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# Transition of hemoglobin between two tertiary conformations: The transition constant differs significantly for the major and minor hemoglobins of the Japanese quail (*Cortunix cortunix japonica*)

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**Abstract**

We demonstrate that 5,5′-dithiobis(2-nitrobenzoate) – DTNB – reacts with only CysF9[93]β and CysB5[23]β among the multiple sulfhydryl groups of the major and minor hemoglobins of the Japanese quail (*Cortunix cortunix japonica*). $K_{equ}$, the equilibrium constant for the reaction, does not differ very significantly between the two hemoglobins. It *decreases* 430-fold between pH ≈ 5.6 and pH ≈ 9: from a mean of 7 ± 1 to a mean of 0.016 ± 0.003. Quantitative analyses of the $K_{equ}$ data based on published X-ray and temperature-jump evidence for a tertiary structure transition in *liganded* hemoglobin enable the calculation of $K_{rt}$, the equilibrium constant for the **r** ⟷ **t** tertiary structure transition. $K_{rt}$ differs significantly between the two hemoglobins: 0.744 ± 0.04 for the major, 0.401 ± 0.01 for the minor hemoglobin. The mean p$K_a$s of the two groups whose ionizations are coupled to the DTNB reaction are about the same as previously reported for mammalian hemoglobins.


*Keywords:* Japanese quail hemoglobin; Tertiary conformational transition; Multiple reactive sulfhydryl group; Reaction with 5,5′-dithiobis(2-nitrobenzoate); Equilibrium constant

## 1. Introduction

The CysF9[93]β sulfhydryl group of hemoglobin has been employed as a probe of tertiary and quaternary structure change [1–11]. In *liganded* hemoglobin the *cis*-to-amino and *cis*-to-carbonyl conformations of this sulfhydryl group are coupled to two tertiary isomeric forms of hemoglobin, **r** and **t**, in dynamic equilibrium [9–11]. We have used the fact that the reaction of this sulfhydryl with 5,5′-dithiobis(2-nitrobenzoate) – DTNB – is reversible [12,13] to determine the equilibrium constant, $K_{rt}$, for the tertiary **r** ⟷ **t** structure transition in three mammalian hemoglobins [11]. These mammalian hemoglobins have considerably different primary structures [14]. Nevertheless, our analyses indicated that $K_{rt}$ is the same for all the three hemoglobins: a value of about 0.22 at 25 °C [11]. We now wish to extend this study to non-mammalian hemoglobins to determine whether $K_{rt}$ will be the same or will be different for non-mammalian hemoglobins.

In a previous report [11], we determined the equilibrium constant, $K_{equ}$, for hemoglobins containing CysF9[93]β as the only sulfhydryl group reacting with DTNB. In the present report we extend our study to hemoglobins containing a further reacting sulfhydryl group, CysB5[23]β. This sulfhydryl group is present in the hemoglobins of all avian species but one — that of the guinea fowl. We have undertaken a comprehensive equilibrium study of the reaction of DTNB with two *avian* hemoglobins to test the general validity of the findings reported for mammalian hemoglobins. For this purpose we have selected the Japanese quail (*Cortunix cortunix japonica*); its hemolysate contains two hemoglobin components [15]. We find that these hemoglobins have significantly different $K_{rt}$ values: 0.744 ± 0.04 and 0.401 ± 0.01 for the major and minor hemoglobins, respectively.

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## 2. Experimental

Japanese quail chicks were obtained from a veterinarian. We subsequently maintained them until they became mature (6 months). Blood samples were collected from the birds into bottles containing freshly prepared acid–citrate–dextrose anticoagulant. The method used for the preparation of the hemolysate has been described in detail elsewhere [11]. The major and minor hemoglobins were separated on a carboxymethylcellulose column (Whatman CMC-52, microgranular, preswollen), as previously described for cat and chicken hemolysates [12,16]. The preparation and characterization of the oxy, carbonmonoxy and aquomet derivatives have been described before [11]. The number of reacting sulfhydryl groups in each hemoglobin was determined with *p*-chloromercuri(II) benzoate (*p*-CMB) and DTNB, as previously described [17]. The method employed for the determination of equilibrium constants, $K_{equ}$, has been described in detail before [13]. The standard error involved in these determinations was about 10%. All samples were deionized on a Dintzis ion exchange column [18] prior to use for experiments. $K_{equ}$ data were fitted with theoretical curves using programs written on a MicroMath Scientist software (Salt Lake City, Utah).

