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Spectral and interferometrical study of the interaction of haemin with glutathione

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Abstract

The interaction of haemin with reduced glutathione (GSH) was investigated in vitro and the association constant was determined by both spectrophotometric and interferometric methods. In order to elucidate the nature of this interaction, the reaction of haemin with a typical reductive agent (sodium dithionite) and a typical chelating agent for hem–iron (histidine) was also studied. Haemin-induced haemolysis in the presence and absence of GSH was monitored spectrophotometrically. The results outline the role of GSH at physiological concentration in preventing erythrocyte damage by haemin.

Keywords: Blood; Haemin; Glutathione; Spectrophotometry; Interferometry; Erythrocytes

1. Introduction

Haemolytical anaemia is characterized by the presence of unstable haemoglobins tending to release haemin, which, by its interaction with the membrane proteins or by intercalation into the membrane lipid bilayer [1–3], leads finally to premature red blood cell (RBC) lysis. Reduced glutathione (GSH) is present in high concentrations in normal erythrocytes (1–4 mM), and is well known for its protective role against different substances. For this reason, it has been implicated as an agent in the "trapping" of free haemin from the cell cytosol [4]. In order to get a deeper insight into this area, more experimental work is necessary. Therefore the interaction of haemin with reduced glutathione was investigated in the present study by using both spectrophotometric and interferometric methods.

As haemin is well known to dimerize in aqueous solutions and the dimerization equilibrium can influence the binding capacity of haemin to glutathione, a preliminary study of the aggregation of haemin was performed under our working conditions.

The following aspects will be outlined in the present study:

- the dimerization equilibrium of haemin and determination of its constant, K_D;
- the interaction of haemin with GSH, in order to determine the association constant and to establish the nature of this interaction;
- the association of haemin with red blood cell (RBC) ghosts;

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- the effect of GSH on the haemolysis of RBC induced by haemin.

2. Experimental

The haemin stock solution was prepared by dissolving haemin in 10 mM NaOH (half of the necessary volume) and diluted with a phosphate buffer solution of pH 7.6 to obtain a concentration of about 10^{-4} M in 5 mM NaOH. The obtained solution was filtered and centrifuged and its precise concentration was determined spectrophotometrically from the absorbance at 385 nm (molar absorption coefficient $\varepsilon = 58\,400$ l mol^{-1} cm^{-1} [1]).

The absorption and difference spectra were recorded on a Specord UV–Vis spectrophotometer (Carl Zeiss, Jena).

The interferometric measurements were performed on an Rayleigh–Haber–Lowe interferometer (Carl Zeiss, Jena), using liquid cells of maximum capacity (20 ml). All measurements were performed at room temperature.

Haemoglobin-free ghosts were prepared by a 1:40 hypotonic lysis in 5 mM phosphate buffer at pH 8.0 as described in the literature [4].

3. Results and discussion

3.1. Aggregation of haemin

3.1.1. Spectrophotometric results

The visible absorption spectrum of haemin is presented in Fig. 1, and was deconvoluted using a literature computer program, based on the Levenberg method of damped least squares [5]. The two overlapping bands were assigned, respectively, to the monomer (band at 385 nm) and dimer (342 nm), in agreement with previous data [6].

The dimerization of haemin in solution may be represented by the equilibrium

$$2M \rightleftarrows D + nH^+ \qquad (1)$$

$$K_D = \frac{[D][H^+]^n}{[M]^2} = K'_D[H^+]^n$$

$$K'_D = K_D/[H^+]^n = [D]/[M]^2$$

In order to determine the dimerization constant at constant pH, K'_D, the influence of concentration was followed, maintaining the product of the concentration and the path length constant ($c \times l$ = const.). The absorption curves obtained do not present any

