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Titration Behavior of Histidines in Human, Horse, and Bovine Hemoglobins

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SUMMARY

The titration curves of the human hemoglobins A₂ and F, the slow and fast components of horse hemoglobin, and bovine hemoglobin B have been analyzed, mainly to obtain information about the titration behavior of the histidines in these hemoglobins. The results were also compared with earlier data concerning human hemoglobin A and bovine hemoglobin A.

In both hemoglobin A₂ and F, 18 titratable histidines were found, two less than in hemoglobin A. Assuming that those histidyl residues which in the three-dimensional model occupy the same sites will show similar titration behavior, we reached the conclusion that in human hemoglobin histidine G19 and histidine H21 are titratable and that histidine G18 is not titratable.

Both the slow and fast components of horse hemoglobin were found to contain 22 titratable histidines and 70 titratable carboxyl groups. Combining these results with the known number of amides in the α chain we calculated that the β chain should contain 11 amides.

It appeared that in bovine hemoglobin B the histidines at B1(18) β , which are absent in bovine hemoglobin A, are titratable. The pK of these histidines, as estimated from the difference titration curve, is about 7.8, both in oxy- and deoxyhemoglobin. This high pK is probably caused by the formation of a saltbridge with the carboxyl group of aspartic acid B3(20) β in the same chain. Both hemoglobins contain 72 titratable carboxyl groups.

Comparing the human, horse, and bovine hemoglobins, assuming again similar titration behavior for structurally identical histidines, we were able to correlate the titration results of human and horse hemoglobin. The human and bovine hemoglobins failed to show an equally good correlation, although the discrepancy was small.

24 groups liberated, at least 12 were imidazole groups. An estimation of the number of groups unmasked at acid pH is complicated by the fact that the electrostatic interaction factor w changes simultaneously with unmasking. Therefore to find the number of titratable histidines in hemoglobin we have followed a different approach which consists of a direct measurement or counting of the total number of histidines which are titrated in the neutral pH region where the protein is in its native state. A direct counting procedure, although different from ours, was also followed by Tanford and Nozaki (4) for human and horse hemoglobin and by Bucci *et al.* (5) for human hemoglobin and its subunits. The accuracy of their analyzing procedure was about one-half histidine per heme or two groups per tetramer. In earlier reports on bovine and human hemoglobin (6, 7) we have introduced a measuring and analyzing procedure for titration curves which permits a greater accuracy in the determination of the number of titratable histidines. In this paper we have extended our study to human hemoglobins A₂ and F, horse hemoglobin, including the slow and fast components, and bovine hemoglobin B.

In our analyses of the several hemoglobins we also present an effort to correlate the data of the several hemoglobins in order to get information about the titratability and pK values of particular histidines.

EXPERIMENTAL PROCEDURE

Hemoglobin solutions were prepared following the toluene method of Drabkin (8).

The two components of horse hemoglobin were isolated as described by Perutz *et al.* (9). By this method the so-called slow and fast components are obtained as methemoglobin. The two fractions were concentrated by means of a Diaflo ultrafiltration cell and subsequently converted to the cyanomet derivatives. The separation was checked with polyacrylamide gel electrophoresis at pH 8.

The method of Bernini (10) for the isolation of human hemoglobin A₂ was somewhat modified in order to obtain larger quantities. As buffers were used 0.2 M Tris to which 0.2 M NaH₂PO₄ was added until the pH was 8.5, and a solution of 0.035 M Tris adjusted to pH 8.5 with 0.035 M NaH₂PO₄. Chromatography was performed on DEAE-Sephadex type A-50. Ten grams of this resin were allowed to swell in the first buffer for 12 hours and subsequently washed several times with the second buffer. Before operating the column (4 × 20 cm) was equilibrated with

It has been established by Steinhardt and Zaiser (1), Steinhardt and Hiremath (2), and Geddes and Steinhardt (3) that at acid pH groups are becoming titratable in hemoglobin which are masked when the protein is in its native state. In the case of horse carboxyhemoglobin it was found (3) that out of a total of

the second buffer. The column was charged with 40 ml of a hemoglobin solution, concentration about 100 g per liter, previously dialyzed against the second buffer. Subsequently the column was eluted with this buffer. The hemoglobin A₂ moved rapidly through the column and could satisfactorily be separated from the main component. The solution containing hemoglobin A₂ was concentrated by means of ultrafiltration and dialyzed against distilled water. The separation was checked by polyacrylamide gel electrophoresis at pH 8. The whole procedure was carried out at 4°. To one sample of hemoglobin A₂, Na₂S₂O₄ was added and the solution immediately deionized (11) in order to reduce any methemoglobin which might have been formed during the whole procedure. The titration curve obtained from this sample was not significantly different from that of an untreated sample.

