-Ringer phosphate (pH 7·4) with or without added lactate or glucose at a final concentration of 12 mM. The washed cells were finally suspended in the appropriate buffer–substrate solution, and the concentration was adjusted to about 5·0 mM heme as assayed by the cyanmethemoglobin method.[6] In experiments where hemolyzed cells were required, the suspension was treated with a minimal amount of saponin to transparency. Equal aliquots of the preparations were then incubated in tubes immersed in a constant temperature bath at 37°.

Fresh solutions of sodium nitrite were prepared each day. Concentrations of the stock solutions were adjusted so that 0·05-ml aliquots added to the incubating cell suspensions (5·0 ml) resulted in final nitrite/heme ratios of 0·05/1, 0·15/1, 0·45/1, and 1·35/1 (final nitrite concentrations of about 0·25, 0·75, 2·25 and 6·75 mM). A control tube of blood not treated with nitrite was included with each experimental set. Except where specifically indicated, control methemoglobin levels have been subtracted from each experimental result. In some experiments methylene blue (Nutritional Biochemicals Corp). or new methylene blue N (Allied Chemical Corp., methylene blue NN) was added to the incubating cells to a final concentration of 10^{-5} or 10^{-4} M 15 min before adding nitrite.

Equal aliquots of the reaction mixtures were withdrawn 20, 40, 60, and 120 min after nitrite treatment. Whole-cell suspensions were centrifuged and washed three to four times with physiologic saline solution before hemolysis in 0·066 M phosphate buffer (pH 6·6) with saponin. Aliquots of lysates were analyzed directly without washing. The percent methemoglobin was determined spectrophotometrically as described previously.[5] Table 1 illustrates the reproducibility *in vitro* of the system. This particular nitrite/heme molar ratio was selected because it is the one found to be most useful in the interpretation of our data. The standard deviations are comparable in magnitude to those *in vivo* (data in Table 2).

TABLE 1. PER CENT OF THE TOTAL BLOOD PIGMENT CONVERTED TO METHEMOGLOBIN BY SINGLE DONOR HUMAN LYSATES IN GLUCOSE AT 0·45 $NaNO_2$/HEME

N	Minutes after addition of nitrite			
	20	40	60	120
6	53·1 ± 8·1	66·6 ± 5·2	68·8 ± 5·0	86·7 ± 4·7

TABLE 2. PER CENT OF THE TOTAL BLOOD PIGMENT CONVERTED TO METHEMOGLOBIN IN MICE AFTER 75 MG $NaNO_2$/KG, I.P.*

Pretreatment	Minutes after injection				
	0	20	40	60	120
None†	1·3 ± 0·6	33·4 ± 6·2	34·1 ± 8·4	25·2 ± 7·2	8·1 ± 6·4
Methylene blue‡		3·4 ± 1·1	3·1 ± 0·2	2·6 ± 1·1	1·8 ± 1·1

* Values are means ± S.D. for six mice.
† Data of Abbanat and Smith.[5]
‡ Fifty mg/kg given i.p. 15 min prior to nitrite.

RESULTS

Table 2 shows the control methemoglobinemic response to sodium nitrite in mice. About a third of the total circulating pigment is converted to methemoglobin within 20 min, and levels decline to near normal in 2 hr. Therefore, mouse blood *in vivo* possess a fairly brisk rate of reductase activity, but this rate can be stimulated further by pretreating the animals with methylene blue. Under these circumstances the methemoglobinemic response to the given dose of sodium nitrite is almost totally blocked (Table 2). These data are interpreted as evidence that the mouse possesses a NADPH-dependent methemoglobin reductase which can be accelerated by methylene blue *in vivo*.

