

AD-A163 658

REPORT DOCUMENTATION PAGE

2

2a. SECURITY CLASSIFICATION AUTHORITY		1b. RESTRICTIVE MARKINGS	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE		3. DISTRIBUTION/AVAILABILITY OF REPORT UNLIMITED	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 85-85		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION Naval Med Rsch Institute Detachment (Toxicology)	6b. OFFICE SYMBOL (If applicable) NMRI/TD	7a. NAME OF MONITORING ORGANIZATION Naval Medical Command	
6c. ADDRESS (City, State, and ZIP Code) Area B, Bldg 433 (NMRI/TD) Wright-Patterson AFB, OH 45433-6503		7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington D.C. 20372-5120	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Med Rsch and Dev Command	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) Bethesda, MD 20814-5044		10. SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO. 62758N	PROJECT NO. F58524
		TASK NO. MF58524001	WORK UNIT ACCESSION NO. DN377025
11. TITLE (Include Security Classification) Interspecies Variability in Propylene Glycol Dinitrate-Induced Methemoglobin Formation(U)			
12. PERSONAL AUTHOR(S) Wyman, J.F., Gray, B.H., Coleman, J., Flemming, C., and Uddin, D.E.			
13a. TYPE OF REPORT Journal Article	13b. TIME COVERED FROM TO	14. DATE OF REPORT (Year, Month, Day) 85 12	15. PAGE COUNT 12
16. SUPPLEMENTARY NOTATION TOXICOLOGY AND APPLIED PHARMACOLOGY 1985 Nov;81(2):203-212 (REPRINT)			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
		Keywords: Propylene glycol dinitrate, methemoglobin, interspecies, dog, rat, guinea pig, human, erythrocytic enzymes	
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Interspecies variability of propylene glycol dinitrate (PGDN)- induced methemoglobin formation was studied in vitro employing erythrocytes from four separate species. The net rate of methemoglobin formation was significantly different among species with dog>guinea pig>rat>human. This order of susceptibility was maintained in stroma-free hemolysates, indicating that interspecies variability was not a reflection of differences in red cell membrane permeability or intracellular transport of PGDN. The erythrocytic enzymes, catalase, superoxide dismutase, glutathione peroxidase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, methemoglobin reductase, and glutathione-S-transferase, were assayed by adaption of existing methods to a centrifugal analyzer. The above enzymes were removed from hemoglobin derived from each species and the order of susceptibility to PGDN-induced methemoglobin formation remained essentially the same with dog>guinea pig>human = rat. However, the net rate of PGDN-mediated oxidation of hemoglobin to methemoglobin increased in purified hemoglobin preparations from each species. These results demonstrate (See reverse)			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION	
22a. NAME OF RESPONSIBLE INDIVIDUAL Rosemary Spitzen		22b. TELEPHONE (Include Area Code) (202) 295-2188	22c. OFFICE SYMBOL ISB/ADMIN/NMRI

DD FORM 1473, 84 MAR

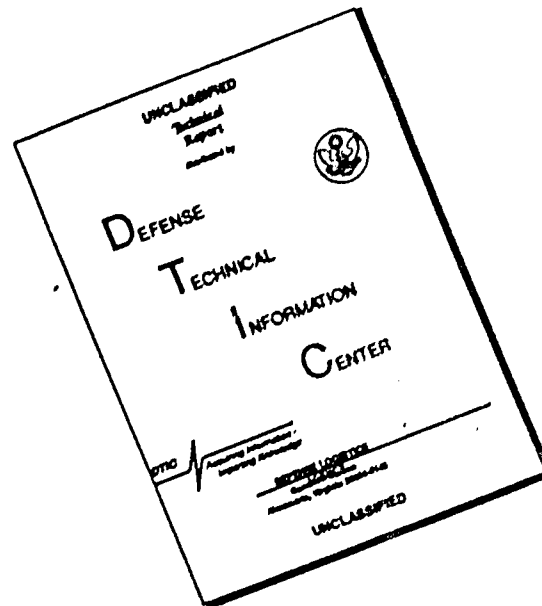
83 APR edition may be used until exhausted.

SECURITY CLASSIFICATION OF THIS PAGE

All other editions are obsolete.

DTIC FILE COPY

DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract (continued) that there is species variability in the net rate of PGDN-mediated methemoglobin formation. Total enzyme activity in erythrocytes may contribute to reduction in the net rate of methemoglobin formation. However, the primary determinant of the net rate of methemoglobin formation induced by PGDN appears to be the structure of each hemoglobin molecule.

Accession For	
DTIC	☒
DTIC	☐
DTIC	☐
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	20

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE

Interspecies Variability in Propylene Glycol Dinitrate-Induced Methemoglobin Formation¹

J. F. WYMAN², B. H. GRAY, L. H. LEE, J. COLEMAN, C. FLEMMING,* AND D. E. UDDIN

Naval Medical Research Institute/Toxicology Detachment, Wright-Patterson Air Force Base, Ohio 45433-6503; and *Toxic Hazards Research Unit, University of California, Irvine, Overlook Branch, P.O. Box 31009, Dayton, Ohio 45431-0009