Bovine hemoglobin B was obtained from a BB genotype of the Limousine breed.

Hemoglobin isolated from umbilical cord blood was used for the hemoglobin F studies. The amount of hemoglobin F was determined by the alkali denaturation test (12).

Before titration all hemoglobin solutions were dialyzed against distilled water and subsequently deionized by repeated passing through a mixed bed ion exchange column.

The concentrations of the hemoglobin solutions were determined by drying to constant weight at 105°. The titrations were performed at 25° at an ionic strength of 0.1 (KCl) as described earlier (7, 13).

All reagents were analytical grade.

Treatment of Titration Data—The titration results are presented as normal titration curves (pH versus Z_H), differential titration curves ($-dpH/dZ_H$ versus Z_H) or difference titration curves (ΔZ_H versus pH), in which Z_H is the mean proton charge of the protein with the isoionic point ($Z_H = 0$) as reference, dpH/dZ_H the reciprocal of the buffer capacity and ΔZ_H the difference in protons bound by two proteins at the same pH. The analysis of differential titration curves has been given by De Bruin and Van Os (13) and we will give here the main features without going into details.

In the analysis it is assumed that the Linderström-Lang equation may be used to describe the titration behavior of each class of groups i .

$$\text{pH} = \text{p}K_i + \log (\alpha_i/1 - \alpha_i) - 0.868 wZ \quad (1)$$

where K_i is the intrinsic dissociation constant of the n_i groups of Class i , α_i its degree of dissociation, w the electrostatic interaction factor, and Z the protein charge (see also Reference 14). If only protons are bound, then $Z = Z_H$. The value of Z_H is given by

$$Z_H = Z_{\text{max}} - \sum n_i \alpha_i \quad (2)$$

where Z_{max} is the maximum positive charge of the native protein, so

$$Z_{\text{max}} = n_{\text{His}} + n_{\alpha\text{-NH}_2} + n_{\text{Lys}} + n_{\text{Arg}} \quad (3)$$

in which n_{His} , $n_{\alpha\text{-NH}_2}$, n_{Lys} , and n_{Arg} represent the number of titratable histidines, α -amino groups, lysines, and arginines. Equations 1 and 2 describe the normal titration curve of a protein. From these equations the following expression can be derived.

$$-d \text{pH}/dZ_H = 1/[2.303 \sum n_i \alpha_i (1 - \alpha_i)] + 0.868 w \quad (4)$$

The inflection points in a normal titration curve of a protein, terminating the neutral region at the acid and alkaline side, are found in a differential titration curve as peaks. The positions of these peaks are indicated by Z_I and Z_{II} . In the case of hemoglobin the second peak at Z_{II} is found near pH 9 and is always a sharp one. Calculations have shown that for Z_{II} the following relation holds.

$$Z_{II} = Z_{\text{max}} - n_{\text{COOH}} - n_{\text{His}} - n_{\alpha\text{-NH}_2} \quad (5)$$

in which n_{COOH} is the number of titratable carboxyl groups. With the help of Equation 3 this relation can be written as

$$Z_{II} = n_{\text{Lys}} + n_{\text{Arg}} - n_{\text{COOH}} \quad (6)$$

The first peak at Z_I (near pH 6) represents the titration end point of the carboxyl groups, at least when the peak is sharp. So for Z_I we have in first approximation the equation

$$Z_I = Z_{\text{max}} - n_{\text{COOH}} \quad (7)$$

Combining the Equations 6 and 7 we have

$$Z_I - Z_{II} = n_{\text{His}} + n_{\alpha\text{-NH}_2} \quad (8)$$

Calculations have shown that Equation 7 may be applied to a good approximation when the first peak is well resolved. In that case the number of carboxyl groups is mostly found only one group too large. As a consequence the number of histidines and α -amino groups, obtained with the help of Equation 8, is found one group too small. This implies that to the number of titratable histidines estimated in this way about one group has to be added. By means of calculated curves the result can be checked. Therefore, in view of the fact that the first peak in the differential titration curve of deoxyhemoglobin is much better resolved than in oxyhemoglobin, we have studied the deoxy form of the several hemoglobins to find the number of titratable histidines.

In deoxyhemoglobin the acid Bohr groups are mainly titrated at the acid side of the first peak. In our calculations we have assumed that there are four acid Bohr groups and, moreover, that they are carboxyl groups (15).

