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## pK Change of Imidazole Groups in Bovine Serum Albumin Due to the Conformational Change at Neutral pH\*

B. J. M. Harmsen,† S. H. De Bruin, L. H. M. Janssen, J. F. Rodrigues De Miranda, and G. A. J. Van Os

**ABSTRACT:** The pH-dependent change in conformation of bovine serum albumin, located between pH 6 and 9 (neutral transition), was studied by means of optical rotation measurements at 313 nm and hydrogen ion titration experiments, both in the presence of KCl or CaCl<sub>2</sub>. The optical rotatory dispersion measurements revealed that with CaCl<sub>2</sub> the transition proceeds at lower pH values and within a smaller pH range than without calcium. From an analysis of the titration curves, combined with the observed influence of calcium ions on the neutral transition, it could be concluded that the transition causes a pK shift of certain groups, probably imidazole groups,

since as a consequence of the pK shift protons are released in the neutral region. That these groups are indeed imidazole groups was further confirmed by measuring the apparent heat of proton dissociation. The highest pK was found in the low pH conformation. This suggests that in this conformation several histidyl residues are involved in salt bridges. The effect of calcium on the neutral transition indicates that the affinity of albumin for protons decreases upon calcium binding. The relation between calcium and proton binding to albumin shows much resemblance with the Bohr effect of hemoglobin, *i.e.*, the relation between oxygen and proton binding.

It has been established by several methods that bovine serum albumin (hereafter referred to as the albumin) shows a conformational change between pH 3.5 and 4.5, which is called the normal-fast or NF transition (Aoki and Foster, 1956, 1957; Foster, 1960; Sogami and Foster, 1968) and a change in conformation roughly between pH 6 and 9 (Leonard *et al.*, 1963), the neutral transition. The NF transition can be explained (Foster, 1960) by assuming that the albumin molecule contains four compact parts or "subunits," held together by the peptide backbone itself. Later on evidence has been presented for a three- or four-subunit model (Bloomfield, 1966; Peters and Hawn, 1967; Franglen and Swaniker, 1968; Pederson and Foster, 1969). When the net charge of the molecule increases a rearrangement of the subunits occurs, resulting in an exposure of the interfaces between the compact folded parts to the solvent and consequently in unmasking of certain groups. Only a small rearrangement is supposed to happen at the neutral transition because hydrodynamic parameters remain nearly constant (Tanford and Buzzell, 1956; Leonard *et al.*, 1963). At the NF transition which is accompanied by an appreciable change in these parameters (Sogami and Foster, 1968), presumably a more drastic rearrangement occurs which probably reflects an increase in distance between the subunits.

Experimental data point to the idea that besides tyrosyl residues (Herskovits and Laskowski, 1962; Ohkubo, 1969) also carboxylate groups are involved in the subunit interactions since it has been found by Vijai and Foster (1967) that in the native form of the protein only about 60 of the approximately 100 carboxylate groups are available for protonation. These authors suggest that probably  $\epsilon$ -amino groups partici-

pate as cationic partners of the masked carboxylate groups in the electrostatic interactions between the subunits in the pH region between the NF transition and the neutral transition. This would be in accord with the results of Goldfarb (1966), who found indications for the presence of masked  $\epsilon$ -amino groups. However, it cannot be ruled out that other cationic groups such as imidazole or guanidinium groups are also involved in this mechanism. Some evidence for the participation of guanidinium groups is presented by Barré and Van Huot (1965). So far, however, the presence of masked imidazole groups has not been shown, although Decker and Foster (1967) had to assume, in order to explain their titration results, the presence of 10 histidines with a fairly high pK of about 7.5. This increase in pK can be caused by negative charges located near the imidazole groups and therefore could be an indication that these groups are involved in the formation of salt bridges. On the other hand, De Bruin (1969) found a somewhat anomalous temperature dependence of the hydrogen ion titration curve of the albumin in the pH region of the neutral transition, which could not be explained merely by assuming the existence of two classes of histidine residues.

Because this abnormal behavior might be linked to the neutral transition, we made a more detailed study of this transition by means of optical rotation measurements at 313 nm (*cf.* Leonard *et al.*, 1963) and differential hydrogen ion titration curves. Also the influence of the binding of Ca<sup>2+</sup> ions (Katz and Klotz, 1953; Harmsen, 1970) on the neutral transition was studied. The results strongly indicate the existence of imidazole groups, which show a pK shift due to the change in conformation during the neutral transition.

