PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **147**, 245–249 (1974)

Interactions between Methylene Blue and Erythrocytes of Several Mammalian Species, *in Vitro*[1] (38319)

JOHN W. HARVEY AND J. J. KANEKO

Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, California 95616

Heinz bodies (HzB) were first reported in cats in 1899 by Schmauch (1) who described them in normal feline erythrocytes. Nearly all cats have demonstrable HzB in some of their red blood cells (RBC) and the cat is generally recognized as the species most susceptible to HzB formation from a variety of oxidant drugs given experimentally (2, 3). Methylene blue (MB) is one of these drugs which will readily produce HzB in cats by oxidatively denaturing hemoglobin (Hb). Rentsch and Wittekind (4) studied HzB production by MB in six laboratory animal species, *in vivo*. They attributed the greater susceptibility of cats to HzB formation to a difference in liver metabolism of MB rather than an inherent difference in the susceptibility of cat erythrocytes to oxidation. While the association of MB and HzB formation has been recognized for many years, its clinical importance was only recently recognized when HzB hemolytic anemias were reported in cats after treatment with urinary antiseptic–antispasmodic drugs containing MB (5).

When erythrocytes are incubated with MB, there is a marked stimulation of glucose oxidation via the pentose phosphate pathway (PPP) as a result of the oxidation of NADPH by MB. Electrons are transferred from NADPH to MB with the formation of leukomethylene blue (LMB). Human RBC are relatively impermeable to LMB and as a result it accumulates within the RBC (6). The amount of LMB which accumulates intracellularly is dependent on the inherent capability of the RBC to increase the PPP and the rate of spontaneous oxidation of LMB by molecular oxygen. Sass *et al.* (6) used this phenomena of "reductive trapping" to develop a screening test (methylene blue uptake test) for glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes. After the addition of MB to dilute erythrocyte suspensions, he noted that the blue color in the supernate disappeared at a slower rate in G6PD deficient cells compared to normal cells. This was attributed to an inability to increase PPP flow in the G6PD deficient.

Reduced glutathione (GSH) is a tripeptide which is important in protecting RBC from oxidative damage. Although GSH appears to have some direct protective effects when associated with Hb and certain red cell enzymes, its most important function in erythrocytes appears to be as a substrate for glutathione peroxidase (GPx) in the enzymatic degradation of peroxides. The oxidized glutathione (GSSG) produced by this reaction can be reduced back to GSH via NADPH and glutathione reductase (GR). The ability to maintain GSH at normal levels in the presence of an oxidant, *e.g.*, acetylphenylhydrazine, has been used as another *in vitro* screening test (glutathione stability test) for G6PD deficiency (7).

This report describes several *in vitro* experiments undertaken to study interactions between MB and intact erythrocytes. Red blood cells from the dog and man were included in each study for comparison to feline erythrocytes. Canine RBC were of particular interest since the dog ranked second to the cat in ease of HzB formation with MB *in vivo* (4).

Methods. Heparinized blood samples were collected from healthy dogs and cats of mixed breeding and from laboratory personnel. All samples were immediately centrifuged at 1000*g* and plasma and buffy coats were removed. Each sample was washed three times in the buffer to be used for the respective incubation procedure.

[1] Supported in part by a Public Health Training Grant No. GM-633 from the National Institute of General Medical Sciences.

For the *in vitro* production of HzB with MB, erythrocytes were suspended in a buffer containing 130 m*M* NaCl, 4.5 m*M* KCl, 2.7 m*M* $MgCl_2$, 20 m*M* Na_2HPO_4, 4 m*M* NaH_2PO_4, and 11.1 m*M* glucose (pH 7.4). The test was started by adding 1 ml of RBC suspension, with a packed cell volume (PCV) of 40%, to 5.5 ml of buffer (containing MB) in an 11 × 110 mm test tube. Methylene blue[2] was present at a final concentration of 6.7 μM. The test tube was stoppered and incubated horizontally at 37° in a shaking water bath. At 2 hr intervals, a small aliquot was removed and centrifuged at 1000*g*. The supernate was removed and the packed cells were suspended (1:1) in homologous plasma saved from the initial blood sample centrifugation. Coverslip smears were prepared and HzB were demonstrated by treating the smears with 0.5% copper sulfate followed by Wright–Leishman staining (8).

