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R and R2 quaternary structures of carbonmonoxyhemoglobins: Differential effect of inositol hexakisphosphate on their affinity for Ellman's reagent

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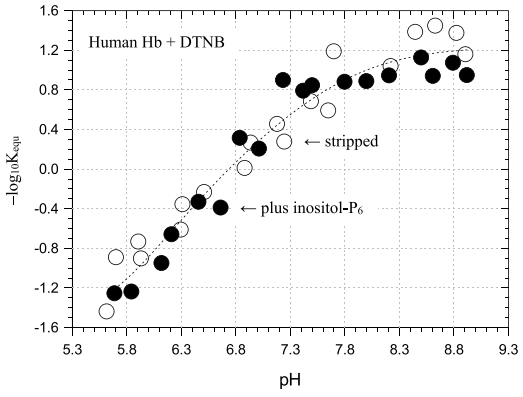
HIGHLIGHTS

- Guinea pig Hb has the R2 quaternary structure and His2β; and inositol-P_6*decreases* its affinity for Ellman's reagent.
- Ungulate and cat Hbs have R quaternary structure, *lack* His2β, and inositol-P_6*increases* their affinity for Ellman's reagent.
- Human and dog Hb have His2β and structures between R2 and R. Inositol-P_6 has no effect on their Ellman's reagent affinities.
- Avian Hbs have His2β and structures between R2 and R. Inositol-P_6 has *no effect* on their Ellman's reagent affinities.
- Chicken major Hb has His2β. Inositol-P_6*decreases* its Ellman's reagent affinity; it has the R2 quaternary structure.

GRAPHICAL ABSTRACT



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ABSTRACT

The reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with hemoglobin sulfhydryl groups is linked to three negatively contributing Bohr effect groups: His2β is present in all avian hemoglobins but absent in some mammalian hemoglobins; His77β and His143β are absent in avian but present in nearly all mammalian hemoglobins. To probe the consequences of these differences, we determined the influence of inositol hexakisphosphate (inositol-P_6) on the DTNB affinities of avian and mammalian *carbonmonoxyhemoglobins*. Inositol-P_6*decreases* by two orders of magnitude the DTNB affinity of guinea pig hemoglobin, which has His2β and the R2 quaternary structure. It decreases, or has no effect on, the DTNB affinities of hemoglobins that have His2β and whose structures lie along the R2 ⇌ R quaternary equilibrium. Finally, inositol-P_6*increases* by one to two orders of magnitude the DTNB affinities of hemoglobins that *lack* His2β. Thus His2β, DTNB and inositol-P_6, in combination, distinguish the R2 from the R quaternary structure.

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1. Introduction

Since the discovery of the R2 quaternary structure of human hemoglobin A by Arnone and co-workers [1], it has been difficult to distinguish functionally between it and the R quaternary structure of the Monod-Wyman-Changeux model [2]. The x-ray crystal structure of guinea pig methemoglobin has been determined and the structure was found to exist entirely in the R2 quaternary state [3]. In the guinea pig R2 state a salt bridge, $NH_3^+ \ldots {}^-OOC$, is formed between the NH_3^+ terminal group of one β-chain and the terminal COO^- group of the partner β-chain in the same molecule [3]. These findings open up the possibility of distinguishing in some way between the R and R2 quaternary structures through a reaction that is non-cooperative and therefore allows for the detection of some distinct property of the R2 state.

The reaction of the Cys93β sulfhydryl group of hemoglobin with various chemical reagents has been employed as an indicator of tertiary and quaternary structure [4–14]. Among all these reagents Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoate) (abbreviated DTNB), is not only stable at room temperature but also reacts reversibly with Cys93β of various hemoglobins [15–18] and, in addition, with Cys23β of avian hemoglobins [19]. Here we report our equilibrium binding studies on the reaction of DTNB with seven avian hemoglobins and twelve mammalian hemoglobins. These hemoglobins may be divided into two groups: (i) those that have histidine at the 2β position [20] and also have the full complement of the basic groups at the organic phosphate binding site [21,22]; and (ii) those in which the 2β position is occupied not by histidine but by methionine, by glutamine or by phenylalanine [20]; in addition, members of this group do not have the full complement of the basic groups at the organic phosphate binding site. The seven avian hemoglobins and four of the twelve mammalian hemoglobins, including guinea pig hemoglobin, belong to the first group. The second group includes six ungulate hemoglobins and the two hemoglobins of the domestic cat. We selected these two groups because they differ at positions in the molecule that are important for hemoglobin function: His2β is responsible for the major part of the so-called additional Bohr effect [23]; the organic phosphate binding groups [21,22] are important for the control of oxygen binding in vivo. Unless otherwise stated, all further references to hemoglobin are to the carbonmonoxy derivative.

We found that at pH 7.3 inositol hexakisphosphate, inositol-P_6, *decreases* by about two orders of magnitude the DTNB affinity of guinea pig hemoglobin, which has (i) the R2 quaternary structure; (ii) the NH_3^+ group at the 1β position and histidine at the 2β position; and (iii) the full complement of the basic groups at the organic phosphate binding site. By contrast, inositol-P_6*increases* the DTNB affinity of the ungulate and cat hemoglobins, which lack histidine at the 2β position and also lack the full complement of the basic groups at the organic phosphate binding site. Furthermore, we found that inositol-P_6 has no effect on, or weakly *decreases*, the DTNB affinity of hemoglobins (i) whose structures lie at various points along the R2 ⇌ R quaternary equilibrium, (ii) which have NH_3^+ at the 1β and histidine at the 2β position, and (iii) which have the full complement of the basic groups at the organic phosphate binding site.

2. Experimental

Blood samples of the various animal species were used to prepare hemolysates as previously detailed [14–19]. For hemolysates containing more than one type of hemoglobin, we separated the different hemoglobins on a column of carboxymethylcellulose (Whatman CMC-52, microgranular, pre-swollen), as previously described [15–19]. We previously reported in detail the method we employed to determine the equilibrium constant for the reaction of DTNB with various animal hemoglobins [14,16,17]. Since the oxygen affinities of the various hemoglobins used in the present study vary widely, we considered it necessary to employ the carbonmonoxy derivatives for determining the DTNB affinities of the hemoglobins used in this study. Carbon monoxide is difficult to dissociate off from carbonmonoxyhemoglobin, whereas in the presence of inositol-P_6 oxygen may dissociate off from some hemoglobins that we employed in this study which may have rather low O_2 affinities. All equilibrium constants were determined at 25 °C in phosphate and borate buffers of ionic strength 50 mmol dm^{-3}.

3. Results

The reaction of DTNB with a hemoglobin sulfhydryl group is given by the equation:

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

In Eq. (1) PSH is hemoglobin with a sulfhydryl group in its protonated form, which does not react with DTNB; PS^- is the anion form of the sulfhydryl that reacts with DTNB; PS.ST is the mixed disulfide formed after the 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 and of TNBH, respectively; K_{equ} is the equilibrium constant for the formation of the mixed disulfide, that is, the DTNB reaction step. The relationship between K_{equ} and the species and terms in Eq. (1) is:

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

In Eq. (2) $[P]_{tot}$ is the total hemoglobin concentration in terms of sulfhydryl groups. All other terms are defined as in Eq. (1). We previously presented a detailed derivation of Eq. (2) [16].

3.1. Effect of inositol-P_6 on the reaction of mammalian hemoglobins with DTNB

In previous reports [24,25] we presented evidence indicating that the reaction of DTNB with human hemoglobin is linked to three Bohr effect groups, each with $pK^{oxy} > pK^{deoxy}$ [26]. These groups are His2β, His77β and His143β. In Table 1 we list all the Bohr effect groups, with their Bohr group positions, as they appear in various mammalian hemoglobins. It is seen in columns 2 to 8 that the following hemoglobins lack His2β: goat (major and minor), horse (major and minor), donkey, and cat (major and minor). Each of these hemoglobins has His143β. However, while each of the other hemoglobins in columns 2–8 has histidine at position 77β, the two cat hemoglobins have asparagine at this position. Also presented in Table 1 (last four rows) are the groups that appear at the organic phosphate binding site [21,22] of the various hemoglobins.