The left side of Fig. 1 shows the results obtained when cat, human, and dog erythrocytes are incubated at 37° in Krebs-Ringer phosphate with 12 mM glucose in the presence of four concentrations of sodium nitrite. Since the methemoglobin levels show no appreciable decline from peak values, it may be inferred that reductase

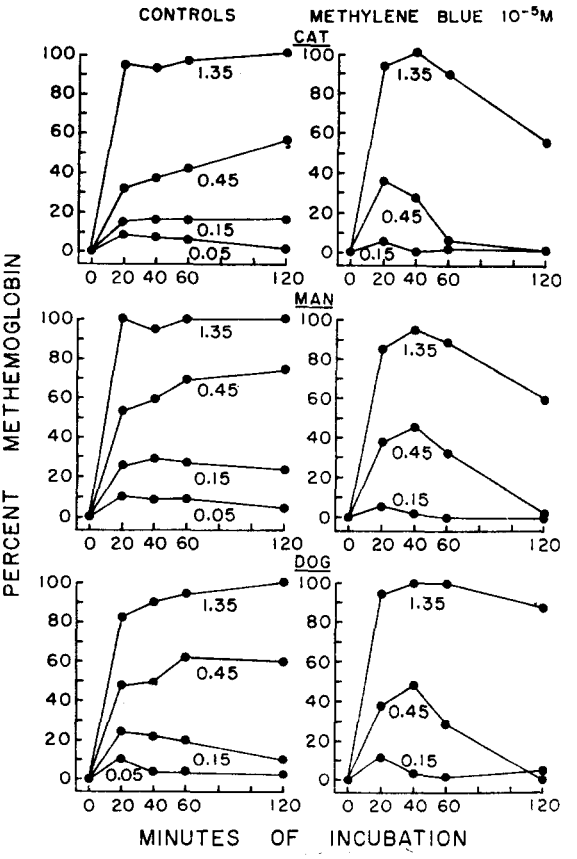

FIG. 1. Per cent of the total blood pigment converted to methemoglobin when cat, human, and dog erythrocytes in Krebs-Ringer phosphate–glucose (pH 7·4) are exposed to four concentrations of sodium nitrite with and without methylene blue. Numerals refer to nitrite/heme molar ratios.

activity is either minimal or absent over the 2-hr period. If glucose is omitted as a substrate, the response of human red cells to nitrite is not altered; therefore, ongoing diaphorase activity[1] in these cells is not detectable by the present method within this time span. The cat and dog erythrocytes closely resemble those of man, and all three species respond similarly to methylene blue by net reductions of methemoglobin after the initial 20 to 40 min. If glucose is omitted, methylene blue is without effect as tested in red cells of human and dog.

In contrast, Fig. 2 shows a rapid rate of reductase activity in mouse and rabbit red cells in the presence of glucose even when methylene blue is absent. With the lowest molar ratio of nitrite to heme (0·05, not shown in the figure), methemoglobin levels are no higher than in the control tubes. The pattern for mouse cells at the 0·45 molar ratio closely resembles the data (*in vivo*) in Table 2. The rabbit data are the means of two experiments on red cells from different albino animals. In accord with the results in Table 2, the mouse exhibits an accelerated reductase activity *in vitro*, comparable to that obtained in the whole animal with methylene blue. Rabbits, however, respond much less dramatically, if at all, to methylene blue.

