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The Specific Carboxymethylation of the N-Terminal Amino Groups of Human Hemoglobin: Structural and Functional Implications

Wendy Jane Fantl

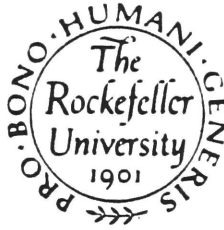
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The Specific Carboxymethylation of the N-Terminal
Amino Groups of Human Hemoglobin:
Structural and Functional Implications

A thesis submitted to the faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Wendy Jane Fantl

May, 1986

The Rockefeller University

New York, NY 10021

DEDICATION

This thesis is dedicated to the memory of my father, Zdenek Fantl, Dr. Juris (Prague), LL.M (London), 1911-1965. His example as a fine human being and brilliant scholar has and always will be my inspiration.

ACKNOWLEDGEMENTS

It has been both a privilege and an honor to have worked with my advisor Dr. James M. Manning and to have benefited from his great scientific acumen. I wish to extend my thanks for his tremendous patience and kindness throughout the course of these studies.

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List of Abbreviations

<u>Name</u>	<u>Abbreviation</u>
Adenosine Triphosphate	ATP
Bicarbonate	HCO_3^-
Bis(2-Hydroxyethyl) Imino-Tris(Hydroxymethyl) Methane	bistris
Butyloxycarbonyl	boc
Carbamyl	Cbm
Carboxymethyl	Cm
2,3-Diphosphoglyceric Acid	2,3-DPG
Electron Paramagnetic Resonance	EPR
Ethylenediaminetetraacetic Acid	EDTA
N-Ethylmaleimide	NEM
N-Ethylsuccinamide	NES
Ferri-cyanide	$[\text{Fe}(\text{CN})_6]^{4-}$
Guanosine Triphosphate	GTP
Hemoglobin	Hb
High Performance Liquide Chromatography	HPLC
4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid	HEPES
p-Hydroxymercuribenzoate	p-HMB
Inositol Hexaphosphate	IHP
Inositol Hexasulfate	IHS
2-Methyl-2,4-Pentanediol	MDP
Myoglobin	Mb
Nuclear Magnetic Resonance	NMR
Partial Pressure of Oxygen at Which Hb is Half Maximally Saturated with Oxygen	P_{50}
Pyridoxal Phosphate	PLP
Relaxed Conformation	R

Sodium Hydroxide	NaOH
Tense Conformation	T
L-(1-Tosylamido-2-Phenyl) Ethyl Chloromethyl Krtone	TPCK
Trichloroacetic Acid	TCA
Tris(Hydroxymethyl)-Aminomethane	Tris

INTRODUCTION

A) The Technique of Carboxymethylation

The studies reported in this thesis describe the conditions elucidated for the selective reductive carboxymethylation of the α -amino termini of hemoglobin (Hb)¹ and the structural and functional consequences of such a modification. The initial premise for such a modification ($\text{HbNHCH}_2\text{COO}^-$) was to test its usefulness as a carbon dioxide (CO_2) or carbamino analogue (HbNHCOO^-). The latter compound is formed reversibly (which is physiologically necessary) and cannot be isolated. The former is irreversibly formed and, when prepared in sufficient amounts, could lead to a wealth of information concerning the binding of CO_2 to Hb as well as the interplay between this effector and other important physiological modulators of hemoglobin.

The first question to be addressed concerned the choice of reagent to be used as the carboxymethylating agent. As early as 1911 Thunberg (1, 2) suggested that the toxicity of certain haloacids (in his study iodoform, CH_3I) was a result of their reactivity with tissue sulfhydryl groups. Indeed, later studies (3) proved that the carboxymethyl group could be introduced into various amino acid side chains such as those of cysteine, histidine, methionine, and lysine depending upon the conditions employed (Figure 1).

The mechanism is usually an $\text{S}_\text{N}2$ reaction: a nucleophilic substitution that is bimolecular (2, 4). The α -carbonyl group enhances the leaving capacity of the halide which is usually iodide or bromide. These two are more efficient than chloro- or fluoroacetate. Haloacetamides may also be used. Carboxymethylation by α -haloacetates

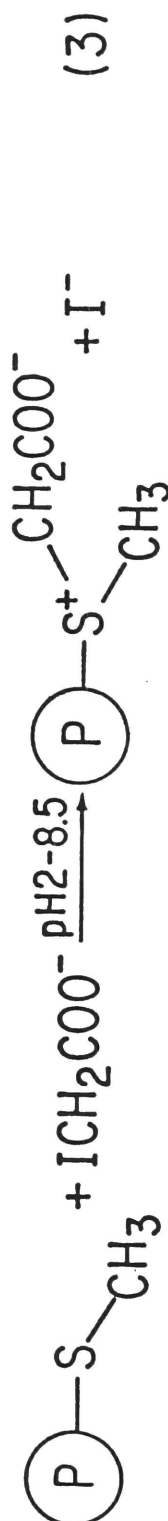
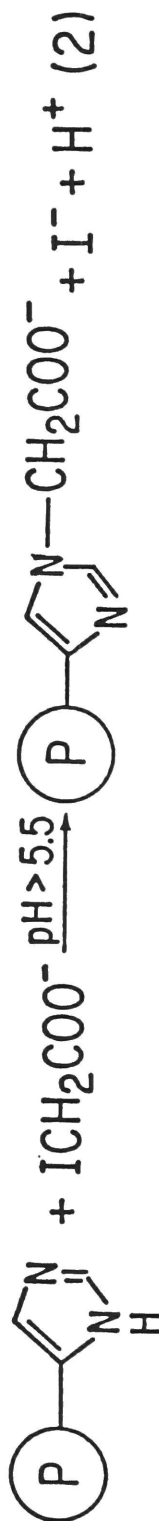
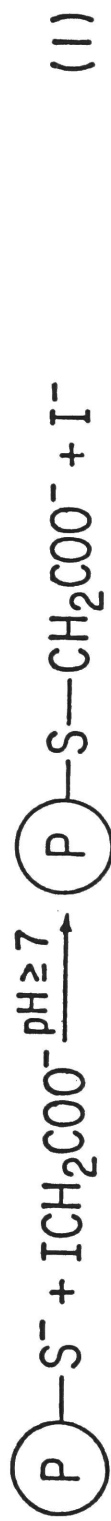


Figure 1 - Carboxymethylation of various amino acid side-chains using iodoacetate. Redrawn from reference 2.

can give valuable information regarding the degree of exposure of certain protein residues as well as information regarding, for example, the active site of an enzyme, since the carboxymethyl group is identical to the side-chain of aspartic acid and should cause minimal perturbation upon introduction into a protein. This proved to be the case in studies involving ribonuclease carried out in the laboratory of Moore and Stein (5-10). When RNase-A is carboxymethylated by iodoacetate, two inactivated enzyme products, 1-carboxymethylhistidine-119 and 3-carboxymethylhistidine-12 ribonuclease, are formed in a ratio of 8 to 1 (5). The salient features of the reaction are firstly a pH optimum of 5.5 to 6.0, which is lower than expected for histidine residues with normal exposure. The unprotonated imidazole is the reactive species. Also, the extent of reaction in each case is limited to monocarboxymethylation. The hypothesis put forward to account for the differential activities of His-12 and His-119, proposed that the two imidazoles were close enough (5 \AA) for the iodoacetate anion to interact with the protonated form of one imidazole and so orientate it correctly to react with the unprotonated form of the other (6). The difference in the yields of the two alkylation products may be explained on steric grounds and indeed Heinrickson et al (7) were able to demonstrate that the ratio of one inactivated product to the other at pH 5.5 could be altered by use of a haloacid which differed with respect to chain length, optical configuration, additional functional groups, as well as the position of the halogen relative to the carboxylate group. In addition, RNase-A was also inactivated by carboxymethylation with bromoacetate at pH 8.5. At this pH, however, 70% of the reaction product was due to the carboxymethylation of Lys-41, with His-12 and

His-119 accounting for the remaining 30% (8). These results were in good agreement with earlier studies (9) that implicated the role of Lys-41 in the active site of RNase-A. It was shown further in this study (8) that while the presence of carboxymethyllysine-41 did not inhibit the carboxymethylation of His-12 and His-119 at pH 5.5, it reduced carboxymethylation of the former to a greater extent than for His-119. From these studies valuable information was derived with regard to the residues Lys-41, His-12, and His-119 of the active site of RNase-A.

In the case of the reaction of sperm whale metmyoglobin, with bromoacetate at pH 7.0, the reactivities of the histidine residues were correlated with the crystalline structure (3, 11, 12). By careful choice of conditions, α -haloacetates can yield valuable information regarding the active center of an enzyme or structural features of a protein. However, the possibility of using an α -haloacetate for the carboxymethylation of only the amino groups of Hb seemed remote for the following reason. Reaction with an α -haloacetate requires the amino function to be unprotonated and since the pK_a of this moiety is about 9, or lower at the N-terminus of a protein, reaction conditions must necessarily employ a higher pH. Even under such conditions the rate of carboxymethylation of an unprotonated amino group with an α -haloacetate is 1/100 that of a sulfhydryl group (2), which is too slow to be particularly useful. Without much doubt, the carboxymethylation by an α -haloacetate of the α -amino groups of Hb would be accompanied by the unwanted carboxymethylation reaction of other residues.

An alternate method was developed by Means and Feeney (13, 14) for modifying protein amino groups. Reductive alkylation (14, 15)

(Figure 2) converts the amino groups of proteins to their alkylamine derivatives (13) by reduction of the Schiff base intermediate formed between the amino group and an added aldehyde, such as formaldehyde (reductive methylation). Sodium cyanoborohydride is usually the reducing reagent of choice (15-19) since it is specific for the reduction of a protonated Schiff base. This is in contrast to sodium borohydride (Figure 2) which will also reduce any aldehyde directly, thereby decreasing the efficiency of a reaction. This latter reducing agent is unstable at neutral pH, reduces disulfide bonds and can cleave peptide bonds (20).

In numerous cases reductive alkylation does not significantly impair the biological properties of a protein (13-17). For example, extensive methylation ($\geq 50\%$) of the lysyl groups of alkaline phosphatase, trypsin, β -galactosidase, or lactate dehydrogenase allows 50-80% of the full activity to remain (16). The activities of chymotrypsin (13), trypsin (21), ovine pituitary luteinizing hormone (22, 23) and hen egg white lysozyme (24) are not appreciably changed upon reductive methylation. The activity of horse alcohol dehydrogenase appears to increase upon reductive methylation (25). On the other hand, yeast hexokinase P_1 and glucose-6-phosphate dehydrogenase were inactivated by the same modification (16). This is also the case for pancreatic ribonuclease in which the catalytic activity of the protein is reduced by 99% upon reductive methylation without any significant structural change (2, 13, 14, 17). In another study, reductive methylation using ^{14}C or ^3H label, those lysine residues in the bovine brain tubulin dimer essential for polymerization were identified (26). Because many proteins retain their native physico-chemical and

Reductive Methylation with Either NaBH_4 or NaCNBH_3

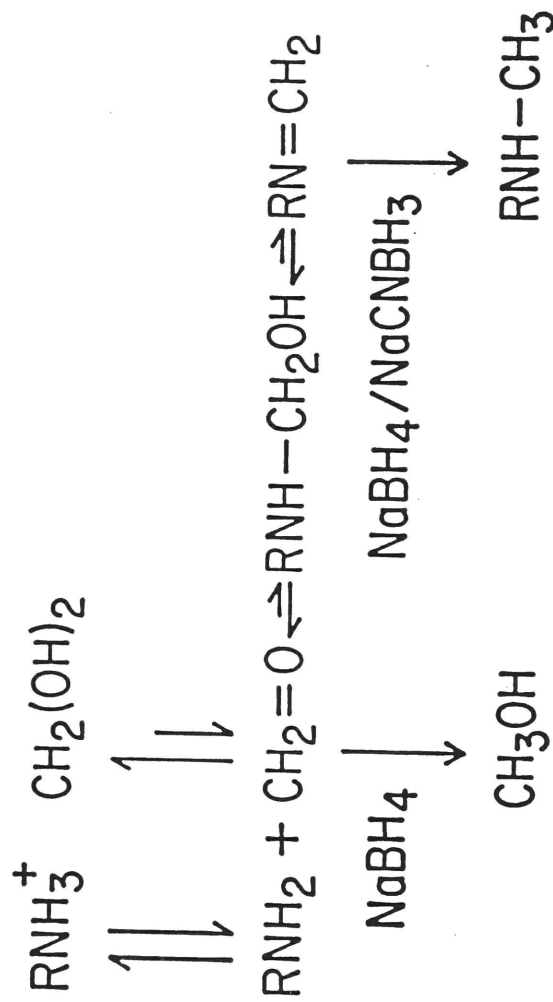


Figure 2 - The technique of reductive alkylation using formaldehyde and sodium borohydride or sodium cyanoborohydride. Redrawn from reference 16.

biological properties upon reductive alkylation, especially methylation, labeled proteins can be prepared of high specific activity as in the case of the radioimmunoassay reported by Tack et al (27). The introduction of the ^{13}C label affords a means to study lysyl group function by ^{13}C -NMR (28, 29).

So far, the carbonyl compound of choice in the studies described above has been formaldehyde, which has many advantages over other carbonyl compounds. The methyl group which is of small size and neutral charge can be introduced readily into protein amino functions under mild conditions. The resultant secondary or tertiary amines retain their positive charge and often have pK_a values that are quite similar to the unmodified amino group. The methyl groups once incorporated into a protein appear to have minimal effects on the protein conformation. Moreover, reductive alkylation provides an efficient method to introduce ^3H , ^{13}C or ^{14}C radioactive label into a protein. The mechanism of reductive methylation is such that the dimethylation predominates (17). In certain cases it may be advantageous to use different carbonyl compounds for the reductive alkylation of protein amino groups (17). Since such a compound would necessarily be bulkier, the rates of reaction are predictably slower, and formation with the resultant secondary amine to the dialkyl derivative should usually be precluded. Tsai (30) studied the effect of introducing various alkyl substituents into those lysine residues of horse liver alcohol dehydrogenase which interact with the nicotinamide coenzyme. He wished to examine what factors other than the net charge effect could influence the reactions of the enzyme. Reductive ethylation of lysine residues using acetaldehyde and sodium borohydride enhanced enzymatic activity 3-4

fold. Introduction of a negative charge through carboxymethylation of the enzyme, utilizing sodium glyoxylate and sodium borohydride, decreased enzyme activity by half. The reaction mechanism is shown in Figure 3.

In a study carried out by King et al (31), ragweed pollen antigen E was reductively carboxymethylated using sodium glyoxylate and sodium cyanoborohydride. The purpose of their study was to produce derivatives of the native antigen with reduced allergic activities, yet with retention of the original immunogenic properties of the native molecule. Such derivatives could have therapeutic potential since greater amounts could be safely used with allergic patients. The studies of Tsai et al (30) and King et al (31) demonstrated that a carboxymethyl (Cm) group could be introduced into a protein without severe conformational perturbation. It therefore seemed possible that the introduction of the carboxymethyl group into the Hb tetramer could be brought about using the same reagents with careful monitoring as to whether carboxymethylation was N-terminal or whether the modification was on lysine residues. This conclusion would allow us to determine whether such a carboxymethylated derivative may be used as a structural analogue for carbamino Hb. In addition, it could provide more information concerning the interaction and regulation of Hb with anions, CO_2 , and protons (Bohr effect).

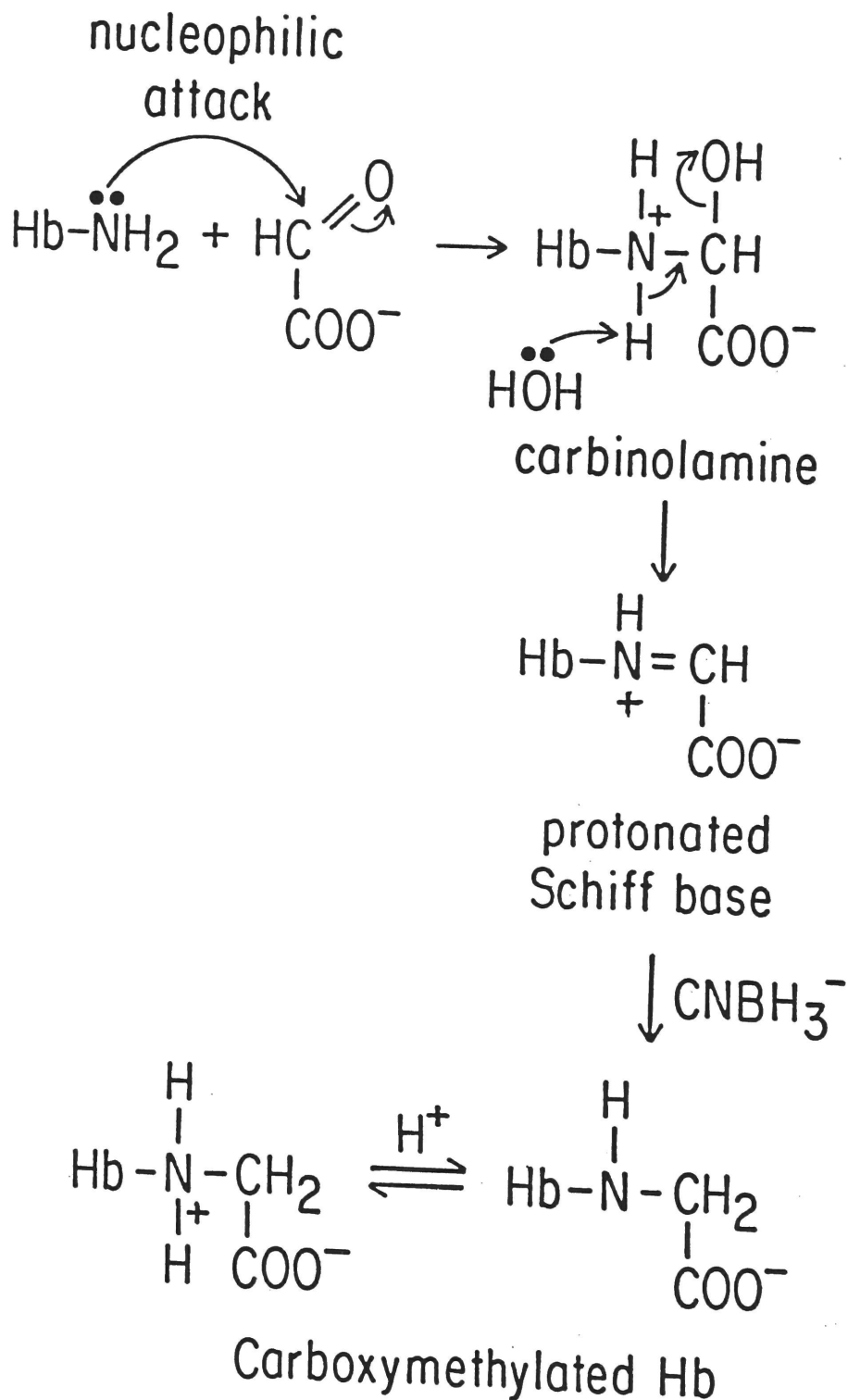


Figure 3 - The mechanism for reductive alkylation using sodium glyoxylate and sodium cyanoborohydride.

B) The Binding of Anions to Hb

In this section of the Introduction an attempt is made to review what is known about inorganic anion binding to Hb, Section B-1, and organic anion binding which will be reviewed in section B-2.

B-1) Inorganic Anion Binding to Hb

The profound influence of salt on the oxygenation of Hb has been recognized for a long time (32-37). In general, as the concentration of salt is increased, the oxygen affinity decreases (34-37). This necessarily means that there is a preference for the binding of salt to the deoxy as opposed to the oxy conformation of Hb (36). There is a wide range in the effectiveness of different anions on the oxygen affinity of Hb (34, 35, 37). However, at high enough salt concentration the oxygen affinity is similar in solutions of different salts. At concentrations greater than 1 M, however, the P_{50} (defined as the partial pressure of oxygen at which Hb is half maximally saturated with oxygen) may increase or decrease depending upon the nature of the anion (37). It is important to note, however, that the more highly charged organic anions are more efficacious in lowering the oxygen affinity of human HbA: the most profound effects being elicited by inositol hexaphosphate (IHP) and 2,3-diphosphoglyceric acid (2,3-DPG) (36).

Two physical techniques have been utilized for the identification of inorganic anion binding sites: $^{35}\text{Cl}^-$ nuclear magnetic resonance (NMR) studies (38-41) and X-ray crystallographic studies (42-44). Chloride binding to human HbA could be measured directly by NMR since $^{35}\text{Cl}^-$ with a nuclear spin of 3/2 would make such a study feasible. Studies carried out by Chiancone et al (38) measured chloride binding to oxy,

carbonmonoxy, and deoxy Hb, as well as to myoglobin. The results obtained from quadrupole relaxation experiments on the excess line-width of the nuclear magnetic resonance signals of $^{35}\text{Cl}^-$ when it is bound to Hb were suggestive of at least two classes of chloride binding sites. The high affinity sites are oxygen-linked, since, over a range of chloride concentration from 0.1 to 2.5 M, deoxy Hb binds more chloride than oxy Hb. This effect is maximal at pH 7 when the chloride concentration is 0.1 M. The difference in the amount of chloride bound to deoxy compared to oxy Hb is 2 chloride ions per tetramer. Interestingly, no such difference was apparent in the myoglobin counterparts.

The well-known effect that the organic phosphates, 2,3-DPG, adenosine triphosphate (ATP) and IHP and to a somewhat lesser degree inorganic phosphates have in lowering the oxygen affinity of Hb suggests preferential binding of these modulators to deoxy Hb (36, 45, 46). This will be discussed in more detail later. At this stage it is interesting to note that high concentrations of salt are able to suppress the effects on oxygen affinity of these organic modulators (36). This suggests the possibility of competition for a common binding site. Indeed Chiancone et al (38) tested this hypothesis by examining the effect of ATP on $^{35}\text{Cl}^-$ line-width in the presence of various sodium chloride concentrations at different pH values. They observed a maximal effect of ATP on both oxy and deoxy Hb at pH values between 6.0 and 6.7 and a chloride concentration of 0.2 M. Increasing the chloride concentration to 0.5 M or elevating the pH to 7.5 diminished or abolished, respectively, the effect of ATP on the $^{35}\text{Cl}^-$ line-width. These investigators proposed that this effect was due to high affinity

anion binding sites, since the concentration of chloride that inhibits ATP binding is relatively low. Chiancone et al (38) then provided evidence for low affinity chloride binding sites. From $^{35}\text{Cl}^-$ NMR experiments where Cys-93(β) has been modified by p-hydroxymercuribenzoate p-HMB, the suggestion was proposed that these low affinity sites may involve the N-terminal regions of the β -chains (38). In a later publication (41), Chiancone et al extended their $^{35}\text{Cl}^-$ nuclear magnetic resonance studies to either HbA digested with carboxypeptidase A or with carboxypeptidase B or with a mixture of these two enzymes. Again they are able to show the presence of two different classes of chloride binding sites. Under their experimental conditions it is the high affinity sites that are studied in detail. In the single digests a strong oxygen-linked chloride binding persists as in unmodified HbA. In the mixture of the two digestions, even though chloride still binds, it is no longer oxygen-linked. Such an observation indicates the importance of quaternary structure in the binding of chloride. Indeed Perutz and Ten-Eyck (47) showed by X-ray crystallography that both des-(Arg-141(α)), (carboxypeptidase B digest), and des-(His-146(β)) (carboxypeptidase A digest) are able to assume either an oxy or a deoxy quaternary conformation. However, an Hb tetramer treated with both carboxypeptidase A and B can only be crystallized in the oxy conformation.

As to the actual assignment of binding sites for chloride, Chiancone et al (41) carried out competition studies using IHP and found results suggestive of some common sites for both organic phosphates and chloride. In the same communication (41) the results from a $^{35}\text{Cl}^-$ NMR experiment using unmodified HbA, or HbA digested with carboxypeptidase

B, suggested a chloride binding site 'at or near Val-1(α_1)-Arg-141(α_2)'. Results using HbA digested with carboxypeptidase A were suggestive of a chloride binding site 'at or near His-146(β)'.

As mentioned above, X-ray crystallographic studies (42-44) have also been of great importance in determining the sites for anion binding. At a resolution of 3.5 Å, an electron density map of human deoxy Hb indicated the possibility of a salt bridge between the amino group of Val-1(α_1) to the carboxyl group of Arg-141(α_2) as well as to the symmetrical counterpart (48, 49). The interaction was absent in both Met HbA (50) and in carbonmonoxy Hb (51). At the time it was postulated that the increased pK_a that such an interaction would necessarily confer in deoxy Hb could account for that percentage of the alkaline Bohr effect attributed to Val-1(α) (49, 52, 53) (This will be discussed more fully later.) In a later study, Fermi (54) refined a 2.5 Å resolution electron density map of human deoxy Hb and showed that the intermolecular distance of 5.3 Å between the carboxyl and amino moieties was too far for the formation of a direct salt bridge in deoxy Hb. He speculated that there may be a water molecule between the two groups (54).

Later X-ray diffraction studies of deoxy Hb specifically modified either by carbamylation (42) or reaction with pyridoxal sulfate (55) provided some new insight into the location of two inorganic anion binding sites. In these studies evidence was given suggestive of an inorganic anion binding site between the amino terminus of Val-1(α_1) and the positively charged guanidinium group of Arg-141(α_2). A second site was thought to occur between the amino terminus of Val-1(α_1) and the hydroxyl group of Ser-131(α_1). A subsequent study (43) presented direct

evidence for the above conclusions by showing that selenate anions could bind to the proposed sites. The distance between the amino group of Val-1(α_1) and the hydroxyl group of Ser-131(α_1) (as well as that of the symmetrical counterpart) is very similar in horse Met Hb (50) (5.5 \AA) and in human deoxy Hb (5.2 \AA). However, the amino group of Val-1(α_1) is 10 \AA away from the guanidinium group of Arg-141(α_2) in horse met Hb and decreases to 8 \AA in human deoxy HbA. This finding suggests that the latter anion binding site may only exist in the deoxy conformation, making this locus an oxygen-linked binding site (43).

Indeed, Rollema et al (56) proposed that the contribution to the alkaline Bohr effect (to be discussed in more detail later) by Val-1(α) is due to the differential binding of chloride to deoxy versus oxy Hb. Support for this hypothesis was reported by Nigen et al (57). They measured the rate of carbamylation of both oxy and deoxy Hb in the absence and presence of chloride. They observed a 3-fold enhancement in the rate of reaction with deoxy Val-1(α) compared to the oxy form and that chloride was a better competitive inhibitor of the reaction under deoxy conditions. These observations were explained in the following manner. The isocyanate anion, the reactive form of cyanate (58), forms a complex between the protonated amino terminus and other cationic groups closeby, (Arg-141(α), Lys-127(α)). That the site of this complex formation is an anion binding site is supported by two further pieces of experimental evidence. Firstly, the enhanced rate of reaction did not occur in des-(Arg-141(α)) HbA and secondly, increasing concentrations of chloride could effectively reduce the rate of carbamylation. Thus, this kinetic study fits in well with the results reported from the X-ray data (43). In a later and more detailed study (44) of the preliminary data

presented on carbamylated Hb (42), X-ray data in conjunction with solution studies again implied that the amino terminus of Val-1(α) was associated with two inorganic anion binding sites. Three significant features were evident from the X-ray data of deoxy $\alpha_2^{Cb\text{m}}\beta_2$ (where $\alpha^{Cb\text{m}}$ refers to carbamylation of the amino terminus of the α -chain). A positive electron density peak was contiguous with the native electron density peak of the α -chain amino terminus indicating the position of the carbamyl group. Two negative peaks were seen, one between the Val-1(α) NH_2 -terminus and the guanidinium group of Arg-141(α_2) and the other between Val-1(α_1) NH_2 and the hydroxyl of Ser-131(α_1). These negative electron density peaks were thought to represent the displacement of an inorganic anion from these sites. In the same report O'Donnell et al (44) measured the alkaline Bohr effect of $\alpha_2^{Cb\text{m}}\beta_2$. The value for the Bohr coefficient of $\alpha_2^{Cb\text{m}}\beta_2$ was similar in both 5 mM and 100 mM chloride. These results differed from the values obtained for unmodified HbA under similar conditions as measured by Rollema et al (56) and confirmed by O'Donnell et al (44). The alkaline Bohr effect of unmodified HbA was observed to increase by about 30% when the chloride concentration was increased from 5 mM to 100 mM (44, 56). The interpretation of these data proposed that chloride binding to Val-1(α) in deoxy HbA was responsible for the contribution made by this residue to the alkaline Bohr effect. Carbamylation at Val-1(α) abolishes the binding of chloride at this locus which consequently diminishes the alkaline Bohr effect (44). The results from this X-ray crystallographic study (44) allowed the chloride binding sites associated with Val-1(α) to be identified directly. Moreover, the results were consistent with the $^{35}\text{Cl}^-$ NMR experiments described earlier in the Introduction (41).

In addition, the solution studies from the laboratory of Manning (57, 59) showed that chloride was a competitive inhibitor of carbamylation at Val-1(α), more so in the deoxy than the oxy form. Also, at chloride concentrations below 10 mM the difference in oxygen affinities of unmodified HbA and $\alpha_2^{Cb\text{m}}\beta_2$ is less (44, 59) than at higher concentrations of chloride (44). These data may be interpreted with respect to a functional equivalence of unmodified HbA and $\alpha_2^{Cb\text{m}}\beta_2$. In contrast, however, the results of Poyart et al (60) showed that the oxygen affinity of $\alpha_2^{Cb\text{m}}\beta_2$ was increased with respect to that for unmodified HbA even at chloride concentrations below 10 mM. They interpreted these data with respect to their different experimental conditions and also suggested that the carbamylation of Val-1(α) may somehow destabilize the T conformation at sites other than those involved in the binding of chloride.

It may be interesting at this point to consider how specific carboxymethylation at the α -chain NH_2 -terminus could influence the binding of anions to this site. One could extrapolate from the X-ray studies of the $\alpha_2^{Cb\text{m}}\beta_2$ derivative and postulate that the modification at the Val-1(α) amino terminus has eliminated the binding sites for inorganic anions. However, in this case we are dealing with the carboxymethyl group which is a charged moiety. Since chloride bound to Val-1(α) causes a decrease in oxygen affinity, the 'permanently bound' $-\text{CH}_2\text{COO}^-$ group at Val-1(α) could elicit a similar effect. Also, that percentage of the Bohr effect normally attributed to the binding of chloride at Val-1(α) would be unchanged if the carboxymethyl moiety acts in an analogous manner with chloride.

The observation cited earlier (36, 38, 41) that chloride and organic phosphates can compete with each other suggests that these two types of anions may share a common binding site/sites on the Hb tetramer. Indeed, in a later study (61) the effect of chloride on unmodified HbA in the presence or absence of IHP showed that at a sufficiently high concentration of chloride the effects of the polyanion could be abolished. Thus, initially, another site to which chloride or inorganic phosphate was presumed to bind included some of the residues identified in the 2,3-DPG binding cleft (62, 63). The effect of increasing concentrations of chloride on the oxygen binding properties of different Hb mutants gave a great deal of insight as to which residue(s) within the 2,3-DPG binding cleft were involved in anion binding. Hb Abruzzo (His-143(β) \longrightarrow Arg) (64), Hb Little Rock (His-143(β) \longrightarrow Gln) (65), Hb Deer Lodge (His-2(β) \longrightarrow Arg) (66), Horse Hb (His-2(β) \longrightarrow Gln) (67), Hb Raleigh (Val-1(β) \longrightarrow Acetylala) (68) and $\alpha_2\beta_2^{\text{Cbm}}$ (69) (where the β -chain is carbamylated specifically on the amino terminus) all show similar oxygen binding patterns in the presence of increasing concentrations of chloride (61). In contrast to these results are those from the chloride titrations of Hb Providence Asn (Lys-82(β) \longrightarrow Asn) and Hb Providence Asp (Lys-82(β) \longrightarrow Asp) (61, 70). There is a dramatic reduction in the oxygen-linked chloride binding for both mutant hemoglobins but the reduction is greater for Hb Providence Asp (61). In both mutants the net positive charge within the 2,3-DPG binding cleft is reduced. Likewise, Hb Rahere (Lys-82(β) \longrightarrow Thr) (71) also exhibits a reduced response to oxygen-linked chloride binding. The explanation for this behavior is as follows. In the complete absence of chloride, the positive charges in the 2,3-DPG binding crevice will repel

each other, creating an energetically unfavorable deoxy T structure. The R conformation, being of lower energy, will be preferred and this will increase the oxygen affinity of Hb under these conditions. Removal of the charge at Lys-82(β) in the mutants described above should serve to lower the energy of the T conformation, thereby reducing the oxygen affinity of Hb in the absence of chloride. Presumably, the electrostatic repulsion caused by Lys-82(β) is greater than the repulsion at the other 2,3-DPG binding residues. Addition of chloride to unmodified HbA removes electrostatic repulsion and lowers the energy of the deoxy T state, thus favoring this quaternary structure.

At this stage one might consider what response Hb, specifically carboxymethylated at the residue Val-1(β), would have to increasing concentrations of chloride. As noted above, Hb Raleigh (Val-1(β) \rightarrow Acetylala (68) showed a normal response in its oxygen linked chloride binding as did $\alpha_2\beta_2^{\text{Cbm}}$ (69).

In another study Manning et al (59) and Nigen et al (72) studied the properties of a Hb hybrid, $\alpha_2^{\text{Cbm}}\beta_2^{\text{Prov Asn}}$. In this derivative the α -chains have NH_2 -terminal residues that have been blocked specifically by carbamylation and the β -chains have been derived from Hb Providence Asn. Here there was a small but significant dependence of its oxygen equilibrium curve on the concentration of inorganic anions. This necessarily implicates a third inorganic anion binding site. Since it is not known whether one or both the anion binding sites are lost when the α - NH_2 -terminus is carbamylated, it is possible that one oxygen-linked anion binding site per half-molecule might remain between Ser-131 of one α -chain and Arg-141 of the other. In an NMR study by Wiechelman

et al (73), additional support was provided for the role of Lys-82(β) in the binding of inorganic anions.

Another possibility for the identification of a third inorganic anion binding site was provided in a communication by Adachi et al (74). They demonstrated that the chloride effect on the P_{50} values of Hb York (His-146(β) \rightarrow Pro) was only 20% of that found for HbA. In addition, they measured the same parameters in three types of Hb cross-linked by Bis 3,5-dibromosalicylfumarate (75) between Lys-82(β_1) and Lys-82(β_2). In HbS-f (where the nomenclature 'f' indicates Hb that is cross-linked) cross-linking the two lysines reduced the chloride effect by 60% and confirmed the earlier reports that identified Lys-82(β) as an inorganic anion binding site (59, 61, 70, 72). In Hb York-f the chloride effect is reduced to the same extent as in the native mutant Hb York (20% of the value obtained for unmodified HbA). This supposedly indicates that the mutation at His-146(β) simultaneously abolishes the chloride binding effect at Lys-82(β). The manner in which this is manifested is unclear, especially since Lys-82(β) and His-146(β) are fairly far apart on the molecular model, although both are part of the DPG cleft. These workers found a reduction of 60% for the chloride effect of HbSY-f. In this derivative a β -chain from Hb York and a β -chain from HbS are cross-linked with Walder et al's reagent (75). Since it is well established (44, 60) that Val-1(α) contributes 20% to the total chloride binding effect, Adachi et al (74) allocate 40% of the chloride effect to the β -chain carboxyl terminal histidines. Carbamylated HbY showed no oxygen-linked chloride binding.