From the experimental differential titration curves Z_I and Z_{II} can be read off with an accuracy of about 0.1.

All data and discussions are based on hemoglobin as a tetramer.

RESULTS

Human Hemoglobin A₂—This minor component of normal adult hemoglobin has two α chains identical with those of human hemoglobin A. The other chains of hemoglobin A₂ are different from the β chains and are called δ chains. These δ chains have alanine B4(22) δ , arginine G18(116) δ , and asparagine G19(117) δ , whereas in the β chains at the same positions, glutamic acid B4(22) β , histidine G18(116) β , and histidine G19(117) β are found (Reference 16 and Table I). Besides these three polar replacements there are seven other differences between the β and δ chains involving only nonpolar residues (18).

In Fig. 1 the differential titration curve of human hemoglobin A₂ is given. Z_{II} for hemoglobin A₂ is -6.0 while it is -10.0 for hemoglobin A (Table II). This difference corresponds with the fact that hemoglobin A₂ has two carboxyl groups less and two arginines more per tetramer than hemoglobin A (see Equation 6). Z_I is 15.0 so $Z_I - Z_{II}$ is 21.0 as compared with 23.0

TABLE I
Partial amino acid composition of human and horse hemoglobins

The numbers given for horse hemoglobin refer to the slow component. In the fast component 1 lysine residue has been replaced by a glutamine (16). Data from Dayhoff (17).

Nature of groups	Human				Horse	
	α	β	γ	δ	α	β
α -COOH.....	1	1	1	1	1	1
Asn.....	4	6	5	8	4	14
Asp.....	8	7	8	7	9	
Gln.....	1	3	4	5	1	14
Glu.....	4	8	8	7	3	
Heme-COOH.....	2	2	2	2	2	2
His.....	10	9	7	7	10	9
α -NH ₂	1	1	1	1	1	1
Lys.....	11	11	12	11	11	11
Tyr.....	3	3	2	3	3	3
Cys.....	1	2	1	2	1	1
Arg.....	3	3	3	4	3	4

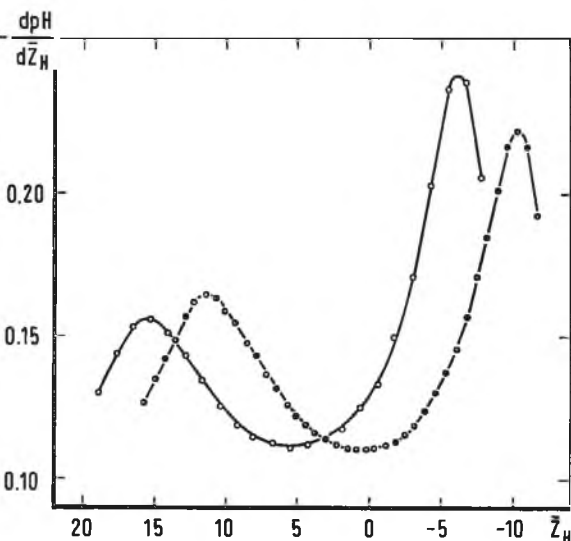


FIG. 1. Differential titration curves of human deoxyhemoglobin A₂ (O) and F (⊙).

for the A component. This means that in hemoglobin A₂ two histidines less are titratable than in hemoglobin A.

Human Hemoglobin F—This hemoglobin contains two α chains identical with those of hemoglobin A and two γ chains which differ from the β chains on 37 positions (18). Among these differences are histidines G18(116) β and H21(143) β which are replaced by isoleucine and serine, respectively, in the γ chains. From Table I we find $n_{\text{Lys}} + n_{\text{Arg}} = 58$ and $n_{\text{COOH}} = 68$; therefore we expect $Z_{II} = -10$, as is found experimentally (see Fig. 1). Z_I is 11.6, so $Z_I - Z_{II}$ is 21.6 as compared with 23.0 for hemoglobin A. Correcting for the fact that the sample studied consisted of 75% hemoglobin F and 25% hemoglobin A we find $Z_I - Z_{II} = 21.1$ and so we conclude that in hemoglobin F two histidines less are titratable than in hemoglobin A. The equal number of titratable histidines in hemoglobins A₂ and F

TABLE II
Survey of some of results obtained from differential titration curves of several deoxyhemoglobins and comparison with amino acid composition