## 3. Results

### 3.1. Titrations with sulfhydryl reagents

#### 3.1.1. p-Chloromercuri(II)benzoate titration: p-CMB

In Fig. 1 we present the results of the titration of the major and minor quail carbonmonoxyhemoglobins with *p*-CMB. It is seen that the major hemoglobin gives eight sulfhydryl groups titrated per hemoglobin tetramer, while the minor hemoglobin gives six. These results are readily understood on the basis of the primary structures of the α and β subunits of the hemoglobins of the Japanese quail [15]. These show that, whereas the major hemoglobin has a cysteine residue at the H13[130]α position, the minor hemoglobin has a serine residue at the same position [15]. In all other positions containing sulfhydryl groups the two hemoglobins are exactly equivalent [15]. Since each position appears as a pair per hemoglobin molecule, it is not surprising that the major hemoglobin has two sulfhydryl groups more than the minor.

According to the amino acid sequences of its subunits, the major hemoglobin of the quail has sulfhydryl groups at the following positions: G11[104]α, H13[130]α, H4[126]β, F9[93]β and B5[23]β [15]. Since there are two α and two β subunits per (tetramer) molecule, this gives a total of ten sulfhydryl groups per tetramer. The minor hemoglobin has sulfhydryl groups at the same positions, except that serine replaces cysteine at position H13[130]α. The minor hemoglobin therefore has a total of eight sulfhydryl groups per tetramer.

CysG11[104]α is at the $\alpha_1\beta_1$ subunit interface; it is known to be masked and therefore unreactive toward any sulfhydryl reagent [19]. Organic mercurial reagents, including *p*-CMB, are known to react with all unmasked sulfhydryl groups in

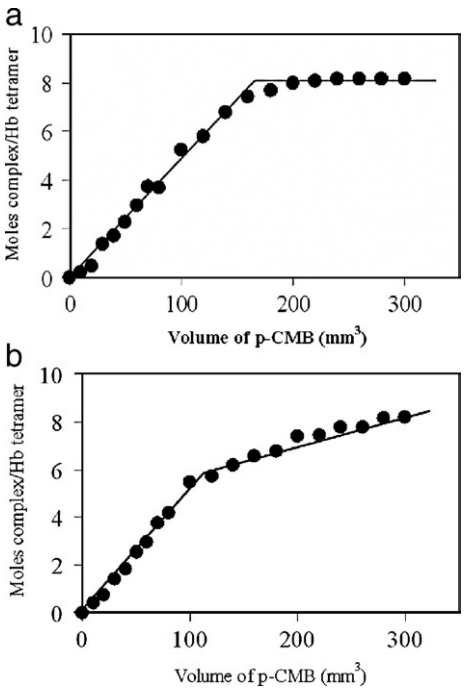



Fig. 1. Titration of the hemoglobins of the Japanese quail with *p*-chloromercuri(II)benzoate (*p*-CMB): ratio of the concentration of the complex formed to the concentration of hemoglobin tetramers. (a) major, (b) minor carbonmonoxyhemoglobin. Conditions: phosphate buffer pH 7.6, ionic strength, 50 mmol $dm^{-3}$ (added salt, NaCl); hemoglobin concentration, 25 μmol (heme) $dm^{-3}$; stock *p*-CMB concentration, 840 μmol $dm^{-3}$; working wavelength, $\lambda = 250$ nm; total volume of hemoglobin used, 3 $cm^3$.