Methylene blue uptake by RBC was studied as previously described (6). One ml of erythrocyte suspension (PCV 40–50%) was added to 10 ml of buffer containing 11.1 m*M* glucose, 154 m*M* NaCl and 4 m*M* phosphate buffer (pH 7.4) in a 19 × 150 mm cuvette. After the addition of 1 ml MB (0.006% in buffer), the mixture was stoppered, mixed, and centrifuged within 1 min. The optical density (O.D.) of the supernate was measured at 615 nm in a Coleman, Jr., spectrophotometer. Immediately after the o.d. measurement, the erythrocytes were resuspended and incubated with horizontal shaking at ambient temperatures. This procedure of centrifugation, o.d. measurement, and resuspension was performed at 10 min intervals. A control tube was prepared daily by substituting 1 ml of buffer for the erythrocyte suspension. The o.d. of the control tube was compared to the 0 time o.d. measurement of each sample to evaluate the rapid MB uptake during the initial mixing and centrifugation.

For glutathione stability measurement, erythrocytes were suspended to a PCV of 20% in the buffer described for HzB production experiments. Each individual's erythrocytes were incubated with and without MB addition, in the presence and absence of glucose. When glucose was omitted, an additional 5 m*M* of NaCl was added. MB was present in a final concentration of 6.7 μM. Reduced glutathione was assayed using the DTNB method (9). After preincubation samples were taken for GSH analysis, 2.5 ml of RBC suspension was incubated in a stoppered 11 × 110 mm test tube placed in a horizontal position in a shaking water bath at 37°. The GSH level was again assayed after 6 hr of incubation and expressed as percent of the preincubation GSH level.

Results. Phase contrast photomicrographs of coverslip smears prepared to demonstrate HzB are given in Fig. 1. Small HzB began to appear in the erythrocytes of the cat after 2 hr of incubation, and were present in high concentrations at 4 hr. The HzB continued to increase in size for the duration of the incubation. In contrast, HzB in dog and human erythrocytes did not appear until after 6 hr incubation and were considerably smaller than those found in feline red cells even after 8 hr incubation.

Feline erythrocytes incubated under identical conditions, without the addition of MB, did not develop HzB. Although the erythrocytes from the cat used in this particular experiment contained approximately 90% Hb A and 10% Hb B, other experiments using cats with approximately 50% Hb A and 50% Hb B also showed the same increased susceptibility of feline erythrocytes to HzB formation relative to canine and human red cells.

Results from the MB uptake studies are given in Fig. 2. Human erythrocytes concentrated significantly more MB ($P < 0.001$) than red blood cells of either the dog or cat. The line of best fit was calculated for each species using the least squares method. The 30 min points were excluded from this calculation in man since virtually all MB was taken up in 20 min. A thin coating of erythrocytes on the cuvette walls prevented the o.d. from reaching zero using human RBC. Based on the calculated regression lines, there was an average decrease in o.d. of 0.379/10 min in man compared to 0.078 for the cat and 0.014 for the dog. Feline red blood cells took up significantly more MB than those of the dog. However, this difference may not be significant since variable degrees of hemolysis were apparent in canine samples beginning at the 10 min o.d. readings. Although the significance of the rapid uptake of MB during the initial mixing of samples is obscure, it is interesting to note that there was a significantly greater decrease in o.d. in feline and human samples (0.094 and 0.140 respectively) compared to the dog (0.053). There

[2] Basic Blue 9, Matheson Coleman and Bell, NJ.