Hemoglobin	Z_I	Z_{II}	Number of histidines			$n_{\text{Lys}} + n_{\text{Arg}}^c$	n_{COOH}^f
			$Z_I - Z_{II}^a$	Titratable ^b	From amino acid composition ^c		
Human A ^e	13.0	-10.0	19.0	20 ^f	38	56	66 ^g
Human A ₂	15.0	-6.0	17.0	18	34	58	64 ^g
Human F ^h	11.6	-10.0	17.6	18	34	58	68 ^g
Horse.....	11.6	-13.0	20.6	22 ^f	38	57	70
Bovine A ⁱ	11.3	-10.3	17.6	18 ^f	32	62	72
Bovine B.....	9.5	-14.0	19.5	20	34	58	72
α Chain ^e	6.0	-2.0		7	10	14	16 ^j
β Chain ^e		-4.7		6	9	14	19 ^j

^a Approximated number of titratable histidines obtained by using Equation 8, subtracting four α -amino groups.

^b Obtained by rounding off upward to the next even integer the numbers in the preceding column.

^c Data from Dayhoff (17).

^d Calculated with Equation 6 and rounded off to the next even integer.

^e From Reference 7.

^f Checked by comparing with calculated curves.

^g In accord with amino acid composition.

^h The hemoglobin studied contained 75% hemoglobin F and 25% hemoglobin A.

ⁱ From Reference 6.

^j Taking into account one extra carboxyl group from the *p*-mercuribenzoate.

is also shown by the nearly identical shape of the two curves. The main difference is the expected shift of four groups.

Horse Hemoglobin—Horse hemoglobin consists of two components roughly in equal amounts. They are called the slow and fast components according to their electrophoretic behavior (19, 20). In Table I the amino acid composition of the slow and fast components is given. In the fast component lysine E9(60) α has been replaced by a glutamine residue (16). Crystallographically the two components are indistinguishable (9).

The differential titration curves of the two components are shown in Fig. 2. The curves are nearly identical in shape and shifted about two groups with respect to each other; Z_{II} is -12.4 for the slow and -14.6 for the fast component, so the shift is 2.2. This means that, as expected, the two components have the same number of titratable histidines. The differential titration curve reveals in addition information about the total number of titratable carboxyl groups. Applying Equation 6 with the data of Table I we find $n_{\text{COOH}} = 70.4$ for the slow and 70.6 for the fast component. Rounding off these numbers to the next even integer we obtain a value of 70 titratable carboxyl groups in both components.

The isoionic points of the cyanomet derivatives of the slow and fast components are about 7.2 and 7.0, respectively. This difference of 0.2 is what has to be expected because at $Z_H = 0$ $-dpH/dZ_H$ is near 0.1 and the difference in charge between the two components is 2.

From the results described it can be concluded that for a de-

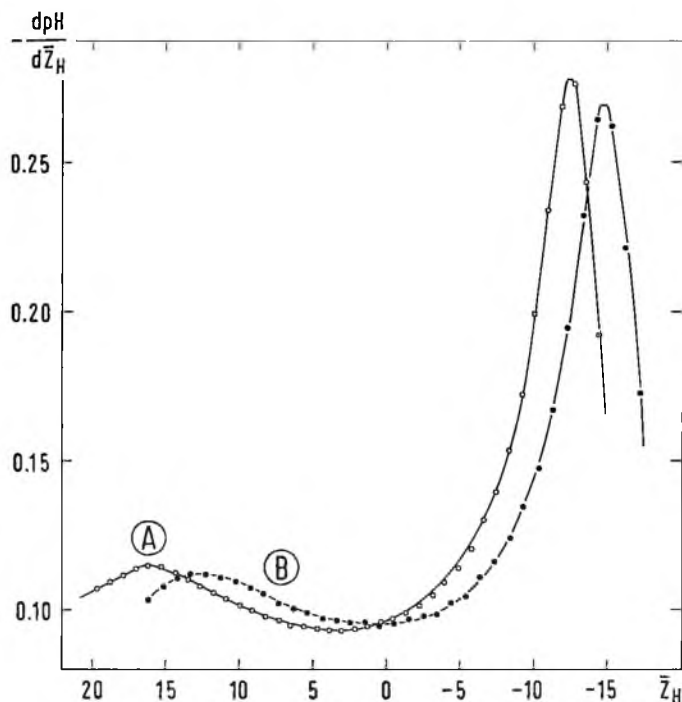


Fig. 2. Differential titration curves of the two components of horse hemoglobin. Curve A, slow component. Curve B, a fast component. The hemoglobins were titrated in the cyanomet form.