Received November 9, 1984; accepted July 3, 1985

Interspecies Variability in Propylene Glycol Dinitrate-Induced Methemoglobin Formation. WYMAN, J. F., GRAY, B. H., LEE, L. H., COLEMAN, J., FLEMMING, C., AND UDDIN, D. E. (1985). *Toxicol. Appl. Pharmacol.* 81, 203-212. Interspecies variability of propylene glycol dinitrate (PGDN)-induced methemoglobin formation was studied *in vitro* employing erythrocytes from four separate species. The net rate of methemoglobin formation was significantly different among species with dog > guinea pig > rat > human. This order of susceptibility was maintained in stroma-free hemolysates, indicating that interspecies variability was not a reflection of differences in red cell membrane permeability or intracellular transport of PGDN. The erythrocytic enzymes, catalase, superoxide dismutase, glutathione peroxidase, 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, methemoglobin reductase, and glutathione-S-transferase, were assayed by adaptation of existing methods to a centrifugal analyzer. The above enzymes were removed from hemoglobin derived from each species and the order of susceptibility to PGDN-induced methemoglobin formation remained essentially the same with dog > guinea pig > human > rat. However, the net rate of PGDN-mediated oxidation of hemoglobin to methemoglobin increased in purified hemoglobin preparations from each species. These results demonstrate that there is species variability in the net rate of PGDN-mediated methemoglobin formation. Total enzyme activity in erythrocytes may contribute to reduction in the net rate of methemoglobin formation. However, the primary determinant of the net rate of methemoglobin formation induced by PGDN appears to be the structure of each hemoglobin molecule. © 1985 Academic Press, Inc.

Propylene glycol dinitrate (PGDN, 1,2-propanediol dinitrate) is the primary component

of the Naval torpedo propellant, Otto Fuel II. Symptoms of PGDN intoxication, including vasodilation, hypotension, headache, and methemoglobin formation (Jones *et al.*, 1972; Stewart *et al.*, 1974), are similar to those caused by other nitrated esters (Ellis *et al.*, 1984; Strein *et al.*, 1984; Andersen and Mehl, 1973; Clark and Litchfield, 1969; Carmichael and Lieben, 1963). Acute intoxication of animals with PGDN produces methemoglobinemia, and the resulting anoxia is proposed as the immediate cause of death (Clark and Litchfield, 1969; Jones *et al.*, 1972; Andersen and Mehl, 1973).

¹ This work was supported by the Naval Medical Research and Development Command, Research Task MR0412201 0003. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. The experiments conducted herein were performed according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

² Correspondence should be addressed to John F. Wyman, LCDR, MSC, USN, ASD/NMRI/TD, Building 433, Area B, Wright-Patterson AFB, Ohio 45433.

The specific mechanism of the reaction of nitrate esters with hemoglobin is quite complex and as yet not well understood. Oxidation of human and rat hemoglobin by PGDN has been attributed to direct interaction of the dinitrate with oxyhemoglobin (Andersen and Smith, 1973). Other nitrate esters produce methemoglobin by cleavage of nitrite from the ester, followed by hemoglobin oxidation by nitrite (Strein *et al.*, 1984). The reaction of PGDN with hemoglobin does release nitrite within the erythrocyte (Clark and Litchfield, 1969) and this nitrite may further stimulate methemoglobin formation. Moreover, denitrication of nitrate esters may occur enzymatically through glutathione-S-transferase or nonenzymatically by reaction with glutathione (Heppel and Hilmoe, 1950; Needleman and Hunter, 1965; Chasseaud *et al.*, 1978). Mechanisms potentially exist for both direct interaction of PGDN with hemoglobin and nitrite oxidation of hemoglobin to produce methemoglobin with this nitrate ester. Once initiated by nitrite, oxidation of hemoglobin to methemoglobin proceeds autocatalytically as a result of superoxide production (Watanabe and Ogata, 1981; Kosaka *et al.*, 1979; Misra and Fridovich, 1972; Lynch *et al.*, 1976, 1977).

There is *in vivo* species variability in the rate of methemoglobin formation following PGDN exposure, with dogs and monkeys being more susceptible than rats or guinea pigs (Jones *et al.*, 1972). Similarly, interspecies variability was reported for nitrite-induced methemoglobin formation in isolated erythrocytes from rats, sheep, and humans (Calabrese *et al.*, 1983). Both of the above studies suggested that the observed differences in susceptibility to methemoglobin formation may result from variable activities of erythrocyte methemoglobin reductase. Indeed, human erythrocytes congenitally impaired by insufficient methemoglobin reductase activity often contain elevated methemoglobin amounts (Scott, 1960). Factors other than total methemoglobin reductase enzyme activity in erythrocytes may also contribute to observed species variability in net rates of hemoglobin oxidation. It is pos-

sible that antioxidants in erythrocytes from different species, including amounts of antioxidant enzymes, may contribute to reduction in the net rate of methemoglobin formation. Also, variations in amounts of glutathione-S-transferase may be important in releasing nitrite from nitrate esters, subsequently affecting net oxidation rates. Finally, species variation in hemoglobin structure may contribute to the variability of PGDN-mediated oxidation of hemoglobins.

In this study we determined if variability existed in the net rate of PGDN-mediated formation of methemoglobin in isolated erythrocytes from four mammalian species. In addition, cell-free and partially purified hemoglobin preparations from each species were examined to determine if net rates of methemoglobin production varied among species. Selected enzyme activities in erythrocytes from each species were also compared in an attempt to explain variations observed in PGDN-mediated hemoglobin oxidation. Enzyme profiles plus results with cell-free and partially purified hemoglobin preparations were examined to determine the relative contribution of enzymes and the hemoglobin molecule itself in maintenance of oxyhemoglobin upon exposure to PGDN.

METHODS

Chemicals

PGDN was graciously provided by Mr. Robert Sarncomb, Naval Surface Weapons Center, Indian Head, Maryland.

PGDN was stored as a 10% solution in ethanol until needed, at which time 1 to 3 ml of PGDN was separated from ethanol by extraction of ethanol in 10 to 30 ml water. The purity of PGDN isolated in this manner was verified by gas-liquid chromatography employing an electron capture detector (Erk *et al.*, 1982).

Tris(hydroxymethyl)aminomethane and 1-chloro-2,4-dinitrobenzene were purchased from Aldrich Chemical Company, sodium citrate was obtained from J. T. Baker, and disodium ethylenediaminetetraacetate (EDTA) was from Fisher Scientific. β -D-Glucose 6-phosphate, β -NAD⁺, β -NADP⁺, β -NADPH, cysteine, glutathione reductase, glycylglycine, maleimide, phosphogluconic acid, pyrogal-

lol, and sodium azide were purchased from Sigma Chemical Company. All other chemicals used in this study were of reagent-grade quality.