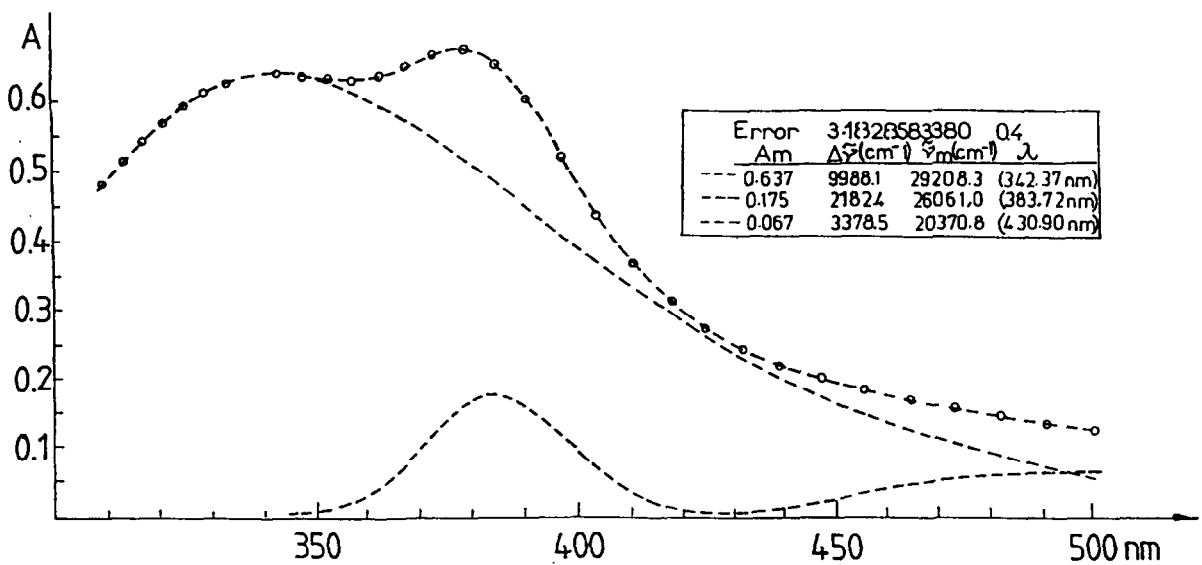

Fig. 1. Visible absorption spectrum of haemin (C_H = 10 μM in phosphate buffer pH 7.4): (○) experimental; (– – –) deconvoluted.

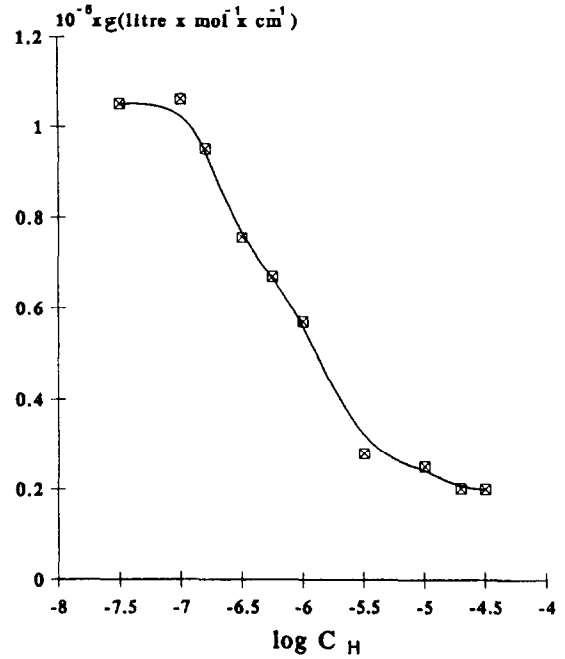

Fig. 2. Concentration dependence of ε_{385} for haemin solutions at 20°C.

isosbestic point and attest the predominance of the dimeric form (band at 342 nm) even at low haemin concentrations.

By plotting ε at 385 nm against the logarithm of haemin concentration (C_H), the sigmoid curve in Fig. 2 was obtained. In order to avoid the difficulties presented by the sigmoid extrapolation, the method described by Tipping et al. [7] was used to determine the molar absorption coefficient of the monomeric haemin.

Starting from the equilibrium given in Eq. 1, at constant pH, the absorbance at a fixed wavelength is given by:

$$A = \varepsilon_{obs} C_H = [M]\varepsilon_M + [D]\varepsilon_D \qquad (2)$$

where ε_{obs}, ε_M and ε_D are the apparent, monomeric and dimeric molar absorption coefficients respectively, and C_H is the total concentration of haemin ($C_H = [M] + 2[D]$).

