SOME ASPECTS OF STRUCTURE-FUNCTION RELATIONS OF HUMAN HEMOGLOBIN

H.S. ROLLEMA

SOME ASPECTS OF STRUCTURE-FUNCTION RELATIONS OF HUMAN HEMOGLOBIN

SOME ASPECTS OF STRUCTURE-FUNCTION
RELATIONS OF HUMAN HEMOGLOBIN

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN
DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE
KATHOLIEKE UNIVERSITEIT VAN NIJMEGEN, OP GEZAG
VAN DE RECTOR MAGNIFICUS PROF. DR. A.J.H. VENDRIK,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP DONDERDAG
18 NOVEMBER 1976 DES NAMIDDAGS TE 4.00 UUR

DOOR

HARRY SYBREN ROLLEMA

GEBOREN TE RIGA

Aan mijn ouders
Aan Henneke, Olav en Mariecke

CONTENTS

[1] Reproduction of Biochem. Biophys. Res. Commun. 58, 204-209 (1974),
    permitted by Academic Press Inc., New York.

[2] Reproduction of Biochem. Biophys. Res. Commun. 58, 210-215 (1974),
    permitted by Academic Press Inc., New York.

[3] Reproduction of J. Biol. Chem. 250, 1333-1339 (1975), permitted
    by The American Society of Biological Chemists, Inc.

[4] Reproduction of Biophys. Chem. 4, 223-228 (1976), permitted
    by North-Holland Publishing Company, Amsterdam.

[5] Reproduction of FEBS Lett. 61, 148-150 (1976), permitted by North-
    Holland Publishing Company, Amsterdam.

[6] Reproduction of Biochem. Biophys. Res. Commun. (1976), in the press,
    permitted by Academic Press Inc., New York.

[7] Submitted for publication.

CHAPTER 1

1.1   GENERAL INTRODUCTION

Since the 19th century the properties of the respiratory protein hemoglobin
have been studied extensively in order to obtain information on the relation-
ship between its structure and function. At the present a vast amount of
data is available.

In this chapter a number of subjects which have relevance to the follow-
ing chapters are presented. For a more detailed treatise it is referred to
a number of extensive reviews covering several fields of hemoglobin research
(1-6).

Since in this thesis studies concerning only human hemoglobin are pre-
sented the term hemoglobin will be used to denote human hemoglobin.

1.1.1   Structure of hemoglobin

Hemoglobin is a tetrameric globular protein with a molecular weight of
64,500. The tetramer consists of two types of polypeptide chains denoted by
α and β. Using this notation, hemoglobin can be represented by $\alpha_2\beta_2$. The α
and β chains differ in primary structure. The α chain contains 141 amino
acid residues, the β chain 146 residues. The amino acid composition of both
chains is given in Table 1.
Each polypeptide chain carries a heme group, an iron-protoporphyrin IX complex,
the structure of which is shown in Fig. 1. The iron atom of each heme group
is covalently bound to the polypeptide chain and ligands are bound to the
iron atom at the sixth coordination site. The heme iron can occur either
in the ferrous or in the ferric state. In both states several types of
ligands can be bound. A number of derivatives are summarized in Table 2.
From the X-ray crystallographic studies of Perutz and his colleagues the
three-dimensional structure of hemoglobin has become known in great detail
(9-15). The secondary structure of both the α and β chains shows eight
helical regions separated by random coil segments. The tertiary structure
of the α and β chains of hemoglobin resembles very much the tertiary struc-
ture of myoglobin, a muscle heme protein, the structure of which is shown in
Fig. 2.
The heme group is found in a cleft between the E and F helices, the so called

TABLE 1

The amino acid composition of the
α and β chain of human hemoglobin (7).

Amino acid	α chain	β chain
Ala	21	15
Arg	3	3
Asn	4	6
Asp	8	7
Cys	1	2
Gln	1	3
Glu	4	8
Gly	7	13
His	10	9
Leu	18	18
Lys	11	11
Met	2	1
Phe	7	8
Pro	7	7
Ser	11	5
Thr	9	7
Trp	1	2
Tyr	3	3
Val	13	18
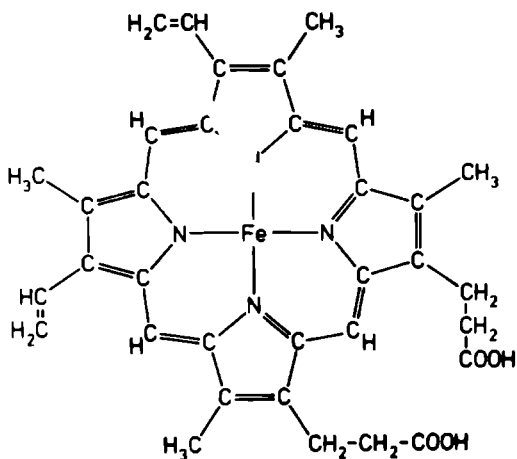
Fig. 1. Heme group

TABLE 2

Some hemoglobin derivatives

	ligand	nomenclature
ferrous derivatives	none	deoxyhemoglobin
	O_2	oxyhemoglobin
	CO	carboxyhemoglobin
	NO	nitrosylhemoglobin
ferric derivatives	H_2O, OH^-	aquo-, hydroxymethemoglobin*
	CN^-	cyanomethemoglobin
	F^-	fluoromethemoglobin
	N_3^-	azidomethemoglobin

*for the ionization of the watermolecule a pK value of 8.1 is found (8)
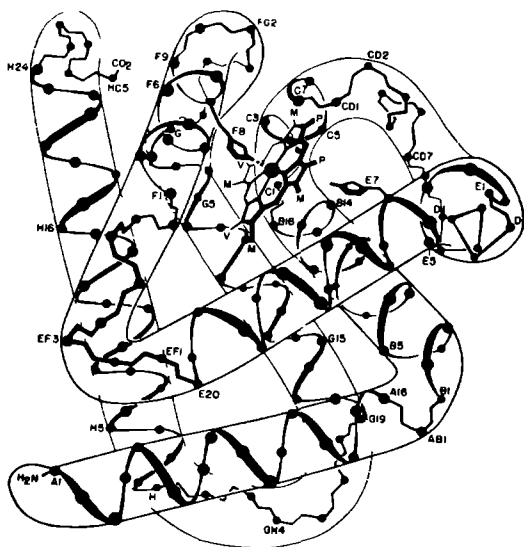
Fig. 2. The tertiary structure of sperm whale myoglobin (reproduced by permission from R.E. Dickerson, The Proteins 2, 603-778, H. Neurath ed., Academic Press, New York, London). The α-carbon positions are represented by dots. Helical regions are indicated by letters. Random coil segments are indicated by two letter symbols corresponding with the two adjacent helical regions. The position of the residues NA1 and NA2 is not given.

11

heme pocket. Apart from the covalent bond between His F8 (i.e. the eighth residue in helix F) and the heme iron a number of van der Waals contacts exists between the heme and the polypeptide chain.

A schematic representation of the spatial arrangement of the four chains (the quaternary structure) in hemoglobin is given in Fig. 3, showing that the molecule has a two-fold axis of symmetry.
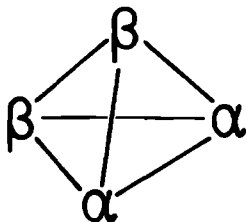
Fig. 3. Schematic representation of the spatial arrangement of the four polypeptide chains in hemoglobin.

The four heme groups are placed at the corners of an irregular tetrahedron. X-ray crystallographic data have shown that hemoglobin is able to adopt two different quaternary conformations which are commonly designated R and T. The R quaternary structure was observed for the first time in crystals of horse methemoglobin (10,11). Unligated hemoglobin possesses the T quaternary structure (12-16). Recently it has been shown that although different ligated forms have the same quaternary structure, the subunits show differences in tertiary structure dependent on the type of ligand bound (17-19). The transition from the T state to the R state involves a rotation of the individual subunits concomitant with small translations of the subunits relative to each other. In the T structure a number of salt bridges are present which break upon the transition to the R structure. Differences between the R and T conformations are also observed in the interchain contacts.

The trigger for the change in quaternary structure occurring upon ligation of deoxyhemoglobin has been postulated to be a displacement of the iron atom relative to the plane formed by the four pyrrollic nitrogens of the heme group (20). This hypothesis is based on the crystallographic studies on ferric porphyrin complexes of Hoard et al. (21), in which it is shown that upon a low spin-high spin transition the iron is displaced from the

plane of the porphyrin. According to Perutz, in deoxyhemoglobin the heme
iron is high spin and is situated 0.7 Å out of the plane of the heme group,
while in oxyhemoglobin the iron is low spin and has an in plane position.
However, recently Eisenberger et al. (22) have reported that there are
strong indications that in oxy- and deoxyhemoglobin the iron atom is in the
same position relative to the plane of the heme group. Consequently the
trigger for the change in quaternary structure should be explained in terms
of differences in heme-subunit interactions between deoxy- and oxyhemoglobin.

1.1.2   Oxygen binding properties

In oxygen binding studies the degree of saturation Y, is measured as a
function of the oxygen pressure, p_{O_2} . Binding curves of this type obtained
for hemoglobin and for the isolated α chain are shown in Fig. 4.
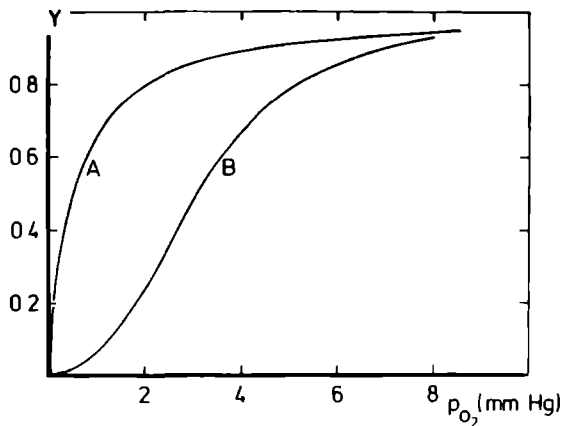
Fig. 4.   Oxygen binding curves of isolated α chains (curve A) and
          hemoglobin (curve B).

Clearly the curves have quite different shapes. The binding of oxygen
to the α chain is represented by a hyperbolic curve, which can be de-
scribed by one binding constant. The curve for hemoglobin has a sigmoidal
shape, which implies that an increase of the number of ligands bound
causes an increase in oxygen affinity. In other words after oxygen is bound
to one binding site the affinity of the other sites increases resulting in
cooperative ligand binding. This kind of interactions between binding sites
for the same ligand are generally referred to as homotropic interactions.

Commonly the log p_{50} value (where p_{50} is the oxygen pressure at half satura-
tion) is used as a measure of the oxygen affinity. Alternatively oxygen
binding data are often presented according to Hill (23), by plotting
log Y/(1-Y) versus log p_{O_2}. An example of such a Hill plot is shown in Fig. 5.
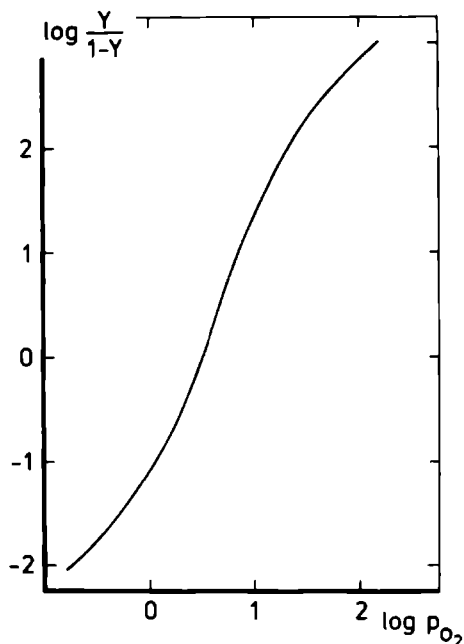
Fig. 5. Hill plot for the oxygen binding to hemoglobin.

The slope of the curve at Y = 0.5 is known as the Hill parameter n. When
n>1 there is positive cooperativity. In the absence of any interaction
between equivalent binding sites the Hill plot shows a straight line with
unit slope.
The oxygen affinity of hemoglobin depends on a number of effectors, e.g.
hydrogen ions and organic phosphates like DPG (2,3-diphosphoglycerate) and
IHP (myo-inositolhexaphosphate).
These interactions, between binding sites for different ligands, are known
as heterotropic interactions. A protein showing homo- and heterotropic
interactions which are mediated by structural changes is called an
allosteric protein.
The dependence of the log p_{50} value on the pH, known as the Bohr effect,
is shown in Fig. 6.
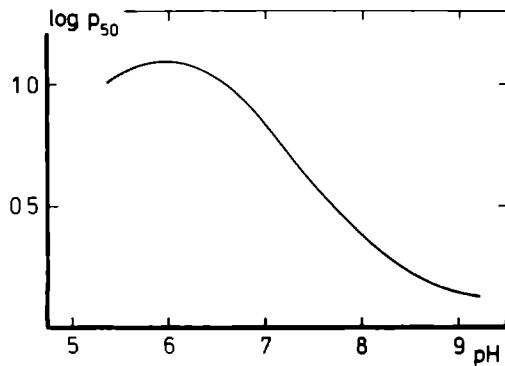
Fig. 6.   The pH dependence of the oxygen affinity
          of hemoglobin.

The dependence of the oxygen affinity on pH means that deoxy- and oxy-
hemoglobin have different proton affinities. In other words at constant
pH deoxy- and oxyhemoglobin differ in the number of protons bound. This
difference is shown in Fig. 7.
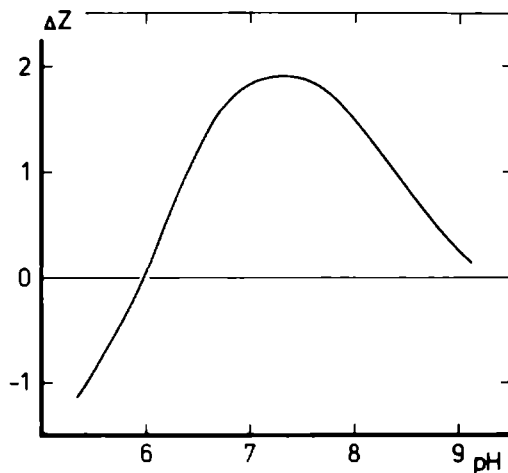
Fig. 7.   The difference in number of protons bound
          by deoxy- and oxyhemoglobin (ΔZ) as a
          function of pH.

At pH values above 6.0 oxygenation results in proton release (alkaline Bohr effect), below pH 6.0 proton uptake is observed (acid Bohr effect). The curves shown in Fig. 6 and Fig. 7 are related by the equation (24)

$$\frac{\partial \log p_{50}}{\partial pH} = -\frac{1}{4} \Delta Z$$

where ΔZ is the difference in the number of protons bound by deoxy- and oxyhemoglobin.

Organic phosphates like DPG and IHP (DPG is found in human erythrocytes with a molar ratio of 1:1 to hemoglobin) are known to lower the oxygen affinity of hemoglobin by preferential binding to the unligated form (25-28). Crystallographic studies have shown that the binding site for these phosphates in deoxyhemoglobin is formed by a cluster of eight positively charged groups of the β chains, located at the entrance of the central cavity (29,30).

The binding properties of other heme ligands like carbon monoxide and nitric oxide are similar to those observed for oxygen. There are however distinct differences in affinity.

1.1.3   Kinetics of ligand binding

The cooperativity in the binding of heme ligands, observed in equilibrium studies, will be reflected in the kinetics of the ligand binding. The binding of oxygen and carbon monoxide shows an autokatalytic time course, while the dissociation rate constants increase with decreasing degree of ligation (1).

The association rate constant for the binding of carbon monoxide to the R state is about ten times larger than the association rate constant for the T state. In rapid mixing experiments a rate constant for the carbon monoxide binding to deoxyhemoglobin of about 10^5 $M^{-1}s^{-1}$ is observed (31). Flash photolysis studies show that after hemoglobin has three ligands bound the fourth binds with a rate constant of about 10^6 $M^{-1}s^{-1}$ (32).

For nitric oxide cooperativity is observed in the kinetics of the dissociation reaction only (33).

1.1.4   Allosteric models for the functional behaviour of hemoglobin

In 1965 Monod, Wyman and Changeux (34) presented a model for the allosteric behaviour of enzymes. In this model it is assumed that the protein consists of a number of equivalent subunits and that the protein occurs in two con- formations with different ligand affinities. Within a given conformation all binding sites have the same intrinsic affinity towards ligands.
Applying this model to hemoglobin the two conformations are the T and the R quaternary state. In both quaternary states hemoglobin can bind up to 4 ligands. Characterizing the molecule in the T or R conformation having i ligands X bound by T_i or R_i (i=0,...4) respectively the system can be represented by the following set of equilibria:

$$R_i \rightleftharpoons T_i$$

$$R_{j-1} + X \rightleftharpoons R_j \qquad\qquad T_{j-1} + X \rightleftharpoons T_j \quad (j=1,...4)$$
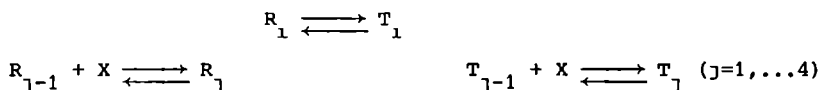
A quantitative description is obtained by introducing the microscopic ligand dissociation constants K_R for the R state and K_T for the T state and the equilibrium constant L for the equilibrium between R_O and T_O:

$$L = [T_O]/[R_O]$$

With $c = K_R/K_T$ and $\alpha = [X]/K_R$ the following saturation function is obtained

$$Y = \frac{(1+\alpha)^3 + L\alpha c(1+\alpha c)^3}{(1+\alpha)^4 + L(1+\alpha c)^4}$$

Using this equation ligand binding curves of hemoglobin can be fitted satis- factorily.
The switch-over point i_s, i.e. the value at which $[T_i] = [R_i]$, is given by

$$i_s = - \frac{\log L}{\log c}$$

A number of extensions of this two state model are known. Extensions have been proposed where the nonequivalence of the α and β chains is taken into account (35-37) or where a third conformational state is assumed to provide a satisfactory description of the influence of allosteric effectors on the oxygenation properties of hemoglobin (38).
In contrast to the model of Monod, Wyman and Changeux (MWC model) in which

cooperativity in ligand binding is assumed to be caused by a change in quaternary conformation in 1966 Koshland, Némethy and Filmer have presented a model (KNF model), where the assumption is made that ligand binding to a subunit introduces a conformational change in the subunit only (39). In this model the affinity of the other subunits is affected by a change in inter-actions between subunits.

Experiments carried out in the period after the presentation of both models have led to a preference for the two state MWC model.

A stereochemical mechanism explaining the functional properties of hemoglo-bin in terms of detailed conformational changes has been presented by Perutz (20). The mechanism is based on the two quaternary structures observed for hemoglobin in X-ray crystallographic studies. Perutz assumes that within a given quaternary state the subunits can exist in two tertiary structures corresponding to the ligated and the unligated state.

A thermodynamic treatment based on this stereochemical mechanism has been presented by Szabo and Karplus (40).

1.1.5   Artificial intermediates

In order to obtain information about the properties of the half ligated state of hemoglobin the so called artificial intermediates are of great interest. Artificial intermediates or hybrids are hemoglobin molecules in which the hemes of the α and the β chains are in different states. The term hybrid is also used for hemoglobin molecules with polypeptide chains of different mammalian hemoglobins. It became possible to prepare artificial intermediates after Bucci and Fronticelli reported that chemical modification of the -SH groups in hemoglobin with p-chloromercuribenzoate results in a dissociation of the tetramer into monomers (41). After separation, the α and β chains can be prepared in different states. Recombination of the chains yields the desired intermediate.

Intermediates with carbon monoxide or oxygen as ligand are not stable be-cause the rate of dissociation for these ligands is too fast.

The only relatively stable intermediates in which both types of chains are in the ferrous form are $\alpha_2^{NO}\beta_2$ and $\alpha_2^{NO}\beta_2$ (42). However, since it has been shown that the behaviour of nitrosylhemoglobin differs significantly from the behaviour of oxy- or carboxyhemoglobin (43-45) the nitrosyl intermediates can no longer be taken as being representative of hemoglobin half ligated with CO or O_2.

Very stable artificial intermediates are obtained by mixing one chain in the ferric form with its partner chain in the ferrous form. These intermediates are called valency hybrids. The functional properties of the valency hybrids has been the subject of many studies (46-56). In the absence of inorganic or organic phosphates valency hybrids show little cooperativity in oxygen binding but a significant Bohr effect (48,49,54). Experiments on the kinetics of the binding of carbon monoxide to valency hybrids (53) show that in the absence of phosphates the hybrids react predominantly with a rate characteristic of the R state.

Using NMR techniques, Ogawa and Shulman (50) have shown that in the absence of phosphates the cyanomet valency hybrids with unligated ferrous heme groups have the quaternary R structure. Upon addition of DPG $\alpha_2^{+CN}\beta_2$ assumes the T state while for $\alpha_2\beta_2^{+CN}$ the R to T transition occurs only upon addition of IHP.

1.2 INTRODUCTION TO THE FOLLOWING CHAPTERS

1.2.1 Influence of organic phosphates on the Bohr effect

In addition to the effect on the oxygen affinity, organic phosphates have been shown to influence the Bohr effect of hemoglobin (57-59). The change in Bohr effect induced by organic phosphates is due to a difference in interaction of these organic phosphates with deoxy- and oxyhemoglobin. Up to 1973 it was assumed that only the interaction of phosphates with deoxy-hemoglobin was important, while that with oxyhemoglobin could be neglected. In contrast to this commonly accepted opinion it has recently been shown that DPG binding to oxyhemoglobin contributes significantly to the DPG induced change in the Bohr effect (60,61). Furthermore strong indications have been presented that oxy- and deoxyhemoglobin have the same binding site for organic phosphates (62).