Blood Collection

Fresh blood was obtained from Fisher 344 rats and Hartley guinea pigs by cardiac puncture, under ether anesthesia, and from the jugular veins of beagle dogs. All animals were housed in cages with water and laboratory chow provided *ad libitum*. Human blood was collected from brachial veins of volunteers within our laboratory. The anticoagulant used in all collections was citrate-phosphate-dextrose (Lee and Henry, 1979).

Erythrocyte Isolation and Hemoglobin Preparation

Erythrocytes were separated from plasma by centrifugation and washed three times in Dulbecco's phosphate-buffered saline (Dulbecco and Vogt, 1954) or physiological saline. Hemolysates were prepared by lysing red cells in an equal volume of 1 mM phosphate buffer (pH 7.8). Crystallization of hemoglobin solutions was prevented by adjusting the pH to 7.8 (dog, guinea pig, and human) or 8.5 (rat). Red cell stroma was removed by centrifugation at 30,000g max for 30 min.

Partial purification of dog, guinea pig, and human hemoglobins was accomplished by ion-exchange chromatography with carboxymethyl cellulose cation exchange resin, Whatman CM 52 (Lynch *et al.*, 1977). Stroma-free hemoglobin solutions were dialyzed overnight against 2 liters of distilled water. The dialysate (approximately 20 to 40 ml) was diluted to 500 ml with 5 mM phosphate buffer (pH 6.8) and added to a column containing CM 52 resin which had been equilibrated with the same phosphate buffer (bed volume approximately 75 to 125 ml). Under these conditions the hemoglobin readily bound to the resin with only small amounts eluting off the column. The column was developed overnight with 5 mM phosphate buffer to ensure a clear column effluent after which hemoglobins were eluted from the resin with 1 M phosphate buffer (pH 6.8). The hemoglobin effluent was collected, the pH adjusted to 7.8 with 1 N NaOH, and the solution concentrated by ultrafiltration using an Amicon stirred cell concentrator with a PM 30 membrane filter.

Rat hemoglobin crystallized at neutral pH, preventing purification of this hemoglobin by the method employed for the other species. Therefore, 30,000g max supernatant fractions from rat hemolysates were adjusted to pH 7 to allow crystallization. Solutions were stored in an ice bath for approximately 2 hr, after which crystals were collected by centrifugation at 500g. The supernatant fraction was decanted, and the crystalline pellet was resuspended and washed in an equal volume of distilled water (three repetitions). Crystals of rat hemoglobin were dissolved by adjusting the pH to 9.3 with 1 N NaOH, and insoluble ma-

terial was removed by centrifugation at 30,000g max for 10 min. Resultant supernatant fractions were dialyzed overnight against distilled water.

In Vitro Incubations

Erythrocytes, hemolysates, and partially purified hemoglobin preparations were incubated in polypropylene tubes at 37°C in a shaking water bath. The hemoglobin concentration for all incubations was approximately 775 μ M (total volume = 5 ml). Amounts of PGDN were measured gravimetrically ($d = 1.6$ g/ml at 20°C) and added neat (10 mM) or in dilution: ethanol (3, 1, and 0.3 mM), with control preparations containing ethanol only. Samples (0.2 ml) were removed from erythrocyte-PGDN mixtures at specified times during the 1-hr incubations and lysed in 0.8-ml volumes of 1 mM phosphate buffer (pH 7.8). From these hemolysates and from partially purified hemoglobin solutions, 0.3-ml aliquots were removed, diluted in 0.4 ml of 50 mM phosphate buffer (pH 6.6), and assayed for methemoglobin as described below.

Analytical Methods

Spectrophotometric assays, with the exception of the catalase enzyme assay, employed a Cobas-Bio centrifugal analyzer (Roche Analytical Instruments, Inc.). Assays were adapted to the centrifugal analyzer from previously reported methods. Unless otherwise stated, reagents were prepared in distilled water and concentrations listed represent the final concentration in the reaction cuvette. The specific program parameters employed are listed in Table 1. The concentration of hemoglobin in samples assayed for enzymatic activity was initially about 50 mg/ml. The specific dilutions prepared for each assay are described below.

Hemoglobin and methemoglobin. The concentration of hemoglobin in whole-cell preparations, in hemolysates, and in partially purified hemoglobin solutions was determined with Drabkins reagent (van Kampen and Zijlstra, 1961). Prior to assay washed whole-cell preparations were lysed by osmotic shock by diluting 0.1 ml red cells with 0.9 ml 1 mM phosphate buffer (pH 7.8).

Methemoglobin was determined by modifying the method of Evelyn and Malloy (1938) to permit use of the centrifugal analyzer. The optical density (630 nm) of hemoglobin solutions was measured with four separate reagents. All reagents (total volume 10 ml) contained 9.375 ml 0.1 M phosphate buffer (pH 6.6); reagents 1 and 3 contained 0.625 ml distilled water, while reagents 2 and 4 contained 0.625 ml 0.4 M $K_3Fe(CN)_6$. Finally, 0.61 mmol KCN was dissolved in reagents 3 and 4. Total hemoglobin concentrations were adjusted to about 0.5 μ M with 50 mM phosphate (pH 6.6) or 1 mM phosphate (pH 7.8) for rat, and analysis was performed with each reagent by the pro-