What could one speculate about $\alpha_2\beta_2^{Cm}$? In this derivative the negative charge introduced directly at Val-1(β) should serve to lower

the repulsive energy within the 2,3-DPG binding cleft. This negative charge could interact to different extents with His-2(β), Lys-82(β), or His-143(β). Any such interactions could stabilize the deoxy quaternary structure and the oxygen affinity of this derivative could be lowered. Extrapolating back to the titration data obtained with Hb's Providence Asn and Asp and Hb Rahere (61), one could expect reduced oxygen-linked chloride binding if there were an interaction between the carboxymethyl moiety with Lys-82(β). This could further substantiate the identity of Lys-82(β) as an inorganic anion binding site.

To summarize, it appears that there are at least three and perhaps four inorganic anion binding sites. Numerous studies have been consistent with two sites at Val-1(α), where one anion interacts with the amino group and the positively charged guanidinium group of Arg-141 on the opposite α -chain, and the other with the amino terminus and the hydroxyl group of Ser-131 of the same α -chain. In addition, numerous studies have substantiated evidence for an anion binding site at Lys-82(β). A recent study measuring the oxygen-linked chloride binding of the mutant Hb York (His-146(β) \longrightarrow Pro) assigns His-146(β) as a possible binding site. It will be of interest to investigate the interaction of chloride with Hb specifically carboxymethylated to see what effect this modification has on the functional properties of Hb.

B-2) Organic Anion Binding to Hb

It had been known for at least fifty years that the oxygen affinity of blood increases when the red cell contents are diluted by hemolysis. This led Barcroft to speculate as early as 1921 as to whether there was 'some third substance present...which forms an integral part of the oxygen hemoglobin complex?' (76). The actual presence of 2,3-DPG in human red blood cells was found in 1941 (77). However, it was not until the mid 1960's that the identification and importance of this 'third' substance was fully realized as 2,3-DPG (77-80). The key result to emerge, with its tremendous ramifications concerning the physiology of blood gas transport, was that the affinity of Hb for oxygen and 2,3-DPG is a reciprocal relationship (45, 46). This necessarily implies that binding of 2,3-DPG to Hb decreases oxygen affinity and vice versa. Among the organic phosphates found in the red cell that lower the oxygen affinity of Hb, 2,3-DPG is the most abundant. It is nearly equimolar (5 mM) with the concentration of Hb found within the red cell (45). Although ATP could modify the oxygen affinity of Hb (its concentration is about 1.3 mM), its high affinity for magnesium eliminates this effect (81). Other organic phosphates which modify the oxygen binding behavior of Hb are found in other species. In some birds and turtles, 2,3-DPG is replaced by either inositol hexaphosphate (77) or inositol pentaphosphate (82).

A consideration of any of the data published before 1966 concerning the functional properties of Hb must take into account the presence of variable amounts of 2,3-DPG in the sample. For complete removal of the organic phosphate, dialysis against chloride of at least 0.1 M is necessary to yield 'stripped' Hb (46, 83). An oxygen binding

curve of this Hb at low ionic strength shows a high oxygen affinity: indeed the value approaches that for myoglobin (46). Subsequent studies by many groups (36, 83-87) demonstrated conclusively the effect of 2,3-DPG on the oxygen affinity of Hb, namely, a decrease. The suggestion was made (84) that 2,3-DPG somehow stabilizes the deoxy T conformation, since there is increasing resistance to oxygenation as the concentration of 2,3-DPG is raised. The Benesch laboratory then went on to measure the stoichiometry of 2,3-DPG-Hb complex formation (83, 84). A single molecule of DPG was bound by deoxy Hb but not by oxy Hb, under their conditions of ionic strength, pH and temperature. Other experimentors reported significant binding of 2,3-DPG and other organic phosphates to oxy Hb (36, 87-91). The relevance of this is unclear at present. However, non-specific binding should not be unexpected since there are many positively charged residues to which 2,3-DPG could bind. As far as their involvement in regulating oxygen affinity is concerned, the residues in the 2,3-DPG binding cleft are the only ones of importance (62).

It was clear from the DPG-Hb binding study mentioned above (83, 84) that one mole of 2,3-DPG was bound per mole of deoxy Hb tetramer. The same was found to be true for the β_4 tetramer (45, 92) which has a deoxy like conformation even in the presence of oxygen (93). Furthermore, the oxygen affinity of the β_4 tetramer was uninfluenced by the binding of 2,3-DPG, indicating that the molecular basis by which 2,3-DPG regulates oxygen affinity in unmodified HbA depends on its differential affinity for two different quaternary conformations.

The electrostatic nature of the interaction between 2,3-DPG and Hb precludes isolation and identification of the 2,3-DPG-containing

fragment. Both Perutz et al (50) and Benesch et al (92) did postulate correctly that the entrance to the central cavity on the diad axis of Hb would be a suitable binding site for DPG. This cavity expands upon deoxygenation and closes up on oxygenation (94-96), thereby expelling the DPG. The pyridoxal phosphate (PLP) studies of Benesch et al (97-99) showed that Val-1(β) was a possible 2,3-DPG binding site since PLP was an effective competitor of 2,3-DPG binding to deoxy Hb and conferred the same decrease in oxygen affinity. Several other biochemical studies were carried out in attempts to identify the DPG binding site (85, 92, 100-102). Of the aforementioned studies, the one reported by Bunn and Briel (85) investigated the interaction of 2,3-DPG with various human hemoglobins. Glycosylated HbA, where Val-1(β) is glycosylated, HbF, which possesses Gly-1(γ) and Ser-143(γ) (as opposed to His-143 in the β -chain), and acetylated HbF, where Gly-1(γ) is acetylated, all showed diminished responsiveness toward 2,3-DPG. At that time a consideration of these observations (85) with respect to the X-ray data of human deoxy HbA (95) suggested that Val-1(β) and His-143(β) may be residues involved in the binding of 2,3-DPG.

The definitive identification of the residues involved in the binding of 2,3-DPG came from the X-ray data of Arnone (62). He used 2-methyl-2,4-pentanediol to avoid the use of high salt concentrations used in previous crystal preparations, which would dissociate the DPG-deoxy Hb complex. One molecule of 2,3-DPG bound to one molecule of deoxy Hb tetramer and the site was seen to lie at the entrance to the central cavity between the N-termini of the β -chains. This confirmed the earlier predictions obtained from biochemical and model building experiments (50, 46). The site is surrounded by four pairs of basic

groups contributed by the residues Val-1(β), His-2(β), Lys-82(β), and His-143(β). The arrangement of these groups is such that they are able to form seven salt bridges with the anionic groups of 2,3-DPG. Upon oxygenation the N-terminal valines move apart and the binding cavity closes up and thereby facilitates the expulsion of the organic phosphate.

Several other multivalent anions are also able to bind to the central cavity described above, stabilize the deoxy T conformation and thereby facilitate the release of oxygen. These anions include ATP, guanosine triphosphate (GTP), ferri-cyanide ($[\text{Fe}(\text{CN})_6]^{4-}$), IHP (103), myo-inositol hexasulfate (104) and polycarboxylic acids (105-108) (to be discussed later). The most effective of the above is IHP which is very similar to inositol pentaphosphate found in birds and takes the place of 2,3-DPG in regulating the oxygen affinity of their erythrocytes (77, 82). The X-ray analysis of the IHP-deoxy Hb complex (103) reveals that the mode of binding in this case is similar to that of 2,3-DPG binding. However, since IHP has eight negative charges compared with the three or four for DPG at neutral pH, the IHP-deoxy Hb complex will have a much higher binding energy (the dissociation constant was estimated to be at least 10^{-8} M (109) compared to a value of 1.5×10^{-5} M for 2,3-DPG binding (46)). At this stage it may be worth reiterating an observation described earlier in this introduction - high salt concentrations are able to decrease oxygen affinity (32, 37). However, much lower concentrations of 2,3-DPG than of salt can elicit the same decrease in oxygen affinity. More specifically, the concentration of chloride must be about 10^3 times higher than that of DPG to observe the same shift in oxygen affinity (36, 46).

The identification of the residues involved in the binding of 2,3-DPG was certainly a landmark in that it was able to facilitate the interpretation of the abnormal functional properties manifested by some Hb mutants, especially mutations in the binding site per se. It may be useful to consider a few such variants and some of the different functional properties they possess, especially toward organic anions. This could perhaps facilitate interpretation of functional studies of a modified Hb, where a carboxymethyl group has been covalently attached to Val-1(β). Not only is a 2,3-DPG binding residue modified directly (i.e., Val-1(β)) but possibly the interaction of this negative charge with other residues within the 2,3-DPG binding cleft may also change the functional properties of this Hb derivative.

In Hb Little Rock (LR) (65) and Hb Abruzzo (64), His-143(β) has been substituted by glutamine and arginine respectively. In HbF, on the other hand, the position of 143 on the γ -chain is occupied by serine (110, 111). These three Hb's all exhibit an altered response toward DPG compared with unmodified HbA. In Hb Abruzzo (64), although the positive charge at position 143(β) is still maintained, the mutant is more sensitive toward DPG than unmodified HbA, an effect that may be attributed to both the increased pK_a as well as to the increased repulsive energy within the binding cleft due to the longer chain length of arginine compared to histidine. The substitution of His-143(β) for a neutral residue in both HbLR, (Gln-143(β)) (65) and HbF, (Ser-143(γ)) (85) diminishes the response of these Hb's toward 2,3-DPG. It is also of interest to examine the oxygen affinities of the aforementioned Hb's. In HbF, the decrease observed (111) is interpreted with respect to a stabilization of the T state, a consequence of the removal of two

positively charged residues from the 2,3-DPG binding cavity. The oxygen affinities of HbLR (65) and Hb Abruzzo (64) are both elevated compared with unmodified HbA, though for two different reasons. In the former mutant the suggestion was made by Perutz (112) that the oxy conformation was stabilized by an additional hydrogen bond between Gln-143(β) and Asn-139(β). In Hb Abruzzo, on the other hand, increased repulsive energy within the 2,3-DPG binding cavity due to the replacement of His-143(β) by Arg destabilizes the T state to a greater extent than for HbA. The allosteric constant L, defined as the ratio between the T state to the R state (113), is decreased.

Any mutation in which Lys-82 is substituted by an alternative residue should also exhibit an altered response toward organic anions. Indeed, the Hb variants Rahere (Lys-82(β) \longrightarrow Thr) (71, 114) and Hb Helsinki (Lys-82(β) \longrightarrow Met) (115) both exhibit a very diminished response towards organic phosphates. Hb Providence (61, 70, 116) is a Hb variant where Lys-82(β) has been substituted for by asparagine or aspartate. The aspartate is probably formed through in vivo deamidation of the asparagine (116-119). The introduction of either a neutral (Asn) or negative (Asp) charge into the DPG binding site at Lys-82 has several functional consequences. Both 'stripped' Hb Providence Asn and Hb Providence Asp have reduced oxygen affinities as a result of a diminished value of L. In addition, there is a diminished response of these mutants to organic phosphates and inorganic anions (greater for Hb Providence Asp than Hb Providence Asn): an effect attributable to the loss of the positive charge at position 82(β).

Study of Hb Deer Lodge (His-2(β) \longrightarrow Arg) (66) was of interest in order to elucidate the relative role and relative importance of the

His-2(β) residue in modulating the functional properties of Hb. As previously mentioned (63), His-2(β) is involved in the binding of 2,3-DPG. As in Hb Abruzzo (64) (where there is also a substitution of histidine for arginine, but at the 143(β) position), 'stripped' Hb Deer Lodge has an elevated oxygen affinity. The presence of arginine at position 2(β) does not alter small anion binding at neutral pH. However, due to enhanced anion binding at high pH, Hb Deer Lodge has a slightly lower oxygen affinity than HbA at pH 9 in the presence of 2,3-DPG or IHP.

Hb Raleigh (68) is a Hb mutant where not only is the N-terminal amino acid value substituted by alanine, but this residue is acetylated thereby reducing the effective positive charge within the 2,3-DPG binding crevice by two units. This variant in the absence of organic and inorganic anions exhibits a decreased oxygen affinity over the pH range 5.5 to 9.0 when compared to HbA. The reduction in the net positive charge might stabilize the deoxy conformation as discussed previously. This lower oxygen affinity is also comparable to other Hb derivatives blocked N-terminally (85) which include glycosylated Hb (120), a minor component of normal HbA. The response to 2,3-DPG and IHP on the oxygen affinity of Hb Raleigh is 50% and 70%, respectively, compared to that value found for unmodified HbA.

The above discussion concerning the Hb variants where the mutation has occurred within the 2,3-DPG binding cavity is by no means complete. Suffice it to say that there are other variants with substitutions in the 2,3-DPG binding site. Also, there are other Hb mutants where amino acid substitutions at other sites can affect the response to 2,3-DPG presumably by subtle changes in tertiary and quarternary structure.

Examples include Hb Zurich (His-63(β) \longrightarrow Arg) (121), Hb Leiden (β Glu 6 or 7 deleted) (122), and Lemur Hb (His-2(β) \longrightarrow Leu) (123). The main concepts to evolve from the Hb variants discussed above are that, in general, a removal of cationic groups within the 2,3-DPG binding cavity serves to shift the allosteric equilibrium in favor of the T conformation. This is due to a decrease in the electrostatic repulsion between the positively charged residues. In general these mutants Hb's have a lowered oxygen affinity and a decreased responsiveness toward organic phosphates.

In light of the above discussion concerning Hb's mutated within the 2,3-DPG binding cleft, it is of interest to forecast how a covalently attached carboxymethyl group at either Val-1(α) or Val-1(β) would affect the oxygen affinity as well as the binding of organic phosphates. A Hb modified only at the terminus of each α -chain ($\alpha_2^{Cm}\beta_2$) could have a reduced oxygen affinity since an oxygen-linked anion binding site is occupied as discussed earlier. Since the modification is at a site removed from the 2,3-DPG binding site, a normal response to organic phosphates would be predicted. On the other hand Hb, specifically carboxymethylated at the NH_2 -terminus of each β -chain ($\alpha_2\beta_2^{Cm}$), would be expected to have a lowered oxygen affinity and a decreased response to organic phosphates. Since Val-1(β) is a residue involved in the binding of 2,3-DPG, any substitution or modification of this residue may serve to reduce organic phosphate binding. The introduction of the carboxymethyl moiety could lessen electrostatic repulsion within the positive charge cluster and so stabilize the T conformation. Two positive charges have been removed directly and there is a possibility that positive charge could be further reduced

indirectly by interaction of other residues with the carboxymethyl moiety. This would serve to lower the oxygen affinity. However, the possibility should not be neglected that the introduction of a negative charge at either terminus could also stabilize the oxy structure in some unforeseen manner.

B-3) Interaction of Hb with Polycarboxylic Acids

In this part of the Introduction brief mention will be made of studies that involved the interaction of Hb with polycarboxylic acids (105-108), since these modulators might have similar effects to the carboxymethyl derivatives in general. As discussed previously, the models proposed by Perutz (49) and Arnone (63) for the interaction of 2,3-DPG with human Hb showed that Val-1(β), His-2(β), Lys-82(β), and His-143(β) of the two β -chains formed salt bridges with the five negative charges of 2,3-DPG. This interaction is of key importance in that it regulates oxygen affinity and CO₂ transport (36, 100, 124, 125). In addition, the increased uptake of protons when 2,3-DPG binds to deoxy HbA increases the pH dependency of the oxygen affinity, the so-called 'additional Bohr effect' (36, 126-129). It would be very informative to be able to measure the binding constant for 2,3-DPG to Hb under a variety of conditions such as pH, temperature, ionic strength, as well as in the presence of different concentrations of O₂ and CO₂. The experimental approach taken by Bucci et al (105-108) was to measure directly the number of protons that were absorbed by Hb upon interaction with the effector under a variety of experimental conditions. However, there was a complication involved with this approach: namely an overlap in the ionization of some of the negative charges of 2,3-DPG with those of the positive charges of unmodified HbA. The values for pK₄ and pK₅ of 2,3-DPG were reported as 7.46 and 7.99 (130). The simultaneous proton absorption and release that would necessarily occur would preclude a true measurement of the protons absorbed upon interaction of Hb with 2,3-DPG (105, 106). For this reason, a structural analogue of

2,3-DPG was sought. The polycarboxylic acids, which are fully ionized at pH 7, were chosen (105-108) and in particular benzenepentacarboxylic acid (105, 106) and benzenhexacarboxylic acid (108) had the greatest effect on Hb. Of great importance was the observation that the stoichiometry of binding between Hb and this modulator was 1:1. Also, these polycarboxylic acids modulated many aspects of the functional behavior of HbA in much the same way as 2,3-DPG (104, 105).

Since the polycarboxylic acids are fully ionized below pH 7, as stated above, any protons absorbed or released upon the interaction of the analogue with HbA will be due to pK_a shifts of certain amino acid residues per se of HbA. Between pH 6 and 9, the protons absorbed by the interaction between deoxy HbA and benzenhexacarboxylate were ascribed to the fact that three pairs of groups had a pK_a shift of 1.26 pH units (108). Moreover, at high enough concentrations of the polycarboxylate, considerable interaction with oxy Hb was demonstrated (106).

Thus, to summarize briefly, the pK_a values of the carboxyl groups of either benzenepentacarboxylic acid or benzenhexacarboxylic acid are all low enough to allow a direct measurement of the absorption or release of protons from Hb when it is deoxygenated or oxygenated respectively in the presence of this DPG analogue. Although not done, a study of proton binding of carboxymethylated hybrids in the presence of these polycarboxylates could provide details for pK_a changes that could be extrapolated to carbamino Hb.

C) The Bohr Effect of Human Hb

C-1) Alkaline Bohr Effect

As stated earlier, the primary function of Hb is to transport oxygen from the lungs to the tissues and allow the CO_2 excreted to be evolved through the lungs into the atmosphere. In mammalian blood this function is precisely controlled by the complex interplay of other molecules and ions, the binding of which is related to the degree of oxygenation of Hb. The molecules which affect the oxygen-carrying capacity of Hb are described as 'oxygen-linked', a phenomenon discussed earlier. Such 'oxygen-linked' ligands have been the subject of many studies. In this section of the Introduction an attempt will be made to discuss the effect of hydrogen ions upon the oxygen-carrying properties of Hb. It should be stressed that it is impossible to study the effect of one cofactor on Hb in isolation from the others. Therefore, when necessary, reference will be made to other factors that influence the behavior of hydrogen ions and the overall effect on Hb.

The relationship between pH and oxygen affinity is termed the Bohr effect (131). It is important to distinguish the Bohr effect from the Classical Bohr effect (132). Bohr, Hasselbach, and Krogh (131) showed that the oxygen equilibrium curve of Hb was shifted to the right in the presence of increasing concentrations of CO_2 . At the time there was considerable discussion as to whether this shift was due to the concomittant decrease in pH caused by CO_2 or whether CO_2 per se was influencing the oxygen equilibrium curve. It turned out that the right shift was due to both an increase in the number of protons and to a direct effect of CO_2 . This latter effect will be discussed in the next sub-section of the Introduction. Thus, a distinction is made between

the Classical Bohr effect and the Bohr effect, the former being a composite of the specific effect of CO_2 and pH, the latter, the inverse relationship of pH on oxygen affinity.

It took ten years after the discovery of the Bohr effect for the reciprocal effect to be shown. Since as stated above, protons lower the affinity of Hb for oxygen, it necessarily follows by the laws of mass action that increasing the partial pressure of oxygen lowers the affinity of Hb for protons, which are released. This is the Haldane effect (133, 134). Both the Bohr effect and the Haldane effect may be interrelated by the linkage equations derived by Wyman (135), shown below

$$\left(\frac{\delta H^+}{\delta Y_{O_2}} \right)_{pH} = - \left(\frac{\delta \log pO_2}{\delta pH} \right)_{Y_{O_2}}$$

HALDANE
COEFFICIENT

BOHR
COEFFICIENT

where H^+ refers to the number of protons released per heme, Y_{O_2} the fractional saturation with oxygen of Hb, and pO_2 the partial pressure of oxygen. The above equation may then be expressed as:

$$\Delta H^+ = - \frac{\Delta \log P_{50}}{\Delta pH}$$

where ΔH^+ is a measure of the difference in the protons released or bound in oxy or deoxy Hb respectively, and the P_{50} value is the half-fractional saturation-with-oxygen value. The above expression assumes that the Haldane effect, ΔH^+ is equivalent to the Bohr coefficient,

$-\text{dlog } P_{50}/\text{dpH}$. Tyuma and Ueda (136) tried to verify experimentally the above relationship. They observed a reasonable equivalence in the absence of allosteric ligands. However, in the presence of an allosteric modulator, which has a different affinity for either the R or T conformation, the equivalence of the relationship no longer exists and the assumption that the Bohr effect approximates the Haldane effect no longer holds (137-139).

In an interesting study, Benesch et al (139) examined the effect of four different polyanions, inositol hexaphosphate (IHP), inositol pentaphosphate (IPP), inositol hexasulfate (IHS), and 2,3-DPG, on the relationship defined in the above equation. Under their conditions, Hb (1×10^{-5} M) Cl^- (0.1 M), and varying the concentration of cofactor from 0 to 2 mM, the inositol esters increased the Bohr effect much more than they do the Haldane effect at low effector concentrations. At an ester concentration of about 1.5 mM the differential disappears. Comparison of this data with that obtained for 2,3-DPG reveals that the Bohr effect and the Haldane effect are equivalent at low concentrations of organic phosphate. Above about 0.6 mM, however, the Haldane effect becomes larger than the Bohr effect.

In the presence of an allosteric modulator both the Bohr and Haldane coefficients are a composite of two components (36, 105, 126-129, 140). One of these is due to the increased pK_a of certain residues in deoxy compared to oxy Hb resulting in proton uptake by deoxy Hb (to be discussed later). The second component is due to the additional proton uptake when an allosteric modulator binds to residues in the cleft between the two β -chains. The increase in the Haldane effect will depend on the relative affinity of the allosteric modulator for oxy or

deoxy Hb. ΔH^+ will be greater if the difference in affinity of cofactor is larger between the two states. The additional Bohr effect (105) is due to the pH dependence of cofactor binding which will necessarily increase at decreasing pH. Thus, the additional Bohr effect in the presence of low concentrations of inositol esters can be attributed to an increased number of negative charges of these cofactors and their consequent stronger binding to Hb. The elevated Haldane coefficient at high 2,3-DPG concentrations reflects the relative low affinity of this modulator for oxy Hb. However, the ratios used are not physiological. As reported by Perutz (111), under physiological conditions both the Bohr effect and the Haldane effect are equivalent and are referred to as the alkaline Bohr effect.

As discussed previously, part of the Haldane effect and the Bohr effect is necessarily due to a group or groups that change their pK_a upon oxygenation. This molecular mechanism for the Bohr effect was first suggested by Christiansen et al (133). In order to test the above hypothesis a direct measure of proton binding behavior of both oxy and deoxy Hb can be ascertained from the differential titration curve of the two forms. It was only with the advent of the glass electrode that German and Wyman were able to perform the titrations (141). They demonstrated conclusively that oxy Hb is more acidic than deoxy Hb when the pH is greater than 6. Below this pH, however, they discovered the reverse to be true. In other words, protons are absorbed upon oxygenation. The positive effect above pH 6 is the alkaline Bohr effect, the negative effect below pH 6 is the acid or reverse Bohr effect (to be discussed later in this sub-section of the Introduction). The linkage equations developed by Wyman (135) and described earlier

showed that two oxygen-linked groups per heme changed their pK_a value on oxygenation. One changed its pK_a from 5.25 to 5.75 (acid Bohr groups), the other from 7.81 to 6.8 (alkaline Bohr groups). In order to identify more exactly what groups might change their pK_a in oxy compared to deoxy Hb, Wyman (142) measured the effect of temperature on the position of the differential titration curve of Hb. From these measurements a value of 6.5 Kcal for ΔH , the enthalpy of ionization, was calculated for the oxygen-linked acid group, consistent with the identity as an imidazole of histidine. (It was technically more difficult to measure this value for the group contributing to the acid Bohr effect.) This was contrary to the hypothesis of Roughton (143) who felt that since the formation of carbamate at some amino groups of Hb was oxygen-linked, the ionization of these same amino groups was also oxygen-linked. He suggested that these groups may contribute to the alkaline Bohr effect. At first Wyman (142) speculated that the Bohr groups were the proximal and distal histidines above and below the iron heme, since these bond lengths change upon oxygenation. However, since this same change of bond length occurs in myoglobin (144), whereas the alkaline Bohr effect is absent, such an assignment did not appear likely. This led Wyman and Allen (145) to propose that a change in conformation caused by oxygenation could bring about the ionization of a group at a site well removed from that of ligand binding. The results described above, as well as those from other studies (146-149) could only provide fairly general information concerning the nature of the Bohr effect. It was only through chemical modification studies and X-ray crystallography that more precise information became available (150).

The rest of the discussion in this section concerning the Bohr effect will describe the specific residues involved and the state of the field today.

As mentioned earlier, there was some dispute as to the identity of the groups involved in the Bohr effect. The first line of thought, supported by Ferguson and Roughton (151) and Roughton (143), suggested the involvement of amino groups. The evaluation of the enthalpy of ionization for the Bohr groups by Wyman (142) suggested the involvement of the imidazole group of histidine. It turned out that both groups would be involved in the alkaline Bohr effect.

The involvement of amino groups was shown through chemical modification studies. More specifically, the α -amino groups were subject to modification since the ϵ -amino group of the lysines have pK_a values that are out of the pH range at which the alkaline Bohr effect occurs. There were a number of studies where modification of the α -amino groups was carried out with reagents such as trinitrobenzene sulfonate (152) and 1-guanyl-3,5-dimethylpyrazole nitrate (37). However, such studies were unable to identify whether it was the α -chain or β -chain amino groups that contributed to the alkaline Bohr effect. Kilmartin and Rossi-Bernardi (52, 53, 153) reacted the α -amino groups with cyanate (52, 53). The ionization of the resultant carbamylated amino groups was precluded.



In the initial studies carried out with horse Hb and later extended to human Hb (154), the Bohr effect was measured for the four purified tetramers $\alpha_2\beta_2$, $\alpha_2^{Cb_m}\beta_2$, $\alpha_2\beta_2^{Cb_m}$, and $\alpha_2^{Cb_m}\beta_2^{Cb_m}$, where Cbm denotes carbamylation of the amino terminus of that particular chain.

The resultant derivatives retained normal heme-heme interaction and no significant structural changes were detected by X-ray crystallography (52, 53). The derivative carbamylated on the β -chain amino terminus $\alpha_2\beta_2^{\text{Cbm}}$ produced a normal Bohr effect. The Bohr effect of $\alpha_2^{\text{Cbm}}\beta_2$ and $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$ was diminished by 25% in the horse derivative (52, 53, 153) and 30% in the human (154).

In separate experiments, the pK_a of the α -chain amino group was measured directly by determining the pH dependence of dinitrophenylation using Sanger's reagent (155). This reagent only reacts with uncharged amino groups. Hill and Davies (155) obtained a pH value of 6.7 for the α -chain amino terminus of liganded Hb which is lower than expected for a free amino group (156). A more 'normal' value of between 7.7-8.2 was calculated for the pK_a of this amino group in unliganded Hb by Tanford and Nozaki (157). However, the pK_a of this group in liganded Hb was consistent with the report of Hill and Davies (155). A measurement of the pK_a of the α -chain amino group in isolated subunits (158) also gave a value in the 'normal' range, 7.4. In a later study (159) the pK_a of the NH_2 -terminal groups was measured indirectly by measuring the relative reactivity of these groups toward cyanate. The pK_a value of the α -chain amino terminus was elevated by about 0.8 pH units in deoxy compared with oxy Hb.

The interpretation as to the reason for the elevated pK_a of the α -chain amino terminal group in deoxy compared to oxy Hb came from the X-ray analysis of Perutz and his co-workers. At the time a comparison of the low resolution X-ray crystallographs (48, 49, 160, 161) of oxy horse and deoxy human or horse Hb showed that in oxy Hb the amino terminus was free. In addition, the C-terminus of the α -chain was also able to take

up several positions. Inspection of the 5.5 Å (160) deoxy human or horse and the 3.5 Å deoxy human Hb (48) electron density maps indicated the possibility of a salt bridge extending between the Val-1(α) amino terminus to the carboxylate group of Arg-141(α_2). The presence of these salt bridges should necessarily elevate the pK_a of the amino terminus of the α -chain in deoxy compared to oxy Hb. This could stereochemically account for that portion of the Bohr effect contributed to by Val-1(α) (49). As discussed in B-1, however, the 2.5 Å high resolution electron density maps of human deoxy Hb (54) showed that the distance between the amino and carboxyl moieties was too great for the two groups to interact directly. At the time the suggestion was made that a water molecule was lodged between the two charged groups. The subsequent X-ray studies of Arnone (43, 44) identified Val-1(α) as an anion binding site. The distance between the amino terminus and the carboxyl group of Arg-141 on the opposite α -chain increases from 8 Å in deoxy Hb to 10 Å in oxy Hb. The proposal was made that anion binding to this site should be oxygen-linked, with the result of increasing the pK_a of the Val-1(α) amino groups. Indeed, Rollema et al (56) were able to show an increase in the alkaline Bohr effect when the chloride concentration was increased from 5 mM to 100 mM.

With about one quarter of the Bohr effect accounted for, from where could the remaining 75% come? As previously mentioned, the thermodynamic linkage calculations of Wyman (142) indicated the participation of an imidazole group in the alkaline Bohr effect. A tentative identity was assigned to His-146(β). This came from examining the electron density map of N-ethyl succinamide HbA (NES-Hb). A number of studies (162-164) described the conditions under which Cys-93(β)

reacts with N-ethylmaleimide to form the above derivative. The alkaline Bohr effect of this modified Hb is reduced by 50% (162, 163). Since a modification at Cys-93(β) by iodoacetamide did not produce a similar decrease (162), Cys-93(β) itself was not a participant in the alkaline Bohr effect. The electron density map of this derivative was able to reveal the mechanism by which this modification had inhibited the Bohr effect. The difference map of oxy Hb and oxy NES-Hb showed one positive peak identified as the NES group at Cys-93(β) (160). On the other hand, a difference map of the deoxy states revealed three peaks: one of positive density corresponds to the NES group. Of the remaining two peaks, one was of positive and the other of negative density indicating the displacement of a group by the presence of the bulky NES group. The X-ray analysis of deoxy Hb Rainier (Tyr-145(β) \longrightarrow Cys) (165) facilitated an elucidation of the above results. In this mutant, a negative peak coincided with the one described above. This was consistent with the displacement of His-146(β) in both the chemically modified and mutant Hb. Model building (160) based on data reported by Muirhead et al (95) predicted that in normal deoxy Hb the positively charged imidazole would interact with the carboxyl group of Asp-94 of the same β -chain. Such an interaction would elevate the pK_a of this group in deoxy compared to oxy Hb (160, 161). It appears that the formation of this salt bridge is precluded when either the bulky NES group is attached to Cys-93(β) or when tyrosine is substituted by cysteine as in Hb Rainier (165). In normal deoxy Hb, His-146(β) is held in position by virtue of the adjacent tyrosine and the salt bridge between the carboxyl group of His-146(β) and the ϵ -amino group of Lys-40(α).

In a separate study (166), the role of His-146(β) in the alkaline Bohr effect was reemphasized. Des-(His-146(β)) Hb, in which the carboxy terminal histidine has been enzymatically removed with carboxypeptidase B (and where heme-heme interaction was intact), displayed a 50% reduction in the alkaline Bohr effect in 0.2 M phosphate. This result was consistent with the proposal made by Perutz and his co-workers (160, 161) described above. A direct measurement of the pK_a value of His-146(β) was made in both liganded and unliganded Hb (167) using 1H -NMR. The assignment of the resonance contributed by His-146(β) was made by comparing the spectrum from unmodified HbA with des-(His-146(β)) HbA in the aromatic region. The change in position of the relevant peak was measured as a function of pH in 0.2 M phosphate and 0.2 M chloride. The titration data for oxy Hb gave a pK_a of 7.1 for His-146(β) which increased to 8.0 upon removal of ligand.

At this stage a brief summary might be useful. The difference in ligand affinity between the R (high affinity) and T (low affinity) conformations of Hb is due to salt bridges present in the latter (49) but absent in the former. These salt bridges include those responsible for the Bohr effect, namely Val-1(α) whose amino group forms a salt bridge via an inorganic anion to the guanidinium of Arg-141(α), and His-146(β) whose imidazole forms a salt bridge with the carboxyl group of Asp-94 (discussed above). The kinetic experiments of Antonini (168) showed that CO uptake and proton release were synchronous. This supported the proposal made by Perutz that binding of ligand ruptured the Bohr group salt bridges, with the result of a T to R quaternary transition. Contrary to this proposed stereochemical mechanism were the results of X-ray crystallographic studies on Hb Iwate (169), HbA (170),

and Hb Kansas (171, 172). In the case of HbA (170) the X-ray structure was examined after lattice strengthening with acrylamide in the deoxy state followed by air oxidation to the aquomet state. By filling the Hb crystals water spaces with an inert matrix (acrylamide), the shattering of the crystal (173) that usually accompanies the change from the T to the R quaternary conformation could be avoided. This allowed a closer examination of changes in tertiary structure. Of relevance to this discussion, was the observation that the Bohr group salt bridges remained intact. However, ligation to the high-spin aquomet ligand only shifts the iron atom about half-way from its out-of-plane position in deoxy to its in-plane position in oxy Hb. Thus, the changes in tertiary structure may not have been sufficient to break the salt bridges within the confines of the matrix. With this in mind another system was sought whereby a fully ligated Hb could be crystallized in the T state. If such a structure could exist, the Bohr group salt bridges should be ruptured. Anderson selected Hb Kansas (Asn-102(β) \longrightarrow Thr) as a suitable candidate (171) for the system mentioned above. This Hb variant, first described by Reissman et al (174) and studied later by Bonaventura and Riggs (175), has a low oxygen affinity and, moreover, exhibits an alkaline Bohr effect of normal magnitude (175). Since IHP can maintain Hb Kansas in the T state even when ligated with carbon monoxide (176), this variant was selected by Anderson in order to examine the changes in tertiary and quaternary structure when deoxy crystals of Hb Kansas in the presence of IHP were exposed to carbon monoxide (171). The result of this study was that the T state salt bridges were not observed to break. This was interpreted with respect to increased quaternary constraint in the T state due to the changed crystal lattice, due to the

mutation, as well as to the presence of IHP. Such increased quaternary constraints appeared to prevent breakage of the salt bridges. Indeed, Kilmartin et al (172) extended the study and found by NMR, that the pK of His-146(β) was unaltered on ligation of T state Hb Kansas with nitrous oxide (a high spin ligand) in the absence or presence of IHP. Moreover, in this liganded T state, the Bohr effect was minimal. The data presented above (171, 172) contradict the proposal of Perutz (49): whereby, uptake of ligand is coupled to breakage of a salt bridge and release of Bohr protons. Kilmartin et al (172) suggested that the Perutz mechanism was applicable to 'stripped' HbA but not to the T states of Hb's with low ligand affinity.

