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# ON THE MECHANISM OF THE OXIDATION OF HUMAN AND RAT HEMOGLOBIN BY PROPYLENE GLYCOL DINITRATE*

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**Abstract**—The kinetics and stoichiometry of the oxidation of human and rat oxyhemoglobin ($O_2Hb$) by propylene glycol dinitrate (PGDN) have been investigated *in vitro* in both hemolysate and in intact red blood cell preparations. In hemolysate fractions from both these species, the rate constant for oxidation is nearly constant between pH 7·0 and 9·0, but it increases dramatically at lower pH values. The reaction is molecular and approximately first-order in both [PGDN] and in [$O_2Hb$]. The rate of oxidation is related complexly to the $O_2$ concentration. No oxidation occurs at zero $O_2$ concentration or at very high $O_2$ concentrations. Maximal rates are observed at $O_2$ concentrations where the hemoglobin is only partially saturated with $O_2$. The stoichiometry appears to be 1·5 hemes oxidized per ester bond broken. In whole cells, the reaction is still molecular, is approximately first-order in both reactants, and has a stoichiometry of 1·9 to 2·3 moles heme oxidized per mole of reacted ester.

THE ABILITY of organic polynitrates to oxidize hemoglobin was recognized before the turn of the century.[1,2] Numerous investigations of the pharmacologic and toxicologic properties of these nitrates have been conducted since that time, yet it is not known whether the hemoglobin is oxidized by the nitrate itself or by nitrite produced upon the hydrolysis of the nitrate ester. Even the question of whether the reaction is molecular or enzymatic is still open.[3] On the basis of the amounts of methemoglobin (met-Hb) formed as a function of the amount of organic nitrate administered to the cat, Wilhelmi[4] suggested that the reaction was molecular. However, no systematic chemical analysis of this reaction has yet appeared and Wilhelmi's data are by no means conclusive.

The metabolism of polynitrates in blood has received considerable attention.[3] Clark and Litchfield[5] found that propylene glycol dinitrate (PGDN) when incubated with rat blood was broken down to inorganic nitrite ($NO_2^-$), inorganic nitrate ($NO_3^-$), and propylene glycol mononitrate (PGMN), with the 2-isomer predominating. Ethylene glycol dinitrate (EGDN) was known to be metabolized in a similar fashion,[6] and with both of these dinitrates, the hydrolysis occurred in the erythrocytes, not in the plasma.

We recently conducted a detailed comparison of the toxicities of two organic dinitrates, PGDN and triethylene glycol dinitrate (TEGDN).[7] In preliminary experiments with rats, it was observed that PGDN produced larger amounts of met-Hb

* The opinions expressed herein are those of the authors and do not necessarily reflect the view o the Navy Department or the naval service at large.

*in vivo* than did equivalent doses of TEGDN. In order to determine the biochemical basis of this difference in heme-oxidizing potency and to provide a better understanding both of this oxidative process and of the metabolism of these organic dinitrates, we have studied *in vitro* the kinetic and stoichiometric characteristics of the oxidation of human and rat hemoglobins by these organic dinitrates.

## METHODS

*Hemoglobin.* Outdated human blood was obtained from the Blood Bank at the U.S. Naval Hospital, Bethesda, Md., and fresh blood at intervals from the authors. Blood was also obtained from NMRI:O(SD) Sprague–Dawley rats weighing approximately 300 g. Erythrocytes were spun down at 5000 rev/min in a Sorvall RC-2B centrifuge for 5–10 min, and then washed three times with 0·9% saline. For experiments using whole cells, washed erythrocytes prepared only from freshly drawn blood were taken up in two parts 0·9% saline, stored at 4°, and used within a week of their preparation. Hemolysates were prepared from either fresh or outdated erythrocytes* by adding five parts distilled water to one part of packed red cells. After stirring for several min and standing for 5 min at 4°, the stroma were removed by centrifugation at 15,000 rev/min for 15 min. Potassium phosphate buffer pH 7·0 was added to the supernatant to a concentration of 0·01 M. After 30 min the hemolysate was cloudy and was again centrifuged at 15 000 rev/min for 30 min to clear the solution. Because crystals often formed in concentrated hemolysates of rat oxyhemoglobin ($O_2Hb$), these solutions were always filtered before they were used in any experiments.