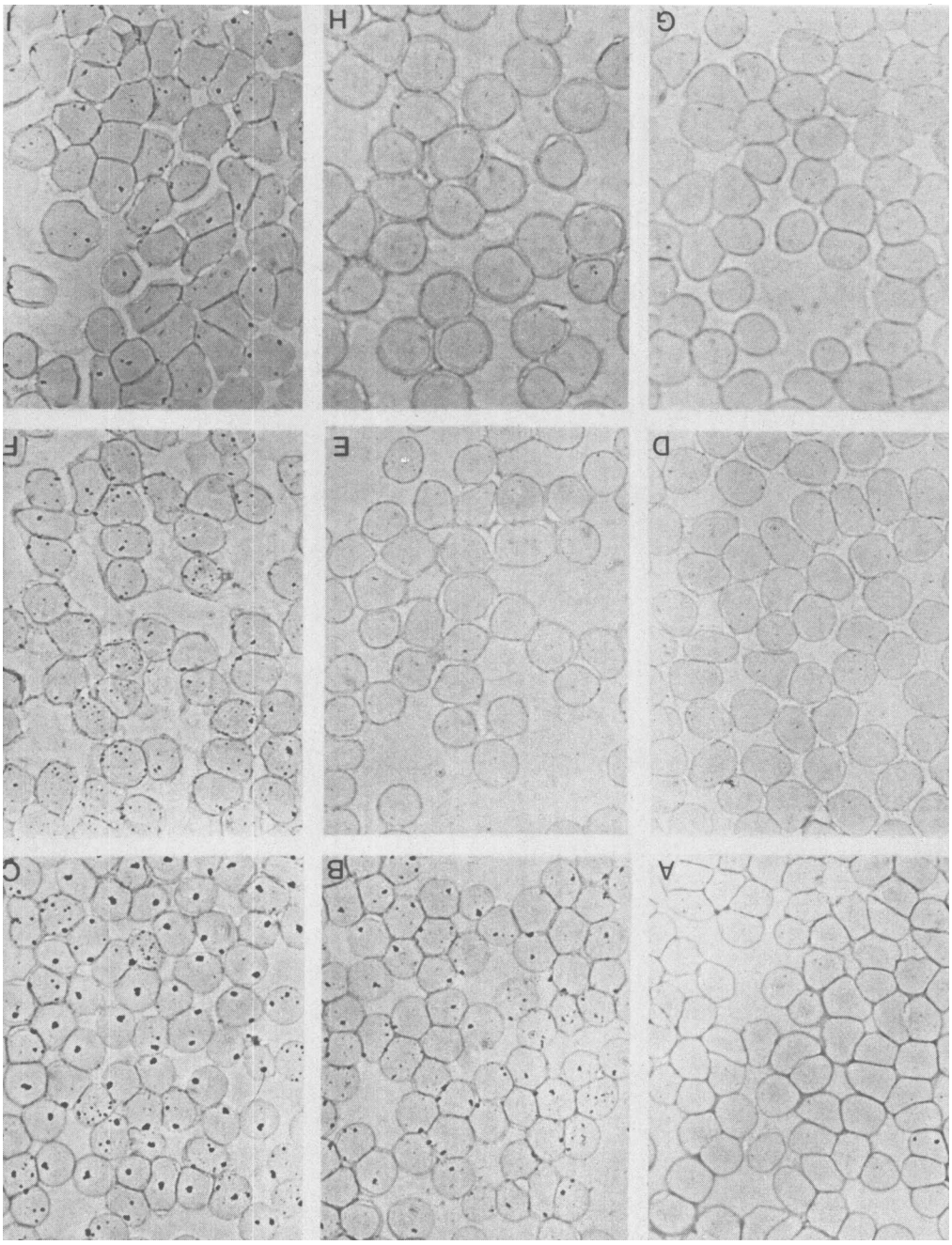

FIG. 1. Production of Heinz bodies with methylene blue *in vitro* at 37° in the presence of glucose. Feline (A, B, C), canine (D, E, F), and human (G, H, I) erythrocytes are shown after 0, 4, and 8 hr of incubation, respectively.

was no hemolysis in canine samples after this first (0 time) centrifugation.