Rearrangement of Eq. 2 gives

$$C_H = \frac{[M]\varepsilon_M + [D]\varepsilon_D}{\varepsilon_{obs}}$$

At very low haemin concentrations the monomer concentration greatly exceeds that of the dimer and in the limit $C_H = [M]$ and $\varepsilon_M = \varepsilon_{obs}$. Therefore extrapolation of a plot of $1/\varepsilon_{obs}$ against C_H to $C_H = 0$ gives $1/\varepsilon_M$ as the intercept on the ordinate (Fig. 3). Either linear extrapolation or sigmoid extrapolation (Fig. 2) leads to the same value: $\varepsilon_M = 1.04 \times 10^5$ M^{-1} cm^{-1}.

In the range 10^{-4}–10^{-6} M the absorption spectrum attests the predominance of the dimeric form, $[D] \gg [M]$, and $C_H = 2[D]$. Substitution into Eq. 2 gives

$$\varepsilon_{obs} = \frac{\varepsilon_M}{\sqrt{2K'_D}} \times \frac{1}{\sqrt{C_H}} + \frac{\varepsilon_D}{2} \qquad (3)$$

Plotting $\varepsilon_{obs} = f(1/\sqrt{C_H})$ (see Fig. 4), K'_D may be determined from the slope and ε_D from the intercept. A value of $(3.92 \pm 0.05) \times 10^6$ M^{-1} is obtained for K'_D.

3.1.2. Interferometrical study

The study of the refractive index n of a solution as a function of its concentration leads generally to a linear dependence in the case of dilute aqueous

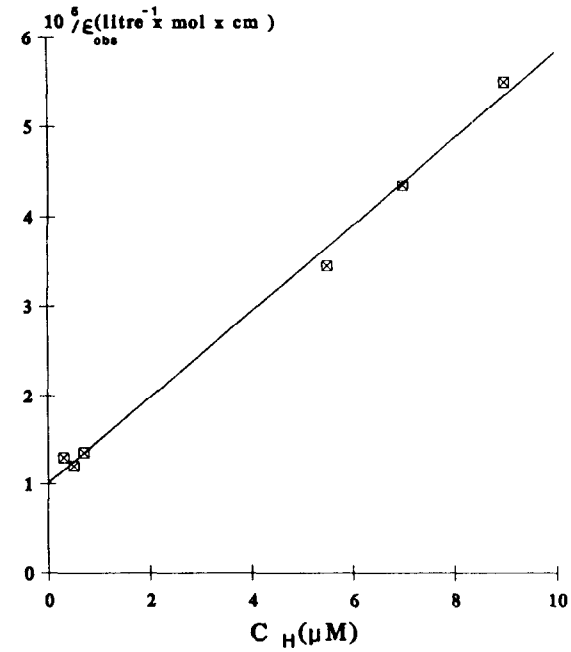

Fig. 3. Plot of $1/\varepsilon_{obs}$ vs. C_H in order to determine ε_M.

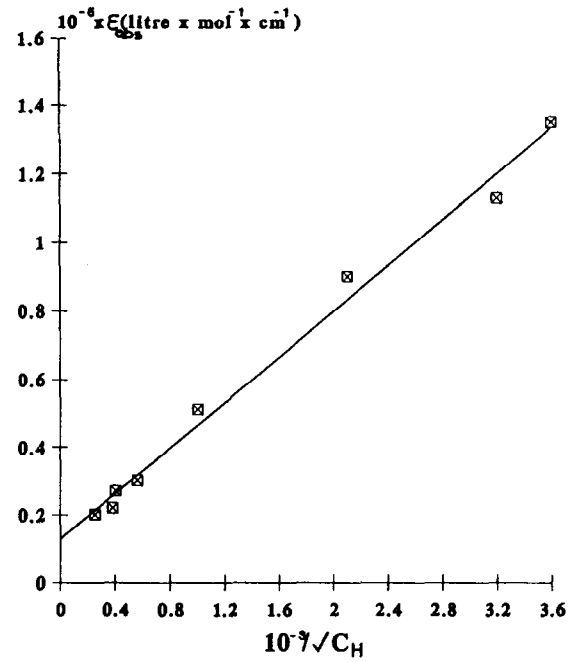

Fig. 4. Plot of ε_{obs} vs. $1/\sqrt{C_H}$ according to Eq. 3.

protein solutions [8,9] or of binary mixtures of hydrocarbons [10]. For larger ranges of concentrations, different polynomial expressions with higher powers of n were established [11]. In solution, where intermolecular interactions are supposed to occur, graphical representations of the form $n^2 = f(c)$ have also been employed [12].