The amino acid sequences of goat and sheep hemoglobin are closely similar, and they have identical groups at the organic phosphate binding site [20]. In previous studies on the reaction of DTNB with the major and minor sheep hemoglobins we found that, compared to stripped hemoglobin, their DTNB affinities are increased by about an order of magnitude in the presence of the allosteric effector inositol hexakisphosphate, inositol-P_6. In view of the close similarity of the goat and sheep hemoglobin sequences, we expected that the effect of inositol-P_6 on the goat hemoglobins would be similar to its effect on the sheep hemoglobins. Fig. 1 shows that this is indeed the case: At pH 7.3, the physiological pH, inositol-P_6 increases the DTNB affinity of the major goat hemoglobin by about two orders of magnitude (Fig. 1a) and that of the minor hemoglobin by about an order of magnitude (Fig. 1b). Similarly, at pH 7.3 inositol-P_6 increases the DTNB affinity of the major horse hemoglobin by about two orders of magnitude (Fig. 1c) and that of the minor hemoglobin by about one order of magnitude (Fig. 1d). At the same pH, the DTNB affinity of donkey hemoglobin is also increased

Table 1
Bohr effect groups and their Bohr group positions in various mammalian hemoglobins.

Bohr group position	Goat major	Goat minor	Horse major	Horse minor	Donkey	Cat major	Cat minor	Dog	Human A	Rabbit	Guinea pig
Negative contributors											
20α	*Asn*	*Asn*	His	His	*Asn*	His	His	His	His	His	His
45α	His	His	His	His	His	His	His	His	His	His	His
112α	His	His	His	His	His	His	His	His	His	His	His
2β	*Met*	*Met*	*Gln*	*Gln*	*Gln*	*Phe*	*Phe*	His	His	His	His
77β	His	His	His	His	His	*Asn*	*Asn*	*Asn*	His	His	His
143β	His	His	His	His	His	His	His	His	His	His	His
Positive contributors											
1α	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+
50α	His	His	His	His	His	His	His	*Pro*	His	His	*Pro*
72α	His	His	His	His	His	His	His	His	His	His	His
89α	His	His	His	His	His	*Tyr*	*Tyr*	*Tyr*	His	His	His
97β	His	His	His	His	His	His	His	His	His	His	His
116β	*Arg*	*Arg*	*Arg*	*Arg*	*Arg*	His	His	His	His	His	His
117β	His	His	His	His	His	His	His	His	His	His	His
146β	His	His	His	His	His	His	His	His	His	His	His
Inositol-P_6 binding groups											
1β	_	_	NH_3^+	NH_3^+	NH_3^+	NH_3^+	*Acetylated Ser*	NH_3^+	NH_3^+	NH_3^+	NH_3^+
2β	*Met*	*Met*	*Gln*	*Gln*	*Gln*	*Phe*	*Phe*	His	His	His	His
82β	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
143β	His	His	His	His	His	His	His	His	His	His	His

by a little less than one order of magnitude in the presence of inositol-P_6 (Fig. 1e). Each of these five ungulate hemoglobins (Table 1; columns 2–6) lacks a histidine at the 2β position. Furthermore, the goat hemoglobins, like the sheep hemoglobins, have a deletion at the 1β position [20].

In Fig. 2 we report the effect of inositol-P_6 on the DTNB affinities of two hemoglobins from a carnivore: the major and minor hemoglobins of the domestic cat. At pH 7.3 inositol-P_6 increases the DTNB affinity of the major hemoglobin by about two orders of magnitude (Fig. 2a) and that of the minor hemoglobin by about three orders of magnitude (Fig. 2b). Each of these two hemoglobins lacks a histidine at the 2β position and, in addition, lacks a histidine at the 77β position. Instead they have phenylalanine and asparagine at these respective positions (see Table 1; columns 7 and 8). We suggest that the larger effect produced by inositol-P_6 on the DTNB affinities of the cat hemoglobins, compared to the affinities of the hemoglobins in columns 2–6 of Table 1, may not be unconnected with the replacement of histidine by asparagine at the 77β position. Furthermore, the order of magnitude greater effect produced in the minor cat hemoglobin (compare Fig. 2b with Fig. 2a) is likely because the residue at the beginning of its β-chain is uncharged, since it is an acetylated serine residue. We recall that this acetylation led to the conversion of the complex pH dependence profile of the second-order forward rate constant of the major cat hemoglobin to a simple form in the minor hemoglobin (compare Figs. 2 and 3 of [15]).

Unlike the hemoglobins listed in columns 2–8 of Table 1 that lack a histidine at the 2β position, those in columns 9–12 (dog, human, rabbit and guinea pig) have a histidine at this position. In the last four rows of Table 1 we compare the residues present at the organic phosphate binding site of dog, human, rabbit and guinea pig hemoglobin with those of the other hemoglobins listed in Table 1. It is seen that dog, human, rabbit and guinea pig hemoglobin have the full site complement of positively charged residues at the organic phosphate binding site: the β-chain terminal NH_3^+ group, His2β, Lys82β and His143β. By contrast, each of the hemoglobins in columns 2–8 lacks either one or two of these positively charged residues. We therefore expect the negatively charged inositol-P_6 to bind with greater affinity to those hemoglobins that have the full site complement (dog, human A, rabbit and guinea pig) than to those that lack the full site complement (the ungulates and cat). Consequently, if all the hemoglobins in Table 1 had the same quaternary structure, we would expect inositol-P_6 to produce a greater increase in the DTNB affinities of dog, human A, rabbit and guinea pig hemoglobin compared to those seen in Figs. 1 and 2 for the hemoglobins listed in columns 2–8 of Table 1. This expectation is based purely on electrostatic grounds, and it is justified by the fact that inositol-P_6 has a greater effect in decreasing the oxygen affinity of human A compared to horse hemoglobin (see Fig. 1 of Giardina et al. [27]).

In Fig. 3 we report the effect of the addition of inositol-P_6 on the DTNB affinities of dog, human A, rabbit and guinea pig hemoglobin. It is seen that the organic phosphate produces no change in the DTNB affinities of dog and human hemoglobin (Fig. 3a and b). Moreover, inositol-P_6*decreases* the DTNB affinity of rabbit hemoglobin (Fig. 3c) and, even more poignantly, that of guinea pig hemoglobin (Fig. 3d). These results are in sharp contrast to the effect of inositol-P_6 on the hemoglobins that lack histidine at the 2β position and also lack the full complement of the basic groups at the organic phosphate binding site (Figs. 1 and 2). Considering the fact that dog, human, rabbit and guinea pig hemoglobin must bind inositol-P_6 more tightly than these other hemoglobins (see last 4 rows in Table 1), the results seen in Fig. 3 are indeed the reverse of what we had good reason to expect, going by the results seen in Figs. 1 and 2 of this paper and in Fig. 1 of [27] in which the effects of 2,3-BPG and inositol-P_6 on the oxygen affinities of human and horse hemoglobin are compared. The results in Figs. 1 and 2 of this paper suggest, at least tentatively, that the presence of histidine at the 2β position gives rise to the *neutralization* (Fig. 3a and b) or even the *reversal* (Fig. 3c and d) of the organic phosphate effect seen in Figs. 1 and 2. The results also suggest that the hemoglobins in columns 9–12 of Table 1 do not have the same quaternary structure as those in columns 2–8.

3.2. Effect of inositol-P_6 on the reaction of avian hemoglobins with DTNB

So far, we have considered only mammalian hemoglobins. In Table 2 we show the amino acid residues present at various Bohr group positions in seven avian hemoglobins: turkey (major and minor), pigeon, quail (major), guinea fowl (major) and chicken (major and minor). For reference purposes only, we have also included in Table 2 similar data for human hemoglobin A. The avian hemoglobins, like all avian hemoglobins, are seen to possess histidine at the 2β position. Furthermore, in each case a more positively charged arginine residue replaces the histidine found at position 143β in human hemoglobin. However, position 77β is occupied in each case by asparagine, which replaces the histidine found in that position in human hemoglobin. In the last four rows of Table 2 we show the residues present at the organic