The crystallographic studies discussed above were biased toward the mechanism whereby specific residues contributed to the alkaline Bohr effect. Not apparent from the X-ray work was that the magnitude of the Bohr effect seems to be sensitive to solvent conditions (36, 56, 147, 177-179) especially salts of inorganic anions. Debruin et al (178, 179) and Rollema et al (56) found that the alkaline Bohr effect increased to a maximum as the chloride concentration rose from 5 mM to 100 mM and thereafter became suppressed as the chloride concentration continued to be raised to 2.0 M. Cooperativity and heme-heme interaction were not appreciably altered. This finding prompted a number of studies, where the Bohr effect was measured under a variety of different solvent conditions. Russu et al (180-182) used 250 MHz high resolution ^1H -NMR to measure the pK_a values of His-146(β) (180) and the 22 (per tetramer) surface histidine residues under a variety of solvent conditions. Chemical shift measurements of the C₂ proton of His-146(β) in low salt (0.1 M bistris chloride, where the chloride concentration varies from 5

to 60 mM) gave a pK_a value of the imidazole group that was 7.98 in deoxy Hb and 7.85 in carbonmonoxy Hb. These values are in contrast to the 0.9 differential of the pK_a values between the two forms measured in 0.2 M phosphate and 0.2 M chloride (167) and discussed above. The suggestion was put forward (180-182) that since the magnitude of the Bohr effect, as measured by proton titration, was the same under both sets of conditions, i.e., high and low salt, respectively, (167, 180) the molecular mechanism differed under different solvent conditions. The contribution of His-146(β) was calculated to be 50% in the former (0.2 M phosphate) (167) and only 5% in the latter set of conditions (low salt) (180). Under conditions of low salt the suggestion was put forward for a mechanism that did not involve breakage of the salt bridge between His-146(β) and Asp-94(β). In a subsequent report the same investigators (181) measured the pK_a values of the 22 (per tetramer) surface histidine residues under the low salt conditions employed in their previous study (180). They reached the conclusion that the sum of the small pK_a changes of these surface histidyl residues could account for the observed magnitude of the Bohr effect under their conditions of low salt. In agreement with these experimental determinations were the theoretical calculations of Matthew et al (183-185). These workers applied the Tanford-Kirkwood (186, 187) discrete-charge electrostatic theory to Hb and modified it to compensate for the non-spherical nature of the protein. Very simply, this theory considers any dissociable proton binding group or any bound ion as a point charge, which interacts with other point charges in the microenvironment. In Hb, the resultant electrostatic charge array can be altered by the allosteric modulators that are known to regulate oxygen uptake and release. Incorporation of

atomic distances known from X-ray crystallography data (54, 188) allowed calculation of the pK_a values of eleven histidine residues. The results were in agreement with the experimentally determined values of Russu et al (181). Thus, it appears from the above results that different groups can change their pK_a depending upon the environment and contribute to the alkaline Bohr effect to varying degrees.

From the above discussion, it seems that the percentage contribution made by His-146(β) to the Bohr effect varies depending upon the solvent conditions. In the presence of chloride, carbonates, and cyanates (56, 129, 138, 158, 159, 179, 189-192) Val-1(α) contributes to the Bohr effect. Of note is the fact that at low chloride concentrations (56) the Bohr effect is reduced. As previously described, the elevated pK_a of Val-1(α) in deoxy Hb is due to occupation of the space between the Val-1(α) amino terminus and the guanidinium group of Arg-141(α) by chloride (44). The lack of contribution to the Bohr effect by Val-1(α) in the presence of phosphate (192) may be attributed to the larger diameter of this molecule compared to the anions listed above. Deuterium exchange studies (193) suggested that under physiological conditions His-89(α) and His-146(β) were the groups principally involved in the alkaline Bohr effect. Inclusion of the contribution of Val-1(α) gave a value for proton release that was larger than the observed value. Indeed, Kaplan et al (194) using acetic anhydride and Sanger's reagent in an $^3\text{H}/^{14}\text{C}$ competitive labeling approach concluded that the contribution of Val-1(α) to the alkaline Bohr effect was minimal under physiological conditions. Bresciani (195), on the other hand, used the same technique with acetic anhydride and obtained results consistent with the involvement of Val-1(α) in the

alkaline Bohr effect. In an attempt to reconcile some of these differences Bucci and Fronticelli (196) measured the Bohr effect of Hb after treatment with bromoacetate. They also examined the Bohr effect of unmodified HbA in Goode buffers. Regardless of the state of ligation, treatment with bromoacetate resulted in the same extent of alkylation at both Val-1(α) and His-146(β). However, alkylation of only the liganded state of HbA abolished the Bohr effect. Why this difference occurred could be due to the persistence of one disubstituted histidine per $\alpha\beta$ dimer when alkylation was carried out in the presence of carbon monoxide. Under the conditions of the alkylation reaction, moreover, high salt could have favored tetramer-dimer dissociation of the carbonmonoxy form. Thus, a residue at the $\alpha_1\beta_2$ subunit could have been alkylated preferentially whereas in the deoxy form such a residue may have remained hidden. The suggestion was put forward that the differences observed could have been due to an interaction with anions. Bohr titrations carried out in Goode buffers, whose pH does not require adjustment with anions such as chloride, acetate, etc, were represented by titration curves whose shapes were very different from those usually found. A new mathematical formula for the alkaline Bohr effect was presented that included interaction with the solvent components.

Perutz and his co-workers accommodated or disproved some of the models just described, which were at variance with the original stereochemical mechanism proposed for the alkaline Bohr effect. They tried to resolve the discrepancy that concerned the involvement of His-146(β) in the alkaline Bohr effect. The chemical study, described above (166), in addition to the ^1H -NMR titration data (167) both carried out in 0.2 M phosphate, attributed a 40% contribution of His-146(β) to

the alkaline Bohr effect. Russu et al on the other hand (180-182) assigned a percentage value of 5% when they measured the pK_a values of His-146(β) in low salt concentrations under oxy and deoxy conditions. However, when Kilmartin et al (197) measured the Bohr effect of des-(His-146(β)) Hb or Hb N-ethyl succinamide in the absence of phosphate and low chloride the alkaline Bohr effect was diminished by at least 60%. The salt bridge between the imidazole of His-146(β) to the carboxy group of Asp-94(β) cannot form in this latter derivative. Moreover, inspection of the electron density map of met Hb crystallized under salt-free conditions showed the absence of the salt bridge discussed above. This is contrary to the proposition (181) that this salt bridge remains intact upon transition to the R structure under conditions of low salt. Thus, it appears that the central issue concerning the controversy over the mechanism of the Bohr effect is either whether the contribution is due to a few specific residues or to small contributions from a larger number of groups inclusive of interactions with solvent components. The extensive study of mutant Hb's by Perutz (111) supports the former hypothesis and disproves the latter. According to several articles (150, 198, 199), 20 to 30% of the Bohr effect remains unaccounted for. In an attempt to find the missing Bohr group(s), Perutz et al (111) reported investigations of many Hb variants. They were able to give a full interpretation of the Bohr effect under both physiological and 'stripped' conditions.

There seems to be fairly strong evidence supporting the role of Val-1(α) in the alkaline Bohr effect. As discussed earlier, Arnone and Williams (43) and O'Donnell et al (44) were able to show that binding of chloride between the amino terminus of Val-1(α) and the guanidinium

moiety of Arg-141(α) in deoxy, but not in oxy Hb, elevated the pK_a of Val-1(α) in the former but not in the latter conformation. In des-(Arg-141(α)), or low chloride, the contribution of Val-1(α) to the alkaline Bohr effect disappears (44, 200). Moreover, in the specific carbamylation studies, the Bohr effect was reduced by 25% in $\alpha_2^{Cbm}\beta_2$ (44). pK_a measurements per se (159, 191, 200) showed the pK_a of Val-1(α) to be increased ~ 0.8 pH units in deoxy compared with oxy Hb.

What about the role of His-146(β), about which there is considerable controversy? The X-ray crystallographic studies of Perutz and his colleagues (54, 160, 201, 202) in conjunction with chemical studies showed that in deoxy HbA there was a salt bridge between the positively charged imidazole of His-146(β) with the carboxyl moiety of Asp-94(β). Conditions under which salt bridge formation is prohibited such as des-(His-146(β)) Hb (167, 203), or in the mutants Hb Hiroshima (His-146(β) \longrightarrow Asp) (204, 205), Hb Cochin-Port Royal (His-146(β) \longrightarrow Arg) (206), Hb York (His-146(β) \longrightarrow Pro) (207), Hb Cowntown (His-146(β) \longrightarrow Leu) (208-212), and Hb Barcelona (Asp-94(β) \longrightarrow His) (213) all have a considerably reduced alkaline Bohr effect (50% or 25% of the value for Hb). In contrast to the $^1\text{H-NMR}$ pK_a determinations for His-146(β) by Russu et al (180-182), and the calculations of Matthew et al (183-185), several other studies (167, 214, 215) confirmed an elevated pK_a value for this residue in deoxy compared to oxy HbA, regardless of the solvent conditions. In fact a greater role was attributed to His-146(β) where chloride concentrations were low (197). A more detailed study as to the role of His-146(β) in the alkaline Bohr effect was afforded by Hb Cowntown, a mutant Hb, in which His-146(β) has been replaced by leucine (208-212). Studies (208, 209) showed that the increased oxygen

affinity and halved alkaline Bohr effect of this variant were due directly to the replacement of Leu for His, as opposed to the more widespread structural rearrangements throughout the Hb tetramer that this substitution might incur (180-185). Indeed, X-ray analysis of this deoxy Hb mutant (209) compared with deoxy unmodified HbA, showed localized electron density changes only in the immediate vicinity of the β -chain carboxy terminus. Structural perturbations failed to be recognized anywhere else in the molecule. This also proved to be the case when electron density maps from other Hb's, mutated at the same site, were examined (209).

That specific residues are involved in the alkaline Bohr effect, as opposed to small structural and electrostatic changes throughout the molecule was further substantiated by the result that in NES-HbA, NES-Hb Cowntown, and Hb Cowntown the reduction in the alkaline Bohr effect was 50% in all three cases (207). Moreover, Perutz et al (209) recalculated the values obtained by Matthew et al (183-185) taking into account tertiary structural changes and were able to calculate pK_a values consistent with the experimental determinations (167). In a recent study Perutz et al (210) repeated the $^1\text{H-NMR}$ determinations of Russu et al (180-182) for HbA and des-(His-146(β)) in both the carbonmonoxy and deoxy forms under identical conditions of ionic strength but at higher resolution (500 MHz (208)) as opposed to 250 MHz (180-182)). In addition, Perutz et al (210), measured the resonance spectra of Hb Cowntown (His-146(β) \rightarrow Leu), Hb Malmo (His-97(β) \rightarrow Gln), and Hb Wood (His-97(β) \rightarrow Leu) under the same experimental conditions as described by Russu et al (180-182). In the interpretation of their data they provide evidence that the resonance assigned by Russu et al (180-182) to

His-146(β) under conditions of low ionic strength is in fact due to His-97(β). By use of the mutants, it was possible to assign the resonance peaks that were due to His-97(β) and His-146(β) respectively. Evidently, under their conditions Russu et al were unable to detect the fact that under conditions of low ionic strength, the resonance due to His-97(β) in carbonmonoxy HbA splits into two peaks, the first of which moves further upfield and the second is coincidental with the position of His-146(β). In des-(His-146(β)) Hb, therefore, the incorrect assignments were made under conditions of low ionic strength. Under the high ionic strength conditions used by Kilmartin et al (167) the His-97(β) split does not occur. Perutz et al (210) also found that, whereas the pK_a of His-146(β) in deoxy Hb remains constant regardless of ionic strength, the pK_a of this residue in carbonmonoxy Hb falls as the ionic strength diminishes. These findings corroborate the involvement of His-146(β) in the alkaline Bohr effect.

Thus, it appears that there is strong evidence favoring a contribution of His-146(β) to the alkaline Bohr effect (167, 197, 203, 210). However, the relative extent of this residue's contribution is dependent upon solvent conditions. So far therefore, about 60% of the Bohr effect is accounted in conditions of moderate ionic strength by Val-1(α), (25%) and His-146(β), (40%) (197, 198) and in conditions of low ionic strength by His-146(β) (60%) (197).

The hydrogen-tritium exchange method utilized by Nishikura (199) identified His-122(α) as having a pK_a of 6.1 in carbonmonoxy Hb and 6.6 in deoxy Hb. It was calculated to contribute 20% to the alkaline Bohr effect. In support of this finding was the reduced alkaline Bohr effect of Hb Tacoma (216, 217) where the Arg-30(β) residue in the vicinity of

His-122(α) is replaced by serine (218). Also, embryonic Hb Portland (219) and Llama Hb (111) have a replacement of His-122(α) by aspartate and both exhibit a diminished alkaline Bohr effect. However, it seems hard to reconcile the above results with the fact that the microenvironment of His-122(α) seems to be the same in deoxy, met, and carbonmonoxy Hb (54, 220, 221) as seen by X-ray crystallography. It is possible that, at low ionic strength, His-146(β) and, possibly, His-122(α) account fully for the alkaline Bohr effect. Under these solvent conditions the Bohr coefficient is reduced compared to its value in 0.1 M chloride (56). It would be interesting to measure the alkaline Bohr effect of Hb Cowtown under conditions of low ionic strength (≤ 5 mM chloride). As stated previously, when the chloride concentration is increased to 0.1 M chloride (and in the absence of organic phosphates) the Bohr effect attains a maximum value which then decreases as chloride increases to 2 M (56). As previously discussed (44), chloride is able to increase the pK_a of Val-1(α) which contributes about 25% to the alkaline Bohr effect. Thus, even if one includes His-122(α), there is still at least another 20% to be accounted for in the presence of 0.1 M chloride and in the absence of organic phosphates.

The studies reported by Perutz (111) suggest that the 'missing' Bohr group is Lys-82(β) which acts in a chloride-dependent manner. The evidence leading to this conclusion is based upon the 20% reduction observed for the alkaline Bohr effect in which there is a mutation at Lys-82(β) by a neutral or anionic amino acid. These mutants include Hb Providence, (Lys-82(β) \longrightarrow Asn or Asp) (116), Hb Helsinki, (Lys-82(β) \longrightarrow Met) (115), Hb Rahere (Lys-82(β) \longrightarrow Thr) (109). All these mutants were observed to have a diminished response to chloride when the

concentration of this anion was increased to 0.1 M (111). These observations were also extended to Hb Andrew Minneapolis, (Lys-144(β) \longrightarrow Asn) (222), and Hb Little Rock (His-143(β) \longrightarrow Gln) (65). The mutations have a common feature: they lie in or close to the 2,3-DPG binding cleft and in this way could interfere with chloride binding to Lys-82(β). The reduction in the alkaline Bohr effect of Hb Andrew Minneapolis and Hb Little Rock could also be due to a weakened salt bridge between His-146(β) and Asp-94(β).

Referring back to part B-1 of the Introduction, Chiancone et al (41) found evidence for high and low affinity oxygen-linked chloride binding sites. Substantial evidence has identified the Val-1(α) to Arg-141(α) locus as the high affinity site (41, 44, 59). The chloride titration of Hb Helsinki (Lys-82(β) \longrightarrow Met) (111) seems to indicate an absence of low affinity binding sites. This should also be the case with the mutants discussed above. Thus, to conclude briefly on this section, under 'stripped' conditions, Lys-82(β) contributes about 20% to the alkaline Bohr effect due to its low affinity binding for chloride. His-146(β), Val-1(α), and perhaps His-122(α) make up the balance. Caution should be exercised here since the involvement of His-122(α) appears tenuous. Also, perhaps solvent components other than chloride have a dominant role (196). Under physiological conditions in the presence of 2,3-DPG, Val-1(α), His-146(β), His-122(α), as well as the protons absorbed upon binding of organic phosphate account fully for the alkaline Bohr effect.

What kind of predictions and what new information could be obtained from a measurement of the Bohr effect of Hb specifically carboxymethylated at the α -NH₂-termini? Under conditions of low salt

(5 mM) and in the absence of 2,3-DPG, the Bohr effect for unmodified Hb is reduced and the major (60%) contribution comes from His-146(β) (197) with a less sure contribution from His-122(α). It seems that under these conditions the contribution from Val-1(α) does not occur (44, 197). Under 'stripped' conditions in 0.1 M chloride, however, Val-1(α) and Lys-82(β) both contribute to the alkaline Bohr effect due to high and low affinity binding of chloride respectively, to each of these sites (111). In the $\alpha_2^{Cm}\beta_2$ hybrid, the carboxymethyl moiety is covalently attached to the NH_2 -terminus of the α -chain in both the oxy and deoxy conformation. In conditions of low salt, if the pK_a of the resultant secondary amine is the same in the oxy and deoxy conformation, the $\alpha_2^{Cm}\beta_2$ hybrid may be functionally equivalent to unmodified HbA with respect to the alkaline Bohr effect. If, however, the presence of the negative charge of the carboxymethyl moiety results in an electrostatic interaction with Arg-141(α) in the deoxy but not in the oxy hybrid, an elevated Bohr coefficient may be predicted compared with unmodified HbA under low salt conditions. Alternatively the attachment of the carboxymethyl group at Val-1(α) could therefore be functionally equivalent to the presence of chloride at this site. If on the other hand, carboxymethylation at Val-1(α) caused an increased pK_a value of the secondary amine in oxy compared to deoxy Hb through interaction with some residue other than Arg-141(α) (the distance between Val-1(α) and Arg-141(α) is probably too great in the oxy conformation), the absorption of protons upon oxygenation would act in opposition to the alkaline Bohr effect and result in a decreased value. Under solvent conditions of 0.1 M chloride there could be three possible results for the $\alpha_2^{Cm}\beta_2$ hybrid. In the first, the maximum value achieved for

unmodified HbA may not occur due to the inability of chloride to bind between Val-1(α) and Arg-141(α) (44). Thus, the Bohr coefficient could be the same in low salt and 0.1 M chloride. Alternatively, if the carboxymethyl moiety is orientated away from Arg-141(α) (by analogy with carbamino Hb (223)) chloride would still be able to bind and as a consequence elevate the alkaline Bohr coefficient. In the third possibility, carboxymethylation at Val-1(α) may have resulted in a conformational change such that chloride interacts with some other residue of the protein and this could increase or decrease the alkaline Bohr coefficient under conditions of 0.1 M chloride. Assuming that the conformation of the 2,3-DPG binding site has not been altered by the presence of the carboxymethyl moiety at Val-1(α), the additional Bohr effect (36, 126-129) due to the absorption of protons upon interaction of Hb with the organic phosphate should not be changed. Thus, the usefulness of the determination of the alkaline Bohr coefficient for $\alpha_2\text{Cm}\beta_2$ under various conditions of ionic strength will be apparent when the data are interpreted with respect to the X-ray data. X-ray crystallography will allow us to see the orientation of the carboxymethyl moiety, and especially to see if we have a carbamino analogue.

X-ray crystallography of the modified β -chain termini will also allow a clearer interpretation of the alkaline Bohr coefficient as measured for the hybrid $\alpha_2\beta_2\text{Cm}$. Carboxymethylation of the β -chain amino terminus has introduced negative charge directly into the 2,3-DPG binding cleft. Dependent upon the X-ray data it may also have removed positive charge indirectly by interactions with the 2,3-DPG binding residues. Carbamylation of Val-1(β) does not cause a reduction in the

alkaline Bohr effect (44, 52) when measured in 0.1 M chloride. Replacement of other positively charged residues in the DPG cleft, however, with a neutral or anionic amino acid diminishes the Bohr effect, i.e., Hb Providence Asn or Asp (61). These measurements were made in low concentrations of chloride. Two alternative results could be predicted for the $\alpha_2\beta_2^{\text{Cm}}$ hybrid in low salt. The first result could consider that an interaction with Lys-82(β) in the deoxy form could be functionally equivalent to the presence of chloride at this site. In this case the Bohr coefficient could be elevated by about 20% compared with the value for unmodified HbA under low salt conditions. On the other hand neutralization of part of the positive charge within the binding cleft could be compared with Hb Providence Asn or Asp where a reduction of 30% and 50% of the alkaline Bohr effect respectively was reported, at low chloride concentrations. X-ray data should identify any conformational alterations that could account for either of these possibilities. Under conditions of moderate ionic strength (0.1 M chloride), a 25% increase in the alkaline Bohr effect of $\alpha_2\beta_2^{\text{Cm}}$ could be manifested due to chloride binding between Val-1(α) and Arg-141(α) (44), assuming that carboxymethylation at Val-1(β) has not exerted an impairment of chloride binding at Val-1(α). The response of this carboxymethylated hybrid to 2,3-DPG would be expected to be minimal since Val-1(β) is blocked.

Alternative predictions may also be made for the $\alpha_2\beta_2^{\text{CmCm}}$ hybrid in which all four amino termini are carboxymethylated. A full Bohr effect of similar magnitude to unmodified HbA in 0.1 M chloride (56) may be measured under conditions of low ionic strength, having made the assumption that the presence of the carboxymethyl group is functionally

equivalent to the presence of chloride. If, however, a reduced Bohr effect manifests itself for either $\alpha_2^{Cm}\beta_2$ or $\alpha_2\beta_2^{Cm}$ this would be reflected in $\alpha_2^{Cm}\beta_2^{Cm}$ in some way which would also be dependent on any conformational changes that may have resulted from the introduction of four negative charges onto the Hb tetramer.

If indeed, the X-ray data substantiates our initial premise for carboxymethyl Hb as a carbamino analogue we will be in a unique position whereby we can examine the influence that the presence of CO_2 ($-CH_2COO^-$) at one terminus can have on the alkaline Bohr effect.

C-2) Acid Bohr Effect

The pH titration results of German and Wyman (141) of oxy and deoxy Hb showed that although above pH 6 protons are absorbed upon deoxygenation, below this pH protons are released upon removal of ligand. This negative part of the Bohr effect (below pH 6) is referred to as the acid or reverse Bohr effect. Whereas the proposal was made (142) that the implication of the alkaline Bohr effect necessitated a group or groups that changed their pK_a from about 6.8 to 7.8, the implication for the acid Bohr effect necessitates a group or groups whose pK_a increases from about 5.25 to 6.25 (150) or 4.9 to 5.5 (111) upon oxygenation. Therefore, the acid Bohr effect may not be of physiological importance but an understanding of the phenomenon will contribute to our knowledge of protein chemistry.

Antonini et al (149) calculated the enthalpy of ionization of the acid Bohr groups and found this value to be small. This is consistent with the identity of a carboxyl group (156), which was also confirmed in a later study (224). Identification of the specific carboxyl group contributing to the acid Bohr effect has been elusive in the past due to the difficulty associated with carboxyl group modification. Recently, however (225), specific modification of three carboxyl groups per HbS tetramer was accomplished using the method of Hoare and Koshland (226). Conceivably such a method could be useful in the future for identification of the acid Bohr group(s) provided that the necessary selectivity of modification could be achieved.

X-ray crystallography, unlike that employed successfully for the alkaline Bohr effect, has not really provided any clear-cut evidence as

to which specific residues may be involved in the acid Bohr effect (50, 54, 161, 201, 220, 227, 228). The suggestion was made (160) that the carboxylate of Arg-141(α) and of Asp-94(β) could make a contribution. The carboxylate of the former is salt-bridged to Lys-127 of the opposite α -chain, that of the latter is salt-bridged with His-146 of the same β -chain. The involvement of either of these residues would necessarily mean that the carboxyl pK_a would have to be raised in oxy Hb. In other words, it would be protonated. The same proposal could also be made for the carboxylate of His-146(β) which forms a salt-bridge with Lys-40(α). It is important to note that carboxyl groups with pK_a 's greater than 5.7 do exist in proteins (229). For example, 2 out of the 52 carboxyl moieties in β -lactoglobulin have a pK_a value of 7.3. In addition, at least 6 out of the 38 carboxyl groups of pepsin remain protonated at pH values greater than 6.5. In order to test the above hypothesis, that either of the C-terminal residues might be involved in the acid Bohr effect, this coefficient has been measured for des-(Arg-141(α)) Hb and des-(His-146(β)) Hb as well as for a tetramer with both Arg-141(α) and His-146(β) removed. The acid Bohr effect of des-(His-146(β)) Hb is largely unaltered (166). This also shows that the acid Bohr effect is not linked to that part of the alkaline Bohr effect associated with His-146(β). On the other hand, such linkage does occur with the Arg-141(α) residue, since a diminished acid and alkaline Bohr effect is observed with des-(Arg-141(α)) Hb (230).

Before discussing the more recent attempts to identify the residues responsible for the acid Bohr effect, especially from mutant Hb's, brief mention should be made of an earlier hypothesis put forward by Perutz (49). He suggested that the change in crystal structure of

horse oxy Hb at pH 5.9 could account for the acid Bohr effect. This change in crystal structure was accompanied by a reduction in the distance between the two Cys-93's of the β -chain as measured by the mercury atoms attached to each cysteine. This distance increases in the R \rightarrow T transition (49). Unlike the alkaline Bohr effect, which is relatively constant within mammalian species, the acid Bohr effect varies. Therefore, different amino acids result in a greater variety of quaternary structures below pH 6.

Despite the above theory, the search for that residue or residues involved in the acid Bohr effect has continued. The two α -chains of human fetal Hb (HbF) are identical with those of human adult Hb, but the composition of the two γ -chains differ (39 differences (231)). The acid Bohr effect of HbF is only about 50% that of adult HbA (232, 233). A comparison of the different ionizable groups present in the β - and γ -chains (111) suggested that the difference in magnitude of the acid Bohr effect between adult and fetal Hb was due to the substitution of His-143(β) by Ser-143(γ). In deoxy compared to oxy adult Hb the pK_a of His-143(β) may be lowered by the proximity with its positively charged neighbors, Lys-82(β) and Lys-144(β). Thus, the elevated pK_a of His-143(β) in oxy compared to deoxy Hb could in theory allow this residue to contribute to the acid Bohr effect. In a recent report, Matsukawa et al (203) measured a pK_a value for His-143(β) that was 5.2 in deoxy and 6.0 for carbonmonoxy Hb. All mutant Hb's in which residues are within or at close proximity to the 2,3-DPG binding cleft have shown a reduced acid Bohr effect, even though the extent of the reduction is different. Thus Hb Helsinki (Lys-82(β) \rightarrow Met) (115), Hb Rahere (Lys-82(β) \rightarrow Thr) (114), Hb Providence (Lys-82(β) \rightarrow Asn or Asp) (61,

116), Hb Andrew Minneapolis (Lys-144(β) \longrightarrow Asn) (222) and Hb Little Rock (His-143(β) \longrightarrow Gln) (65) all exhibit a reduced acid Bohr effect. In addition, Van Beek et al (189) were able to show the chloride dependence of the acid Bohr effect. They showed that there was a larger chloride-induced proton uptake for oxy than deoxy Hb below pH 6 in 0.1 M chloride. These sites to which chloride binds below pH 6 are as yet unidentified.

To briefly summarize therefore: under 'stripped' conditions in 0.1 M chloride, the main contributions to the acid Bohr effect could come from the carboxyl group of Arg-141(α), the imidazole of His-143(β) and/or to the differential binding of chloride to the oxy and deoxy conformations. In addition to the quaternary changes suggested by Perutz (49), there could be some as yet undiscovered mechanism.

It would certainly be of interest to speculate as to the magnitude of the acid Bohr effect in a Hb tetramer that is specifically carboxymethylated at the α -amino termini. In the tetramer specifically carboxymethylated at the α -chain terminus, the $-\text{CH}_2\text{COO}^-$ moiety could perhaps interact with the guanidinium group of Arg-141(α) in a manner analogous to chloride in deoxy Hb. The larger size of this anion and its close proximity to the carboxyl of Arg-141(α_1) would create repulsive interactions. This could perhaps raise the pK_a of the Arg-141(α) carboxyl group in the deoxy state. In other words, the pK_a of this residue in the deoxy $\alpha_2^{\text{Cm}}\beta_2$ tetramer would be higher than in unmodified deoxy HbA. Assuming then that the contribution of the Arg-141(α) carboxyl group is due to a higher pK_a value in oxy compared to deoxy Hb, if this differential were lowered, one could predict a reduced acid Bohr effect, in conditions of low salt. In 0.1 M chloride

this reduction may be compensated for by a differential binding of chloride to the oxy and deoxy forms of this modified tetramer, if the chloride binding site in oxy Hb is removed from the site of modification.

For the Hb tetramer carboxymethylated only at the β -chain amino terminus, under conditions of low salt, a reduction in the acid Bohr effect may be predicted. This is based upon the assumption that the negative charge of the $-\text{CH}_2\text{COO}^-$ moiety interacts with some of the cations in the 2,3-DPG binding crevice, as previously discussed. This may serve to increase the pK_a of His-143(β) which has been shown to be an acid Bohr group (111), and thereby lower the differential pK_a between the oxy and deoxy states. If the modification has not interfered with those sites that bind chloride preferentially in oxy Hb as opposed to deoxy Hb, addition of 0.1 M chloride could serve to elevate the acid Bohr effect in the $\alpha_2\beta_2^{\text{Cm}}$ tetramer (189).

A tetramer carboxymethylated at all four amino termini in low salt could display a reduction in its acid Bohr effect equal to a composite of any of the reductions that may be observed in $\alpha_2^{\text{Cm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$, respectively.

Thus, carboxymethylation at Val-1(α) might allow the attainment of further data involving the contribution of Arg-141(α) (230) to the acid Bohr effect, while carboxymethylation at Val-1(β) might allow further assessment of the role played by His-143(β) in this effect (111).

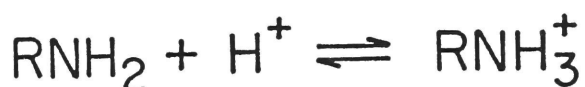
D). Interaction of Hemoglobin with CO₂

A detailed historical account of the interaction of CO₂ with Hb is given by Roughton (234) and by Edsall (134). As mentioned in C-1 of the Introduction, Bohr, Hasselbach and Krogh (131) were able to show that increased pCO₂ shifted the oxygen equilibrium curve to the right and this fact, coupled with the resultant decrease in pH, is the classical Bohr effect (132). Moreover, the reciprocal phenomenon, the release of CO₂ in the presence of increasing pO₂ was shown by Christiansen et al (133). The results of Bohr et al (131) and Christiansen et al (133) were linked together by the thermodynamic equations of Adair (235).

Bohr believed in the existence of a carbon dioxide-Hb complex but was unable to show this experimentally. By 1928 it was generally thought that the CO₂ in the blood was fully accounted for by dissolved CO₂ in the form of carbonic acid and bicarbonate (HCO₃⁻). Henriques (236) was able to provide the first evidence that CO₂ could directly combine with Hb. He suggested that the free amino groups were involved and formed a carbamino derivative. The results of Henriques were later reproduced by Meldrum and Roughton (237), only in the presence of cyanide which inhibits carbonic anhydrase. For some unapparent reason, the activity of the carbonic anhydrase in Henriques' experiments must have been inhibited. Ferguson and Roughton (151, 238) measured carbamino formation with Hb directly using the barium precipitation method of Faurholt (239, 240). They concluded that carbamino formation with Hb was oxygen-linked. In other words, this reaction occurred to a greater extent with deoxy than with oxy Hb under the same conditions. Moreover, they also concluded that about 20% of the CO₂ evolved from the lungs had been transported there as carbamino Hb. The high pO₂ (100 mm

Hg) in the lungs (241) oxygenates the Hb and allows the release of CO₂ attached to Hb.

That the binding of CO₂ to Hb was oxygen-linked was challenged by Wyman (135). The two fundamental reactions that occur when carbamino formation occurs are:



$$K_z = \frac{[\text{H}^+][\text{RNH}_2]}{[\text{RNH}_3^+]}$$



$$K_c = \frac{[\text{RNHCOO}^-][\text{H}^+]}{[\text{RNH}_2][\text{CO}_2]}$$

where k_z is the ionization constant for the protein amino group and K_c is the association constant for carbamino formation. Wyman postulated that if carbamino formation occurred with the amino termini then these same groups were also alkaline Bohr groups. Moreover, he calculated the pK_a of these groups to be 6.8 and 7.8 in the oxy and deoxy conformations respectively (142). Therefore, at neutral pH, in deoxy Hb, these groups would be predominantly protonated and therefore, unable to participate in carbamino formation. With reference to the equations described above, Wyman suggested that carbamino formation would be pH dependent but not oxygen-linked (142). The dispute remained unsettled until

Rossi-Bernardi and Roughton (224, 242-244) developed a refined technique for the measurement of carbamate CO_2 that was bound to Hb. They measured this parameter as a function of pH under conditions of constant pCO_2 . The data conclusively demonstrated that under identical conditions (pH and pCO_2) more CO_2 was bound as carbamate to deoxy than to oxy Hb. In addition they showed that the buffering capacity of Hb under physiological conditions was 20 to 30% greater than had previously been estimated, presumably due to carbamate modification of Hb. In their studies Rossi-Bernardi and Roughton (234, 244) showed also that physiological concentrations of CO_2 had an antagonistic effect on the value of the Bohr coefficient. Examination of the equation above shows that carbamate formation is accompanied by the release of two protons, one released from the protonated amino group and the second from the carbamate group ($\text{pK}_a \sim 6$) (143). Since more CO_2 binds to deoxy than to oxy Hb, the release of protons by deoxy Hb will counteract the uptake of protons due to the Bohr effect. It should be emphasized, however, that a greater portion of the CO_2 produced by respiring cells is hydrated by carbonic anhydrase to bicarbonate and protons. These protons are taken up by deoxy Hb. Thus, the two mechanisms for CO_2 transport are antagonistic to each other, carbamino formation with deoxy Hb releases one or two protons which opposes the formation of bicarbonate. It was suggested, however (52), that carbamate formation can account for 60% of CO_2 transport. It was shown (52) that as the pH increased from 7.0 to 7.4 carbamate formation increased, such that the protons absorbed upon deoxygenation were those released due to the modification of the α -amino groups by CO_2 . This markedly reduces the Bohr effect. Other estimates

of the total contribution of carbamate formation to CO_2 transport state a value of 30% (132, 245).

Although calculations (244) revealed that the pK_a values of the α -amino groups of Hb would fall into the range where reaction with CO_2 was feasible, it was only by the chemical modification studies that this theory was substantiated (52, 53). Specific carbamylation of the α -amino groups (58) would provide a means to identify the contribution made by the α -amino groups to carbamate formation as well as to the alkaline Bohr effect (discussed in section C-1). In summary, Kilmartin and Rossi-Bernardi prepared three derivatives of horse Hb, $\alpha_2^{\text{Cbm}}\beta_2$, $\alpha_2\beta_2^{\text{Cbm}}$, $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$, where superscript 'Cbm' denotes carbamylation (52, 53). They measured the interaction of these derivatives with CO_2 by measuring the total CO_2 in their oxy and deoxy forms. In addition, the P_{50} values of these derivatives were measured in the absence and presence of CO_2 . As discussed in section C-1, both $\alpha_2^{\text{Cbm}}\beta_2$ and $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$ showed a reduction of 25% in their alkaline Bohr effect. There did not appear to be any effect of CO_2 upon the derivative $\alpha_2\beta_2^{\text{Cbm}}$, as there was no difference in the total CO_2 between the carbonmonoxy and deoxy forms of this derivative. In addition, CO_2 did not cause a further change in the P_{50} value of $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$. These results indicate that the α -terminal amino groups are the sole source of the oxygen-linked CO_2 effects due to carbamate formation. Also since CO_2 reduces the oxygen affinity of both $\alpha_2^{\text{Cbm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cbm}}$, both the α -chain and β -chain amino termini are capable of carbamate formation. This is in contrast to the measurements for the Bohr effect where a reduction in proton binding was observed only for $\alpha_2^{\text{Cbm}}\beta_2$.