This influence of organic phosphates on the Bohr effect can be described according to the following scheme (60):

$$Hb \xrightarrow{\Delta z_1} HbO_2$$

$$\Bigg\downarrow \Delta z_2 \qquad\qquad \Bigg\downarrow \Delta z_3$$

$$Hb\ P \xrightarrow{\Delta z_4} HbO_2\ P$$
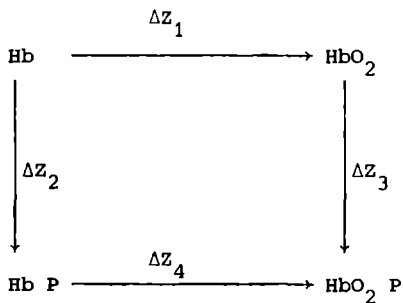
where P stands for the organic phosphate and $\Delta z_1, \ldots \Delta z_4$ represents the
number of protons released per tetramer in the several reaction steps as
indicated. From the scheme it follows that

$$\Delta z_4 - \Delta z_1 = \Delta z_3 - \Delta z_2$$

It is seen that the additional phosphate induced Bohr effect $(\Delta z_4 - \Delta z_2)$
is caused by a difference in interaction of the phosphate with oxy- and
deoxyhemoglobin $(\Delta z_3 - \Delta z_2)$.
The influence of DPG on the Bohr effect of human hemoglobin is shown in
Fig. 8.
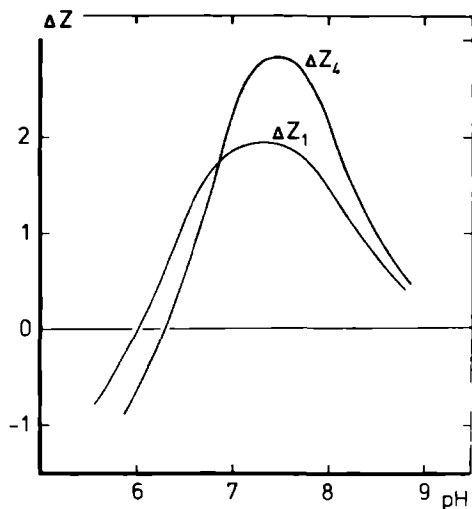
Fig. 8. The Bohr effect in the presence (Δz_4) and
the absence of DPG (Δz_1).

In Fig. 9 ΔZ_2 and ΔZ_3 are given as a function of pH.
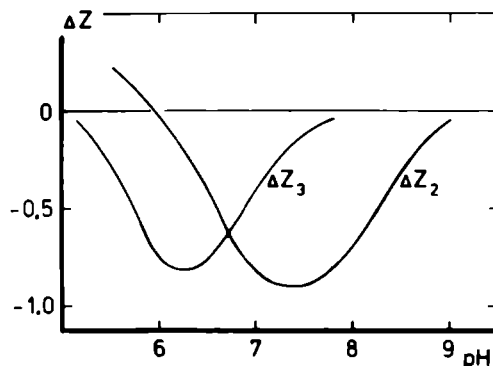
Fig. 9. Number of protons released upon the binding of DPG to oxy- (ΔZ_3) and deoxyhemoglobin (ΔZ_2).

In chapter 2 and 3 of this thesis it is shown that a model analogous to the one outlined above can also describe the influence of chloride ions on the Bohr effect.

1.2.2 Molecular mechanism of the Bohr effect

Since the discovery of the Bohr effect a large number of studies have been performed in order to clarify the molecular mechanism of this effect. With respect to the acid Bohr effect it has been suggested that a number of carboxyl groups are responsible for it (20,63,64). However, no direct crystallographic or chemical evidence has been presented as to the identity of the acid Bohr groups. It will be shown in chapter 3 and 4 of this thesis, that the acid Bohr effect observed between pH 5.5 and 6.0 is predominantly due to a difference in interaction of chloride ions with oxy- and deoxyhemoglobin.

On the other hand in relation to the alkaline Bohr effect a number of groups have been identified as Bohr groups. Chemical modification of the α-amino groups of the α chain results in a reduction of about 25% in the alkaline Bohr effect, suggesting that this group is partly responsible for the Bohr effect (65). Moreover X-ray crystallographic data (14,15) show that in deoxyhemoglobin the α-amino group of Val 1α forms a salt-bridge with the carboxyl group of Arg 141α of the opposite α chain. This

saltbridge is disrupted when deoxyhemoglobin is converted to the ligated
state. As a result the pK of the α-amino group is lowered leading to a
release of protons.

In 1973 Kilmartin et al. (66) presented evidence that His 146β is involved
in the Bohr effect. From a comparison of the NMR spectra of normal hemo-
globin with those of des His-hemoglobin (i.e. hemoglobin where the His
146β residues have been removed by digestion with carboxypeptidase B) they
were able to determine the pK value of His 146β in deoxy- and oxyhemoglobin.
The pK changes for Val 1α and His 146β observed upon ligation of deoxyhemo-
globin can account for about 75% of the alkaline Bohr effect.

So far only suggestions about the identity of the missing Bohr group re-
sponsible for the remaining part of the alkaline Bohr effect have been
presented (20). Since chloride ions contribute substantially to the alkaline
Bohr effect (chapters 3 and 4 of this thesis) it can be hypothesized that a
difference in interaction of chloride ions with oxy- and deoxyhemoglobin is
responsible for this remaining part of the Bohr effect.

Besides the problem of the identity of the Bohr groups the question exists
whether the T to R transition is the only mechanism for the release of
Bohr protons. It is not yet clarified whether the change in tertiary struc-
ture, which the subunits show upon ligation, may also cause a release of
protons. Experiments with mutant and chemically modified hemoglobins in which
the stability of one of the two quaternary structures is affected, point to
a linkage of the Bohr effect to the change in quaternary structure (2, 67).

In 1975 Imai and Yonetani (68) showed that the four Adair constants used
for the description of the binding of oxygen to hemoglobin appear to have
different pH dependences. This result indicates that the number of protons
released is not the same for the four stages of oxygenation. Nevertheless
in equilibrium studies it has been observed that at neutral pH the release
of Bohr protons is linear in the degree of ligation (69,70). This must
be attributed to the strong cooperativity of the oxygen binding as a result
of which the populations of the non ligated and fully ligated states
dominate the populations of intermediate states.

Kinetic experiments, where the contribution of partially ligated states
is significant have also shown that the release of Bohr protons is linear
in the degree of ligation (71-73). These experiments therefore suggest
that the Bohr effect is related to changes in tertiary structure of the
subunits rather than to the change in quaternary structure. Based on an

investigation of the Bohr effect of valency hybrids in the presence and the absence of organic phosphates an identical conclusion is arrived at in chapter 5 of this thesis.

For the monomeric Chironomus thummi thummi hemoglobin also a Bohr effect has been found, indicating that this effect is not an exclusive property of tetrameric hemoglobins (74). Moreover the isolated α and β chains of human hemoglobin possess a small but definite Bohr effect (chapter 6 of this thesis). The acid Bohr effect of the chains is comparable in magnitude to that observed for tetrameric hemoglobin.

A general representation of the Bohr effect within a two state MWC model has been presented by Shulman et al. (14). In this model n_T protons are released upon binding of a ligand to hemoglobin in the T state, n_R protons upon binding of a ligand to the protein in the R state, while n_i protons are released upon the transition from T_i to R_i. The parameters n_T, n_R and n_i can be expressed in the MWC parameters as follows:

$$n_T = - \frac{d \log K_T}{d \, pH}$$

$$n_R = - \frac{d \log K_R}{d \, pH}$$

$$n_O = - \frac{d \log L}{d \, pH}$$

$$n_i = n_O + i(n_R - n_T) \qquad (i=0,\ldots.4)$$

Following this scheme, experiments of Imai and Yonetani (67) indicate that n_R is small. This is in contrast to the observed Bohr effect for the cyanomet valency hybrids (48,49,54, chapter 5 of this thesis) which are believed to be for the greater part in the R state when unligated (50). For aquomet hybrids, which have a Bohr effect nearly identical to that observed for the cyanomet valency hybrids this problem does not exist because from kinetic experiments presented in chapter 7 of this thesis indications are obtained that ligand free aquomet valency hybrids are in the T state.

1.2.3 Kinetic properties of partially ligated states of human hemoglobin

In chapter 7 of this thesis a study of the kinetic behaviour of methemo-globin partially reduced by hydrated electrons is presented. The kinetics of the binding of carbon monoxide to these molecules provide information about their quaternary structure. The results show that at neutral pH reduction of two heme groups is required to induce the change from the R to the T state. This indicates that unligated aquomet valency hybrids are in the T state.

In chapter 8 of this thesis the kinetic properties of partially and fully reduced valency hybrids are reported. Total reduction of these hybrids results in the formation of the carbon monoxide intermediates $\alpha_2\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2$. These are real intermediates in the sense that they occur during the ligation process of deoxyhemoglobin with carbon monoxide.

Comparison of the kinetic behaviour of these intermediates with the kinetic properties of cyanomet valency hybrids, which up to now have been used as model systems for the half ligated state of hemoglobin, shows that there are differences in behaviour between the carbon monoxide intermediates and the cyanomet valency hybrids.

REFERENCES

1. Antonini, E. and Brunori, M. (1970) Ann. Rev. Biochem. $\underline{39}$, 977-1042.

2. Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland Publishing Company, Amsterdam.

3. Kilmartin, J.V. and Rossi-Bernardi, L. (1973) Physiol. Rev. $\underline{53}$, 836-890.

4. Weissbluth, M. (1974) Hemoglobin, Cooperativity and Electronic Properties, Springer-Verlag Berlin, New York.

5. Shulman, R.G., Hopfield, J.J. and Ogawa, S. (1975) Quart. Rev. Biophys. $\underline{8}$, 325-420.

6. Edelstein, S.J. (1975) Ann. Rev. Biochem. $\underline{44}$, 209-232.

7. Dayhoff, M.O. (1969) Atlas of protein sequence and structure 4, National Biomedical Research Foundation, Silver Spring, Maryland, USA.

8. Brunori, M., Amiconi, G., Antonini, E., Wyman, J., Zito, R. and Rossi Fanelli, A. (1968) Biochim. Biophys. Acta, $\underline{154}$, 315-322.

9. Perutz, M.F., Rossmann, M.G., Cullis, A.F., Muirhead, H., Will, G. and North, A.C.T. (1960) Nature $\underline{185}$, 416-422.

10. Perutz, M.F., Muirhead, H., Cox, J.M., Goaman, L.C.G., Mathews, F.S., McGandy, E.L. and Webb, L.E. (1968) Nature $\underline{219}$, 29-32.

11. Perutz, M.E., Muirhead, H., Cox, J.M. and Goaman, L.C.G. (1968) Nature $\underline{219}$, 131-139.

12. Muirhead, H., Cox, J.M., Mazzarella, L. and Perutz, M.F. (1967) J. Mol. Biol. $\underline{28}$, 117-156.

13. Bolton, W., Cox, J.M. and Perutz, M.F. (1968) J. Mol. Biol. $\underline{33}$, 283-297.

14. Muirhead, H. and Greer, J. (1970) Nature $\underline{228}$, 516-519.

15. Bolton, W. and Perutz, M.F. (1970) Nature $\underline{228}$, 551-552.

16. Fermi, G. (1975) J. Mol. Biol. $\underline{97}$, 237-256.

17. Deatherage, J.F., Loe, R.S., Anderson, C.M. and Moffat, K. (1976) J. Mol. Biol. $\underline{104}$, 687-706.

18. Heidner, E.J., Ladner, R.C. and Perutz, M.F. (1976) J. Mol. Biol. $\underline{104}$, 707-722.

19. Deatherage, J.F., Loe, R.S. and Moffat, K. (1976) J. Mol. Biol. $\underline{104}$, 723-728.

20. Perutz, M.F. (1970) Nature $\underline{228}$, 726-739.

21. Hoard, J.L., Hamor, M.J., Hamor, T.A. and Caughey, W.S. (1965) J. Amer. Chem. Soc. $\underline{87}$, 2312-2319.

22. Eisenberger, P., Shulman, R.G., Brown G.S. and Ogawa, S. (1976) Proc. Nat. Acad. Sci. USA $\underline{73}$, 491-495.

23. Hill, A.V. (1910) J. Physiol. $\underline{40}$, IV-VII.

24. Wyman, J. (1948) Advan. Prot. Chem. $\underline{4}$, 407-531.

25. Benesch, R. and Benesch, R.E. (1967) Biochem. Biophys. Res. Commun. 26, 162-167.

26. Chanutin, A. and Curnish, R.R. (1967) Arch. Biochem. Biophys. 121, 96-102.

27. Benesch, R., Benesch, R.E. and Yu, C.I. (1968) Proc. Nat. Acad. Sci. USA 59, 526-532.

28. Tyuma, I., Imai, K. and Shimizu, K. (1971) Biochem. Biophys. Res. Commun. 44, 682-686.

29. Arnone, A. (1972) Nature 237, 146-149.

30. Arnone, A. and Perutz, M.F. (1974) Nature 249, 34-36.

31. Gibson, Q.H. (1959) Progr. Biophys. Biophys. Chem. 9, 1-54.

32. De Young, A., Tan, A.L., Pennelly, R.R. and Noble, R.W. (1975) Biophys. J. 15, 80a.

33. Moore, E.G. and Gibson, Q.H. (1976) J. Biol. Chem. 251, 2788-2794.

34. Monod, J., Wyman, J. and Changeux, J.P. (1965) J. Mol. Biol. 12, 88-118.

35. Ogata, R.T. and McConnell, H.M. (1971) Cold. Spr. Harb. Symp. Quant. Biol. 36, 325-335.

36. Ogata, R.T. and McConnell, H.M. (1972) Proc. Nat. Acad. Sci. USA, 69, 335-339.

37. Ogata, R.T. and McConnell, H.M. (1972) Biochemistry 11, 4792-4799.

38. Minton, A.P. and Imai, K. (1974) Proc. Nat. Acad. Sci. USA 71, 1418-1421.

39. Koshland, D.E., Némethy, G. and Filmer, D. (1966) Biochemistry 5, 365-385.

40. Szabo, A. and Karplus, M. (1972), J. Mol. Biol. 72, 163-197.

41. Bucci, E. and Fronticelli, C. (1965) J. Biol. Chem. 240, PC551-PC552.

42. Antonini, E., Brunori, M., Wyman, J. and Noble, R.W. (1966) J. Biol. Chem. 241, 3236-3238.

43. Cassoly, R. (1975) J. Mol. Biol. 98, 581-595.

44. Salhany, J.M., Ogawa, S. and Shulman, R.G. (1975) Biochemistry 14, 2180-2190.

45. Maxwell, J.C. and Caughey, W.S. (1976) Biochemistry 15, 388-396.

46. Ogawa, S., McConnell, H.M. and Horwitz, A. (1968) Proc. Nat. Acad. Sci. USA 61, 401-405.

47. Banerjee, R. and Cassoly, R. (1969) J. Mol. Biol. 42, 337-349.

48. Banerjee, R. and Cassoly, R. (1969) J. Mol. Biol. 42, 351-361.

49. Brunori, M., Amiconi, G., Antonini, E., Wyman, J. and Winterhalter, K.H. (1970) J. Mol. Biol. 49, 461-471.

50. Ogawa, S. and Shulman, R.G. (1972) J. Mol. Biol. 70, 315-336.

51. Maeda, T. and Ohnishi, S. (1971) Biochemistry 10, 1177-1180.

52. Maeda, T., Imai, K. and Tyuma, I. (1972) Biochemistry 11, 3685-3689.

53. Cassoly, R. and Gibson, Q.H. (1972) J. Biol. Chem. 247, 7332-7341.

54. Banerjee, R., Stetzkowski, F. and Henry, Y. (1973) J. Mol. Biol. 73, 455-467.

55. Bauer, C., Henry, Y. and Banerjee, R. (1973) Nature New Biology 242, 208-209.

56. Ogawa, S. and Shulman, R.G. (1971) Biochem. Biophys. Res. Commun. 42, 9-15.

57. Bailey, J.E., Beetlestone, J.G. and Irvine, D.H. (1970) J. Chem. Soc. (A), 756-762.

58. de Bruin, S.H., Janssen, L.H.M. and van Os, G.A.J. (1971) Biochem. Biophys. Res. Commun. 45, 544-550.

59. Kilmartin, J.V. (1973) Biochem. J. 133, 725-733.

60. de Bruin, S.H., Janssen, L.H.M. and van Os, G.A.J. (1973) Biochem. Biophys. Res. Commun. 55, 193-199.

61. Kilmartin, J.V. (1974) FEBS Lett. 38, 147-148.

62. Brygier, J., de Bruin, S.H., van Hoof, P.M.K.B. and Rollema, H.S. (1975) Eur. J. Biochem. 60, 379-383.

63. Antonini, E., Wyman, J., Brunori, M., Fronticelli, C., Bucci, E. and Rossi-Fanelli, A. (1965) J. Biol. Chem. 240, 1096-1103.

64. Rossi-Bernardi, L. and Roughton, F.J.W. (1967) J. Biol. Chem. 242, 784-792.

65. Kilmartin, J.V. (1972) in Oxygen affinity of Hemoglobin and Red Cell Acid-Base Status: Alfred Benzon Symp. IV, 93-100, Munskgaard, Copenhagen.

66. Kilmartin, J.V., Breen, J.J., Roberts, G.C.K. and Ho, C. (1973) Proc. Nat. Acad. Sci. USA 70, 1246-1249.

67. Kilmartin, J.V., Hewitt, J.A. and Wootton, J.F. (1975) J. Mol. Biol. 93, 203-218.

68. Imai, K. and Yonetani, T. (1975) J. Biol. Chem. 250, 2227-2231.

69. Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronticelli, C. and Rossi-Fanelli, A. (1963) J. Biol. Chem. 238, 2950-2957.

70. Tyuma, I. and Ueda, Y. (1975) Biochem. Biophys. Res. Commun. 65, 1278-1283.

71. Antonini, E., Shuster, T.M., Brunori, M. and Wyman, J. (1965) J. Biol. Chem. 240, PC2262-PC2264.

72. Gray, R.D. (1970) J. Biol. Chem. 245, 2914-2921.

73. Olson, J.S. and Gibson, Q.H. (1973) J. Biol. Chem. 248, 1623-1630.

74. Sick, H. and Gersonde, K. (1969) Eur. J. Biochem. 7, 273-279.

CHAPTER 2

THE INTERACTION OF 2,3-DIPHOSPHOGLYCERATE WITH HUMAN
DEOXY- AND OXYHEMOGLOBIN

Simon H. de Bruin, Harry S. Rollema, Lambert H.M.
Janssen and Gerard A.J. van Os

Department of Biophysical Chemistry,
University of Nijmegen, Toernooiveld, Nijmegen,
The Netherlands

SUMMARY: Binding of 2,3-diphosphoglycerate (DPG) to both deoxy-
hemoglobin (Hb) and oxyhemoglobin (HbO$_2$) is accompanied by an
uptake of protons. A study of this proton uptake as a function
of n, the mole to mole ratio of DPG and hemoglobin, yielded
adsorption isotherms which could be described with one single
association constant. It appeared that at pH 6.8 the proton
uptake per molecule of DPG bound is larger for HbO$_2$ than for Hb.
The data showed that the binding of DPG to HbO$_2$ is functionally
significant.

DPG has a remarkable effect on the oxygen affinity of human
hemoglobin; P_{50} the oxygen pressure at half saturation increases
strongly on addition of DPG (1-3). It is now known that
in addition to this effect DPG also increases both the alkaline
(4-7) and acid Bohr effect (7). In a recent report (8) we have
shown that the increase in alkaline Bohr effect is due to an
uptake of protons which occurs upon binding of DPG to Hb and
that the increase in acid Bohr effect is surprisingly due to an
proton uptake occurring upon binding of DPG to HbO$_2$. These two
results were confirmed by the observations of Kilmartin (9). The
data showed however that at n=1.3 the influence of the binding
of DPG to HbO$_2$ on the Bohr effect could almost be neglected at
pH values above pH 7.3. In this paper we extend our study of
the DPG effect to higher n values, up to a DPG concentration of
5×10^{-3} M. We measured the number of protons taken up upon
a) mixing solutions of Hb and DPG, b) oxygenation of Hb in the
presence of DPG, c) oxygenation of Hb in the absence of DPG
and d) mixing solutions of HbO$_2$ and DPG. Indicating the number

of protons bound per tetramer along the several pathways by ΔZ_a, ΔZ_b, ΔZ_c and ΔZ_d the following equation will hold:

$$\Delta Z_a + \Delta Z_b = \Delta Z_c + \Delta Z_d \tag{1}$$

Since ΔZ_a and ΔZ_d will be proportional to the number of DPG molecules bound, a determination of these quantities as a function of n will yield the association constants of the binding of DPG to Hb and HbO$_2$.

The pH stat procedure has been outlined in our previous report (8). In all experiments the concentration of hemoglobin was 2.5×10^{-4} M per tetramer. The highest DPG concentration used was 5×10^{-3} M (i.e. n=20), which is equal to the DPG concentration in vivo. When protons were bound ΔZ values were given a positive sign.

