Methemoglobin formation and reduction in relation to hemoglobin oxygen affinity[1]

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Summary. It is suggested that although the high oxygen affinity hemoglobin (Hb LR) (β143(H21)His → Gln) with stabilization of R quaternary conformation is not more susceptible to the oxidizing effect of nitrites in vitro, in an in vivo situation where hemoglobin is partly deoxygenated, it might be more susceptible to methemoglobin formation due to higher oxidation and lower reduction rate.

It has been recently reported that methemoglobin Wood (β97 (FG4) His → Leu) a high oxygen affinity hemoglobin whose quaternary conformational equilibrium is shifted towards R conformation, has a significantly lower reduction rate than methemoglobin A[3]. Due to the fact that nitrites oxidize hemoglobin at a much faster rate when it is in the R than in the T conformation, it was hypothesized that red cells containing a high oxygen affinity hemoglobin which is stabilized in the R conformation must be more susceptible to methemoglobin formation by nitrites not only due to high oxidation rate but also due to low reduction rate. For this reason oxidation and reduction rates of hemoglobin Little Rock (LR) (β143 (H21) His → Gln)[4] a high oxygen affinity hemoglobin with stabilization of R conformation[5] were measured and compared to those of hemoglobin A.

Methods. Hemoglobin A was purified by ion exchange chromatography[6]. Hemoglobin LR was purified by the same method as was originally reported[4]. NADH methemoglobin reductase was purified by the method of Sugita et al.[7] up to the 4th step which results in about 1500-fold enzyme purification.

Hemoglobin oxidation by sodium nitrite under oxy and deoxy conditions was carried out following exactly the same procedure which has been reported in a recent communication[8]. Methemoglobin was prepared by dialysis of hemoglobin against 0.15 M NaCl solution containing 0.1% sodium nitrite[9].

Methemoglobin reduction was carried out by placing 2 ml of 0.2 mM solution of methemoglobin in a quartz cuvette. After addition of Ferrocyanide and reductase the reaction was started by the addition of β-NADH to the sample solution and the decrease of absorbance at 631 nm which is proportional to the reduction of methemoglobin was measured.

Results and discussion. Figure 1 shows that contrary to the hypothesis the oxidation rate of hemoglobin LR by sodium nitrite is significantly slower than that of hemoglobin A in spite of the shift of its conformational equilibrium towards R conformation. This indicates that the R conformation is not necessarily the factor which affects the rate of oxidation by nitrite. In fact high affinity towards O_2 decreases the rate of oxidation indicating that the dissociation of heme ligand is necessary before the oxidation takes place. This is also demonstrated by the very slow oxidation of carboxyhemoglobin by sodium nitrite even in the presence of oxygen (unpublished data). On the other hand when heme is not ligated (deoxy condition) as is shown in figure 2 the oxidation of hemoglobin LR is significantly faster than that of hemoglobin A indicating that a conformational shift towards R is an important rate determining factor in case of deoxyhemoglobin oxidation by nitrite. It should be noted further that due to similar spectral properties of hemoglobin and methemoglobin A and LR, the spectral differences between these hemoglobins in figures 1 and 2 are solely related to their different oxidation rates. Finally, figure 3 demonstrates that the enzymatic reduction

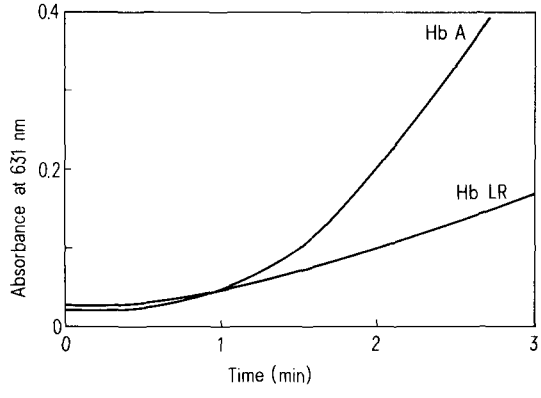

Fig. 1. Hemoglobin A and LR oxidation (in air) by sodium nitrite at pH 6 and 25 °C. Nitrite/heme molar concentration = 1.

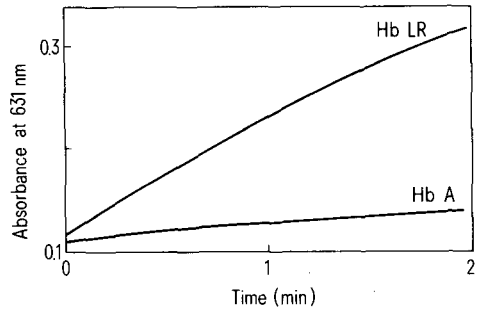

Fig. 2. Hemoglobin A and LR oxidation (in the absence of oxygen) by sodium nitrite under the same conditions as in figure 1 except that nitrite/heme molar concentration was = 5.