### Materials and Methods

Bovine serum albumin was obtained from the Nutritional Biochemicals Corp. (four-times crystallized) and deionized

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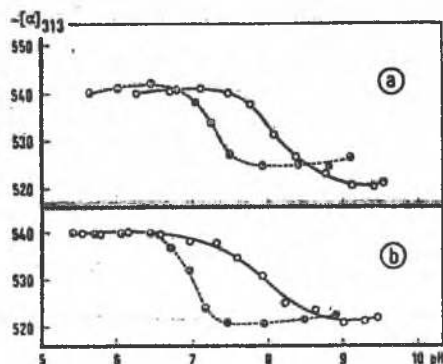


FIGURE 1: Specific rotation at 313 nm as a function of pH in the presence of KCl (O) or  $\text{CaCl}_2$  (●). Ionic strength 0.06 (a) or 0.20 (b). Protein concentration 0.5%; temperature 25°.

by passing through a mixed-bed ion-exchange column (Amberlite IRA 400 and IR 120). Concentrations were determined spectrophotometrically using  $E_{1\%}^{1\text{cm}} = 6.67$  at 279 nm or by drying at 105° in air to constant weight. All other materials were analytical grade (Merck, Darmstadt, Germany).

Differential titration curves were determined as described by De Bruin and Van Os (1968) and Janssen *et al.* (1970). The solutions were brought to the desired ionic strength with KCl or  $\text{CaCl}_2$  and titrated under nitrogen with carbonate-free NaOH at 24.9 or  $5.0 \pm 0.05^\circ$ . From the titrations at the two temperatures the apparent heat of the proton dissociation was calculated (Tanford, 1966). The protein concentrations were 1–2%. The titration charge of the protein was calculated assuming a molecular weight of 69,000.

The optical rotation at 313 nm was measured with a Jasco spectropolarimeter, Model ORD-CD-UV-5, equipped with a 450-W xenon lamp. Cells with a path length of 10 mm were employed. Since the change in optical rotation at the neutral transition is only about 4%, the cell containing the protein solution was placed in series with a cell filled with a solution of sucrose of such a concentration that the rotation of the albumin solution at pH 5–6 was nearly compensated. In this way it was possible to measure the small change in optical rotation with good accuracy. The pH of a sample was varied using small increments of base. The observed rotations were corrected for dilution caused by these additions. The specific rotation,  $[\alpha]$ , was calculated as  $[\alpha] = 100\alpha/cl$ , where  $\alpha$  is the rotation angle,  $c$  the protein concentration (g/100 ml), and  $l$  the optical path length (dm).

## Results and Discussion

Figure 1 shows the specific rotation at 313 nm as a function of pH in the presence of KCl or  $\text{CaCl}_2$  at  $I = 0.06$  and 0.20. The small decrease in specific rotation,  $[\alpha]$ , with increasing pH reflects (Leonard *et al.*, 1963) the change in conformation occurring at neutral pH. It is striking, as the figure shows, that in presence of  $\text{Ca}^{2+}$  ions the neutral transition steepens and shifts to lower pH values.

To characterize the transition curves we use the midpoint of the curve  $\text{pH}_{50}$ , *i.e.*, the pH value at which the change in  $[\alpha]_{313}$  is 50% of the maximum change and the steepness of the curve  $\Delta\text{pH}_{10,90}$ , *i.e.*, the pH difference between the points where the change in  $[\alpha]_{313}$  is 10 and 90% of the total amplitude. In Table I these parameters are given for the measurements shown in Figure 1. It is seen that if KCl is replaced by  $\text{CaCl}_2$  without changing the ionic strength a decrease in  $\text{pH}_{50}$  of

TABLE I: Midpoint and Steepness of the  $[\alpha]_{313}$  vs. pH Curves (see Figure 1).<sup>a</sup>

$I$	Salt	$\text{pH}_{50}$	$\Delta\text{pH}_{10,90}$
0.06	KCl	8.10	1.2
0.06	$\text{CaCl}_2$	7.25	0.7
0.20	KCl	7.90	1.5
0.20	$\text{CaCl}_2$	7.00	0.6

<sup>a</sup> The accuracy of the tabulated values is about  $\pm 0.05$  for  $\text{pH}_{50}$  and  $\pm 0.1$  for  $\Delta\text{pH}_{10,90}$ .  $I$  is the ionic strength of the solution.

about 0.85 unit is observed, and that  $\Delta\text{pH}_{10,90}$  is about twice as low.

