# Interaction of mammalian hemoglobins with dehydroascorbic acid[1]

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*Summary.* Reactive sulfhydryl groups of major hemoglobins from guinea-pig, rat and cat reduced dehydroascorbic acid to ascorbic acid leading to formation of intrachain disulfide bonds. Hybridization experiments indicated that the reduction was carried out by the $\alpha$ chain of cat hemoglobin.

In human erythrocytes dehydroascorbic acid (DHA) is reduced to ascorbic acid (AA) by glutathione (GSH) and this reduction is dependent on the erythrocyte glucose-6-phosphate dehydrogenase and glutathione reductase[2]. Further investigation with other mammalian erythrocytes indicated that besides GSH, reactive sulfhydryl groups (SH) of the major hemoglobins (Hbs) of guinea-pig, rat and cat reduced DHA to AA, resulting in the formation of intrachain disulfide bonding in the hemoglobin molecule.

*Materials and methods.* Major guinea-pig Hb was isolated at 4 °C from a CM-cellulose column (1.75 × 20 cm) equilibrated with sodium phosphate buffer (0.014 M, pH 6.65) following the method of Huisman et al.[3]. Major rat and cat Hbs were isolated according to the methods described by Chua et al.[4] and Taketa et al.[5], respectively. The homogeneity of the Hb was determined by gel electrophoresis on starch using Tris-EDTA-Borate buffer, pH 8.9[6]. Solutions of Hbs of other species were prepared by gel filtration of hemolysates (1:9) using a column of Sephadex G-25 equilibrated with 0.01% KCl solution. The KCl was later removed by dialysis. The concentration of Hbs were determined as cyanomethamoglobin[7] taking the mol. wts of Hbs to be 65,000.

DHA reduction was measured by formation of AA as described previously[2]. The incubation system contained 1 ml of Hb solution (15–25 mg), 20 µmoles of sodium phosphate buffer pH 7.0, and 8.5 µmoles of DHA in a total volume of 1.3 ml.

DHA-treated Hbs were prepared by incubating Hb solutions with DHA as stated above for 30 min at 37 °C followed by removal of AA and excess DHA by gel filtration.

Hbs were freed from small molecules like p-hydroxymercuribenzoate (PMB), N-ethylmaleimide (NEM), diamide (diazenedicarboxylic acid bis-N, N-dimethylamide), oxidized glutathione (GSSG), GSH, DHA, AA, 2-mercaptoethanol (2-ME) and urea by gel filtration using a column of Sephadex G-25 as stated before.

SH groups of Hbs were estimated by spectrophotometric titration[8] using PMB. The plot was made according to Birchmeier et al.[9].

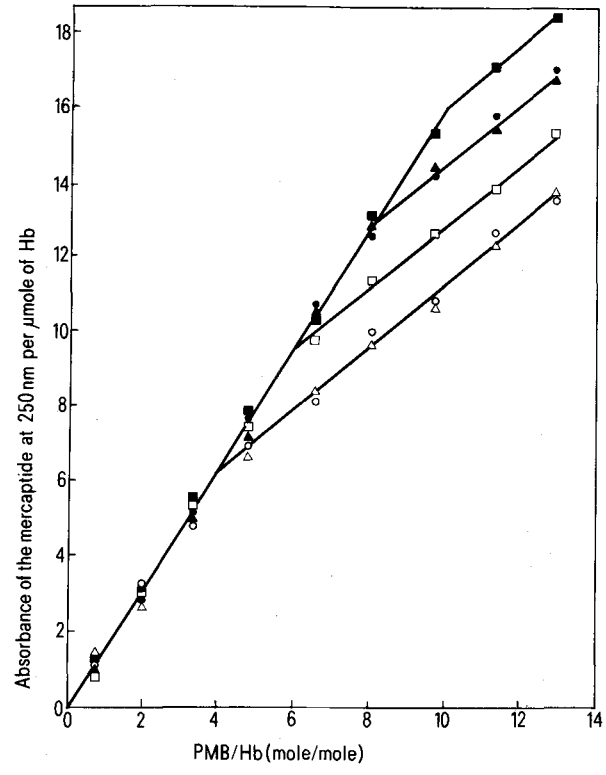
Electrophoresis of Hbs in 7% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) was performed according to the method of Weber and Osborn[10].