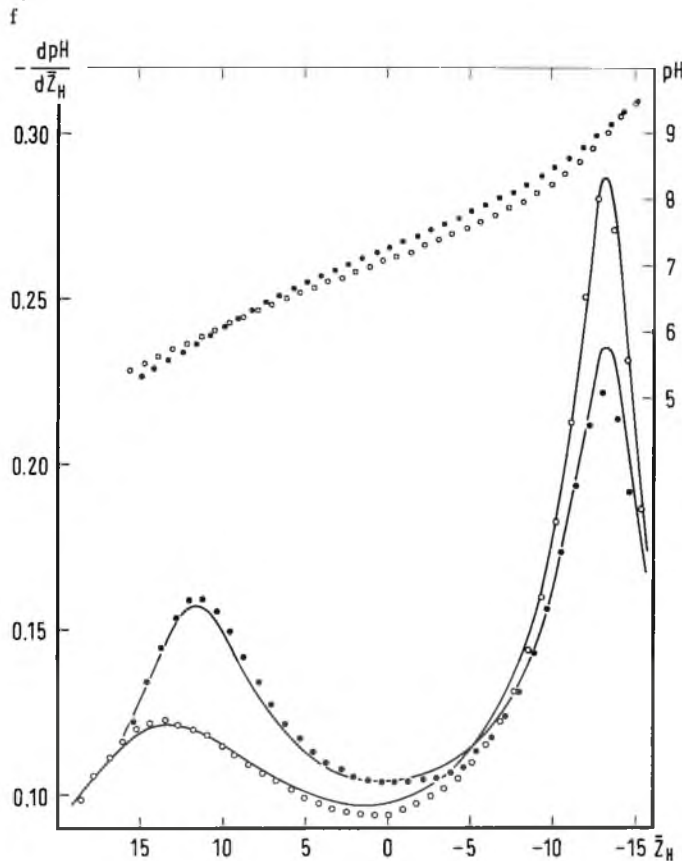


Fig. 3. Normal and differential titration curves of horse deoxyhemoglobin (●) and oxyhemoglobin (○). Drawn lines have been calculated with the parameters of Table III.

TABLE III
Parameters used to calculate the normal and differential titration curve of horse hemoglobin

Number of groups	pK in deoxy-hemoglobin	pK in oxyhemoglobin	Function	Identity
66	4.25	4.25	Acid Bohr groups Normal Bohr groups	Carboxyl groups
4	5.25 ^a	5.85 ^a		Carboxyl groups
4	8.0 ^a	6.65 ^a		Histidines and α -amino groups
14	7.1	7.1		
2	6.2	6.2		
2	7.8	7.8		Lys, Tyr, Cys
4	8.0	8.0		
55	10.7	10.7		

$$Z_{\max} = 82.7 \quad 0.868w = 0.018$$

^a From Reference 15.

termination of the number of titratable imidazole groups in horse hemoglobin it is permitted to use the naturally occurring mixture of the two components.

The normal and differential titration curve of the deoxygenated and oxygenated form of this mixture are shown in Fig. 3. When both components occur in equal amounts the calculated value for Z_{II} , based on Equation 6, with $n_{\text{COOH}} = 70$, is -13.0 as is found experimentally. The distance between the two peaks in the curve of the deoxy form is 24.6; subtracting from this number the four titratable α -amino groups we obtain in first approximation 21 titratable histidines. Calculations showed that a good fit could only be obtained with 22 titratable histidines. This means that out of 38 histidines present, 16 are masked. The observed shift to the left of Z_I upon oxygenation and the increase of the peak at Z_{II} is due to the change in pK of the acid and normal Bohr groups and resembles the situation in bovine and human hemoglobin (6, 7). Table III gives the parameters which were used to obtain the calculated curves of Fig. 3. The table shows, besides the total number and pK value of each class, the nature and presumed identity of the groups titrated. The lysines, tyrosines, and cysteines were considered as one class. From the 12 tyrosines present 8 were assumed to be titratable (21); the four cysteines and all lysines were considered as being titratable. The number of lysines is the mean value of the number of lysines occurring in the two components.

Bovine Hemoglobin B—Two kinds of bovine hemoglobin called A and B are known (19, 22). In a previous report we studied the titration behavior of bovine hemoglobin A (6). This molecule differs only at three sites from hemoglobin B: the residues glycine-15, lysine-18, and lysine-119 in the β chain of hemoglobin A are replaced by serine, histidine, and asparagine in hemoglobin B (17). Fig. 4 shows the differential titration curves of the deoxy form of both hemoglobins. The second peak is found at -14.0 for the B component, while in hemoglobin A Z_{II} is -10.3 . This difference arises from the different number of lysines (see Equation 6 and Table II). For hemoglobin A we found $Z_I - Z_{II} = 23.5$ while for hemoglobin B this value is 21.6. This result clearly indicates that the two extra histidines in hemoglobin B are titratable.