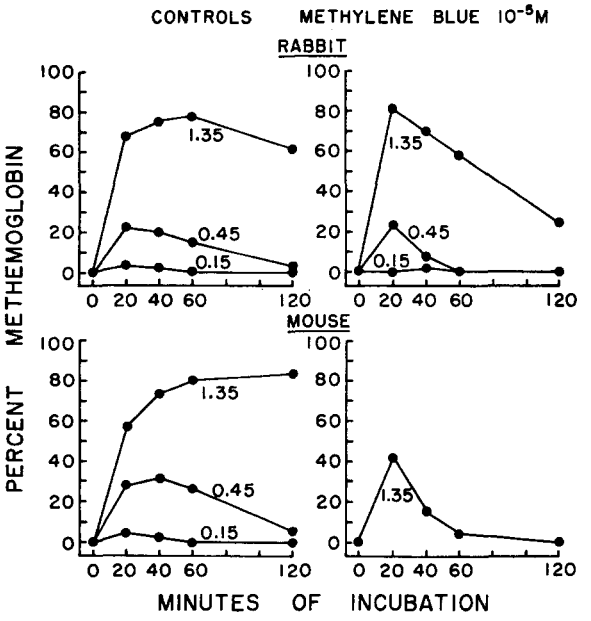

FIG. 2. Per cent of the total blood pigment converted to methemoglobin when albino rabbit and mouse erythrocytes in Krebs Ringer phosphate–glucose (pH 7·4) are exposed to four concentrations of sodium nitrite with and without methylene blue. Numerals refer to nitrite/heme molar ratios.

Since the spontaneous reduction of methemoglobin in mouse and rabbit cells in the presence of glucose might be due to either NADH- or NADPH-coupled enzymes, the same experiments were repeated with 12 mM lactate substituted for glucose, as seen in Fig. 3. The pattern for human cells is essentially the same with lactate as with glucose or no substrate. However, the cells no longer respond to methylene blue. Rabbit and mouse cells with lactate have reductase activities which are essentially the same as with glucose. With lactate as a substrate, however, the mouse responds only weakly, if at all, to methylene blue. These results indicate that the high rates of spontaneous reductase activity in the rabbit and the mouse are mediated through a NADH-linked system.

As evidence that the different patterns observed are in fact due to differences in reductase activity and not to inherent differences in the sensitivity of the various hemoglobins to nitrite, experiments performed with lysates are illustrated in Fig. 4. Reductase activity is seen to be virtually abolished by hemolysis, and the hemoglobins of man, mouse, and rabbit show equaivalent sensitivity to oxidation by sodium nitrite. Similarly, reductase activity in mouse cells is abolished if both glucose and lactate are omitted.

Figure 5 shows the response of human red cells in glucose to new methylene blue N at 10^{-5} M. The results are similar to those with methylene blue at the same concentration (cf. Fig. 1). At 10^{-4} M, the two dyes show further similarity in that they rapidly produce significant levels of methemoglobin before nitrite is added. The stippled areas in the two graphs on the right (Fig. 5) depict levels of methemoglobin in control tubes, upon which the nitrite responses are superimposed. These levels

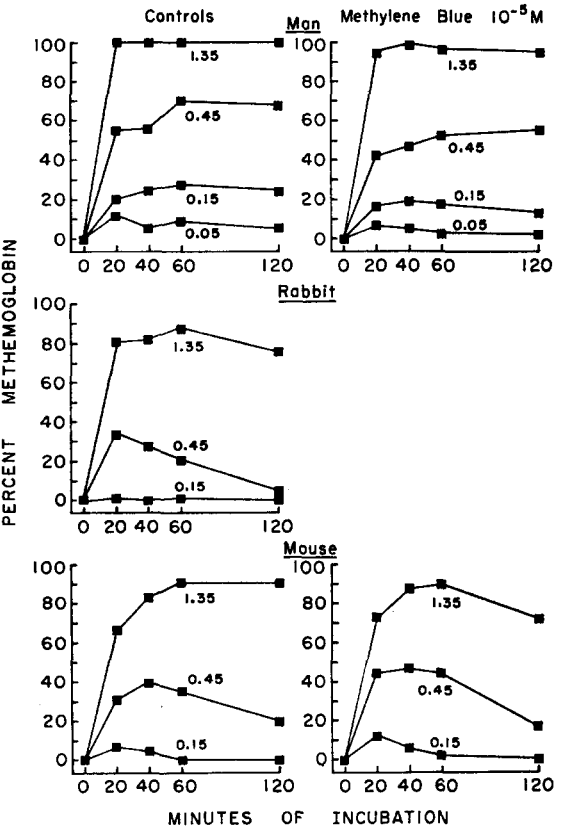

FIG. 3. Per cent of the total blood pigment converted to methemoglobin when human, rabbit, and mouse erythrocytes in Krebs Ringer phosphate–glucose (pH 7·4) are exposed to four concentrations of sodium nitrite with and without methylene blue. Numerals refer to nitrite/heme molar ratios.