Concentrations of hemoglobin, calculated on a heme basis, were determined by measuring the absorbance of dilutions of stock $O_2Hb$ preparations in 1 cm square cuvettes in a Gilford model 240 spectrophotometer, and then using the extinction coefficients reported by Banerjee *et al.*[8] Lyophilized, twice-recrystallized bovine met-Hb, obtained from CalBiochem, was reduced to deoxyhemoglobin (deoxyHb) with 0·1% solutions of dithionite (Hardman & Holden, Miles Platting, England). Immediately after reduction it was passed over a column of G-25 Sephadex to remove all small molecule reaction products, and then reoxygenated by gentle swirling.

*Nitrates.* Since the pure dinitrates are unstable, stock solutions contained small amounts of a physiologically inert, water-insoluble stabilizer.[9] Aqueous solutions of these dinitrates were made by adding weighed aliquots of the stock solution to saline. These mixtures were stirred for 72 hr, after which they were centrifuged for 15 min at 15,000 rev/min and then filtered. These last two steps removed all the insoluble stabilizer and produced clear solutions.

The absorbance of these dinitrate solutions, diluted 1:99 with ethanol, was measured and concentrations were determined from a standard curve of absorbance at 220 nm versus the amount of dinitrate in alcoholic solution. Spectrophotometric and gravimetric analyses always gave closely similar estimates of dinitrate concentration. By spectrophotometric analysis we obtained a linear relation between the concentration of PGDN in saline solution and the PGDN added up to 10 mM, and found a maximum solubility in normal saline of 13 mM (2·16 g/l).

*Chemicals and apparatus.* A boric acid–NaOH buffer was used at pH 9·0 and

* In all instances where similar experiments were performed on hemolysate $O_2Hb$ prepared both from fresh and from outdated blood, equivalent results were obtained.

$KH_2PO_4$–$K_2HPO_4$ buffers were used between pH 6·0 and 8·5. Routine absorbance measurements were made with a colorimeter in 1·2 cm round cuvettes and kinetic data were obtained with a spectrophotometer with analog output. Met-Hb formation was followed at 630 nm[10] and the per cent of met-Hb in solution was estimated via the method of Evelyn and Malloy.[11] Analysis for $NO_3^-$ and $NO_2^-$ was accomplished using the methods of Litchfield.[12]

A tonometer was custom-made for regulating the $O_2$ concentration in reactant solutions. It consisted of a 500-ml round-bottomed flask which had a side arm fitted with a stopcock, a 1 cm square cuvette fused to the bottom, and a ground glass neck. The tonometer was sealed by means of a male ground glass fitting through which passed a 5 mm O.D. glass tube connected to another stopcock. Hemoglobin solutions were deoxygenated by passing a stream of water-saturated, high-purity grade $N_2$ (Matheson Gas Produtcs, East Rutherford, N.J.) through the tonometer. Stock dinitrate solutions, in enclosed tubes, were deoxygenated by bubbling with $N_2$ for 10 min. The deoxygenated dinitrate solution was then injected through an air-tight septum on the end of the side arm into the partially evacuated tonometer, containing a pure $N_2$ atmosphere, and measured amounts of air or pure $O_2$ were then injected. The solution was equilibrated with the gas phase by vigorous swirling for 2–5 min and the oxidation reaction then monitored continuously.

## RESULTS

*Role of enzymatic catalysis.* Reactions of PGDN with human hemolysate $O_2Hb$ at pH 7·0 and at 37° were performed at constant PGDN and varying $O_2Hb$ concentrations, and at constant $O_2Hb$ and varying PGDN concentrations. From the observed initial rates of oxidation, Lineweaver–Burk reciprocal plots were constructed (Fig. 1, panels A and C). Instead of giving y-intercepts of some positive value, as is expected in the case in which an enzymatically catalyzed reaction is the rate-limiting step, these lines pass through the origin. Thus, there is no finite limit to the reaction rate at infinite dinitrate concentration. Since these plots are linear, the reaction is approximately first-order in both dinitrate and $O_2Hb$.