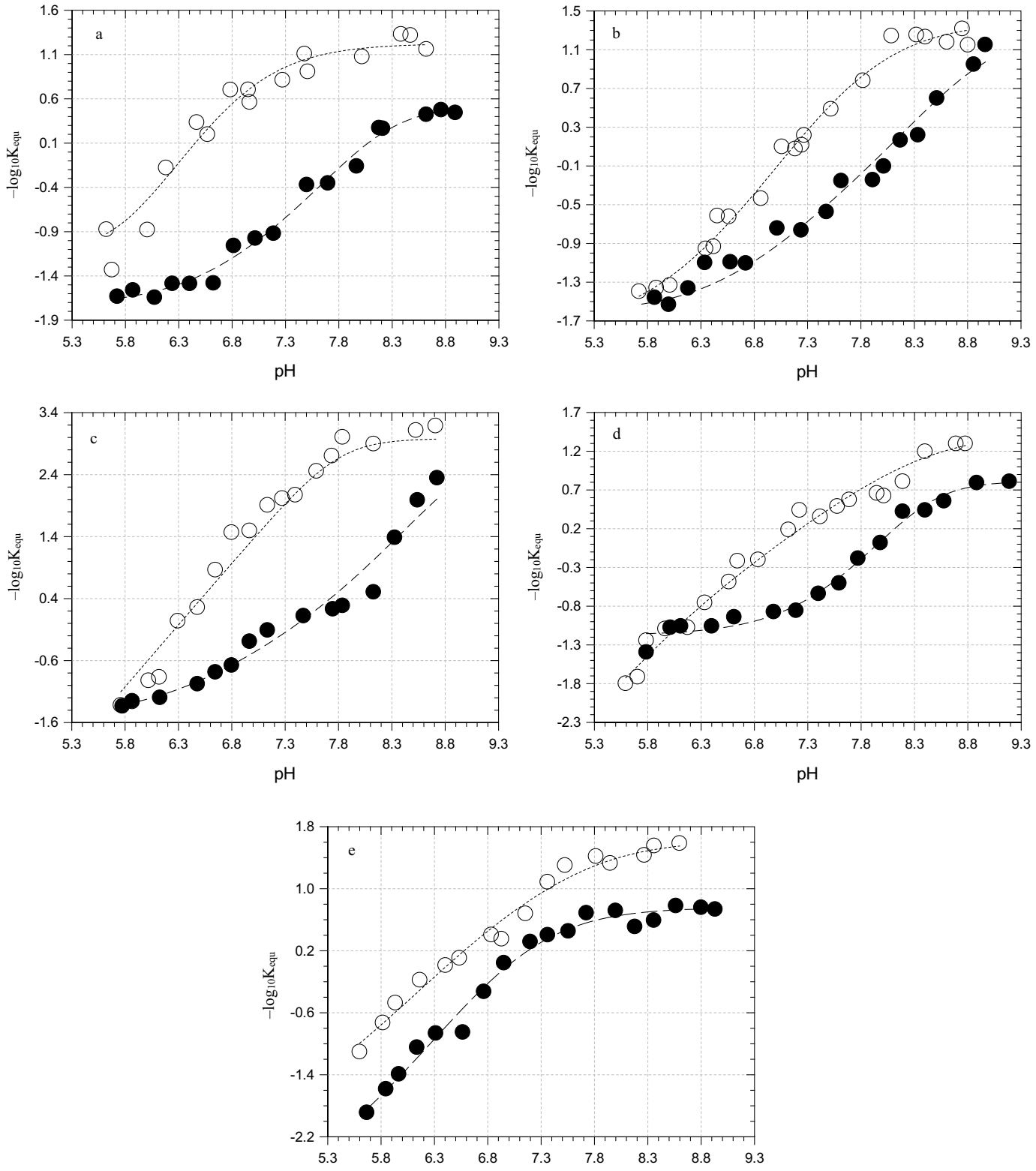

Fig. 1. Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of ungulate carbonmonoxyhemoglobins with 5,5′-dithiobis(2-nitrobenzoate), DTNB: Effect of inositol-P_6 on the affinity. (a) goat major; (b) goat minor; (c) horse major; (d) horse minor; (e) donkey. Open symbols, stripped hemoglobin; filled symbols, hemoglobin plus inositol-P_6; [Hb_4]: [inositol-P_6] = 1:4. Conditions: phosphate buffers, pH 5.6–8.0; borate buffers, pH 8.0–9.2; ionic strength, 50 mmol dm^{-3} (added salt, NaCl); temperature, 25 °C; hemoglobin concentration, 12.5 μmol (tetramer) dm^{-3}. Each data point is subject to a standard error of *ca* ± 0.1 in the log.

phosphate binding site [21,22] of the avian hemoglobins. The full complement of residues is present: the terminal NH_3^+ group of the β-chain, His2β, Lys82β and Arg143β. In addition to these, two further residues face the cavity between the β-chains: Arg135β and His139β. (Arg135β and His139β respectively substitute the alanine and asparagine that appear at these positions in human hemoglobin.) Since the positive charge at the organic phosphate binding site is higher in avian compared to human hemoglobin, it is clear that organic phosphates

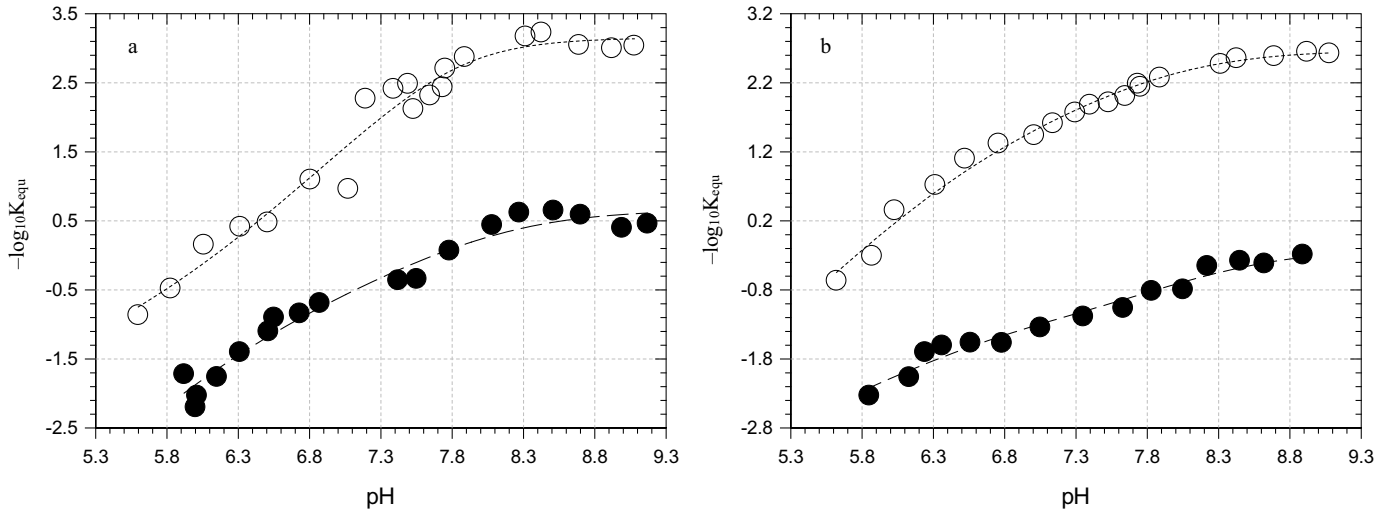

Fig. 2. Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of cat carbonmonoxyhemoglobins with 5,5′-dithiobis(2-nitrobenzoate), DTNB: Effect of inositol-P_6 on the affinity. (a) cat major; (b) cat minor. Conditions as in Fig. 1. Open circles, stripped hemoglobin; filled circles, hemoglobin + inositol-P_6.