In Fig. 1 ΔZ_a and ΔZ_d values measured at pH 6.8 have been plotted vs. n. The shape of the two curves corresponds to normal binding isotherms and can be described with a single association constant for both Hb and HbO$_2$ (see below). Without doing a quantitative analysis the data indicate that at pH 6.8 Hb binds DPG stronger than HbO$_2$ and that at this pH the maximum proton uptake upon binding of DPG is at least twice as large for HbO$_2$ as for Hb.
From eqn. 1 it follows that the DPG induced Bohr effect $(\Delta Z_b - \Delta Z_c)$ should be equal to $(\Delta Z_d - \Delta Z_a)$. These two difference quantities have been plotted in Fig. 1 too (lower part); it can be seen that the agreement between the two independent sets of data is very good. The difference curve shows that at low values of n the DPG induced Bohr effect is negative; this is due to the large affinity of DPG to Hb; at high values of n, where the binding of DPG to HbO$_2$ becomes increasingly important the induced Bohr effect is positive.

The full lines of curves a and d were calculated using a non linear least squares fitting procedure. The curves were fitted using two parameters, viz. the association constants for the hemoglobin DPG complex and the maximum values for ΔZ_a and ΔZ_d for n going to infinity. Only one binding site was assumed to be present in both Hb and HbO$_2$. At pH 6.8 we found
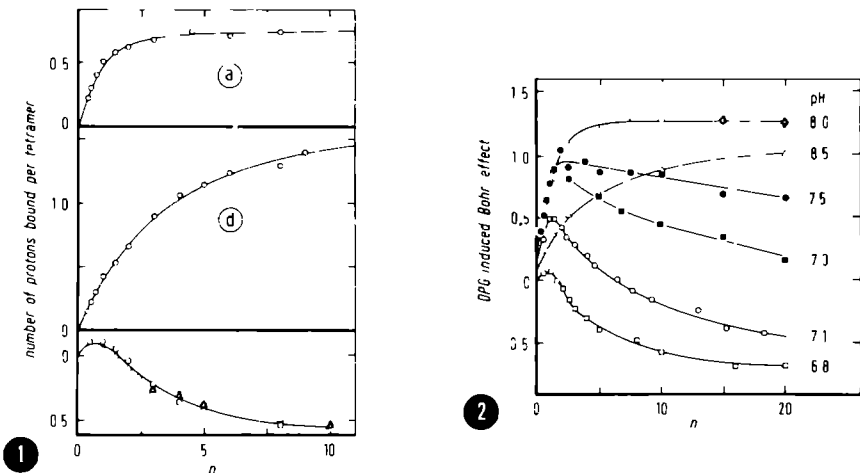
Fig. 1   Number of protons bound upon binding of DPG to Hb
           (curve a) and HbO_2 (curve d); n is the mole to mole
           ratio of DPG and hemoglobin; full lines were calculated
           (see text). In the lower part the DPG induced Bohr
           effect has been plotted: ($\triangle$), directly observed values;
           ($\square$), obtained by subtracting curve a from curve d;
           pH = 6.8, hemoglobin concentration 2.5×10^{-4} M (tetramer
           basis), KCl concentration 0.1 M, temp. 25°C.

Fig.2.   The DPG induced Bohr effect ($\Delta z_b - \Delta z_c$) observed at
           various pH values; these pH values are indicated in
           the figure. KCl concentration 0.1 M; temp. 25°C.

---

for Hb $K_{ass} = 1.7 \times 10^4$ M^{-1}, $\Delta z_a^{max} = 0.77$; for HbO_2 we calculated
$K_{ass} = 1.2 \times 10^3$ M^{-1}, $\Delta z_d^{max} = 1.64$. The fact that our data proved
to be consistent with the assumption of one binding site in both
Hb and HbO_2 is in agreement with the results obtained in direct
binding studies (10-12), although some additional weaker binding
sites have been observed (13, 14). The relatively large difference
of more than a factor 10 between the two association constants is
in better agreement with the results of Benesch and Benesch (10)
and Benesch et al. (11) than with the results reported by Chanutin
and Herman (13) and Garby and Verdier (14).

     In Fig. 2 the DPG induced Bohr effect is shown at various
pH values. The data show that in going from low to high pH the
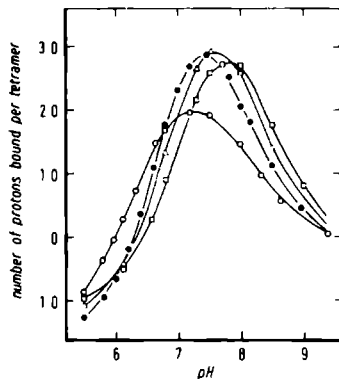contribution to the Bohr effect of the binding to HbO_2 decreases

Fig. 3. The Bohr effect as observed at various values of n:
(o) n=o; (●) n=1.3; (Δ) n=5; (□) n=20; KCl con-
centration 0.1 M; temp. 25°C.

in proportion to the increasing contribution of the binding of
DPG to Hb. Above pH 8 only the latter is observed. It must be
noticed here that our data prove that the mechanism of the enhance-
ment of the alkaline Bohr effect by DPG as proposed by Riggs (15)
was partly correct; in this model it is assumed that both HbO_2
and Hb bind DPG under uptake of protons. However it was also
assumed that the pH dependence of this proton uptake was equal
for both Hb and HbO_2; according to this mechanism only the alkaline
Bohr effect would be affected by the DPG binding to Hb and HbO_2.
This is evidently not the case.

     The curves obtained near the physiological pH show that at
high n values the induced Bohr effect tends to go to zero. This
is consistent with the observation of Benesch et al. (14) that
the values for $\Delta \log p_{50}/\Delta pH$ were identical at high and zero DPG
concentration, while at intermediate concentrations larger values
were observed than at n=0; this phenomenon was explained by
assuming that it was caused by the pH dependence of the binding
of DPG to Hb; our data show that the actual reason is that at
pH 7.3 the two contributions to the Bohr effect of the binding
of DPG to Hb and HbO_2 cancel out.

     Our results invalidate the assumption made in reports on
the influence of DPG on the oxygen saturation curves of hemoglobin
(16, 17) viz. that DPG only binds to Hb and to hemoglobin partial-
ly saturated with one or two ligands. The hemoglobin and DPG con-

centrations used in these oxygenation experiments were such that
comparison is possible to conditions existing at n=10 in the
experiments described in this paper. Fig. 3 clearly shows that
at pH 7.3 the binding of DPG to HbO_2 cannot be considered as
functionally insignificant; from this it follows that the Adair
constants are affected by this binding, which is in contrast
to the assumption mentioned above.

In Fig. 3 Bohr curves are shown at various values of n.
The data show that the curves get displaced to the right when n
increases. At high n values the curve is considerably different
from previously reported curves (6-9) obtained at n values
near one. It might be noted again, that, if the interaction of
DPG with HbO_2 would have been negligible, the increase in alkaline
Bohr effect would have been much larger at neutral pH than
actually is observed. In view of this the conclusion is inevitable
that log p_{50} is strongly influenced by the interaction of DPG
with HbO_2 - note: the difference in log p_{50} between pH 9 and any other
pH can be calculated by integrating the curves shown in Fig. 3
from pH 9 to that pH -.

At pH values below pH 6 we see that at high n values the
curves tend to coincide with the curve measured at n=0; we think
that this is due to the fact that in this pH region protons are
released when DPG binds to Hb, whereas above this pH protons are
taken up (8).

The nature of the DPG binding site in Hb is well established. It
is at the entrance of the central cavity, where a cluster of
positively charged groups form saltbridges with the negatively
charged groups of DPG (18). In preliminary experiments we studied
the influence of the presence of DPG on the reactivity of the
$\alpha-NH_2$ group of the α chain. In the presence of DPG we found a
diminished reactivity. As a result we are inclined to think
that the $\alpha-NH_2$ group of the α chain is involved in the binding
of DPG in HbO_2. If this is true, HbO_2 has two identical binding
sites for DPG. It will be obvious that the simulated curve for
the binding of DPG to HbO_2 as shown in Fig. 1 can be fitted
equally well assuming two identical sites with a maximum value
for Δz_d^{max} of 0.82 per site instead of 1.64 in the case of one
binding site.

In a previous paper we have shown that the DPG binding site
in HbO_2 must be absent in Hb (8). The $\alpha-NH_2$ group of the α chain

fulfills this requirement; for in HbO_2 this group is free to move, whereas in Hb it forms a saltbridge with the carboxyl group of Arg HC3 (141)α(18). More experiments will be needed to establish the nature of the DPG binding site in HbO_2.

REFERENCES

1. Benesch, R., and Benesch, R.E. (1967) Biochem. Biophys. Res. Commun., 26, 162-167.
2. Chanutin, A., and Curnish, R.R. (1967) Arch. Biochem. Biophys. 121, 96-102.
3. Tyuma, I., Shimuzu, K., and Imai, K. (1971) Biochem. Biophys. Res. Commun. 43, 423-428.
4. Benesch, R.E., Benesch, R., and Yu, C.I. (1969) Biochemistry 8, 2567-2571.
5. Tomita, S., and Riggs, A. (1971) J. Biol. Chem. 246, 547-554.
6. Bailey, J.E., Beetlestone, J.C., and Irvine, D.H. (1970) J. Chem. Soc. Sect. A, 756-762.
7. De Bruin, S.H., Janssen, L.H.M., and Van Os, G.A.J. (1971) Biochem. Biophys. Res. Commun. 45, 544-550.
8. De Bruin, S.H., Janssen L.H.M., and Van Os, G.A.J. (1973) Biochem. Biophys. Res. Commun. 55, 193-199.
9. Kilmartin, J.V. (1974) FEBS Letters 38, 147-148.
10. Benesch, R., and Benesch, R.E. (1969) Nature 221, 618-622.
11. Benesch, R.E., Benesch R., Renthal, R., and Gratzer, W.B. (1971) Nature, New Biol. 234, 174-176.
12. Caldwell, P.R.B., Nagel, R.L., and Jaffe, E.R. (1971) Biochem. Biophys. Res. Commun. 44, 1504-1509.
13. Garby, L., Gerber, G., and De Verdier, C.H. (1969) Eur. J. Biochem. 10, 110-115.
14. Chanutin, A., and Hermann, E. (1969) Arch. Biochem. Biophys. 131, 180-184.
15. Riggs, A. (1971) Proc. Nat. Acad. Sci. U.S.A. 68, 2062-2065.
16. Tyuma, I., Imai, K., and Shimuzu, K. (1973) Biochemistry, 12, 1491-1498.
17. Imai, K., and Tyuma, I. (1973) Biochim. Biophys. Acta 293, 290-294.
18. Perutz, M.F. (1970) Nature 228, 726-739.

CHAPTER 3

THE INTERACTION OF CHLORIDE IONS
WITH HUMAN HEMOGLOBIN

Simon H. de Bruin, Harry S. Rollema, Lambert H.M.
Janssen and Gerard A.J. van Os

Department of Biophysical Chemistry,
University of Nijmegen, Toernooiveld, Nijmegen,
The Netherlands

SUMMARY: Studying the effect of KCl on the Bohr effect of human
hemoglobin, it appeared that at low Cl$^-$ concentration the alkaline
Bohr effect is considerably smaller than it is at a Cl$^-$ ion concen-
tration near 0.1 M. The data show that at least part of the Bohr
effect, that thus far could not be attributed to a particular
residue in hemoglobin, is due to interaction of hemoglobin with
anions. The effect of KCl on the Bohr effect shows a striking
similarity with the effect of 2,3-diphosphoglycerate (DPG) on the
Bohr effect. Based on this a mechanism is proposed which satisfacto-
rily explains the observed salt effect.

Recently (1) we have shown that the effect of DPG on the Bohr
effect can be attributed to the fact that binding of DPG to both
deoxyhemoglobin (Hb) and oxyhemoglobin (HbO$_2$) is accompanied by an
uptake of protons. It was established that the binding of DPG to
HbO$_2$ increases the acid Bohr effect (or decreases the alkaline
Bohr effect) whereas the binding to Hb enhances the alkaline Bohr
effect. These results were confirmed by Kilmartin (2). Studying
the influence of high salt concentrations on the proton dissocia-
tion behaviour of Hb and HbO$_2$, we recently observed (unpublished
results) that surprisingly the free energy of saltbridges occurring
in Hb and thought to be responsible for the Bohr effect was not in-
fluenced by high concentrations of univalent salt. This weakening
of the salt bridges at high ionic strength has long been assumed
to occur (3-6). In view of these results and the fact that
high salt concentration decreases the oxygen affinity of hemo-
globin in a way similar to DPG (7), it can be hypothesized that the
influence of salt on the Bohr effect as observed by Antonini et al.
(3) and ourselves (unpublished results) might equally well be

attributed to a different interaction of univalent anions with Hb
and HbO_2, respectively. We present therefore in this paper prelimi-
nary results concerning the influence of chloride ions on the Bohr
effect at various pH values.

The measurements were carried out following the pH stat proce-
dure described earlier (1). With this method the number of protons
released upon oxygenation of Hb are measured. Isoionic solutions
of hemoglobin freed from DPG (1) were adjusted to a known Cl^- ion
concentration (KCl, Merck, suprapur). Starting from the isoionic
point pH values were adjusted with HCl or NaOH. The Cl^- ion concen-
trations were corrected for the small amounts of HCl added. In all
experiments the hemoglobin concentration was 1.6×10^{-4} M on tetra-
mer basis. The measurements were carried out at $25^{\circ}C$.

Fig. 1 shows the dependence on the chloride concentration of
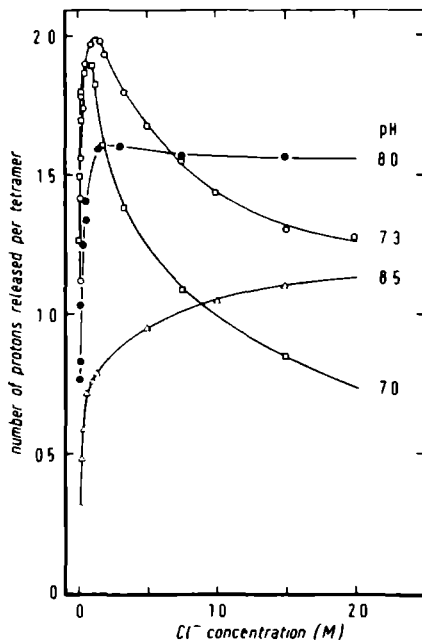

Fig. 1.   The dependence on the chloride ion concentration
           of the number of protons released upon oxygenation of
           deoxyhemoglobin. The experiments were carried out
           at pH 7.0 (□), pH 7.3 (o), pH 8.0 (•) and pH 8.5 (Δ).

the number of protons released upon oxygenation of Hb. All curves
obtained show a striking similarity with the curves obtained
studying the dependence of the Bohr effect on the DPG concentration
(see preceding paper). Also in the presence of Cl^- ions the curves
resulting from measurements at pH 7.0 and pH 7.3 at first show a
sharp increase in the number of protons released followed by a
rather gradual decrease. Similarly at pH 8 and 8.5 a strong increase
in the number of released protons is at first observed at low Cl^-
concentration, but above a certain salt concentration the curves
tend to level off at these pH values. Similar behaviour was seen
when the influence of DPG on the Bohr effect was examined. In the
case of this DPG effect we were able to elucidate the mechanism causing
it. The most important feature of this mechanism is that the binding
of DPG to both Hb and HbO_2 is accompanied by an uptake of protons.
It was possible to prove this since solutions of DPG can be added
to solutions of Hb or HbO_2 while keeping the ionic strength con-
stant. However this kind of experiments cannot be carried out with
KCl. The model we propose for the influence of Cl^- ions on the
Bohr effect will therefore be based on the observed similarity
in behaviour of Cl^- ions and DPG as far as the influence on the
Bohr effect and oxygen affinity (7) is concerned. The model is
identical to that which proved to be valid for the interaction of
DPG with hemoglobin. It can be formulated as follows.
a] Chloride ions bind to positively charged groups in both Hb
and HbO_2; b] due to this binding the pK of these positively
charged groups is increased which means that upon binding of
Cl^- ions protons are taken up; c] the groups to which chloride
ions are bound in HbO_2 have a lower pK than the groups which
are the binding sites in Hb; d] chloride ions are weaker bound
to HbO_2 than to Hb.

The above mechanism can explain satisfactorily the shape
of the curves in Fig. 1. The sharp increase in the number of
protons released observed at all pH values is due to a stronger
binding of Cl^- to Hb as compared to the binding of Cl^- to HbO_2.
The decrease at high ionic strength observed in the curves measured
at pH 7.0 and 7.3 is due to the fact that at high Cl^- concentration
the effect of the binding of Cl^- to HbO_2 is counteracting the
contribution of the binding of Cl^- by Hb to the Bohr effect. The
two curves at pH 8 and 8.5 tend to reach a constant level and show
no decrease at high salt concentrations, because at high pH the

groups in HbO_2 which bind Cl ions are then no longer charged and consequently incapable of binding.

The proposed mechanism is supported by the NMR results of Chiancone et al. (8) and Bull et al. (9) who found that Cl^- ions are bound by Hb and HbO_2 and that the ligand affinity of the binding site in Hb was larger than that of the site in HbO_2. From a NMR study on the chloride binding to hemoglobin Abruzzo, in which His (143)β has been replaced by Arg, Chiancone et al. (10) concluded that this histidine may be involved in binding of Cl^- ions.

In Fig. 2 we enlarged part of Fig. 1 up to a Cl^- concentration

Fig. 2.   Enlarged part of Fig. 1 up to a chloride concentration
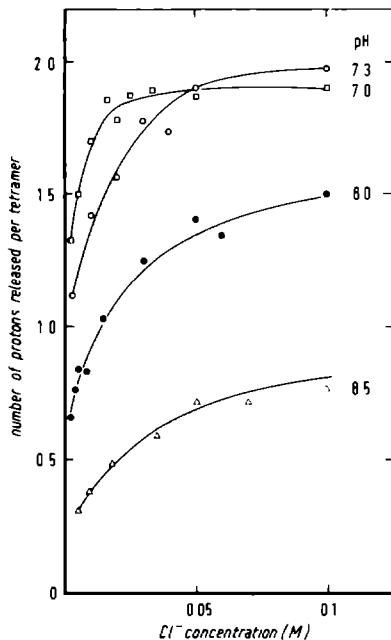   of 0.1 M. For the meaning of the symbols we refer to
           the legend of Fig. 1.

of 0.1 M. The curves drawn for the data obtained at pH 7.3 and 7.0 show the usual value of about two protons released at a KCl concentration near 0.1 M. On going down to KCl concentration of 3×10^{-3} M the number of Bohr protons released decreases strongly

and reaches a value of 60 to 70 percent of the effect measured
at $[Cl^-] = 0.1$ M. The decrease observed at pH 8.0 and 8.5 is
comparatively even larger than observed at the other pH values.
The difference in slope of the curves shown in Fig. 2 support the
proposed mechanism for the interaction of chloride ions with hemo-
globin as outlined above. In going to high pH the slope of the
curves becomes smaller which indicates a decrease in affinity of
Cl^- ions to deoxyhemoglobin upon an increase in pH. This decrease
in affinity has also been observed with DPG. It is caused by the
fact that at high pH groups involved in the binding become ionized
and loose their positive charge so that anion will not be bound
in that pH range.

From the data reported we are led to important conclusion
that part of the Bohr effect measured at $(Cl^-) = 0.1$ M is due
to an interaction of Cl^- ions with deoxyhemoglobin. The effect
measured at $(Cl^-) = 0.1$ M cannot totally be attributed to the
so called Bohr groups, which are positively charged groups forming
saltbridges with negatively charged partners in Hb. In other words
a great part of the Bohr effect is not merely a property of hemo-
globin itself being more or less independent from solvent condi-
tions, but on the contrary a great part of the effect is strongly
related to interaction of hemoglobin with the solute. It might
be noted that our results are consistent with crystallographic
data in so far as up till now a part of the Bohr effect could not
be attributed to any particular saltbridge (11). Perutz has pro-
posed His H5(122)α which forms a saltbridge with Asp H9(126)α
both in Hb and in HbO_2, as a possible Bohr group although they
emphasized that they could not find clear crystallographic evidence
for a change in free energy of the saltbridge upon oxygenation
of deoxyhemoglobin (11).

To conclude we think it should be realized that our conclusion
about the part played by Cl^- and other anions will stand even if
the model proposed would appear not to be correct.

REFERENCES

1. De Bruin, S.H., Janssen, L.H.M., and Van Os, G.A.J. (1973) Biochem. Biophys. Res. Commun. 55, 193-199.
2. Kilmartin, J.V. (1974) FEBS Letters, 38, 147-148.
3. Antonini, E., Wyman, J., Rossi-Fanelli, A., and Caputo, A.J. (1962) J. Biol. Chem. 237, 2773-2777.
4. Perutz, M.F., Muirhead, H., Mazzarella, J., Crowther, R.A., Greer, J., and Kilmartin J.V. (1969) Nature 222, 1240-1243.
5. Thomas, J.O., and Edelstein, S.J. (1973) J Biol. Chem. 248, 2901-2905.
6. Huestis, W.H., and Raftery M.A. (1972) Proc. Nat. Acad. Sci. USA 69, 1887-1891.
7. Benesch, R., and Benesch, R.E. (1967) Biochem. Biophys. Res. Commun. 26, 162-167.
8. Chiancone, E., Nerne, J.E., Forsén, S., Antonini, E., and Wyman, J. (1972) J. Mol. Biol. 70, 675-688.
9. Bull, T.E., Andrasko, J., Chiancone, E., and Forsén, S. (1973) J. Mol. Biol. 73, 251-259.
10. Chiancone, E., Nerne, J.E., Bonaventura, J., Bonaventura, C., and Forsén, S. (1974) Biochim. Biophys. Acta 336, 403-306.
11. Perutz, M.F. (1970) Nature 228, 726-739.