The reactions of $O_2Hb$ in air-saturated buffer with an 8- and 16-fold excess of PGDN relative to heme (pseudo first-order conditions) were carried out at 23 $\pm$ 1°. The time courses of these reactions (Fig. 2) illustrate that through the oxidation of 80 per cent of the hemoglobin the reaction is indeed nearly first-order in $[O_2Hb]$ and slightly accelerating.

Figure 3 shows the effect of pH on the rate of oxidation. Since the absorbance of met-Hb at 630 nm varies with pH, the observed initial rates were corrected to the rate which would be seen if all the met-Hb were present in the more strongly absorbing acid form. We assumed for this calculation that the acid form has an extinction coefficient 2·6 times that of the alkaline form and that the transition between these two forms of met-Hb followed a simple titration curve with a p$K$ of 8·00.[10] These data indicate that the oxidation reaction is nearly zero-order in hydrogen ion above pH 7·0, but markedly pH dependent at lower values.

As it appeared that the reaction was molecular, we undertook experiments to show that no material from the hemolysate other than $O_2Hb$ was required for oxidation. Hemolysate fractions of human $O_2Hb$ were chromatographed on a 2 $\times$ 50 cm column of G-100 Sephadex which separated the various protein molecules on the

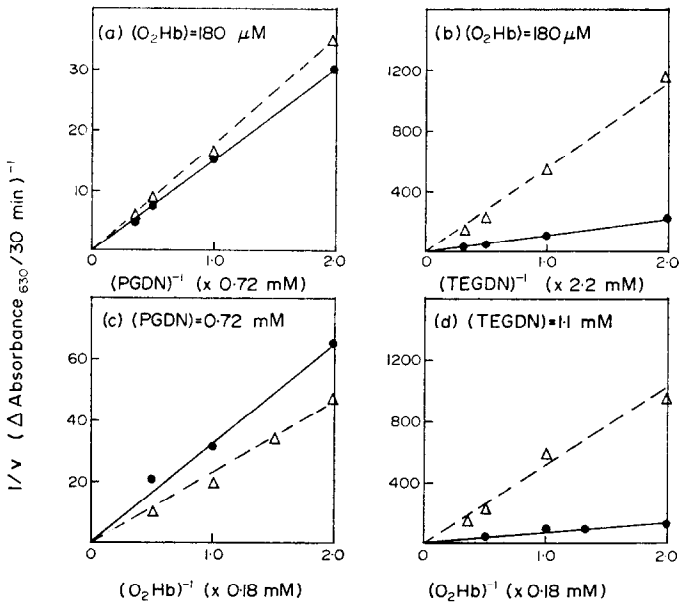


FIG. 1. Lineweaver–Burk plots of the reciprocal of the observed rate of oxidation versus the reciprocal of the substrate concentration in hemolysate preparations. Solutions were incubated for 30 min at 37° in air-equilibrated, 20 mM pH 7·0 phosphate buffer and oxidation was measured as the increase in absorbance at 630 nm. Triangles refer to data with human $O_2Hb$; circles, with rat $O_2Hb$. In panels A and B all reactant solutions contained 180 μM $O_2Hb$; in panel C all contained 0·72 mM PGDN; and in panel D all contained 1·1 mM TEGDN.

