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The kinetic differences between sodium nitrite, amyl nitrite and nitroglycerin oxidation of hemoglobin

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Summary. The effect of sodium nitrite, amyl nitrite and nitroglycerin (glyceryl trinitrate) on the hemoglobin of adult erythrocytes was examined *in vitro*. Both amyl nitrite and nitroglycerin reacted immediately with oxyhemoglobin to effect oxidation into methemoglobin while sodium nitrite required an inductionary period (lag phase) prior to the reaction. Kinetic studies of the bimolecular rate law for each of the preceeding reaction's reactionary periods (log phases) allowed rate constant calculations to be made. The values are $1.14 \times 10^{4} M^{-1} min^{-1}$, $7.45 \times 10^{4} M^{-1} min^{-1}$, and $3.50 \times 10^{1} M^{-1} min^{-1}$ for sodium nitrite, amyl nitrite and nitroglycerin, respecticely. A comparison of the amyl nitrite and nitroglycerin rate constants reveals that amyl nitrite is approximately 2000-fold more toxic to oxyhemoglobin than nitroglycerin. These oxidant's effect on *in vitro* hemoglobin solutions are comparable since both reactions approximate to rectangular hyperbolae. Sodium nitrite reacts about 300-fold faster with oxyhemoglobin than does nitroglycerin. However, the sodium nitrite reaction proceeds in a sigmoidal fashion which makes a strict comparison between these compounds relative toxicities less clear cut.

Key words: Sodium nitrite - Amyl nitrite - Nitroglycerin - Oxyhemoglobin - Methemoglobin

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Introduction

Sodium nitrite has been found in elecated levels in drinking water obtained from wells in the United States and Israel (Shuvall and Gruener, 1977; Craun et al., 1981). Controversy as to its possible effects on well water consumers abounds in the literature; however, at least one case of death by ingestion of well water containing sodium nitrite and its precursor, sodium nitrate, has been reported (Bucklin and Myint, 1960). The mechanism whereby sodium nitrite oxidizes oxyhemoglobin has been a subject of intense study (Kakiraki et al., 1964; Kiese, 1974; Tomoda et al., 1981).

According to Haley (Haley, 1980) amyl nitrite and sodium nitrite affect the blood in a similar fashion. These nitrites readily oxidize hemoglobin (Hb) into methemoglobin, both *in vitro* and *in vivo,* thus inducing methemoglobinemia (Nikerson et al., 1979). Alkyl nitrites, of which amyl nitrite is but one example, hace become widely used in the drug culture as aphrodisiacs (Sigell et al., 1978) and can sometimes result in fatality when ingested (Chilcote et al., 1977; Shesser et al., 1980; Steiner and Manoguerra, 1980). They pose a special threat to the neonate (Tarburton and Metcalf, 1985).

With the wide usage of nitroglycerin in the treatment of coronary heart disease some controversy as to its effect on methemoglobin formation and side effects therefrom has arisen in the medical literature (Marshall and Eklund, 1980; Yamamura et al., 1980; Gibson et al., 1982; Husum et al., 1982).

The present work was undertaken in an attempt to compare the effects of sodium nitrite, amyl nitrite, and nitroglycerin on Hb of lysed human erythrocytes.

Materials and methods

Materials

Isoamyl nitrite, sodium nitrite, and other chemicals (e.g., for solutions, solvents, cleaning, etc.) were obtained from the Aldrich Chemical Company (Milwaukee, Wisconsin, USA). Pharmaceutical grade amyl nitrite was obtained from the Burroughs Wellcome Company (Research Park, North Carolina, USA). Tridil (nitroglycerin/glyceryl trinitrate) was obtained from American Critical Care (McGraw Park, Illinois, USA).

Procedure

Blood samples were drawn by venipuncture into a heparinized tube, with informed consent from thirteen, healthy adult volunteers from the Anatomy Department and Medical School. It was stored for not longer than 24 hours at 2 - 4°C prior to use.

The blood was centrifuged at 2000 g for 20 minutes to allow for removal of the plasma. The remaining packed cells were aerated with filtered air and washed with normal saline at physiological pH 7.2. The procedure of centrifugation, aeration, and washing was repeated twice more. The erythrocytes were resuspended in 10 times their volume of isotonic saline and stored at 2 - 4°C for a maximum of 60 minutes prior to testing. The oxidant, i.e., sodium nitrite, amyl nitrite, or nitroglycerin, having been stored in the cold, was diluted to the strength desired by the addition of isotonic saline immediately prior to use.