The shift of the neutral transition to lower pH as judged by the optical rotation measurements is consistent with the results of Grams *et al.* (1967), who found that at constant ionic strength and pH the hydrogen–tritium exchange was enhanced when  $\text{Ca}^{2+}$  ions were present.

The normal titration curve of liganded and unliganded albumin is shown in Figure 2.  $Z_H$  represents the mean charge of the protein due to proton binding. As is seen the two curves are parallel below pH 6 and above pH 9 and in both pH regions at about the same distance. Between these pH values the affinity of albumin for protons is decreased upon  $\text{Ca}^{2+}$  binding. This means that in this pH region protons are given off by albumin when  $\text{Ca}^{2+}$  is bound. However, the total number of groups titrated between about pH 6 and 9.5 is in both cases nearly the same. This means that in this pH region the integral of the buffer capacity  $\Delta Z_H/\Delta\text{pH}$  is about the same in both conditions. This is also clear from Figure 3, where the differential titration curves,  $-(\Delta\text{pH}/\Delta Z_H)$  vs.  $Z_H$ , are given. The two curves coincide above  $Z_H = -5$  and below  $Z_H = -25$ ; between these two  $Z_H$  values a shift in buffer capacity is found from the region below the crossover point of the two curves to the region above this point. A striking fact is the very low value of  $-(\Delta\text{pH}/\Delta Z_H) = 0.080$  at the minimum in the curve for albumin in the presence of  $\text{Ca}^{2+}$  ions.

Figure 4 presents the difference in protons bound at the same pH by liganded and unliganded albumin, respectively. The curve is a direct measure for the change in proton affinity of the protein upon  $\text{Ca}^{2+}$  binding. The maximum change is found around the physiological pH. A direct consequence of the finding that the proton affinity of the protein is influenced by  $\text{Ca}^{2+}$  ions is that also the  $\text{Ca}^{2+}$  ion affinity should be affected by hydrogen ions (Wyman, 1968). According to Wyman (1968) the total change in  $\text{Ca}^{2+}$  affinity between two pH values is proportional to  $\int \Delta Z \text{d}p\text{H}$  of the curve in Figure 4 between these two pH values. However,  $\text{Ca}^{2+}$  binding experiments are too inaccurate to give good data in this respect.

The interaction between the binding of  $\text{Ca}^{2+}$  and protons is analogous to the interaction found between the oxygen and proton binding sites in hemoglobin. In the case of hemoglobin it is generally accepted (Perutz, 1970) that the conformational change which takes place upon oxygenation breaks up some salt bridges, which results in a lowering in  $\text{pK}$  of the positively charged partners of these salt bridges; this in turn lowers the pH.

We believe that also in the case of albumin the conformational change in the neutral pH region could serve as an

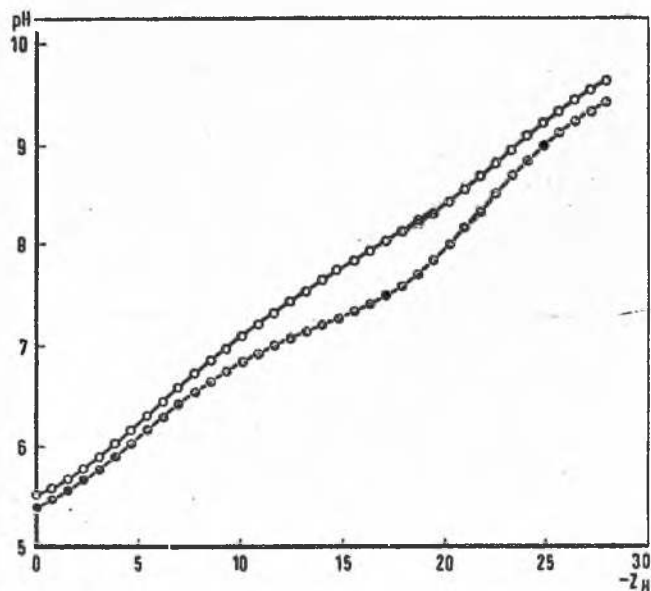


FIGURE 2: Normal titration curves of bovine serum albumin in KCl (O) or CaCl<sub>2</sub> (●). Ionic strength 0.06; protein concentration 2%; temperature 24.9°.

explanation for the allosteric effect shown by the titration curves, assuming that it triggers some pK shifts. Since, however, the change in conformation, as judged by the optical rotation measurements occurs also when Ca<sup>2+</sup> ions are absent, the influence of Ca<sup>2+</sup> binding on the proton affinity must consequently be interpreted as a direct influence of these ions on the conformational change.