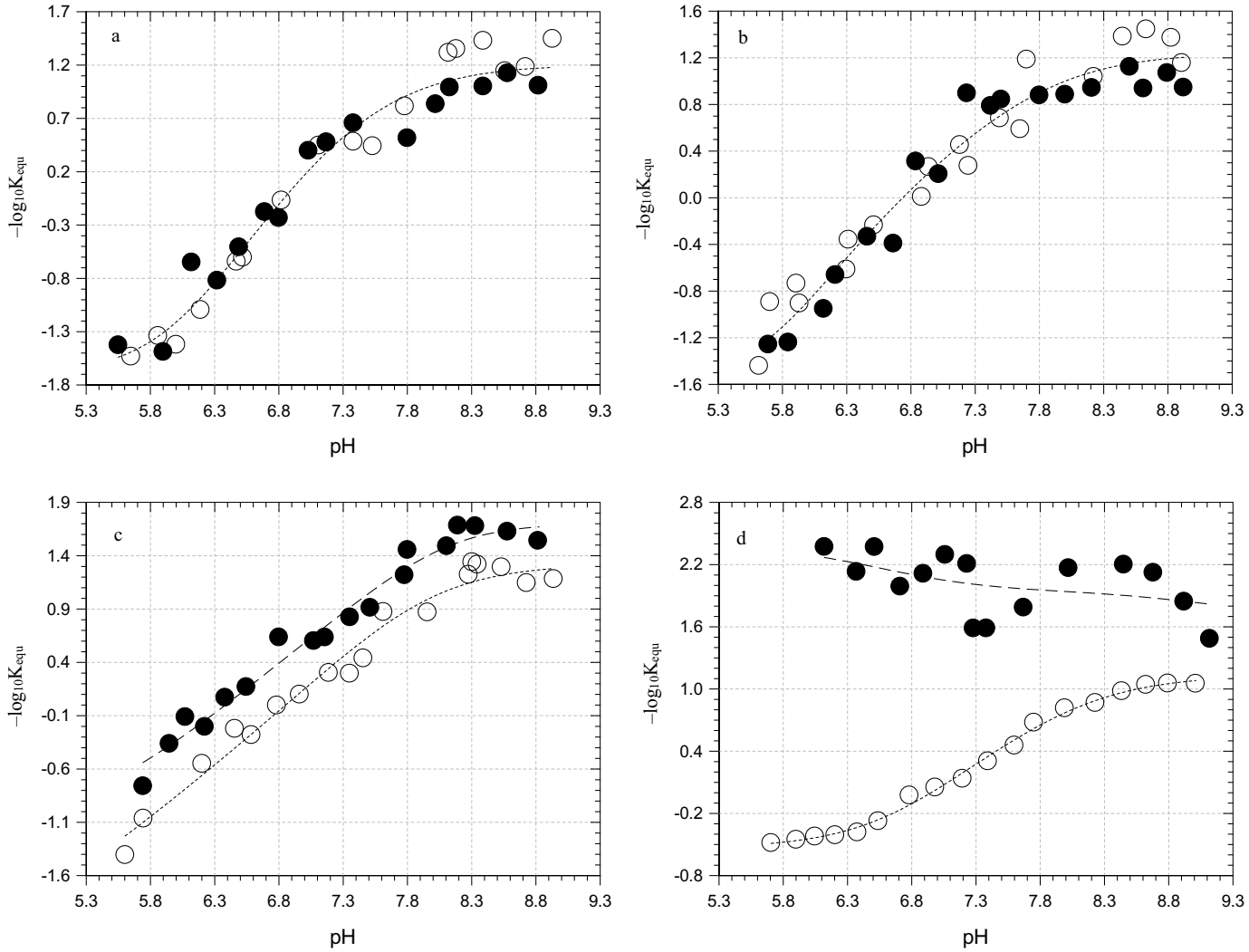

Fig. 3. Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of mammalian carbonmonoxyhemoglobins with 5,5′-dithiobis(2-nitrobenzoate), DTNB: Effect of inositol-P_6 on the affinity. (a) dog; (b) human A; (c) rabbit; (d) guinea pig. Conditions as in Fig. 1. Open circles, stripped hemoglobin; filled circles, hemoglobin + inositol-P_6.

Table 2
Bohr effect groups and their Bohr group positions in various avian and human hemoglobins.

Bohr group position	Turkey major	Turkey minor	Pigeon	Quail major	Guinea fowl major	Chicken major	Chicken minor	Human A
Negative contributors								
20α	His	His	*Gln*	His	His	His	His	His
45α	His	His	His	His	His	His	His	His
112α	His	His	His	His	His	His	His	His
2β	His	His	His	His	His	His	His	His
77β	*Asn*	*Asn*	*Asn*	*Asn*	*Asn*	*Asn*	*Asn*	His
143β	*Arg*	*Arg*	*Arg*	*Arg*	*Arg*	*Aarg*	*Arg*	His
Positive contributors								
Val1α	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	Met1 α NH_3^+	NH_3^+
50α	His	*Pro*	*Leu*	His	His	His	*Pro*	His
72α	His	*Asn*	His	His	His	His	*Asn*	His
89α	His	*Tyr*	*Gln*	*Gln*	His	His	*Tyr*	His
97β	His	His	His	His	His	His	His	His
116β	*Ala*	*Ala*	*Ala*	*Ala*	*Ala*	*Ala*	*Ala*	His
117β	His	His	His	His	His	His	His	His
146β	His	His	His	His	His	His	His	His
Organic phosphate binding groups								
1β	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+	NH_3^+
2β	His	His	His	His	His	His	His	His
82β	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
143β	*Arg*	*Arg*	*Arg*	*Arg*	*Arg*	*Arg*	*Arg*	His

must bind to avian hemoglobins with much higher affinities than to human hemoglobin. For example, at pH 6.8 human oxy- and deoxyhemoglobin bind 2,3-bisphosphoglycerate with association constants of $1.2 \times 10^3\,M^{-1}$ and $1.7 \times 10^4\,M^{-1}$, respectively [28], whereas *chicken* carbonmonoxyhemoglobin binds it with an association constant of $1 \times 10^4\,M^{-1}$ at pH 7 [29], nearly equal to the binding constant for human deoxyhemoglobin. If the avian hemoglobins had the same quaternary structure as the ungulate and cat hemoglobins, one would reasonably expect that inositol-P_6 will produce in avian hemoglobins a much greater increase in DTNB affinities compared to the increases seen in Fig. 1a to e for the hemoglobins of the ungulates and in Fig. 2 for those of the cat.

In Fig. 4 we show the effect of the addition of inositol-P_6 on the DTNB affinities of the major (Fig. 4a) and minor (Fig. 4b) turkey hemoglobins. Surprisingly, the organic phosphate produces absolutely no effect on these affinities. A similar result is seen in Fig. 4c for pigeon hemoglobin, in Fig. 4d for the major hemoglobin of the Japanese quail and in Fig. 4e for the major hemoglobin of the guinea fowl. These results are similar to those seen for the mammalian dog and human hemoglobins (Fig. 3a and b). Unlike these two mammalian hemoglobins, each of the four avian hemoglobins in columns 2 to 5 of Table 2 has DTNB-reactive sulfhydryl groups at two locations: 93β and 23β. It might be argued that this weakens a comparison of the inositol-P_6 effect on their DTNB affinities with those of the mammalian hemoglobins, which have only Cys93β. For this reason, we have included data for guinea fowl (major) hemoglobin, which also has only Cys93β [19]. It is seen that inositol-P_6 also has no effect on the DTNB affinity of this avian hemoglobin (Fig. 4e).

Even more stunning than the data reported so far for the avian hemoglobins are the results seen in Fig. 5 for the chicken hemoglobins: Rather than increase their DTNB affinities, inositol-P_6 actually *decreases* those affinities. At pH 7.3 inositol-P_6*decreases* the DTNB affinity of the major chicken hemoglobin by two orders of magnitude (Fig. 5a) and that of the minor hemoglobin by about half of an order of magnitude (Fig. 5b). The results in Fig. 5a are not merely the opposite of what we expected, going by the results shown in Figs. 1 and 2 for the ungulate and cat hemoglobins; they are even more stunning than what we saw in the case of the other avian hemoglobins (Fig. 4) and for dog, human and rabbit hemoglobin (Fig. 3a, b and c). However, the chicken (major) results (Fig. 5a) are similar to the results reported in Fig. 3d for guinea pig hemoglobin.

3.3. *Is an inter β-chain* NH_3^+ .....$^-$OOC *salt bridge formed in the major chicken hemoglobin?*

The effects of inositol-P_6 on the DTNB affinity of guinea pig hemoglobin and on that of the major chicken hemoglobin are similar in direction and magnitude (compare Fig. 3d with Fig. 5a). While it is definitely known that guinea pig hemoglobin exists in the R2 quaternary structure and has a salt bridge between the NH_3^+ terminal group of one β-chain and the terminalCOO^- group of the partner β-chain in the same molecule [3], the exact nature of the quaternary structure of the major chicken hemoglobin is not known. In view of the similar effect of inositol-P_6 on the DTNB affinities of guinea pig and chicken (major) hemoglobin, it is pertinent to ask whether the latter exists in the R2 quaternary state and also whether it has a NH_3^+.....$^-$OOC salt bridge. This question is pertinent since, to the best of our knowledge, the x-ray structure of the major chicken hemoglobin has not been determined, although that of the minor hemoglobin has been determined [30].