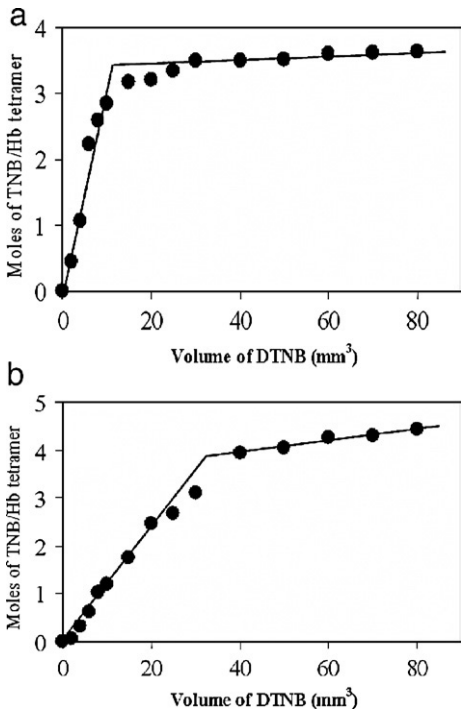



Fig. 2. Titration of the hemoglobins of the Japanese quail with 5,5′-dithiobis(2-nitrobenzoate), DTNB: ratio of the concentration of 5-thio-2-nitrobenzoate (TNB) produced to the concentration of hemoglobin tetramers. (a) major, (b) minor carbonmonoxyhemoglobin. Conditions: phosphate buffer pH 7.6, ionic strength, 50 mmol $dm^{-3}$ (added salt, NaCl); hemoglobin concentration, 10 μmol (heme) $dm^{-3}$; stock DTNB concentration, 0.5 mmol $dm^{-3}$; working wavelength, $\lambda$=412 nm; total volume of hemoglobin used, 10 $cm^3$.

hemoglobin, irrespective of their states of ionization [16,17,20]. On titrating with *p*-CMB, therefore, we expect to obtain eight sulfhydryl groups per tetramer for the major hemoglobin and six for the minor hemoglobin. These expectations are fulfilled by the data in Fig. 1.

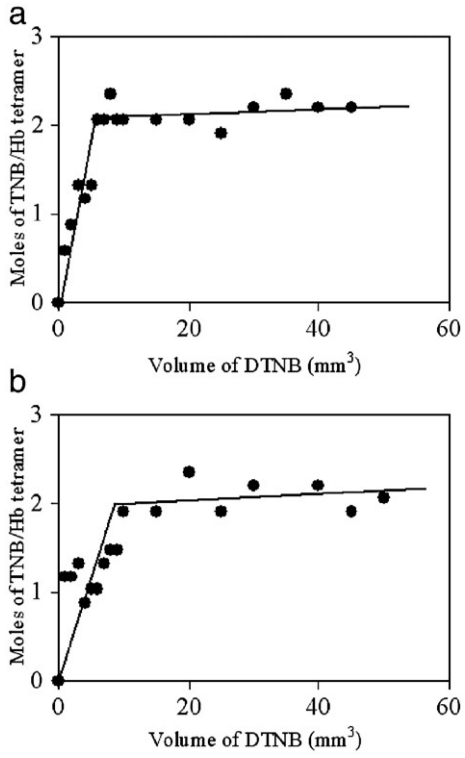



Fig. 3. Titration of the hemoglobins of the guinea fowl with 5,5′-dithiobis(2-nitrobenzoate), DTNB: ratio of the concentration of 5-thio-2-nitrobenzoate (TNB) produced to the concentration of hemoglobin tetramers. (a) major, (b) minor carbonmonoxyhemoglobin. Conditions as in Fig. 2.

*3.1.2. Titration with 5,5′-dithiobis(2-nitrobenzoate): DTNB*

Fig. 2 reports the results of titrations of the major and minor hemoglobins with DTNB. As can be seen (Fig. 2a), the major hemoglobin gives a value of 3.5 (*ca* four) titratable sulfhydryl groups per hemoglobin tetramer; the minor hemoglobin gives 3.8 (*ca* four) (Fig. 2b). It seems clear from these results that CysH13[130]α of the major hemoglobin does not react with DTNB. If it did, the major hemoglobin (Fig. 2a) would have given at least six sulfhydryl groups per tetramer since the minor hemoglobin, which does not have a cysteine but a serine at the H13[130]α position, gave four (Fig. 2b). CysF9[93]β is known to react with all sulfhydryl reagents. With CysG11[104]α, CysH13[130]α and CysF9[93]β accounted for in the major hemoglobin – CysG11[104]α and CysF9[93]β in the minor hemoglobin – this leaves CysH4[126]β and CysB5[23]β yet to be accounted for in both the major and minor hemoglobins. Of the four sulfhydryl groups that react with DTNB in the major and minor hemoglobins, the CysF9[93]β pair accounts for two. The remaining two must be due to either the CysH4[126]β pair or the CysB5[23]β pair.

In order to reach a definite conclusion on this matter, we carried out DTNB titrations on two avian hemoglobins in which, compared to the hemoglobins of other birds, the mutation B5[23]$\beta^{Cys\rightarrow Ser}$ has occurred [15]. These are the major and minor hemoglobins of the guinea fowl (*Numida meleagri*). The results are reported in Fig. 3. It is seen that the major hemoglobin gives two sulfhydryl groups per hemoglobin (tetramer) molecule (Fig. 3a). Fig. 3b shows that the minor hemoglobin also gives two sulfhydryl groups per molecule. Comparison of these results with those for the Japanese quail hemoglobins (Fig. 2) leads to the conclusion that it is the CysB5[23]β pair in the quail hemoglobins that reacts with DTNB, not the CysH4[126]β pair.