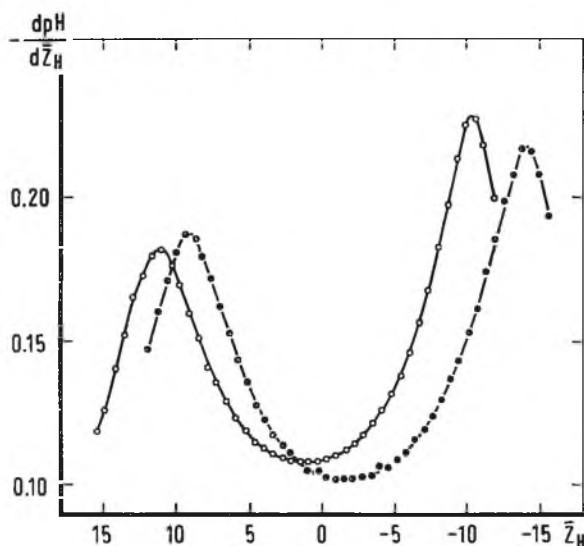


Fig. 4. Differential titration curves of bovine deoxyhemoglobin A (○) and B (●).

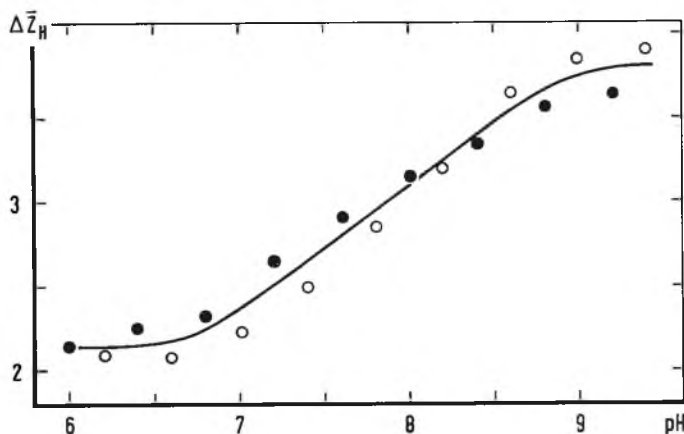


Fig. 5. Difference titration curve of bovine hemoglobins A and B. (●) Deoxy form; (○) oxy form.

Fig. 5 gives the difference titration curve of the A and B type both for the deoxy and the oxy form. Assuming that the pK values of all common titratable residues in both forms are the same, this curve represents the titration of the extra histidines in hemoglobin B. From this curve an apparent pK of about 7.8 can be estimated for this histidine in both the deoxygenated and oxygenated state. We further wish to remark that both hemoglobins A and B should contain 72 titratable carboxyl groups, in contrast with the amino acid analysis which gives a number of 76 (17).

DISCUSSION

Bovine Hemoglobin B—The fairly high pK of 7.8 for histidine B1(18) β in bovine hemoglobin B strongly suggests that this residue shows some interactions with other groups. These groups could be the carboxyl groups of aspartic acid B3(20) β and glutamic acid B4(21) β (see Fig. 3 of Reference 23). Studying the atomic model of horse hemoglobin and assuming structural correspondence with bovine hemoglobin it is even possible to bring both carboxyl groups within saltbridge distance to

histidine B1.¹ The observation that the pK has the same value in both deoxy- and oxyhemoglobin can be understood in view of the fact that the three residues are found nearly in sequence in the peptide chain and are not located at interfaces so that they probably are little sensitive to the structural changes which take place upon oxygenation of deoxyhemoglobin. Assuming that the pK of a normal histidine is near 7 it can easily be calculated that a pK shift to 7.8 is associated with a standard free energy change of about 1 kcal per mole which is a reasonable value for the formation of a saltbridge (24).