A 0.1 ml portion of resuspended red cells was hemolyzed by the addition of 1.0 ml of distilled water and a final volume of 2.6 ml was obtained by the addition of Sorenson's phosphate buffer at physiologic pH 7.2. The hemoglobin solutions were adjusted to a standard initial absorbance **(A,** = 0.72) at the wavelength of 436 nm by the addition of distilled water. A 2.6 ml aliquot of hemoglobin solution was added to a 0.2 ml aliquot of the oxidant in saline. The above was performed at hemoglobin concentrations of 10 or 100µM. In the sodium nitrite series of experiments the final concentration range of oxidant was 5×10^{-4} to 2×10^{-3}M, for amyl nitrite the range was 1.3×10^{-4} to 2.6×10^{-4}M, and for nitroglycerin the range was 1.6×10^{-1} to 3.2×10^{-3}M. The spectrophotometer-chart recorder generated a graphic representation of the conversion of oxyhemoglobin into methemoglobin as a function of time. In all cases the temperature was 20 + 1°C and the pH was 7.2 both before and after each reaction. The terminal period, or asymptotic phase, corresponds to 100% MetHb formation. Final absorbance equals 0.30 ± 0.01 in each instance.

Calculations and Statistics

The rate law for the reaction of oxyhemoglobin with oxidant has been proven to be bimolecular during the log phase for each oxidant (Kakizaki et al., 1964; Tarburton, 1984). Further, the stoichiometry for each individual oxidation reaction has been established (Sung and Rerner, 1949; Tarburton, 1984) so that the rate constants can now be calculated from the following equation (Gardiner, 1969):

$$k(t) = \frac{1}{\nu_B[Hb(O_2)_4]_0 - \nu_A[\text{oxidant}]_0} \ln \left(\frac{\nu_B[Hb(O_2)_4]_0 - \nu_A[\text{oxidant}]_0}{[Hb(O_2)_4]_0} \cdot \frac{\nu_A[\text{oxidant}]_0}{\nu_B[Hb(O_2)_4]_0} \right) \frac{1}{t}$$

where ν_A = moles of oxyhemoglobin reacted; ν_B = moles of oxidant reacted; where oxidant = sodiurn nitrite, amyl nitrite, or nitroglycerin, $[\text{oxidant}]_0$ = initial oxidation concentration, $[Hb(O_2)_4]_0$ = initial oxyhemoglobin concentration, and $T_{1/2}$ = half-life of the log phase, i.e., reactionary period. The half-lives were measured for each oxidation reaction which, therefore, allowed the calculation of the rate constants (k(t)) from the above equation.

The appropriate test to use for these data is the Student's t-test employing the Bonferroni procedure (Wallenstein et al., 1980). The data were analyzed using a statistical packet on an IBM PC computer. The significance level has been considered to be a probability (p) of less than 0.05.

Results

Preliminary investigations were done to determine the effects of sodium nitrite, amyl nitrite, and nitroglycerin on Hb. As expected all ultimately oxidized oxyhemoglobin into methemoglobin wherein a red to dark-brown color change was observed. However, with sodium nitrite a sigmoid curve consisting of an induction period (lag phase), a reactionary period (log phase), and a terminal period (asymptotic phase) resulted (Fig. 1) (Kakizaki et al., 1964; Kosaka et al., 1979; Tomada et al., 1981); while the addition of amyl nitrite to oxyhemoglobin yielded a curve consisting of only a reactionary period and a

Table **1.** Comparison of the reaction rates between hemoglobin and sodium nitrite, amyl nitrite, or nitroglycerin*

	$NaNO_2$	AmONO	GTN
Mean Rate Constant ($M^{-1}min^{-1}$)	1.14×10^4	7.45×10^4	3.50×10^1
Standard Error of the Mean	2.04×10^3	1.31×10^4	1.65

* The reaction rate for nitroglycerin was significantly slower ($p < 0.05$) than for amyl nitrite or sodium nitrite.

terminal period (Fig. 2). Likewise, nitroglycerin addition to oxyhemoglobin gave the same results as amyl nitrite except that the reaction was spread over a much greater period of time (Fig. 3). The shapes of these latter two curves resemble that of a rectangular hyperbolae wherein the reaction commences immediately with the log phase. All of the above oxidation reactions with oxyhemoglobin yielded soluble products without precipitates.