The present work aims to determine the dimerization constant of haemin, K'_D, from the variation of the difference between the refractive index of the solution, n_{sol}, and that of the corresponding solvent (buffer solution), n_{solv}, as a function of the haemin concentration. The plot of k, the number of divisions read on the interferometer vs. $\log C_H$ (Fig. 5a) is characterized by a change in slope in the concentration range 10^{-6}–10^{-7} M.

If this change in the slope is supposed to be due to the dimerization of haemin, then the two linear terminal parts may be associated either with monomer or dimer forms only.

We can define a dimerization degree, θ, so that the initial slope, which reflects no dimerization, would correspond to $\theta = 0$ ($C_H = [M]$), whereas the final straight line, implying complete dimerization ($C_H = 2[D]$) corresponds to $\theta = 1$. For the intermediate region the refractive index of the solution, n, (or the interferometer division, k, which is proportional to it) can be given by: $n = n_M(1-\theta) + n_D\theta$, in terms of the monomer (n_M) and dimer (n_D) refractive indexes, respectively. So any measured point

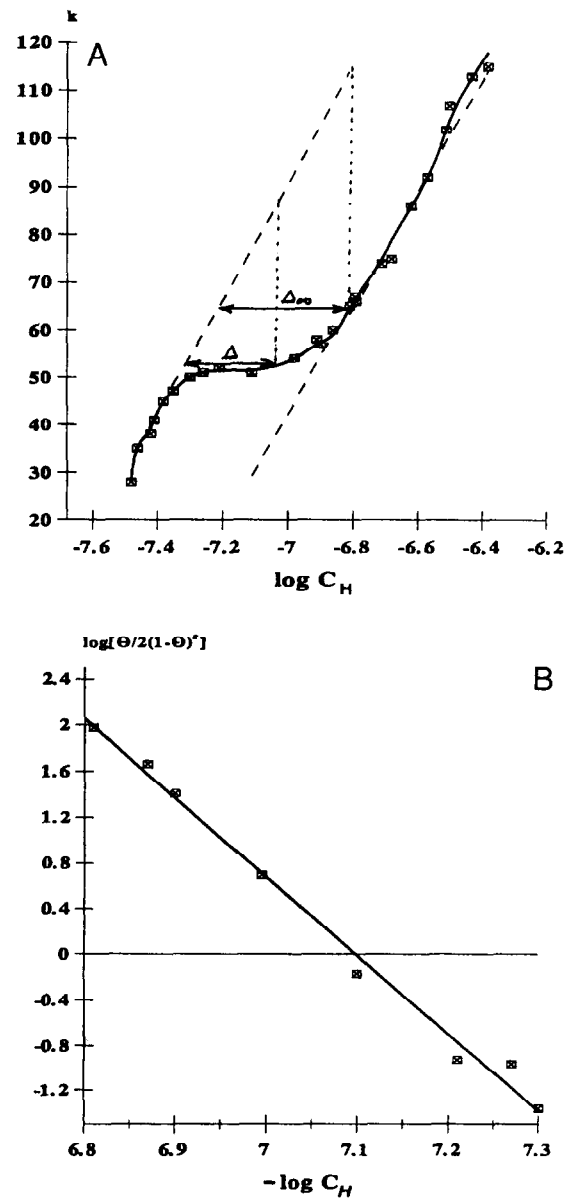

Fig. 5. Interferometrical determination of K'_D. (A) plot of k vs. $\log C_H$; graphical determination of $\theta = \Delta/\Delta_\infty$ at different C_H values. (B) Determination of K'_D according to Eq. 5.

separates the distance between these two parallel lines in a proportion equal to $\theta/(1-\theta)$ and therefore θ can be evaluated graphically (Fig. 5a) as the ratio:

$$\theta = \frac{\Delta}{\Delta_\infty} = \frac{n_M - n}{n_M - n_D}$$

Expressing the monomer and dimer concentrations as functions of θ and replacing in the expression of K'_D gives

$$K'_D = \frac{[D]}{[M]^2} = \frac{\theta}{2C_H(1-\theta)^2} \quad (4)$$

or

$$\log \frac{\theta}{2(1-\theta)^2} = \log K'_D + \log C_H \quad (5)$$

Therefore the plot of $\log(\theta/2(1-\theta)^2)$ vs. $\log C_H$ intersects the $\log C_H$ axis at a value of $\log K'_D$ corresponding to $K'_D = (1.20 \pm 0.03) \times 10^{-7}\ M^{-1}$ (Fig. 5b). This value is about three times greater than the value obtained spectrophotometrically but still in the range encountered in determinations of association constants by different physical methods [13].