The barium hydroxide precipitation method of Ferguson and Roughton (238) used to measure total carbamate formation of Hb was subject to certain technical difficulties. Rossi-Bernardi et al (246) and Perrella et al (247) developed an alternative technique that utilized gel-filtration and ion exchange chromatography. Very simply, the pH of Hb in equilibrium with CO_2 is raised to 11 to stabilize carbamino CO_2 and an ion exchange resin added to remove carbonate and bicarbonate ions. Acidification of the carbamino-Hb solution displaces the bound CO_2 which is measured by gasometric analysis. When this technique for the estimation of total carbamate was applied to human Hb, the data suggested that there were two classes of binding site with different affinities for CO_2 (248). Moreover, in the interpretation of their data these workers suggested that the α -amino groups of the α - and β -chains react with CO_2 with affinities that differ 3-fold. It is not possible from their data to identify which terminus had the higher affinity. In view of the detailed mechanism of action of Hb this result is not surprising. The micro-environments of the two different N-terminal amino groups are different, that of the α -chain is involved in the alkaline Bohr effect (see part C-1) and that of the β -chain in the binding of organic phosphate (see part B-2).

It is important to note that as mentioned above, CO_2 alone does not modulate the function of Hb. It, together with protons and anions, compositely control the action of Hb. From the preceeding discussion in this Introduction it is apparent that CO_2 and 2,3-DPG compete for a common site: namely the α -amino terminus of Val-1(β). It was shown by a number of groups that 2,3-DPG reduced carbamate formation and that the reciprocal relationship was also true (100, 124, 249-252). Indeed, in a

slightly earlier study Brenna et al (125) tried to identify the low and high affinity binding sites for CO_2 by saturating the α -amino groups of the β -chain with ATP. From their CO_2 binding data the α -chain terminal amino group was tentatively assigned as the high affinity site for CO_2 binding.

In view of the existence of two different affinity binding sites for CO_2 (249, 250), Kilmartin et al (253) extended the studies of Brenna et al (125). They examined the individual contribution of the α - and β -chain amino terminus to CO_2 binding when the opposite chain was carbamylated on its amino terminus as in $\alpha_2^{\text{Cbm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cbm}}$. Briefly, the slope (Bohr coefficient) of the change in $\log P_{50}$ versus pH was measured in the presence of combinations of 2,3-DPG and CO_2 . The oxygen affinity of $\alpha_2\beta_2^{\text{Cbm}}$ was decreased by CO_2 to the same extent in the presence or absence of 2,3-DPG. CO_2 increased the oxygen affinity of $\alpha_2^{\text{Cbm}}\beta_2$ to a greater extent than for $\alpha_2\beta_2^{\text{Cbm}}$ in the absence of 2,3-DPG. However, in the presence of 2,3-DPG the oxygen affinity of both hybrids was lowered to a similar degree by CO_2 . It appears from this study that there is preferential binding by CO_2 to Val-1(β), but 2,3-DPG suppresses this because it also binds to Val-1(β).

In light of the above studies, the X-ray data of Arnone (223, 254) was of great importance. In an early report (223) the difference electron density profile between Hb soaked in 70% 2-methyl-2,4-pentanediol (MDP) with or without CO_2 /bicarbonate buffer at pH 7.4 were compared. A peak of positive density corresponding to the carbamate anion was seen to be contiguous with the α -amino group of the β -chain. However, unlike the modification by organic phosphate at this site (43) carbamate did not exert any tertiary structural changes within the

β -subunits. It appeared to have replaced an inorganic anion at the entrance to the 2,3-DPG binding crevice and in so doing removed positive charge. Surprisingly, and in view of the data discussed above (125, 247, 248, 255) in this early study, no peaks of positive density contiguous with the α -chain amino terminus were seen. Positive density was seen, however, behind the β -heme group. In view of the latter two observations, that were a little surprising, X-ray data was collected from crystals grown from solutions of polyethylene glycol, where, unlike in the early study, the concentration of CO_2 was similar to that found in vivo (254). The 4.4 Å resolution electron density map showed electron density contiguous with the β -chain amino terminus, that confirmed the observation seen in the earlier study (223). Moreover, model building studies showed that the orientation of the carbamate was such that it could form an intrasubunit salt bridge with Lys-82(β). An inspection of the electron density pattern at the α -chain amino terminus revealed carbamate at this site. The orientation of this moiety was toward the hydroxyl of Ser-131(α) which is a region of anion binding (43). Of note is the fact that the electron density of carbamate at the locus of the α -chain terminus is less intense than that found at the β -chain terminus.

This later X-ray study was consistent with results from some of the solution studies that preceded it. Bauer et al (255) examined the effect of CO_2 on the P_{50} values of HbA, HbA_{1c}, HbF, HbF^{Acetylated}, and Hb Hope (Gly-136 \rightarrow Asp). In this latter Hb, a salt bridge extends from the aspartate to the terminal valine of the β -chain. In all these cases the effectiveness of CO_2 in lowering the oxygen affinity was considerably reduced in comparison to HbA.

It may be constructive to review the state of affairs regarding oxygen-linked CO₂ binding to Hb discussed so far. From the work of Brenna et al (125) and Kilmartin et al (253) on human Hb, it is evident that there are two classes of binding sites for CO₂. The assignment by Brenna et al (125) that the α -chain terminal amino group is a high affinity site is in contrast to the interpretations made by Kilmartin et al (253). Moreover, the observations of Bauer et al (255) are consistent with the proposal of Kilmartin et al (253). The X-ray work of Arnone (223, 254) seems to provide evidence for carbamate formation at the terminus of each chain. From the electron density patterns the distribution seems to be greater at the β -chain terminus. With these facts in mind what are the next questions to be addressed? Clearly it would be informative to measure CO₂ binding constants to the α - and β -chains directly. It would be of considerable value to study this association both in the absence and presence of physiological concentrations of 2,3-DPG. As mentioned previously and stressed again, the function of Hb is modulated by the complex interplay between protons, CO₂, and anions. Another question to be addressed therefore is, what is the nature of the competition that exists for effectors with a common site?

The ion exchange/gel filtration technique was used to measure the CO₂ binding constants for the carbamylated derivatives $\alpha_2\beta_2^{\text{Cbm}}$, $\alpha_2^{\text{Cbm}}\beta_2$, and $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$ (248). In contrast to the conclusions reached by Brenna et al (125), Perrella et al (256) found that in the absence of 2,3-DPG there was about a 3-fold increase in the amount of CO₂ bound to deoxy $\alpha_2^{\text{Cbm}}\beta_2$ compared to deoxy $\alpha_2\beta_2^{\text{Cbm}}$. The effect of 2,3-DPG upon the CO₂ binding to deoxy $\alpha_2^{\text{Cbm}}\beta_2$ is a 30% reduction in carbamate formation while

this modulator has a negligible effect on $\alpha_2\beta_2^{\text{Cbm}}$. These data are opposite to the measurements recorded by Brenna et al (125) where the α -chain amino terminus is tentatively assigned as a high affinity binding site for CO_2 . Perrella et al (256) suggested that the discrepancies were due to the fact that Brenna et al (125) neglected to recognize the existence of substantial populations of HbCO_2DPG and $\text{Hb}(\text{CO}_2)_2\text{DPG}$. The stereochemical configuration of the HbCO_2DPG and $\text{Hb}(\text{CO}_2)_2\text{DPG}$ complexes are unknown (257).

In a separate series of studies, the application of ^{13}C -NMR was reported in detail from the laboratory of Gurd (258). Matwiyoff and Needham (259) were the first to test the feasibility of ^{13}C -NMR in the quantitation of carbamate formation. This study was extended by Morrow et al (260) who equilibrated either 'stripped' Hb solutions or whole red blood cell suspensions with bicarbonate and ^{13}C -enriched CO_2 . In summary the results showed that a carbamino resonance could be most clearly seen in deoxy Hb at alkaline pH in the absence of 2,3-DPG. Carbamino resonance was also seen for carbonmonoxy Hb. In addition to resonance measurements of deoxy or oxy unmodified Hb, ^{13}C -NMR spectra were also established for the carbamylated derivatives $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$, $\alpha_2\beta_2^{\text{Cbm}}$, and $\alpha_2^{\text{Cbm}}\beta_2^{\text{Cbm}}$ (261, 262). In this way an assignment could be made to either the α - or β -chain terminal carbamino adduct and extended to calculations for pK_z and pK_c . From these studies in the absence of 2,3-DPG, Val-1(β) was shown to be the preferred site of carbamino formation (262).

Thus, the extent of carbamate formation at the two termini differs. As previously stated, this could be expected in view of the different environments. At the α -chain terminus an elevated pK_a of

Val-1(α) in deoxy compared to oxy Hb makes it an alkaline Bohr group. This elevated pK_a might be antagonistic to oxygen-linked carbamate formation which depends on reaction with the unprotonated amine. In contrast, the pK value of Val-1(β) is only slightly elevated on oxy compared to deoxy Hb (258). The lower pK value of Val-1(β) compared to Val-1(α) in deoxy Hb might tend to favor carbamate formation at the former. In vivo at pH 7.4 however, 2,3-DPG is equimolar with Hb and binding of this modulator to Val-1(β) should partially or completely inhibit carbamino formation at this locus. In practice, a calculation of Z , the mole fraction of carbamino derivative per $\alpha\beta$ dimer, for either the α - or β -chain terminus in deoxy Hb showed values of 0.23 and 0.56 respectively at pH 7.2 (261). Addition of 2,3-DPG at pH 6.9 removed the resonance that was due to carbamate formation at Val-1(β). At higher pH values, 2,3-DPG binding weakens due to a reduction in the number of positively charged residues. Nevertheless, a reduction in the peak due to carbamate formation at Val-1(β) was still observed, indicating competition for the same site by CO_2 and DPG. This organic phosphate also influenced the carbamate resonances in liganded Hb. The interpretation of this result is unclear.

Thus, what seems readily apparent is a competition between the allosteric modulators of Hb for certain common sites, which necessarily means that the binding of one modulator influences the binding of another. The studies presented above have essentially discussed CO_2 interaction with Hb that was fully oxy or deoxy, as opposed to intermediates between the two forms. For this reason an investigation was carried out (263) to determine the effect of CO_2 , protons, and anions on the four step oxygenation scheme of Adair (264). This study

showed that the four Adair association constants were affected differently by CO_2 in combination with other allosteric modulators. This again reiterates the complexity of the regulation of the function of Hb by these modulators.

What further information could the carboxymethylated derivatives $\alpha_2^{\text{Cm}}\beta_2$, $\alpha_2\beta_2^{\text{Cm}}$, and $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ provide? The reversible nature of the reaction by which carbamino formation occurs has to date made isolation of this compound impossible. This has necessitated the use of indirect means to determine the nature and amount of adduct formed. Although such techniques have indeed provided a great deal of information, we felt that a structural analogue could further an understanding of these phenomenon especially with regard to the interaction between Hb and its allosteric modulators and their regulatory action on Hb function. The carboxymethyl moiety attached to the α -terminal amino group differs from the carbamino group by an extra methylene group. X-ray data could reveal any structural perturbations that this extra group would confer. On the assumption therefore of negligible disturbance, experimental conditions would have to be elucidated under which modification occurred only at the N-terminals and not at any of the 44 ϵ -amino groups of lysine. Only then would it be possible to use such a derivative for functional and kinetic studies. Derivatives of the type $\alpha_2^{\text{Cm}}\beta_2$, $\alpha_2\beta_2^{\text{Cm}}$, and $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ also differ from their carbamylated counterparts in that two or four extra negative charges have been introduced into the Hb tetramer, a situation more comparable to carbamino Hb. If specifically carboxymethylated Hb is a structural analogue of carbamino Hb, a decreased oxygen affinity could be predicted for this derivative. This is in direct contrast to carbamylation where modification of Val-1(α)

results in an overall increased oxygen affinity for $\alpha_2^{Cbm}\beta_2^{Cbm}$ (265, 266). Thus, a carboxymethylated hybrid might have step-wise oxygen association constants (264) that are more comparable to those of carbamino Hb. It would be of interest to be able to measure the affinity of an unblocked site for a heterotropic ligand (Cl^- , CO_2 , or DPG) in tetramers of the type $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$. CO_2 decreases the alkaline Bohr effect. Would carboxymethylated Hb exhibit a comparable decrease? If so, could this be restored by 2,3-DPG? Also which is more influential, carbamate at Val-1(α) or at Val-1(β) in the modification of the Bohr effect? A carbamino analogue could provide an answer to some of these questions.

E) The 'Trigger' Between the R and T Conformations

A comparison of the oxygen binding curves for myoglobin (Mb) and Hb reveals a hyperbola for the former and a sigmoid curve for the latter. The hyperbolic binding curve for Mb is indicative of a Mb-oxygen association, whereby the Mb molecule absorbs oxygen readily but becomes saturated at low pressure. In the case of Hb, the curve rises gently at first, steepens and then flattens out again. This is interpreted to mean that the binding of the first oxygen molecule occurs with difficulty but that the affinity increases with oxygen uptake. The physiological consequence of this is that at arterial oxygen pressure both molecules are nearly saturated. At the venous partial pressure of oxygen, Mb releases only about 10% of its oxygen whereas Hb releases about half. The higher oxygen affinity of Mb makes it ideal for oxygen storage in the muscles.

In Hb, that the binding of one oxygen to heme depends on the state of the other three heme molecules indicates that each heme is not independent. The first oxygen binds weakly and the last the most strongly. Conversely, in the tissues the tendency to lose the first oxygen is least but the remaining oxygens are released with greater and greater ease. Thus, there must be some kind of communication whereby the behavior of one heme depends on the state of the others, i.e., heme-heme interaction. It is also of note that it is difficult to isolate partially oxygenated Hb. It is either fully oxy or fully deoxy. This allows application of the Monod-Wyman-Changeux theory of allostery to Hb (113), whereby there is an equilibrium between the R (relaxed) state and the T (tense) state defined by L , the allosteric constant. In the R

state there are fewer constraints within the tetramer which allows Hb to bind oxygen. In the deoxy T state, extra salt bridges give Hb a lower oxygen affinity.

Many studies (51, 267), especially in the laboratory of Perutz, have tried to elucidate the mechanism by which the transformation between the T and R state occurs and what causes the breakage of the salt bridges. In extremely simplistic terms, the iron atom in deoxy Hb is high spin, forms a covalent bond with the proximal histidine and is pulled about 0.8 \AA out of the plane of the porphyrin ring (51). In oxy Hb, the binding of oxygen in the space between the iron and distal histidine makes iron low spin and pulls this heavy atom so that it is almost flush with the plane of the porphyrin ring. These changes in position of the iron are transmitted to other regions of the Hb molecule as conformational changes which eventually cause the rupture of the salt bridges. In other words, the change in ionic radius of the iron, due to a change in spin state on binding of ligand, is translated into quaternary structural changes. There are detailed accounts of this phenomenon (51). X-ray crystallography was of paramount importance in formulating the above mechanism.

One of the most crucial questions is what triggers the events that cause the transformation from the T to the R state? It appears from X-ray data (267) that the space between the heme and the distal histidine is not intrinsically large enough to allow the entrance of an oxygen molecule. Somehow the space must be enlarged through changes in the protein structure to allow ligation of the first oxygen molecule. How this is achieved exactly is at present unclear. Does a change in

spin state of the iron elicit changes in the protein conformation, or is the reverse true?

In this section of the Introduction, a discussion will be presented with regard to the techniques available for the study of the relationship between the spin state of the iron and quaternary structure. Is there a possibility that Hb specifically carboxymethylated could be used to provide additional information about the above relationship?

According to the Perutz school, there appears to be a reciprocal relationship between spin state and quaternary structure (267). In other words, while binding of oxygen changes the spin state of the iron atoms, which then results in a change in quaternary structure, a change in quaternary structure will alter the spin state of the iron atoms. A good example of this is found in Hb Milwaukee (268). In this mutant, Val-67(β), found in the hydrophobic binding pocket for oxygen, is replaced by glutamate. The resultant Hb variant contains two β -chains in which the iron is oxidized to the ferric state. The conformation (i.e., R or T) assumed by Hb Milwaukee will be dependent upon the state of ligation of the normal α -subunits. If the α -subunits are ferrous, high spin and deoxy, the tetramer will crystallize isomorphously with deoxy Hb. If on the other hand the α -chains are aquomet and ferric, the crystals of the tetramer will be isomorphous with met Hb. Thus, there is a structural change in the β -subunits, without ligation, which depends upon the conformational state of the tetramer. In addition, it is possible to observe a spectral change of the ferric iron in Hb Milwaukee β -chains when the ferrous iron α -chain combines with CO, indicative of a change in spin state. These observations could

therefore be consistent with the reciprocal relationship between spin state and quaternary conformation as mentioned above. In met unmodified HbA, the iron lies 0.3 \AA out of the plane of the porphyrin ring (51). This distance is intermediate between that seen for oxy and deoxy Hb respectively. Maybe the iron in met HbA could take up the spin state and position of either structure with the resultant changes in tertiary structure. Perutz (267) described spectra of deoxy met hybrids which suggested that the ferric hemes of the met subunits had shifted to a state of higher spin characteristic of the deoxy state. Moreover, spectral changes similar to these were produced when IHP was added to aquomet Hb. Since IHP can induce changes in the conformation of aquomet HbA (267), the spectral changes that showed a change in spin state of the iron, support the proposal of Perutz whereby the spin state of the iron and quaternary conformation are coupled (267).

If a conformational change is precluded, would the spin state of the iron change? This is not the case based on studies with deoxymet hybrids of either (NES-des-Arg) Hb or des-(His-146, Tyr-145) Hb (267). Both derivatives have a structural similarity to oxy Hb even when fully deoxy (47). Ligation of these hybrids with CO failed to produce the spectral changes observed with the deoxymet hybrids. Further credence to the hypothesis that a change in quaternary conformation is necessary for a change in spin state came from some experiments with Hb Milwaukee (268). Addition of IHP to Hb Milwaukee 'locks' the conformation into the T state. Addition of CO to deoxy $\alpha_2\beta_2^{+(\text{Mil})}$ did not produce the spectral changes observed in the absence of this organic phosphate that were due to changes in spin state. To reiterate from the experiments described (267, 268), it appears that the binding of ligand will not

produce a change in spin state of the iron in the neighboring hemes unless this binding is accompanied by a change in quaternary structure.

In a very detailed series of studies Perutz et al investigated further the influence of globin structure on the state of the heme (269-271). They wished to test two main concepts experimentally. First, they wished to establish the inverse relationship between oxygen affinity and the spin state of the iron. Second, they wished to test the proposal made by Perutz (267, 269) namely that 'the low oxygen affinity of the T structure may be related to an increased tension at the heme which pulls the iron atom further away from the plane of the porphyrin ring and thus opposes its movement into the ring on reaction with oxygen'. It would ordinarily not be possible to investigate these hypotheses in the absence of any ligand binding to or disassociated from the heme. However, in the first of three reports, Perutz et al (269) examined the physico-chemical behavior of (NES-des-Arg) Hb, des-(Arg-Tyr) Hb, and Hb Kempsey (Asp-99(β) \longrightarrow Asn). These derivatives assume the oxy R conformation even under deoxy conditions. It is only upon the addition of IHP that there are changes in electronic and nuclear magnetic resonance spectra, ultraviolet absorption, and circular dichroism, characteristic of deoxy HbA. Thus, the R \longrightarrow T transition can be studied in the absence of ligands. Moreover, exactly the same changes were observed when IHP was added to high spin aquo or fluoro met HbA (270). The mechanism by which IHP can stabilize the T conformation may in part be due to the extra salt bridge that exists between Lys-82(β) and a negatively charged phosphate (269). (When 2,3-DPG binds to deoxy Hb only one Lys-82(β) is involved in salt bridge formation (62). The changes in physico-chemical parameters, which it should be

stressed are pH dependent, determined by the techniques described above, appear to be useful diagnostic measures with which to assess the conformational state of the particular Hb under study.

The above results are interpreted with respect to a coupling of spin state with conformational change. The T state promotes the high spin character of the iron to create a diminished affinity for oxygen. Transition to the R state reduces the distances between the iron and the porphyrin nitrogens and the iron and proximal histidine respectively, This creates an increased affinity for oxygen. In reports from other groups, however, the above hypothesis has not been corroborated (57, 272-276). Hensley et al (272) using physico-chemical techniques that included subunit dissociation, haptoglobin binding, reactivity towards p-hydroxymercuribenzoate and electron paramagnetic resonance (EPR) measurements concluded that IHP did cause a conformational change, but the resultant structure was not analogous with the T state. The subunit dissociation studies and -SH reactivity measurements gave data representative of a conformation intermediate between the T and R conformations. Moreover, this 'intermediate' conformation is independent of the spin state of the iron since similar data were produced for subunit dissociation and -SH availability for both high and low spin forms.

In an independent study Gupta and Mildvan (274) showed by magnetic susceptibility and nuclear relaxation experiments that IHP does alter the quaternary structure of met HbA. However, this did not appear to be correlated with a change in spin state of the iron.

In another study carbamylation of Val-1(α) was used as a means to measure the conformational state of Hb (57). This was possible since

Val-1(α) in deoxy Hb is carbamylated at about a threefold higher rate than in the oxy form. The α -chains of met Hb in the presence of IHP were carbamylated to a higher degree than in the absence of this organic phosphate. However, this increase was only 30% of that observed in going from the oxy to deoxy conformation. These data supported a change in conformation caused by IHP. However, it is not possible to ascertain from this study or those cited above (272-276) whether there is a mixture of tetramers with structures intermediate between R and T, or whether there are distinct populations of tetramers that are either fully T or fully R.

The studies described in this section of the Introduction seem to be divided into two schools of thought as regards the ability of IHP to change the conformational state of the Hb tetramer. It thus still remains a matter of intense speculation as to the sequence of events that cause the transition between quaternary states. From the Perutz studies (267-271), it seems that the Hb's examined had their allosteric equilibrium constant (L) tipped in favor of the T state. IHP only elicited changes in derivatives where the iron was high spin initially. So we are still left with the unanswered question - under physiological conditions what factors are responsible for the shift in conformation? If a small change in spin state is necessary to initiate the subsequent changes in quaternary structure, how is this change brought about? Conceivably, binding of allosteric modulators could induce such a change but no evidence of this has been reported so far.

It is confirmed from a number of studies (248, 256, 261, 264) that CO₂ can bind to oxy Hb. With this in mind we decided that it might be of interest to determine whether the specifically carboxymethylated

tetramers $\alpha_2^{\text{Cm}}\beta_2$, $\alpha_2\beta_2^{\text{Cm}}$, $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ could have their R \rightarrow T equilibrium tilted in such a way as to assume the deoxy conformation upon addition of IHP. The results expected are unpredictable. A positive response could permit an additional role to be assigned to CO_2 in modulating the function of Hb.

F. Overall Strategy of Project

This section will describe the overall approach used to study the chemistry of the reductive carboxymethylation of Hb and to evaluate the structural and functional aspects of carboxymethylated Hb.

In the first part of the experimental work, Hb was reacted with glyoxylate and sodium cyanoborohydride. This mixture was then purified to produce the final products $\alpha_2^{\text{Cm}}\beta_2$, $\alpha_2\beta_2^{\text{Cm}}$, $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$, where the notation Cm represents the carboxymethyl group and experimental conditions were found whereby the carboxymethylation was predominantly N-terminal. By a number of chemical and physical criteria, the purity of these derivatives was established such that the carboxymethylation was N-terminal. These derivatized tetramers were then used for functional studies.

It was of great interest to determine the oxygen affinity of these derivatives in the absence or presence of various combinations of allosteric modulators.

In a separate series of functional studies related to the first, both the acid and alkaline Bohr effects were measured and the data interpreted with respect to the difference electron density maps produced in the laboratory of Dr. A. Arnone, University of Iowa.

Competitive binding studies were carried out whereby the affinity of an unmodified amino terminal for radiolabeled glyoxylate in the presence or absence of different combinations of allosteric modulators was measured. The $\alpha_2^{\text{Cm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$ tetramers were used for these studies and $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ was used as a control.

If the carboxymethylated adducts were considered to satisfy certain physical and functional criteria comparable to those of

carbamino Hb, the derivatives in this study could be considered stable analogues of carbamino Hb.

In the final section of the experimental work the possibility that the derivatized tetramers may have their $R \rightarrow T$ equilibrium tipped toward one or the other conformation was examined.

METHODS

a) Preparation of Unmodified Hb

Erythrocytes from normal individuals were obtained by centrifugation of whole blood in ethylenediaminetetraacetic acid (EDTA)-containing tubes at 1000 xg for 5 minutes at 4°C. The plasma supernatant was removed and the cells were washed three times with 0.85% saline (277). The cells were lysed by addition of an equal volume of distilled water and freeze-thawed. The lysate was then centrifuged at 12,000 xg for 20 minutes in order to remove the membranes and particulate fraction of the cells. The resultant supernatant 'lysate' was dialyzed extensively against 0.1 M chloride in order to remove 2,3-DPG. Certain Hb samples were analyzed for the presence of phosphate by elemental analysis (the Molybdenum Blue Method (278) to ensure that the removal of 2,3-DPG had been complete. The Hb was dialyzed against a buffer, the composition of which depended upon the experiment. HbA concentration, which is expressed as tetrameric Hb, was determined by measurement of the absorbance at 540 nm. The molar extinction coefficient for tetrameric HbA at 540 nm is $57.08 \times 10^3 \text{ M}^{-1} \text{ Cm}^{-1}$ (277).

b) Carboxymethylation Reaction

For the carboxymethylation of HbA, sodium [^{14}C]-glyoxylate (8 Ci/mol) was obtained from Amersham Corporation. Unlabeled sodium glyoxylate (Sigma) was added to the [^{14}C]-glyoxylate in 30-fold excess to yield stock solutions of 80 mM [^{14}C]/[^{12}C]-glyoxylate. Portions were stored at -20°C and thawed just before use. The concentration of

glyoxylate was measured as the 2,4-dinitrophenylhydrazone derivative (279).

Sodium cyanoborohydrate (Aldrich) was recrystallized in the following manner (15). After addition of about 11 g of the compound to 25 ml of acetonitrile, insoluble material was removed by centrifugation. Sodium cyanoborohydrate present in the supernatant was precipitated by addition of 150 ml of methylene chloride. After standing overnight at 4°C the precipitate was collected on filter paper by suction through a Buchner funnel and dried in a vacuum desiccator.

2,3-DPG (Sigma), purchased as the pentacyclohexylammonium salt, was subject to ion exchange chromatography to yield the free acid (85). The concentration of the resultant acid was determined by elemental analysis for total phosphate (278).

HbA₀ was purified preparatively by a modification of the conditions described by Schroeder and Huisman (280). All manipulations were carried out at 4°C unless otherwise stated. Usually about 6 g of HbA₀ were separated by DEAE cellulose (Whatman) chromatography in order to remove the minor hemoglobins. Columns 2.2 x 35 cm of DE resin were loaded with the dialyzed lysate and eluted with a linear gradient of 50 mM tris acetate from pH 8.3 to 7.3 (500 ml of each) (280, 281). In all the experimental manipulations that involved tris buffer the counter ion used was acetate. The major peak, HbA₀, was concentrated to 1 mM in an Amicon filtration unit fitted with a YM-10 membrane and dialyzed against 0.1 M chloride. Under either oxy or deoxy conditions, carboxymethylation was carried out with a 10-fold molar excess of [¹⁴C]-glyoxylate and a 100-fold molar excess of sodium cyanoborohydrate at 37°C in 100 mM hepes buffer, pH 7.2, for 40 minutes. Excess reactants

were removed from the protein by gel filtration on a Sephadex G-25 column (2.5 X 35 cm) with 50 mM tTris acetate, pH 8.5 as the eluent. Under liganded conditions, the sample and buffers were saturated with carbon monoxide throughout the course of the experiments. Under deoxy conditions preliminary studies were carried out to determine the extent of deoxygenation by taking advantage of the difference in extinction coefficient between oxy and deoxy Hb at 760 nm (282). A value of 2.86 for the ratio of the deoxy A_{760} to the oxy A_{760} represents 100% deoxygenation. A special flask was designed to measure this ratio in the Aminco DW-2 scanning spectrophotometer. It consisted of a 150 ml cone-shaped flask with a two way stop-cock that controlled a gas inlet and outlet at the top. A side-arm from this flask led to a quartz cuvette. After deoxygenation with humidified nitrogen of a 5 ml sample for 1 hr, the flask was tilted to allow the Hb solution to fill the cuvette. The whole apparatus was placed into the spectrophotometer and the absorption spectrum was measured.

The above method was suitable for determining the extent of deoxygenation of concentrated Hb solutions (0.5 mM to 1 mM in tetramer). When solutions were considerably more dilute (60 μ M in tetramer for measurement of the Bohr effect, which will be described later) a hand spectroscope (Zeiss) was used and the observations made using this instrument were correlated with a measurement of the spectrum on the scanning spectrophotometer of the same Hb sample, taken between 400 and 600 nm (Figure 4, Table 1). When the value of the absorbance at 555 nm, divided by the absorbance at 540 nm was 1.24, the sample was fully deoxygenated (283).

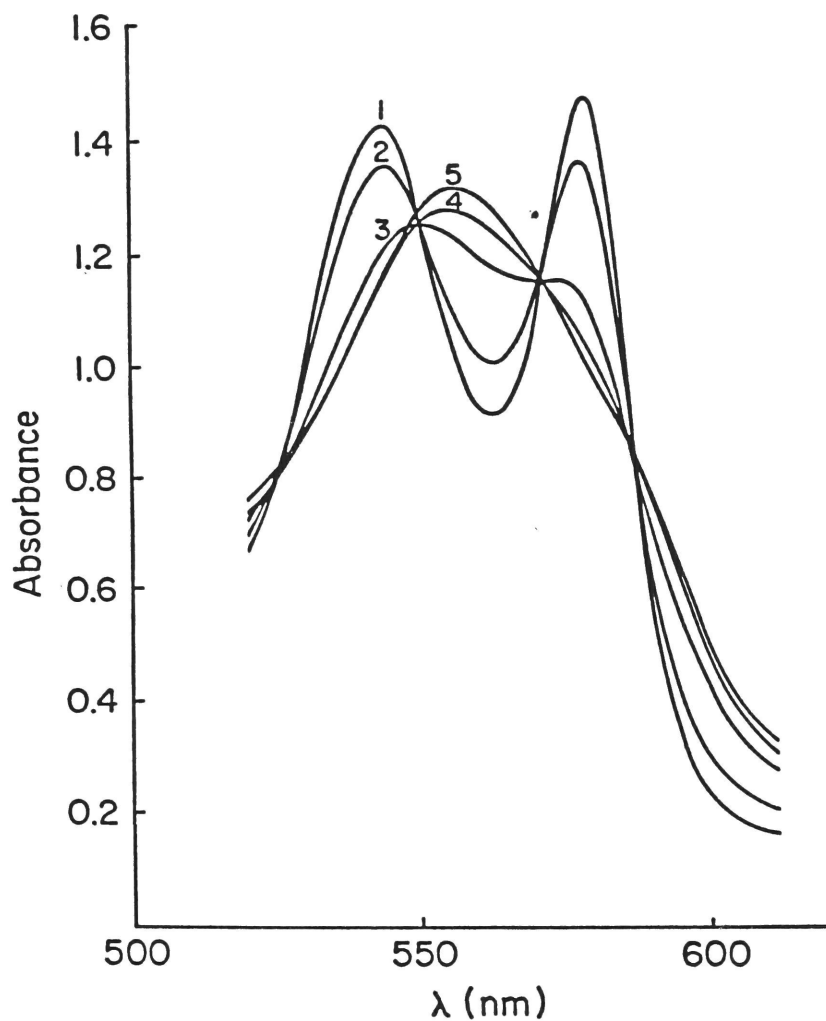


Figure 4 and Table 1 - Correlation between bands visualized on hand spectroscope, and the spectrum obtained for a dilute solution (i.e., 60 μ M) of Hb to measure the extent of deoxygenation. The specialized glassware is described in the text.

<u>Minutes for N₂ Bubbling</u>	<u>Curve Number</u>	<u>Observation from Hand Spectroscope</u>	<u>A₅₅₅/A₅₄₀</u>	<u>% Deoxygenation</u>
0	1	2 distinct dark bands in green part of the visible spectrum	-	0
10	2	The same 2 bands are visible	0.97/1.20	65
23	3	Twin bands becoming less distinct	1.14/1.03	89
26	4	One band only is visible	1.18/0.98	97
36	5	One sharper band is visible	1.22/0.98	100

Table 1

Nitrogen was bubbled through the Hb solution in the specialized glassware described in the Methods section for the length of time shown. After observing the solution through the hand-spectroscope the spectrum was measured on the scanning spectrophotometer (see Figure 4).

The carboxymethylation of deoxy Hb was carried out in a specially modified Ehrlenmeyer flask, with two side-arms (284), one for the glyoxylate solution and the other for the cyanoborohydride solution. The concentration of the reactants was the same as that used for the carboxymethylation of carbonmonoxy-treated Hb. When deoxygenation was complete the flask was tilted in such a way to allow all the reactants to mix. After a 40 minute incubation at 37°C, carbon monoxide was bubbled through the solution. Thereafter, the sample was subjected to some of the same experimental manipulations as the Hb sample that had been reacted with glyoxylate and sodium cyanoborohydride in the carbonmonoxy form.

c) Isolation of Carboxymethylated Hb Derivatives

The gel-filtered Hb sample was chromatographed on a system identical to that described above for the purification of HbA₀. The presence of the various Hb derivatives was determined by their absorbance at 540 nm. In cases where [¹⁴C]-glyoxylate was used, 20 µl aliquots of the fractions were mixed with Liquiscint (New England Nuclear) for measurement of the amount of [¹⁴C]-glyoxylate incorporated into Hb. A quench correction curve was incorporated into the program used in the LKB β-counter.

d) Separation of α- and β-chains of [¹⁴C]-Carboxymethylated Hb and Preparation of Specifically Modified Hybrids

Pooled fractions that represented derivatives Hb₁ and Hb₂ from the DE-52 column (Figure 5) were dialyzed against carbon monoxide-saturated

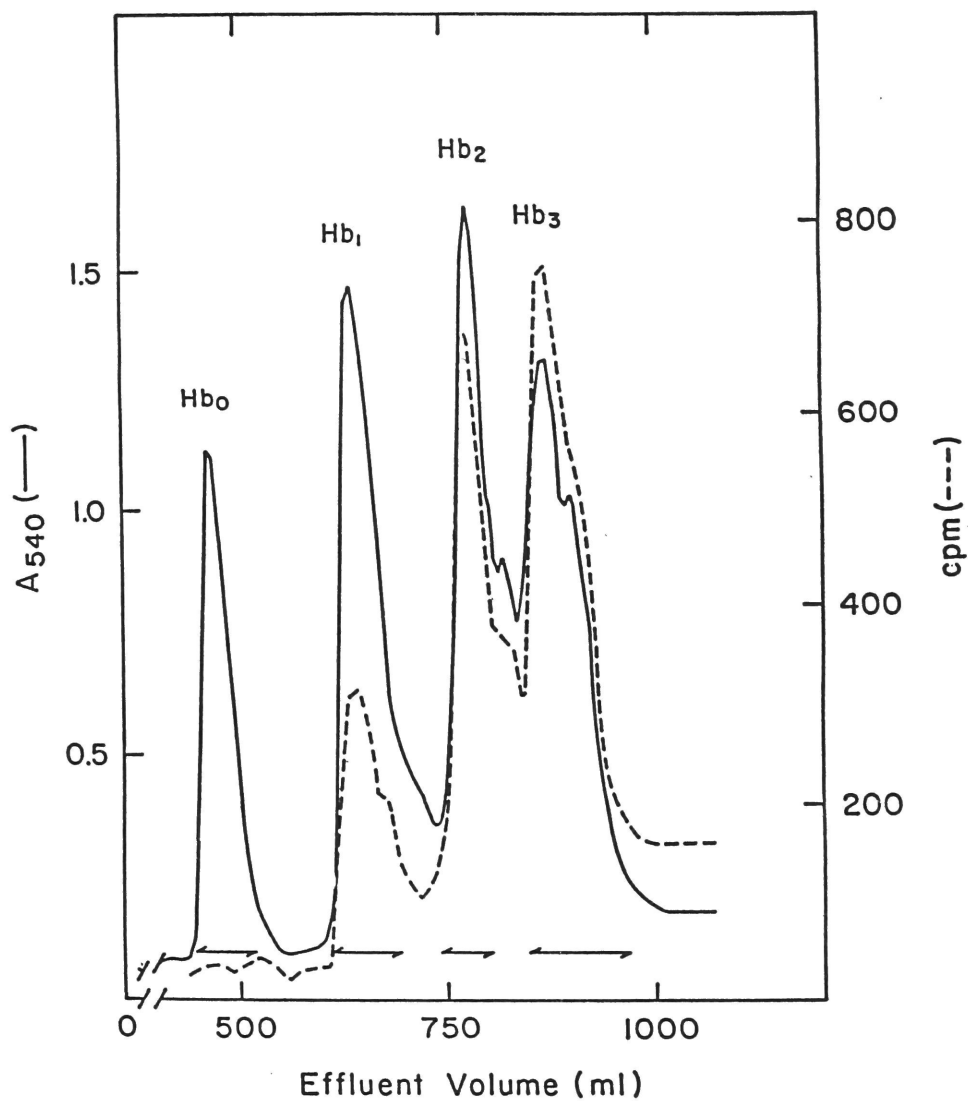


Figure 5 - Separation of carboxymethylated derivatives of Hb on DE-52. The separation was achieved as described in the text. For analysis of the various species, the fractions designated by the arrow were pooled.