*Results and discussion.* Among the Hbs from different species examined, namely, man, guinea-pig, rat, cat, rabbit, goat, cow and dog only the major Hbs from guinea-pig (English, short hair), rat (Charles Foster) and cat reduced DHA to AA. The reduction of DHA was apparently made by exposed SH groups of the Hbs, because the DHA reduction was inhibited 100% by PMB and NEM, 93% by GSSG and 77% by diamide, when each was used at a molar ratio of Hb:SH reagent as 1:6.

Spectrophotometric titration of thiol groups of PMB, both in the presence and absence of 6 M urea revealed that the native guinea-pig Hb molecule contained 8 SH groups (fig.), 6 of them were reactive and 2 unreactive. That guinea-pig Hb had 8 cysteine residues was suggested by amino acid analysis[11]. PMB titrations also showed, as reported by others[12–14], that the total number of SH groups per molecule of rat and cat Hbs were 10 and 8 respectively (fig.).

The observed stoichiometry of reduction of DHA by Hbs from guinea-pig, rat and cat, as measured by AA formation, were Hb:DHA as 1:2, 1:1.9 and 1:1.7 respectively, indicating a molar ratio of 1:2. PMB titrations of DHA-treated Hbs (fig.) from guinea-pig, rat and cat indicated that in each case 4 SH groups of the Hb molecule were consumed for the reduction of 2 molecules of DHA.

These observations suggested that DHA reduction led to disulfide bond formation in the Hb molecule. When 4 SH groups were oxidized by treatment with DHA, the Hb molecule (whether from guinea-pig, rat or cat) became completely ineffective for further reducing DHA. When the DHA treated guinea-pig Hb was incubated with 2-ME at a molar ratio of 1:6, PMB titration in presence of 6 M urea revealed the presence of 8 SH groups per Hb molecule, indicating regeneration of the 4 oxidized SH groups.




PMB titration of Hbs in presence of 6 M urea. ●, Guinea-pig Hb; ■, rat Hb; ▲, cat Hb. The corresponding open symbols represent DHA-treated Hbs from the respective species. Similar results were obtained from 6 animals of each species. Increments of 2 mM solution of PMB were added to a constant amount (0.031 µmoles) of Hb in 0.11 M sodium phosphate buffer, pH 7.0, in a total volume of 2.5 ml. The incubation mixture was 6 M in urea and was kept for 90 min at 37 °C; absorbance at 250 nm was then measured in a Beckman DU 2 spectrophotometer, after 1:1 dilution with 6 M urea solution.

However, the DHA reducing ability was not regained. This might be due to irreversible denaturation of Hb by 2-ME[15]. Hb denaturants like urea also caused complete inhibition of DHA reduction by Hbs from guinea-pig, rat and cat. The inhibition was, however, released when urea was removed by gel filtration. This would suggest that not only the presence of reactive SH groups but also the native conformation of the Hb molecule was needed for DHA reduction. Presumably, in the native conformation 2 reactive SH groups come into suitable proximity for the reduction of a DHA molecule.

In order to find out whether disulfide linkage formation took place within the same peptide chain or between 2 chains, SDS polyacrylamide gel electrophoresis (in presence and absence of 2-ME) of both DHA-treated and untreated guinea-pig Hb was done. This resulted in the appearance of a single band in the same position on both the gels, corresponding to the mol. wt of 16,000, indicating the formation of intrachain disulfide bonding. Similar results were obtained with rat and cat Hbs. The $\alpha$ chain of the major cat Hb has 3 reactive SH groups whereas the $\beta$ chain has only one[14]. This suggests that the DHA reducing SH groups of cat Hb are present in the $\alpha$ chain. This was confirmed by hybridization experiments between cat Hb and human HbA, which is incapable of reducing DHA. Among the hybrids $\alpha_2^{cat}\beta_2^{human}$ and $\alpha_2^{human}\beta_2^{cat}$, prepared according to the method of Taketa et al.[14], only $\alpha_2^{cat}\beta_2^{human}$ was able to reduce DHA to AA.