3.2. Interaction of haemin with glutathione

The absorption spectra obtained by adding a 1–5 mM GSH solution to a haemin solution in the range 1–20 mM are characterized by the decrease in the haemin monomer band at 385 nm and the increase in the absorbance in the range 355–365 nm, due to the binding of GSH to haemin (Fig. 6a). As the bands of the complex and those due to the monomeric and dimeric haemin are strongly overlapped, a difference spectrum was necessary in order to identify the absorption bands of the haemin–GSH complex. Fig. 6b, presenting the absorption difference spectrum of the H–GSH system vs. a haemin solution of the same concentration as that in the complex, allows to locate the new bands due to the binding of GSH to haemin at 365 and 424 nm.

A first problem to elucidate is the nature of the interaction of haemin with GSH: is it a redox type

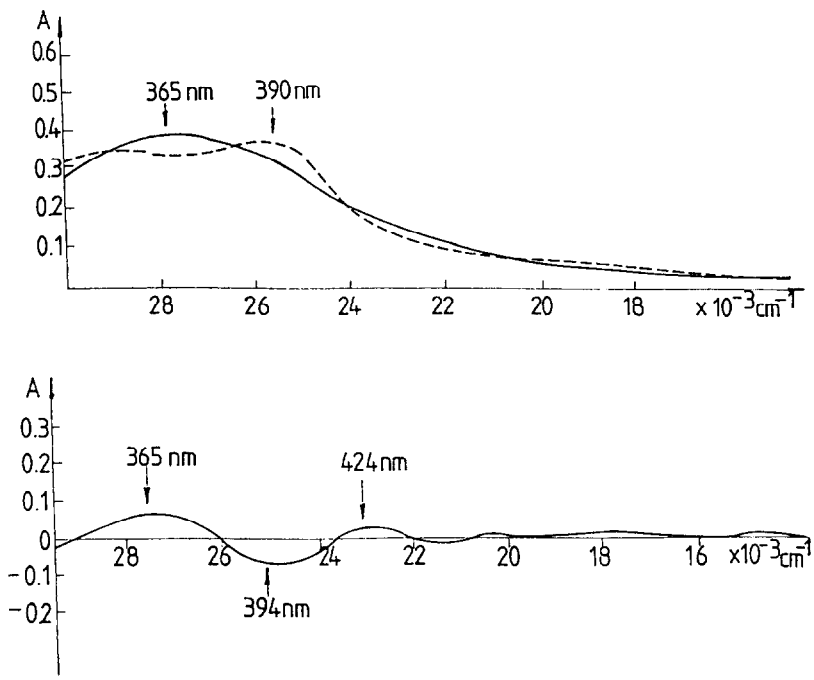

Fig. 6. (A) Absorption spectrum of haemin (- - -) and haemin–GSH complex (—). (B) Difference spectrum of the haemin–GSH complex vs. a haemin solution of the same concentration.

reaction leading to Fe(II)–hem and oxidized glutathione (GS–SG) or a complexation of the hem Fe(III) by GSH?

Therefore the following systems were additionally investigated under the same work conditions:

- haemin–sodium dithionite,
- haemin–histidine,
- haemin–oxidized glutathione.

The following statements may be made:

- The spectral modifications observed when the micromolar haemin solution is titrated with sodium dithionite, i.e., when a reduction of Fe(III)–hem is supposed to take place, are different to those observed in the presence of GSH, both haemin bands decreasing and a new absorption range being apparent at 595 nm.
- The spectral modifications observed in the presence of histidine, which is known as a Fe(III) chelating agent, are quite similar to those produced by GSH.
- The spectrum of haemin is not modified by oxidized glutathione.

Therefore, it may be inferred that reduced glutathione serves as a ligand by its thiol group for the haemin Fe(III), because no interaction is observed when glutathione is oxidized.