0.1 M NaCl. Each tetrameric species was separated into its constituent p-hydroxymercuribenzoate (p-HMB) chains on CM-52 resin by a modification (285) of the procedure of Bucci and Fronticelli (286). The Hb was diluted to 0.8 mM in heme and KCl was added to a final concentration of 2 M. The sample was treated with p-HMB (2 mM) at pH 6.1 overnight. After dialysis against 10 mM potassium phosphate, 1 mM EDTA, pH 5.85 the protein solution was centrifuged to remove any precipitate. After concentration in an Amicon apparatus, the sample was eluted with a gradient of 10 mM potassium phosphate, 1 mM EDTA pH 5.85, to 15 mM potassium phosphate, 1 mM EDTA pH 7.9 at 4°C. About 300 mg of sample (volume < 10 ml) can be loaded onto a column 0.9 cm x 25 cm. In this chromatographic system (285), the negatively charged carboxymethyl groups permit the separation of the modified α -chain from the unmodified α -chain (Figure 6). However, there is no such separation of the carboxymethylated β -chains since both modified and unmodified chains are eluted in the void volume of the column. The modified β - and α -chains from both Hb₁ and Hb₂ were utilized in the preparation of the hybrid hemoglobins. For preparation of these hybrids carboxymethylated α - or β -chains were mixed, either together, in equimolar quantities to generate $\alpha_2^{Cm}\beta_2^{Cm}$, or with an equimolar amount of the unmodified β - or α -chain to yield $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ respectively. A 300-fold molar excess of 2-mercaptoethanol (relative to the concentration of derivatized sulfhydryl groups) was added and after gentle mixing the solution was allowed to stand overnight at 4°C. In this procedure, which is identical with that used for the preparation of specifically carbamylated Hb derivatives, the solutions of the Hb chain are used at the pH and ionic strength at which they are eluted from the CM-52 column

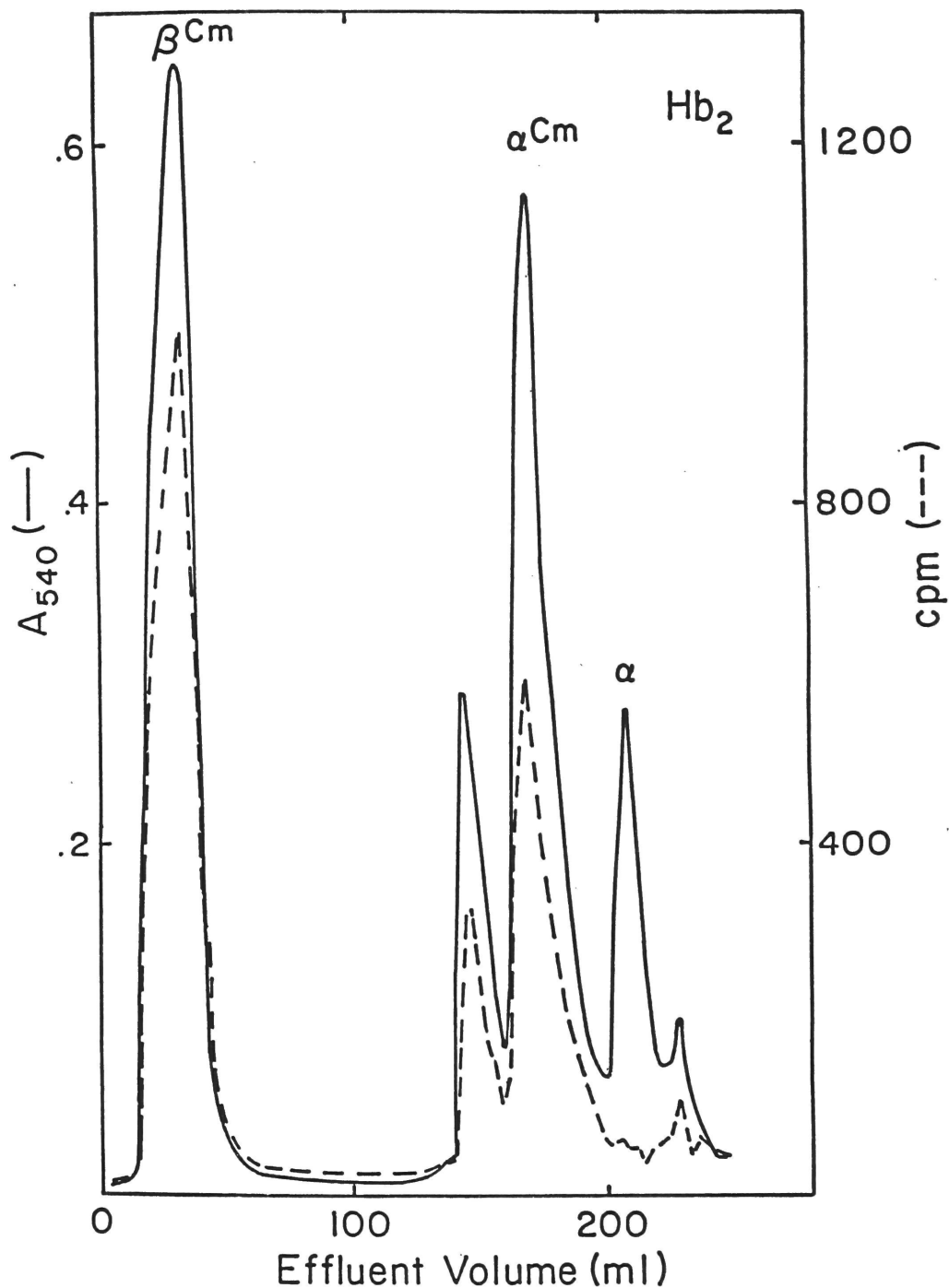


Figure 6 - Separation of the chains of Hb₂. The tetramer was treated with p-hydroxymercuribenzoate and subjected to chromatography on CM-52 as described in the text. The fractions that contained β_{Cm} and α_{Cm} were pooled separately and hydridized with unmodified α- and β-chains, respectively.

(265). The excess 2-mercaptoethanol was removed by extensive dialysis against several volumes of carbon monoxide-saturated 10 mM NaCl.

In order to ensure that the hybrid Hb derivatives were free of minor Hb contaminants, each hybrid tetramer was chromatographed on CM-52 cellulose (0.9 X 30 cm) after concentration to a volume less than 10 ml with an Amicon cell, fitted with a YM-10 membrane. The gradient used for the elution of the derivative from the column was from 10 mM potassium phosphate, pH 5.85, 1 mM EDTA, to 15 mM potassium phosphate, pH 7.9, 1 mM EDTA (150 ml in each gradient chamber). The buffers were saturated with carbon monoxide. This chromatographic system is the same one that was used to separate the chains of tetrameric HbA.

e) Identification of the Sites of Carboxymethylation

HPLC Analysis of Tryptic Peptides - Each carboxymethylated Hb hybrid that was purified on CM-52 as described above was again separated into its α - and β -chains (285). Globin was prepared from the separated chains by acidified acetone precipitation (287). After lyophilization, the globin chains (about 10 mg/ml) were treated with bromoethylamine (Sigma) in order to form trypsin susceptible derivatives of cysteine (288, 289). The reaction was carried out in 8 M urea, 0.1 mM dithiothreitol (Sigma) and 0.1 M tris buffer. Bromoethylamine was added to a final concentration of 0.5 M. The pH was adjusted to 12 with sodium hydroxide since this promotes the conversion of bromoethylamine to ethyleneimine which will then react with the cysteine sulfhydryl groups. After standing 2-5 minutes, the pH was then adjusted with concentrated HCl to 9.2 and 2-mercaptoethanol was added to a final concentration of 0.05 M. After incubation for one hour at 22°C the

sample was desalted by G-25 Sephadex chromatography using 0.1 M acetic acid as the eluent. The sample was then lyophilized and incubated with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin. The trypsin: protein ratio was 1:50. The reaction was carried out for 24 hrs in 0.2 M ammonium bicarbonate at 37°C. The sample was evaporated to dryness in a Savant microconcentrator, dissolved in 0.1 M acetic acid and evaporated to dryness again. The sample was then dissolved in 500 µl of 5% acetonitrile (Burdick and Jackson) and 0.1% trifluoroacetic acid (Pierce). A small aliquot of this mixture, 20 µl, was then applied to a reverse phase analytical column (4.6 x 250 mm), packed with Partisil-10 ODS-2 resin (purchased from Whatman). This column was a part of an HPLC system made up of components as described by Acharya et al (290). The separation of the tryptic peptides of the aminoethylated chain was performed with a linear gradient of 5-70% acetonitrile in 0.1% trifluoroacetic acid as described previously (290). The labeled peptides were detected by the presence of the ¹⁴C-label after counting 500 µl of each 1 ml fraction. The radioactive fractions were pooled, evaporated to dryness in a Savant microconcentrator, and hydrolyzed in 1 ml of 6 N HCl in vacuo for 22 hrs. Amino acid analysis was performed on a Dionex D-500 amino acid analyzer. The purity of the peptides isolated by the high performance liquid chromatography (HPLC) system permitted identification of the sites of modification by glyoxylate.

Detection of Carboxymethyllysine after Performic Acid Oxidation -

By performic acid oxidation of carboxymethylated Hb, methionine is oxidized to the methionine sulfone (291). This avoided the problem of coelution of carboxymethyllysine with methionine. The procedure was

applied to the protein before acid hydrolysis. Performic acid was generated in situ by allowing a solution that was 9 parts formic acid (and 88% solution) and 1 part hydrogen peroxide (a 30% solution) to stand at room temperature for 1 hour. A few milligrams of lyophilized Hb was mixed with 1 ml of the above solution and left overnight at 4°C. The methodology employed was as described by Moore (292). After the sample was treated with performic acid it was evaporated to dryness in a Savant microconcentrator and subjected to acid hydrolysis in 6 N HCl at 120°C for 16 hrs. The percentage modification at lysine, as carboxymethyllysine, was estimated after amino acid analysis.

Dowex-2 Chromatography System for the Quantitation of Mono-carboxymethylvaline, Dicarboxymethylvaline, and Monocarboxymethyllysine

N- α -Monocarboxymethylvaline was prepared by incubation of valine, sodium [^{14}C]-glyoxylate (Amersham Company, specific radioactivity 7.3 mCi/mmol), [^{12}C]-glyoxylate (Sigma), and recrystallized sodium cyanoborohydride. In this mixture 0.25 μCi [^{12}C]-glyoxylate were used. The reagents were incubated in a molar ratio of 1:1:10 in 50 mM hepes, pH 7.0 for 12 hrs at 37°C. N,N- α -Dicarboxymethylvaline was synthesized by a similar incubation but the reagents were in a molar ratio of 1:10:100. In this latter preparation 0.60 μCi of [^{14}C]-glyoxylate were used. The two samples that contained valine were acidified with HCl and lyophilized. N- ϵ -monocarboxymethyllysine was prepared by incubation of α -t-butyloxycarbonyl (boc)-lysine, [^{14}C]/[^{12}C]-glyoxylate and cyanoborohydride in a molar ratio of 1:1:10 in 50 mM hepes, pH 7.0 for 4 hrs at 37°C. After incubation, the t-butyloxycarbonyl group was removed from the α -amino group of lysine by addition of 200 μl 50% trifluoroacetic acid in methylene chloride to the dried down sample (292a). After

standing for 30 minutes at room temperature the sample was evaporated to dryness in a Savant microconcentrator. Each of the three samples were dissolved in 1 N acetic acid. Since mono- and dicarboxymethylvaline eluted in the void volume of the amino acid analyzer, Dowex-2 anion exchange chromatography (Bio-Rad) was deemed suitable for the separation of these acidic species. After application to a column of Dowex-2 (OH-form) column (0.9 X 22 cm), each individual incubation mixture described above was separated into its components by elution with 1 N acetic acid, 3 N acetic acid, and finally, 1 N HCl. (This system was a modification of the one used by Dreze et al (293) for desalting amino acids.) Portions of each fraction (800 μ l) were mixed in scintillation fluid and the radioactivity measured in an LKB scintillation counter. The peaks that contained radioactivity (Figure 7) were pooled and lyophilized. The two valine derivatives were subjected to elemental analysis. The results shown in Table 2 were consistent with the theoretical values calculated for N- α -monocarboxymethylvaline and N,N- α -dicarboxymethylvaline.

In addition, after repeated washing of each sample with D₂O, ¹H-NMR spectra from the Nicolet 300MHz instrument also corroborated the identities of the valine derivatives as the monocarboxymethyl (Figure 8a) and dicarboxymethyl (Figure 8b) derivatives, respectively. The assignments were made as follows, from Figure 8a: 1.09 ppm (d,d) from the methyl protons, 2.38 ppm (q,d) from the β proton, 3.81 ppm (d) from the α proton, 3.86 ppm (d,d) from the methylene proton. The assignments made from Figure 8b were 1.08 ppm (d,d) from the methyl protons, 2.26 ppm (q,d) from the β proton, 3.78 ppm (d) from the α proton and 4.11 ppm (d,d) from the methylene proton. The integration

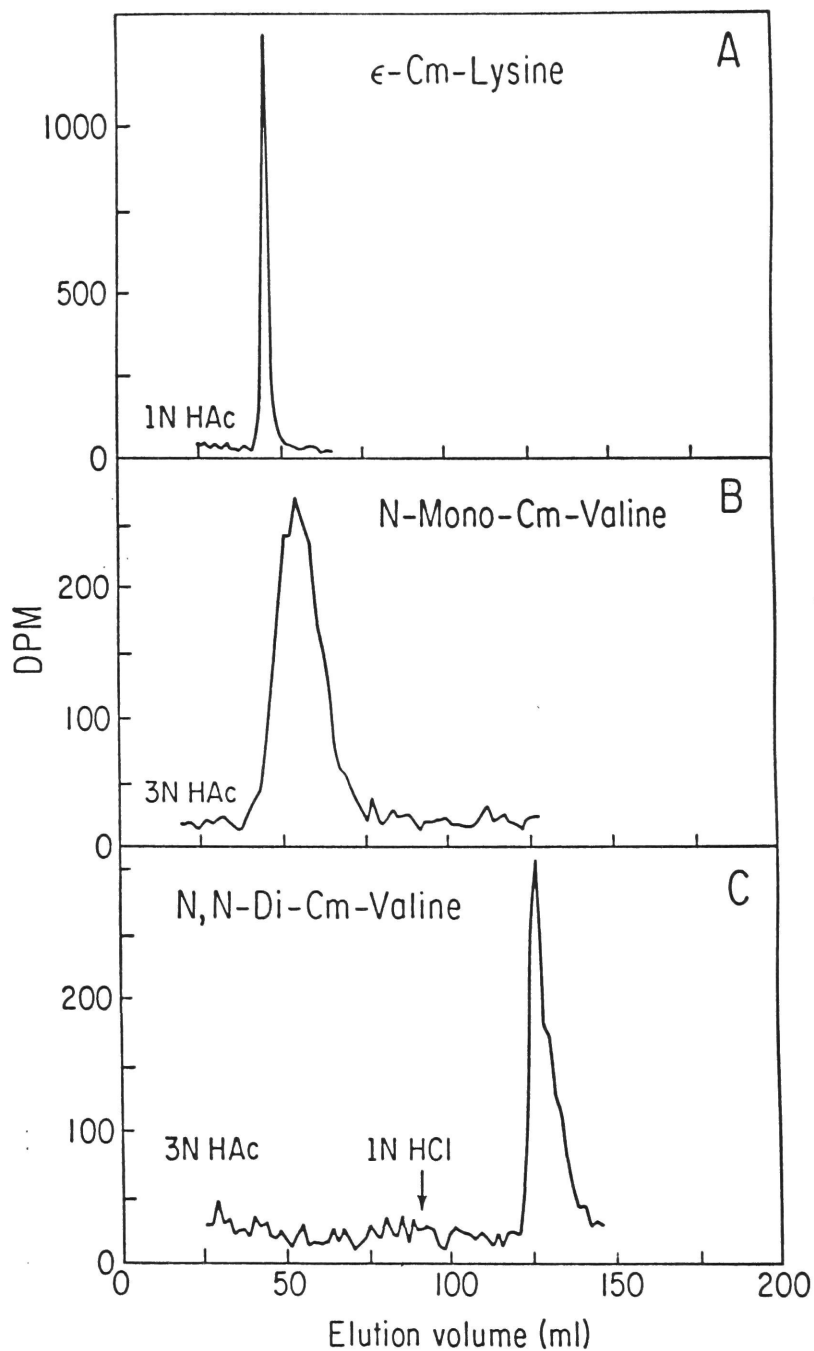


Figure 7 - Dowex-2 chromatography showing elution positions of the carboxymethylated amino acids ϵ -carboxymethyllysine, N-mono, and N,N-dicarboxymethylvaline as determined from the peaks of radioactivity. The peaks from panels B and C were identified by elemental analysis (Table 2) and ^1H -NMR analysis (Figures 8a and 8b).

N-Monocarboxymethylvaline

N	C	H	O	
8.00	47.99	7.48	36.53	theory
7.52	46.56	7.34	-	found

N,N-Dicarboxymethylvaline

N	C	H	O	
5.20	40.30	5.20	-	theory
4.90	39.26	5.10	-	found

Contamination

$C_{0.2}$ $H_{0.56}$ $O_{0.4}$ 1/10 N Acetic acid

(Compound eluted in 3 N Acetic acid)

Table 2

Elemental analysis of the peaks of radioactivity as seen in panels B and C of figure 7. The analysis were performed by Mr. S.T. Bella in our laboratory.

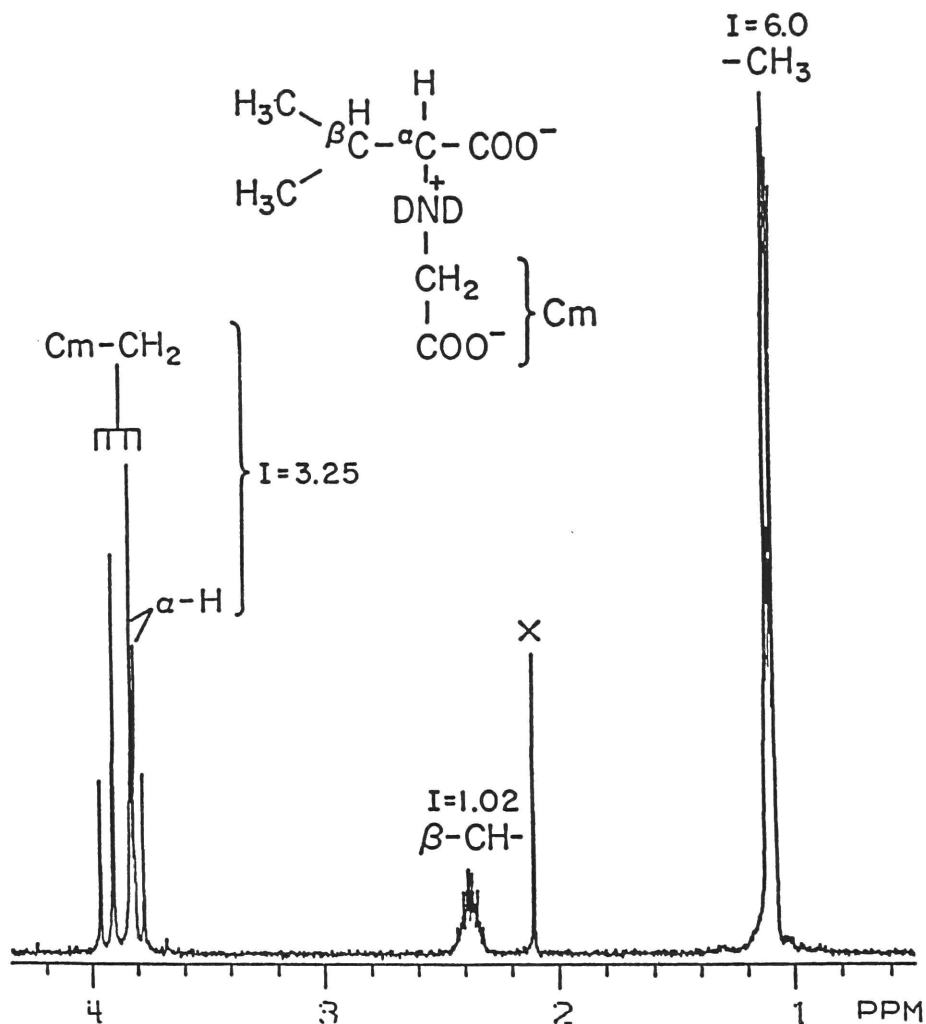


Figure 8a - Proton NMR of carboxymethylated valine adducts. About 1 mg of the material from the radioactive peaks of panels B was dissolved in D₂O and their ¹H-NMR spectrum was measured on a Nicolet 300 MHz instrument. 'X' is the signal due to a component in the external standard CDCl₃.

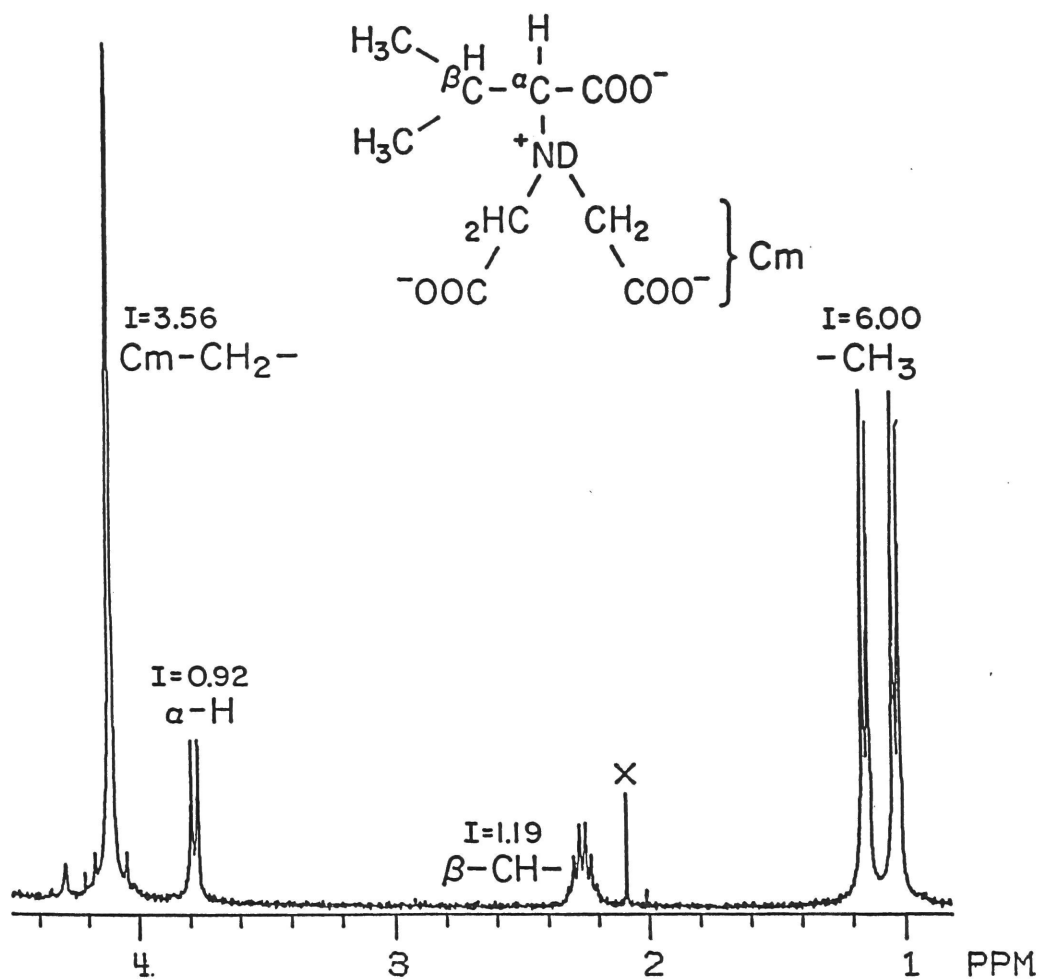


Figure 8b - Proton NMR of carboxymethylated valine adducts. About 1 mg of the material from the radioactive peaks of panels C was dissolved in D_2O and their ^1H -NMR spectrum was measured on a Nicolet 300 MHz instrument. 'X' is the signal due to a component in the external standard.

values shown on the Figures are consistent with the presence of 2 methylene protons from Figure 8a and 4 of these protons from Figure 8b as the notable difference between the two spectra. The lysine derivative, which was eluted in 1 N acetic acid was applied to an amino acid analyzer. Its position coincided with that of a commercial sample of N- ϵ -monocarboxymethyllysine (Sigma) that eluted according to published procedures (294).

f) Non-Reducing Gel of Carboxymethylated Hybrids

In order to assess the purity of the carboxymethylated hybrids the samples were loaded onto a polyacrylamide slab gel. These gels were then subjected to electrophoresis for 70 minutes at 100 volts in a tris-borate-EDTA buffer, pH 8.6 (265) under non-denaturing and non-reducing conditions. p^HMB was added to the gel buffers (i.e., running and sample buffers) to a final concentration of 0.02 mg/ml as described by Rosemeyer and Huehns (295). The gel was stained with Coomassie Blue under standard conditions (277).

g) X-Ray Crystallographic Studies

The X-ray crystallography was carried out for crystals of native HbA and for $\alpha_2^{Cm}\beta_2^{Cm}$, both grown in the deoxy state, from solutions of concentrated ammonium sulfate, using the methodology of O'Donnell et al (44). All manipulations were carried out in a nitrogen-filled glove box.

The diffraction data (3.5 Å⁰) were collected on an Enraf-Nonius CAD4 diffractometer using the ω scan mode. One crystal was used in each

set of data (15,000 reflections). After corrections for radiation damage, difference electron density maps were constructed using the known phases of deoxy HbA and the difference amplitudes ($|F|_{\text{modified Hb}} - |F|_{\text{HbA}}$). The final difference electron density map could then be plotted after symmetry averaging about the molecular 2-fold rotation axis that relates the 2 equivalent $\alpha\beta$ dimers (44).

h) Functional Studies

Determination of the Oxygen Equilibrium Curves of Carboxy-methylated Hb - The Hb derivatives that were purified on CM-52 were concentrated to about 0.1 mM by ultrafiltration with an Amicon YM-10 membrane and then dialyzed against 50 mM bistris, 50 mM tris pH 7.3. Prior to the determination of the oxygen equilibrium curves on an Aminco Hem-O-Scan, each sample was converted from the carbonmonoxy form to the oxy form according to the method described by Kilmartin and Rossi-Bernardi (53). The sample was placed in a round-bottomed flask. The ratio of the flask volume to the sample volume was usually about 100. The interior of the flask was evacuated to about 1 mm Hg. Oxygen was then carefully allowed to enter the flask to a pressure of 1 atmosphere. The flask was then rotated on an ice-water bath under a 200-watt light for 10 minutes. After this procedure had been carried out at least three times, the spectrum of the Hb samples was measured between 400 nm and 700 nm on an Aminco DW-2 scanning spectrophotometer. By measuring the ratio of the absorbance at 576 nm to that at 540 nm the extent of conversion from carboxymonoxy Hb to oxy Hb could be monitored. A ratio of 1.81 indicated that carbonmonoxide removal was complete (284). Prior to determination of the oxygen equilibrium curves on the Aminco Hem-O-

Scan, the concentration of the samples was increased to 1 mM by centrifugation in an Amicon Centricon 10 microconcentrator at 8,000 rpm. The length of time for centrifugation depended on the amount of sample to be concentrated. For example, a 2 ml sample of Hb at $\sim 25 \mu\text{M}$ required ~ 1 hr to be concentrated to 1 mM. Experiments in the presence of 2,3-DPG were performed on Hb samples that had been previously mixed with organic phosphate such that the concentration of Hb was in the range of 0.32 to 0.48 mM and the $[\text{DPG}]/[\text{Hb}]$ ratio varied from 1 to 4. Experiments were also performed on specifically carboxymethylated hybrids in the presence of varying chloride concentrations (0.025 to 1 M). The P_{50} values were determined directly from the graphs of the Hem-O-Scan. For estimation of the Hill coefficient, n , the logarithmic values of the fractional saturation from 40% to 70% were plotted against the logarithmic value of the oxygen tension. The slope of the resulting straight line gave the n value.

Measurement of the Alkaline and Acid Bohr Coefficient - For determination of the alkaline Bohr effect two different methodologies were used in the absence or presence of different effectors. The first method measured the alkaline Bohr coefficient by determining the oxygen affinity of each sample with respect to pH within the range of about 6.8 to 8.0 at 25°C . The $\log P_{50}$ value versus the pH gives a plot whose slope is a measure of the alkaline Bohr coefficient (296). The P_{50} , which represents the value of the partial pressure of oxygen at which the Hb sample is half maximally saturated with oxygen, is a measure of oxygen affinity. This value was read from the oxygen equilibrium curve determined from an Aminco Hem-O-Scan instrument. A solution of the desired hybrid, in the oxy form, at a concentration of 1 mM in H_2O , was

diluted twofold with one of several bis tris solutions (100 mM) whose pH varied within the range of 6.5 to 8.0. The pH of the sample was checked by use of a microelectrode (Microelectrodes Inc.). 4 μ l of the solution were administered onto a glass slide for loading into the Hem-O-Scan instrument and the subsequent measurement of the oxygen equilibrium curve.

The second method that was utilized for the determination of the alkaline Bohr coefficient was the proton titration technique using an autotitrator as described by previous investigators (56, 296). The Hb samples in the oxy form were at a concentration of 4 mg/ml in water. Each was placed in a water-jacketed vessel maintained at 25°C. One drop of octanol, an anti-foaming agent, was added. A rubber stopper was placed over the reaction vessel. It had four holes, a gas inlet, a gas outlet, a third to allow administration of a standardized solution of 10 mM sodium hydroxide (NaOH) into the Hb solution and a fourth for a pH electrode (Radiometer or Microelectrodes Incorporated). The Hb sample was adjusted to a slightly alkaline pH in the range of 7.8 to 8.2 with 0.1 M NaOH. Nitrogen, passed through a humidifier, was bubbled through the solution until no change in pH could be detected. The pH value for the deoxygenated sample was then set on the Titrator II (Radiometer). Humidified oxygen was then bubbled through the sample. The release of protons upon oxygenation led to a decrease in the pH value. The amount of this proton release was calculated from the amount of 10 mM NaOH that had to be added from the autoburette (ABU 12 Radiometer) to raise the pH to the value that had been set on the pH stat when the sample had been in the deoxy state. Addition of 5 μ l 0.1 N acetic acid after each addition of NaOH allowed ΔH^+ to be determined at successively lower pH

values. ΔH^+ corresponds to the difference in protons absorbed between the deoxy and oxy states. A plot of ΔH^+ versus pH gave a curve that rose to a maximum value and then decreased between pH 6.0 and about 8.2. The maximum amplitude was measured, and gave the number of protons released per tetramer.

Below pH 6.0, protons are absorbed upon oxygenation. Thus, the pH value for the oxy Hb sample was set on the Titrator II. Humidified nitrogen was passed through the sample until no further decrease in pH was observed. The amount of protons released, upon deoxygenation was calculated from the amount of 10 mM NaOH that had to be added from the autoburette to restore the pH to the value attained in the oxy state. Extrapolation to pH 5.0 from a plot of ΔH^+ versus pH gave a value for the acid Bohr coefficient. Since Hb is rather unstable at acidic pH, particular care was taken to ensure that the percentage of met Hb never exceeded 10%. The percentage met Hb was determined as described by Benesch et al (297). Estimation of the amount of met formation was made several times during the course of the entire titration curve. Usually each titration curve is a composite of two or three samples with overlapping points.