Liang et al. [31] determined the x-ray structure of bar-headed goose hemoglobin in the deoxy form. They concluded that the Asp94/His146β salt bridge is not formed in the T-state of this hemoglobin. However, one could reasonably argue that if such a very high O_2 affinity hemoglobin has an unstable T-state it might well crystallize in the R-deoxy form. In that case, no Asp94/His146β salt bridge would be formed. In the solution state, the differentiation between the two crystal states will no longer be a point to be debated. Fortunately, Rollema and Bauer [32] have determined the Bohr effect of stripped bar-headed and graylag goose hemoglobins (see Fig. 4 of [32]). Our theoretical analysis of their data showed that at pH 7.3 the Bohr effect of each of these hemoglobins is about 60% that of human hemoglobin A (see Fig. 4a of [33]). Since His146β contributes about 40% of the Bohr effect of human hemoglobin, this result shows clearly that His146β does not contribute to the Bohr effect of the two goose hemoglobins. Consequently, the conclusion of Liang et al. [31] is correct, namely, that the Asp94/His146β salt bridge is not formed in the T-state of the goose hemoglobins.

In our previous report [33] we observed that it was difficult to determine whether His146β of chicken hemoglobin makes any contribution to the Bohr effect. This difficulty arose on two grounds: (i) Unlike the Bohr effect of the goose hemoglobins, the Bohr effect of chicken hemoglobin is almost exactly of the same magnitude as that of human hemoglobin (see Fig. 4b of [33]). (ii) When we assumed that

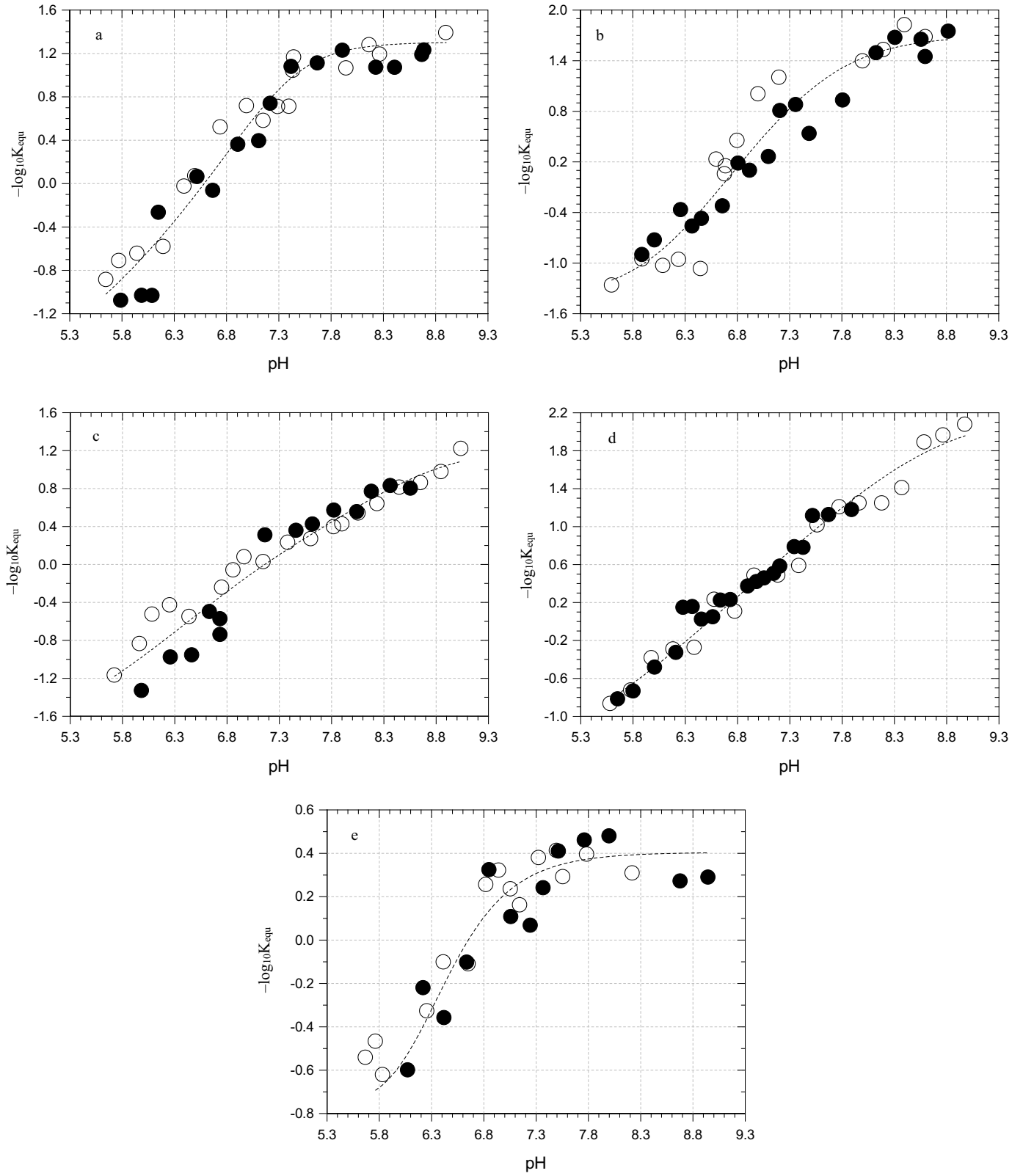

Fig. 4. Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of avian carbonmonoxyhemoglobins with 5,5′-dithiobis(2-nitrobenzoate), DTNB: Effect of inositol-P_6 on the affinity. (a) turkey major; (b) turkey minor; (c) pigeon; (d) quail major; (e) guinea fowl major. Conditions as in Fig. 1. Open circles, stripped hemoglobin; filled circles, hemoglobin + inositol-P_6.

His146β made a contribution to the chicken Bohr effect, this required that the pK_a of the NH_3^+ terminal group of Val1α be higher in oxyhemoglobin than in deoxyhemoglobin (see columns 4 and 5, Table 4 of [33]). This is contrary to experimental observation [34,35].

The close similarity between the effect of inositol-P_6 on the DTNB affinity of guinea pig (Fig. 3d) and chicken (Fig. 5a) hemoglobin suggested to us that an inter β-chain salt bridge most probably exists in chicken hemoglobin, as it does in guinea pig hemoglobin [3]. To check

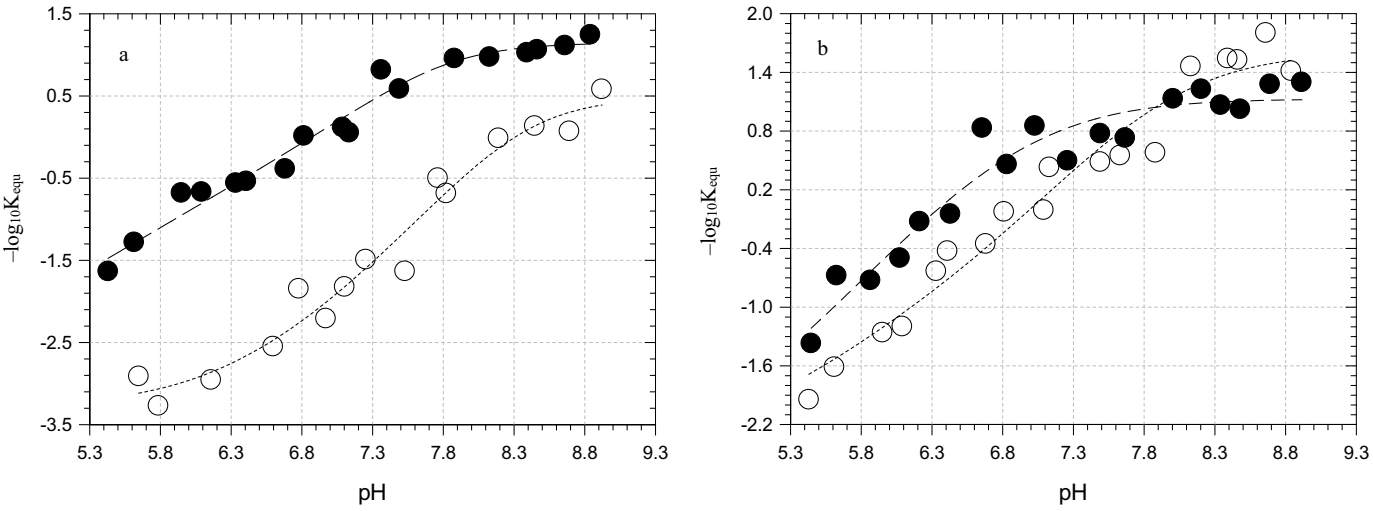

Fig. 5. Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of chicken carbonmonoxyhemoglobins with 5,5′-dithiobis(2-nitrobenzoate), DTNB: Effect of inositol-P_6 on the affinity. (a) Chicken major; (b) chicken minor. Conditions as in Fig. 1. Open circles, stripped hemoglobin; filled circles, hemoglobin + inositol-P_6.