were produced by the dyes within 15 min and remained virtually constant over 2 hr. The methemoglobin reductase system, however, responds differently to the two dyes at 10^{-4} M. Methylene blue still appears to stimulate reductase activity, whereas new methylene blue N does not. Note the peculiar circumstance that the pre-nitrite level of methemoglobin produced by methylene blue actually appears to protect the cells against the action of the lowest nitrite concentration. These results are consistent with the data of Collier and Gray,[7] who report new methylene blue N as a much more potent inhibitor of glucose-6-phosphate dehydrogenase than is methylene blue. The patterns obtained here suggest that the inhibition of glucose-6-phosphate dehydrogenase by new methylene blue N at 10^{-4} M blocks its stimulatory effects on the NADPH-dependent reductase.

That methylene blue possesses both the property of oxidizing hemoglobin to methemoglobin and an ability to stimulate reductase activity is shown by the data in Table 3. Here 10^{-4} M methylene blue in human cell lysates produces higher levels of methemoglobin than in intact cells (Fig. 5), and these levels tend to increase with time.

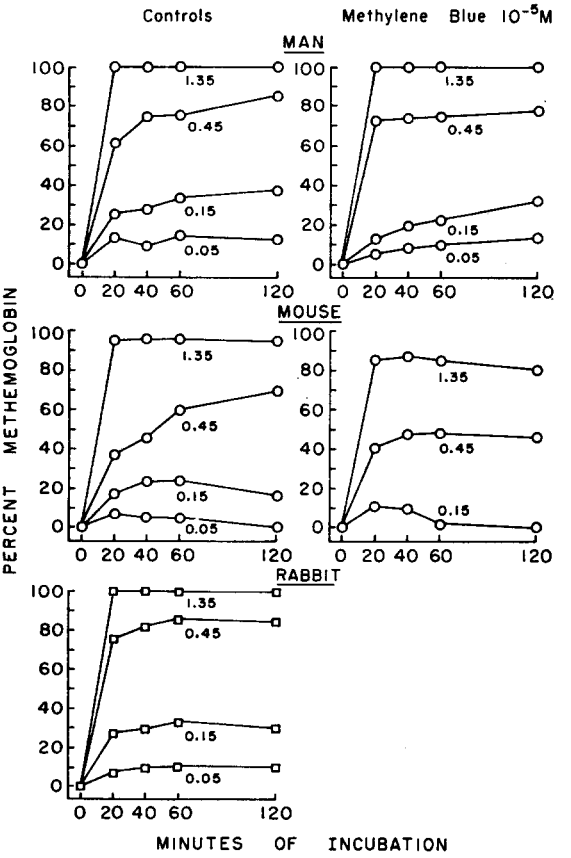

FIG. 4. Per cent of the total blood pigment converted to methemoglobin when human, mouse, and rabbit red cell lysates in Krebs Ringer phosphate containing glucose ○ or lactate □ are exposed to four concentrations of sodium nitrite with and without methylene blue. Numerals refer to nitrite/heme molar ratios.