Oxyhemoglobin from erythrocytes of thirteen blood samples was investigated wherein the Student's t-test for independent samples was applied to the rate constants. The first comparison was that of the rate constant of the nitroglycerin oxidation reaction compared to either sodium nitrite or amyl nitrite. The second comparison was that of the rate constant of the sodium nitrite oxidation reaction vs. the amyl nitrite oxidation reaction (Table 1). The former statistical comparison revealed that nitroglycerin oxidized oxyhemoglobin slower than either sodium nitrite or amyl nitrite ($p < 0.05$). The latter statistical comparison revealed no statistically significant difference between the two nitrites ($p > 0.05$). It must be remembered that these rate constants are indicative of the rate of this oxidation reaction during the reactionary period, i.e., log phase, only. All curves of the oxidation of oxyhemoglobin by sodium nitrite, amyl nitrite, and nitroglycerin were continuous functions of time as illustrated in Figures 1-3.

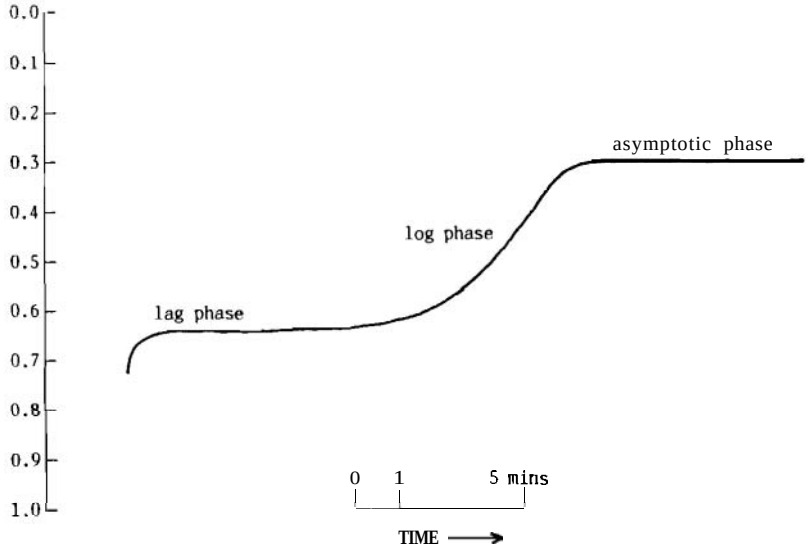

Fig.1. Reaction curve of methemoglobin formation of adult blood (A_i = 0.72) using sodium nitrite as the oxidant

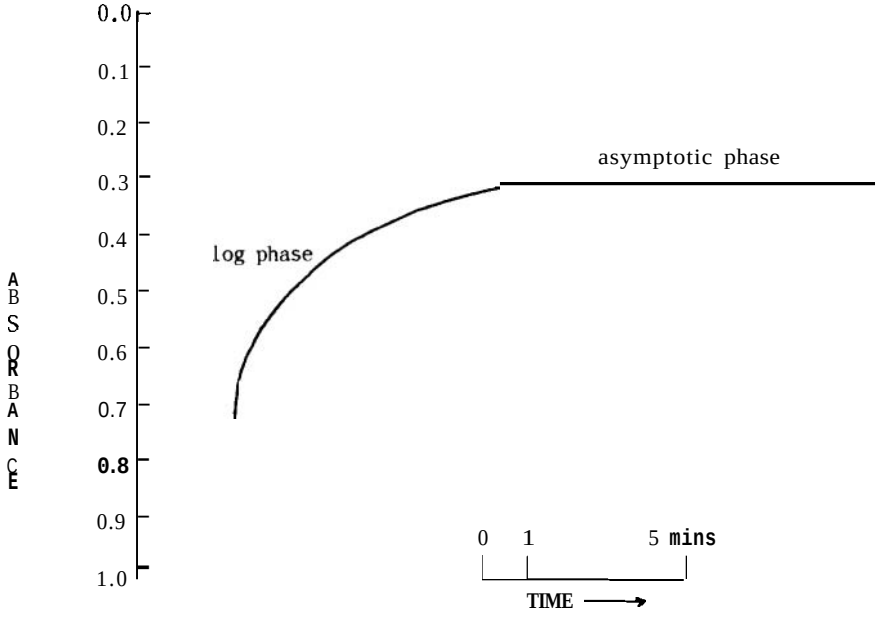

Fig. 2. Reaction curve of methemoglobin formation of adult blood (A_i = 0.72) using amyl nitrite as the oxidant