The groups which show a pK shift are very probably imidazole groups, because this kind of groups are mainly titrated in the neutral region, though the single terminal amino group cannot be excluded.

*A priori* it is also possible that the pK shift of the imidazole groups is only caused by binding of calcium ions and that the conformational change has nothing to do with it. Supposing that the number of binding sites for calcium ions is the same in the two conformations of albumin, this explanation can be ruled out by the following reasoning. According to De Bruin and Van Os (1968) we can write for the reciprocal of the buffer capacity of a native protein the relation

$$-\frac{dpH}{dZ_H} = 0.868w + \frac{1}{2.303 \sum n_i \alpha_i (1 - \alpha_i)} \quad (1)$$

where  $n_i$  is the number of groups of class  $i$ ,  $\alpha_i$  the degree of ionization of these groups, and  $w$  (Tanford, 1966) the electrostatic interaction factor. Suppose now that the only consequence of Ca<sup>2+</sup> binding is that some groups change their pK, then the question arises: Is it reasonable to expect the above mentioned low value for  $-(\Delta pH/\Delta Z_H)$  (*viz.*, 0.080) at the minimum of the curve in Figure 3?

Equation 1 learns in this respect that in the pH region considered (the imidazole region) the lowest possible value for  $-dpH/dZ_H$  is obtained when all imidazole groups are identical, which means that they have the same pK, and when  $\alpha_{Im} = 0.5$ ; in the neutral region the ionizations of other groups are all very near to zero or to one and do not contribute therefore to the sum in the denominator of eq 1;  $n_{Im}$  as given by the amino acid analysis (Spahr and Edsall, 1964) is 18; at  $I =$

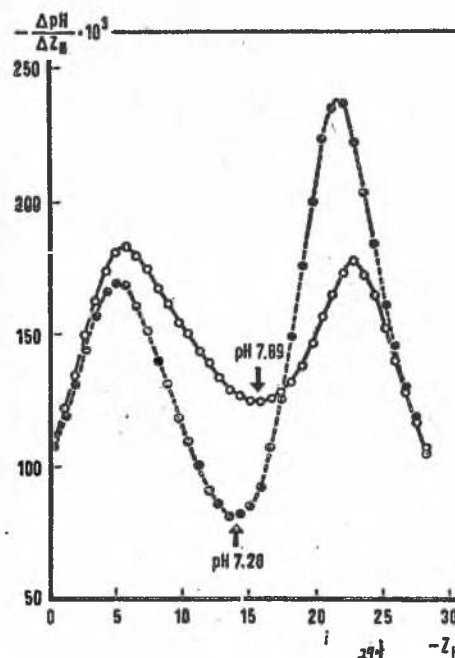


FIGURE 3: Differential titration curves of bovine serum albumin in KCl (O) or CaCl<sub>2</sub> (●). Ionic strength 0.06; protein concentration 2%; temperature 24.9°.

0.06, 0.868 $w$  is about 0.030 (Tanford, 1966). So we calculated that the lowest value of  $-(dpH/dZ_H) = 0.130$ , which is much higher than experimentally is found. This big difference between calculated and experimental buffer capacity rules out in our opinion the above explanation.

It is still possible that Ca<sup>2+</sup> ions do indeed influence directly the pK of imidazole groups, but that due to the fact that Ca<sup>2+</sup> ions bind presumably more to the high pH conformation than to the low pH form, the shift in pK is mainly found in the high pH conformation. In this way the change in conformation remains effectively involved. In fact, this explanation is indistinguishable from our first proposal. However it does not answer the question, why imidazole groups have an abnormally high pK value in the low pH conformation. To answer this question the existence of salt bridges has to be assumed and combined with the change in conformation this makes our first proposal more likely. That the change in conformation is the direct cause of pK shifts is also supported by the fact that

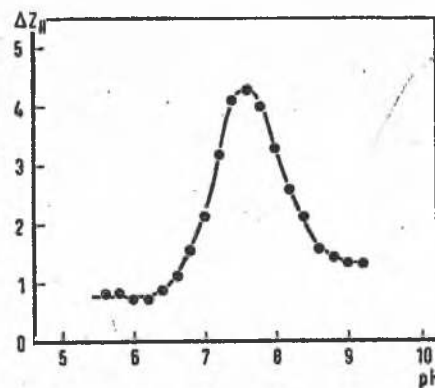


FIGURE 4: Difference in protons bound by bovine serum albumin in the presence and absence of calcium ions. The curve was obtained from the data of Figure 2.