TABLE I
PARAMETER LISTING FOR ENZYMATIC ACTIVITY ASSAYS PERFORMED ON THE COBAS-BIO CENTRIFUGAL ANALYZER

Parameter	Cyanomet- hemoglobin (mol/l)	Methemoglobin (mmol/l)	GSH peroxidase (mol/l)	GSH-S- transferase (mol/l)	Glucose-6- phosphate dehydrogenase (mol/l)	6-Phosphogluconate dehydrogenase	Methemoglobin reductase (U/l)
Calculation factor	227.27	1	2009.6	1041.67	1607.7	1607.7	238.1
Limit	500	3	1	2	1	1	2
Temperature (°C)	25	25	25	25	30	25	37
Type of analysis	1	5	3	3	2	2	3
Wavelength	540	630	340	340	340	340	575
Sample volume (μl)	25	25	20	25	25	25	25
Diluent volume (μl)	25	25	20	30	25	25	25
Reagent volume (μl)	250	200	250	150	200	200	170
Incubation time (sec)	10	0	10	10	0	0	600
Starting reagent volume (μl)	0	0	10	25	0	0	10
Time of first reading (sec)	240	180	10	1	10	10	0.5
Time interval (sec)	240	180	10	10	10	10	10
Number of readings	2	1	9	24	10	10	30
Blanking mode	1	1	1	1	1	1	1
Printout mode	1	3	4, 1	1	1, 3, 4	1, 3	1

gram parameters listed in Table 1. The percentage of methemoglobin was calculated by

$$\% \text{ methHb} = 100 \left[\frac{\text{OD}_1 - \text{OD}_3}{\text{OD}_2 - \text{OD}_4} \right]$$

where methHb = methemoglobin and OD = optical density. The net percentage of methemoglobin formed by PGDN oxidation of hemoglobin was calculated by subtracting the percentage of methemoglobin in control (ethanol-treated) hemoglobin from that of PGDN-treated hemoglobins.

Catalase activity: Catalase was determined by existing methods (Beers and Sizer, 1952; Aebi, 1971). Samples were diluted with 0.1 M phosphate buffer (pH 7.0) so that a ΔOD_{240} from 0.45 to 0.40 occurred in approximately 10 sec (Beckman DK2 uv-vis spectrophotometer). The dilution ratio for dog was 1:10, for the rat it was 1:20, and for guinea pig and human it was 1:30.

Superoxide dismutase (SOD): CuZn SOD activity was measured employing a modified pyrogallol autoxidation method (Gray *et al.*, 1985).

Glutathione peroxidase: Samples were diluted in an equal volume of 0.15 M phosphate buffer (pH 7.0) containing EDTA (0.15 M final concentration). Hemoglobin was oxidized to methemoglobin by adding 4 parts Drabkins reagent about 10 min prior to beginning the assay. The reagent mixture consisted of 50 mM phosphate buffer, 5 mM EDTA (pH 7.0), 3.7 mM sodium azide, 5 mM reduced glutathione (prepared in 50 mM phosphate buffer, pH 7.0), 1 U glutathione reductase, and 0.28 mM NADPH. Hydrogen peroxide (70 μM) was used to start the reaction (Paglia and Valentine, 1967).

Glutathione-S-transferase: Samples were diluted in equal volumes of 1 mM phosphate buffer (pH 7.5). The reagent mixture consisted of 0.75 mM 1-chloro-2,4-dinitrobenzene (prepared in ethanol) and 0.1 M phosphate buffer (pH 6.25). Reduced glutathione was added to start the reaction (Carmagnol *et al.*, 1981).

Glucose-6-phosphate dehydrogenase: Samples prepared from dog, guinea pig, and human hemolysates were diluted with equal volumes of 10 mM Tris buffer (pH 7.5). Rat hemolysates were diluted with 4 vol of 10 mM Tris (pH 7.5). Enzyme activity was assayed utilizing the method of Kachmar and Moss (1976), with a reagent mixture composed of 0.1 M Tris buffer (pH 7.5), 10 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 mM NADP, 0.7 mM β -D-glucose 6-phosphate, and 4 mM maleimide.

6-Phosphogluconate dehydrogenase: Diluted samples prepared for the glucose-6-phosphate dehydrogenase assays were used for this determination. The assay used a reaction mixture composed of 50 mM glycylglycine buffer (pH 8.6), 20 mM MgCl_2 , 2 mM phosphogluconic acid, 6.5 mM cysteine, and 1.5 mM NADP (King, 1971).

Methemoglobin reductase: The dilution ratio for samples, with 1 mM phosphate buffer (pH 7.8), was 1:5 for dog, 1:10 for rat and human, and 1:20 for guinea pig. The reagent mixture for this assay consisted of 0.54 mM EDTA (disodium salt), 5 mM sodium citrate (trisodium salt), 0.16 mM $\text{K}_3\text{Fe}(\text{CN})_6$, and 3.5 μM diaphorase-free hemoglobin

from rat. NADH (0.2 mM) prepared in 1 mM phosphate buffer (pH 7.8) was used as the starter reagent. The assay was adapted from the method of Hagesh *et al.* (1968).

Statistical Analysis

Amounts of PGDN-induced methemoglobin formation in erythrocytes, hemolysates, and partially purified hemoglobins from different species were evaluated for significant differences by two- and three-factorial multivariate repeated-measure designs (Dixon, 1983; Timm, 1975). The factors included species, dose, and type of preparation with time being the repeated measure. A pairwise Scheffé test was used to analyze differences within given significant interactions.