The Bohr effect was determined as described above for unmodified HbA, des-(Arg-141(α)) Hb, $\alpha_2^{Cm}\beta_2$, $\alpha_2\beta_2^{Cm}$, and $\alpha_2^{Cm}\beta_2^{Cm}$. Des-(Arg-141(α)) HbA was prepared by addition of 10 μ l carboxypeptidase B (5 mg/ml, Worthington), after dialysis of the enzyme against 0.2 M ammonium bicarbonate pH 7.4 at 4°C, to about 20 ml of unmodified HbA which was about 60 μ M (57). After incubation for 1 hr at room temperature, a 100 μ l aliquot of the Hb solution was evaporated to dryness in a Savant microconcentrator apparatus. The sample was

dissolved in 100 μ l water and then mixed on a vortex in the presence of 100 μ l of 10% sulfosalicylic acid. After standing for about 5 minutes at room temperature, the sample was centrifuged on a microfuge and the supernatant (which should contain the arginine released by the enzyme) was loaded onto an amino acid analyzer. A control sample incubated in the absence of enzyme, but otherwise under identical conditions was subjected to amino acid analysis after acid hydrolysis. An additional 'enzyme blank' was also analyzed to quantitate any contaminants. The relevant calculations revealed that more than 90% of the carboxy terminal arginine had been released by carboxypeptidase B, and no other amino acids were evident from the amino analysis chromatogram. The remainder of the Hb solution that was not analyzed for release of arginine was passed through a DE-52 (1.5 x 25 cm) column and eluted with a pH gradient of 50 mM tris acetate buffer from 8.3 to 7.3. This procedure separated the des-(Arg-141(α)) Hb from the enzyme. The Bohr effect determinations were then made for samples under the following conditions, no chloride added, 100 mM chloride, 500 mM chloride, and 2 mM 2,3-DPG and 100 mM chloride. It should be noted that even under experimental conditions where no chloride had been added, chloride was still present in the Hb solution and it was derived from two sources. Elemental analysis (278) of a lyophilized sample of Hb, before the start of the experiment revealed that at least 0.2 mM to 0.6 mM chloride remained tightly bound to Hb. This was despite extensive dialysis against water. At the conclusion of a proton titration experiment, elemental analysis (298) confirmed the presence of 5 mM chloride that had leaked out of the Radiometer electrode. Use of the microelectrode reduced this leakage to 1 mM chloride.

i) Competitive Binding Studies

In these experiments it was of interest to determine how effectively glyoxylate and the allosteric modulators, 2,3-DPG, chloride, CO_2 , protons, and various combinations of these would compete with each other for the α -amino termini of Hb. A time course was measured to examine the rate at which $[^{14}\text{C}]$ -glyoxylate was incorporated into unmodified HbA and into the non-radioactive hybrids $\alpha_2^{\text{Cm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$. Conditions were determined to satisfy two important criteria: a) carboxymethylation should be non-saturating with respect to glyoxylate, in order to obtain an initial linear time course and b) carboxymethylation should be predominantly N-terminal, with minimal modification of lysine residues. In order to meet criterion a), 1 mM Hb was carboxymethylated at different concentrations of glyoxylate and a 10-fold molar excess of cyanoborohydride at pH 7.2 in 100 mM Hepes buffer for 1 hour. The resultant graph showing the amount of $[^{14}\text{C}]$ -glyoxylate incorporated (described later) is shown in Figure 9. In order to optimize the linearity of the subsequent kinetic experiments, we chose as low a glyoxylate concentration as possible. Also halving the concentration of the reactants produced identical reaction profiles. Thus, since we wished to conserve the hybrids, the concentration of reactants utilized was, 0.4 mM Hb, 0.1 mM glyoxylate, and 1 mM cyanoborohydride in 100 mM hepes buffer at pH 7.2 incubated for 9 minutes at 22°C. In order to satisfy criterion b), i.e., that carboxymethylation occurred N-terminally, an incubation mixture (500 μl) was set up between either oxy or deoxy unmodified HbA with $[^{12}\text{C}]/[^{14}\text{C}]$ -glyoxylate, and cyanoborohydride in 100 mM hepes buffer at pH 7.2 for 9 minutes at 22°C. After extensive dialysis against water, the mixtures

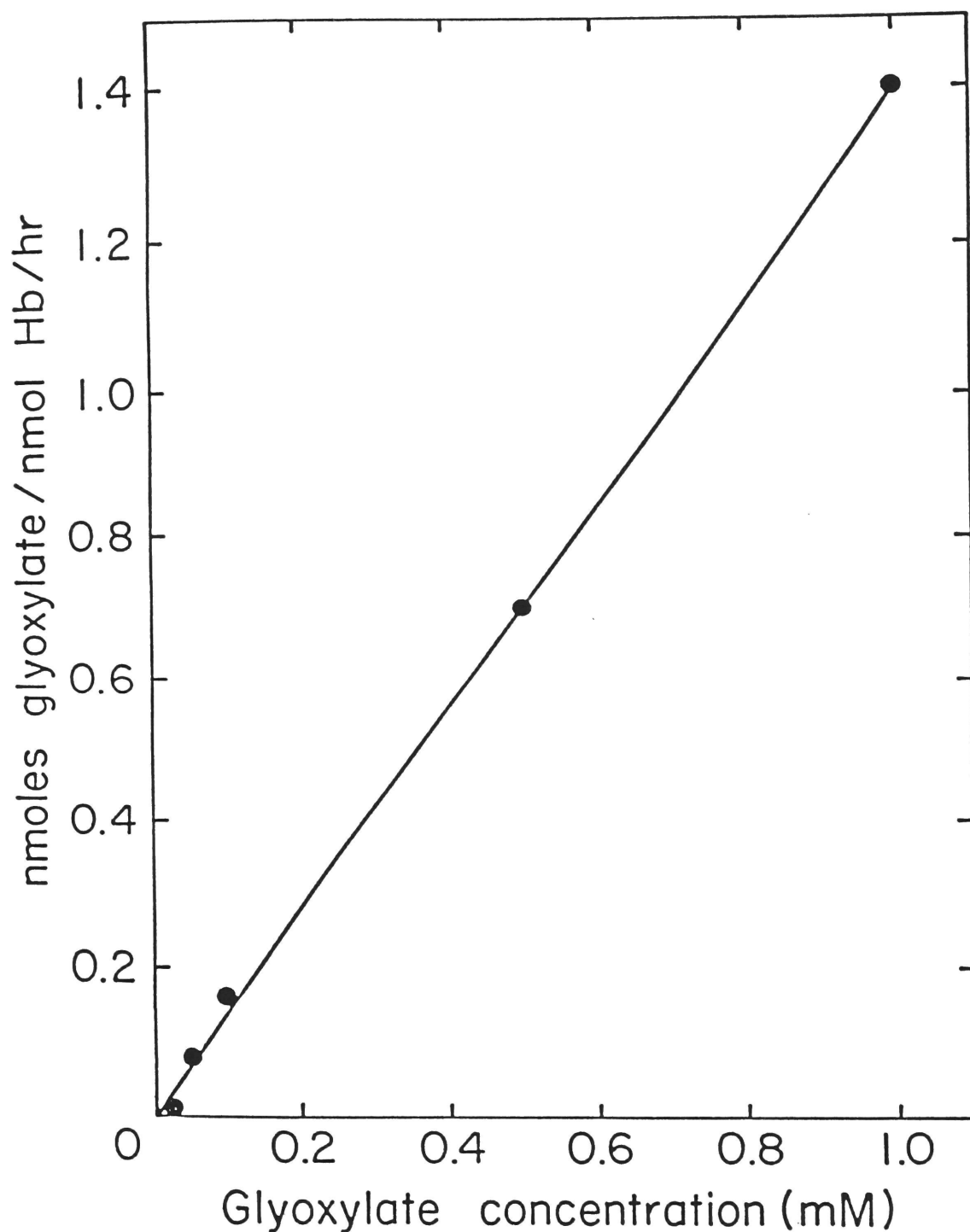


Figure 9 - Incorporation of glyoxylate into a 1 mM solution of oxy unmodified HbA as a function of glyoxylate concentration, at pH 7.2 in 100 mM hepes buffer for 1 hour as described in the text. The extent of carboxymethylation was determined from the radioactivity present on a filter disc containing an aliquot of the precipitated protein, as described in the text.

were subjected to acid hydrolysis in 6 N HCl for 16 hours at 120°C. Each sample was then applied to a Dowex-2 column (0.9 x 30 cm) in order to quantitate monocarboxymethylvaline, dicarboxymethylvaline, and monocarboxymethyllysine, as described earlier in the Methods section. The positions of the radioactive components that eluted from the Dowex-2 column were compared with the positions of the synthesized standards. Table 3 shows that at least 80% of the ^{14}C radioactive label was present in monocarboxymethylvaline. No dicarboxymethylvaline was detected under these conditions. In these studies, moreover, the extent of modification was less than 2% of the total α -amino groups. In a control experiment, a time course was measured for the rate of carboxymethylation into the hybrid $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$. The value obtained for the rate of incorporation of the carboxymethyl group into this hybrid was subtracted from the rates measured for the other hybrids. The reasons for this are described in the Results Section.

For the actual kinetic experiments, the rate of carboxymethylation was measured in an incubation mixture (50 μl) that contained Hb (0.4 mM), [^{14}C]/[^{12}C]-glyoxylate (0.1 mM) and cyanoborohydride (1 mM) in hepes buffer (100 mM) pH 7.2, or bicarbonate buffer for the CO_2 experiments. All mixtures contained on drop of octanol as an anti-forming agent.

The rate at which [^{14}C]-glyoxylate was incorporated into $\alpha_2\beta_2$, $\alpha_2\beta_2^{\text{Cm}}$ and $\alpha_2\beta_2^{\text{Cm}}$ in the absence or presence of 2,3-DPG (5 mM), chloride (0.5 M), CO_2 (5% or 14%), or as a function of pH was determined. For experiments with oxy Hb, all the solutions used were exposed to the air. For experiments with deoxy Hb, preliminary studies were carried out to determine the extent of deoxygenation by taking advantage of the

	<u>% Cm-Val</u>	<u>% Cm-Lys</u>
Oxy unmodified HbA	78%	22%
Specific activity [*]	0.029	0.008
Deoxy unmodified HbA	82%	18%
Specific activity [*]	—	—

* Specific activity expressed as mols [¹⁴C]-glyoxylate incorporated per mol Hb.

Table 3

Oxy or deoxy unmodified HbA (0.4 mM) was incubated in the presence of 0.1 mM [¹²C]/[¹⁴C] glyoxylate and 1 mM cyanoborohydride, pH 7.2 for 9 min. After acid hydrolysis, the sample was loaded onto a Dowex-2 column as described in the text (Methods). The elution positions of the radioactive peaks were compared with those of the synthesised standards (figure 7) and this allowed a calculation of the distribution of the carboxymethyl groups within oxy or deoxy Hb.

difference in extinction coefficient between oxy and deoxy Hb at 760 nm (282) as described in part b) of the Methods section. All the solutions were deoxygenated with nitrogen that had been passed through a humidifier.

For the kinetic studies, deoxygenation under the conditions described above, was carried out in a 5 ml Reacti-Vial (Pierce), fitted with a Teflon seal. To initiate the carboxymethylation time course of a reaction, a solution of [^{14}C]/[^{12}C]-glyoxylate and cyanoborohydride was added through a syringe to the Hb solution, and a time point was taken as early as possible.

Oxy and deoxy experiments in the presence of CO_2 were carried out in reacti-vials which contained bicarbonate buffer, the concentration of which was calculated from the Henderson-Hasselbach equation. In pilot experiments, a microelectrode was placed in the solution to make sure that the pH remained constant throughout the course of the experiment.

After the initiation of each experiment, 5 μl aliquots of the incubation solution were removed over a period of 9 minutes and added to 2 ml 5% trichloroacetic acid (TCA) solution. The samples were mixed on a vortex and stored overnight at 4°C . They were then filtered on Millipore filter discs (Type HAWPO2500, 0.45 μM pore size) washed 5 times with 5 ml of 5% TCA solution and then with one rinse of ether (5 ml). The dried filter discs were counted in vials containing β -Fluor (NEN) in an LKB scintillation counter. The data were plotted as dpm incorporated over a period of 9 minutes. The slopes gave the rate of incorporation and were used to calculate the second order rate constants under each set of conditions.

j) Reaction of Hb with N-Ethylmaleimide as a Probe of Conformation

The reaction between oxy HbA, deoxy HbA, metHbA or the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ with N-ethylmaleimide (NEM) under a variety of experimental conditions was carried out based on the method of Riggs (162). Met HbA was prepared by oxidation of oxy HbA in the presence of a 3-fold molar excess over heme concentration of potassium ferricyanide (57). The conversion to met Hb is observed as a dramatic change in color from bright red to a dark brown color and occurs within minutes after the addition of the potassium ferricyanide. The ferro- and ferricyanide were removed by gel filtration through a G-25 column equilibrated with 0.1 M NaCl.

The study of Riggs (162) applied the spectrophotometric assay for determining the reactivity of NEM toward sulfhydryl groups (298, 299) to Hb. The early reports (298, 299) described how, at pH 6, the loss in absorbance of NEM at 300 nm could be used to quantitate the number of sulfhydryl groups when the NEM was present in an excess. When applied to Hb, Riggs found that NEM reacted rapidly with one available sulfhydryl group per β -chain and that this reaction occurred 8 times more rapidly in the oxy form compared with the deoxy form (162). Under slightly different conditions, the Benesch's (300) found that deoxy Hb was more reactive by a factor of about 3 toward NEM. In the experimental conditions used in the present studies the concentration of NEM and Hb were both chosen to take into account the low millimolar extinction coefficient of NEM (0.62) (162) as well as the desire to conserve the amount of Hb hybrid to be used in the experiments. In all cases the experiments employed Hb (about 80 μ M) in potassium phosphate buffer (100 mM) at pH 6.8. A pH of between 6 and 7 is necessary since

NEM is of low reactivity in acid solution and unstable in alkali (301). Also, conditions were used that were similar to those where IHP elicits spectral changes towards net HbA (see Discussion) for comparative purposes. NEM, dissolved in potassium phosphate buffer (100 mM), pH 6.8, was added to the Hb solution such that the final NEM concentration was 1.5 mM. The final volume of all the reactants was 1.5 ml. The assay was carried out using $\alpha_2\beta_2$, met HbA, $\alpha_2^{Cm}\beta_2$, or $\alpha_2\beta_2^{Cm}$ under a variety of experimental conditions. The profiles of oxy and deoxy HbA were determined. The profiles of met HbA were determined in the absence or presence of a 2-fold molar excess of IHP. The hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ were assayed in the oxy form and also in the absence or presence of a 2-fold molar excess of IHP. Other conditions are stated in Table 15, presented in the Results section. 90 μ l aliquots of the reaction mixture were withdrawn over a period of 90 minutes and precipitated into 20 μ l 50% TCA. The samples were allowed to stand for a few hours at 4°C. They were then centrifuged in a microfuge and the absorbance of each supernatant was measured at a wavelength of 300 nm on a Zeiss spectrophotometer. The reaction profiles were plotted as the absorbance at 300 nm on the ordinate and time along the abscissa.

RESULTS

a) Separation of Carboxymethylated Derivatives of Hb

Carboxymethylated HbA₀ in the carbonmonoxy form, prepared with [¹⁴C]-glyoxylate, was fractionated on DE-52 as described in the Methods section. A typical profile is shown in Figure 5. Four discrete peaks were eluted in 85% yield. Derivative Hb₀, which comprised 9% of the total amount applied, did not contain any ¹⁴C label and represents unreacted Hb. The second peak, Hb₁, which was isolated in 26% yield contained about 2 moles of ¹⁴C label per Hb tetramer. Derivative Hb₂ possessed about 3.5 moles of ¹⁴C label per Hb tetramer and represented about 28% of the Hb applied to the column. The fourth derivative, Hb₃, contained 5-6 moles of [¹⁴C]-glyoxylate per Hb tetramer in 22% yield. The average degree of incorporation for the whole reaction mixture before application the DE-52 cellulose column was 3 moles of [¹⁴C]-glyoxylate per mole of Hb tetramer. This is close to the average degree of incorporation for the fractionated derivatives. Carboxymethylation of deoxy Hb generated essentially the same pattern from DE chromatography but there was a reduced amount of Hb₀ ($\leq 1\%$) and an enrichment of fractions Hb₁, Hb₂, and Hb₃. The greatest enrichment was for Hb₂ (Table 4).

In the procedure used to make the carboxymethylated hybrids, the peaks Hb₁ and Hb₂ from the carbonmonoxy preparation were routinely used. In these fractions the incorporation of 2 or 4 moles [¹⁴C]-glyoxylate per mole Hb, respectively, is perhaps an indication that carboxymethylation here is predominantly N-terminal with only a small amount of modification at the ε-amino groups of the lysine residues.

DE-cellulose chromatography

	Hb ₀	Hb ₁	Hb ₂	Hb ₃
Oxy Hb	9 %	26 %	28 %	22 %
Deoxy Hb	2.5 %	33 %	40 %	26 %

Table 4

Comparison of the DE-cellulose chromatography profiles of unmodified HbA reacted with glyoxylate and cyanoborohydride (as described in the Methods section) under either oxy or deoxy conditions.

Hb₀, where minimal incorporation was observed, and Hb₃, where the incorporation of 5 to 6 moles glyoxylate per mole Hb most likely includes carboxymethyllysine, were not used to prepare the hybrids.

b) Preparation of the Chains from the Carboxymethylated Derivatives

Separation of the Hb chains was carried out for Hb₁ and Hb₂ for the reason stated above. The chains were separated on a CM cellulose column after derivatization of the -SH groups with p-HMB as described in the Methods section. The method used (285) was a variation of the conditions described by Bucci and Fronticelli (286). Figure 6 shows the separation of the p-HMB chains of the Hb₂ fraction. From the separation of the chains from both Hb₁ (not shown) and Hb₂ there were three peaks of radioactivity. The radioactive label is confined to three peaks which contain carboxymethylated β -chains and α -chains, respectively and a third eluting at the position of unsplit tetramer. HPLC analysis of the tryptic peptides of the first and third radioactive peak confirmed the N-terminal carboxymethylation. This will be described later in more detail. Table 5 shows the percentage of reacted chains for Hb₁ and Hb₂ respectively. The peak that eluted with the void volume of the column contains about 70% and 100% of the total β -chains respectively from Hb₁ and Hb₂. The specific activity of the carboxymethylated β -chains from Hb₁ was 0.8 mols [¹⁴C]-glyoxylate per mol Hb monomer. Thus, in this peak there probably is a mixture of β - and β^{Cm} -chains, although carboxymethylation of lysine residues cannot be ruled out. In Hb₂ the specific activity of 1.4 mols [¹⁴C]-glyoxylate per mole Hb monomer in the peak that represented most of the β -chains indicates incorporation of the carboxymethyl moiety into some lysine residues. The differences

<u>DE-component</u>	<u>% of total ¹ β-chains modified</u>	<u>specific activity₂ of peak 1</u>	<u>% of total α-chains modified peak 3</u>	<u>specific activity₂ of peak 3</u>
Hb ₁	74	0.8	20	0.9
Hb ₂	100	1.4	70	1.08

¹ Peaks 1 and 2 from Figure 6 contain all the β-chains. The percentage values given refer to peak 1, the void volume peak.

² Specific activity expressed as nmols [¹⁴C]-glyoxylate incorporated per nmol Hb.

Table 5

The percentage modification of each chain out of the total amount of that chain is calculated from the CM-52 profiles, that allowed the separation of Hb₁ and Hb₂ into their constituent chains. The methodology was described in part d) of the Methods section.

in the quantities of the third major peak, which represent mostly α^{Cm} , differed greatly between Hb_1 and Hb_2 . Table 5 shows that 20–30% of the total α -chains were carboxymethylated in Hb_1 as opposed to 55–70% of the total α -chains in Hb_2 . From the values of their specific activity, only a small amount of chains appear to be totally unmodified in Hb_1 . In Hb_1 , a slight increase in the value for the specific activity of α^{Cm} indicates either total N-terminal modification or an increased incorporation of the carboxymethyl moiety into lysine residues. The results that show the location of the carboxymethyl moiety are described later.

c) Preparation and Purification of Specifically Carboxymethylated Hemoglobin Tetramers

Hybrid tetramers of Hb, specifically carboxymethylated at either the α - or β -chains, were readily constituted as described in the Methods section. The β -chains derived from either Hb_1 or Hb_2 (Figure 6) were hybridized with unmodified α -chains prepared from native unreacted Hb to yield the hybrids $\alpha_2\beta_2^{\text{Cm}}$. Similarly, the carboxymethylated α -chains were combined with unmodified β -chains to produce $\alpha_2^{\text{Cm}}\beta_2$. In order to ensure that these derivatives were not contaminated with either unmodified valine, dicarboxymethylated valine, or carboxymethylated lysine residues, each hybrid was rechromatographed. The system used was CM-52 cellulose and the conditions used were the same as for the chain separations (285). Figure 10 shows the rechromatography of $\alpha_2\beta_2$, $\alpha_2^{\text{Cm}}\beta_2$, $\alpha_2\beta_2^{\text{Cm}}$, and $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$, respectively. The specific activity of the major component of each chromatogram was 1.8 moles [^{14}C]-glyoxylate

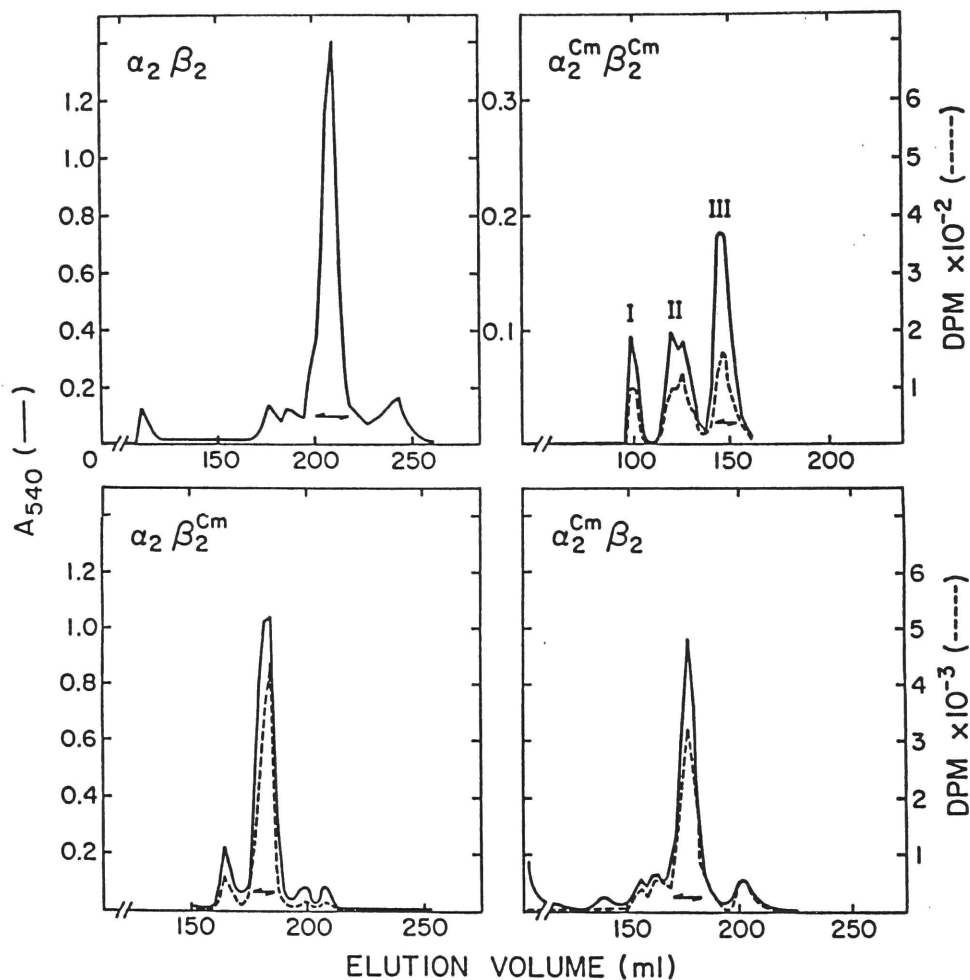


Figure 10 - Rechromatography of the hybrids $\alpha_2\beta_2$, $\alpha_2^{Cm}\beta_2^{Cm}$, $\alpha_2\beta_2^{Cm}$, and $\alpha_2^{Cm}\beta_2$. The system used for the elution of these hybrids was the same as that used to separate the p-IIMB chains from IIb₁ and IIb₂ (Figure 6) as described in the text.

per mol Hb tetramer for $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ and between 3 and 4 moles [^{14}C]-glyoxylate per mol tetramer for the $\alpha_2^{Cm}\beta_2^{Cm}$ hybrid.

d) Further Purification of Carboxymethylated Hybrid Tetramers

The hybrid tetramers shown in Figure 10 from rechromatography on CM-52 cellulose were analyzed for carboxymethyllysine, after treatment with performic acid, as described in the Methods section (292). Table 6 shows that both $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ were essentially free of carboxymethyllysine. Analysis of peaks I, II, and III from the rechromatography of $\alpha_2^{Cm}\beta_2^{Cm}$ (Table 7, and Figure 10 upper right hand panel) showed the presence of 12%, 5%, and between 1% and 3% carboxymethyllysine for each peak respectively. Peak III was considered essentially free of carboxymethyllysine and, therefore, used for the functional and kinetic studies. The rechromatography of the carboxymethylated hybrids, where the carboxymethylated chains are derived from Hb₁ and Hb₂, contain minor amounts of ϵ -carboxymethyllysine, which can be efficiently removed by rechromatography on CM-52 cellulose.

e) Identification of Carboxymethylated Residues Within α - and β -chains by High Pressure Liquid Chromatography (HPLC)

In order to establish the sites and extent of carboxymethylation of the α - and β -chains, respectively, an HPLC system (described in the Methods) was used (290). The α^{Cm} -chain was prepared from the $\alpha_2^{Cm}\beta_2$ hybrid, shown in Figure 10 eluted from CM-52 resin, as described above. The modified chain was subjected to tryptic digestion after

<u>Hybrid</u>	<u>Mono-Cm-Val</u>	<u>Di-Cm-Val</u>	<u>Mono-Cm-Lys</u>
$\alpha_2 \text{Cm} \beta_2$	100%	0%	0%
$\alpha_2 \beta_2 \text{Cm}$	95	0	5
$\alpha_2 \text{Cm} \beta_2 \text{Cm}$	97	0	3

Table 6

Each radiolabeled hybrid was subjected to acid hydrolysis in 6N HCl for 16 hrs. The void volume from the amino acid analyser was eluted on the Dowex-2 chromatography system as described in the Methods, in order to quantitate the amount of monocarboxymethylvaline, dicarboxymethylvaline and monocarboxymethyllysine.

Identification of Carboxymethyllysine from Peaks I, II, and III

of Rechromatography of α_2 -Cm β_2 -Cm (Figure 10)

<u>Peak</u>	<u>Percentage</u>	
	A*,	B*
I	6.0,	9.5
II	3.1,	5.0
III	1.1,	3.3

* A and B represent determinations from two separate preparations.

Table 7

The percentage ϵ -monocarboxymethyllysine was calculated for peaks I, II and III of the chromatogram in the lower left hand panel of Figure 10. 3 ml of each peak was evaporated to dryness and then subjected to performic acid oxidation as described in the Methods Section. After acid hydrolysis, the sample was loaded onto an amino acid analyser and the percentage ϵ -monocarboxymethyllysine was calculated.

aminoethylation (288-290). The mixture was then applied to an HPLC column as described in the Methods section. The peptide map (Figure 11) obtained from the α -chain of $\alpha_2^{Cm}\beta_2$ was similar to that of the α -chain from unmodified Hb except for the appearance of radioactivity, which was confined to two peaks. The amino acid composition of the peak eluting at 26 minutes indicated that it was peptide α T-1 (Table 8), except for the absence of 0.8 residues of valine. In order to purify the second radioactive peak, it was rechromatographed with a shallower gradient and a single labeled peptide eluted (Figure 11, inset). Amino acid analysis of this peptide after acid hydrolysis indicated that its identity was α T(1+2) (Table 8). This peptide has arisen from incomplete tryptic cleavage at Lys-7 of the α -chain. This linkage is known to be relatively resistant to trypsin even in the unmodified α -chain (302). Amino acid analysis of the α T-1 peptide established that Val-1(α) was the site of carboxymethylation under the experimental conditions employed. However, from this HPLC system and subsequent amino acid analysis, it was not possible to detect whether carboxymethylation was limited to only the mono-substituted derivative. This is addressed later in this section.

The peptide map obtained from the β -chain of $\alpha_2\beta_2^{Cm}$ was similar to that of the β -chain of unmodified Hb (Figure 11) and a single peak eluting at the position of β T-1 was found to contain all of the radioactivity. Amino acid analysis of this peptide (Table 8) confirmed its identity as β T-1 except for the absence of 0.7 residue of valine, most likely due to carboxymethylation at this site. Thus, Val-1(β) was the site of carboxymethylation, since there are no other valine residues

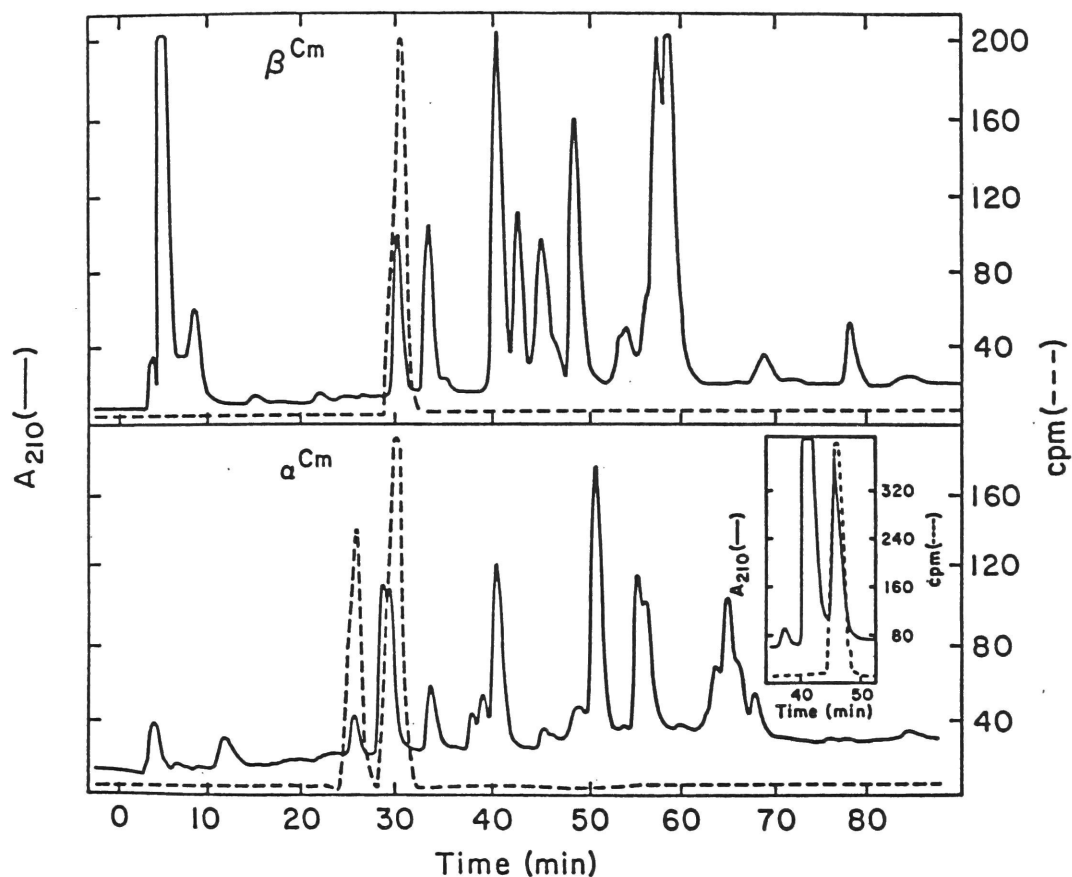


Figure 11 - Peptide map of the tryptic digest of the [^{14}C]-carboxymethylated β - and α -chains from HbA. The digest of each chain was analyzed on an HPLC unit assembled from commercial components (290). The initial gradient used for each digest was 5 to 70% acetonitrile containing 0.1% trifluoroacetic acid. For rechromatography of the peak eluting at 30 minutes (of the digest of the α -chain), the fraction comprising this peak were pooled, concentrated, and applied to the HPLC unit with a gradient of 5 to 50% acetonitrile containing 0.1% trifluoroacetic acid.

Amino Acid Composition of the Labeled Peptides from
the α - and β -Chains of ^{14}C -Carboxymethylated Hemoglobin

<u>Amino Acid</u>	<u>Peptide βT-1</u>		<u>Peptide αT-1</u>		<u>Peptide αT-1+2</u>	
	<u>Found</u>	<u>Theory</u>	<u>Found</u>	<u>Theory</u>	<u>Found</u>	<u>Theory</u>
Asp	0.2	0	1.2	1	2.2	2
Thr	1.2	1	0.1	0	1.1	1
Ser	0.3	0	1.0	1	1.1	1
Glu	2.2	2	0.2	0	0	0
Pro	1.3	1	0.5	1	0.5	1
Gly	0.3	0	0	0	0.1	0
Ala	0.2	0	1.2	1	1.2	1
<u>Val</u>	<u>0.3</u>	<u>1</u>	<u>0.2</u>	<u>1</u>	<u>1.3</u>	<u>2</u>
Met	0	0	0	0	0	0
Leu	1.0	1	1.0	1	1.1	1
Tyr	0	0	0	0	0	0
Phe	0	0	0	0	0	0
His	0.6	1	0	0	0	0
Lys	1.0	1	1.0	1	2.0	2
Arg	0	0	0	0	0	0

Table 8

The tryptic peptides from the α - and β -chains were isolated as described in the text. After acid hydrolysis of the ^{14}C -labeled peptides, the amino acid composition was determined. The values are given relative to lysine.

present in the β T-1 peptide. Whether the carboxymethylation is mono or di, cannot be ascertained from this procedure, but is discussed later.

Although the labeled tryptic peptides obtained from both the α^{Cm} - and β^{Cm} -chains contain an amino group at both the COOH terminus of the peptide in addition to the amino terminus, it is unlikely that the ϵ -NH₂ group of the -COOH terminal lysine is carboxymethylated since it would most likely not have been recognized by the trypsin. Moreover, the rest of the tryptic map would not have resembled the map of unmodified α - or β -chain respectively if extensive modification of other residues had occurred. Since the peptide map of the modified and unmodified chains are identical, except for the labeled peptide, extensive carboxymethylation of lysine and other residues is excluded in the fractions.

The Hb₃ component was also partially characterized on HPLC. This derivative whose chromatographic behavior on anion exchange resin, DE-52, suggests the incorporation of more carboxymethyl groups than in Hb₁ or Hb₂, was separated into its α - and β -chains as described above. The α -chain of Hb₃ was found to contain 1.4 [¹⁴C]-glyoxylate residues per chain. Analysis of the tryptic peptides of each by HPLC showed that peptides α T-1 and β T-1 had 0.91 and 0.95 [¹⁴C]-glyoxylate residues per chain, respectively. Hb₃ completely carboxymethylated at its NH₂-terminal residues, would yield 4 [¹⁴C]-glyoxylate residues per tetramer. The average degree of incorporation in the sample Hb₃ from Figure 5 was 5-6 [¹⁴C]-glyoxylate residues per tetramer. The remaining 1-2 [¹⁴C]-carboxymethyl groups were found to be distributed among 8-9 peptides on the HPLC chromatogram with about twice as much modification in the β -chains compared with the α -chains. These sites most likely

represent lysine residues on Hb and were not further investigated since Hb₃ is probably a mixture of carboxymethylated tetramers.

f) Mono- or Di-Carboxymethylation at the NH₂-Terminal Valine Residues

A system was developed to measure whether the N-terminal carboxymethylation was limited to the state of monocarboxymethylation or whether the resultant secondary amine could be carboxymethylated further to produce the di-derivative. A chromatographic system was developed as described in the Methods section, whereby it was possible to separate mono and dicarboxymethylvaline and carboxymethyllysine: this procedure is a modification of the desalting methodology described by Dreze et al (293) to remove sodium citrate after amino acid analysis of an acid hydrolysate. It was necessary to have such a procedure since the valine derivatives are ninhydrin-negative. Therefore, [¹⁴C]-glyoxylate was used in the preparation for the purposes of detection. The elution patterns of the labeled derivatives are shown in Figure 7 of the Methods section. The identities of the compounds, as confirmed by ¹H-NMR and elemental analysis (Figure 8a, 8b, and Table 2) are consistent with peaks I and II as mono and dicarboxymethylvaline, respectively. The derivatives are eluted in the order; monocarboxymethyllysine, monocarboxymethylvaline, and dicarboxymethylvaline, under progressively increasing acidic conditions. In principle, elution of each modified amino acid should take place when its carboxyl group is fully protonated and there is no net negative charge through which it could bind to the resin. Thus, the increased retention of the carboxymethylated derivatives indicates that each had an additional group whose pK_a becomes successively lower. That carboxymethyllysine can be eluted in

acetic acid (pH ~ 2.5) indicates that the pK_a of the ϵ -carboxymethyl moiety has a value close to this pH. The progression to conditions of increased acidity necessary to elute the mono and dicarboxymethylvaline indicates that the pK_a of these carboxymethyl moieties is even lower than 2.5. These pK_a values could be different within the actual protein.