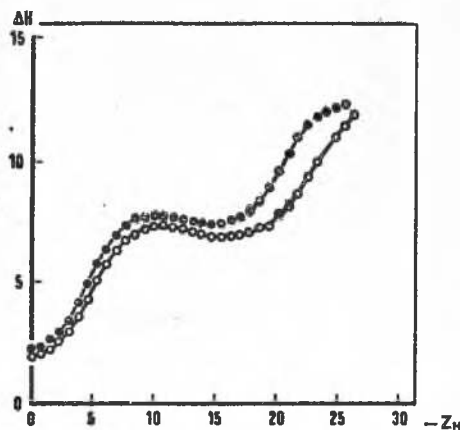


FIGURE 5: Heat of dissociation  $\Delta H$  (kcal/mole) of bovine serum albumin in KCl (O) or  $\text{CaCl}_2$  (●).  $\Delta H$  was calculated from the titration curves measured at 24.9 and 5.0°.  $I = 0.06$ .

in absence of  $\text{Ca}^{2+}$  ions the change in conformation also shifts to lower pH upon increasing the ionic strength only (Leonard *et al.*, 1963) and that also in that case a crossing-over of the differential titration curves is found (S. H. De Bruin *et al.*, unpublished data).

That a change in  $pK$  of imidazole groups due to a change in conformation combined with an influence on it of  $\text{Ca}^{2+}$  ions can explain the anomalously high buffer capacity can easily be understood if it is taken into consideration that an abrupt  $pK$  shift of a group—from a high to a lower value—forces this group to release its proton very suddenly, and this requires an extra amount of base for being neutralized.

This phenomenon is also observed when deoxyhemoglobin is oxygenated; due to the accompanying shift in  $pK$  of the Bohr groups NaOH has to be added to keep the pH constant. In other words, we are confronted in this case with an infinite buffer capacity. The main difference with albumin is that the change in conformation is not induced by the binding of  $\text{Ca}^{2+}$  ions only, as the binding of oxygen does in the case of hemoglobin, but that only the pH region of the transition and its sharpness are influenced by these ions.<sup>1</sup>

That the groups involved are imidazole groups is strongly supported by the curves shown in Figure 5, which represent the heat of proton dissociation  $\Delta H$  vs.  $Z_H$  at constant ionic strength in  $\text{CaCl}_2$  or KCl, respectively. It is seen that in both cases between  $Z_H = -8$  and  $-17$ , where due to the binding of  $\text{Ca}^{2+}$  ions the buffer capacity is sharply increased,  $\Delta H$  is about 7 kcal, a value characteristic for imidazole groups. The slight difference in position of the two curves is probably systematic in origin and does not interfere with our conclusion concerning the identity of the groups, which show a  $pK$  shift.

To show that our explanation given so far can serve as a description of the anomalous titration behavior and especially of the high buffer capacity of albumin after  $\text{Ca}^{2+}$  binding, we

<sup>1</sup> The phenomena described were also studied under more physiological conditions. Two solutions were compared which contained the same concentrations of NaCl and KCl as in bovine serum. To one of them also 5 mequiv of  $\text{CaCl}_2$  and 2.5 mequiv of  $\text{MgCl}_2$  were added per l. while to the other solution so much NaCl was added that the total ionic strength of both solutions was the same ( $I = 0.16$ ). The albumin concentration was 29.3 g/l. The values of  $pH_{50}$ ,  $\Delta pH_{10,90}$ , and  $-\Delta pH/\Delta Z_H$  (in the minimum of the titration curve) were 7.65, 0.8, and 0.103 for the Ca and Mg containing solution and 8.11, 1.6, and 0.126 for the other solution. This indicates that also under physiological conditions similar phenomena are observed as described in the other experiments.