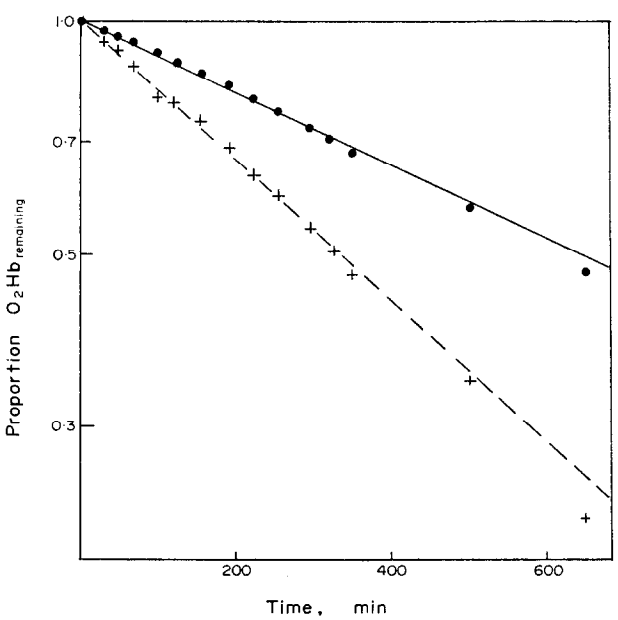


FIG. 2. Time course of the oxidation *in vitro* of human $O_2Hb$ by PGDN. These two reactions were carried out at 23 ± 1° in air-saturated 0·05 M potassium phosphate buffer, pH 7·0, and each contained 280 μM human $O_2Hb$. The concentrations of PGDN were 2·16 mM for the upper curve and 4·32 for the lower. The reaction was followed at 630 nm, and the end point determined by adding several crystals of ferricyanide to the solution to complete oxidation.

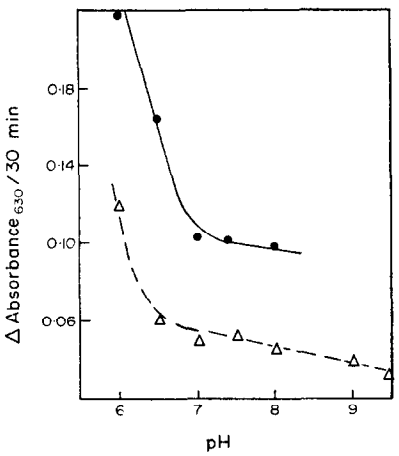


FIG. 3. Rate of oxidation of hemolysate $O_2Hb$ by PGDN as a function of pH. The triangles are data obtained with human $O_2Hb$. These air-saturated solutions contained 0·72 mM PGDN, 0·18 mM $O_2Hb$ and the buffer concentration was 20 mM. Incubations were at 37° for 30 min. The ordinate is the calculated rate which has been corrected as described in the text. The circles are data obtained with 0·4 mM rat hemolysate $O_2Hb$.

basis of molecular weight. The effluent $O_2Hb$ was collected, and a series of reactions were performed between this partially purified hemoglobin and PGDN. These reactions were first-order in both dinitrate and hemoglobin, and the second-order rate constant was within 10 per cent of that obtained with the unchromatographed hemolysate. Furthermore, when a sample of purified bovine hemoglobin was converted to $O_2Hb$ and reacted with PGDN, the reaction was also first-order in each reactant.

In other experiments, reaction mixtures were again prepared as described in the legend to Fig. 1, except they also contained 400 $\mu$M carbon monoxide, an amount more than sufficient to maintain all the hemoglobin as carbonmonoxyhemoglobin (COHb). In none of these solutions did measurable oxidation occur within 3 hr. All these reactions thus far investigated were performed in solutions where hemoglobin exists exclusively in the liganded state, either $O_2Hb$ or COHb. Since the oxidation by PGDN is inhibited when CO occupies the heme site, the rate of this oxidation should also be affected by the $O_2$ concentration. The data in Table 1 illustrate the complex effects of $O_2$ concentration on the observed initial first-order rate constant.

Stoichiometry for this reaction was determined by preparing a series of reaction mixtures at pH 7·0, 0·1 M phosphate buffer, each containing equal hemoglobin concentration and varying amounts of PDGN. These solutions were incubated overnight at 37°, and the absorbance of each was then measured at 630 nm after 16 and again after 18 hr to insure that the reactions were complete. From the first set of results in Table 2, the stoichiometry was calculated as 1·45 moles heme oxidized per mole of PGDN added, and the mean $\pm$ standard deviation of three determinations was $1{\cdot}5 \pm 0\,1$.