The Effect of Potassium Chloride on the Bohr Effect of Human Hemoglobin*

Harry S. Rollema, Simon H. de Bruin, Lambert H. M. Janssen, and Gerard A. J. van Os

From the Department of Biophysical Chemistry, University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands

SUMMARY

The normal and differential titration curves of liganded and unliganded hemoglobin were measured at various KCl concentrations (0 1 to 2.0 M). In this range of KCl concentrations, the curves for deoxyhemoglobin showed no salt-induced pK changes of titratable groups. In the same salt concentration range oxyhemoglobin showed a marked change in titration behavior which could only be accounted for by a salt-induced increase in pK of some titratable groups. These results show that the suppression of the alkaline Bohr effect by high concentrations of neutral univalent salt is not caused by a weakening of the salt bridges in deoxyhemoglobin but is due to an interaction of chloride ions with oxyhemoglobin.

Measurements of the Bohr effect at various KCl concentrations showed that at low chloride ion concentration (5 × 10⁻¹ M) the alkaline Bohr effect is smaller than at a concentration of 0.1 M. This observation indicates that at a chloride ion concentration of 0.1 M, part of the alkaline Bohr effect is due to an interaction of chloride ions with hemoglobin. Furthermore, at low concentrations of chloride ions the acid Bohr effect has almost vanished. This result suggests that part of the acid Bohr effect arises from an interaction of chloride ions with oxyhemoglobin.

The dependence of the Bohr effect upon the chloride ion concentration can be explained by assuming specific binding of chloride ions to both oxy- and deoxyhemoglobin, with deoxyhemoglobin having the highest affinity.

The quaternary structure of unliganded hemoglobin differs considerably from that of liganded hemoglobin (1). In Hb¹ there are a number of salt bridges which are absent in HbO₂. Some of the positively charged partners of these salt bridges are titrated in the neutral pH range. At pH near 9 these groups are no longer charged and are unable to form salt bridges, causing a

¹ The abbreviations used are Hb, deoxyhemoglobin, HbO₂, oxyhemoglobin, HbCO, carboxyhemoglobin, p_{50}, the oxygen pressure at half-saturation

destabilization of the deoxystructure (T state) with respect to the oxystructure (R state) In other words the allosteric constant, L (2), which describes the equilibrium between the R and T state, is pH dependent Consequently the value of log p_{50} is pH dependent Going from pH 6 to pH 9 log p_{50} decreases This effect is known as the alkaline Bohr effect The pH dependence of log p_{50} is related to ΔZ_B, the difference in the number of protons bound by Hb and HbO₂ by (3)

$$\frac{\partial \log p_{50}}{\partial pH} = -\frac{1}{4} \Delta Z_B \tag{1}$$

On a molecular level this change in proton charge upon ligation is explained as follows During the transition from the T to the R state the salt bridges break up, causing a change in pK of the groups involved in the salt bridges This results in a release of protons at neutral pH Up to now the groups which have been identified as alkaline Bohr groups are His HC3(146)β and Val NA1(1)α, they form salt bridges with Asp FG1(94)β and Arg HC3(141)α, respectively (4–10) These Bohr groups are responsible for about 70% of the total alkaline Bohr effect at a KCl concentration of 0.1 M Perutz has suggested that the still missing Bohr group might be His H5(122)α, forming a salt bridge with Asp H9(126)α in Hb (1) He emphasized, however, that there is no clear crystallographic evidence for a pK shift of this group upon ligation Below approximately pH 6 protons are taken up upon ligation of Hb This is known as the acid Bohr effect

Neutral salts have a marked effect on the oxygen affinity and on the Bohr effect of human hemoglobin (11–13) At increasing salt concentration the oxygen affinity decreases and the Bohr effect is strongly suppressed However, no influence on the value of the Hill parameter is observed

Previously, the suppression of alkaline Bohr effect by high concentrations of a neutral salt has been interpreted as a weakening of the salt bridges, resulting in a destabilization of the deoxystructure (5) On the other hand, Huestis and Raftery (14) recently pointed out that hydrophobic interactions might be important in destabilizing the oxytetramer at high salt concentrations This suggestion was based on the invariancy of the Hill parameter with respect to variations of the ionic strength and on the observation that the oxygen affinity decreases upon increasing ionic strength In a recent short report on the dependence of the alkaline Bohr effect on the chloride ion concentration (15), we showed that the number of Bohr protons re-

leased upon ligation increases in going from a chloride ion concentration of 5×10^{-4} M to a concentration of 0 1 M followed by a decrease at higher concentrations. From this observation we concluded that part of the alkaline Bohr effect which could not be attributed to a particular Bohr group actually arises from interactions of chloride ions with hemoglobin. As a result of our observations on the influence of 2 3-diphosphoglycerate on the Bohr effect (16, 17) we have proposed a model which satisfactorily describes the influence of chloride ions on the Bohr effect. The essential features of the model are (a) chloride ions are bound both to Hb and HbO_2 (b) the affinity of Hb towards chloride ions is larger than that of HbO_2 (c) the positively charged groups to which chloride ions are bound undergo an increase in pK and (d) the groups which bind chloride ions in HbO_2 have a lower pK than those in Hb. Since pK shifts introduced by chloride binding will strongly affect the proton binding behavior of hemoglobin we present in this paper a study of hydrogen ion titration curves of Hb and $HbCO$ at KCl concentrations ranging from 0 1 M to 2 0 M, with additional data on the influence of chloride ions on the Bohr effect. The influence of NaCl on the titration curves of HbO_2 and HbCO has been measured by Antonini et al (18) in 1963. However we re examined these data because removal of 2 3 diphosphoglycerate could have been incomplete at that time and this would have interfered with the results. Although ΔZ_B values as defined by Equation 1 can be obtained by subtracting the hydrogen ion titration curves of Hb and HbO_2 the Bohr data presented in this paper were obtained by a more accurate direct measurement using a pH stat technique

The results indicate that the above model can indeed account for the effect of KCl on the proton binding behavior of hemoglobin in every aspect.

Human hemoglobin was prepared by the toluene method of Drabkin (19). The hemoglobin solutions were dialyzed against distilled water and freed from 2 3 diphosphoglycerate and other ions by repeated passing through a mixed bed ion exchange column (Amberlite IRA 400 and IR 120)

Hydrogen ion titration curves were determined at 25° with automatic titration equipment as described elsewhere (20). This equipment has meanwhile been improved by using a pH meter of very high stability which was built with an electrometer operational amplifier (Analog Devices type 311 K)

In each experiment 4 ml of the hemoglobin solution were brought into the titration vessel. In the experiments with Hb the hemoglobin solutions were deoxygenated in a rotating tonometer while argon was continuously passed over the solution. The time needed to reach equilibrium was about 5 min and complete deoxygenation was checked spectrophotometrically using the molar absorption coefficients reported by Benesch et al (21). The samples were transferred anaerobically to the titration vessel. The solutions were brought to the desired concentration of chloride ions with KCl (Merck Suprapur) and KCl was also added to the titrant to ensure a constant KCl concentration during the measurements

The titrations of Hb were performed under argon those of HbCO were performed under oxygen. Replacement of carbon monoxide by oxygen does not influence the results because the titration behavior of HbCO and HbO_2 is identical. As a reference point for counting Z_H the mean proton charge of the protein we took as usual the isoionic pH. Because a difference titration curve of Hb and HbO_2 is less accurate than a direct measurement of the difference in protons bound by Hb and HbO_2 at constant pH the Bohr curves were measured with a pH stat at 25°

For these experiments we have constructed very sensitive pH stat equipment. The sensitivity of the equipment is such that titrant is added as soon as the pH difference between the actual and the chosen pH amounts to only a few ten thousandths of a pH unit while the rate of titrant addition is proportional to this difference

As titrants HCl and NaOH were used. NaOH was stored in wax coated flasks and kept free of carbonate. The hemoglobin concentration of the solutions was determined by drying to constant weight at 105°. All results shown are averages of at least three experiments carried out with different hemoglobin preparations

Ultracentrifugation was performed with a model E Spinco ultracentrifuge. A synthetic boundary valve type cell was used to facilitate an accurate determination of the boundary position. All runs were performed at 25° at a speed of 67 770 rpm

In Table I the Z_H values for both Hb and HbCO are tabulated as a function of the pH at different salt concentrations. The Z_H values listed for HbCO are rather different from those reported by Antonini et al (18). At a salt concentration of 0 1 M our data indicate a value of 7 25 for the isoionic pH in contrast to a value of 6 8 found by Antonini et al. Moreover the data of Antonini et al give a difference in proton charge between pH 6 0 and pH 9 0 where we established a value of 26 2 which is in agreement with the amino acid composition of human hemoglobin. Generally we find fewer titratable groups at different KCl concentrations. This discrepancy might have been caused by incomplete removal of 2 3-diphosphoglycerate from their hemoglobin preparations

The data show that at constant pH the change in mean proton charge upon increasing ionic strength is larger for HbCO than for Hb. The fact that high salt concentrations affect the proton binding behavior of Hb and HbCO in a different way indicated that the salt induced pK changes are different in Hb and HbCO

It has been pointed out that the shape of a differential titration curve is very sensitive to changes in pK of the protonic groups of the protein. This type of curve is obtained by plotting $\Delta pH/\Delta Z_B$ which is the reciprocal of the buffer capacity, against Z_H

Following the Linderstrøm Lang approximation (22) differential titration curves can be described by

$$ -\frac{\Delta pH}{\Delta Z_H} = \frac{1}{2\ 3\ 3\ \Sigma_1\ r\ \alpha_1\ (\ -\alpha_1)} + 0\ 868\ u \qquad 2 $$

where n is the number of titratable groups of a certain class i (having the same pK) α is the degree of ionization and u is the electrostatic interaction factor (23). Of the two terms on the right side of the equation only the second term is ionic strength dependent. u decreases upon an increase in ionic strength. The Linderstrøm Lang approximation predicts that the differential titration curve will show a vertical shift upon variation in ionic strength without any change in the shape

In Figs. 1 and 2 the differential titration curves at different salt concentrations are shown for Hb and HbCO respectively. The experimental points are not shown to avoid overcrowding of the figures. The two figures show indeed that in going to higher salt concentrations the curves for both Hb and HbCO are lowered. In this respect the proton binding behavior of Hb and HbCO follows the behavior predicted by the Linderstrøm Lang approximation. However the presence of shape invariancy is difficult to judge from the curves presented in the two figures. In order to elucidate these aspects the differences between these curves are shown in Figs. 3 and 4. Fig 3 shows that within the experimental error the differential titration curve for Hb retains its shape at all salt concentrations used. This means that the ionic strength dependence of the proton binding behavior of Hb can entirely be described by the electrostatic interaction

Number of protons bound per tetramer (Z_H) by Hb and HbCO as a function of pH at different KCl concentrations

The hemoglobin concentration was 1.5×10^{-4} M on tetramer basis. The standard deviation of the figures presented in the table amounts to 0.15 Z_H unit

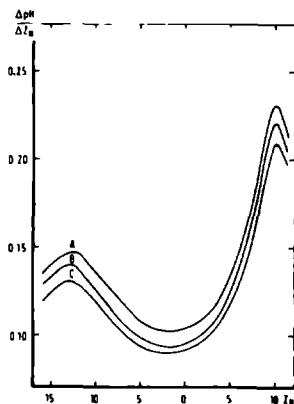| pH	TL				HbCO			
	0.1 M	0.5	1 M	2.0 M	0.1 M	0.5 M	1.0 M	2.0 M
5.4	15.	17.3	16.2	19.0	15.9	18.0	19.1	20.6
5.6	13.8	15.7	16.5	17.1	14.1	16.2	17.2	18.5
6.	12.4	14.1	14.7	15.3	12.3	14.4	15.3	16.4
6.	11.1	12.7	13.1	13.7	10.5	12.6	13.5	14.5
6.4	9.6	11.2	11.6	12.2	8.7	10.7	11.6	12.5
6.6	8.1	9.7	10.0	10.6	6.7	8.8	9.6	10.5
6.8	6.4	7.9	8.3	8.9	4.7	6.7	7.5	8.4
7.	4.6	6.0	6.4	7.0	2.7	4.4	5.3	6.2
7.	2.7	4.0	4.3	4.9	0.8	2.3	2.9	3.8
7.4	0.8	1.6	2.	2.8	-1.1	0.1	0.6	1.4
7.6	-1.1	-0.4	-0.1	0.5	-2.9	-2.1	-1.6	-1.0
7.8	-2.9	-2.3	-2.2	-1.6	-4.5	-3.9	-3.7	-3.2
8.	-4.6	-4.2	-4.1	-3.6	-5.9	-5.6	-5.5	-5.1
8.2	-6.1	-5.8	-5.1	-5.4	-7.	-6.9	-7.0	-6.7
8.4	-7.3	-7.2	-7.	-6.9	-8.1	-8.0	-8.1	-8.0
8.6	-8.1	-8.3	-8.3	-8.1	-8.9	-8.9	-9.1	-9.1
8.8	-9.4	-9.4	-9.	-9.2	-9.7	-9.7	-9.9	-9.9
9.	-10.3	-10.2	-10.3	-10.1	-10.3	-10.5	-10.7	-10.8

FIG 1 The effect of high KCl concentrations on the differential titration curve of Hb *Curve A*, 0 1 M KCl, *Curve B*, 0 5 M KCl, *Curve C*, 2 0 M KCl, hemoglobin concentration is 1.5×10^{-4} M on tetramer basis

factor using the Linderstrøm Lang approximation In other words, it is not necessary to assume that in Hb certain groups show salt induced pK changes The *arrows* in Fig 3 indicate the calculated differences The agreement between the calculated and the measured values is satisfactory. The difference

curves for HbCO are shown in Fig 4 The strong Z_H dependence observed here indicates that the proton binding behavior of HbCO cannot be described by the Linderstrøm Lang equation with the same set of pK values at different salt concentrations It must be stressed here that regardless of any model used the data shown in Fig 4 indicate that in going from low to high salt concentrations a certain amount of buffer capacity is transferred from positive Z_H values to more negative Z_H values In other words, upon an increase in salt concentration, certain titratable groups in HbCO shift their pK to higher values

Since it is known that dissociation of HbCO into dimers occurs at high salt concentrations (24, 25) this anomaly in titration behavior of HbCO might be due to a proton linked dissociation process We therefore measured S_{20} values at different pH values The results presented in Table II show that at all salt concentrations used no pH dependence of the sedimentation coefficient could be detected From these data it can be concluded that there is no proton linked dissociation process that could cause the observed change in proton binding behavior of HbCO

The Bohr curves as obtained by the pH stat method are shown in Figs 5 and 6 The curves measured at KCl concentrations above 0 1 M are shown in Fig 5 Those measured at KCl concentrations below 0 1 M are presented in Fig 6 Fig 5 shows that in going from 0 1 M KCl to higher concentrations, the alkaline Bohr effect decreases while the acid Bohr effect increases As a result, the maximum effect observed is displaced to the right at high salt concentrations Fig 6 on the other hand shows that in going from 0.1 M KCl to 5×10^{-3} M KCl both the acid and the
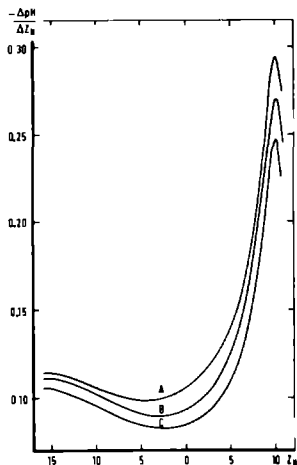
43

FIG 2 The effect of KCl on the differential titration curve of HbCO *Curve A, 0 1 M KCl, Curve B, 0 5 M KCl, Curve C, 2 0 M KCl* Hemoglobin concentration is $1 5 \times 10^{-4}$ M on tetramer basis
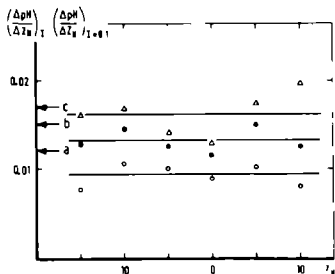

FIG 3 The KCl induced changes in the differential titration curve of Hb The curves were obtained by subtracting the $\Delta pH/\Delta Z_H$ values measured at 0 1 M KCl from those measured at 0 5 M KCl, ○, 1 0 M KCl, ●, and 2 0 M KCl, △ The *arrows* indicate the calculated values based on Equation 2 *a* for 0 5 M KCl, *b* for 1 0 M KCl, and *c* for 2 0 M KCl
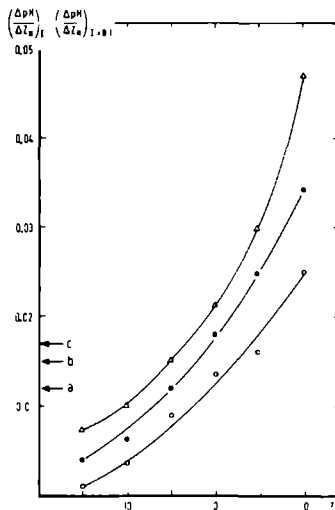

FIG 4 The KCl induced change in the differential titration curve of HbCO The curves were obtained by subtracting the $\Delta pH/\Delta Z_H$ values measured at 0 1 M KCl from those measured at 0 5 M KCl, ○, 1 0 M KCl, ●, and 2 0 M KCl, △ For the meaning of the *arrows* see the legend to Fig 3

TABLE II

Effect of pH and KCl concentration on the S_{20} value of HbCO

Hemoglobin concentration $1 5 \times 10^{-4}$ M on tetramer basis

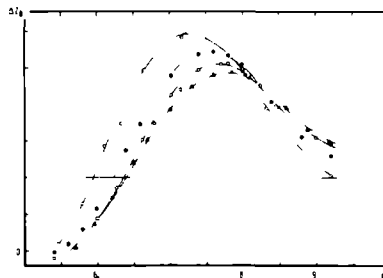| pH	0 1 M	1 0 M	2 0 M
6 3	4 31	3 89	2 74
7 8	4 36	3 63	2 68
9 2	4 53	3 67	2 78

FIG 5 The number of Bohr protons (ΔZ_B) per tetramer as measured at 0 1 M KCl, ○, 0 5 M KCl, ●, 1 0 M KCl, □, and 2 0 M KCl, ▲ Hemoglobin concentration is $1 5 \times 10^{-4}$ M on tetramer basis

alkaline Bohr effect decrease At a KCl concentration of 5×10^{-3} M the maximum alkaline Bohr effect is found near pH 6 8 and amounts to about 70% of the maximum effect observed at pH 7 2 at a KCl concentration of 0 1 M At a chloride ion concentration of 5×10^{-3} M, no acid Bohr effect is observed at pH values greater than 5 5

Fig 7 shows the number of protons released upon oxygenation as a function of the chloride ion concentration at different pH values The curve at pH 5 6 shows that when the salt concentration is lowered ΔZ_B increases and reaches a value near zero at the lowest salt concentration used The curve measured at pH 6 0 shows a similar behavior Here, however, ΔZ_B changes its sign at low salt concentration In going from low to high KCl concentrations the curves measured at the pH values 6 5, 7 0, and 7 4 first show a sharp increase in ΔZ_B followed by a rather slow decrease This decrease is almost absent in the curve

measured at pH 8 0 and is totally absent in the curve obtained at pH 8 5 In Fig 8 we enlarged the part of Fig 7 from zero KCl concentration up to a concentration of 0 20 M Fig 8 clearly shows that the slope of the curves becomes smaller in going to high pH
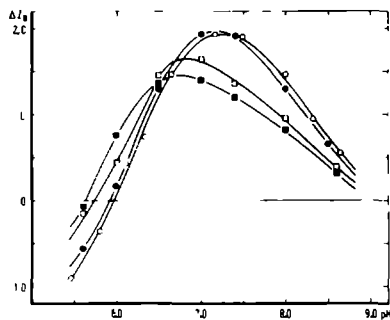
FIG 6 The number of Bohr protons (ΔZ_B) per tetramer as measured at 10^{-1} M KCl ○ 5×10^{-2} M KCl, ● 10^{-2} M KCl □ and 5×10^{-3} M KCl ■ Hemoglobin concentration is 1.5×10^{-4} M on tetramer basis

Fig 9 shows the difference in Bohr effect observed at a salt concentration of 0 1 M and 5×10^{-3} M The curve is very similar to the curve of the additional 2 3 diphosphoglycerate induced Bohr effect, which is the difference in Bohr effect measured in the presence and in the absence of 2,3 diphosphoglycerate (16)

DISCUSSION

It is known that 2 3 diphosphoglycerate and chloride ions at high concentrations have a similar effect on the oxygen affinity of human hemoglobin In order to explain this phenomenon, Benesch et al (12) supposed that chloride ions are more strongly bound to Hb than they are to HbO₂ Chiancone et al (26) and Bull et al (27) showed, using NMR techniques, that chloride ions bind specifically to Hb and HbO₂ and that the binding is proton linked Their results indicate that there are two classes of binding sites differing in affinity toward chloride ions The high affinity sites are oxygen linked in such a way that Hb has a higher affinity towards chloride ions than HbO₂

The curves shown in Figs 7 and 8 indicate that the binding of chloride to hemoglobin is proton linked which is in accordance with the above mentioned NMR experiments In order to show the validity of this view we will assume that due to the negative charge of the chloride ion the positively charged groups of the protein involved in the chloride binding undergo an upward pK shift upon chloride binding This will result in a proton up take by the hemoglobin molecule The maximum effect will occur when the pH is near the pK of the groups involved Indicating the number of protons taken up by Hb and HbO₂ per tetramer upon binding of chloride ions as ΔZ_{deox} and ΔZ_{ox} respectively we can write for the observed Bohr effect ΔZ_B (the number of protons per tetramer released upon oxygenation)

$$\Delta Z_B = \Delta Z_0 + \Delta Z_{decx} - \Delta Z_{ox} \qquad (3)$$

where ΔZ_0 represents the number of protons per tetramer released upon oxygenation of Hb in the absence of salt