In order to determine the association constant of GSH and histidine to haemin, a Benesi–Hildebrand treatment [14] was applied, according to the equation

$$\frac{C_H^0}{A_\lambda^0 - A_\lambda} = \frac{1}{\Delta\varepsilon} + \frac{1}{K\Delta\varepsilon}\frac{1}{C_{GSH}} \qquad (6)$$

where C_H^0 is the starting concentration of haemin, A_λ^0 and A_λ the absorbances of the haemin solutions and of the complex, respectively, at the same wavelength. At $\lambda = 365$ nm, the maximum absorption wavelength of the complex, the plot of $C_H^0/(A_\lambda^0 - A_\lambda)$ vs. the reciprocal of the GSH concentration (Fig. 7) leads to a value of $(2.80 \pm 0.05) \times 10^3$ M^{-1} for the association constant, K, of haemin and GSH.

A similar treatment applied for the haemin–histidine system leads to a K value of 26.61 ± 0.05 M^{-1}.

Analysis of the binding process of GSH to haemin points out the dependence of the binding type on the concentration of GSH. At low GSH concentrations

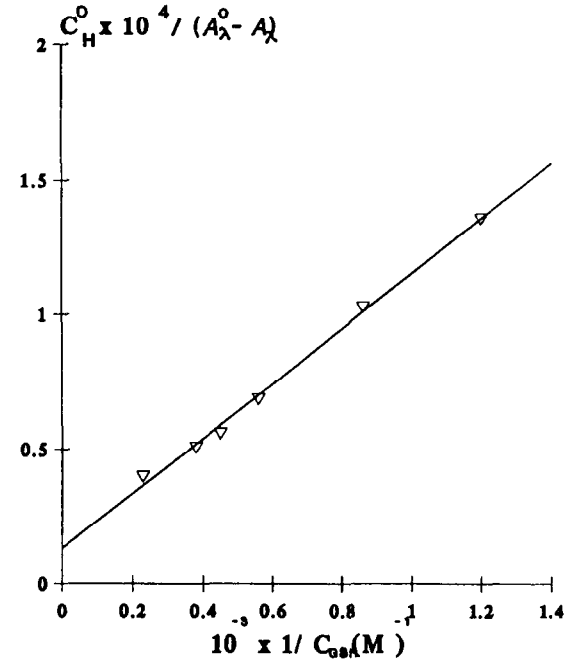

Fig. 7. Spectrophotometric determination of the association constant of haemin with GSH (Eq. 6).

the plot of the absorption difference $(A_\lambda^0 - A_\lambda)$ for $\lambda = 365$ nm vs. the GSH concentration (Fig. 8a) points out that saturation occurs at $C_{GSH} = 2$ mM. At $C_{GSH} > 50$ mM a new binding site is apparent as evidenced by the plot in Fig. 8b, and attesting a more complex binding mechanism, the elucidation of which needs more experimental work.

The interferometrical determination of the association constant between haemin and GSH was performed starting from isorefractible solutions of haemin and GSH, measuring the variation of refractive index at different mixing ratios [15]. It was considered that the maximum deviation of the refractive index from that of the starting solutions corresponds to the complexation ratio and the association constant was calculated. However, it was observed that the value obtained for K decreases markedly with increasing ratio of GSH to haemin. As the spectrophotometric data have indicated that at high GSH to haemin concentration ratios the binding becomes more complex and new binding sites are apparent, we consider as pertinent to retain the K value obtained for the same range of concentrations as that used in the spectrophotometric determination;