RESULTS

In vitro comparison of the relative rates of PGDN-induced methemoglobin formation in intact erythrocytes from dog, guinea pig, rat, and human demonstrated interspecies differences (Fig. 1). At a concentration of 10 mM PGDN the amount of methemoglobin formation in dog erythrocytes was significantly higher ($p < 0.05$) than that of human erythrocytes after 1 hr incubation. After 2 hr, dog methemoglobin was significantly higher than

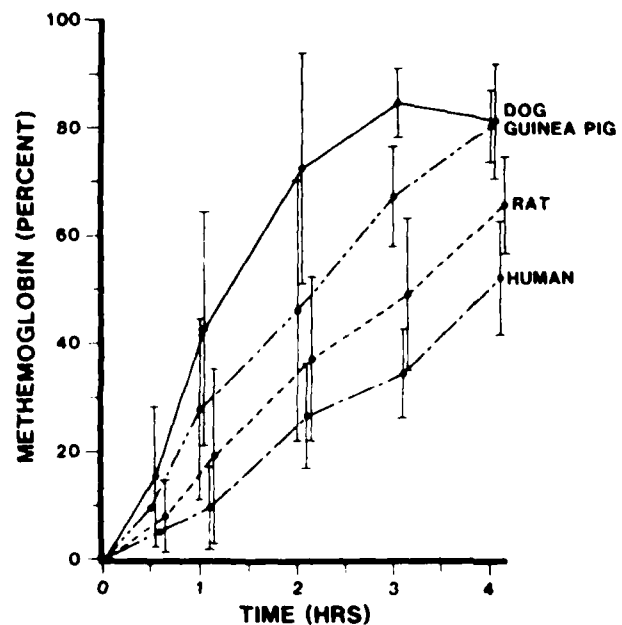


FIG. 1. PGDN-induced methemoglobin formation in erythrocytes from different species. Concentration of PGDN = 10 mM. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details about erythrocyte preparations.

for hemoglobin of all other species. At 3 hr, methemoglobin content of guinea pig was similar to that of dog and different from that of human and rat. Methemoglobin quantities in rat erythrocytes were significantly higher than human after 4 hr ($p < 0.001$) but remained below that observed for dog and guinea pig. Under these experimental conditions, the net rate of PGDN-induced methemoglobin formation was dog > guinea pig > rat $\geq$ human. The relatively high amount of methemoglobin formation in dogs has been previously reported *in vivo*, following chronic inhalation of PGDN vapors (Jones *et al.*, 1972).

The net rate of methemoglobin formation was compared in stroma-free hemolysates to evaluate whether differences in oxidation of hemoglobins might be attributable to differences in the membrane permeability of various red cells to PGDN. Figure 2 shows methemoglobin formation as a function of time for hemolysates (30,000g supernatant fractions prepared from erythrocytes from each species). The concentration of PGDN tested with 775 μ M hemoglobin was 3 mM. Significant differences among species were observed at different times during the incubation. Thirty minutes after the addition of PGDN, the net amounts of methemoglobin was significantly higher in dog and guinea pig as compared to that of human and rat hemolysates. Differences between the amounts of methemoglobin formed for human and rat hemolysates were statistically significant after 3 hr, while differences between dog and guinea pig were significant after 4 hr incubation. Thus, after 4 hr net methemoglobin formation was significantly different in hemolysates prepared from each species with dog > guinea pig > rat > human. The order of susceptibility to methemoglobin formation observed with intact erythrocytes was maintained in hemolysate preparations.

Methemoglobin formation in hemolysates from each species was a function of PGDN concentration (Fig. 3). Significant differences among species at specific times during an incubation were dependent upon the concen-

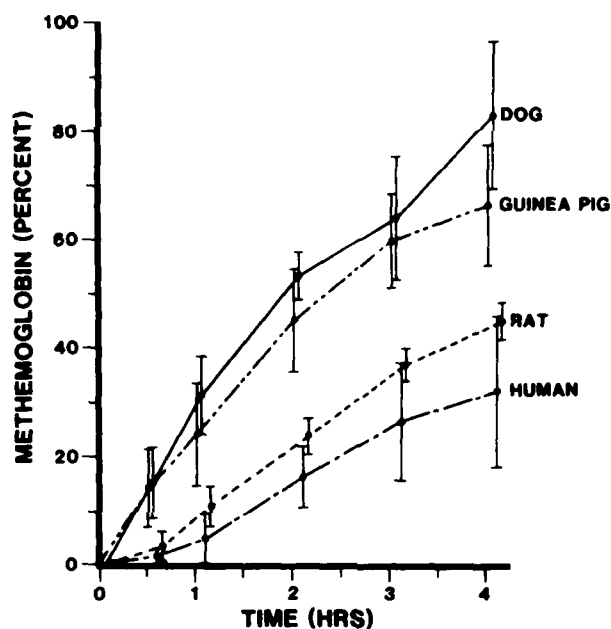


FIG. 2. PGDN-induced methemoglobin formation in stroma-free hemolysates from different species. Concentration of PGDN = 3 mM. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details regarding preparation of hemolysates.

tration of PGDN employed. Human hemoglobin was the least susceptible to oxidation by PGDN when the dose was increased.

Table 2 lists the enzyme activity determined for erythrocytes from each of the four species. Species with high methemoglobin reductase activity are not consequently better protected against hemoglobin oxidation by PGDN (Jones *et al.*, 1972). Methemoglobin reductase activity is three times higher in guinea pig erythrocytes compared to that of human and rat, although the latter were significantly less susceptible to methemoglobin formation (Figs. 1 and 2). Second, glutathione-S-transferase activity in dog and guinea pig erythrocytes is lower than that determined for rat and human. Also, rat erythrocytes possess a more active complement of antioxidant enzymes, including glutathione peroxidase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and superoxide dismutase, as compared with other species. These results suggest that rat erythrocytes may be better protected against the oxidation of hemoglobin to methemoglobin. The lack of measurable