The curves in Figs 7 and 8 measured at pH 7 4 and 7 0, show a ΔZ_B value of about 2 0 at KCl concentrations near 0 1 M, at low salt concentrations ΔZ_0 drops to a value of about 1 0 This means that at these pH values ΔZ_0 has a value near unity From this it will be clear that the part of the Bohr effect which up to now could not be attributed to any particular Bohr group in hemoglobin (5, 10) actually arises from binding of chloride ions to Hb and HbO₂

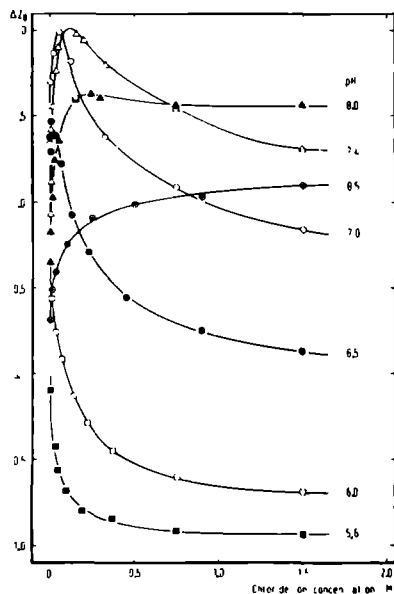
FIG 7 The effect of the chloride ion concentration on the number of Bohr protons measured at pH values as indicated in the figure Hemoglobin concentration is 1.5×10^{-4} M on tetramer basis

The shape of the curves as presented in Figs 7 and 8 can easily be interpreted in terms of Equation 3 Due to the fact that Hb has the highest affinity towards chloride ions ΔZ_{deox} will reach its maximum value at lower chloride ion concentration than will ΔZ_{ox} The chloride binding sites in Hb will be saturated at a much lower KCl concentration than those in HbO₂ As a result ΔZ_B should, after an initial sharp increase, go through a maximum followed by a slower decrease This is indeed observed in all curves except those measured at pH 5 6 and 6 0 apparently ΔZ_{deox} is either too small to be observed or the chloride binding to Hb is so strong that the above mentioned maximum is to be found at KCl concentrations lower than 5×10^{-3} M

The fact that the chloride binding to Hb and HbO₂ becomes weaker at increasing pH values as indicated by the slope of the curves in Figs 7 and 8 is to be expected because the groups to which chloride ions are bound become ionized at high pH, losing their ability to bind chloride ions At pH 8 5, ΔZ_{ox} is even equal to zero

In Fig 9 we plotted the difference in ΔZ_B as measured at 0 1 M and 5×10^{-3} M KCl The shape of the curve is very much like the shape of the one representing the additional 2,3-diphosphoglycerate induced Bohr effect (16) This strongly supports our idea that the action of chloride ions on the Bohr effect is analogous to the influence of 2 3-diphosphoglycerate on the Bohr effect As far as this 2,3-phosphoglycerate influence is concerned we have presented solid evidence that this was due to the binding of 2,3-diphosphoglycerate to both Hb and HbO₂ (16)

Fig 6 shows that the acid Bohr effect decreases at low salt concentrations From this observation it might be concluded
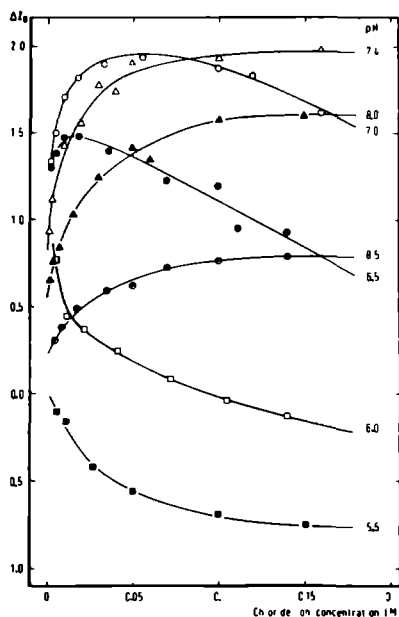
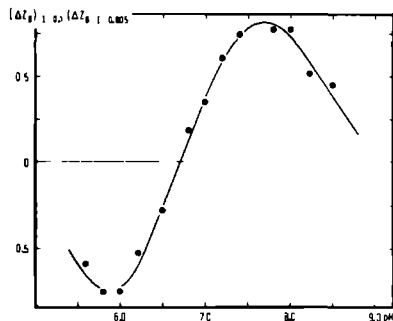FIG 8 Enlarged part of Fig 7 up to a KCl concentration of 0 2 M

FIG 9 Difference in the number of Bohr protons as measured at a KCl concentration of 10^{-1} M and 5×10^{-3} M

that the part of the acid Bohr effect observed between pH 5 5 and 6 0 at 0 1 M KCl arises merely from the interaction of hemo globin with chloride ions

The data shown in Figs 5 and 6 can directly be correlated to the results of a study on the ionic strength dependence of log p_{50} versus pH curves as measured by Antonini et al (13) The Bohr plots presented in this reference show that at pH 6, in going from low to high salt concentrations the slope of the curves changes from positive to negative values According to Equation 1 this is in agreement with our data This agreement is also found at other pH values A similar correspondence is

found with the pH dependence of the p_{50} value at 10^{-3} M and 0 1 M NaCl as reported by Bunn and Guidotti (28)

It is commonly assumed that the carboxyl groups which form salt bridges with the alkaline Bohr groups in Hb are responsible for the acid Bohr effect Our conclusion that part of the acid Bohr effect is due to interaction of HbO_2 with chloride ions is supported by the fact that before now no real explanation has been offered for the abnormally high pK values which these car boxyl groups should have in order to act properly as acid Bohr groups

We now show that the titration data as shown in Figs 3 and 4 can be described in terms of the model proposed It can be shown that if chloride binding occurs, the electrostatic interaction factor w as used in Equation 2 must be replaced by an apparent electrostatic interaction factor w_{app} which is related to w by

$$w_{app} - w \left(1 - \frac{\partial \nu}{\partial Z_H} \right) \tag{4}$$

where ν represents the number of chloride ions bound (29) Considering the pH dependence of the chloride ion binding to HbCO and Hb as shown by Chiancone et al (26), as a first ap proximation in the pH region studied we may write for ν

$$\nu = a Z_H + b \tag{5}$$

where a and b are ionic strength dependent constants Equa tion 4 now becomes

$$w_{app} - w(1 - a) \tag{6}$$

This relation shows that chloride ion binding results in a decrease of the observed electrostatic interaction factor and that w_{app} does not depend on Z_H

To understand the absences of shape invariancy in the differ ential titration curves of HbCO as shown in Fig 4 it must be realized that Equation 2 is also based on the assumption that the intrinsic pK values of the titratable groups do not change upon variation of the salt concentration This is evidently not the case when chloride ions bind to titratable groups The binding of chloride ions to HbCO is still incomplete at 0 1 M KCl A further increase of the chloride ion concentration causes changes in pK values of some titratable groups As a result the quantity plotted in Fig 4 is not independent of Z_H As for Hb however, the chloride binding reaches its saturation level at 0 1 M KCl (Figs 7 and 8) This means that a further increase in chloride ion concentration does not introduce significant pK changes Consequently the difference quantity as plotted in Fig 3 fulfills the Linderström Lang approximation and remains constant throughout the Z_H range studied as indicated by 1 quation 6

To conclude, we should like to point out that apart from any model the data show that upon an increase in KCl concentration from 0 1 M to 2 0 M, in the case of HbCO only, a shift of buffer capacity from low to high pH values takes place This implies that in this range of chloride ion concentrations salt induced pK changes occur in HbCO only

REFFRENCES

1 PERUTZ M F (1970) Nature 228 726 734
2 MONOD J WYMAN J AND CHANGEUX J P (1965) J Mol Biol 12, 88–118

3 WYMAN, J (1948) *Adv Protein Chem* **4**, 407–531
4 PERUTZ, M F (1970) *Nature* **228**, 734 739
5 PERUTZ, M F , MUIRHEAD, H , MAZZARELLA, L , CROWTHER, R A , GREER, J , AND KILMARTIN, J V (1969) *Nature* **222**, 1240–1243
6 MUIRHEAD, H , AND GREER, J (1970) *Nature* **228**, 516–519
7 KILMARTIN, J V , AND WOOTTON, J F (1970) *Nature* **228**, 766–767
8 KILMARTIN, J V , AND ROSSI BERNARDI, L (1969) *Nature* **222**, 1243 1246
9 KILMARTIN, J V , AND ROSSI-BERNARDI, L (1971) *Biochem J* **124**, 31–45
10 KILMARTIN, J V , BREEN, J J , ROBERTS, G C K , AND HO, C (1973) *Proc Nat Acad Sci U S A* **70**, 1246–1249
11 ANTONINI, I , WYMAN, J ROSSI FANELLI, A , AND CAPUTO, A (1962) *J Biol Chem* **237**, 2773–2777
12 BENESCH, R E , BENESCH, R , AND YU, C I (1969) *Biochemistry* **8**, 2567 2571
13 ANTONINI, E , AMICONI, G , AND BRUNORI, M (1972) in *Oxygen Affinity of Hemoglobin and Rea Cell Acid Base Status* (ASTRUP, P , AND RØRTH, M , eds) pp 121–129, Academic Press, New York
14 HUESTIS, W H , AND RAFTERY, M A (1972) *Proc Nat Acad Sci U S A* **69**, 1887–1891
15 DE BRUIN, S H , ROLLEMA, H S , JANSSEN, L H M , AND VAN OS, G A J (1974) *Biochem Biophys Res Commun* **58**, 210–215
16 DE BRUIN S H , JANSSEN, L H M , AND VAN OS, G A J (1973) *Biochem Biophys Res Commun* **55**, 193–199
17 DE BRUIN, S H , ROLLEMA, H S , JANSSEN, L H M , AND VAN OS, G A J (1974) *Biochem Biophys Res Commun* **58**, 204–209
18 ANTONINI, E , WYMAN, J , BRUNORI, M , BUCCI, E , FRONTICELLI, C , AND ROSSI-FANELLI, A (1963) *J Biol Chem* **238**, 2950–2957
19 DRABKIN, D L (1946) *J Biol Chem* **164**, 703–723
20 JANSSEN, L H M , DE BRUIN, S H , AND VAN OS, G A J (1970) *Biochim Biophys Acta* **221**, 214 227
21 BENESCH, R E , BENESCH, R , AND YUNG, S (1973) *Anal Biochem* **55**, 245 248
22 TANFORD, C (1962) *Adv Protein Chem* **17**, 69–165
23 DE BRUIN, S H , AND VAN OS, G A J (1968) *Rec Trav Chim Pays Bas* **87**, 861–872
24 GUIDOTTI, G (1967) *J Biol Chem* **242**, 3685–3693
25 NORÉN, I B E , BERTOLI, D A , HO, C , AND CASASSA, E F (1974) *Biochemistry* **8**, 1683–1686
26 CHIANCONE, E , NORNE, J E , FORSÉN, S , ANTONINI, E , AND WYMAN, J (1972) *J Mol Biol* **70**, 675–688
27 BULL, T F , ANDRASKO, J , CHIANCONE, E , AND FORSÉN, S (1973) *J Mol Biol* **73**, 251–259
28 BUNN, H F , AND GUIDOTTI, G (1972) *J Biol Chem* **247**, 2345–2350
29 JANSSEN, L H M (1970) Ph D Thesis, University of Nijmegen, The Netherlands

CHAPTER 5

THE INFLUENCE OF ORGANIC PHOSPHATES ON THE BOHR EFFECT OF HUMAN HEMOGLOBIN VALENCY HYBRIDS

Harry S ROLLEMA, Simon H DE BRUIN and Gerard A J VAN OS

Department of Biophysical Chemistry University of Nijmegen Toernooiveld Nijmegen The Netherlands

The Bohr effect of hemoglobin and that of the aquomet and cyanomet valency hybrids was measured in the presence and the absence of IHP (inositol hexaphosphate) and DPG (2,3-diphosphoglycerate) In the absence of these organic phosphates the four hybrids show similar, but suppressed Bohr effects as compared to hemoglobin Addition of IHP and DPG results in all cases in an increase of the Bohr effect The additional phosphate induced Bohr effect of the hybrids with the α chain in the oxidized form is almost identical to that of hemoglobin, while this effect of the hybrids with oxidized β chains is slightly lower than that of hemoglobin The results suggest (a) that the Bohr effect is correlated to the ligation state of the hemoglobin molecule rather than to its quaternary structure, (b) that the additional phosphate induced Bohr effect is related to the change in quaternary structure of the tetramer, and (c) that with respect to the Bohr effect of the hybrids there is no difference between high and low spin species

1 Introduction

Since the discovery of the Bohr effect by Bohr et al [1] much work has been done to elucidate the molecular mechanism of this effect (for a review, see ref [2]) It has been well established that the so called alkaline Bohr groups Val 1α and His 146β account for approximately 70% of the alkaline Bohr effect As a candidate for the remaining 30% of the effect Perutz has suggested His 122α [3] Kilmartin and Rossi-Bernardi, however, hypothesized that this remaining part of the Bohr effect could be based on a difference in chloride ion binding between oxy and deoxyhemoglobin [2] Recently we have presented evidence for the validity of this hypothesis [4,5]

The change in pK of the Bohr groups must be caused by a change in structure of the protein upon ligand binding According to the stereochemical model of Perutz [6] this structural change involves both the tertiary structure of the subunits and the quaternary structure of the whole tetramer, the latter going upon ligation from the deoxystructure or T state to the oxystructure or R state At intermediate stages of ligation intermediate structures exist in which the subunits are

in the liganded or unliganded tertiary structure while the whole tetramer is in the R or T state

If the Bohr effect is correlated with a change in tertiary structure of the subunits, one expects a gradual release of Bohr protons as the molecule is saturated with ligand This gradual release should be absent in case the Bohr effect is correlated with a change in quaternary structure In CO recombination studies a linear relationship between the release of Bohr protons and the rate of ligand saturation has been found [7—9] suggesting a correlation with the tertiary structure On the other hand the properties of chemically modified and mutant hemoglobins in which one of the two quaternary structures is destabilized indicate a linkage of the Bohr effect to the quaternary structure [2,10—12]

In this respect the properties of artificial intermediates are of great interest, because these molecules have two chains frozen in the liganded state while the other two are free to bind ligands Banerjee and Cassoly studying oxygenation equilibria have shown [13,14] that the two aquomet hybrids have a suppressed alkaline Bohr effect, although the degree of suppression is quite different for both hybrids The oxygenation studies of Brunori et al [15] indicate, however, that

the cyanomet hybrids possess a suppressed but equal Bohr effect The suppression of the Bohr effect observed for the valency hybrids may be understood in terms of tertiary structural changes, since upon ligation only two of the four subunits change their tertiary structure Relating the Bohr effect to the T—R transition the suppressed Bohr effect of the hybrids can also be explained by assuming that of the intermediates in the unliganded form not all molecules possess the quaternary T-structure In the latter case, however, the full Bohr effect should be restored by addition of an effector such as IHP or DPG, which are known to stabilize the T-structure [16]

To obtain more information on this subject we measured the Bohr effect of the artificial aquomet and cyanomet intermediates in the presence and absence of IHP and DPG The results are compared with those obtained for hemoglobin

2 Materials and methods

Hemoglobin was prepared according to the toluene method of Drabkin [17] The hemoglobin solutions were freed from organic phosphates by passage over a mixed bed ion-exchange column (Amberlite IRA 400 and IR 120) For the preparation of α and β chains carbon monoxide hemoglobin was reacted with p-chloromercuribenzoate [18] The chains were separated on DEAE-Sephadex (start buffer, 0 1 M Tris-HCl, pH 8 0, limit buffer, 0 1 M Tris-HCl, pH 8 0, 0 4 M NaCl) The regeneration of the SH-groups of the α and β chains was achieved by a β-mercaptoethanol treatment on G25-Sephadex [19] After this treatment the α chains contained 1 0 free SH-groups as judged from a Boyer titration [20] A number of 2 0 free SH-groups for the β chains was found after they had been incubated for approximately 12 h with a 5-fold excess of dithiothreitol, which was removed on G25-Sephadex

To check whether this procedure yielded native α^{SH} and β^{SH} chains, the chains were recombined to form hemoglobin The hydrogen ion titration curve of the recombined α and β chains was within the experimental accuracy identical with the titration curve obtained with freshly prepared oxyhemoglobin For the sedimentation coefficient of the recombined hemoglobin an apparent value of 4 1 S (20°C, 0 1 M KCl, pH 7 3) was found, identical to the value we found for oxyhe-

moglobin

After replacement of the bound CO by O_2 (see below) the chains were oxidized by adding a stoechiometric amount of $K_3Fe(CN)_6$ in 0 2 M phosphate buffer pH 6 6 [21] Immediately after chain oxidation the hybrids were prepared by adding the other chain having CO bound If required a small excess of KCN was added to obtain the cyanomet hybrids

Measurements of the apparent sedimentation coefficient of the hybrids yielded a value of 4 5 S (25°C, 0 05 M KCl, 0 05 M bis—tris buffer, pH 7 0) Electrophoresis showed that no single chains were present The percentage of oxidized heme groups was determined by optical spectroscopy, samples which showed a deviation of more than 5% from the theoretical value were discarded ^{13}C-NMR spectra of the hybrids reacted with ^{13}CO (Stohler Isotope Chemicals), recorded one week after preparation, showed one single resonance characteristic for the reduced chain [22,23] indicating that heme exchange from one chain to the other did not occur Nevertheless all experiments were carried out within four days after chain recombination

Ultracentrifugation experiments were performed with a model E Spinco ultracentrifuge at a speed of 67 770 rpm

The ^{13}C-NMR spectra were obtained at 25 2 MHz on a Varian XL-100 spectrometer equipped with a Varian 620/L computer using the pulse Fourier transform technique

Electrophoresis was performed with the Gelman Sepratek electrophoresis system

DPG (Calbiochem), obtained as the pentacyclohexylammonium salt, was converted to the acid form by passage through Amberlite IR 120 The concentration of the DPG stock solution was determined by titration The DPG solutions were neutralized with NaOH

The concentration of the IHP (Sigma) solutions was determined by weight

The Bohr curves in the presence and the absence of IHP and DPG were measured at 25°C with a pH-stat equipment constructed for this type of experiments [24] After the hybrids were freed from the phosphate buffer, removal of CO was achieved in a rotating tonometer, passing oxygen over the solution under constant illumination The tonometer was cooled by ice to 0°C Subsequently the hybrids were deoxygenated under a constant flow of argon From the tonometer a known volume was transferred anaerobically to the

titration vessel of the pH-stat equipment Deoxygenation was checked for completeness by withdrawing anaerobically a small amount of the solution from the titration vessel followed by measurement of the optical spectrum For the measurement of the Bohr effect of hemoglobin the same procedure was followed

3 Results and discussion

Fig 1 shows the Bohr effect of hemoglobin in the absence and the presence of a 6-fold excess of IHP The curves shown are very similar to those presented by Kilmartin [21] The additional IHP induced Bohr effect observed (i e , the Bohr effect in the presence of IHP minus the Bohr effect in the absence of IHP) is due to a difference in interaction of IHP with oxy- and deoxyhemoglobin

Figs 2 and 3 show the Bohr effect of the aquomet

Fig 2 The Bohr effect of $\alpha_2^+\beta_2$ (measured as the number of protons released per tetramer upon ligation) in the presence (▲) and the absence (∆) of IHP, hybrid concentration, $1\ 7 \times 10^{-4}$ M on tetramer basis, IHP concentration, $1\ 0 \times 10^{-3}$ M, $0\ 1$ M KCl, $25°C$
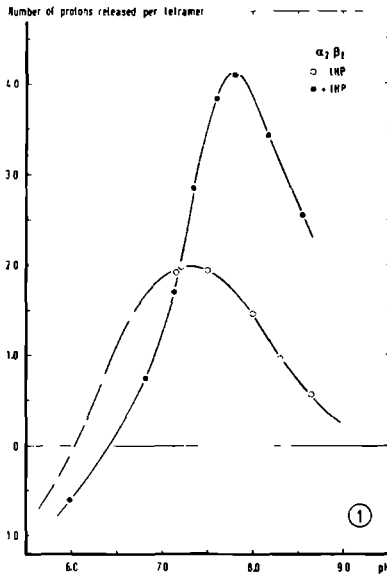

Fig 1 The Bohr effect of hemoglobin (measured as the number of protons released per tetramer upon ligation) in the presence (●) and the absence (○) of IHP, hemoglobin concentration, $1\ 7 \times 10^{-4}$ M on tetramer basis, IHP concentration, $1\ 0 \times 10^{-3}$ M, $0\ 1$ M KCl, $25°C$
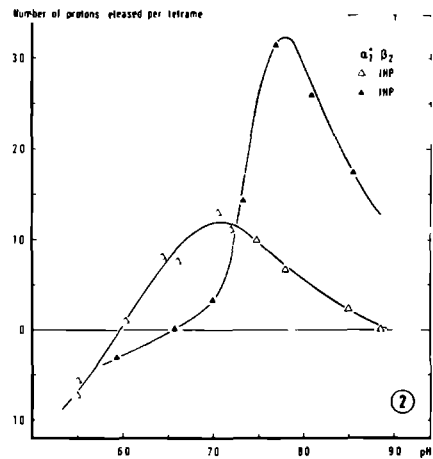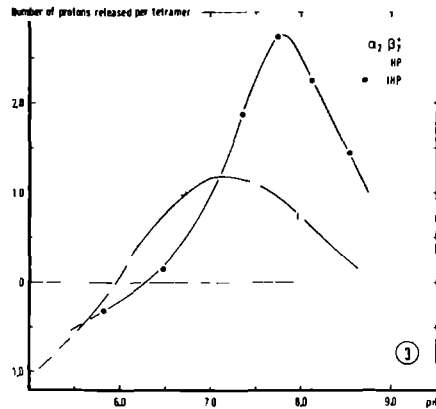