Furthermore, we carried out a preliminary kinetic study on the reaction of DTNB with the major and minor carbonmonoxyhemoglobins of the Japanese quail under pseudo-first order conditions [12]. Each of the hemoglobins displayed biphasic kinetics. By contrast, the major and minor hemoglobins of the guinea fowl displayed monophasic kinetics under the same experimental conditions. We are again led to the conclusion that in the quail hemoglobins two types of sulfhydryl groups react with DTNB, whereas only one type reacts in the guinea fowl hemoglobins.

The guinea fowl hemoglobins appear to be unique among avian hemoglobins in having a serine rather than a cysteine residue at the B5[23]β position [14,15,21]. Other than this they have sulfhydryl groups at exactly the same positions as in the other avian hemoglobins: G11[104]α, H13[130]α, H4[126]β, and F9[93]β in the major hemoglobin; G11[104]α, H4[126]β, and F9[93]β in the minor hemoglobin. CysF9[93]β is known to react with all sulfhydryl reagents. Since the guinea fowl

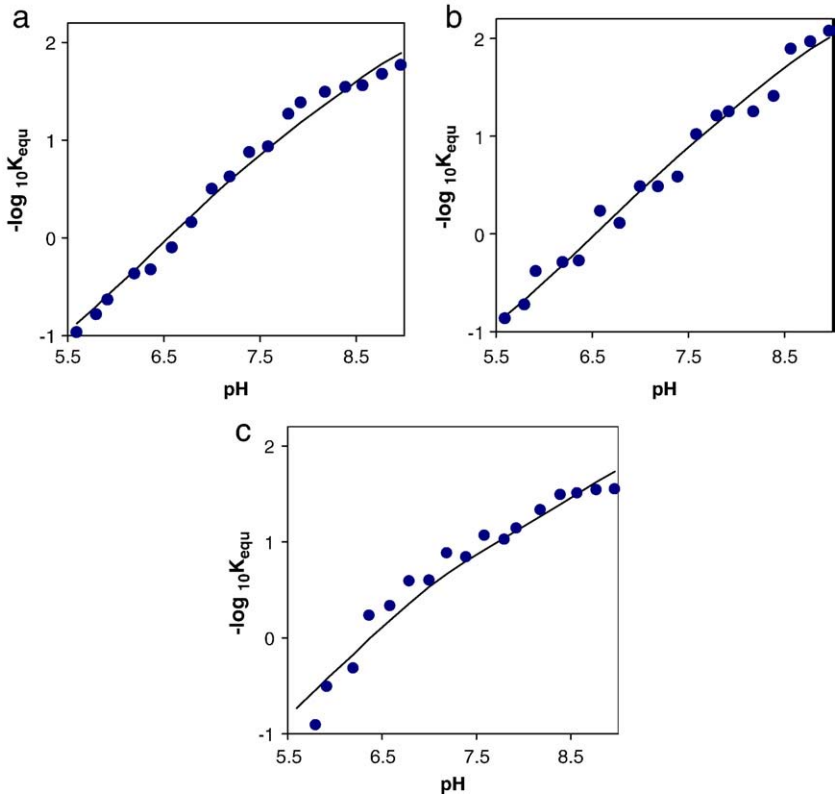



Fig. 4. Variation of $-\log_{10}K_{equ}$ with pH for the reactions of derivatives of the *major* hemoglobin of the Japanese quail with 5,5′-dithiobis(2-nitrobenzoate), DTNB. Conditions: 25 °C; phosphate buffers, pH 5.6–8.0; borate buffers, pH 8.0–9.0, both of ionic strength 50 mmol $dm^{-3}$ (added salt, NaCl). (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Each experimental point is subject to a standard error of about 10%. The lines through the data points are the theoretical best-fit lines drawn with the parameters reported in Table 1 for the *major* hemoglobin (cf. Scheme 1 and Eq. (3) for $n=2$).