*Oxidation of hemoglobin in erythrocytes.* In order to allow for the inactivation of the met-Hb reductase system, due to depletion of utilizable glycolytic substrates, freshly prepared erythrocytes were aged 5 days prior to any experimentation. Incubations

TABLE 1. EFFECT OF $O_2$ CONCENTRATION ON THE RATE OF METHEMOGLOBIN FORMATION BY PGDN*

| $O_2$ (mm Hg) | Rate ($hr^{-1}$) | % $O_2Hb$ |
|---|---|---|
| 0 | 0 | 0 |
| 3·0 | 0·10 | 8·1 |
| 9·0 | 0·34 | 42·0 |
| 18·0 | 0·38 | 86·0 |
| 27·0 | 0·41 | 97·0 |
| 36·0 | 0·55 | 100·0 |
| 45·0 | 0·38 | 100·0 |
| 60·0 | 0·23 | 100·0 |
| 150·0 | 0·17 | 100·0 |
| 304·0 | 0·11 | 100·0 |
| 760·0 | 0 | 100·0 |

* Reactions were performed at 37° in a tonometer in which the reaction volume was 3·0 ml and the gas phase contained known partial pressures of $O_2$. The hemoglobin concentration was 0·46 mM, the PGDN concentration, 1·9 mM, and the buffer was 20 mM pH 7·0 potassium phosphate. Rate constants for the initial part of the reaction were calculated as the slopes of the time course of these oxidations (see Fig. 2) during the first 10 per cent of the curve. The per cent of $O_2Hb$, estimated spectrophometrically following the method of Andersen and Gibson,[13] represents the proportion of total heme sites in the hemoglobin solution which, at the given $O_2$ tension, have $O_2$ bound. The hemoglobin used in these experiments was prepared from outdated blood.

TABLE 2. STOICHIOMETRY DETERMINATIONS WITH PGDN

| Hemolysate* | | | Erythrocytes† | | |
|---|---|---|---|---|---|
| $O_2Hb$ (mM) | PGDN (mM) | % Oxidized | Cellular $O_2Hb$ (mM) | PGDN (mM) | % Oxidized |
| 1·79 | 0 | 21·4 | 7·28 | 0 | 4·0 |
| 1·79 | 0·09 | 36·3 | 7·28 | 0·18 | 9·6 |
| 1·79 | 0·18 | 41·7 | 7·28 | 0·36 | 13·7 |
| 1·79 | 0·36 | 57·5 | 7·28 | 0·72 | 29·4 |
| 1·79 | 0·54 | 67·7 | 7·28 | 1·08 | 40·3 |
| 1·79 | 0·72 | 83·0 | 7·28 | 1·44 | 48·5 |
| 1·79 | 0·90 | 94·7 | 7·28 | 1·8 | 61·7 |
| 1·79 | 1·80 | 100·0 | 7·28 | 3·6 | 88·0 |
| | | | 7·28 | 7·2 | 100·0 |

* Hemolysate: all solutions contained 20 mM pH 7·0 potassium phosphate buffer and were air saturated. The experimental details are in the text. Met-Hb was estimated by adding cyanide and measuring the decrease in absorbance at 630 nm.[11]

† Erythrocytes: cells were suspended in saline and reacted overnight as described in the text. At the end of the reaction a 2-ml aliquot of the reaction mixture was added to 5 ml of a cell-dissolving solvent, pH 6·6, 0·1 M potassium phosphate buffer containing 1·0% Sterox-E detergent. The absorbance of this solution in the presence and absence of cyanide was then measured and the difference used as a measure of methemoglobin in solution.[11]

with PGDN were performed at 37° with constant, gentle swirling and with intermittent inversion of the tubes to provide a homogeneous suspension. In none of these incubations did appreciable hemolysis occur. These reactions (Table 3) were first-order in dinitrate and in $O_2Hb$, and the stoichiometry was 2·30 moles heme oxidized per mole of PGDN added (Table 2). Again, the rate of oxidation increases linearly with dinitrate concentration and does not approach a limit, which indicates that the contribution, if any, to this oxidation reaction from enzymatic mechanisms must be minimal.