Fig 3 The Bohr effect of $\alpha_2\beta_2^+$ (measured as the number of protons released per tetramer upon ligation) in the presence (●) and the absence (○) of IHP, hybrid concentration, $1\ 7 \times 10^{-4}$ M on tetramer basis, IHP concentration, $1\ 0 \times 10^{-3}$ M, $0\ 1$ M KCl, $25°C$

hybrids with and without IHP In the absence of IHP both aquomet hybrids show a Bohr effect which is about half the Bohr effect of hemoglobin This result seems to contradict the observation of Banerjee and Cassoly [13,14], that the hybrid with the β chain in the oxidized form has a Bohr effect twice as large as that of the other hybrid In our opinion this apparent discrepancy can be accounted for by a difference in solvent conditions

If the Bohr effect is related to the T—R transition, the observed suppression of the Bohr effect for the hybrids would suggest that in the deoxygenated form about half of the molecules possess the R quaternary structure In this event it is likely that addition of IHP will result in a Bohr effect comparable to that found for hemoglobin in the presence of IHP Figs. 2 and 3 show that this is definitely not so The maximum value for the Bohr effect measured in the presence of IHP is significantly lower than the value measured for hemoglobin in the presence of IHP

The influence of IHP on the Bohr effect of the cyanomet hybrids is shown in the figs 4 and 5. In the absence of IHP a decreased Bohr effect is observed of about half the effect of normal hemoglobin. It should
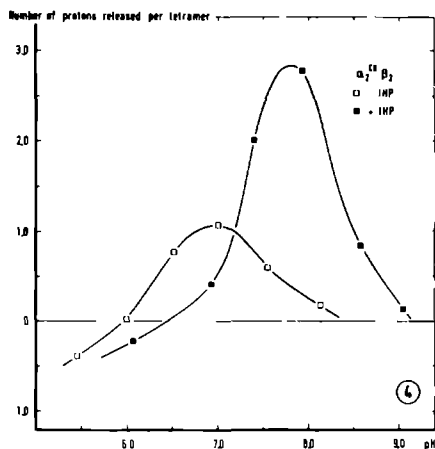
Fig 5 The Bohr effect of $\alpha_2\beta_2^{+CN}$ (measured as the number of protons released per tetramer upon ligation) in the presence (●) and the absence (○) of IHP, hybrid concentration, 1 7 × 10^{-4} M on tetramer basis, IHP concentration, 1 0 × 10^{-3} M KCl, 25°C

be stressed here that this Bohr effect is observed while it is known from NMR studies [25] that in the absence of phosphates the deoxy cyanomet hybrids are for the greater part in the R quaternary state As is seen the effect of IHP on the Bohr effect of the cyanomet hybrids
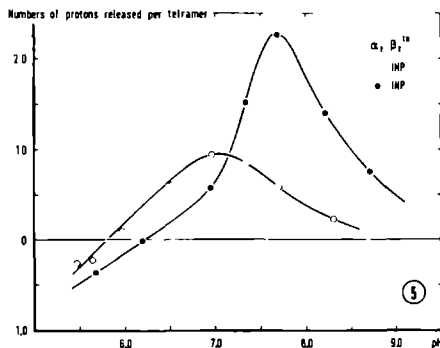
Fig 4 The Bohr effect of $\alpha_2^{+CN}\beta_2$ (measured as the number of protons released per tetramer upon ligation) in the presence (■) and the absence (○) of IHP, hybrid concentration, 1 7 × 10^{-4} M on tetramer basis, IHP concentration, 1 0 × 10^{-3} M, 0 1 M KCl, 25°C
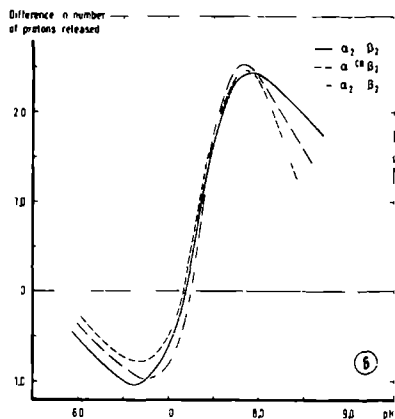
Fig 6 The additional IHP induced Bohr effect (i e the Bohr effect with IHP minus the Bohr effect without IHP) of $\alpha_2^+\beta_2$ (— –), $\alpha_2^{+CN}\beta_2$ (– – –) and hemoglobin (———) Experimental conditions as in figs 1, 2, and 4
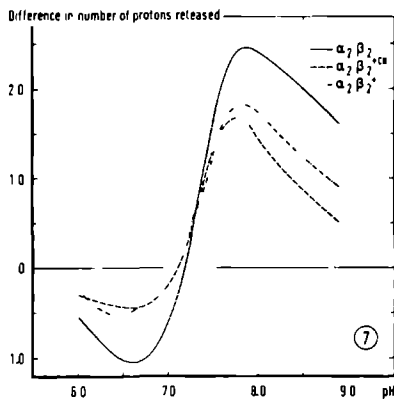
Fig 7 The additional IHP induced Bohr effect of $\alpha_2\beta_2^+$ (— —), $\alpha_2\beta_2^{+CN}$ (– – –) and hemoglobin (——) Experimental conditions as in figs 1, 3 and 5

Fig 8 The additional DPG induced Bohr effect of hemoglobin (——), $\alpha_2^+\beta_2$ (– – –) and $\alpha_2^{+CN}\beta_2$ (— —), protein concentration, 1 3 × 10^{-4} M on tetramer basis, DPG concentration, 1 7 × 10^{-3} M, 0 1 M KCl, 25°C

is similar to that observed for the aquomet hybrids

Figs 6 and 7 show the additional IHP induced Bohr effect of hemoglobin and of the aquo- and cyanomet hybrids The figures clearly demonstrate that the additional Bohr effect of the hybrids resembles very much that found for hemoglobin This result suggests that in the presence of IHP the deoxy hybrids possess a quaternary structure very similar to that of deoxy hemoglobin The fact, however, that the additional Bohr effect of the hybrids does not exceed that observed for hemoglobin indicates that upon addition of IHP the suppressed Bohr effect observed without IHP does not become restored Otherwise the additional Bohr effect of the hybrids should have exceeded that of hemoglobin

Figs 8 and 9 show the additional DPG induced Bohr effect of hemoglobin and of the aquo- and cyanomet hybrids It is seen that DPG has an influence on the Bohr effect of the hybrids analogous to the effect of IHP From these data the same conclusions are reached as from the results obtained with IHP

The results presented so far strongly suggest that the alkaline Bohr effect is related to the state of ligation of the subunits within the tetramer rather than to the change in quaternary structure of the hemoglobin tetramer This conclusion is supported by the observation that the sum of the Bohr effects of the two cya-
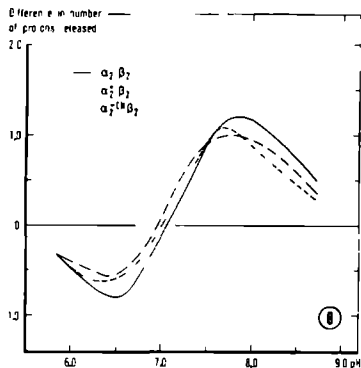
nomet and the two aquomet spin state hybrids is about equal to the Bohr effect of normal hemoglobin

Our observations are in accordance with the kinetic studies of the Bohr effect [7–9] and with the stereochemical model presented by Perutz [6] The observations of Olson and Gibson (9) that in case of n-butyl isocyanide binding the β chains contribute 20% to the Bohr effect and the α chains 80% cannot be readily
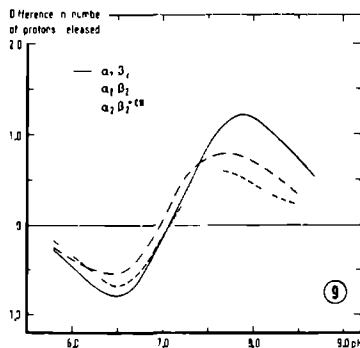

Fig 9 The additional DPG induced Bohr effect of hemoglobin (——), $\alpha_2\beta_2^+$ (—) and $\alpha_2\beta_2^{+CN}$ (— —), experimental conditions as in fig 8

understood unless there are ligand specific effects, because the four hybrids show similar Bohr effects, suggesting an equal contribution of both chains to this effect in case of oxygen binding

In addition to the things we discussed some other features of the data presented need some further comment. First figs 6—9 show that the intermediates with the β chains in the ferric form have a lower additional Bohr effect than the intermediates with the β chain in the ferrous form, which have an additional Bohr effect which is almost identical to the one of hemoglobin. This observation can be understood taking into account that in the T state IHP and DPG are bound at the entrance of the central cavity by a cluster of positively charged groups located on the β chains [26,27] In the intermediates the geometry of this binding site is more likely to be similar to that in deoxyhemoglobin when the β chains are in the reduced form, than when they are in the ferric form. A similar difference in behaviour of the hybrids has been observed for the binding of DPG and some spin labels [28,29]

Secondly no difference is found between the low spin cyanomet hybrids and the high spin aquomet hybrids even at low pH. In other words the spin state of the heme iron of the two chains in the ferric form does not influence the Bohr effect of the intermediates studied

Finally, since part of the Bohr effect of hemoglobin is due to difference in interaction of chloride ions with the T and R state [4,5], the question remains which part of the Bohr effect of the intermediates might be due to differences in interactions of these ions with the unligated and ligated hybrid. Studies on this subject and on the interaction of IHP separately with the oxy- and deoxy hybrids are in progress

Acknowledgement

References

[1] C Bohr, K A Hasselbalch and A Krogh, Skan Arch Physiol 16 (1904) 402
[2] J V Kilmartin and L Rossi-Bernardi, Physiol Rev 53 (1973) 836
[3] M F Perutz, Nature 228 (1970) 734
[4] S H de Bruin, H S Rollema, L H M Jansen and G A J van Os Biochem Biophys Res Commun 58 (1974) 210
[5] H S Rollema, S H de Bruin, L H M Janssen and G A J van Os, J Biol Chem 250 (1975) 1333
[6] M F Perutz, Nature 228 (1970) 726
[7] E Antonini, T M Schuster, M Brunori and J Wyman, J Biol Chem 240 (1965) PC2262
[8] R D Gray, J Biol Chem 245 (1970) 2914
[9] J S Olson and Q H Gibson, J Biol Chem 248 (1973) 1623
[10] M F Perutz, P D Pulsinelli and H M Ranney, Nature New Biology 237 (1972) 259
[11] H I Bunn, R C Wohl, T B Bradley, M Cooley and Q H Gibson, J Biol Chem 249 (1974) 7402
[12] J V Kilmartin, J A Hewitt and J F Wootton, J Mol Biol 93 (1975) 203
[13] R Banerjee and R Cassoly, J Mol Biol 42 (1969) 351
[14] R Banerjee, F Stetzkowski and Y Henry, J Mol Biol 73 (1973) 455
[15] M Brunori, G Amiconi, E Antonini, J Wyman and K Winterhalter, J Mol Biol 49 (1970) 461
[16] R Benesch, R F Benesch and C I Yu, Proc Nat Acad Sci USA, 59 (1968) 526
[17] D L Drabkin J Biol Chem 164 (1946) 703
[18] F Bucci and C I ronticelli, J Biol Chem 240 (1965) PC551
[19] I Tyuma, R E Benesch and R Benesch, Biochemistry 5 (1966) 2957
[20] P D Boyer, J Am Chem Soc 76 (1954) 4331
[21] J V Kilmartin Biochem, J 133 (1973) 725
[22] F Antonini M Brunori, I Conti and G Geraci, F I BS Lett 34 (1973) 69
[23] R B Moon and J H Richards, Biochemistry 13 (1974) 3437
[24] S H de Bruin, L H M Jansen and G A J van Os, Biochem Biophys Res Commun 55 (1973) 193
[25] S Ogawa and R G Shulman J Mol Biol 70 (1972) 315
[26] A Arnone and M F Perutz Nature 249 (1974) 34
[27] A Arnone, Nature 237 (1972) 146
[28] R T Ogata and H M McConnell, Proc Nat Acad Sci USA 69 (1972) 335
[29] C Bauer, Y Henry and R Banerjee, Nature New Biology 242 (1973) 208

CHAPTER 6

THE BOHR EFFECT OF THE ISOLATED α AND β CHAINS OF HUMAN HEMOGLOBIN

H S ROLLEMA, S H de BRUIN and G A J van OS

Department of Biophysical Chemistry University of Nijmegen Toernooiveld Nijmegen The Netherlands

1 Introduction

Although the Bohr effect (i e the linkage between oxygen and proton binding sites) has been discovered already in 1904 [1], still the molecular mechanism of this effect is not completely understood A number of groups responsible for a part of the Bohr effect has been identified [2] Recently we have shown that part of the Bohr effect is not an intrinsic property of the hemoglobin tetramer, but due to a difference in inter action of chloride ions with oxy and deoxyhemo globin [3] A question which is still unanswered is whether the Bohr effect is related to the change in quaternary structure only or also to the change in tertiary structure of the subunits

Experiments carried out in our laboratory on the Bohr effect of valency hybrids (submitted for publi cation) suggest that the Bohr effect is related to the state of ligation rather than to the change in quaternary structure This observation is in accor- dance with the results of kinetic studies on the rate of proton release upon ligand binding [4—6] which suggest a relation between the Bohr effect and changes in tertiary structure of the subunits If so, then it becomes difficult to understand why the isolated α and β chains do not show any Bohr effect at all as has been suggested in oxygenation studies [7,8]

We present therefore a study of the Bohr effect of the α and β chains of hemoglobin using the very sensitive pHstat equipment we constructed [9] This method is more suited to observe small effects than measuring oxygen binding curves

Horse heart myoglobin was studied for comparison

*Abbreviation Ins P_6, inositolhexaphosphate

It appeared that the α and β chains have a small but significant Bohr effect while myoglobin shows no Bohr effect In the case of β chains a marked influence of Ins P_6^* on the Bohr effect is found

2 Experimental

Human hemoglobin was isolated according to the toluene procedure of Drabkin [10] Organic phosphates were removed by passage over a mixed bed ion exchange column (Amberlite IRA 400 and IR 120) Preparation of α^{PMB} and β^{PMB} chains was achieved by incubating carbon monoxide hemoglobin with β chloromercuribenzoate [11] followed by a chromato- graphic separation on DEAE Sephadex

The α^{PMB} and β^{PMB} chains were demercurated by a β mercaptoethanol treatment [8], resulting in a complete regeneration of the SH group of the α-chain To regain the theoretical number of free SH groups, the β chains had to be incubated with a five fold excess of dithiothreitol for 12 h

Horse heart myoglobin (obtained from Sigma) was converted to the ferrous form by dithionite in the presence of CO

The removal of CO from the α and β chains and from myoglobin was achieved by light The concentra- tion of the Ins P_6 (Sigma) solutions was determined by weight The Bohr curves were measured with a pH stat equipment described elsewhere [9]

3 Results

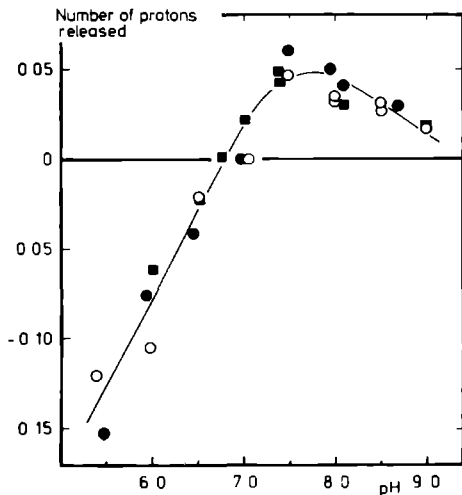Fig 1 shows the Bohr effect of the α chains in the presence and absence of Ins P_6 It is seen that the

Fig.1 The Bohr effect (measured as the number of protons released per heme upon ligation with O_2) of α chains in the presence (o) and absence (● and ■) of Ins P_6 Circles and squares refer to different preparations Protein concentration $8\,0 \times 10^{-4}$ M on heme basis, Ins P_6 concentration $2\,5 \times 10^{-3}$ M 0 1 M KCl 25°C
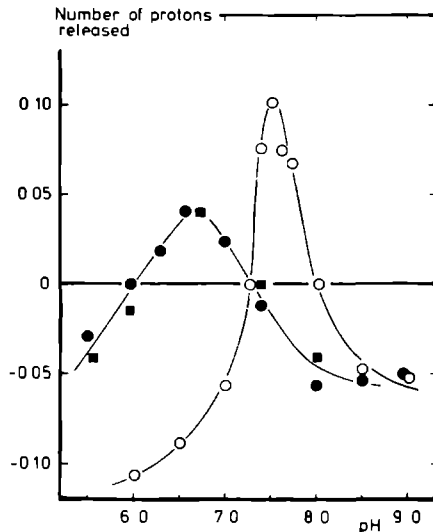

Fig 2 The Bohr effect (measured as the number of protons released per heme upon ligation with O_2) of β chains in the absence (● and ■) and presence ( ) of Ins P_6 Protein concentration $1\,2 \times 10^{-3}$ M on heme basis Ins P_6 concentration $2\,5 \times 10^{-3}$ M, 0 1 M KCl 25°C Circles and squares refer to different preparations

α chain has a small alkaline Bohr effect amounting to about 10% of the effect of hemoglobin The acid Bohr effect of the α chain, however, is about 30% of that found for hemoglobin Fig 1 shows that within the experimental accuracy Ins P_6 has no influence on the Bohr effect of the α chain

Fig 2 shows the Bohr effect of the β chain with and without Ins P_6 The β chain has a small Bohr effect with a quite different shape as compared to hemo globin For this chain we found a marked influence of Ins P_6 on the Bohr effect

Experiments carried out with horse heart myo globin in the pH range 5 5–9 0 showed that this protein has no Bohr effect Upon ligation the changes in the number of protons bound by myoglobin in the presence and the absence of Ins-P_6 did not exceed the value of 0 01 and can be regarded as not significant

4 Discussion

The observed Bohr effect for the α and β chains of

human hemoglobin shows that in the case of human hemoglobin the occurrence of heterotropic allosteric interactions is not restricted to the tetramer formed by the two different chains There is however a difference in magnitude of these effects in the hemo globin tetramer and the α and β chains The relative large acid Bohr effect observed with the α chains suggests that the tertiary structural change of the α chain in hemoglobin could be responsible for a significant part of the acid Bohr effect of the whole tetramer The fact that myoglobin does not show any Bohr effect at all indicates that the effects observed with the isolated chains cannot be considered as non specific

Since the experiments presented in this paper are performed in the presence of 0 1 M KCl there is a possibility that part of the observed Bohr effect of the chains can be explained by a difference in interaction of chloride ions with the oxy and deoxy form of the chains

Finally the observation of an Ins-P_6 induced change in Bohr effect in the case of the β chain leads to the conclusion, that there is a difference in interaction of oxy and deoxy β_4 with Ins-P_6. This can be explained by assuming that the oxy and deoxy form of the β_4 tetramer have a different structure, which is in accordance with recent oxygenation studies of Bonaventura et al. [12].

Acknowledgement

References

[1] Bohr, C, Hasselbalch, K A. and Krogh, A. (1904) Scand Arch. Physiol. 16, 402–412.

[2] Kilmartin, J. V. and Rossi-Bernardi, L (1973) Physiol. Rev. 53, 836–890.

[3] Rollema, H. S , de Bruin, S H., Janssen, L. H. M. and van Os, G. A J (1975) J Biol Chem 250, 1333-1339

[4] Antonini, F., Shuster, T M , Brunori, M. and Wyman, J (1965) J. Biol Chem 240, PC2262–PC2264

[5] Gray, R D (1970) J. Biol Chem 245, 2914–2921.

[6] Olson, J S. and Gibson, Q. H (1973) J. Biol. Chem 248, 1623–1630.

[7] Antonini, E , Bucci, E , Fronticelli, C , Wyman, J. and Rossi-Fanelli, A (1965) J Mol Biol. 12, 375 384

[8] Tyuma, I , Benesch, R E. and Benesch, R (1966) Biochemistry 5, 2957- 2962.

[9] de Bruin, S H., Janssen, L H. M and van Os, G A. J. (1973) Biochem. Biophys Res Comm. 55, 193–199.

[10] Drabkin, D. L (1946) J. Biol. Chem. 164, 703–723.

[11] Bucci, E. and I ronticelli, C (1965) J Biol. Chem 240, PC551–PC552

[12] Bonaventura, J , Bonaventura, C , Amiconi, G., Tenton, L , Brunori, M. and Antonini, E. (1975) J. Biol Chem. 250, 6278- 6281.

CHAPTER 7

THE KINETICS OF CARBON MONOXIDE BINDING

TO PARTIALLY REDUCED METHEMOGLOBIN

Harry S. Rollema, Harry P.F. Scholberg and Simon H. de Bruin
Department of Biophysical Chemistry,
University of Nijmegen, Toernooiveld, Nijmegen
The Netherlands

Adriaan Raap
Department of Molecular Biophysics,
State University of Utrecht,
Sorbonnelaan 4, Utrecht, The Netherlands

SUMMARY: The pulse radiolysis technique has been used to study the kinetics of the CO binding to partially reduced methemoglobin. Experiments with horse heart metmyoglobin show that this technique gives results which are in good agreement with those obtained by other methods. The kinetics of the CO binding to partially reduced methemoglobin show two phases, whose amplitudes appear to depend on the degree of reduction in such a way that they can be attributed to methemoglobin molecules with one or two reduced heme groups. In the presence of inositol hexaphosphate the rate of CO binding to partially reduced methemoglobin decreases strongly. With inositol hexaphosphate a slight biphasic behavior is observed independent of the degree of reduction.