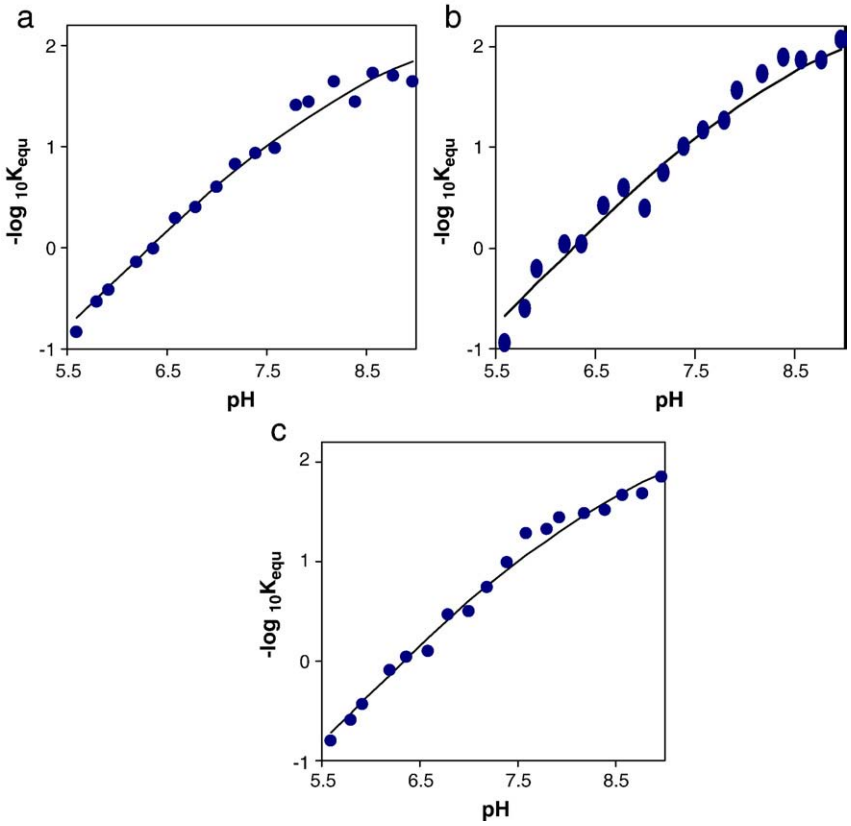



Fig. 5. Variation of $-\log_{10}K_{equ}$ with pH for the reactions of derivatives of the *minor* hemoglobin of the Japanese quail with 5,5′-dithiobis(2-nitrobenzoate), DTNB. Conditions as in Fig. 5. (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Each experimental point is subject to a standard error of about 10%. The lines through the data points are the theoretical best-fit lines drawn with the parameters reported in Table 1 for the *minor* hemoglobin (cf. Scheme 1 and Eq. (3) for $n=2$).

hemoglobins gave two sulfhydryl groups on titration with DTNB, and also gave single phase kinetics, it follows that in the guinea fowl hemoglobins CysF9[93]β is the only sulfhydryl group that reacts with DTNB; the others do not react. By contrast, the quail hemoglobins have sulfhydryl groups in the same positions as in the guinea fowl hemoglobins but, in addition, have a cysteine at the B5[23]β position [15]. On titration with DTNB they give four sulfhydryl groups per (tetramer) molecule; and they display biphasic kinetics. This proves that CysB5[23]β provides the second pair of DTNB-reactive sulfhydryl groups in the quail hemoglobins.

### 3.2. Variation of equilibrium constants with pH

The reaction between the sulfhydryl groups of hemoglobin and DTNB may be depicted [13]:

$$\mathrm{PSH} + \mathrm{DTNB} \overset{Q_{SH}}{=\!=} \mathrm{H^+} + \mathrm{PS^-} + \mathrm{DTNB} \overset{K_{equ}}{=\!=} \mathrm{H^+} + \mathrm{PS.ST} + \mathrm{TNB^-} \overset{Q_{TNB}}{=\!=} \mathrm{PS.ST} + \mathrm{TNBH}. \quad (1)$$

In Eq. (1) PSH is hemoglobin with its sulfhydryl groups in their protonated, unreacting (with DTNB) form; $PS^-$ are the corresponding (reacting) anion forms; PS.ST are the mixed disulfides formed after reaction with DTNB; $TNB^-$ is 5-thio-2-nitrobenzoate, the anionic, chromophoric product of the reaction; TNBH is the protonated form of $TNB^-$; $Q_{SH}$ and $Q_{TNB}$ are the ionization constants of the sulfhydryl groups and TNBH, respectively; $K_{equ}$ is the equilibrium constant for the formation of the mixed disulfides (PS.ST), that is, the DTNB reaction step. The equation relating $K_{equ}$ to the parameters and species in Eq. (1) is:

$$K_{equ} = \frac{[\mathrm{TNB^-}]^2\left\{1+\frac{[\mathrm{H^+}]}{Q_{TNB}}\right\}\left\{1+\frac{[\mathrm{H^+}]}{Q_{SH}}\right\}}{\left\{[\mathrm{P}]_{total}-[\mathrm{TNB^-}]\left(1+\frac{[\mathrm{H^+}]}{Q_{TNB}}\right)\right\}\left\{[\mathrm{DTNB}]_{total}-[\mathrm{TNB^-}]\left(1+\frac{[\mathrm{H^+}]}{Q_{TNB}}\right)\right\}}. \quad (2)$$

A full derivation of Eq. (2) has been reported elsewhere [13].