Amides in Horse β Chain—Table I shows that the α chain of horse hemoglobin contains 15 titratable carboxyl groups in both components; in view of our titration results this means that there are 20 titratable carboxyl groups per β chain, which number includes the terminal COOH and the two heme carboxyl groups. Therefore we may conclude that out of a total number of 28 asparagine and glutamine residues per β chain, 17 are present as aspartic and glutamic acids and the remaining 11 as amides. Smith and Chung (25) have located nine amides in the residues 1 to 82 and 117 to 146 of the horse β chain. These nine amides are found at the same sites both in the human and in the horse β chains. Therefore according to our titration results the Region 83 to 116 should contain the two remaining amides. Because the human β chain has asparagine at positions 102 and 108 (17), it is very likely that the two additional amides in the horse β chains will also be found at the positions 102 and 108.

Masked Imidazole Groups in Horse Hemoglobin—The number of 16 masked imidazole groups we found in horse hemoglobin is four larger than reported by Geddes and Steinhardt (3) and Tanford and Nozaki (4) who found 12 masked groups. The experimental approach of Geddes and Steinhardt was quite different from ours and this could be an explanation for the difference in outcome. Tanford and Nozaki, however, also followed a procedure in which they measure the number of titratable histidines, so a better correspondence with our reported value could be expected. In view of the fact that the number of titratable histidines calculated from the titration data in their way of analysis is greatly influenced by the number of titratable carboxyl groups, the observed discrepancy can be explained as probably caused by the fact that they assumed a number of 78 titratable carboxyl groups while our results indicate a number of 70. Moreover the value of 9.1 they assumed for pK_{SH} seems to be too low (26) and has influenced their results too. In their analysis of the titration data of human hemoglobin A Bucci *et al.* (5) and Tanford and Nozaki (4) reported a number of 16 masked imidazole groups, only two less than we found (7). This better agreement is probably due to the fact that they use for hemoglobin A the proper number of ionizable carboxyl groups.

It is further noteworthy to mention here that Geddes and Steinhardt (3) suppose that at acid pH, besides imidazole groups, also other groups such as neutral ϵ -amino or ionized phenoxy groups, are getting unmasked. However, an objection to this supposition could be made. In the case of human hemoglobin A we found that Equation 6 could be applied. If neutral ϵ -amino groups exist at the pH of the second peak (pH about 9)

¹ Dr. M. F. Perutz informed us that histidine B1(18) β is more likely to interact with aspartic acid B3(20) β rather than with glutamic acid B4(21) β .

TABLE IV

Position of histidine residues in chains of human, horse, and bovine hemoglobins^a

H denotes that a histidyl residue is found at that position.

Position in polypeptide chain	Human horse bovine	Human			Horse	Bovine	Remarks concerning non- α chains ^b
	α	β	γ	δ	β	β	
NA2.....		H	H	H			t; external
B1.....	H					H ^c	
CD3.....	H						
D6.....	H						
E7.....	H	H	H	H	H	H	t; distal histidine
E13.....					H		t; external
E20.....					H		t; external
EF1.....	H	H	H	H	H	H	
F8.....	H	H	H	H	H	H	n; proximal histidine
FG1.....	H						
FG4.....		H	H	H	H	H	
G10.....	H						
G18.....		H					n
G19.....	H	H	H		H		t
H5.....	H						
H21.....		H		H	H	H	t; external
HC3.....		H	H	H	H	H	t; Bohr group

^a From References 17, 27, and 28.

^b t = titratable; n = nontitratable. For details see text.

^c Only in bovine hemoglobin B.

n_{Lys} would be smaller and so Z_{IT} would have a value different from that we found experimentally. The same is true if ionized phenoxy groups are masked. In that case Equation 6 must be extended with a term representing the number of ionized phenoxy groups and that would also result in a shift of Z_{IT} . It seems more likely to us that the extra masked groups are ionized carboxyl groups being partners in saltbridges which are broken up by the acid denaturation.

Masked Imidazole Groups in Human Hemoglobins—From the differential titration curve of hemoglobins A and A₂ we concluded that of the four histidines which the A component has in excess only two are titratable. Because the α chains in hemoglobins A and A₂ are identical it can be expected that these chains show the same number of titratable histidines. It is further reasonable to assume that histidines which are found at the same structural places in the non- α chains also will show the same titration behavior. Based on these assumptions we are led to the conclusion that either histidine G18 or histidine G19 is not titratable in the β chains (Table IV). When the titration results of hemoglobins A and F are compared in the same way the conclusion can be drawn that either histidine G18 or histidine H21 will be not titratable. Combining these two observations the conclusion can be reached that in the human β chain histidine G18 is masked and both histidines G19 and H21 are titratable. It should be stressed in this respect that for these conclusions the identity of the acid Bohr groups is not relevant; only when the exact number of titratable histidines of a particular hemoglobin is the object of the titration analysis this identity is of importance. The titratability of histidine G19 is consist-