Until now the kinetics of the binding of CO to hemoglobin have been studied by rapidly mixing deoxyhemoglobin with CO or by following the CO recombination after removal of the ligand by flash photolysis (1). A new approach to the CO binding kinetics of hemoglobin is offered by the pulse radiolysis technique. Using this technique a methemoglobin solution is irradiated with a short pulse of high energy electrons. The irradiation mainly results in the formation of hydrated electrons, OH and H radicals. Of these primary radicals OH and H can be removed by an appropriate scavenger. The hydrated electrons reduce methemoglobin within a few microseconds. Two secondary processes which are complete in about 500 μs are observed after reduction (2). When the reduction is carried out in the presence of CO, the kinetics of the CO binding to the reduced heme groups can be followed.

Abbreviations used: e_{aq}^-, hydrated electron; IHP, inositol hexaphosphate; bis-tris, 2,2'-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol, CO, carbon monoxide.

Partial reduction of methemoglobin by hydrated electrons produces a number of intermediates, the concentrations of which can be calculated assuming that e_{aq}^- reacts randomly with the ferric heme groups.

An other aspect in the investigation of the kinetics observed for the CO binding to partially reduced methemoglobin is in that the quaternary structure of methemoglobin can be altered by addition of IHP (3).

Experimental

Materials. Human hemoglobin was isolated according to Drabkin (4). After extensive dialysis against destilled water the hemoglobin solutions were freed from organic phosphates by passage through a mixed bed ion-exchange column (Amberlite IRA 400 and IR 120), Methemoglobin was prepared by adding 50% excess of $K_3Fe(CN)_6$ to a solution of oxyhemoglobin. The excess of $K_3Fe(CN)_6$) was removed on a G-25 Sephadex column or by dialysis, followed by passage through a mixed bed ion-exchange column.

Horse heart metmyoglobin (Sigma) was used without further purification. Pulse radiolysis. The irradiation was achieved by a 2 MV Van de Graaf accelerator (High Voltage Engineering Europe), using pulse lengths of 0.5 or 5 μs with a maximum current of 1 A. To ensure homogeneous irradiation of the sample a cell with dimensions of 9 x 5 x 1 mm (optical pathway 9 mm, electron pathway 1 mm) was used. The optical detection system consisted of a xenon arc (XBO 450 W/1, Osram), two Bausch and Lomb grating monochromators (1350 grooves/mm) and a RCA 1P28 photomultiplier; one monochromator was placed between the light source and the cell, the other one between the cell and the detector. The photomultiplier signal was recorded by means of a 7904 Tektronix oscilloscope.

To avoid denaturation of the protein during the removal of oxygen from the solutions the following procedure was used. The buffers were freed from oxygen by passing pure argon through the solutions for 1 h. A concentrated protein solution was deoxygenated in a rotating tonometer by passing argon over it for 15 minutes. A known volume of the concentrated protein solution was transferred anaerobically to the buffer solution. The protein solution was subsequently equilibrated with a mixture of argon and CO. During the experiments a constant flow of this gas mixture was passed over the solution. The mixture was obtained from a gas mixing pump (Wösthoff M300/a-F). The concentration of carbon monoxide in the solution was calculated using a solubility coefficient of 1.36 μM/mm Hg (1). The concentrations of methemo-

globin and metmyoglobin were determined spectrophotometrically. The protein concentrations are given on heme basis.

Static difference spectra were recorded on a Cary 118 spectrophotometer.

As radical scavenger methanol was added up to a concentration of 0.1 M. All kinetic experiments were carried out at room temperature ($22 \pm 1^{\circ}$C). Pseudo-first order conditions were satisfied in all experiments.

In cases where a biphasic behavior for the CO binding kinetics was observed, the data were analyzed according to the equation:

$$F(t) = \alpha \exp(-k_1.[CO].t) + (1-\alpha)\exp(-k_2.[CO].t)$$

where $F(t) = (A_{\infty} - A_t)/(A_{\infty} - A_o)$; A_{∞}, A_t and A_o being the absorbances at the end of the reaction, at time t and at the beginning of the reaction respectively; k_1 and k_2 the CO binding rate constants; α the fractional contribution of the fast phase to the change in absorbance.

RESULTS AND DISCUSSION

It has been shown before that ferrous hemoglobin obtained by reduction of methemoglobin by means of e^{-}_{aq} shows the same functional properties as normal hemoglobin (2). The finding that irradiation does not influence the functional properties of the protein is supported by the kinetics observed for the CO binding to horse heart deoxymyoglobin, produced by the reaction of e^{-}_{aq} with metmyoglobin. The first order plot shown in fig. 1 demonstrates clearly that the CO binding follows pseudo-first order kinetics with a rate constant of $(4.5 \pm 0.5) \times 10^5 M^{-1}s^{-1}$. This value is in good agreement with the results obtained with stopped flow and flash photolysis experiments (5).

The kinetic difference spectrum for the CO binding to reduced heme groups of human methemoglobin is shown in fig. 2 together with the static difference spectrum of carboxy and deoxyhemoglobin. The figure shows that no significant differences are observed.

The kinetics of the CO binding to partially reduced methemoglobin are strongly dependent on the degree of reduction. Fig. 3 shows the first order plot for the binding of CO to partially reduced methemoglobin at two degrees of reduction. At a low degree of reduction, i.e. under conditions where the predominant reaction product is a methemoglobin molecule with one reduced heme group, CO binding is monophasic and fast with a rate constant of $(7 \pm 1) \times 10^6 M^{-1}s^{-1}$.
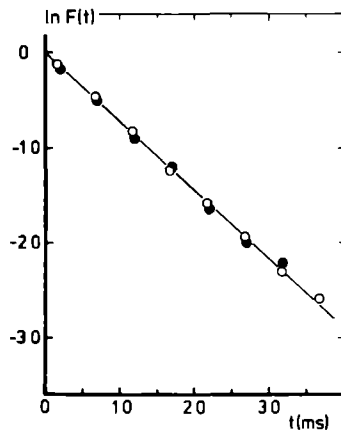
Fig. 1.   First order plots for the reaction of CO with myoglobin
          followed at 435 (o) and 450 (•) nm, after the reduction
          of metmyoglobin by hydrated electrons. 10 μM metmyoglobin,
          degree of reduction 0.40; 155 μM CO; 5 mM bis-tris, pH 7.0;
          0.1 M. methanol; 22°C.
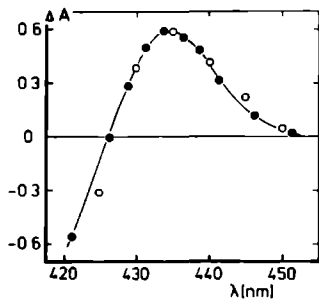
Fig. 2.   Kinetic difference spectrum for the reaction of partially
          reduced methemoglobin with CO (o). Static difference spec-
          trum between deoxy and carboxy hemoglobin (•); 20 μM
          methemoglobin, degree of reduction 0.34; 25 mM bis-tris,
          pH 7.0; 0.1 M methanol; 22°C.

Assuming that e_{aq} reacts at random with the heme groups of methemoglobin
this result indicates the absence of chain heterogeneity with respect to
the CO binding. Furthermore, the fast CO binding suggests that a methemo-
globin molecule with one reduced heme group is in the R state or the R
to T transition is too slow to interfere with the CO binding. This conclu-
sion is not in agreement with an earlier report (2) where the faster of the

Fig. 3. First order plots for the reaction of CO with partially reduced methemoglobin at a degree of reduction of 0.01 (●) and 0.08 (o). 100 μM methemoglobin; 143 μM CO; 25 mM bis-tris, pH 7.0; 0.1 M methanol; 29°C.
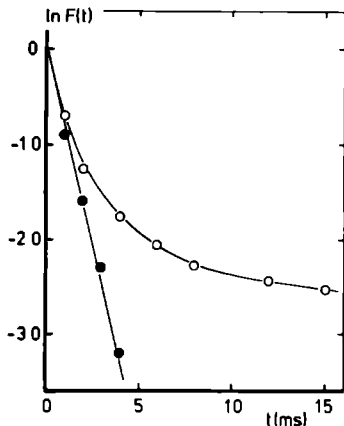
secondary processes following the reduction of methemoglobin by e_{aq}^- has been assigned to a change in quaternary structure. Recently (unpublished results) this process has been observed under solvent conditions (in 2 M KCl) where methemoglobin is largely dissociated into dimers (6), invalidating the assigment mentioned above.

At higher degrees of reduction where the concentration of molecules with two reduced heme groups becomes significant; a slower phase in the CO binding is observed. In this case the first order plot can be fitted using two exponentials with $k_1 = (7 \pm 1) \times 10^6 M^{-1} s^{-1}$ and $k_2 = (3 \pm 0.5) \times 10^5 M^{-1} s^{-1}$.

The values for the rate constants are within the experimental accuracy independent of the degree of reduction, CO concentration and protein concentration and agree rather well with values observed for fast and slow reacting forms of hemoglobin (1).

In fig. 4 the fractional contribution of the slow phase to the change in absorbance is shown as a function of the degree of reduction. The line in fig. 4 gives the contribution to the change in absorbance of molecules with two reduced groups, calculated under the assumption that the reduction of the ferric heme groups by e_{aq}^- proceeds at random and that the dissociation constant for the tetramer-dimer equilibrium for methemoglobin has a value of 1 μM. It can be seen that the experimental points are in reasonable agreement with the calculated curve. This strongly suggests that a hemoglobin tetramer
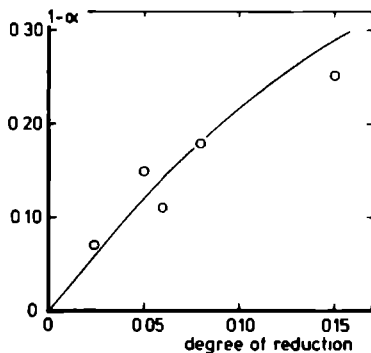
63

Fig. 4. Fraction slow reacting material found in the reaction of
CO with partially reduced methemoglobin as a function of
the degree of reduction. The solid line is a theoretical
curve representing the fractional contribution of molecules
with two reduced heme groups to the change in absorbance.
The curve has been calculated assuming that the reduction
proceeds at random and that the dissociation constant for
methemoglobin has a value of 1 μM. 100 μM methemoglobin;
25 mM bis-tris; pH 7.0; 0.1 M methanol, 22°C.

with two ferrous and two ferric heme groups has the T quaternary structure
and that under the experimental conditions used the R to T transition for
this intermediate is too fast to interfere with the CO binding. This finding
is in contrast with the results of Cassoly and Gibson (7) who did not find
fast exchange between a slow and fast reacting species observed in the reac-
tion of CO with cyanomet hybrids.

Fig. 5 shows the effect of the presence of 1 mM IHP on the CO binding
kinetics. A slow biphasic CO binding is observed independent of the degree
of reduction. The first order plots can be fitted using two exponentials:
k_1 = (4.2 $\pm$ 1.8) x $10^5 M^{-1} s^{-1}$ and k_2 = (1.2 $\pm$ 0.3) x $10^5 M^{-1} s^{-1}$. The contribu-
tion from each phase is approximately 50% (α = 0.47 $\pm$ 0.08). This equality
in amplitude of the two phases suggests IHP induced chain heterogeneity as
a possible explanation. The fact that in the presence of IHP the CO binding
characteristics do not show any dependence on the degree of reduction indi-
cates that as far as the kinetics of the CO binding are concerned methemo-
globin tetramers with one or two reduced heme groups are in the same con-
formational state.

The rate constants found for the two phases differ slightly from those
observed for deoxyhemoglobin in the presence of IHP (8). This difference is
in accordance with the results of Hensley et al. (9,10), who have observed

64
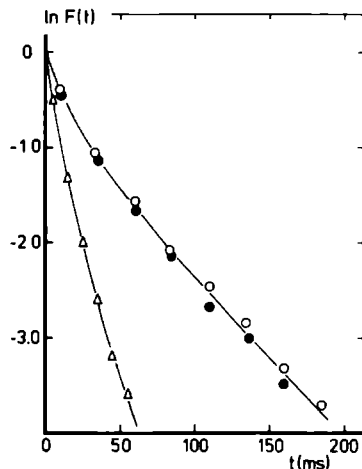
Fig. 5. First order plots for the reaction of CO with partially
reduced methemoglobin in the presence of 1 mM IHP: 100 μM
methemoglobin, 143 μM CO, degree of reduction 0.02 (●) and
0.07 (o); 100 μM hemoglobin, 450 μM CO, degree of reduction
0.14 (Δ); 25 mM bis-tris, pH 7.0; 0.1 M methanol, 22°C.

differences in T structure between deoxyhemoglobin and methemoglobin in the
presence of IHP.

In the presence of IHP no influence is found of the protein concentra-
tion on the CO binding kinetics. This is in agreement with the fact that in
the presence of IHP the dissociation of methemoglobin into dimers is greatly
suppressed (11,12).

In conclusion we can say that the pulse radiolysis technique offers a
new versatile method for studying ligand binding kinetics to hemoglobin with
submillisecond time resolution. Preliminary experiments have shown that this
method is also applicable to the study of oxygen binding kinetics to partially
reduced methemoglobin.

ACKNOWLEDGEMENT

REFERENCES

1. Antonini, E. and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, Amsterdam, North-Holland Publishing Co.

2. Wilting, J., Raap, A., Braams, R., de Bruin, S.H., Rollema, H.S., and Janssen, L.H.M. (1974) J. Biol Chem. 249, 6325-6330.

3. Perutz, M.F., Fersht, A.R., Simon, S.R. and Roberts, G.C.K. (1974) Biochemistry 13, 2174-2186.

4. Drabkin, D.L. (1946) J. Biol. Chem. 164, 703-723.

5. Gibson, Q.H. (1959) Progr. Biophys. Biophys, Chem. 9, 1-53.

6. Kirshner, A.G. and Tanford, C. (1964) Biochemistry 3, 291-196.

7. Cassoly, R. and Gibson, Q.H. (1972) J. Biol. Chem. 247, 7332-7341.

8. Gray, R.D. and Gibson, Q.H. (1971) J. Biol. Chem. 246, 7268-7174.

9. Hensley, P., Edelstein, S.J., Wharton, D.C. and Gibson, Q.H. (1975) J. Biol. Chem. 250, 952-960.

10. Hensley, P., Moffat, K. and Edelstein, S.J. (1975) J. Biol. Chem. 250, 9391-9396.

11. White, S.L. and Glanser, S.C. (1973) Fed. Proc. 32, 551.

12. White, S.L. (1975) J. Biol. Chem. 250, 1263-1268.

CHAPTER 8

KINETICS OF CARBON MONOXIDE BINDING TO FULLY AND PARTIALLY
REDUCED HUMAN HEMOGLOBIN VALENCY HYBRIDS

H.S. Rollema [*], A. Raap [†] and S.H. de Bruin [*]

[*] Department of Biophysical Chemistry,
University of Nijmegen, Toernooiveld, Nijmegen
The Netherlands

and

[†] Department of Molecular Biophysics,
State University of Utrecht,
Sorbonnelaan 4, Utrecht, The Netherlands

SUMMARY: The kinetics of the carbon monoxide binding following fast
reduction of the valency hybrids $\alpha_2^+ \beta_2^{CO}$ and $\alpha_2^{CO} \beta_2^+$ by hydrated electrons
have been studied at different degrees of reduction. The results show
that at pH 6.0 and 7.0 reduction of one heme group yields a species which
reacts fast with carbon monoxide (rate constant in the order of
10^6 $M^{-1}s^{-1}$). At pH 6.0 the intermediates $\alpha_2^{CO} \beta_2$ and $\alpha_2 \beta_2^{CO}$, bind carbon
monoxide with a rate characteristic of the T state. It appears that
at pH 7.0 $\alpha_2^{CO} \beta_2$ is for the greater part in the T state, while in the
case of $\alpha_2 \beta_2^{CO}$ the R and the T state are about equally populated.

INTRODUCTION

Recently we have shown that pulse radiolysis can be used to study the
kinetics of the carbon monoxide binding to partially reduced methemoglobin
(1). The use of pulse radiolysis to study the kinetics of CO binding to
hemoglobin derivatives is based on the fact that hydrated electrons,
generated upon irradiation of aqueous solutions, are able to reduce ferric
heme groups within a few microseconds. When this reduction is carried out
in the presence of carbon monoxide the kinetics of the carbon monoxide

Abbreviations used: α^+, α^{+CN}, α, α^{CO} and β^+, β^{+CN}, β, β^{CO}, the aquomet,
cyanomet, unligated and CO ligated form of the α and β chain of human
hemoglobin; bis-tris, 2,2-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol;
e_{aq}^-, hydrated electron.

binding to the reduced heme groups can be investigated.

An interesting feature of this method is that by irradiation of solutions of valency hybrids the carbon monoxide intermediates $\alpha_2\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2$ are obtained. These intermediates are identical with two of the four possible configurations, in which half ligated hemoglobin occurs in the course of the reaction of deoxyhemoglobin with CO. Since this type of intermediates cannot be isolated, in earlier studies the properties of artificial intermediates like valency hybrids have been considered as being characteristic of the half ligated state of hemoglobin.

In this paper we present a study on the kinetics of the carbon monoxide binding observed after irradiation of solutions of valency hybrids. From the dependence of the binding kinetics on the degree of reduction information is obtained about the properties of the carbon monoxide intermediates. The results show that a distinct difference exists between the kinetic behaviour of these intermediates and the ligand binding kinetics of the valency hybrids as reported by Cassoly and Gibson (2).

MATERIALS AND METHODS

The isolation of human hemoglobin and the preparation of valency hybrids has been described elsewhere (3).

As a source of high energy electrons a 2 MV Van de Graaff accelerator (High Voltage Engineering, Europe) was used. Prior to irradiation the protein solutions were deaerated with pure argon (1). Afterwards the solutions were equilibrated with a mixture of carbon monoxide and argon. This gas mixture was obtained using a gas mixing pump (Wosthoff M 300/a-F). The concentration of carbon monoxide was calculated using a value of 1.36 μM/mmHg for the solubility coefficient.

The rate constant for the reaction of e_{aq}^- with CO has a value of 10^9 $M^{-1}s^{-1}$ (4), while for the reaction of e_{aq}^- with methemoglobin values in the order of 10^{10} $M^{-1}s^{-1}$ are found (5,6). Therefore, under the experimental conditions used the former reaction can be neglected.

To ensure homogeneous sample irradiation a small cell was used (1 x 5 x 9 mm: electron pathlength 1 mm, optical pathlength 9 mm).

As radical scavenger methanol was added to the solutions.

The optical detection system has been described elsewhere (1).

The concentration of the valency hybrids, measured spectrophotometrically

after reduction with sodiumdithionite in the presence of carbon monoxide, is given on heme basis.

The experiments were performed at room temperature ($22 \pm 1°C$).

For the carbon monoxide binding pseudo-first order conditions were satisfied. The carbon monoxide binding data were analysed using two exponentials according to the following equation:

$$F(t) = a \exp(-k_1.[CO].t) + (1-a)\exp(-k_2.[CO].t) \qquad [1]$$

where $F(t)$ represents the fraction unreacted heme groups, k_1 and k_2 the two second order rate constants ($k_1 > k_2$) and $(1-a)$ the fractional contribution to the change in absorbance of the slow phase. $F(t)$ was calculated according to:

$$F(t) = \frac{A(t) - A(\infty)}{A(o) - A(\infty)}$$

with $A(t)$ the absorbance at time t.

The change in absorbance due to the binding of carbon monoxide was measured at 435 nm, using a bandwidth of 2 nm.

The degree of reduction was calculated using a value of 90 mM^{-1} cm^{-1} for the difference in absorbance between unligated and CO ligated heme groups at 435 nm.

RESULTS AND DISCUSSION

Fig. 1 shows typical first order plots for the CO binding observed after fast reduction of valency hybrids by hydrated electrons. From the figure it is seen that the kinetics of the CO binding strongly depend on the degree of reduction. At low degrees of reduction the time dependence of the carbon monoxide binding can be described by a single exponential. At high degrees of reduction biphasic binding kinetics are observed. The time course of the reaction can be described according to equation $\left[1\right]$.

The values observed for k_1 and k_2 are summarized in Table 1. The table shows that k_1 and k_2 do not differ significantly from the values characteristic of the R and the T state (7-9). No indications for chain heterogeneity with respect to the rate of carbon monoxide binding are found.

It appears that the values for the second order rate constants, k_1 and k_2 are independent of the degree of reduction and the carbon monoxide con-
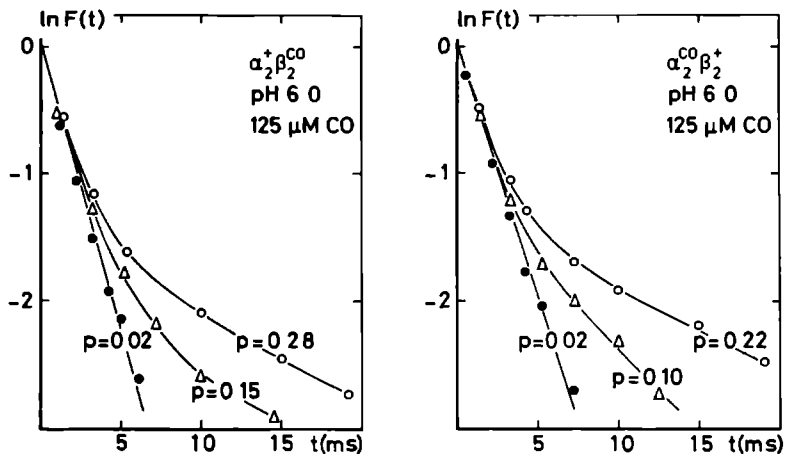
Fig. 1. Carbon monoxide binding observed after partial reduction of valency hybrids at different degrees of reduction (p); 95 μM valency hybrids, 125 μM CO, 30 mM phosphate buffer pH 6.0, 0.1 M methanol, 435 nm, 22°C.