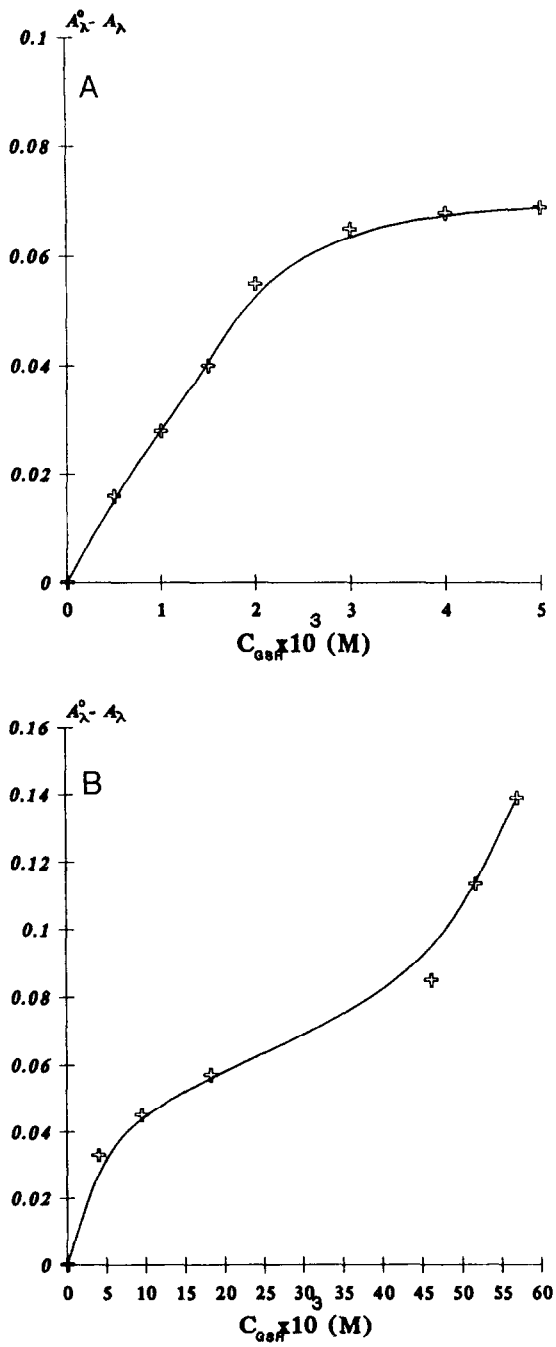

Fig. 8. Plot of $(A^0_\lambda - A_\lambda)$ vs. C_{GSH} at constant concentration of haemin (10 mM). (A) C_{GSH} in the range 0–5 mM; (B) C_{GSH} in the range 0–60 mM.

this leads to $K = (2.31 \pm 0.03) \times 10^3$ M, in good agreement with the value determined spectrophotometrically.

3.3. Association of haemin with red cell membrane

The affinity of haemin and red cell membrane was analyzed in order to estimate the amount of haemin that the RBC membrane is able to bind. Fresh washed red cells were suspended in isotonic phosphate buffer at pH 7.3 to yield a 0.05% cell suspension. The cells were incubated in the dark at 37°C. RBC ghosts obtained from a suspension with a defined concentration were separated as described in the literature [4] and then were mixed with haemin solutions of various concentrations. The mixtures were shaken for 5 min and then centrifuged for 20 min at 43000 g. The supernatants were collected and free aqueous haemin (H_C, unbound haemin) was determined spectrophotometrically (A^{385}) to obtain quantitative measurements of binding. The amount of haemin in the completely bound state (H_B), was determined as the difference between the initial haemin concentration (H_T, total haemin), and unbound haemin (H_C) and can be used to calculate the quantity of haemin per million ghosts ($H_B/10^6$ ghosts). Under stoichiometric conditions the added amount of haemin could be considered totally bound and a linear dependence on added haemin should exist. The results in Fig. 9 demonstrate that the amount of haemin bound to the RBC membranes depends on H_T.

It is apparent that at sufficiently low concentrations of haemin the curve is linear. At higher concentrations of haemin, when not all added haemin is bound, a deviation from linearity (a saturation) occurs.

3.4. Effect of glutathione on the haemolysis of red blood cells induced by haemin

Haemin is known to cause red cell haemolysis as reported in the literature [16,17]. In the present work we employed a different and more accurate approach to the study of RBC lysis induced by haemin alone and in the presence of GSH, using experiments with RBC membranes as "control".