Sequence studies of the $\alpha$ and $\beta$ chains of major rat Hb[12,13] indicate $\alpha$ cys at positions 13, 104 and 111 and $\beta$ cys at 93 and 125. From looking at the Hb model one observes that $\beta$ 93 and 125 are very distant from each other. Therefore, as in cat Hb, in rat Hb also the DHA reducing SH groups are tentatively suggested to be present in the $\alpha$ chain. Considerable reactivity of the cys at $\alpha$ 13 has been reported[12]. Dog Hb also posses 2 cys at $\alpha$ 104 and 111, of which $\alpha$ 111 is reactive to mercurals, but $\alpha$ 104 is not[16]. Considering these facts it would appear that in rat Hb $\alpha$ 111 and $\alpha$ 13 may be involved in DHA reduction. Indirectly, in rat Hb $\alpha$ 111 and $\alpha$ 13 are presumably in close proximity.

The results presented in this communication indicate that DHA may be used as a preliminary probe to estimate vicinal intrachain reactive SH groups in mammalian Hbs.

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## Effect of glucocorticoids on the appearance of gamma-glutamyl transpeptidase activity in primary cultures of adult rat hepatocytes[1]

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*Summary.* Primary cultures of adult rat liver parenchymal cells showed a progressive rise of gamma-glutamyl transpeptidase (GGT) activity (E.C. 2.3.2.2.) after the first 5 days of culture. The presence of dexamethasone and other synthetic glucocorticoids in the culture medium partially prevented this increase.

On adaptation to culture conditions, adult rat liver cells lose many specific functions, and show phenotypic characteristics of fetal liver[3,4]. Thus, it has been shown that GGT activity increases in primary cultures of adult rat hepatocytes[5]. This enzymatic activity, widely distributed in mammalian tissues, is low in adult liver but is present at higher levels in the fetal liver and in hepatomas[6,7].

Attempts to protect cultured hepatocytes from this dedifferentiation have led to the use of different support systems[5,8,9] and culture media supplemented with serum and/or hormones[10-13]. Glucocorticoids, which are known to inhibit fibroblastic cell growth (14, 15), seem to have a beneficial effect on cultured hepatocytes, increasing their survival and maintaining their polygonal epithelial morphology[16-18]. In this work we have studied the effect of several glucocorticoids on the expression of GGT activity in primary cultures of adult rat hepatocytes. The results show that these hormones maintain some adult liver characteristics of cultured hepatocytes for a longer period of time.

*Materials and methods.* Hepatocytes were isolated from livers of normally fed male adult Sprague-Dawley rats (200–300 g), by perfusion with collagenase (type I, Sigma Chemical Co., St. Louis, Mo. USA) according to Hue et al.[19]. Rats were anesthetized with ketamine clorhydrate (25 mg per 100 g b.wt). Ham's F-12 medium (Flow Laboratories, Irvine, U.K.), supplemented with 20% heat-inactivated fetal calf serum and antibiotics, was used[4]. Monolayers were maintained in plastic tissue culture dishes (Falcon Plastics, Los Angeles, Cal., USA) at 37 °C. Culture medium was changed daily. Glucocorticoids were added to the culture medium at the moment of seeding. Hepatocytes were harvested at the indicated times, and were homogenized in a medium containing 5 mM phosphate buffer pH 7.4, 10% glycerol and 1% Triton X-100. GGT activity was assayed at 25 °C according to the procedure described by