TABLE 3. OXIDATION OF HUMAN OXYHEMOGLOBIN BY PGDN IN ERYTHROCYTE PREPARATIONS*

| PGDN (mM) | Rate ($\Delta$ Abs.$_{630}$/ 30 min) |
|---|---|
| 0 | 0 |
| 0·09 | 0·006 |
| 0·18 | 0·022 |
| 0·36 | 0·052 |
| 0·54 | 0·067 |
| 0·72 | 0·087 |

* Packed erythrocytes were taken up in normal saline and PGDN-saturated saline which together diluted the original cell suspension 1:10. Incubations were carred out at 37° for 30 min and the reaction was terminated by adding 2·5 vol. of the cell-dissolving solvent (see Table 2). The change in absorbance was determined by measuring the absorbance of the solution at 630 nm with and without sodium cyanide present. The difference between these two absorbances was used to calculate the rate of oxidation. (Using the changes in absorbance upon the addition of cyanide to determine met-Hb proved more reproducible in experiments with erythrocytes than did using simply the increase in absorbance at 630 nm.)

*Intermediates.* Stock dinitrate solutions in 0·9% saline were analyzed for $NO_2^-$ by the method of Litchfield,[12] and none was found even though several tenths of a $\mu$g/ml (6 $\mu$M) would have easily been detected. In reaction mixtures in which the oxidation of 500 $\mu$M $O_2Hb$ was half complete, less than 20 $\mu$M nitrite was present.

The chemical nature of the met-Hb produced by oxidation with PGDN was determined by preparing two solutions each containing equal amounts of met-Hb, formed in one case by reaction with a slight molar excess of potassium ferricyanide and in the other by reaction with PGDN. To each of these preparations enough $NO_2^-$ was

added to give a final concentration of 10 mM. It is well known that $NO_2^-$ forms an ion-pair complex with met-Hb, the dissociation constant of which is quite large, 3 mM.[14,15] The presence of this complex may be determined spectrally by observing the decrease in the absorbance at 630 nm. The addition of $NO_2^-$ to the met-Hb produced by PGDN produced a similar decrease in absorbance (20 per cent) as did its addition to ferricyanide-generated met-Hb, suggesting that the met-Hb produced by PGDN is not complexed with $NO_2^-$.

*Other hemoglobins and dinitrates.* We studied the reaction of rat $O_2$Hb with PGDN (Figs. 1 and 3). It showed the same kinetics, and was affected by pH in the same fashion as the reaction of this dinitrate with human $O_2$Hb. Its stoichiometry was 1·4 and 1·9, respectively, for hemolysate and erythrocytic preparations of $O_2$Hb.

The oxidation of human and rat $O_2$Hb by TEGDN was also investigated. In both species this reaction was markedly slower than oxidation by PGDN (Fig. 1, panels B and D). Furthermore, the reaction between rat $O_2$Hb and TEGDN was seven to eight times faster than that between human $O_2$Hb and TEGDN. The rate of oxidation by TEGDN was affected by $O_2$ concentration and by pH just as was the rate of oxidation by PGDN (Fig. 3 and Table 1); however, no accurate estimates of stoichiometry of the TEGDN-oxidation could be made since extensive auto-oxidation of the $O_2$Hb occurred before the reactions could go to completion.

## DISCUSSION

The present data are not sufficient to elucidate completely the entire mechanism of the oxidation of $O_2$Hb by PGDN. However, they do establish certain characteristics of this reaction which must be considered in formulating any detailed mechanism. For instance, this oxidation reaction is molecular, requires no erythrocytic component other than $O_2$Hb, and in air-equilibrated solutions where all the available ferrous heme sites are saturated with $O_2$, is first-order in both PGDN and $O_2$Hb (Figs. 1 and 2). At the pH values of the mammalian erythrocyte, the oxidation is zero-order in $[H^+]$, while at lower pH values the rate of oxidation increases markedly with increasing $[H^+]$ (Fig. 3).