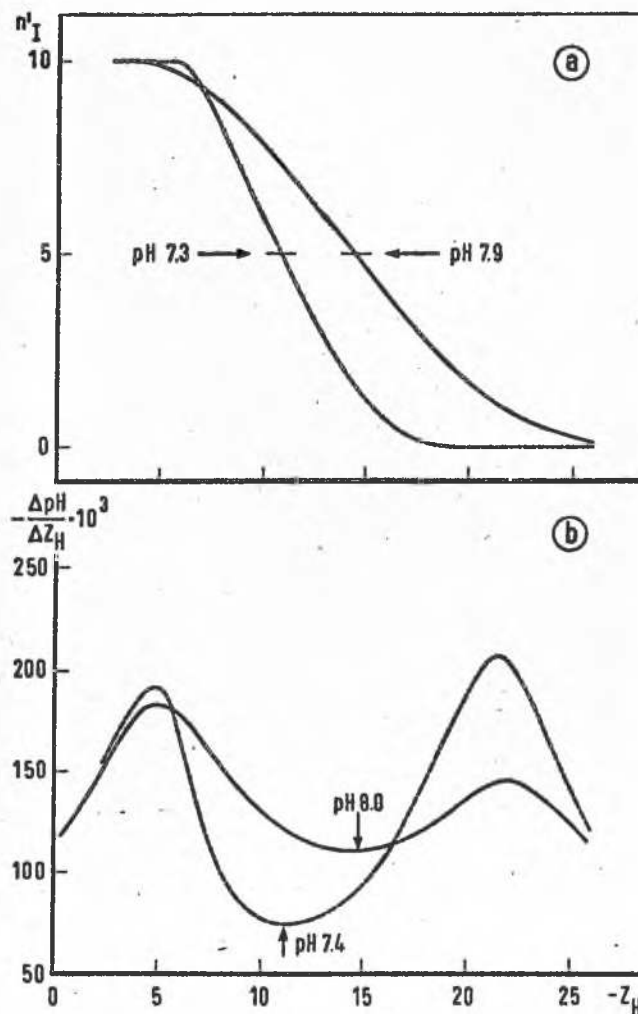


FIGURE 6: Calculation of the influence on the differential titration curves of the unmasking of imidazole groups in the presence and absence of calcium ions. The unmasking was supposed to proceed as given in part a. (a) The number of masked imidazole groups ( $n'_I$ ) as a function of  $Z_H$ . The drawn line and the broken line represent the situation in KCl and in  $\text{CaCl}_2$ , respectively. (b) Differential titration curves calculated according to eq 2 and based on a change of  $n'_I$  with  $Z_H$  as given by the corresponding curve in part a.

have calculated differential titration curves in presence and absence of  $\text{Ca}^{2+}$  ions based on this explanation.

As model equation we used the relation<sup>2</sup>

$$-\frac{dpH}{dZ_H} = 0.868w + \frac{1 + (\alpha_B - \alpha_A) \frac{dn_{Im}'}{dZ_H}}{2.303 \sum_i n_i \alpha_i (1 - \alpha_i)} \quad (2)$$

<sup>2</sup> The way eq 2 was derived can be indicated as follows. At each pH the proton charges  $Z_H^A$  and  $Z_H^B$  of the low and high pH components, respectively, are given by

$$Z_H^A = Z_{max} - \sum_i \alpha_i n_i - \alpha_A n'_{Im} \quad (3)$$

and

$$Z_H^B = Z_{max} - \sum_i \alpha_i n_i - \alpha_B n'_{Im} \quad (4)$$

where  $Z_{max}$  is the maximum positive proton charge, which is the same for both conformations and  $n'_{Im}$  is the real number of imidazole groups of one macromolecule that undergoes a  $pK$  shift during the conforma-

This equation differs from eq 1 by the term  $(\alpha_B - \alpha_A)dn_{Im}'/dZ_H$ ;  $dn_{Im}'$  is the number of imidazole groups, which change their pK upon a change in proton charge  $dZ_H$ ;  $\alpha_A$  and  $\alpha_B$  stand for the degree of ionization of these groups in the low pH conformation (A) and high pH conformation (B), respectively. Because nothing is known about the pK shift, we assumed  $\alpha_A = 0$ , which means that we supposed that the groups involved are really masked in conformation A. We further chose, as is shown in Figure 6a, the pH region of the "unmasking" of the imidazole groups in the liganded and unliganded state in correspondence with the optical rotation data of Figure 1a. Taking a number of 10 imidazole groups as being involved and assigning a pK of 6.9 to both the normal imidazole groups as to those becoming unmasked, the curves in Figure 6b were obtained. In the calculations  $0.868w$  was again chosen equal to 0.030 (Tanford, 1966). It is clear that the curves show very much resemblance to the experimental ones of Figure 3. As the minimum value for  $-(dpH/dZ_H)$  in the presence of  $Ca^{2+}$  ions, we calculate 0.077, while experimentally 0.080 is found. It has to be noted finally that we assumed that  $w$  is not affected by the change in conformation. There is, indeed, no reason to expect a dependence of  $w$  on this change in conformation, because as is said the hydrodynamic parameters of albumin do not change between pH 5 and 10.