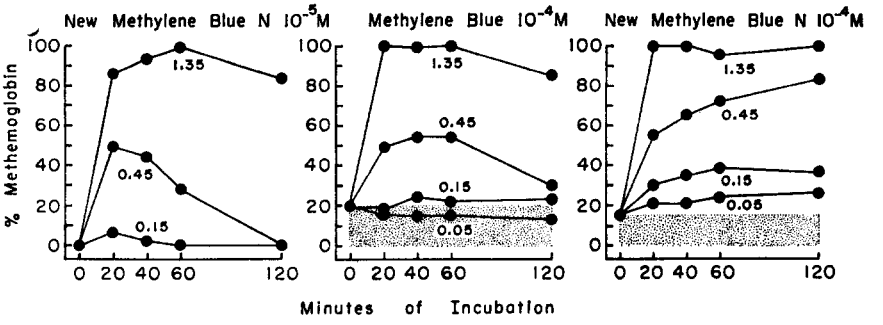

FIG. 5. Per cent of the total blood pigment converted to methemoglobin when human erythrocytes in Krebs Ringer phosphate–glucose (pH 7·4) are exposed to four concentrations of sodium nitrite in the presence of 10^{-5} and 10^{-4} M new methylene blue N or 10^{-4} M methylene blue. Stippled areas show methemoglobin levels in control tubes not exposed to nitrite. Numerals refer to nitrite/heme molar ratios.

TABLE 3. PER CENT OF THE TOTAL BLOOD PIGMENT CONVERTED TO METHEMOGLOBIN BY 10^{-4} M METHYLENE BLUE (HUMAN ERYTHROCYTE LYSATES WITH GLUCOSE)

Minutes after addition of methylene blue			
35	55	75	135
32·4	44·5	61·2	63·8

DISCUSSION

In experiments similar to the ones presented here, Harley and Robin[8] incubated human erythrocytes at a tenfold lower heme concentration (0·5 mM) with various molar ratios of nitrite. They obtained complete conversion to methemoglobin within 2 hr only at molar ratios of 16 or higher. We have confirmed their observations, but our data at 5·0 mM heme would appear to indicate an approximately mole-for-mole conversion to methemoglobin. Both these findings, however, may be artifactual in that an examination of these data reveals that within the limits of heme concentration employed, the amount of pigment oxidized to methemoglobin was a function of the absolute nitrite concentration and not the molar ratio of nitrite to heme.

Data presented here suggest that the hemoglobins of five vertebrate species are about equally sensitive to oxidation by sodium nitrite. Thus the cat does not entirely deserve its reputation for being particularly sensitive to methemoglobin-producing agents. It is likely that the cat metabolizes aromatic amino compounds to methemoglobin-forming intermediates more actively than the other species tested by Lester[2]. On the other hand, a rational basis for the well-known resistance of rodents to methemoglobin-producing agents has been uncovered, namely that they have higher rates of methemoglobin reductase activity, not hemoglobins that are more resistant to oxidation. Perhaps mice also metabolize aromatic amines to active forms more slowly than do other species, but at least *p*-aminopropiophenone produces peak methemoglobin levels more rapidly than does nitrite in mice.[5]

No reasons are known for the marked differences in the NADH-dependent reductase activity between the mouse and the rabbit on the one hand and the cat, dog, and human on the other. If a common enzyme, "diaphorase," exists in all five species, its activity may be inhibited by some natural factor in the latter three species, or the diaphorase system may be more efficient in the rodent and the lagomorph. Conflicting reports[3, 4] regarding the actions of methylene blue on rabbit cells suggest that some but not all strains may possess the NADPH-dependent reductase system. No teleological reasons for the high rates of methemoglobin reductase activity in mice or rabbits have been suggested.

The data in Fig. 5 support the assertion that new methylene blue N is a more potent inhibitor of glucose-6-phosphate dehydrogenase than is methylene blue. Both dyes, however, are capable of slowly oxidizing NADPH. The relative contributions of these two phenomena to the overall patterns observed are not known. At least the pre-nitrite levels of methemoglobin (Fig. 5) are probably unrelated to dehydrogenase inhibition, since these levels are essentially equal even through the two dyes differ greatly in potency as dehydrogenase inhibitors.[7] These data are consistent with the

hypothesis that methylene blue can directly oxidize hemoglobin to methemoglobin (Table 3) and also function as an intermediate electron carrier between methemoglobin and the NADPH-dependent reductase.[1]

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