Carbon monoxide and $O_2$ have pronounced effects on this reaction. PGDN-oxidation was completely inhibited by concentrations of CO which were sufficient to convert all the available ferrous heme sites to COHb, and it was also inhibited by $O_2$ concentrations above 35 mm Hg (Table 1). The dependence of the rate of oxidation on $[O_2]$ over the larger range of $O_2$ concentrations was, however, very complex. In the absence of $O_2$ no oxidation occurred and the highest rates of PGDN-oxidation were observed at $O_2$ concentrations at which some of the ferrous heme sites were vacant. This inhibition of oxidation by high $[O_2]$ is reminiscent of the effects of this ligand in replacement reactions.[16,17] In these reactions $O_2$Hb is mixed with solutions of CO, and COHb is formed as CO replaces $O_2$ as the heme-ligand. Gibson and Roughton[16] have shown that this reaction proceeds according to a mechanism in which CO reacts with the deoxyHb formed as $O_2$ dissociates from $O_2$Hb. Since the short-lived deoxyHb species can react with CO or recombine with $O_2$, the rate of appearance of COHb at a constant [CO] decreases as the $[O_2]$ is raised.[16,17]

On the basis of all these data we propose the following plausible, though admittedly preliminary, mechanism for the oxidation of $O_2$Hb by PGDN. The dinitrate interacts at or near the heme site with deoxyHb, not with $O_2$Hb. This PGDN–deoxyHb

complex in the presence of $O_2$ is oxidized, and the dinitrate is converted to the mononitrate and other products. Such a scheme would explain the effects of $O_2$ and CO on the reaction rates and the first-order dependencies on both $O_2$Hb and PGDN. Attempts were made to obtain spectral evidence for a PGDN–deoxyHb complex by taking spectra between 500 and 650 nm of solutions of deoxyHb and of $O_2$Hb in the presence and absence of 10 mM PGDN. In neither case did the spectrum obtained in the presence of PGDN differ from that obtained in its absence. If this complex does exist, it must then have a very large dissociation constant. Thus only small amounts of the complex would be present at any time.

Upon incubating rat blood with PGDN *in vitro* only one of the nitrate esters of the PGDN is reactive.[5] The oxidation reactions of rat and human $O_2$Hb are similar in every respect (see Figs. 1 and 3) and the possibility that both ester bonds are broken in the reaction with human $O_2$Hb appears remote. The stoichiometry then is more correctly written as 1·5 moles heme oxidized per mole of ester converted. Order in PGDN and stoichiometry are not identical, suggesting that some reactive, oxidative intermediate participates in this oxidation. Nitrite could be such a reactant since it also oxidizes $O_2$Hb.[10] Nitrite is produced when PGDN is incubated with whole rat blood,[5] but unlike $NO_3^-$ which increases monotonically during the incubation, $NO_2^-$ initially increases to a low level, maintains that level until the reaction is virtually complete, and then decreases toward the end of the incubation: behavior expected if $NO_2^-$ were a reactive intermediate. Indeed, in studying the metabolism of EGDN, Clark and Litchfield[6] concluded that this dinitrate was broken down by blood *in vitro* yielding ethylene glycol mononitrate and $NO_2^-$ which is subsequently converted to $NO_3^-$.

In erythrocytes, the oxidation reaction is still molecular (Table 3). Hemoglobin *in vivo* together with the methemoglobin reductase system act catalytically to metabolize these dinitrates to nitrite (or nitrate) and the mononitrates which are further broken down, presumably by the denitrifying enzymes found in various tissues.[18,19] For toxic effects caused by the intact dinitrate, hemoglobin would fulfill an important role in detoxifying these compounds. Thus, it is expected that humans, because of the slow rate of methemoglobin formation by TEGDN (Fig. 1, panels B and D), will be less susceptible to methemoglobinemia from this dinitrate than the rat is. However, humans may be more susceptible to TEGDN-produced nervous disorders which are apparently caused by the intact dinitrate.[7]

In order to determine if this difference in heme-oxidizing ability between PGDN and TEGDN might be due to steric factors limiting the access of the nitrate esters in TEGDN to the heme, space-filling models of these dinitrates were constructed. The remote nitrate esters in an extended form of TEGDN were observed to be less constrained than the vicinal esters of PGDN. On the basis of purely steric considerations then, one would predict, contrary to experiment, that TEGDN would be the more favorable oxidant. One remaining possibility is that the rate enhancement with PGDN may be due to the vicinal position of the esters in PGDN in some way favoring denitrification. This difference in susceptibility to denitrification is also observed in the alkaline hydrolysis of these dinitrates where PGDN is destroyed five times faster than TEGDN.*

* M. E. Andersen, unpublished observations, U.S. Navy Toxicology Unit, 1973.

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