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tional change. Suppose that we have  $m_A$  molecules in the low pH conformation and  $m_B$  molecules in the high pH conformation. For the mean proton charge, we can write then

$$Z_H = \frac{Z_H^A m_A + Z_H^B m_B}{m} \quad (5)$$

where  $m = m_A + m_B$  represents the total number of macromolecules.

When at constant pH,  $dm_A$  molecules change their conformation, the corresponding change in proton charge  $dZ_H$  becomes

$$dZ_H = (Z_H^B - Z_H^A) \frac{dm_A}{m} \quad (6)$$

substitution of eq 3 and 4 in 6 gives

$$dZ_H = (\alpha_A - \alpha_B) dn_{Im}' \quad (7)$$

in which  $dn_{Im}' = n_{Im}' dm_A/m$ .  $dn_{Im}'$  is the average number of imidazole groups per macromolecule which undergo a pK shift when  $dm_A$  molecules change their conformation. Equation 2 can now be derived from eq 7 according to the procedure as outlined by De Bruin and Van Os (1968).

#### References

- Aoki, K., and Foster, J. F. (1956), *J. Amer. Chem. Soc.* **78**, 3538.
- Aoki, K., and Foster, J. F. (1957), *J. Amer. Chem. Soc.* **79**, 3385, 3393.
- Barré, R., and Van Huot, N. (1965), *Bull. Soc. Chim. Biol.* **47**, 1399.
- Bloomfield, V. (1966), *Biochemistry* **5**, 684.
- De Bruin, S. H. (1969), Ph.D. Thesis, Nijmegen, The Netherlands.
- De Bruin, S. H., and Van Os, G. A. J. (1968), *Recl. Trav. Chim. Pays-Bas* **87**, 861.
- Decker, R. V., and Foster, J. F. (1967), *J. Biol. Chem.* **241**, 1526.
- Foster, J. F. (1960), in *The Plasma Proteins*, Vol. I, Putnam, F. W., Ed., New York, N. Y., Academic Press, p 179.
- Franglen, G., and Swaniker, G. R. E. (1968), *Biochem. J.* **109**, 107.
- Goldfarb, A. R. (1966), *Biochemistry* **5**, 2574.
- Grams, R., Hallaway, B. E., Neiderloh, J., and Benson, E. S. (1967), *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **26**, 777.
- Harmsen, B. J. M. (1970), Ph.D. Thesis, Nijmegen, The Netherlands.
- Herskovits, T. T., and Laskowski, Jr., M. (1962), *J. Biol. Chem.* **237**, 2481.
- Janssen, L. H. M., De Bruin, S. H., and Van Os, G. A. J. (1970), *Biochim. Biophys. Acta* **221**, 214.
- Katz, S., and Klotz, I. M. (1953), *Arch. Biochem. Biophys.* **44**, 351.
- Leonard, W. J., Vijai, K. K., and Foster, J. F. (1963), *J. Biol. Chem.* **238**, 1984.
- Ohkubo, A. (1969), *J. Biochem. (Tokyo)* **65**, 879.
- Pederson, D. M., and Foster, J. F. (1969), *Biochemistry* **8**, 2357.
- Perutz, M. F. (1970), *Nature (London)* **228**, 726.
- Peters, T., and Hawn, C. (1967), *J. Biol. Chem.* **242**, 1566.
- Sogami, M., and Foster, J. F. (1968), *Biochemistry* **7**, 2172.
- Spahr, P. F., and Edsall, J. T. (1964), *J. Biol. Chem.* **239**, 850.
- Tanford, C. (1966), *Physical Chemistry of Macromolecules*, New York, N. Y., Wiley, p 526.
- Tanford, C., and Buzzell, J. G. (1956), *J. Phys. Chem.* **60**, 225.
- Vijai, K. K., and Foster, J. F. (1967), *Biochemistry* **6**, 1152.
- Wyman, J. (1968), *Quart. Rev. Biophys.* **1**, 35.