Results of the glutathione stability studies are given in Table I. There was a significant decrease in GSH ($P < 0.01$) in feline erythrocytes incubated with glucose, even in the absence of MB. In contrast to the 15% decrease in GSH in feline RBC, there was no change in GSH content

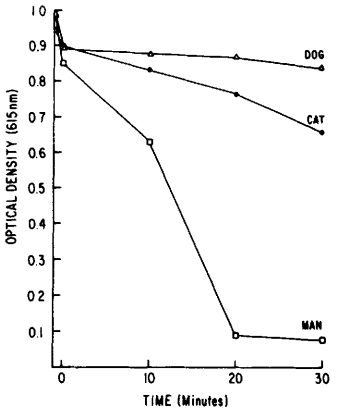

FIG. 2. Disappearance of methylene blue in the supernatant of erythrocyte suspensions of the dog ($N=5$), cat ($N=5$), and man ($N=4$) incubated in the presence of glucose at ambient temperatures.

in canine or human erythrocytes under the same conditions. When MB was included in the incubation media containing glucose, there was a significant decrease in GSH in RBC of both the dog ($P < 0.01$) and cat ($P < 0.001$) but no significant decrease in man, *i.e.*, 95% of the GSH was still present at the end of the incubation in man compared to 64% in the cat and 76% in the dog. The difference between the dog and cat was not significant. In the absence of glucose, there was no significance difference in the %GSH remaining between any of the species, whether MB was present or not.

Discussion. Although there are undoubtedly species and individual differences in the metabolism and excretion on MB *in vivo*, erythrocytes of the cat *in vitro* appear inherently more susceptible to HzB formation with MB than the dog or man (Fig. 1). Increased susceptibilities to HzB formation in man have been associated with either a decreased ability of the PPP to protect erythrocyte Hb from oxidative denaturation (*e.g.*, G6PD deficiency) or with hemoglobinopathies where the abnormal Hb is more susceptible to denaturation.

The MB uptake test has been used in man as a screening test to measure the ability of an individual's erythrocytes to increase PPP flow in the presence of an oxidant stress. The uptake of MB, by RBC of the dog and cat (Fig. 2) is similar to the decreased uptake reported in G6PD deficiency in man (6). This decreased ability to concentrate MB intracellularly, in the reduced form (LMB) could be due to either a decrease in MB reduction or an increase in LMB oxidation. Less MB would be reduced to LMB if RBC of the carnivores were relatively impermeable to MB, if they were deficient in the enzyme NADPH-diaphorase, which accelerates the reduction of MB (10, 6), or if they were unable to increase PPP flow under oxidant stress comparable to that of normal human erythrocytes. None of these explanations adequately explain the decreased uptake, since radioactive tracer studies have shown a greater stimulation of PPP flow by MB in the dog and cat than seen in man (11, 12). Intracellular LMB is spontaneously oxidized to MB by molecular oxygen and species differences in the rate of LMB oxidation could be accounted for by a phenomena reported by Sass *et al.* (6). He noted that, for unexplained reasons, when LMB is placed in the incubation medium on the outside of erythrocytes (or in the presence of washed red cell stroma) it is immediately oxidized to MB even in the absence of oxygen. In contrast, when LMB is "trapped" within red cells, it remains in the reduced form under anaerobic conditions even in a glucose free medium. Leukomethylene blue does not diffuse easily through the erythrocyte membrane in man (6). If red cells of the carnivores were more "permeable" to LMB and it diffused out more quickly and was rapidly reoxidized to MB, it would appear as if less MB was taken up from the supernate. Differences in LMB permeability

TABLE I. Erythrocyte Glutathione Stability Expressed as Percent of Preincubation Reduced Glutathione (GSH) Remaining After 6 hr *in vitro* Incubation at 37° in the Presence and Absence of Glucose and Methylene Blue (MB).

	Glucose		No glucose	
Species	Without MB	With MB	Without ND	With MB
Cat ($N=63$	85 ± 3[a]	64 c 3	76 ± 4	41 c 5
Dog ($N=6$)	99 ± 3	76 c 6	89 ± 6	35 c 5
Man ($N=6$)	101 ± 2	96 c 2	80 ± 2	37 c 1

[a] % GSH remaining ± SEM.

could well account for some of the marked species variations reported in the dye-linked reduction of methemoglobin (13). Although the oxidant effect of red cell stroma is not understood, it might also explain why there was a greater PPP stimulation with MB in the dog and cat versus man with the same molar ratio of MB to Hb (11, 12).