this, we re-fitted the chicken Bohr data [29], assuming that such a salt bridge indeed exists. We employed Eq. (3) and the method we used previously [33] for fitting the chicken data.

$$\Delta h^{+}_{total} = \frac{1}{2}\sum_{j=1}^{n}\left\{\frac{10^{-pK_j^R}}{10^{-pH}+10^{-pK_j^R}}\cdot\frac{10^{-pK_j^T}}{10^{-pH}+10^{-pK_j^T}}\right\} \quad (3)$$

As initial parameter estimates for pK^R (i.e., pK^{oxy}) and pK^T (i.e., pK^{deoxy}) of the NH_3^+ terminal group of Val1α, we employed the values 6.482 and 7.758 (see columns 6 and 7, Table 4 of [33]). As initial parameters for pK^R and pK^T of the NH_3^+ terminal group of Val1β, which forms the inter β-chain salt bridge with the terminal COO^- group of the partner β-chain in the same molecule, we used the values 6.327 and 4.590 calculated for guinea pig hemoglobin (see column 6 and 7 of Table 4 of [36]). Keeping all other pK parameters in Table 4 (columns 4 and 5) of [33] constant, we allowed only those of the terminal NH_3^+ group of Val1α and the NH_3^+ terminal group of Val1β to vary. Following the fit, we activated the statistics subprogram of MicroMath Scientist to obtain the standard deviations in the pK_as obtained for the NH_3^+ terminal groups of Val1α and Val1β. Fig. 6 shows the very good fit to the chicken data. The best-fit parameters are reported in Table 3. It is seen in Table 3 that the values of pK^{oxy} and pK^{deoxy} for the NH_3^+ terminal group of Val1β are 8.170 ± 0.50 and 6.525 ± 0.72. This makes it a negatively contributing Bohr group ($pK^{oxy} > pK^{deoxy}$), just like that of guinea pig hemoglobin [36]. Furthermore, $pK^{oxy} < pK^{deoxy}$ for the NH_3^+ terminal group of Val1α, in line with the experimentally determined values [34,35]. It is very likely that when the x-ray structure of the major chicken hemoglobin is finally determined it will be found that the molecule contains the inter β-chain NH_3^+.....$^-$OOC salt bridge.

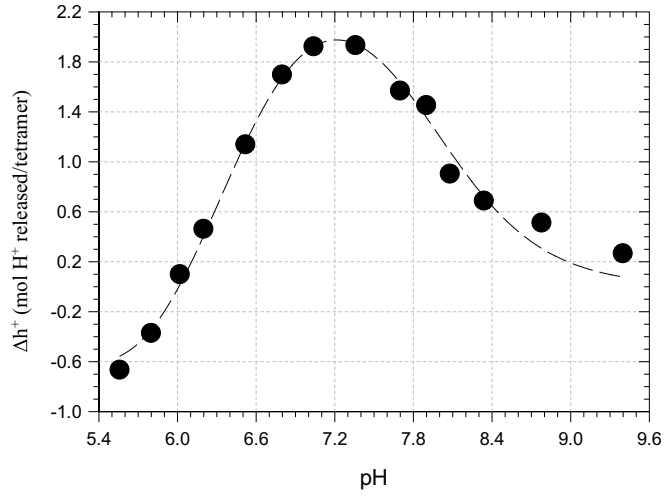

Fig. 6. Bohr effect of chicken (major) hemoglobin (data of Brygier et al. [28]). The curve through the data points was calculated with Eq. (3) of the text, using the parameters listed in Table 3.

Table 3
Chicken (major) hemoglobin (compare with Fig. 6): Parameters employed to fit the Bohr data of Brygier et al. [27] with Eq. (3) of the text, assuming that a salt bridge, NH_3^+.....$^-$OOC, is formed between the NH_3^+ terminal group of Val1 of one β-chain and the COO^- terminal group of His146 of the partner β-chain in the same molecule. The pK values are from Table 1 of [25]. All parameters were fixed during the fitting procedure, except those written in **bold**, *italicized* lettering. Errors quoted are standard deviations. R^2, the square of the correlation coefficient of the fit, was 0.992.

Chicken hemoglobin Bohr groups	pK^{oxy}	pK^{deoxy}
Negative contributors		
Terminal NH_3^+ group of β-chain	***8.170 ± 0.50***	***6.525 ± 0.72***
His20α	7.08	7.02
His45α	6.12	5.25
His112α	7.53	7.49
His2β	6.39	6.17
His77β		
His143β		
Positive contributors		
Terminal NH_3^+ group of α-chain	***6.614 ± 0.75***	***8.017 ± 0.51***
His50α	6.90	7.14
His72α	7.27	7.47
His89α	6.25	6.80
His97β	7.75	8.01
His116β		
His117β	6.39	6.43
His146β	6.42	7.93

A comparison of Fig. 5a and b shows that inositol-P_6 had a much greater effect on the DTNB affinity of the major chicken hemoglobin (Fig. 5a) compared to the effect it had on the minor hemoglobin. This result is in line with the fact that no NH_3^+.....$^-$OOC salt bridge was detected in the x-ray structure of the minor hemoglobin [30]. Most probably, the quaternary structure of the liganded minor hemoglobin lies at an intermediate position along the R2 ⇌ R quaternary equilibrium. Since the two hemoglobins have identical β-chains, the basis of the difference in the results seen in Fig. 5a and b must be sought in the

α-chains. According to Takei et al. [37], there are 65 amino acid differences between the two α-chains. Probably the most important are the following α-chain amino acid substitutions on going from the major to the minor chicken hemoglobin (see Fig. 1 of [37]): Val1 → Met1; Glu30 → Thr30; His50 → Pro50; His72 → Asn72; and His89 → Tyr89. With the exception of the 30α position, the other positions are Bohr group positions (see Table 1 of [26]).

3.4. Structural implications of the effect of inositol-P_6 on the affinities for DTNB

How is the R2 ⇌ R quaternary equilibrium related to the DTNB affinity of hemoglobin? According to Silva et al. [1], "Our crystals of human carbonmonoxyhemoglobin were grown at pH 5.8, and the R-to-R2 transition results in a decrease in the distance between the β-carbons of Cys-93β_1 and Cys-93β_2 of 2.9Å, if the hHB.02.HS structure is taken as the R-state, or 3.8 Å, if the eHB.MT.HS structure is taken as the R-state." This statement clearly indicates that the quaternary R2 ⇌ R transition affects the positions of the two Cys93β sulfhydryls in the hemoglobin molecule and therefore their affinity towards DTNB.

The results obtained on the effect of inositol-P_6 on the DTNB affinities of various hemoglobins appear to contradict one another: (i) The affinities of the ungulate and cat hemoglobins are increased (Figs. 1 and 2); (ii) those of rabbit, guinea pig, and chicken hemoglobin are decreased (Figs. 3c, d and 5); and (iii) those of dog, human and the avian hemoglobins appear to be unchanged (Figs. 3a, b and 4). It is obvious that these three sets of hemoglobin cannot have the same quaternary structure. We now discuss the various possible structures of the three sets of hemoglobin, bearing in mind that x-ray data show that guinea pig hemoglobin has the R2 quaternary structure [3].

(i) Bovine hemoglobin is an ungulate hemoglobin, the crystal structure of which has been determined by Mueser et al. [38]. According to these authors, "The quaternary structure of bovine carbonmonoxyhemoglobin varies considerably in the three crystal forms described above. The low-salt/low-pH crystal form, bHb,CO,PEG5.0, does not have the quaternary R2 structure observed for human hemoglobin crystallized under very similar conditions ……" Since bovine hemoglobin is an ungulate hemoglobin, this conclusion strongly suggests that the ungulate hemoglobins that we studied (Fig. 1) do not have the R2 quaternary structure. Instead, they exist in the R quaternary structure. Therefore, the *increase* in their DTNB affinities arising from the binding of inositol-P_6 (Fig. 1) must be the consequence of a change in tertiary structure of R-state hemoglobin caused by the organic phosphate. By inference, the cat hemoglobins (Fig. 2) must also have the R quaternary structure. In Fig. 7 we present the effect of inositol-P_6 on the DTNB affinity of bovine oxyhemoglobin. As would be expected for hemoglobin in the R-state, the DTNB affinity is increased in the presence of inositol-P_6 (compare Fig. 7 with Figs. 1 and 2).