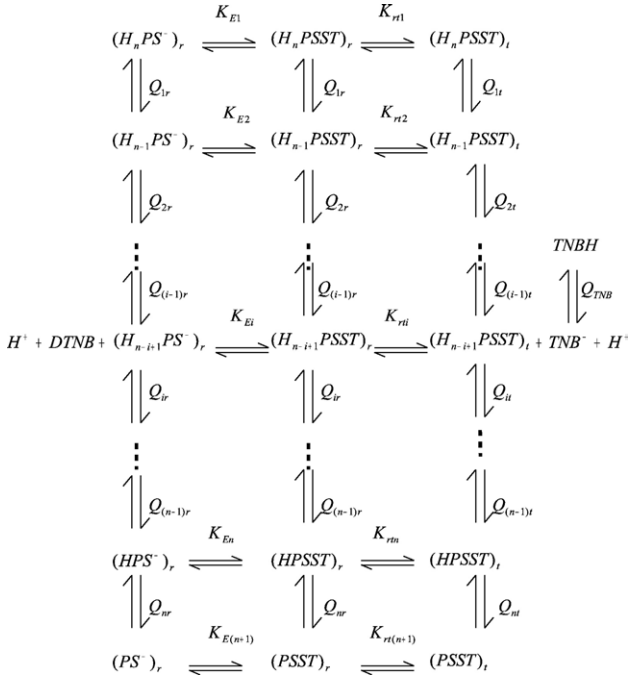
In calculating $K_{equ}$ for the quail hemoglobins, cognizance was taken of the fact that, at the same hemoglobin concentration, the concentration of the DTNB-reactive sulfhydryl groups in these hemoglobins is twice that of the mammalian hemoglobins, which have only two DTNB-reactive sulfhydryl groups per tetramer. Previous analyses of kinetic data on chicken and pigeon hemoglobins show that $pQ_{SH}$ is the same for CysF9[93]β and CysB5[23]β [16,17]. We therefore assumed this in the present analyses. In calculating $K_{equ}$, an absorption coefficient of 14,000 $mol^{-1}$ $dm^3$ $cm^{-1}$ was assumed for $TNB^-$ at 412 nm.

In Fig. 4 we report the variation of $-\log_{10}K_{equ}$ in the pH range 5.6 to 9 for three derivatives of the major hemoglobin of the Japanese quail. It is seen that $K_{equ}$ varies by about 400-fold over this pH range. Similar results are reported for the minor hemoglobin in Fig. 5. The fairly strong pH dependences seen in Figs. 4 and 5 imply that the DTNB reaction (Eq. (1)) is coupled to the ionizations of groups on the hemoglobin molecule. To enable us to determine the nature and the number of such groups, we previously analyzed curves like those in Figs. 4 and 5 on the basis of Scheme 1 [11]. In this scheme the protons arising from the various ionization steps have been omitted for clarity. The species $H_{n-i+1}PSH$ ($i=1, 2,\ldots, n$) are hemoglobin species in which the sulfhydryl groups are in their protonated forms, which do not react with DTNB. These species are therefore not shown in Scheme 1. $H_{n-i+1}PS^-$ ($i=1, 2,\ldots, n$) are species in which the sulfhydryls are in their thiolate anion form, the form that reacts with DTNB [20,22,23]; $H_{n-i+1}PS.ST$ ($i=1, 2,\ldots, n$) are the mixed disulfide species formed after the reaction of the sulfhydryls with DTNB. Species marked with subscripts r and t are those in which the sulfhydryls are in the **r** and **t** tertiary isomeric forms of hemoglobin, respectively. The various proton ionization constants are represented as $Q_{ir}$ and $Q_{it}$ ($i=1, 2,\ldots, n$) to differentiate them from the equilibrium constants $K_{Ei}$ ($i=1, 2,\ldots, n+1$) for the reaction of DTNB; and $K_{rt(n+1)}$ is the equilibrium constant at high pH for the **r**⟷**t** isomerization. The relationship between $K_{equ}$ and the parameters of Scheme 1 is given by the equation:

$$K_{equ}=\frac{K_{E(n+1)}\left\{1+\sum_{i=1}^{n}[H^+]^{n-i+1}\left(\prod_{j=i}^{n}Q_{jr}\right)^{-1}+K_{rt(n+1)}\left(1+\sum_{i=1}^{n}[H^+]^{n-i+1}\left(\prod_{j=i}^{n}Q_{jt}\right)^{-1}\right)\right\}}{1+\sum_{i=1}^{n}[H^+]^{n-i+1}\left(\prod_{j=i}^{n}Q_{jr}\right)^{-1}}. \quad (3)$$

If the equilibrium constants for the **r**⟷**t** isomerization steps in Scheme 1 are assumed to be pH-independent, then $K_{rt1}=K_{rt2}=\ldots K_{rti}=\ldots=K_{rtn}=K_{rt(n+1)}=K_{rt}$ [11].

We have employed Eq. (3), with $K_{rti}$ ($i=1, 2,\ldots, n+1$)$=K_{rt}$, to fit the data in Figs. 4 and 5. The best-fits to all the data were obtained with an $n$ value of 2. The lines through the data points in Figs. 4 and 5 are the best-fit lines drawn with the parameters reported in Table 1. It is seen that the fits are quite good. The mean values of the best-fit parameters are also reported in Table 1. It is seen that the mean $pQ_{ir}/pQ_{it}$ ($i=1, 2$) values are similar to the values obtained previously [11] for mammalian hemoglobins. The $K_{E3}$




Scheme 1.

Table 1
Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β and CysB5[23]β sulfhydryl groups of various derivatives of the major and minor hemoglobins of the Japanese quail: Best-fit parameters employed to fit the equilibrium data using Scheme 1 and Eq. (3) of the text (c.f. Figs. 4 and 5)

| | $pQ_{1r}$ | $pQ_{1t}$ | $pQ_{2r}$ | $pQ_{2t}$ | $K_{E3}$ | $K_{rt}$ |
|---|---|---|---|---|---|---|
| *Major hemoglobin* | | | | | | |
| Oxy | 4.80 | 7.62 | 7.94 | 9.45 | 0.0033 | 0.762 |
| Carbonmonoxy | 5.04 | 7.66 | 7.87 | 9.52 | 0.0025 | 0.668 |
| Aquomet | 4.49 | 7.37 | 8.09 | 9.53 | 0.0040 | 0.802 |
| Mean | 4.78±0.2 | 7.55±0.1 | 7.97±0.07 | 9.50±0.02 | 0.0033±0.0005 | 0.744±0.04 |
| *Minor hemoglobin* | | | | | | |
| Oxy | 4.48 | 7.50 | 7.90 | 9.29 | 0.0067 | 0.393 |
| Carbonmonoxy | 3.99 | 7.58 | 7.91 | 9.31 | 0.0050 | 0.388 |
| Aquomet | 4.30 | 7.53 | 7.93 | 9.35 | 0.0056 | 0.422 |
| Mean | 4.26±0.2 | 7.54±0.03 | 7.91±0.02 | 9.32±0.04 | 0.0058±0.001 | 0.401±0.01 |

Conditions as in Fig. 5. Errors quoted are standard errors.

value for the major quail hemoglobin is a factor of 1.76 lower than that of the minor. Furthermore, and more interestingly, the $K_{rt}$ values are significantly different: 0.725±0.03 for the major and 0.401±0.01 for the minor hemoglobin.

## 4. Discussion

### 4.1. Titration of hemoglobins with 5,5′-dithiobis (2-nitrobenzoate): DTNB

An interesting observation with regard to hemoglobins with multiple reactive sulfhydryl groups, including avian hemoglobins, is that the number of sulfhydryl groups reacting with DTNB is always less than the number reacting with organic mercurials [12,16,17,24]. Whereas organic mercurials react with all unmasked sulfhydryl groups, DTNB reacts only with those that can form the thiolate anion [20,22,23].