In order to demonstrate that carboxymethylation was predominately N-terminal and that only the mono derivative was formed, an oxy or deoxy sample of unmodified HbA was incubated with [^{14}C]-glyoxylate and cyanoborohydride as described in the Methods section. The conditions chosen were those that would later be used for the competitive binding studies. These conditions, where the amount of glyoxylate used was one-tenth the amount for preparation of the hybrids, resulted in a low amount of glyoxylate incorporated. Whether calculated from the data of Tables 3 and 9 or from the data of Figure 20, less than 2% of the total amino groups were modified (Table 9 is discussed in more detail later). However, there is no reason to expect that the pattern and sites of modification should differ compared to the hybrids $\alpha_2^{Cm}\alpha_2$, $\alpha_2\beta_2^{Cm}$, and $\alpha_2^{Cm}\beta_2^{Cm}$ where carboxymethylation was 50-100%. After acid hydrolysis, the Hb sample was applied directly to a Dowex-2 column and the components therein eluted according to the system described previously in the Methods. When a similar sample was loaded onto the amino acid analyzer, collected and loaded onto the Dowex-2 system, the peaks of radioactivity were in the same place and of the same size as those obtained from an acid hydrolysate which was not first separated on an amino acid analyzer. The specific activity of the radioactive peaks, which coeluted with any of the three synthesized compounds mentioned

	<u>% Cm-Val</u>	<u>% Di-Cm-Val</u>	<u>% Cm-Lys</u>
	43 %	14 %	43 %
Specific activity (nmols/nmol Hb)	0.02	0.0032	0.02

Table 9

Deoxy $\alpha_2^{Cm}\beta_2^{Cm}$ was incubated with 0.1 mM [^{12}C]/[^{14}C]-glyoxylate and 1 mM cyanoborohydride for 9 minutes at pH 7.2 as described in the text. After acid hydrolysis the sample was applied to the Dowex-2 chromatography system in order to quantitate the modifications at valine and lysine. The total incorporation was considered as a 'blank' value and was subtracted from the rates of the kinetic experiments carried out with the samples $\alpha_2\beta_2$, $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$.

above was calculated (Table 3). It was found that the total amount of carboxymethylation was limited to the mono-derivative of either valine or lysine and that there was at least 80% monocarboxymethylvaline in either the oxy or deoxy state (Table 3). There was no detectable amount of dicarboxymethylvaline in either sample, unlike reductive methylation where the aldehyde is formaldehyde and the dimethylated compound is formed preferentially (15, 17). Presumably, there could be steric and, what is more likely, electrostatic factors that preclude dicarboxymethylation when glyoxylate is the aldehyde employed.

g) Non-Reducing Gel of Carboxymethylated Hybrids

The polyacrylamide gel run under non-denaturing and non-reducing conditions, shown in Figure 12, verified the purity of each hybrid. Only one band was detected for each sample after the gel was stained with Coomassie Blue as described in the Methods section. From the gel it can be seen that $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ migrated the same distance toward the positive (anode) pole of the gel and that this distance was further than it was for $\alpha_2\beta_2$. The incorporation of negative charge ($-\text{CH}_2\text{COO}^-$) has increased the mobility of $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$. Moreover, $\alpha_2^{Cm}\beta_2^{Cm}$, the hybrid tetramer with four additional negative charges migrates even faster than $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$.

Thus to summarize briefly, carboxymethylated hybrids of the type $\alpha_2^{Cm}\beta_2$, $\alpha_2\beta_2^{Cm}$, and $\alpha_2^{Cm}\beta_2^{Cm}$, where 'Cm' denotes N-terminal carboxymethylation have been prepared. The following criteria established the relative purity of each derivatized tetramer. First only 1% to 3% ϵ -carboxymethyllysine was detected by amino acid analysis after performic acid treatment, acid hydrolysis, and amino acid analysis

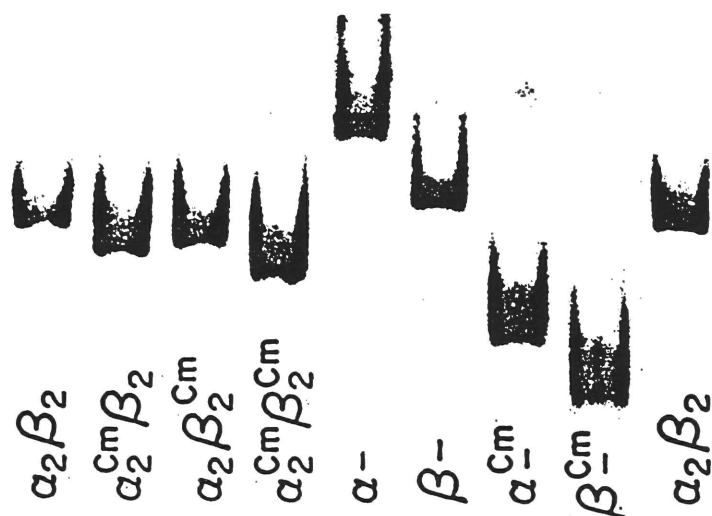


Figure 12 - Polyacrylamide slab gel electrophoresis of native samples to verify the purity of the hybrids $\alpha_2\beta_2$, $\alpha_2^{\text{cm}}\beta_2$, $\alpha_2\beta_2^{\text{cm}}$, and $\alpha_2^{\text{cm}}\beta_2^{\text{cm}}$. The gel 7.5% cross-linked was subjected to electrophoresis at 100 volts for 70 minutes at pH 8.6 as described in the Methods.

of $\alpha_2^{Cm}\beta_2^{Cm}$. Second, HPLC analysis of the tryptic peptides of the α^{Cm} -chain and β^{Cm} -chain from the relevant hybrids showed the presence of the carboxymethyl moiety at Val-1(α) and Val-1(β), respectively. The third criterion by which the purity of each hybrid was checked, was the Dowex-2 chromatography system. Through this technique, monocarboxymethylation at the N-terminal valines was shown to be the predominant reaction product for the hybrids (Table 6). The fourth criterion of purity of the hybrids was the detection of only a single band of each sample after a polyacrylamide gel run under non-denaturing conditions was stained with Coomassie Blue.

X-Ray Diffraction Studies - Figures 13 and 14 show the difference electron density map for the carboxymethylated hybrid $\alpha_2^{Cm}\beta_2^{Cm}$ in the deoxy state. Figure 13 shows the difference density at the DPG binding site in the vicinity of the β -chain termini. The main features in this figure can be interpreted as follows: 1) three alternating negative/positive pairs of difference density peaks show that a movement of Val-1 towards Leu-3 shifts that residue and the beginning of the A-helix resulting in a perturbation of Glu-6(β), 2) normally there is an anion bound between the N-terminal amino group of Val-1(β) and Lys-82(β). The large negative difference peaks at this position (labeled X) along with the very strong positive peak next to it indicates that the newly formed carboxymethyl group of the modified Hb replaces the inorganic anion at approximately the same location. This permanently bound anion is probably much more effective than the non-covalently bound anion in reducing the oxygen affinity of Hb, 3) the only other significant features in the difference map are a pair of negative/positive peaks which indicate that the side chain of His-2(β)

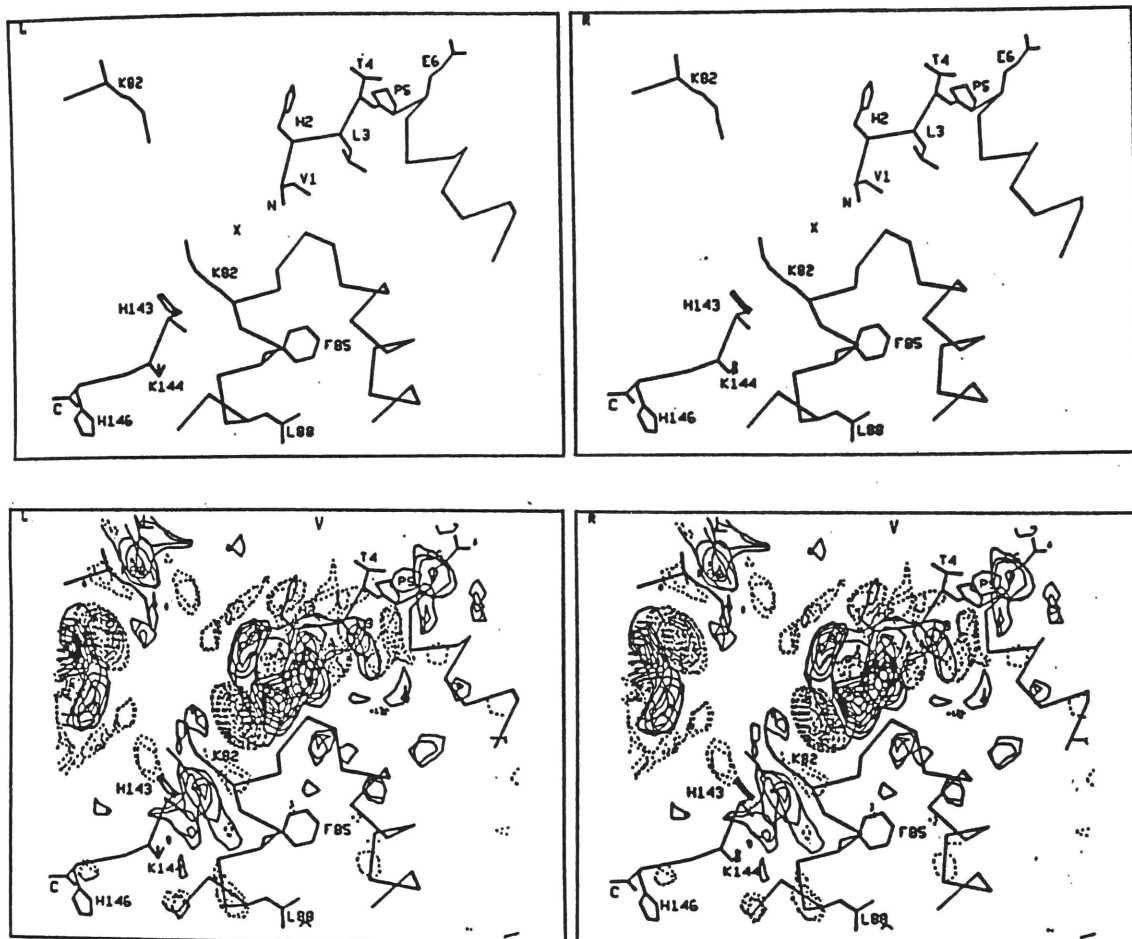


Figure 13 - Stereo plot showing half of the DPG binding site and some adjacent residues. This plot consists of connections between the α -carbon atoms of residues 1-15, 67-90, and 142-146 on the β_1 -subunit as well as residues 81-83 on the β_2 -subunit. The complete side chains for a number of selected residues have also been plotted and labeled. The label 'X' marks the location of a tightly bound anion (probably sulfate) which is clearly seen in the electron density map of native deoxy hemoglobin A. Six two-dimensional sections (spaced 1 \AA apart) of the difference electron density map superimposed on the atomic model shown in A. Solid contours indicate positive difference density, dashed contours indicate negative difference density. The difference density has been contoured at $\pm 1.5 \sigma$, where σ is defined as the root-mean-square density of the entire map.

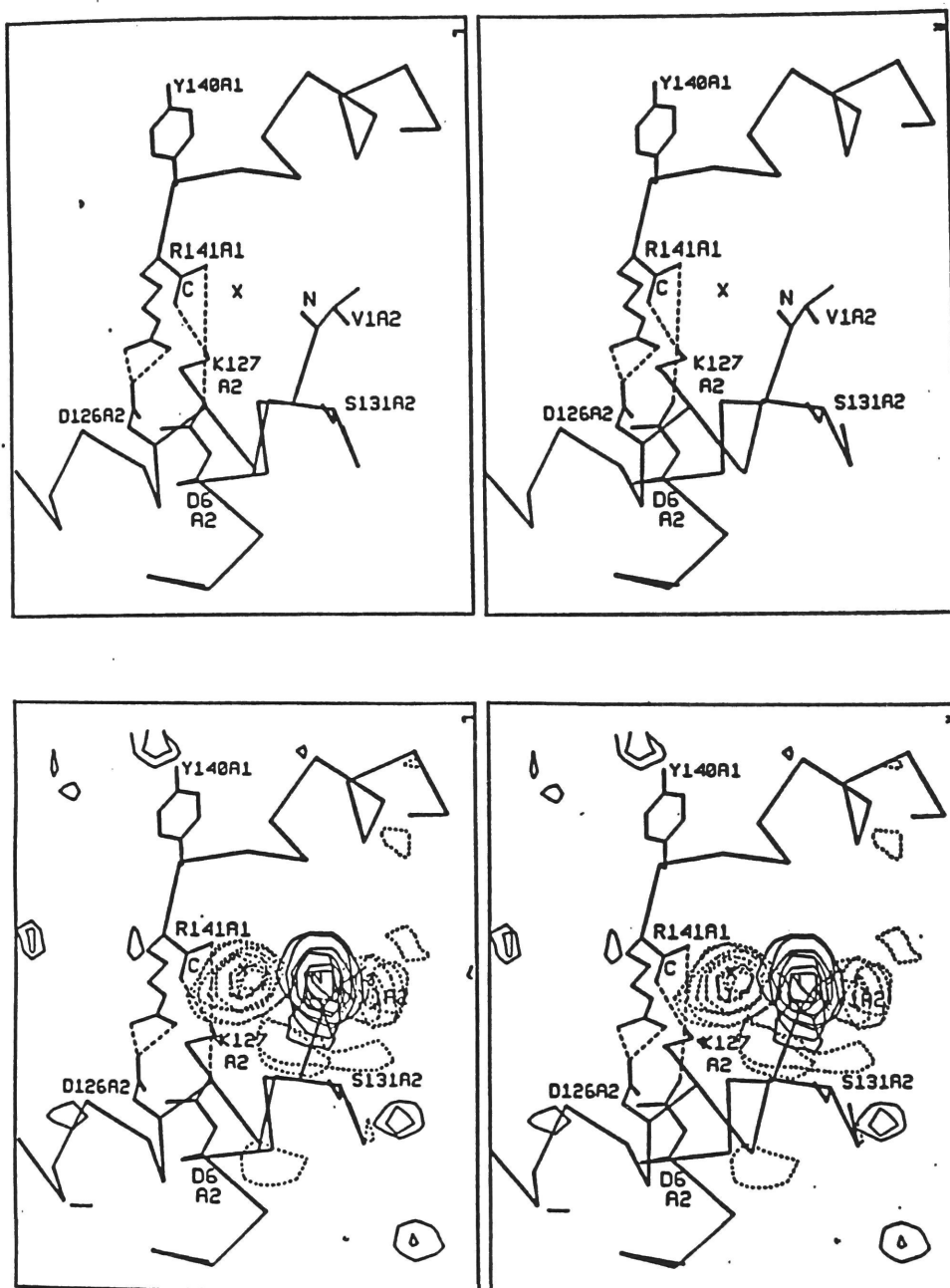


Figure 14 - Stereo plot of the NH_2 -terminal region of the α_2 subunit. This plot was prepared by drawing lines between the α -carbon atoms of residues 1-10 and 120-133 of the α_2 subunit and residues 130-140 of the α_1 subunit. The complete side chains of some selected residues have also been plotted and labeled. The dotted lines indicate the presence of strong ionic interactions between the guanidinium side chain of Arg-141(α_1) and Asp-126(α_2), the COOH -terminal carboxyl group of the α_1 subunit and Lys-127(α_2), and the side chains of Lys-127(α_2) and Asp-6(α_2). The label 'X' marks the location of an inorganic anion (probably sulfate) which is normally bound between NH_2 -terminal amino group of Val-1(α_2) and the guanidinium side chain of Arg-141(α_1). Six two-dimensional sections (spread 1 Å apart) of the difference electron density map superimposed on the atomic model shown in A. Solid and dashed contours refer to positive and negative difference density, respectively, and the contour levels are drawn at $\pm 2 \sigma$.

rotates approximately 180 degrees about its $C_{\alpha}-C_{\beta}$ bond so that it can also interact with the carboxymethylated amino terminus.

The difference density at the N-terminus of an α -subunit is shown in Figure 14. Here the main features are: 1) displacement of the inorganic anion normally bound between the N-terminal amino group and the side chain of Arg-141 on the opposite α -chain, and 2) the lack of any disruption of the salt bridges that involve Arg-141(α). The carboxymethyl group itself is probably interacting with Ser-131(α). Of particular note, when CO_2 reacts to form a carbamate group it interacts at this same site (254). This would tend to lower the oxygen affinity of Hb without reducing cooperativity.

i) Effect of Carboxymethylation on the Functional Properties of Hb

Effect on Oxygen Affinity - The data shown in Figure 15 show that the unmodified and modified hybrid tetramers that had been subjected to the procedures of chromatography, chain separation, reconstitution, and re-chromatography retained the properties of the native molecule with respect to cooperativity (average Hill coefficient for the four hybrids = 2.4). This result imparts confidence that the manipulations and procedures used in the studies do not adversely affect the properties of the protein and that the hybrid tetramer reflects the properties of native Hb.

The hybrid derivative $\alpha_2^{Cm}\beta_2$ has an intrinsic oxygen affinity ($P_{50} = 12$ mm Hg), that is considerably lower than that of the native protein ($P_{50} = 7$ mm Hg) (Figure 15). Initially, this result was explained by the properties of small anionic moieties for the region around Val-1 of the α -chain (281). As discussed in the Introduction,

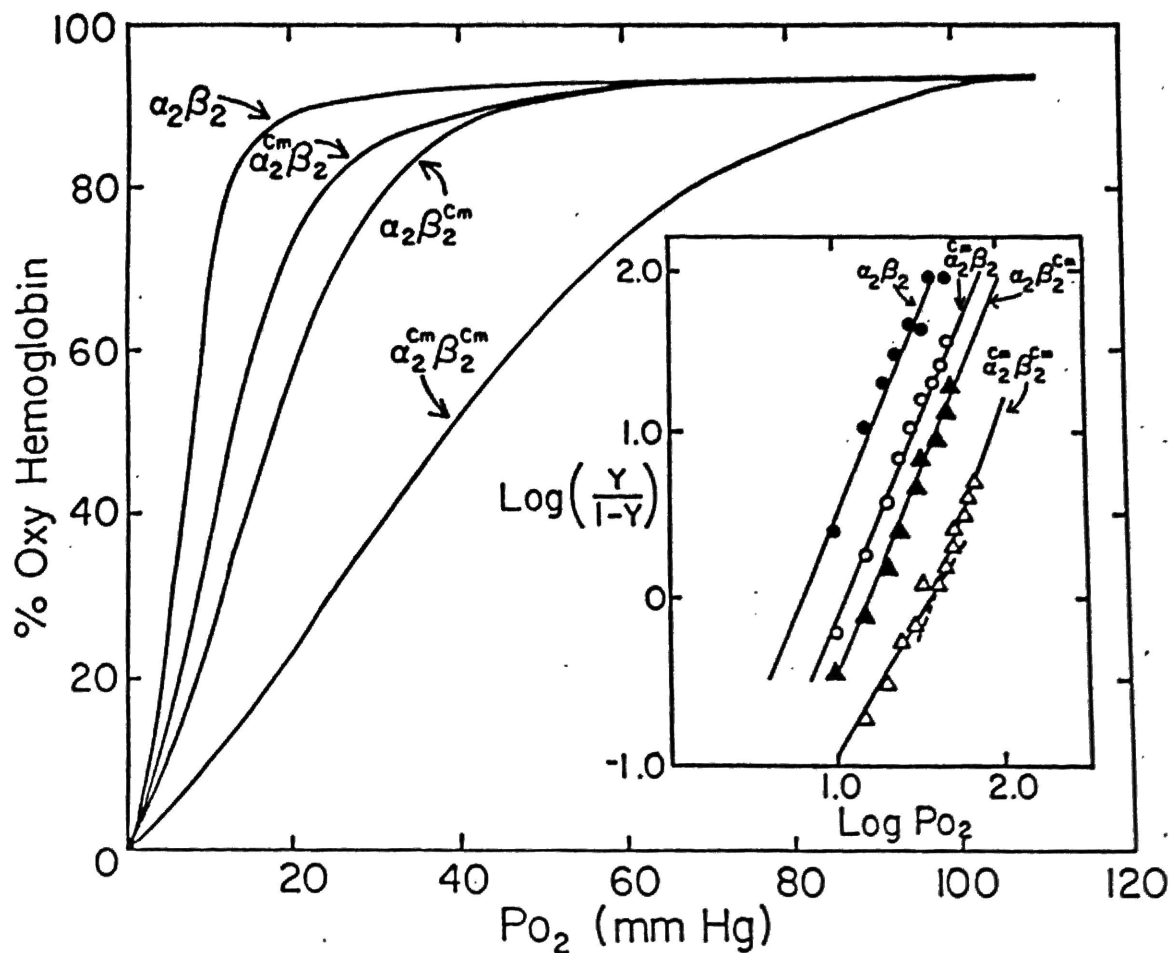


Figure 15 - Oxygen equilibrium curves of unmodified and specifically carboxymethylated hybrids of HbA. The samples were prepared as described in the text, and 4 μ l portions were used for determination of the oxygen equilibrium curve in a Hem-O-Scan instrument.

there is an anion binding site between Val-1(α) and Arg-141(α_2) (43, 44). Possibly the introduction of the negatively charged carboxymethyl moiety at this site creates a high local density of negative charge and mimics the effect of very high concentrations of chloride in lowering the oxygen affinity of Hb. From the electron density patterns, however, the carboxymethyl moiety appears to interact with Ser-131(α) in a manner similar to that observed with carbamate formation at Val-1(α) (254). CO₂ binding is associated with a diminished oxygen affinity and this explanation could be extrapolated to carboxymethylated Hb.

The hybrid derivative $\alpha_2\beta_2^{\text{Cm}}$ has an oxygen affinity ($P_{50} = 17$ mm Hg) that is lower than either that of the unmodified tetramer or of $\alpha_2^{\text{Cm}}\beta_2$ (Figure 15). The result is consistent with the known avidity for anions in the region comprising the cleft between the two β -chains and the consequent lowering of the oxygen affinity of Hb upon binding of such anions.

The intrinsic oxygen affinity of the tetramer that is fully carboxymethylated at its NH₂-terminal residues $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ ($P_{50} = 37$ mm Hg) is much lower than the additive effects of such a modification at the individual chains (Figure 15). The biphasic Hill plot perhaps indicates some change in heme-heme interaction. The presence of the four carboxymethyl moieties seems to stabilize the T structure. How this is achieved is unclear.

Interaction of 2,3-DPG with Specifically Carboxymethylated Hybrids - The 2,3-DPG titrations for all the hybrids are shown in Figure 16. Addition of 2,3-DPG to the hybrid carboxymethylated at the α -chain amino terminus lowers its oxygen affinity about 4-fold (to 48 mm Hg). This is about the same degree to which addition of 2,3-DPG lowers

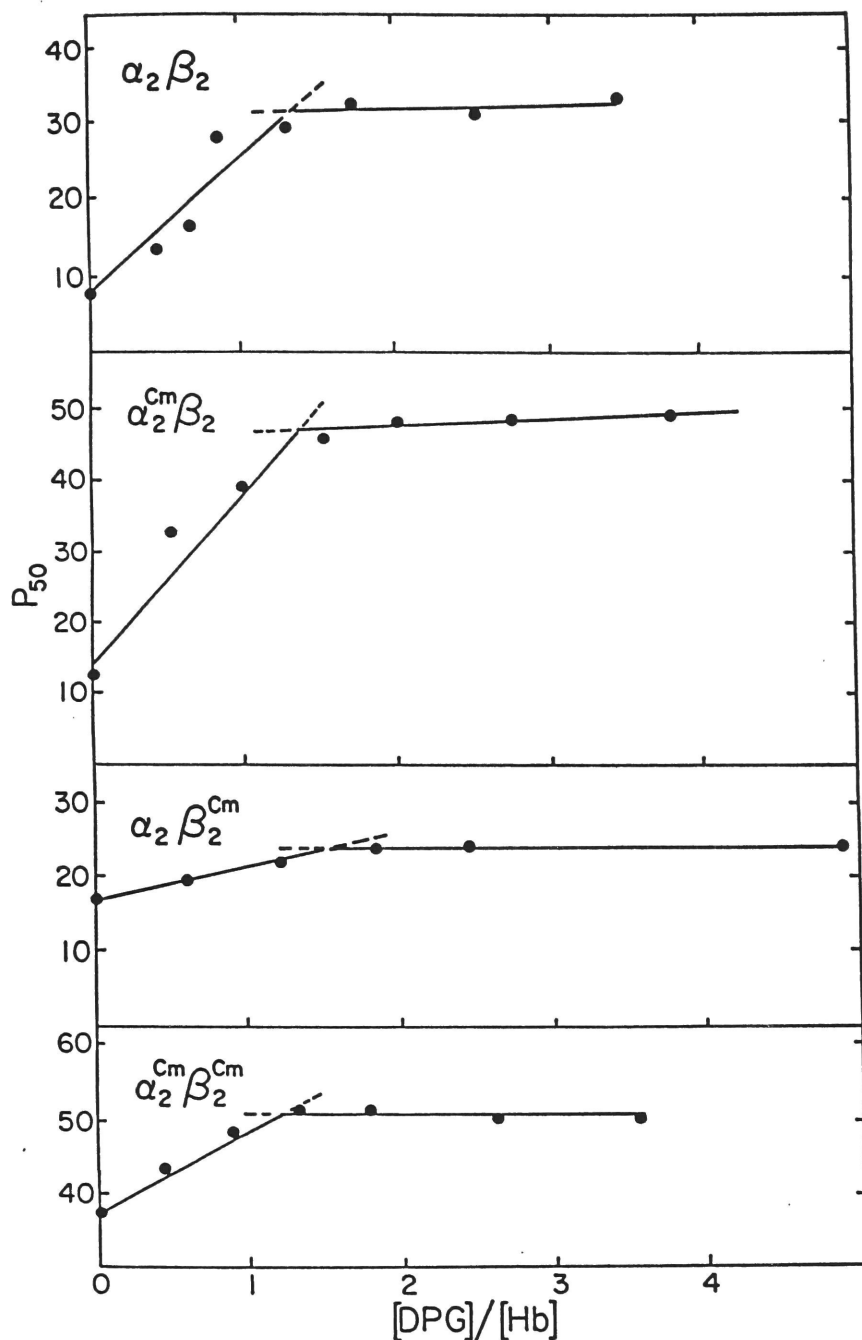


Figure 16 - Effect of 2,3-DPG on the oxygen equilibrium properties of carboxymethylated hybrids of HbA. The samples prepared as described in the text, were mixed with varying amounts of 2,3-DPG to achieve the indicated DPG/Hb ratios. The oxygen equilibrium curves were then determined in the Hem-O-Scan. No chloride was added to these samples. The lines were drawn by linear regression analysis.

the oxygen affinity of unmodified Hb (from 7 mm Hg in the absence to 33 mm Hg in the presence of 2,3-DPG). The equivalence point in the titration of unmodified $\alpha_2\beta_2$ and $\alpha_2^{Cm}\beta_2$ with 2,3-DPG is slightly greater than 1, perhaps because these experiments were done in the absence of 0.1 M NaCl so there may have been some non-specific binding of 2,3-DPG (36). The absolute P_{50} values are, however, greater for this hybrid, $\alpha_2^{Cm}\beta_2$. This indicates that the presence of the carboxymethyl moiety causes an additional decrease in oxygen affinity in the presence of 2,3-DPG compared to the value for the oxygen affinity of unmodified Hb in the presence of 2,3-DPG.

The covalent attachment of the carboxymethyl moiety to the β -chain amino terminus prevents a further lowering of the oxygen affinity by 2,3-DPG to any significant degree. In this respect the covalently bound carboxymethyl group mimics the effect of very high concentrations of chloride (0.5 M) in obviating the effect of 2,3-DPG (36).

The addition of 2,3-DPG to the hybrid $\alpha_2^{Cm}\beta_2^{Cm}$ results in a further lowering of the oxygen affinity ($P_{50} = 50$ mm Hg), although the magnitude of this effect is far less than that observed for unmodified Hb in the presence of 2,3-DPG.

Effect of Chloride on Carboxymethylated Hybrids - Chloride titration data for each hybrid tetramer are shown in Figure 17. The effect of chloride on the hybrid $\alpha_2^{Cm}\beta_2$ shows a slight lowering of oxygen affinity in response to the increasing anion concentrations. Calculations show a 13% decrease in the effect of chloride for $\alpha_2^{Cm}\beta_2$ compared with the unmodified tetramer. In view of the X-ray data, the chloride binding site between Val-1(α_1) and Arg-141(α_2) is still vacant, since the carboxymethyl group seems to be interacting with Ser-131(α_1).

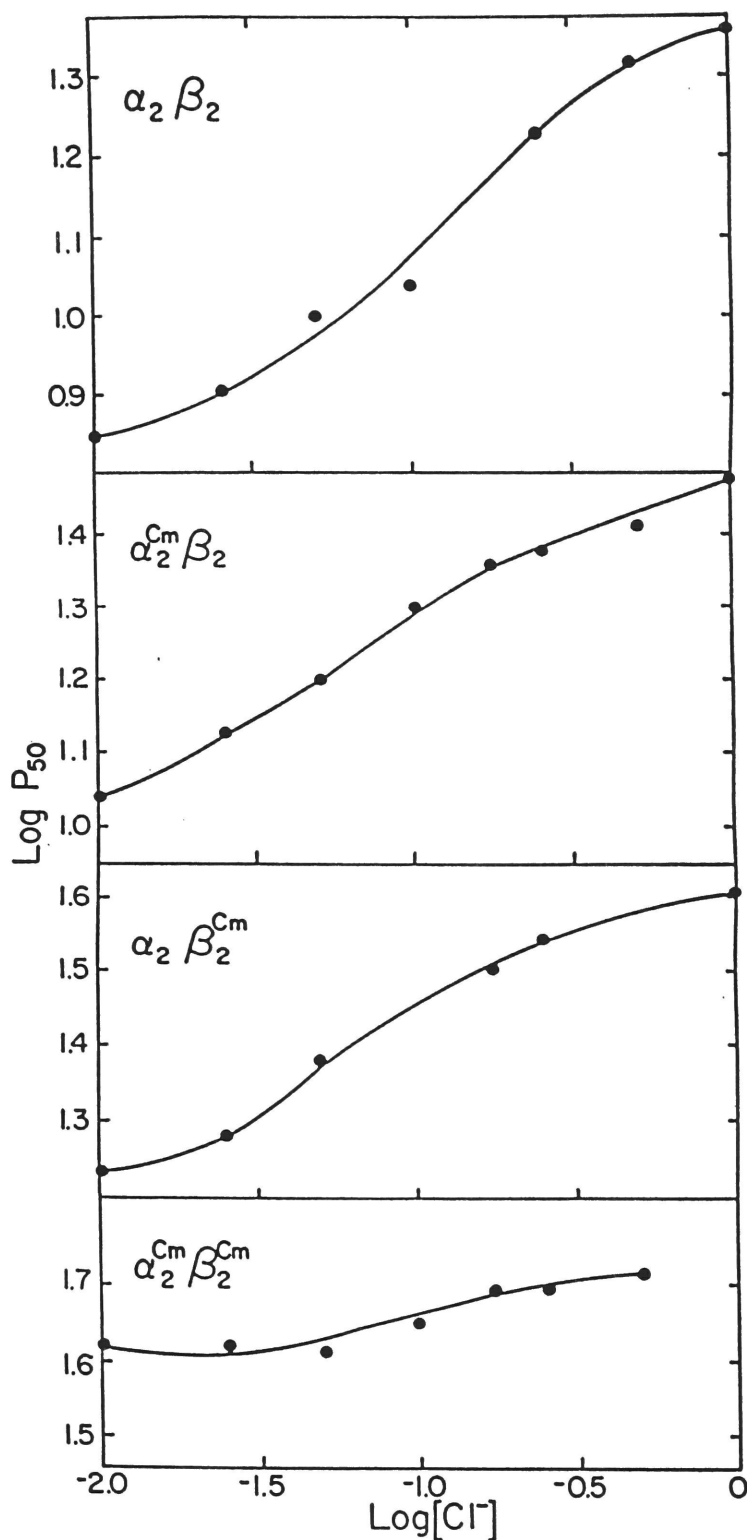


Figure 17 - Effect of chloride on the oxygen equilibrium properties of carboxymethylated hybrids of HbA. The samples, prepared as described in the text, were mixed with varying amounts of chloride and the oxygen equilibrium curves were determined in the Hem-O-Scan. Each P₅₀ value is ± 1 mm Hg.

The X-ray data represent a static state of affairs, however. In solution perhaps there is a dynamic equilibrium whereby the carboxymethyl moiety could interact with the guanidinium side chain of Arg-141(α_2) part of the time. This could be responsible for the observed 13% reduction in the effect of chloride on oxygen affinity due to a decreased ability of chloride to bind at this site. Also, assuming that the carboxymethyl moiety at Val-1(α) has not perturbed the Lys-82(β) site, chloride binding at this site should not be impaired.

The addition of chloride to the $\alpha_2\beta_2^{\text{Cm}}$ hybrid and the resultant titration curve shows about a 28% reduction in the effect of chloride on the oxygen affinity compared to unmodified $\alpha_2\beta_2$. Many reports have provided evidence for the involvement of Lys-82(β) in oxygen-linked inorganic anion binding under stripped conditions (59, 61). The X-ray data for $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ in the region of the β -chains show an electrostatic interaction between the covalently attached $-\text{CH}_2\text{COO}^-$ moiety with Lys-82(β). Lys-82(β) may be unable to bind chloride and this may contribute to the decreased reduction observed for the change of oxygen affinity as the concentration of chloride is increased. If Val-1(α) is devoid of any structural perturbations in $\alpha_2\beta_2^{\text{Cm}}$, it should still be able to bind chloride. The 28% reduction observed here is less than the 60% or 40% contribution of Lys-82(β) to chloride binding assigned by Manning et al (72) and Adachi et al (74).

The reduction in chloride binding to the hybrid $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ is about 80% and this is about 2-fold higher than the sum of the reductions observed for $\alpha_2^{\text{Cm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$. This could be related to decreased heme-heme interaction at low oxygen tensions.