It may be wondered why inositol-P_6 should increase the DTNB affinities of the ungulate hemoglobins when it is known that organic phosphates have little or no effect on their O_2 affinities. Fronticelli et al. [39] have studied the effect of Cl^- ion on the O_2 affinities of bovine and human hemoglobin. In Fig. 2 of [39] they show that Cl^- ion causes a higher pH sensitivity of the O_2 affinity of bovine compared to human hemoglobin. This means that under the same experimental conditions, and at the same Cl^- ion concentration, the O_2 affinity of bovine hemoglobin is lower than that of human hemoglobin. In another report by the same group [40], the authors show (Fig. 2) that in the absence of Cl^- ion the O_2 affinities of bovine and human hemoglobin are equal and are equally sensitive to 2,3-BPG. So, in the absence of Cl^- ion bovine hemoglobin binds 2,3-BPG as strongly as human hemoglobin does. In the determination of O_2 affinities under most experimental conditions, the Cl^- ion concentration

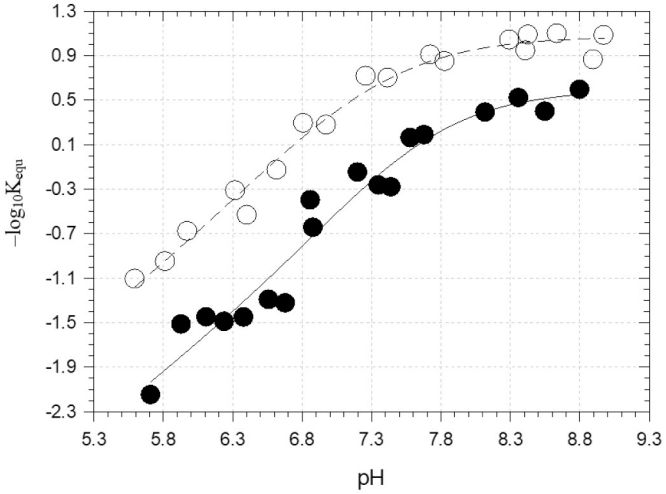

Fig. 7. Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of bovine oxyhemoglobin with 5,5′-dithiobis(2-nitrobenzoate), DTNB: Effect of inositol-P_6 on the affinity. Conditions as in Fig. 1. Open circles, stripped hemoglobin; filled circles, hemoglobin + inositol-P_6.

used in the presence of organic phosphates usually is in the physiological range (*ca* 150 mmol dm^{-3}). Under these conditions, Cl^- ion has so reduced the O_2 affinity of ungulate hemoglobins (see Fig. 2 of [39]) that organic phosphates appear to have no effect. In our DTNB experiments, however, the concentration of Cl^- ion was less than 50 mmol dm^{-3}. So, inositol-P_6 could effectively bind to the ungulate hemoglobins at this low Cl^- ion concentration and cause a reduction in O_2 affinity. There is therefore no doubt that inositol-P_6, which binds hemoglobin with a higher affinity than 2,3-BPG, will bind to the ungulate hemoglobins under our experimental conditions and thus cause them, as R-state hemoglobins, to have a higher affinity for DTNB compared to stripped hemoglobin.

(ii) Guinea pig hemoglobin belongs to the second group. The x-ray study of guinea pig hemoglobin shows that it has the R2 quaternary structure [3]. According to Silva et al. [1], "The magnitudes of the spatial differences between the R- and R2-states are as large as those between the R- and T-states. Of particular interest are the structural changes that occur as a result of R-T and R-R2 transitions at the so-called "switch" region of the critical $\alpha_1\beta_2$ interface. In the R-state, His-97β_2 is positioned between Thr-38α_1 and Thr41α_1 …. In the R2-state, His-97β_2 simply rotates away from threonines 38α_1 and 41α_1, breaking contact with these residues and allowing water access to the center of the $\alpha_1\beta_2$ interface. With the switch region in an open position in the R2-state, His-97β_2 should be able to move by Thr41α_1 and make the transition to the T-state with a steric barrier that is less than that for the R-T transition. Thus, the R2-state may function as a stable intermediate along a R-R2-T pathway." These statements clearly show that the R and R2 states have different structures. It therefore comes as no surprise that inositol-P_6 produces a *decrease* in the DTNB affinity of guinea pig hemoglobin (Fig. 3d), in contrast to the affinity increases it produces in the ungulate and cat hemoglobins (Figs. 1 and 2), which have the R quaternary structure.

The question that now arises is whether guinea pig hemoglobin can be transformed to the R state, that is, "Does the guinea pig R2 state exist in equilibrium with the R state?" To answer this question, we compare the conditions under which the guinea pig R2 crystals were prepared with the conditions under which the human R2 crystals were prepared. According to the generally accepted view, as outlined by Tame [41], the R2 structure is favored by low salt concentrations

during the formation of the crystal employed for x-ray work. The guinea pig structure [3], by contrast, was determined with crystals prepared under high salt concentration (2.6 mol dm^{-3} $(NH_4)_2SO_4$, 100 mmol dm^{-3} sodium phosphate buffer, pH 6.5). In spite of this "R2-unfavorable" condition, the guinea pig structure turned out to be R2. When Shaanan [12] used a similar condition (2.5 mol dm^{-3} sodium/potassium phosphate, pH 6.7) he obtained R-state human hemoglobin. Compare this with the mild "R2-favorable" condition used by Silva et al. [1] to prepare R2 for human hemoglobin (16% polyethylene glycol 6000,100 mmol dm^{-3} sodium cacodylate, 75 mmol dm^{-3} chloride, pH 5.8). There is no doubt in our mind that under the "R2-favorable" conditions of our experiments (total ionic strength, 50 mmol dm^{-3}) stripped guinea pig hemoglobin retains the R2 structure that it has at the high, "R2-unfavorable", ionic strength employed for its crystallization. Furthermore, according to its x-ray structure, guinea pig hemoglobin has a $\beta_1\beta_2$ inter-subunit salt bridge between the terminal NH_3^+ group of one β-chain and the terminal COO^- group of the partner β-chain in the same molecule. This salt bridge should confer some extra stability to the guinea pig R2-state. We conclude that the R2 ⇌ R equilibrium of guinea pig hemoglobin in solution lies completely to the left, unlike human hemoglobin in which the R and R2 structures exist in dynamic equilibrium [42]. We also conclude that the rather large *reduction* in the DTNB affinity of guinea pig hemoglobin caused by inositol-P_6 (Fig. 3d) arises from a tertiary structure change in its R2 state.

As we demonstrated in Section 3.3, chicken (major) hemoglobin, like guinea pig hemoglobin, has a $\beta_1\beta_2$ inter-subunit salt bridge between the terminal NH_3^+ group of one β-chain and the terminal COO^- group of the partner β-chain in the same molecule. Like for guinea pig hemoglobin, the reduction of the chicken (major) DTNB affinity by inositol-P_6 is quite large (Fig. 5a). This large reduction in affinity, coupled with the existence of a $\beta_1\beta_2$ inter-subunit salt bridge in chicken hemoglobin, strongly suggests that chicken (major) hemoglobin exists almost entirely in the R2 quaternary state, with the R2 ⇌ R equilibrium lying almost completely to the left, in favor of the R2-state.

Unlike guinea pig and chicken (major) hemoglobin, rabbit hemoglobin (Fig. 3c) and chicken (minor) hemoglobin (Fig. 5b) have their DTNB affinities decreased by inositol-P_6, but to a minor extent. We suggest that in these hemoglobins there exists an R2 ⇌ R equilibrium that slightly favors the R2 state. Bearing in mind that inositol-P_6 increases the DTNB affinity of the R-state but *decreases* that of the R2-state, the two effects will oppose each other, and only a small effect of inositol-P_6 will be seen.