When RBC suspensions were incubated with MB in the presence of glucose, the carnivores were unable to maintain erythrocyte GSH at preincubation levels under conditions where human erythrocyte GSH remained unchanged (Table I). This might simply be the result of a greater oxidant stress on the erythrocytes of the carnivores versus man by the same concentration of MB as discussed above. A difference in the maintenance of GSH between species would not be anticipated in the absence of glucose and no significant difference was found with or without MB. The mechanism for the inability of feline erythrocytes to maintain GSH when incubated with glucose in the absence of MB is unclear. Information on normal GSH turnover rates in feline erythrocytes is not available.

A difference in metabolism would not seem to explain the greater susceptibility for HzB formation in erythrocytes of the cat versus the dog since their RBC appeared similar with respect to MB uptake and GSH stability. Species differences in Hb structure may, at least in part, account for the observed variations in HzB susceptibilities. Although feline Hb are not heat labile (5), as are the unstable hemoglobinopathies in man, it is conceivable that they are more susceptible to oxidative denaturation than normal human Hb. Feline Hb are particularly susceptible to partial precipitation with sulfhydryl-blocking agents (14), and tend to autoxidize more readily than those of other species (15). The oxidation of "reactive" (readily oxidizable) sulfhydryl groups of Hb molecules is considered a critical step in the oxidative denaturation of Hb to form HzB (16). Feline Hb molecules are reported to have at least eight (and possible ten) reactive sulfhydryl groups (16). No other species has been reported to have more than four reactive groups per molecule, with two reported in man and four in the dog (17). In the presence of oxidant stress, feline Hb would have more sulfhydryl groups to be maintained in the reduced form, which could account for their greater susceptibility to oxidative denaturation.

Summary. Interactions between methylene blue (MB) and erythrocytes of the cat, dog, and man were studied *in vitro*. Feline red blood cells (RBC) were more susceptible to Heinz body formation in the presence of MB than those of the other species. Erythrocytes of the cat and dog were unable to concentrate MB intracellularly as readily as human RBC. In addition, feline and canine RBC were unable to maintain their reduced glutathione (GSH) at preincubation levels when incubated with MB at a concentration which produced no significant decrease in GSH in human cells. These differences in dye concentration and glutathione "instability" are discussed in relation to the greater susceptibility of feline erythrocytes to Heinz body formation.

1. Schmauch, G., Arch. Path. Anat. **156,** 201 (1899).
2. Beritic, T., Blood **25,** 999 (1965).
3. Fertman, M. H., and Fertman, M. B., Medicine **34,** 131 (1955).
4. Rentsch, G., and Wittekind, D., Tox. Appl. Pharmacol. **11,** 81 (1967).
5. Schechter, R. D., Schalm, O. W., and Kaneko, J. J., J. Amer. Vet. Med. Ass. **162,** 37 (1973).
6. Sass, M. D., Caruso, C. J., and Axelrod, D. R., J. Lab. Clin. Med. **69,** 447 (1967).
7. Beutler, E., J. Lab. Clin. Med. **49,** 84 (1957).
8. Jain, N. C., Brit. Vet. J. **125,** 437 (1969).
9. Prin, H. K., and Loos, J. A., *in* "Biochemical Methods in Red Cell Genetics" (J. J. Yunis, ed.), p. 115. Academic Press, New York (1969).
10. Sass, M. D., Caruso, C. J., and Axelrod, D. R., Clin. Chim. Acta **24,** 77 (1969).
11. Harvey, J. W., and Kaneko, J. J., Fed. Proc. **3,** 413 Abs. (1973).
12. Harvey, J. W., Ph.D. Thesis., Univ. of Calif., Davis (1974).
13. Robin, H., and Harley, J. D., Aust. J. Exp. Biol. Med. Sci. **44,** 519 (1966).
14. Paniker, N. V., and Iyer, G. Y. N., Can. J. Biochem. **47,** 405 (1969).
15. Bunn, H. F., Science **172,** 1049 (1973).
16. Mauk, A. G., and Taketa, F., Arch. Biochem. Biophys. **150,** 376 (1972).
17. Snow, N. S., Biochem. J. **84,** 360 (1962).

Received Feb. 26, 1974. P.S.E.B.M., 1974, Vol. 147.