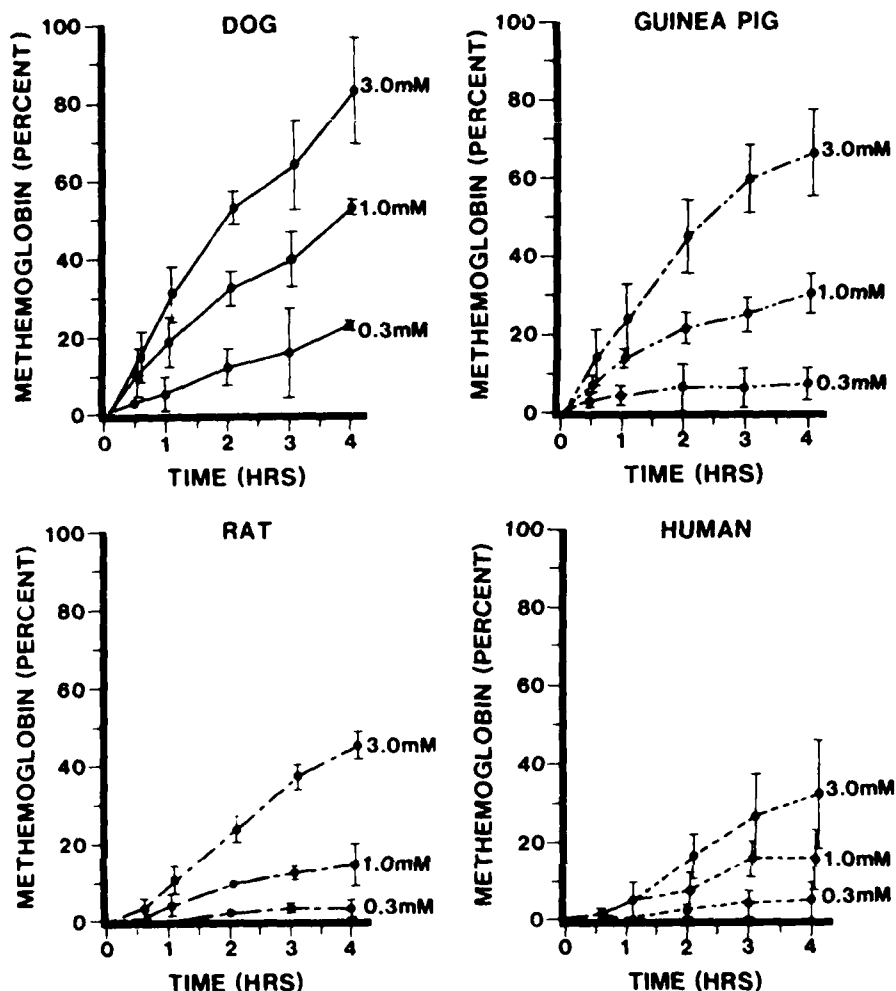


FIG. 3. Formation of methemoglobin in stroma-free hemolysates from different species at varying PGDN concentrations. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details.

catalase activity in dog erythrocytes is consistent with this suggestion. In conflict with this suggestion is the finding that the antioxidant enzyme profile of human erythrocytes resembles that of the guinea pig, even though human hemoglobin preparations are relatively resistant to oxidation by PGDN.

Assays of the rate of oxidation of partially purified hemoglobin solutions from each species were performed with 1 mM PGDN to determine the effect of absence of these enzymes on methemoglobin formation. There was no detectable enzyme activity in hemoglobin preparations prepared by column chromatography as described above. In addition to the removal of enzymes, partial purification of hemoglobin eliminated small molecules which

allosterically affect stability within erythrocytes. Removal of these compounds may have influenced the extent of oxidation of individual hemoglobin molecules. The amount of methemoglobin formation in these partially purified hemoglobin preparations appears elevated for all species, compared to that observed for hemolysates (Fig. 4). Differences were statistically significant only for dog and guinea pig. Therefore, those species most susceptible to PGDN-induced methemoglobin formation appear to be afforded the greatest degree of protection by their erythrocytic enzyme complement. Also, the order of susceptibility observed in hemolysates, as well as whole cells, is not altered by the removal of erythrocytic enzymes.

TABLE 2
COMPARATIVE ENZYME ACTIVITY IN ERYTHROCYTES FROM DIFFERENT SPECIES^a

Enzyme	Animal species			
	Dog (n = 8) ^b	Guinea pig (n = 7)	Human (n = 8)	Rat (n = 6)
Methemoglobin reductase ($\mu\text{mol}/\text{min}/\text{g Hb}^c$)	2.18 ± 0.82^d	15.5 ± 1.88	4.66 ± 1.29	3.59 ± 0.90
Glutathione-S-transferase ($\mu\text{mol}/\text{min}/\text{mg Hb}$)	0.73 ± 0.24	2.57 ± 0.83	3.88 ± 1.28	3.86 ± 1.32
Catalase ($\mu\text{mol}/\text{min}/\text{mg Hb}$)	ND ^e	133 ± 40	188 ± 31	121 ± 33
Superoxide dismutase (unit/mg Hb)	2.39 ± 0.44	1.66 ± 0.41	1.90 ± 0.34	3.98 ± 0.31
Glutathione peroxidase ($\mu\text{mol}/\text{min}/\text{g Hb}$)	104 ± 20	66 ± 16	18 ± 9	606 ± 182
6-Phosphogluconate dehydrogenase ($\mu\text{mol}/$ $\text{min}/\text{mg Hb}$)	2.64 ± 0.62	3.07 ± 0.51	3.85 ± 0.29	5.25 ± 3.02
Glucose-6-phosphate dehydrogenase ($\mu\text{mol}/$ $\text{min}/\text{mg Hb}$)	5.31 ± 0.99	5.42 ± 1.04	5.46 ± 1.54	19.1 ± 6.45

^a Activity for enzymes (except catalase) was determined with a Cobas-Bio centrifugal analyzer (Roche Analytical Instruments). Catalase activity was determined at 240 nm in a Beckman DK-2 spectrophotometer.

^b n = number of determinations.

^c Hb = hemoglobin

^d Values represent the $\bar{x} \pm$ standard deviation.

^e ND = not detected.

DISCUSSION

PGDN reacts with oxyhemoglobin in solution, resulting in oxidation of the molecule to methemoglobin. The rate of methemoglobin formation in hemoglobin solutions upon addition of PGDN reflects a direct interaction with oxyhemoglobin, oxidation of hemoglobin by nitrite released from PGDN, and autocatalytic oxidations due to active oxygen. These reactions collectively produce the methemoglobin observed upon addition of PGDN. Enzymatic cleavage by glutathione-S-transferase of PGDN with release of nitrite is not required to initiate methemoglobin formation in hemoglobin preparations.