(iii) Inositol-P_6 shows no effect on the DTNB affinities of dog (Fig. 3a), human (Fig. 3b) and avian (Fig. 4) hemoglobins. According to Lukin et al. [42], human hemoglobin in solution exists as a mixture of R and R2 states in dynamic equilibrium. With respect to human hemoglobin, Shibayama et al. [43] state as follows, "Interestingly, as shown in Fig. 3, the dotted straight line corresponding to the fourth oxygen equilibrium constant of human hemoglobin in solution (K_4 = 0.19 Torr at 20 °C) … falls right in the middle between the Hill plots of the R and R2 crystals, suggesting that the oxyhemoglobin solution may contain nearly equal amounts of the R and R2 state." We recall that inositol-P_6 increases the DTNB affinity of the R state (Figs. 1 and 2) but *decreases* that of the R2 state (Figs. 3d and 5a). We suggest that the apparent lack of an inositol-P_6 effect seen in the case of human hemoglobin (Fig. 3b) is attributable to the 'nearly equal amounts' of the R and R2 states, with their opposing effects on the DTNB affinity. By extension, this accounts for the apparent lack of an inositol-P_6 effect in dog and avian hemoglobins (Figs. 3a; 4). This is not the case with rabbit and chicken (minor) hemoglobin (Figs. 3c and 5b) for which the amount of the R2 state must be slightly higher than that of the R state.

4. Discussion

4.1. Role of His2β in the reaction of DTNB with carbonmonoxyhemoglobins

The hemoglobins employed in this work include those of various mammalian species (see Table 1): ungulates (columns 2–6; Fig. 7), carnivores (columns 7–9), a primate (column 10) and two lagomorphs (columns 11 and 12). The list also includes seven avian hemoglobins (Table 2), making a total of 19 different hemoglobins. Given the fact that inositol-P_6*increased* the DTNB affinities of the sheep hemoglobins by about an order of magnitude (see Figs. 3 and 4 of [17]), we expected that it would do the same for the ungulate hemoglobins listed in Table 1 (columns 2–6). This expectation was based on two grounds: the fact that (i) sheep and other ungulate hemoglobins (including bovine hemoglobin) do not have histidine at the 2β position; and (ii) they do not have the full complement of positively charged organic phosphate binding groups (see last four rows in columns 2–6 of Table 1). As seen in Figs. 1 and 7, our expectation was fully borne out. The cat hemoglobins (Table 1; columns 7 and 8) are not ungulate hemoglobins. Nevertheless, like the ungulates they lack histidine at the 2β position and also do not have the full complement of positively charged organic phosphate binding groups (see last four rows in columns 7 and 8 of Table 1). It is therefore not surprising that inositol-P_6 increased their DTNB affinities (Fig. 2).

What is surprising and noteworthy is our finding that the DTNB affinities of those hemoglobins in Table 1 that do have His2β and the full complement of positively charged organic phosphate binding groups (see columns 9 and 10) are not affected by inositol-P_6, whereas we had expected it to cause much larger increases in their DTNB affinities than those seen in Figs. 1 and 2. Even more contrary to our expectation, in the case of rabbit and guinea pig hemoglobin (columns 11 and 12 of Table 1) inositol-P_6 actually *decreases* their DTNB affinities. At pH 7.3 these decreases amount to about half of an order of magnitude for rabbit hemoglobin (Fig. 3c) and to about two orders of magnitude for guinea pig hemoglobin (Fig. 3d).

Each of the six avian hemoglobins listed in Table 2 not only has a histidine at the 2β position but also has the full complement of the basic groups at the organic phosphate binding site (last four rows in Table 2). These properties are identical with those of dog, human, rabbit and guinea pig hemoglobin (last 4 columns in Table 1). The data in Fig. 4 show that the DTNB affinities of turkey (major and minor), pigeon, the major quail and guinea fowl hemoglobin are not altered upon binding of inositol-P_6 (compare with Fig. 3a and b). Most surprising, however, are the data for the chicken major and minor hemoglobins (Fig. 5). Not only does inositol-P_6*not* increase their DTNB affinities, at pH 7.3 it *decreases* that of the major chicken hemoglobin by two orders of magnitude (Fig. 5a) and that of the minor hemoglobin by approximately half of an order of magnitude (Fig. 5b).

The above considerations lead us to the conclusion that the binding of inositol-P_6 to a hemoglobin having histidine at the 2β position results in either (i) no effect or even (ii) a *decrease* in its DTNB affinity. Those hemoglobins that do *not* have histidine at the 2β position have their DTNB affinities increased by inositol-P_6. We note in passing that His2β is one of the negatively contributing Bohr groups whose absence accounts for the high pH sensitivity of the oxygen affinity of Root effect hemoglobins [44].

4.2. Basis of inositol-P_6 effect on DTNB affinities of carbonmonoxyhemoglobins

Recently [23] we reported the quantitative analyses of Bohr effect data for stripped human hemoglobin [45] and for human hemoglobin to which 2,3-bisphosphoglycerate (2,3-BPG) was bound [46]. Among all the Bohr effect groups listed in Table 5 of [23] what is most significant is that the organic phosphate transformed His2β from a *negatively* contributing Bohr group, with $pK^{oxy} > pK^{deoxy}$, to a *positively*

contributing Bohr group, with $pK^{oxy} < pK^{deoxy}$ (see Fig. 8b of [23]). This change in the role of His2β could not have occurred without a change in structure, either tertiary or quaternary, especially when one considers that His2β is one of the groups to which organic phosphates bind [21,22].

Of particular relevance to our discussion is the fact that, according to its x-ray crystal structure [3], guinea pig hemoglobin exists in the quaternary R2 state. In the guinea pig R2 state the terminal NH_3^+ group of one β-chain is linked by a salt bridge, NH_3^+.....$^-$OOC, to the COO^- terminal group of the partner β-chain in the same molecule [3]. In a study on the Bohr effect of various hemoglobins [36], we noted that we were unable to account quantitatively for the Bohr effect of guinea pig hemoglobin until we took this salt bridge into consideration. Consequently, one may conclude that the NH_3^+.....$^-$OOC salt bridge formed in guinea pig hemoglobin crystals also exists in solution.

We carried out our DTNB binding studies in solution on hemoglobins in their carbonmonoxy form. Consequently, we expect that the hemoglobin molecule in each case should exist as a dynamic intermediate between the two quaternary structures R and R2, provided the structure close to the NH_3^+ terminal end of the β-chain is not altered. We note, however, that this structure is altered in the case of the hemoglobins of the ungulates (including the cow) and of the cat (Table 1, columns 2–8): (i) in the goat (major and minor) hemoglobins, and in bovine hemoglobin, because position 1β is deleted and position 2β is occupied by methionine rather than histidine; (ii) in the horse and donkey hemoglobins because position 2β is occupied by glutamine; (iii) in the cat hemoglobins because their 2β position is occupied by phenylalanine. Consequently, these hemoglobins cannot exist in the R2 quaternary structure but only in the R quaternary structure.

We are led to conclude that carbonmonoxyhemoglobins that *cannot* exist in the R2 quaternary state, because of a change in their structure near the NH_3^+ terminal end of the β-chain, can only exist in the R quaternary state. Consequently, the effect of inositol-P_6 on these hemoglobins is to increase their DTNB affinities (Figs. 1, 2 and 7). On the other hand, hemoglobins that have their structures preserved at the NH_3^+ terminal end of the β-chain exist as a dynamic mixture of the R2 and R states. Therefore, inositol-P_6 may *decrease* their DTNB affinities or have no effect on those affinities, depending on their position in the R2 ⇌ R quaternary equilibrium. Hemoglobins in which the R2 ⇌ R equilibrium lies more in favor of the R2 state will have their DTNB affinities *decreased* by inositol-P_6. These include rabbit (Fig. 3c), guinea pig (Fig. 3d), chicken major (Fig. 5a) and, to a lesser extent, chicken minor (Fig. 5b) hemoglobin. In each case, the magnitude of the decrease in DTNB affinity will depend on how far to the left the R2 ⇌ R equilibrium lies. Those hemoglobins in which the R2 ⇌ R equilibrium lies at an intermediate state will have their DTNB affinities virtually unaffected by the presence of inositol-P_6: dog (Fig. 3a); human (Fig. 3b); turkey, major and minor (Fig. 4a and b); pigeon (Fig. 4c); quail major (Fig. 4d); and guinea fowl major (Fig. 4e).

5. Conclusion

The effect of inositol-P_6 on the DTNB affinity of a given carbonmonoxyhemoglobin depends on where its quaternary structure lies along the R2 ⇌ R quaternary equilibrium and on whether it has His2β. If, like guinea pig hemoglobin, its quaternary structure is R2, inositol-P_6 will greatly *decrease* its DTNB affinity. If, like human hemoglobin, its quaternary structure is a dynamic intermediate between R and R2, inositol-P_6 will have little or no effect on its DTNB affinity. If it *lacks* His2β and its quaternary structure is pure R, as seems likely with the ungulate and cat hemoglobins, inositol-P_6 will *increase* its DTNB affinity.

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