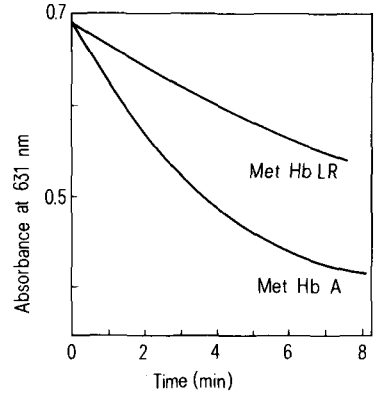

Fig. 3. Reduction of methemoglobin A and LR by NADH methemoglobin reductase. The reduction was carried out in 0.05 M bis-Tris buffer, pH 6 at 25 °C.

rate of hemoglobin LR is about 2-fold slower than that of hemoglobin A similar to the case of hemoglobin Wood. These studies suggest that in spite of slower reduction of hemoglobin LR, cells containing such hemoglobin are not more susceptible to the oxidizing effect of nitrites. However, this does not guarantee that under physiological conditions when part of the hemoglobin circulates in the deoxy form this may not happen.

The conclusion is that although the carriers of those high oxygen affinity mutant hemoglobins which are stabilized in the R conformation might develop methemoglobinemia after drug ingestion (due to slow reduction rate), they might be as well more sensitive to the oxidizing effect of nitrites than carriers of normal hemoglobin although this has not been tested in vivo.

1 Supported by NIH grant No. 5 RO1 AM 20181-02 and VA grant No. 5455-002.
2 Acknowledgments. The technical assistance of Ms Carol A. Perry is highly appreciated.
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Platelet heterogeneity and dense tubular system changes on activation

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Summary. Platelets impregnated with heavy metals appeared as 3 distinct morphological types: 'reticular' cells with a polygonal dense tubular network and stained granules, dark metallophilic cells, and pale metallophobic cells with microvesicles and non-staining granules. On stimulation, type 1 cells decreased while type 3 cells increased, suggesting that with activation dense tubules break up into microvesicles and granules become metallophobic. In the type 2 cells a different functional mechanism may exist.

The dense tubular system (DTS) of platelets is considered to be the pivotal structure in cell activation. Its functions are concerned with enzyme and prostaglandin metabolism and also, in analogy to muscle sarcoplasmic reticulum, with control of intracellular calcium fluxes[1-5].

The evidence for this is largely circumstantial. While various microanalytical and histochemical methods have succeeded in demonstrating calcium stores in dense bodies, nucleoids of alpha granules, plasmalemma and its extensions[6-11], they have so far failed to detect DTS-related calcium. In fact, not even the proper structure of the DTS has been described.

The technique of heavy metal impregnation has been employed in several cell types to study the endoplasmic reticulum[12,13]. It has been applied here to platelets with the hope that it will reveal the dense tubules at rest and following stimulation.

Materials and methods. The cases studied comprised 14 healthy volunteers, 9 females and 5 males. 4 cases were 15–19 years old, 8 were 20–30 and 2 were over 40. The blood was collected by venipuncture into 0.1 volume of 3.8% trisodium citrate. Platelet rich plasma (PRP) was separated by standing for 1 h at 37 °C. It was fixed with 0.2% glutaraldehyde in 0.1 M cacodylate buffer for 30 min at 37 °C, ph 7.4. The fixed PRP was then centrifuged at 300 × g for 15 min and the pellet was further fixed for 30 min in 2% glutaraldehyde in the above buffer. The pellet was next treated with a 5% solution of uranyl acetate at a pH of 3.5 for 1 h at 37 °C. After quick rinses in water a double solution of lead and copper citrate was added and this was followed by overnight postfixation in osmium tetroxide at 4 °C. The pellet was then dehydrated in graded alcohols, cut into small pieces and embedded in araldite.

In several cases the uranyl acetate solutions were adjusted to pH's of 1, 5.5, and 7[13]. Sections 0.1–0.5 µm thick were cut onto formvar coated grids which were examined unstained with a Philips 400 electron microscope using accelerating voltages of 60–100 kV.

Platelet type distribution at rest and after stimulation

	Type 1 cells (%)	Type 2 cells (%)	Type 3 cells (%)
Resting cells (14 cases)	36 ± 18	29 ± 12	35 ± 20
Activated cells (10 cases)*	7 ± 5**	22 ± 15	66 ± 12**

Cumulative results from all the cases, average ± SD. * 60 and/or 120 sec after 1 U/ml thrombin and 2 µM ADP added to PRP at 37 °C. ** Statistically significant (Student's t-test) $p < 0.001$.

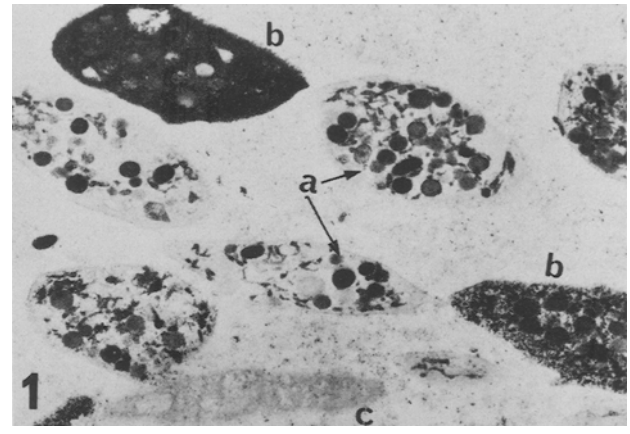

Fig. 1. Platelets impregnated with heavy metals, showing heterogeneous staining. Type 1 cells (a) have a reticular dense tubular system and dark alpha granules. Type 2 cells (b) are very electron opaque. A type 3 cell (c) is very pale. × 6000.