Interspecies variability in PGDN-mediated methemoglobin formation has previously been reported *in vivo* (Jones *et al.*, 1972). The variability among species *in vitro*, demonstrated in the present investigation, indicates that fac-

tors responsible for varied susceptibility to methemoglobin formation are primarily attributed to factors associated with the erythrocyte. In partially purified hemoglobin solutions, stroma-free hemolysates, and erythrocytes, the net rate of PGDN-mediated methemoglobin formation follows the sequence dog > guinea pig > rat $\geq$ human. Thus, effects of variation in erythrocyte membrane permeability to PGDN among species does not account for varied susceptibility to methemoglobin formation. The observed decrease in net amounts of methemoglobin formed in dog and guinea pig stroma-free hemolysates compared to partially purified hemoglobin solutions probably reflects the contribution of enzymes and/or small molecular weight allosteric effectors present in the latter preparations. Although there was a similar, slight decrease in methemoglobin formation in human and rat stroma-free hemo-

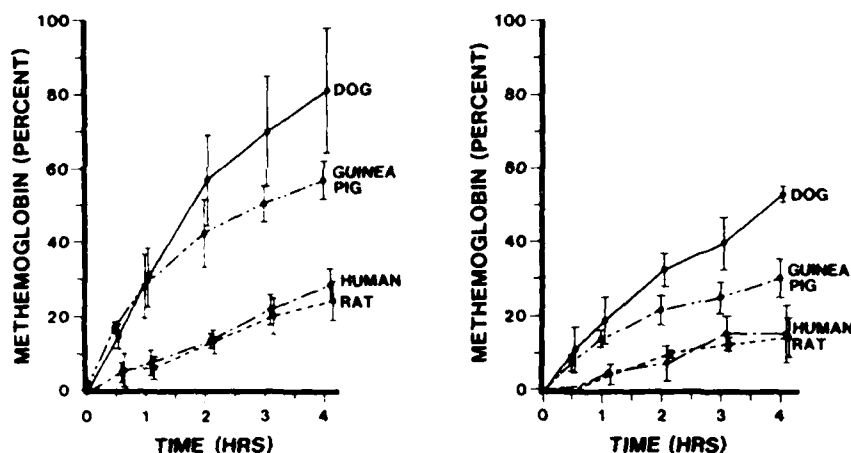


FIG. 4. Comparison of methemoglobin formation in enzyme-free hemoglobin solutions and stroma-free hemolysates from different species. Concentration of PGDN = 1 mM. Values represent the $\bar{x} \pm$ standard deviation, and individual data points for each time period are offset for purposes of presentation. See Methods for specific details.

lysates compared to hemoglobin solutions, the decrease was not statistically significant. Thus, the contribution of antioxidant enzymes in human and rat stroma-free hemolysates does not appear to alter net methemoglobin formation substantially, even though the rat erythrocyte is comparatively well-equipped to handle oxidant stress. However, incubation mixtures were not fortified with excess reductants and cofactors which, upon depletion from the reaction mixtures, may have limited the antioxidant enzyme activity. Further studies are required to outline contributions of specific enzymes, as well as allosteric stabilizers, to maintenance of oxyhemoglobin amounts in erythrocytes.

The primary determinant of the relative susceptibility of different species to PGDN-induced hemoglobin oxidation appears to be the molecular structure of each hemoglobin. Maintenance of the order of susceptibility to methemoglobin formation in each type of preparation, erythrocyte, stroma-free hemolysate, and enzyme-free hemoglobins, lends support to specific hemoglobin structure controlling PGDN-mediated oxidation rates. Differences in hemoglobin structure reflect differences in globin chains of the molecule since heme moieties are identical. That structural differences among animal hemoglobins result in different susceptibilities to oxidation is

somewhat analogous to observations with abnormal human hemoglobins of the M and S types, which, because of congenital alterations in the amino acid sequence of globin chains, are more labile to oxidation. The results of this study point out the need for careful selection of an animal model when studying toxicant-induced oxidation of hemoglobin. Assuming nitrate esters, in general, behave like PGDN, the animal model most closely resembling man is rat.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the excellent technical assistance of W. Burns, L. Gilbert, and J. Lawson. The assistance of D. Paquette and S. Young of the Veterinary Sciences Division and the clerical assistance of P. Gergely, Toxic Hazards Division, Air Force Aerospace Medical Research Laboratory, Wright-Patterson AFB, are greatly appreciated.

REFERENCES

- AEHL, H. (1971). Catalase. In *Methods in Enzymatic Analysis* (H. U. Bergmeyer, ed.), Vol. 2, pp. 673-684. Academic Press, New York.
- ANDERSEN, M. E., AND MEHL, R. G. (1973). A comparison of the toxicology of triethylene glycol dinitrate and propylene glycol dinitrate. *Amer. Ind. Hyg. Assoc. J.* **34**, 526-532.