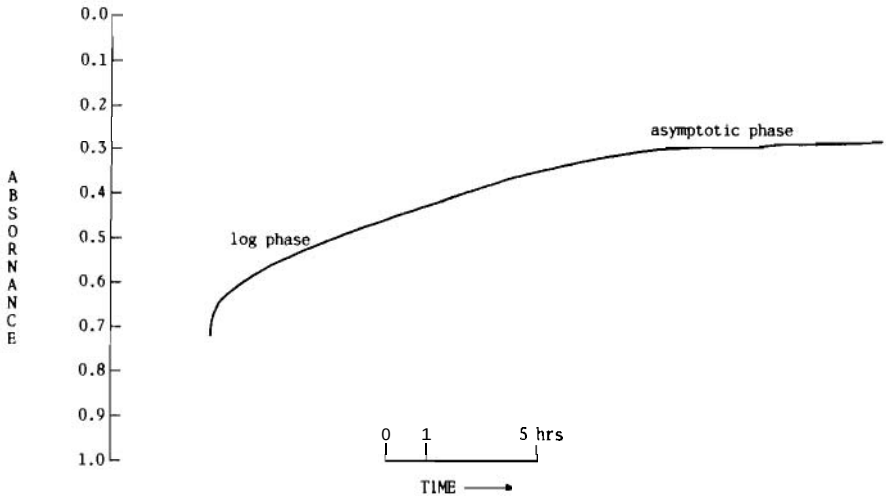

Fig. 3. Reaction curve of rnethernoglobin formation of adult blood (A_i = 0.72) using nitroglycerin as the oxidant

Discussion

The most striking finding in this investigation is the remarkable increased speed of oxidation of adult hemoglobin by amyl nitrite as compared to nitroglycerin ($p < 0.05$). A ratio of the rate constants reveals that the reaction with amyl nitrite is approximately 2000 times faster than that with nitroglycerin. Inspection of Figures 2 and 3 reveal that both oxidation reactions proceed as "rectangular hyperbolae" which means that their only inherent difference is the relative rates of oxidation.

Interestingly, the fast oxidation reaction of amyl nitrite helps to explain a number of cases of either fatality or near fatality when an alkyl nitrite, of which amyl nitrite is but one example, is ingested (Chilcote et al., 1977; Shesser et al., 1980; Steiner and Manoguerra, 1980). On the other hand, the slower oxidation of oxyhemoglobin by nitroglycerin perhaps explains why high dose intravenous nitroglycerin given to treat myocardial infarction showed no fatality due to elevated methemoglobin levels (Yamamura et al., 1980), and standard doses do not normally induce methemoglobin formation in clinically significant amounts (Husum et al, 1982).

A comparison of sodium nitrite oxidation of oxyhemoglobin vs. nitroglycerin reveals a rate constant which is approximately 300-fold faster for sodium nitrite. However, this comparison appears muddled by the fact that one is comparing the sigmoid reaction curve of sodium nitrite with the "rectangular hyperbolic" reaction curve of nitroglycerin. In terms of the log phase proper, however, sodium nitrite is significantly faster ($p < 0.05$) at oxidizing oxyhemoglobin.

Sodium nitrite is found in elevated quantities in well water and water supplies near fertilized fields (Shuvall and Gruener, 1977) and poses a special threat to pregnant women, children, and advanced cancer patients (Keohane and Metalf, 1960; Metcalf, 1961) in whom the lag phase is diminished. In contrast, nitroglycerin is not widely used by groups especially susceptible to sodium nitrite poisoning but is taken mainly by older individuals, i.e., over 50 years, to treat coronary heart disease (Shuvall and Gruener, 1977).

Essentially, then, the greatly enhanced reactivity of amyl nitrite (vs. nitroglycerin) with oxyhemoglobin is clearly defined in these *in vitro* studies. On the other hand, a strict comparison between the relative toxicities of sodium nitrite (vs. nitroglycerin) on oxyhemoglobin based on these findings is not as clearly defined.

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