ent with the difference in electrophoretic mobility between hemoglobins A and P (29). In the latter abnormal hemoglobin histidine G19 is substituted by an arginine. The experiments reported showed that at pH 8.5 hemoglobin P shows a smaller anodic mobility than hemoglobin A, while the mobility is equal at pH 6. This behavior can only be understood if titratability of histidine G19 is assumed. The titratability of histidine H21 is supported by the fact that this histidine is very probably involved in the binding of 2,3-diphosphoglycerate by deoxyhemoglobin A (24). Moreover histidine H21 is external and therefore probably titratable (30). The question remains which other particular histidine residues in the human β chain are titratable or not titratable. We have shown that most probably G19 and H21 are titratable and that G18 is not titratable. From electrophoretic studies on hemoglobin Zürich it can be concluded that histidine E7 is titratable (31). The titratability of histidine HC3 is without doubt because of its involvement in the alkaline Bohr effect (24, 32). The proximal histidine F8 is most likely not titratable while histidine NA2, which is the 2nd residue in the β chain with its imidazole groups external (23), is likely to be titratable. The titratability of histidines EF1 and FG4 probably might be elucidated from a study of hemoglobin J Iran, which has aspartic acid at EF1 (33) and of hemoglobin Malmö, which has glutamine at FG4 (34).

According to our previously reported experiments both the α and β chains have three nontitratable histidines (7). It is nearly without doubt that these residues are also not titratable in the $\alpha_2\beta_2$ tetramer. On the other hand titratable residues in the tetramer are expected to be titratable in the isolated chains too. Therefore from the data of Table II it can easily be calculated that the formation of the $\alpha_2\beta_2$ tetramer from two isolated α and β chains leads to the masking of six imidazole groups. In view of the findings and assumptions mentioned above, this means that upon assembling the $\alpha_2\beta_2$ tetramer either two or three of the titratable histidines in the isolated α chains become nontitratable; the exact number depends on whether one or no histidine becomes masked in the β chains. The question as to which particular residues in the α chains are titratable or not titratable and which residues might become masked in the tetramer is more difficult to answer than in the case of the β chains because the kind of information described above for the β chains is lacking for the α chains. The estimation of the number of histidines which are masked upon formation of the tetramer critically depends on the correctness of the figures used. The determination of the number of titratable histidines in the free β chains is fairly complicated (7). Moreover the absolute number of titratable histidines in hemoglobin depends on the assumption about the nature of the acid Bohr groups. We have assumed, as already mentioned, that they are carboxyl groups, but if these groups are histidyl residues only one group in the α or β chains becomes masked in the tetramer. From studies of the atomic model of hemoglobin it is as yet impossible to see where all the nontitratable residues in the isolated chains might be found, let alone to locate the six histidines which might become masked upon formation of the tetramer.

As mentioned in the previous section, Tanford and Nozaki (4) and Bucci *et al.* (5) have reported that most probably 22 imidazole groups are titratable in human hemoglobin. Moreover Bucci *et al.* reported that 7 histidine residues are titratable in the β chains. With these values we arrive at the same conclusions concerning the number of imidazole groups which be-

come masked upon formation of the $\alpha_2\beta_2$ tetramer as with our values.

Comparison of Human, Horse, and Bovine Hemoglobins—For the comparison of the human hemoglobins with horse and bovine hemoglobin we will start from the same basic assumption that residues at structurally identical positions will show similar titration behavior; in addition we will use the knowledge about the titratability of special groups obtained by comparing the several human hemoglobins with each other. Because the α chains of the hemoglobins studied are all alike as far as the positions of histidines are concerned, the difference in titration behavior of the hemoglobins should be found in the β chains. For this reason the difference in titratable histidine content of two groups between horse and human hemoglobins A can only be understood if histidines E13 and E20 are titratable; this interpretation is supported by the fact that both residues are external (35). A similar comparison between human hemoglobin A and bovine hemoglobin A failed to give the right answer; based on the data of Table IV human hemoglobin is expected to have four titratable histidines in excess, while actually only two are found. As yet we have no explanation for this. One may wonder, however, whether it is justified to compare hemoglobins of different mammals in the way we did, because they show besides differences in histidine content many other amino acid replacements which might introduce some structural differences. This could make our basic assumption less valid. Although some doubt is justified in this respect we do not think that this qualification is needed when hemoglobins of the same mammal are compared because the amino acid replacements in these cases are not very numerous, which makes structural correspondence more likely.

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