- ANDERSEN, M. E., AND SMITH, R. A. (1973). On the mechanism of oxidation of human and rat hemoglobin by propylene glycol dinitrate. *Biochem. Pharmacol.* **22**, 3247-3256.
- BEERS, R. E., AND SIZER, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195**, 133-140.
- CALABRESE, E. J., MOORE, G. S., AND MCCARTHY, M. S. (1983). The effect of ascorbic acid on nitrite-induced methemoglobin formation in rats, sheep, and normal human erythrocytes. *Regul. Toxicol. Pharmacol.* **3**, 184-188.
- CARMAGNOL, E., SINET, P.-M., RAPIN, J., AND HENRI, J. (1981). Glutathione-S-transferases of human red blood cells—Assay values in normal subjects and in two pathological circumstances: Hyperbilirubinemia and impaired renal function. *Clin. Chem. Acta* **117**, 209-217.
- CARMICHAEL, P., AND LIEBEN, J. (1963). Sudden death in explosive workers. *AMA Arch. Environ. Health* **7**, 50-65.
- CHASSEAUD, L. F., DOWN, W. H., AND SACHARIN, R. M. (1978). Effect of repeated oral administration of isosorbide dinitrate on hepatic glutathione-S-transferase activity in the rat. *Biochem. Pharmacol.* **27**, 1695-1697.
- CLARK, D. G., AND LITCHFIELD, M. H. (1969). The toxicity, metabolism, and pharmacologic properties of propylene glycol 1,2-dinitrate in experimental animals. *Toxicol. Appl. Pharmacol.* **22**, 128-137.
- DIXON, W. J. (ed.) (1983). *BMDP Statistical Software Manual*. Univ. of California Press, Berkeley.
- DUTBECCO, R., AND VOGT, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **98**, 167-182.
- ELLIS, H. V., HONG, C. B., LEE, C. C., DACRE, J. C., AND GLENNON, J. P. (1984). Subacute and chronic toxicity studies of trinitroglycerine in dogs, rats, and mice. *Fundam. Appl. Toxicol.* **4**, 248-260.
- ERK, S. D., JARBOE, C. H., NEWTON, P. E., AND PELEDERER, C. (1982). Gas chromatographic determination of 1,2-propanediol dinitrate in blood. *J. Chromatogr.* **240**, 117-123.
- EVELYN, K. A., AND MALLOY, H. T. (1938). Microdetermination of oxyhaemoglobin, methaemoglobin, and sulphaemoglobin in a single sample of blood. *J. Biol. Chem.* **126**, 655-662.
- GRAY, B. H., LEE, L. H., AND WYMAN, J. F. (1985). An automated analysis for superoxide dismutase enzyme activity. *J. Anal. Toxicol.* **9**, 36-39.
- HAGESH, E., CALMONOVICI, N., AND AVRON, M. (1968). New method for determining ferrihemoglobin reductase (NADH-methemoglobin reductase) in erythrocytes. *J. Lab. Clin. Med.* **72**, 339-344.
- HEPPEL, L. A., AND HILMOE, R. J. (1950). Metabolism of inorganic nitrite and nitrate esters. *J. Biol. Chem.* **183**, 129-138.
- JONES, R. A., STUBBS, J., AND SUGG, J. (1972). Toxicity of propylene glycol 1,2-dinitrate in experimental animals. *Toxicol. Appl. Pharmacol.* **22**, 128-137.
- KACHMAR, J. F., AND MOSS, D. W. (1976). Enzymes. In *Fundamentals of Clinical Chemistry* (N. W. Tietz, ed.), pp. 666-672. Saunders, Philadelphia.
- KING, J. (1971). 6-Phosphogluconate dehydrogenase. In *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.), Vol. 2, pp. 632-635. Academic Press, New York.
- KOSAKA, H., IMAIZUMI, K., IMAI, K., AND TYUMA, I. (1979). Stoichiometry of the reaction of oxyhemoglobin with nitrite. *Biochim. Biophys. Acta* **581**, 184-188.
- LEE, C. L., AND HENRY, J. B. (1979). Immunohematology, blood banking, and hemotherapy. In *Clinical Diagnosis and Management by Laboratory Methods* (J. B. Henry, ed.), 16th ed., chap. 43, pp. 1465-1466. Saunders, Philadelphia.
- LYNCH, R. E., LEE, G. R., AND CARTWRIGHT, G. E. (1976). Inhibition by superoxide dismutase of methemoglobin formation from oxyhemoglobin. *J. Biol. Chem.* **251**, 1015-1019.
- LYNCH, R. E., THOMAS, J. E., AND LEE, G. R. (1977). Inhibition of methemoglobin formation from purified oxyhemoglobin by superoxide dismutase. *Biochemistry* **16**, 4563-4567.
- MISRA, H. P., AND FRIDOVICH, I. (1972). The generation of superoxide radical during the autoxidation of hemoglobin. *J. Biol. Chem.* **247**, 6960-6962.
- NEEDLEMAN, P., AND HUNTER, F. E. (1965). The transformation of glyceryl trinitrate and other nitrates by glutathione organic nitrate reductase. *Mol. Pharmacol.* **1**, 77-86.
- PAGLIA, D. E., AND VALENTINE, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**, 158-169.
- SCOTT, F. M. (1960). The relation of diaphrase of human erythrocytes to inheritance of methemoglobinemia. *J. Clin. Invest.* **39**, 1176-1179.
- STEWART, R. D., PETERSON, J. E., NEWTON, P. E., HAKE, C. L., HOSKO, M. J., LEBRUN, A. J., AND LAWTON, G. M. (1974). Experimental human exposure to propylene glycol dinitrate. *Toxicol. Appl. Pharmacol.* **30**, 377-395.
- STREIN, K., BARTSCH, W., SPONER, G., MULLER-BECKMAN, B., AND LEXA, P. (1984). Differences in the nitrite ion formation and the toxicological findings between isosorbide dinitrate and isosorbide-5-mononitrate. *Toxicol. Appl. Pharmacol.* **72**, 142-147.
- TIMM, N. H. (1975). *Multivariate Analysis with Applications in Education and Psychology*. Brooks/Cole, Monterey, Calif.
- VAN KAMPEN, E. J., AND ZIJLSTRA, W. G. (1961). Standardization of hemoglobinometry. II. The hemoglobin-cyanide method. *Clin. Chem. Acta* **6**, 538-545.
- WATANABE, S., AND OGATA, M. (1981). Generation of superoxide and hydrogen peroxide during interaction of nitrite with human hemoglobin. *Acta Med. Okayama* **35**, 173-178.