TABLE 1

Rate constants for the carbon monoxide binding observed after fast reduction of human hemoglobin valency hybrids. Conditions: hybrid concentration, 95 μM; buffer concentration 30 mM; wavelength 435 nm; temperature 22°C.

			$k_1 \ \mu M^{-1} s^{-1}$	$k_2 \ \mu M^{-1} s^{-1}$
$\alpha_2^+ \beta_2^{CO}$	pH 6.0	phosphate	3.4 ± 0.6	0.33 ± 0.1
		bis-tris	4.0 ± 1	0.41 ± 0.1
	pH 7.0	phosphate	4.3 ± 0.6	0.34 ± 0.07
		bis-tris	5.6 ± 1	0.36 ± 0.08
$\alpha_2^{CO} \beta_2^+$	pH 6.0	phosphate	3.1 ± 0.5	0.29 ± 0.06
		bis-tris	3.4 ± 0.4	0.27 ± 0.04
	pH 7.0	phosphate	4.7 ± 0.6	0.41 ± 0.1
		bis-tris	6.1 ± 0.8	0.35 ± 0.09

70

centration. Only the ratio of the amplitudes of the two phases depends on the degree of reduction.

The fast monophasic carbon monoxide binding observed at low degrees of reduction, where the predominant reaction product is a hybrid molecule with one reduced heme group, indicates that after reduction of one heme group the hybrid remains in the R quaternary state. This conclusion is in accordance with results of flash photolysis studies which show that a hemoglobin molecule having three ligands bound possesses the R structure (7). Moreover it has been reported that reduction of one heme group in methemoglobin does not cause the molecule to change its structure from R to T (1).

The fact that the slow phase in the carbon monoxide binding is only observed at degrees of reduction where an appreciable amount of fully reduced valency hybrids is formed justifies the conclusion that this phase can be attributed to the fully reduced hybrids. In order to obtain more information about the fraction of the intermediates $\alpha_2\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2$ reacting slowly with CO, the contribution of the slow phase to the change in absorbance was measured as a function of the degree of reduction (Fig. 2 and Fig. 3).

Since the hydrated electrons react with ferric heme groups with a rate constant approaching the diffusion controlled limit (5,6) it is reasonable to assume that all ferric heme groups have the same probability to react with e_{aq}^-. This results in a binomial distribution of the reduced heme groups among the hybrids. According to this assumption the contribution of fully reduced hybrids to the change in absorbance can be calculated (line A in Figs. 2 and 3). Line B in these figures is obtained by taking into account the presence of dimers, which are known to react fast with CO (10). At pH 6.0 and 7.0 the concentration of dimers has been calculated using a value of 10^{-5} M for the dissociation constant of the tetramer-dimer equilibrium. Fig. 2 shows that at pH 6.0 the experimental values correspond reasonably well with line B. This leads to the conclusion that at this pH $\alpha_2\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2$ react with CO predominantly with a rate characteristic of the deoxy or T quaternary structure.

From the data presented in Fig. 3 it can be estimated that at pH 7.0 about 70% of the $\alpha_2^{CO}\beta_2$ intermediates react slowly while for $\alpha_2\beta_2^{CO}$ this percentage is about 50%. This difference in the amount of slowly reacting material indicates a slight but distinct chain heterogeneity. A similar conclusion has

Fig. 2. The dependence of the relative amplitude of the slow
phase (1-a) on the degree of reduction (p) at pH 6.0
in 30 mM phosphate (●) and 30 mM bis-tris (o); 95 μM
valency hybrids, 125 μM CO, 0.1 M methanol, 435 nm,
22°C. Line A and B represent the calculated contribution
to the change in absorbance of fully reduced hybrids
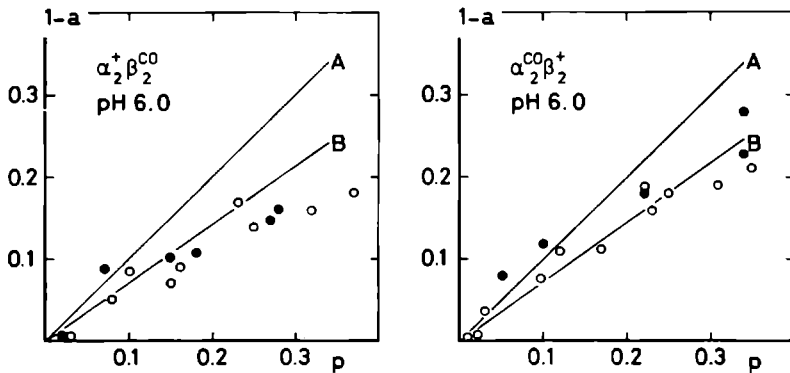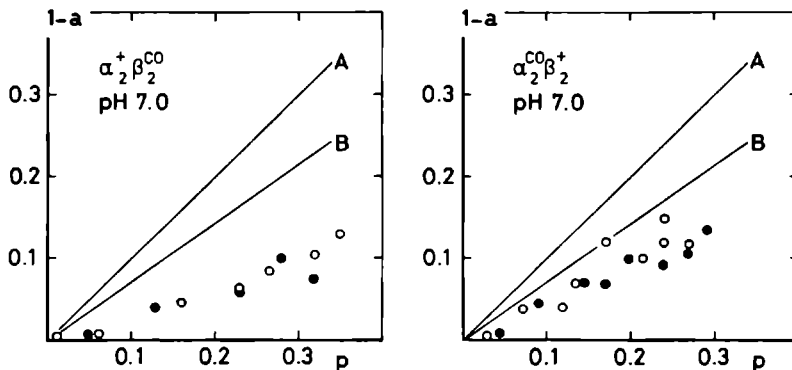(for further details see text).

Fig. 3. The dependence of the relative amplitude of the slow
phase (1-a) on the degree of reduction (p) at pH 7.0
in 30 mM phosphate (●) and 30 mM bis-tris (o); 95 μM
valency hybrids, 125 μM CO, 0.1 M methanol, 435 nm,
22°C for line A and B see legend to figure 2.

been reached by Ogawa and Shulman from NMR studies on the cyanomet valency hybrids (11).

Our results differ from the CO binding data for the valency hybrids $\alpha_2\beta_2^{+CN}$ and $\alpha_2^{+CN}\beta_2$ as obtained by Cassoly and Gibson (2). Their results show that at pH 6.6 about 80% of both hybrids react fast with CO. Moreover both hybrids behave nearly identical indicating absence of chain heterogeneity.

The main conclusion which can be drawn from our results is that at pH 6.0 deoxyhemoglobin having two carbon monoxide molecules bound still possesses the T quaternary structure, while at pH 7.0 the R and the T state are about equally populated.
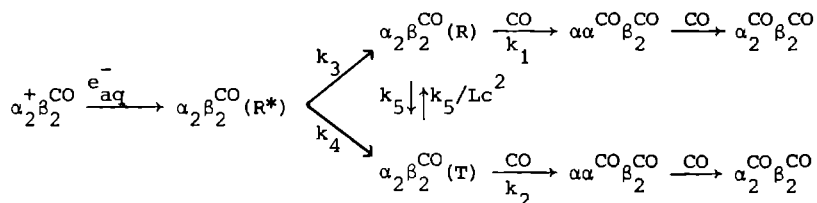
In terms of the model of Monod, Wyman and Changeux (12) these results mean that at pH 6.0 the equilibrium constant of the R-T equilibrium for a hemoglobin molecule with two CO molecules bound, Lc^2 has a value larger than 10 while at pH 7.0 Lc^2 has a value ranging from 1 to 2.

In other words the switch-over point has a value near 3 at pH 6.0 and a value slightly larger than 2 at pH 7.0.

At pH 6.0 and 7.0 for both hybrids the amplitude of the slow phase is not affected by an increase of the CO concentration up to 625 µM. This indicates that the rate of the change in quaternary structure induced by total reduction is faster than the rate of carbon monoxide binding.

On the other hand the observation that at pH 7.0 the amount of slowly reacting material of $\alpha_2\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2$ and the second order rate constant do not depend on the CO concentration, leads to the conclusion that the rate of interconversion between the T and R state of these intermediates is slower than the rate of ligand binding. A similar conclusion has been given by Cassoly and Gibson for the cyanomet valency hybrids (2).

Our observations imply that the quaternary structure of the fully reduced hybrid immediately after reduction is different from the R state of the hybrid during the CO binding. Accordingly the following reaction scheme can be proposed:

$$\alpha_2^+\beta_2^{CO} \xrightarrow{e_{aq}^-} \alpha_2\beta_2^{CO}(R^*) \left\langle \begin{array}{l} \xrightarrow{k_3} \alpha_2\beta_2^{CO}(R) \xrightarrow[k_1]{CO} \alpha\alpha^{CO}\beta_2^{CO} \xrightarrow{CO} \alpha_2^{CO}\beta_2^{CO} \\[2mm] k_5\downarrow\uparrow k_5/Lc^2 \\[2mm] \xrightarrow{k_4} \alpha_2\beta_2^{CO}(T) \xrightarrow[k_2]{CO} \alpha\alpha^{CO}\beta_2^{CO} \xrightarrow{CO} \alpha_2^{CO}\beta_2^{CO} \end{array} \right.$$

The rate by which R* decays to the normal T and R state (this decay is

schematically represented by two rate constants) is too high to interfere with the carbon monoxide binding kinetics. In other words k_3 and k_4 are much larger than $k_1 \cdot [CO]$. Finally it should be noted that the scheme does not account for the fact that before the binding of CO, the R and T structures are in a state of equilibrium. However, in the absence of CO all spectral changes observed after reduction are completed within 500 μs (unpublished results). This indicates that after this time period no redistribution of the intermediates between the R and T states occurs.

REFERENCES

1. Rollema, H.S., Scholberg, H.P.F., de Bruin, S.H. and Raap, A. (1976) Biochem. Biophys. Res. Commun., in the press.

2. Cassoly, R. and Gibson, Q.H. (1972) J. Biol. Chem. 247, 7332-7341.

3. Rollema, H.S., de Bruin, S.H. and Van Os, G.A.J. (1976) Biophys. Chem. 4, 223-228.

4. Hart, E.J., Thomas J.K. and Gordon, S. (1964) Radiation Res. Suppl. 4, 74-88.

5. Wilting, J., Raap, A., Braams, R., de Bruin, S.H., Rollema, H.S. and Janssen, L.H.M. (1974) J. Biol. Chem. 249, 6325-6330.

6. Clement, J.R., Neville, T.L., Klapper, M.H. and Dorfman, L.M. (1976) J. Biol. Chem. 251, 2077-2080.

7. De Young, A., Tan, A.L., Pennelly, R.R. and Noble, R.W. (1975) Biophys. J. 15, 80a.

8. Gibson, Q.H. (1959) Progr. Biophys. Biophys. Chem. 9, 1-54.

9. Sawicki, C.A. and Gibson, Q.H. (1976) J. Biol. Chem. 251, 1533-1542.

10. Schmelzer, U., Steiner, R., Mayer, A., Nedetzka, T. and Fasold, H. (1972) Eur. J. Biochem. 25, 491-497.

11. Ogawa, S. and Shulman, R.G. (1972) J. Mol. Biol. 70, 315-336.

12. Monod, J., Wyman, J. and Changeux, J.P. (1965) J. Mol. Biol. 12, 88-118.

SUMMARY

     This thesis reports on the results obtained from studies on i) the
influence of organic phosphates and chloride ions on the Bohr effect of
human hemoglobin, ii) the Bohr effect of valency hybrids and isolated
chains and iii) the kinetics of carbon monoxide binding to partially
reduced methemoglobin and valency hybrids.

     In chapter 1 an introduction is given to the following chapters.

     In chapter 2 the influence of DPG(2,3-diphosphoglycerate) on the Bohr
effect of hemoglobin is described in terms of differences in interaction
of DPG with oxy- and deoxyhemoglobin.

     Chapters 3 and 4 present a study on the influence of chloride ions
on the Bohr effect. It is shown that the acid Bohr effect observed in the
presence of 0.1 M KCl can be attributed to a difference in interaction of
chloride ions with oxy- and deoxyhemoglobin. This difference in interaction
is also responsible for about 25% of the alkaline Bohr effect.

     Chapter 5 reports the results obtained from a study on the Bohr effect
of valency hybrids in the presence and the absence of organic phosphates.
In the absence of organic phosphates the number of Bohr protons released
by the valency hybrids per ligand bound is equal to the value found for
hemoglobin. Upon addition of DPG or IHP (inositol hexaphosphate) an increase
in the Bohr effect is observed. However the phosphate induced additional
Bohr effect is very similar to that found for hemoglobin. These results
lead to the conclusion that the Bohr effect is linked to changes in tertiary
structure of the subunits rather than to the change in quaternary structure,
which hemoglobin shows upon ligation.

     In chapter 6 it is shown that the isolated α and β chains of human
hemoglobin have a small but significant Bohr effect. The magnitude of the
acid Bohr effect of the α chain is comparable to that of hemoglobin. In
the case of the β chain IHP interacts differently with the ligated and un-
ligated form.

     The kinetic properties of a number of hemoglobin derivatives is dis-
cussed in chapters 7 and 8. Pulse radiolysis has been utilized to study the
kinetics of carbon monoxide binding to partially reduced methemoglobin
(chapter 7) and to partially and fully reduced valency hybrids (chapter 8).

     In chapter 7 it is shown that at neutral pH after reduction of one
heme group methemoglobin remains in the R quaternary structure. Reduction

of two heme groups in methemoglobin causes a transition from the R to the T state. In the presence of IHP partially reduced methemoglobin reacts with carbon monoxide with a rate characteristic of the T state.

Experiments with valency hybrids (chapter 8) demonstrate that at pH 6.0 the carbon monoxide intermediates $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ are in the T quaternary structure. At pH 7.0 these carbon monoxide intermediates show about equal populations of the R and the T state.

In dit proefschrift wordt verslag gedaan van studies over i) de invloed van organische fosfaten en chloride ionen op het Bohr effect van menselijk hemoglobine, ii) het Bohr effect van valentie hybriden en de α en β ketens van hemoglobine en iii) de kinetiek·van de koolmonoxide binding aan partieel gereduceerde methemoglobine en valentie hybriden.

Hoofdstuk 1 geeft een inleiding tot een aantal onderwerpen die van belang zijn voor een goed begrip van de daarop volgende hoofdstukken.

In hoofdstuk 2 wordt aangetoond dat de invloed van DPG (2,3-difosfoglyceraat) op het Bohr effect terug te voeren is op een verschil in interactie van DPG met oxy- en deoxyhemoglobine.

De hoofdstukken 3 en 4 beschrijven de invloed van chloride ionen op het Bohr effect. Het blijkt dat het zure Bohr effect dat in de aanwezigheid van 0.1 M KCl waargenomen wordt, toegeschreven moet worden aan een verschil in interactie van chloride ionen met oxy- en deoxyhemoglobine. Dit verschil in interactie is tevens verantwoordelijk voor ongeveer 25% van het alkalische Bohr effect.

In hoofdstuk 5 wordt ingegaan op het Bohr effect van valentie hybriden en de invloed van organische fosfaten hierop. In afwezigheid van fosfaten vertonen de valentie hybriden een Bohr effect dat de helft kleiner is dan het Bohr effect van hemoglobine. In aanwezigheid van DPG of IHP (inositol hexafosfaat) wordt een toename van het Bohr effect geconstateerd. Deze toename in Bohr effect, is ongeveer gelijk aan de toename die bij hemoglobine wordt waargenomen. Hieruit kan geconcludeerd worden dat het Bohr effect niet zo zeer gecorreleerd is aan de veranderingen in de quaternaire structuur, die hemoglobine ondergaat tengevolge van ligatie, als wel aan de verandering van tertiaire structuur, die de subeenheden ondergaan als gevolg van ligand binding.

Uit hoofdstuk 6 blijkt dat de α en β ketens van menselijk hemoglobine ook een Bohr effect vertonen. Het zure Bohr effect dat voor de α ketens wordt waargenomen, is wat grootte betreft te vergelijken met het zure Bohr effect van hemoglobine. Het blijkt verder dat IHP een verschil in interactie vertoont met de geligandeerde en de niet geligandeerde vorm van de β ketens.

De hoofdstukken 7 en 8 bevatten een onderzoek naar de kinetische eigenschappen van een aantal hemoglobine derivaten. Er is gebruik gemaakt van de mogelijkheden die pulsradiolyse biedt om de kinetiek te be-

studeren van de binding van koolmonoxide aan partieel gereduceerd methemo-globine (hoofdstuk 7) en aan partieel en volledig gereduceerde valentie hybriden (hoofdstuk 8).

Uit het onderzoek beschreven in hoofdstuk 7 blijkt dat bij neutrale pH reductie van een heem groep in methemoglobine niet leidt tot een ver-andering in de quaternaire structuur. Reductie van twee heem groepen heeft de overgang van de R naar de T conformatie tot gevolg. In de aanwezigheid van IHP blijkt dat partieel gereduceerd methemoglobine voorkomt in de T structuur. Tevens worden aanwijzingen gevonden voor het bestaan van keten heterogeniteit met betrekking tot de kinetiek van de koolmonoxyde binding.

Experimenten met valentie hybriden (hoofdstuk 8) tonen aan dat bij pH 6.0 hemoglobine moleculen, waarvan ofwel de α ofwel de β ketens in de carboxy vorm verkeren, zich in de T conformatie bevinden. Bij pH 7.0 wordt voor deze partieel geligandeerde moleculen een significante populatie van de R toestand waargenomen.

CURRICULUM VITAE

De schrijver van dit proefschrift werd in 1946 in Riga geboren. Van 1959 tot 1964 bezocht hij de Christelijke H.B.S. te Amsterdam-N. In 1964 begon hij zijn studie scheikunde aan de Vrije Universiteit te Amsterdam.

In januari 1968 werd het kandidaatsexamen (S_3) afgelegd. Het doctoraal-examen met als hoofdvak Fysische Chemie en als bijvakken Natuurkunde en Electronica werd in maart 1972 behaald.

Van 1 april 1972 tot 1 december 1975 was hij als wetenschappelijk ambtenaar, in dienst van Z.W.O., werkzaam op de afdeling Biofysische Chemie van de Katholieke Universiteit te Nijmegen. Sedert 1 december 1975 is hij in dienst van de Katholieke Universiteit.

STELLINGEN

I

De door Imanaka et al. voorgestelde vlakke driegecoordineerde structuur voor een rhodium(II) complex is aanvechtbaar.

T. Imanaka, K. Kaneda, S. Teranishi and M. Terasawa (1976), Proceedings 6th International Congress on Catalysis, London 12-16 July, A41.

II

Bij de veronderstelling van Elligsen et al. dat een verhoging in lysosomale enzymaktiviteit aanleiding kan geven tot een verlies aan celgroeiregulering bij getransformeerde cellen, is geen rekening gehouden met het feit dat deze verhoging ook na de mitose kan plaats vinden.

J.D. Elligsen, J.E. Thomson and H.E. Frey (1975), Exp. Cell Res. 92, 87-94.

III

Op grond van de experimenten van Obermeier en Geiger met betrekking tot de semisynthese van menselijk insuline, zou het aanbeveling verdienen, de koppelingsplaats tussen het natuurlijke en het synthetische fragment te kiezen tussen Glu^{B21} en $Arg.^{B22}$.

R. Obermeier and R. Geiger (1976), Hoppe-Seyler's Z. Physiol. Chem. 357, 759-767.

IV

De bewering van Benesch en Rubin dat bij hoge DPG concentraties de Haldane en de Bohr coëfficient in getalwaarde verschillen, is niet in overeenstemming met het door henzelf gegeven theoretische verband tussen de Haldane en de Bohr coëfficient.

R.E. Benesch and H. Rubin (1975), Proc. Nat. Acad. Sci. USA 72, 2465-2467.

V

Het reactiemechanisme dat door Hendrickson et al. wordt voorgesteld voor de electrochemische reductie bij -0.22 V van het tris(N,N-di-n-butyldithio-carbamato)nikkel(IV) complex, is aan bedenkingen onderhevig.

A.R. Hendrickson, R.L. Martin and N.M. Rohde (1975), Inorg. Chem. 14, 2980-2985.

VI

De interpretatie die door Mieyal en Freeman gegeven wordt van de verzadigingscurve voor de binding van aniline aan methemoglobine, is niet voldoende gefundeerd

J J Mieyal and L S Freeman (1976), Biochem. Biophys Res Commun 69, 143 148

VII

De door Arai et al berekende dissociatieconstante voor de binding van GDP aan EF-Tu is strijdig met hun experimentele gegevens

K Arai, M Kawakita and Y Kaziro (1974), J Biochem. 76 293 306

VIII

Bij de keuze van de kleur glas van flessen voor het bewaren van geneesmiddelen dient meer aandacht besteed te worden aan de lichtgevoeligheid van bepaalde geneesmiddelen

Nederlandse Farmacopee 1966, zesde uitgave, tweede druk, Staatsdrukkerij 's-Graven hage p 34
Nederlandse Farmacopee 1973, zevende uitgave, Staatsdrukkerij, 's Gravenhage, p 268

Nijmegen, 18 november 1976   H S Rollema