One of the intriguing problems regarding avian hemoglobin sulfhydryl groups has been to determine which of the sulfhydryl groups react with DTNB [16,17]. By examining the environment of each sulfhydryl group in the 3-D structure of hemoglobin, we were able to arrive at the conclusion that CysF9 [93]β and CysB5[23]β are the only reacting groups [16,17]. This conclusion has now been confirmed experimentally. The guinea fowl hemoglobins are the only avian hemoglobins with a serine instead of a cysteine at the B5[23]β position [14,15,21]. This finding has made it possible to prove experimentally (cf. Figs. 2 and 3) that CysB5[23]β is the second sulfhydryl that reacts with DTNB in avian hemoglobins. It is therefore clear that in avian hemoglobins the DTNB-reactive sulfhydryl groups are CysF9[93]β and CysB5[23]β. The replacement of a cysteine residue by a serine at the H13[130]α position [15] has also contributed to an understanding of the differences in the *p*-CMB and DTNB titration results of the major and minor avian hemoglobins.

### 4.2. Does CysB5[23]β, like CysF9[93]β, exist in two conformations?

The evidence for the **r**⟵⟶**t** isomerization process comes from the 2.1 Å X-ray crystallographic data on human oxyhemoglobin [9]. This shows that CysF9[93]β exists in two conformations. Additional evidence comes from temperature-jump studies on human deoxy- and carbonmonoxyhemoglobin, which demonstrated that the equilibrium between the two conformations is dynamic and that the transition is abolished on modification of CysF9[93]β with iodoacetamide [10]. X-ray data have been reported for two liganded avian hemoglobins: the major hemoglobin of bar-headed goose [25] and the minor hemoglobin of chicken [26]. Although the authors did not lay emphasis on the position of CysF9[93]β, the structures they reported do not differ significantly from that reported for human oxyhemoglobin [9]. Consequently, it is reasonable to assume that CysF9[93]β exists in two conformations in avian hemoglobins.

Our analyses tacitly assume that CysB5[23]β also undergoes a transition between two conformations, and the question arises as to whether this assumption can be justified. In a kinetic study of the reaction of DTNB with CysF9[93]β and CysB5[23]β of the major hemoglobin of the domestic chicken we found that the reactivity of CysB5[23]β is highly sensitive to the nature of the heme ligand [16]: In oxyhemoglobin CysB5[23]β is about two orders of magnitude *less reactive* than CysF9[93]β; in carbonmonoxyhemoglobin and aquomethemoglobin this order is reversed and CysB5[23]β is two orders of magnitude *more reactive* than CysF9[93]β. These results indicate quite strongly that CysB5[23]β does not have a fixed structure but, like CysF9 [93]β [9,10], can alternate between two positions: one in which it reacts faster than CysF9[93]β, the other in which it reacts more slowly.

### 4.3. Values of $K_{rt}$

In contrast to the value $K_{rt}\approx 0.2$ observed for mammalian hemoglobins [11], the $K_{rt}$ values of the two quail hemoglobins investigated here are quite different: 0.744±0.04 for the major and 0.401±0.01 for the minor hemoglobin. The major and minor hemoglobins have identical β chains, but their α chain primary structures differ by 57 substitutions [15]. The DTNB-reactive sulfhydryl groups are both on the β chain of the major

and minor hemoglobins. Yet their mean $K_{rt}$ values differ by a factor of 1.8 (see Table 1). $K_{rt}$, a measure of the **r**←→**t** tertiary structure transition in the β chain, appears to be subject to the influence of the α subunit, possibly through one of the subunit interfaces, $\alpha_1\beta_1$ or $\alpha_1\beta_2$.

It seems clear from this report that although $K_{rt}$ has the same value for the mammalian hemoglobins we have investigated [11], its value can be different for other hemoglobins. It is intriguing that $K_{rt}$ is different for two hemoglobins that originate from the same red blood cell: that of the Japanese quail. In the same way, it is intriguing that chicken hemoglobins from the same red blood cell have different oxygen affinities [27,28]. Whatever the role of the **r**←→**t** transition in hemoglobin may be, differences in $K_{rt}$, as observed here, are bound to affect that role. We are currently carrying out equilibrium experiments on the reaction of DTNB with the major and minor hemoglobins of the domestic chicken, for which it is known that the minor hemoglobin has a higher oxygen affinity than the major [27,28].

## 5. Conclusion

The results presented in this paper are quite gratifying with respect to the experimental evidence we have obtained for the role we had previously assigned to CysB5[23]β on the basis of the 3-D structure of hemoglobin. It is now clear that in avian hemoglobins the sulfhydryls that react with DTNB are CysF9 [93]β and CysB5[23]β. It is also clear that $K_{rt}$ can vary from one hemoglobin to another, even for hemoglobins from the same animal species.

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