Freshly washed red cells were suspended in isotonic phosphate buffer at pH 7.3 to yield a 0.05% cell suspension. The cells were incubated in the dark at 37°C, exactly as for the experiments with membranes, and suspensions with defined cell concentrations were mixed with haemin solutions in the ab-

sence or presence of GSH. The mixture was shaken for 5 min and then centrifuged for 20 min at 43 000 *g*. To keep all haemin complexed, ligand was added in a concentration which was shown to yield maximal absorption change; so the final concentration of GSH was 3.3 mM. The supernatants were collected and haemoglobin concentration was determined by measuring the absorbance at 578 nm (the visible peak of haemoglobin where minimal interference with liganded haemin exists). We considered as "control" the experiments with Hb-free membranes (which were realized under similar conditions), i.e., we subtracted the contribution of unbound haemin ($A_{H_U}^{578}$) from the absorbance measured at 578 nm in the experiments with red cells. With this final value of absorbance at 578 nm we calculated, more accurate, the Hb concentration.

The percentage of haemolysis was determined by comparing the haemoglobin absorbance obtained from RBC lysed by haemin alone or in presence of GSH, with that of cells totally lysed in a hypotonic solution (also centrifuged for 20 min at 43 000 *g*).

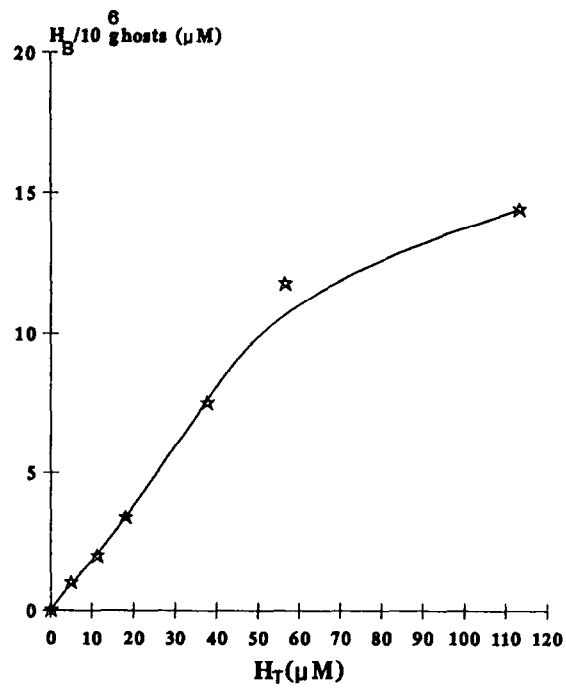

Fig. 9. Association of haemin with RBC ghosts at pH 7.3, buffered by isotonic PBS. The solutions were thermostated at 37°C. Each point represents an average of three measurements deviating from the reported value by 5%.

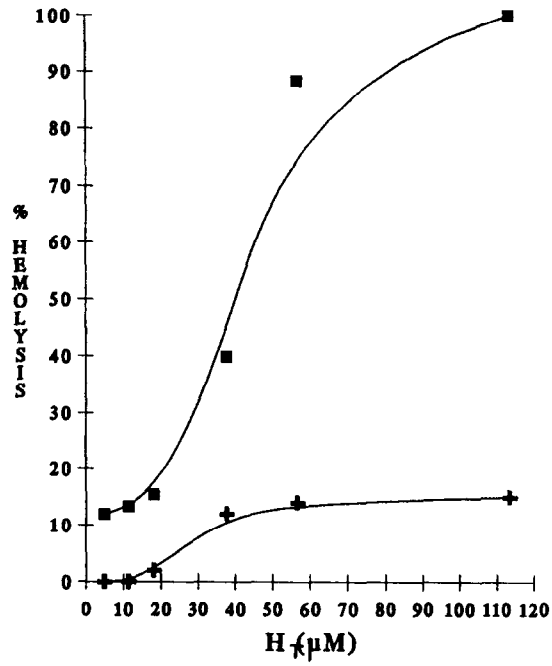

Fig. 10. Haemolysis of intact erythrocytes induced by haemin (■) and by haemin in the presence of GSH (+) at various hemin concentrations. Each point represents an average of three experiments deviating from the reported value by 5%.

The results presented in Fig. 10 demonstrate that haemin alone causes a much larger haemolysis than in the presence of GSH. Therefore GSH at a physiological concentration significantly prevents the lytic effect of haemin, probably due to the competition between GSH and the membrane for haemin.

By binding GSH the hydrophobicity of haemin is much reduced and, as a result, the complex may have a lower affinity to the membrane. On the other hand GSH is implicated in another indirect defense mechanism of the red cell by keeping occupied haemin which can no longer cause inhibition of the cytosolic key enzymes responsible for maintenance of the red cell function [18,19].

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