Alkaline Bohr Effect - The alkaline Bohr effect was measured for the unmodified or the specifically carboxymethylated Hb tetramers as well as for a Hb tetramer in which Arg-141 was cleaved from the carboxyl-terminus of each α -chain, des-(Arg-141(α)) Hb. The Bohr coefficient was determined both by the proton release technique and by measurement of the change in oxygen affinity with respect to pH. Figure 18 shows the actual titration data measured for the four derivatives described above in 0.1 M chloride which for these studies was considered as the maximum Bohr effect as oppose to the additional alkaline Bohr effect (in the presence of 2,3-DPG to be described later). Figure 19 shows a plot of $\log P_{50}$ versus $\log \text{pH}$ under the same conditions. The value for P_{50} was determined from the oxygen equilibrium curve of a sample as measured on a Hem-O-Scan instrument.

In order to measure the Bohr coefficient by proton titration in the presence of as low a chloride concentration as possible, it was necessary to use a microelectrode. The Radiometer electrode, that was used initially, was found to leak about 5 mM total chloride during the course of the experiment, as determined by elemental analysis of the sample at the end of the experiment (278). In addition, even after extensive dialysis, at least 0.2 mM to 0.6 mM chloride seems to remain tightly associated with the Hb. Even the microelectrode leaked chloride, about 1 mM, as determined by elemental analysis (278) at the end of the titration.

The Hem-O-Scan apparatus, on the other hand, measures the oxygen affinity of a particular Hb sample. The oxygen affinity or P_{50} is measured from the oxygen equilibrium curve of Hb. This curve is plotted by exposing the sample to varying oxygen partial pressures, $p\text{O}_2$, and

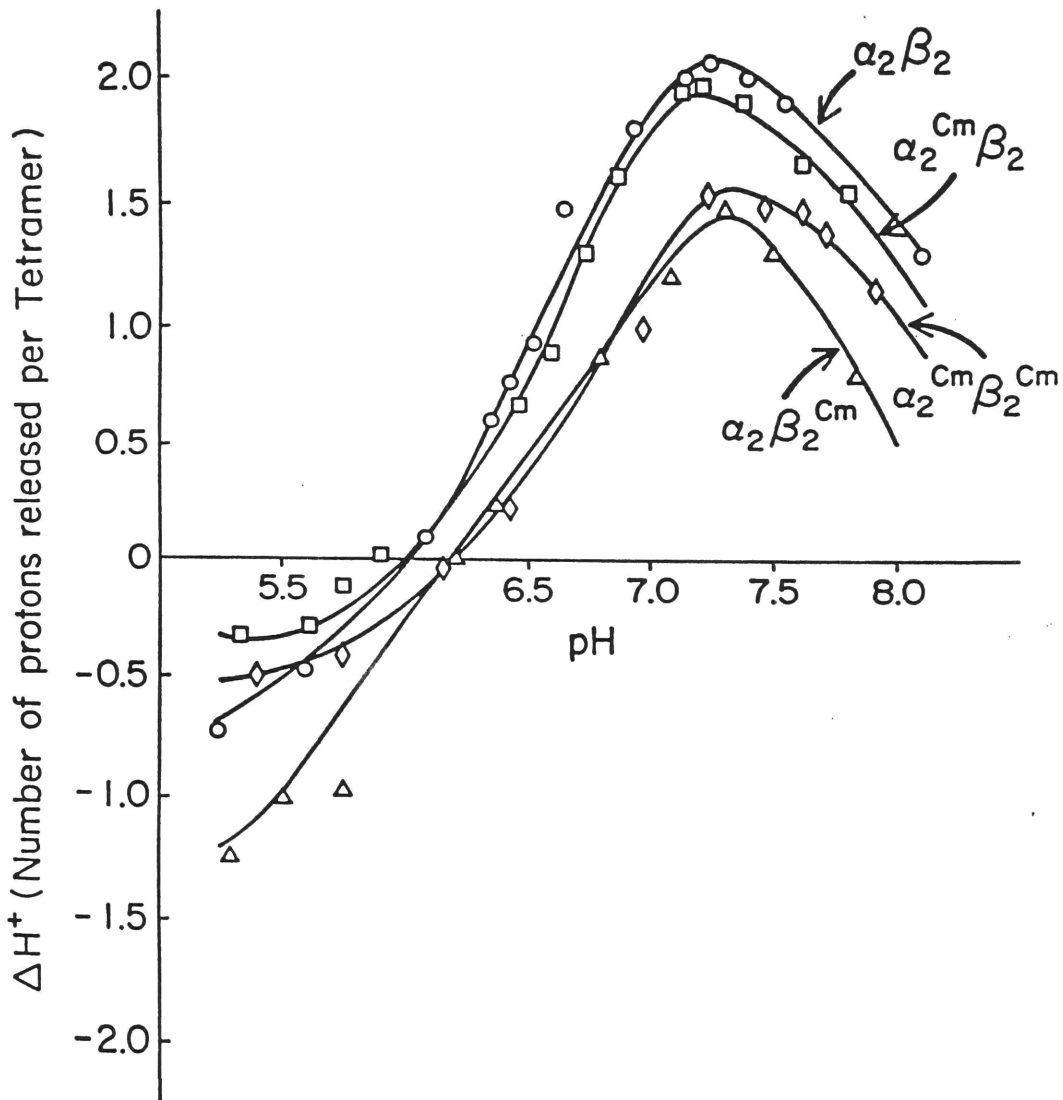


Figure 18 - Alkaline and acid Bohr effect measured according to the proton release technique as described in the Methods. Samples at a concentration of 60 μM in tetramer were deoxygenated in the presence of 0.1 M chloride. After oxygenation a standardized solution of 10 mM NaOH was added from an autoburette to raise the pH of the oxy Hb solution to the value measured in the deoxy form.

Below pH 6, since the pH was greater in oxy than deoxy Hb, the NaOH was added to the deoxy Hb solution until the value was raised to the value of the pH registered by the oxy Hb solution. Thus, ΔH^+ was calculated from the known aliquot of base added. The concentration of met HbA never exceeded 10%.

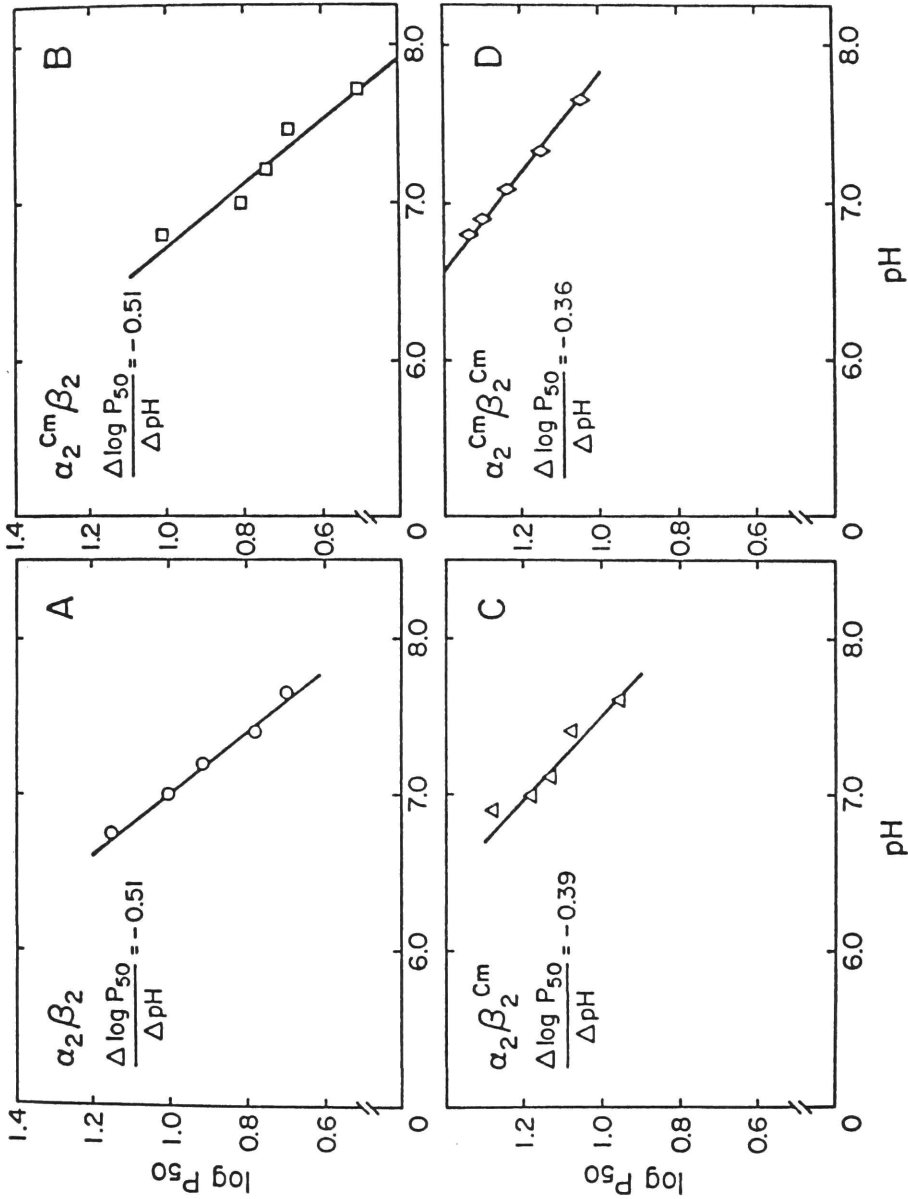


Figure 19 - Alkaline Bohr effect measured as a function of $\log P_{50}$, measured from the Hem-O-Scan versus pH. Aliquots (4 μ l) of the Hb sample (0.5 mM) in 50 mM bis-tris acetate was administered to a glass slide which was then placed in the Hem-O-Scan instrument. The pH of each sample was checked on a microelectrode.

measuring the response of the sample spectrophotometrically. The resultant graph of fraction oxy Hb as a function of pO_2 is a fundamental description of the oxygen transport capacity of Hb. This method circumvents the problem of chloride leakage, the only chloride present is that which is intrinsically bound and cannot be removed by dialysis. The experiments, either proton titration or Hem-O-Scan measurements, were carried out in either 1 mM chloride, 0.1 M chloride, 0.5 M chloride or 2 mM 2,3-DPG, and 0.1 M chloride. Table 10 shows the Bohr effect determinations under conditions of various chloride and/or DPG concentrations. For the most part, agreement between the two techniques is good. Where discrepancies exist, however, we placed more emphasis on the proton titration data, since the Hem-O-Scan readings can vary ± 1 mm Hg and this makes a large difference to $\log P_{50}$ versus pH curve.

Referring to Table 10, the Bohr coefficient (+0.39 protons per heme) for unmodified Hb (60 μ M) as determined by proton titration is in reasonable agreement with values reported from other groups who use low salt (5 mM) (56). The 1.2 to 1.6 mM chloride used here was due to tightly-bound chloride as well as leakage from the microelectrode. However, the results from other labs do have a large excess of chloride over Hb under conditions of 'low salt'. We do not know whether other investigators have taken into account any chloride leakage from their electrodes.

Alkaline Bohr Effect for $\alpha_2\text{---}\beta_2^{\text{Cm}}$ Under Conditions of Low Salt -

Under our conditions of low salt 1.2 to 1.6 mM chloride the Bohr coefficient determined by the proton titration technique for $\alpha_2\text{---}\beta_2^{\text{Cm}}$ appears to have a value +0.40 protons per heme that is close to the value for unmodified Hb with the measurement made in the absence of this

	$\frac{\alpha_2\beta_2}{\alpha_2\beta_2}$	$\frac{C_m\beta_2}{\alpha_2\beta_2}$	$\frac{C_m}{\alpha_2\beta_2}$	$\frac{C_m}{\alpha_2\beta_2}$	$\frac{Des-(Arg-141(a))Hb}{\alpha_2\beta_2}$
0.2mM to 0.6mM chloride that is intrinsically bound to Hb	1*	-0.44	-0.35	-0.22	-
	2*	+0.39	+0.40	+0.26	-
0.1 M chloride	1	-0.51	-0.51	-0.39	-
	2	+0.53	+0.50	+0.38	+0.20
0.5 M chloride	1	-0.47	-0.37	-0.43	-
	2	+0.51	+0.47	+0.39	-
2mM 2,3-DPG 0.1 M chloride	1	-0.60	-0.54	-	-
	2	+0.62	+0.49	+0.27	-

1* Value determined as $d\log P_{s_0}/dpH$

2* Value determined as ΔH^+

Table 10

Alkaline Bohr coefficient comparing the values measured from the 'change in P_{s_0} with respect to pH' technique with the 'proton release technique'. The conditions used are described in the text.

moiety. Although the actual pK_a value of the Val-1(α) amino group could have changed, the alkaline Bohr effect was not affected. This suggests that the carboxymethyl group covalently attached to Val-1(α), and as previously described, it probably interacts with Ser-131(α), does not lower the alkaline Bohr effect further, under conditions of low salt. This could be rationalized in terms of an absence of change in the differential of the pK_a 's of Val-1(α) with a $-\text{CH}_2\text{COO}^-$ group attached, between the oxy and deoxy states as compared with the differential in unmodified HbA.

Alkaline Bohr Effect for $\alpha_2\beta_2^{\text{Cm}}$ Under Conditions of Low Salt -

Under the same conditions of low ionic strength, the alkaline Bohr effect of $\alpha_2\beta_2^{\text{Cm}}$ (+0.26 protons released per heme) is decreased by about 33% compared to $\alpha_2\beta_2$ (Table 10). The difference electron density map has suggested an electrostatic interaction between the carboxymethylated Val-1(β) with Lys-82(β). This is completely analogous to the difference electron density map obtained for carbamino Hb (254) in the region of Val-1(β). In this regard therefore the carboxymethylated Hb can be considered as a carbamino analogue. Moreover, this represents a unique situation since we are able to measure the effect of $-\text{CH}_2\text{COO}^-$ (analogous to CO_2) attached to the terminus of the β -chain only, without any modification at Val-1(α). Although earlier studies that examined CO_2 binding to an N-terminal amino group provided a wealth of important information, the opposite terminus had to be chemically blocked to prevent CO_2 binding to it. Here it is possible to measure for the first time how the $-\text{CH}_2\text{COO}^-$ (CO_2) moiety bound at one terminus with the other terminus free changes the Bohr effect. A detailed interpretation as to

how a modification at Val-1(β) decreases the alkaline Bohr effect will be provided in the Discussion.

Alkaline Bohr Effect for $\alpha_2\beta_2^{CmCm}$ Under Conditions of Low Salt -

The Bohr coefficient of $\alpha_2\beta_2^{CmCm}$ (+0.28 protons per heme) as measured by proton titration was decreased by about 30% compared with unmodified Hb (Table 10). Since, as reported above, $\alpha_2\beta_2^{Cm}$ and unmodified HbA have the same Bohr coefficient, for all intents and purposes, $\alpha_2\beta_2^{CmCm}$, under these conditions of ionic strength, could be considered equivalent to $\alpha_2\beta_2^{Cm}$.

Alkaline Bohr Effect for unmodified Hb in 0.1 M Chloride - The

alkaline Bohr effect was measured for unmodified HbA and the specifically carboxymethylated hybrids in the presence of 0.1 M chloride. Both the Hem-O-Scan and proton titration data are in good agreement (Figures 19, 20, Table 10). Under these conditions of ionic strength Rollema et al (56) measured the alkaline Bohr effect and found it to be maximal compared with their determinations carried out in less or more chloride than 0.1 M. In the case of unmodified Hb, Table 10 shows that there is a substantial increase (about 25%) in the alkaline Bohr effect in the presence of 0.1 M chloride (+0.39 to +0.53 protons per heme). This is most likely due to the part of the Bohr effect attributed to chloride binding between Val-1(α_1) and Arg-141(α_2) as well as to Lys-82(β) (44, 111). Why such a vast excess of chloride is required to manifest the 'full' alkaline Bohr effect by binding to both of these sites is not readily apparent. It is presumably related to the chloride binding association constant to the high affinity (Val-1(α)) and the low affinity (Lys-82(β)) (111) sites, respectively.

Alkaline Bohr Effect for $\alpha_2\text{Cm}\beta_2$ in the Presence of 0.1 M Chloride

The same increase in the alkaline Bohr effect is observed for $\alpha_2\text{Cm}\beta_2$, 20% in the presence of 0.1 M chloride, as for unmodified HbA (Figures 19, 20, and Table 10). This presumably indicates that the Val-1(α_1) to Arg-141(α_2) binding site is still able to bind chloride. Indeed, as shown from the X-ray data (Figure 14), the orientation of the carboxymethyl moiety attached to Val-1(α_1) is toward the hydroxyl group of Ser-131(α_1). Thus, it appears that $\alpha_2\beta_2$ and $\alpha_2\text{Cm}\beta_2$ are functionally equivalent. Moreover, after a review of the literature, it appears that this is the first time that a Bohr group has been modified but yet the normal alkaline Bohr effect has been retained. Also apparent from this result is the fact that chloride binding to Lys-82(β) should not have been impaired.

Alkaline Bohr Effect for $\alpha_2\beta_2\text{Cm}$ in the Presence of 0.1 M Chloride

In contrast to the similarity of the alkaline Bohr coefficient for $\alpha_2\beta_2$ and $\alpha_2\text{Cm}\beta_2$, in conditions of low salt and at 0.1 M chloride, the respective coefficients for the $\alpha_2\beta_2\text{Cm}$ hybrid are reduced in comparison to $\alpha_2\beta_2$ (Figures 19, 20, and Table 10). Even though there was a 35% increase in the alkaline Bohr effect as measured for $\alpha_2\beta_2\text{Cm}$ (+0.26 to +0.38 protons per heme) in the presence of 0.1 M chloride, this final value was still 25% below the value for unmodified HbA. Presumably, carboxymethylation at Val-1(β) did not interfere with chloride binding at Val-1(α) and the increased alkaline Bohr effect observed for $\alpha_2\beta_2\text{Cm}$ when the chloride concentration is raised to 0.1 M most probably reflects this. Chloride binding to Lys-82(β) may be greatly impaired due to the interaction between the carboxymethyl moiety attached to Val-1(β) with Lys-82(β) as seen from the electron density patterns

(Figure 13). A reason as to why the actual value for the Bohr coefficient of $\alpha_2\beta_2^{Cm}$ is decreased in low concentrations of chloride as well as in 0.1 M chloride compared to $\alpha_2\beta_2$ will be presented in the Discussion section.

Alkaline Bohr Effect for $\alpha_2\beta_2^{Cm}$ in 0.1 M Chloride - The 26% increase in the alkaline Bohr effect for $\alpha_2\beta_2^{Cm}$ (+0.28 to +0.38 protons per heme) in the presence of 0.1 M chloride can be rationalized with respect to chloride binding between Val-1(α_1) and Arg-141(α_2), since unmodified HbA and $\alpha_2\beta_2^{Cm}$ appear to be functionally equivalent (Figures 19, 20, and Table 10). Thus, the reduced alkaline Bohr effect for $\alpha_2\beta_2^{Cm}$ under conditions of low chloride and 0.1 M chloride can be an indication of a functional equivalence between $\alpha_2\beta_2^{Cm}$ with $\alpha_2\beta_2^{Cm}$.

Alkaline Bohr Effect for Samples in 0.5 M Chloride - Rollemma et al (56) reported a 14% decrease in the alkaline Bohr effect for unmodified Hb under 'stripped' conditions at a chloride concentration of 0.5 M. The Bohr coefficient reported in this study for unmodified HbA in 0.5 M chloride is +0.51 protons per heme. This result is at variance with their results. Moreover, no significant decrease in the alkaline Bohr effect is observed for $\alpha_2\beta_2^{Cm}$ (+0.47) (Table 10) which continues to respond in a manner analogous to that of unmodified HbA in the presence of various amounts of chloride. There is also no change in the value for $\alpha_2\beta_2^{Cm}$ which is +0.38 and +0.39 protons per heme in the presence of 0.1 M or 0.5 M chloride, respectively.

Alkaline Bohr Effect for Samples in the Presence of 2,3-DPG and 0.1 M Chloride - It is well-known from studies discussed in the Introduction of this thesis (36, 126-129), that the presence of 2,3-DPG elevates the Bohr effect. This is due to the formation of salt bridges

when 2,3-DPG binds to the positively charged residues of the 2,3-DPG binding cleft. At physiological pH, the pK_a values of these groups are increased. Since this organic anion does not bind with such a great affinity to oxy Hb, the difference in protons bound between deoxy and oxy Hb is greater than in the absence of 2,3-DPG. In Table 10 this elevation in the Bohr effect is clearly seen. The value for the Bohr effect increases from +0.39 to +0.62 protons released per heme in the presence of 2,3-DPG and 0.1 M chloride. (Chloride eliminates non-specific binding (36).) That 2,3-DPG appears to be less efficacious in increasing the alkaline Bohr effect of $\alpha_2^{Cm}\beta_2$ than $\alpha_2\beta_2$ in the presence of 0.1 M chloride could perhaps be due to increased binding of 2,3-DPG to the oxy form of the hybrid thereby reducing the difference in protons bound between the two conformations of this modified tetramer. That the oxy form of $\alpha_2^{Cm}\beta_2$ is more 'deoxy-like' is suggested by preliminary studies described later in this section.

The carboxymethylation of Val-1(β) seems to have precluded the binding of 2,3-DPG since the alkaline Bohr coefficient is essentially unaltered in the presence of this effector compared to the value measured for $\alpha_2\beta_2^{Cm}$ at low salt. Of note is the fact that the presence of 2,3-DPG diminishes the value for the alkaline Bohr effect measured in the presence of 0.1 M chloride. Perhaps there is an increased amount of non-specific binding of 2,3-DPG to oxy Hb and this masks the contribution of chloride to Val-1(α). The data shown for $\alpha_2^{Cm}\beta_2^{Cm}$ essentially reflects those measurements for $\alpha_2\beta_2^{Cm}$ and will not be elaborated further.

Other Characteristics from the Titration Curves - The shifts in pH at which the maximum proton release occurs are shown Table 11. The

pH Values of Maximum Amplitudes

	[CHLORIDE] mM		
	1 mM	100 mM	500 mM
			100 mM + 2 mM 2,3-DPG
$\alpha_2\beta_2$	7.0	7.3	7.4
$C_m\beta_2$	7.2	7.3	7.4
$\alpha_2\beta_2$	7.0	7.3	7.5
$C_m\beta_2$	7.2	7.3	7.4
Des-(Arg-141(α)) HbA	-	7.2	-

Values of pH given are an average of at least two separate determinations and are corrected to one decimal place.

Table 11

The pH values at which the maximum amplitude occurs are read from the proton titration curves for each sample (Figure 18).

trend observed is the same for all the samples studied. The pH at which the maximum proton release occurs, shifts to a higher value in the presence of increasing concentrations of chloride. The presence of 2,3-DPG also causes a marked increase in the pH of the maximum amplitude of the titration curve. These shifts have also been observed by other workers and a clear example is shown by Rollema et al (56). These shifts could perhaps be attributed to an elevation in the pK_a values of certain titratable groups in the presence of increasing concentrations of anion. Alternatively, at high concentrations of salt, the accumulation of microscopic pH changes becomes more important in contributing to the overall alkaline Bohr effect (258).

The pH at which $\Delta H^+ = 0$ was read from the proton titration curves and is shown in Table 12. An increase in pH is observed for this measurement as the ionic concentration increases. The pH at which $\Delta H^+ = 0$ implies that the number of protons bound by deoxy Hb is the same as the number of protons bound by oxy Hb. No protons are released on oxygenation and none are absorbed upon deoxygenation. The pK_a 's of the alkaline Bohr groups therefore have the same value in the oxy and deoxy states. Perhaps this can be interpreted with respect to either a lack of breakage of salt bridges, or to a lack of formation of salt bridges at this pH, or else to some previously unrecognized quaternary structure that is neither oxy or deoxy. Ionic strength appears to influence the pH at which this effect is manifested.

The Acid Bohr Effect - The determinations made for the acid Bohr effect in 0.1 M chloride are shown in Figure 18 and Table 13. As discussed in C-2 of the Introduction, the acid Bohr effect is a

pH Values Where $\Delta H^+ = 0$

	[CHLORIDE] mM		
	1 mM	100 mM	500 mM
		100 mM + 2 mM 2,3-DPG	
$\alpha_2\beta_2$	5.5	6.0	6.1
$C_m\beta_2$	5.9	6.1	6.4
$\alpha_2\beta_2$	5.5	6.3	6.4
$C_m\beta_2$	6.0	6.2	6.3
Des-(Arg-141(α)) HbA	-	6.2	-

Values of pH given are an average of at least two separate determinations and are corrected to one decimal place.

Table 12

The pH values at which $\Delta H^+ = 0$ are read from the titration curves of Figure 18.

Acid Bohr Effect ΔH^+ When pH = 5

	<u>[CHLORIDE] mM</u>	
	<u>1 mM</u>	<u>100 mM</u>
$\alpha_2\beta_2$	-	-0.26
$\alpha_2^{Cm}\beta_2$	-0.10	-0.12
$\alpha_2\beta_2^{Cm}$	-	-0.35
$\alpha_2^{Cm}\beta_2^{Cm}$	-0.08	-0.15
Des-(Arg-141(α)) HbA	-	-0.08

Values given by extrapolating data to pH = 5.0.

Table 13

The Acid Bohr effect was measured for the samples shown by calculating the protons released upon oxygenation below pH 6.0.

measurement of the uptake of protons upon oxygenation below pH 6. The value of -0.26 which is an average of several determinations, for unmodified Hb is in good agreement with the value published by Kilmartin et al (255). Of note here, is the diminished value measured for $\alpha_2^{Cm}\beta_2$, the reduction of which is greater than 50% of the value for $\alpha_2\beta_2$. The suggestion has been made (160) that perhaps the carboxyl group of Arg-141(α) could be an acid Bohr group. In other words its pK_a value would be elevated in oxy compared to deoxy Hb. The salt bridge between the carboxyl group of Arg-141(α_1) to Lys-127(α_2) in deoxy Hb and its absence in oxy Hb could allow this elevation in pK_a to occur. We reasoned that the presence of a carboxymethyl moiety attached to Val-1(α) in the vicinity of this salt bridge could perhaps increase the pK_a of this carboxyl group in the deoxy $\alpha_2^{Cm}\beta_2$ hybrid and weaken the salt bridge with Lys-127(α). This could lessen the differential pK_a of the Arg-141(α) carboxyl group in deoxy compared to oxy $\alpha_2^{Cm}\beta_2$ below pH 6. Indeed, total removal of the carboxyl terminal arginine from unmodified HbA after carboxypeptidase B treatment yielded des-(Arg-141(α)) Hb whose acid Bohr effect was -0.08. This is in good agreement with a similar determination made by Kilmartin et al (230). Both these pieces of experimental evidence attribute a role to the acid Bohr effect by the carboxyl group of Arg-141(α).

In contrast to the decrease in the acid Bohr effect observed for $\alpha_2^{Cm}\beta_2$, the hybrid $\alpha_2\beta_2^{Cm}$ exhibits an increased coefficient, -0.35, which is not as great an increase as that observed by Kilmartin et al (255) for a hybrid carbamylated at Val-1(β). The influence of carboxymethylation at Val-1(β) upon the positively charged residues within the 2,3-DPG binding cleft is given as a possible explanation, and

especially in the influence of $-\text{CH}_2\text{COO}^-$ upon the residue His-143(β). This hypothesis will be developed fully in the Discussion of the Thesis.

The acid Bohr coefficient of -0.15 for $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ is for the greater part a reflection of the decreased acid Bohr coefficient of $\alpha_2^{\text{Cm}}\beta_2$ and is not an average of the acid Bohr effect measured for $\alpha_2^{\text{Cm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$.

j) Competitive Binding Studies

Competitive binding studies were carried out between $[^{14}\text{C}]$ -glyoxylate and anions in combination with CO_2 for the N-terminal amino group of either chain. Conditions were selected such that the reaction between Hb and glyoxylate was a) non-saturating, in order to allow the initial rates of reaction to be calculated and b) permitted N-terminal modification predominantly, with minimal modification of the ϵ -amino groups of lysine. Figure 9 shows that between 0 and 1 mM glyoxylate the amount of incorporation into oxy Hb (1 mM), pH 7.2 is linear and therefore the conditions are non-saturating at least up to this concentration of glyoxylate. In addition, it can be seen from Table 3 that under conditions of 0.4 mM unmodified HbA, 0.1 mM $[^{14}\text{C}]$ -glyoxylate and 1 mM cyanoborohydride at pH 7.2, carboxymethylation was identified as about 80% monocarboxymethylvaline, under both oxy and deoxy conditions. The di-derivative was not detected. A small amount of carboxymethyllysine (20%) was detected by this technique. Also that deoxygenation was essentially complete was determined spectrophotometrically as described in the Methods section.

Since, under the conditions described above, the reaction occurred mainly at the NH_2 -terminal amino group of each chain, it was possible to

examine the dependency of the rate of carboxymethylation at an unblocked terminus in the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$, on the presence of allosteric modulators.

The first two striking observations to be seen in the early experiments were a) the increase in rate of carboxymethylation for deoxy as compared to oxy unmodified HbA (about 3 to 4-fold increase, to be discussed a little later in the Results section) and b) the decrease in rate of carboxymethylation with increased pH for oxy and deoxy unmodified HbA, with greater rates for deoxy HbA. These two features are seen from the data plotted in Figures 20, 21, 22, respectively.

Two control experiments were carried out. The first ensured that the decrease in rate with increasing pH was not due to reduction of the aldehyde by cyanoborohydride. The second was concerned with contaminating amounts of cyanide in the cyanoborohydride which could give rise to N-cyanomethyl formation. In the first control experiment the concentration of a glyoxylate solution as a function of pH was measured in the presence of cyanoborohydride. In this assay the glyoxylate was measured at 510 nm as the 2,4-dinitrophenylhydrazone derivative (279). The results indicated that in the presence of cyanoborohydride the concentration of glyoxylate remained constant regardless of pH. The second control experiment was concerned with cyanide as a possible contaminant. It has been shown (303) that low yields of products in reductive methylation reactions can be caused by the reaction between cyanide and a protonated Schiff base (shown in the equation on the next page). The resultant N-cyanomethyl compound is formed irreversibly at neutral pH, but is hydrolyzed to regenerate the starting materials at low pH, which would have occurred when the samples

were precipitated in TCA (see Methods). Thus, as the pH is raised, more unprotonated amino groups would be available for Schiff base formation and, as a consequence, would react with cyanide to form the cyanomethyl compound.

Addition of Ni^{2+} will remove free cyanide due to complex formation. Thus, the rate of carboxymethylation was measured in the presence or absence of Ni^{++} as well as with recrystallized cyanoborohydride. The presence of Ni^{++} did enhance the rate of reaction two-fold. However, there was no enhancement in reaction rate when recrystallized cyanoborohydride was used, and free cyanide should have been absent. In view of the control experiments, therefore, how can the decrease in reaction rate observed as the pH is raised be explained? It is of note that King et al (31) observed an increase in reaction rate at high pH when they incubated ragweed pollen antigen E in the presence of glyoxylate and cyanoborohydride. The mechanism proposed for reductive carboxymethylation involves nucleophilic attack by the unprotonated amine on the aldehydic function of the glyoxylate, followed by reduction of the protonated Schiff base intermediate (18). Thus, an increased pH should increase the amount of unprotonated amine while the concentration of the Schiff base intermediate should decrease. Indeed, the overall reaction rate must somehow reflect a compromise between these two opposing factors. The results reported here suggest that the concentration of the Schiff base intermediate is the limiting factor at high pH. It is also important to consider that the ratio of Cm-Lys to Cm-Val may also be a function of pH.

A typical time course for the incorporation of glyoxylate was linear for the first five minutes but did not quite pass through the

origin. The value at which the line intercepted the ordinate was subtracted from each value of the slope from a kinetic profile. Since carboxymethylation of lysine residues can be minimized but not eliminated, a time course was measured for the carboxymethylation of $\alpha_2^{Cm}\beta_2^{Cm}$. This experiment yielded a 'blank' value of 50 dpm/minute, (or 0.005% of total glyoxylate) from oxy $\alpha_2^{Cm}\beta_2^{Cm}$ and 103 dpm/minute (or 0.01% of total glyoxylate) from deoxy $\alpha_2^{Cm}\beta_2^{Cm}$ which were subtracted from the kinetic rates of the relevant experiments. When an acid hydrolsate of $\alpha_2^{Cm}\beta_2^{Cm}$, after reaction with [^{14}C]-glyoxylate under deoxy conditions, was applied to a Dowex 2 column the results showed (Table 9) that of the total [^{14}C]-glyoxylate incorporated, 14% was dicarboxymethylvaline, which was only seen for $\alpha_2^{Cm}\beta_2^{Cm}$ and not in any other case. Thus, a 'blank value' may be an overestimate. This is because no dicarboxymethylvaline was detected from the experiment described in the legend to Table 1. It is, however, possible that carboxymethylation of a hybrid blocked at Val-1(α) or Val-1(β) might yield the di-derivative at the blocked terminus. However, the extent of dicarboxymethylation for the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ was not determined. Moreover, the remaining 86% of the [^{14}C]-glyoxylate was distributed equally between monocarboxymethylvaline and monocarboxymethyllysine (43% of each). The presence of monocarboxymethylvaline indicates that there were some free, unmodified N-terminal amino groups available for reaction. When oxy or deoxy unmodified HbA were incubated in the presence of [^{14}C]-glyoxylate under the same conditions described above for $\alpha_2^{Cm}\beta_2^{Cm}$, i.e., 0.4 mM Hb, 0.1 mM glyoxylate, and 1 mM cyanoborohydride, 3% (for oxy) and 10% (for deoxy), respectively, of the total glyoxylate was incorporated into Hb per minute after subtraction

of the 'blank' value. This low level of incorporation corresponds to a modification of less than 2% of the total free α -amino groups in oxy and deoxy unmodified HbA, respectively. Calculations also reveal that 0.005% and 0.01% of the total glyoxylate are incorporated into oxy and deoxy $\alpha_2^{Cm}\beta_2^{Cm}$, respectively. The relevant calculations that take into account the 43% monocarboxymethylvaline or the 14% dicarboxymethylation, after reaction between deoxy $\alpha_2^{Cm}\beta_2^{Cm}$ and 0.1 mM [^{14}C]-glyoxylate (discussed above) make very little difference to the actual value obtained.

Thus, to reiterate under the conditions of 0.4 mM 'stripped' unmodified HbA, 0.1 mM [^{14}C]/[^{12}C]-glyoxylate and 1 mM cyanoborohydride at pH 7.2, the primary species was monocarboxymethylvaline at a yield of about 80% both under oxy and deoxy conditions. These same experimental conditions were employed to carboxymethylate the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ in the absence or presence of the allosteric modulators - 5 mM 2,3-DPG, 0.5 M chloride, 5% CO_2 , 14% CO_2 or combinations of these effectors. The data is represented in Figure 20 as the rate of incorporation of [^{14}C]-glyoxylate into the total number of free α -amino groups per minute. In addition the second order kinetic rate constant ($k \text{ mM}^{-1} \text{ h}^{-1}$) was calculated for each reaction in the presence of the allosteric modulators (Tables 14a to 14i). The equation used was the same one derived by Jensen et al (304) who calculated the same rate constant for carbamylation of the α - and β -N-terminal amino groups. It is $dNH_2/dt = K_{NH} [NH_2] [\text{glyoxylate}]$, where dNH_2/dt is the rate of incorporation of the Cm group at the N-terminals per minute measured from a linear slope at about 5 minutes, k_{NH_2} is the second order rate constant for the carboxymethylation of the free α - NH_2 groups, $[NH_2]$ is

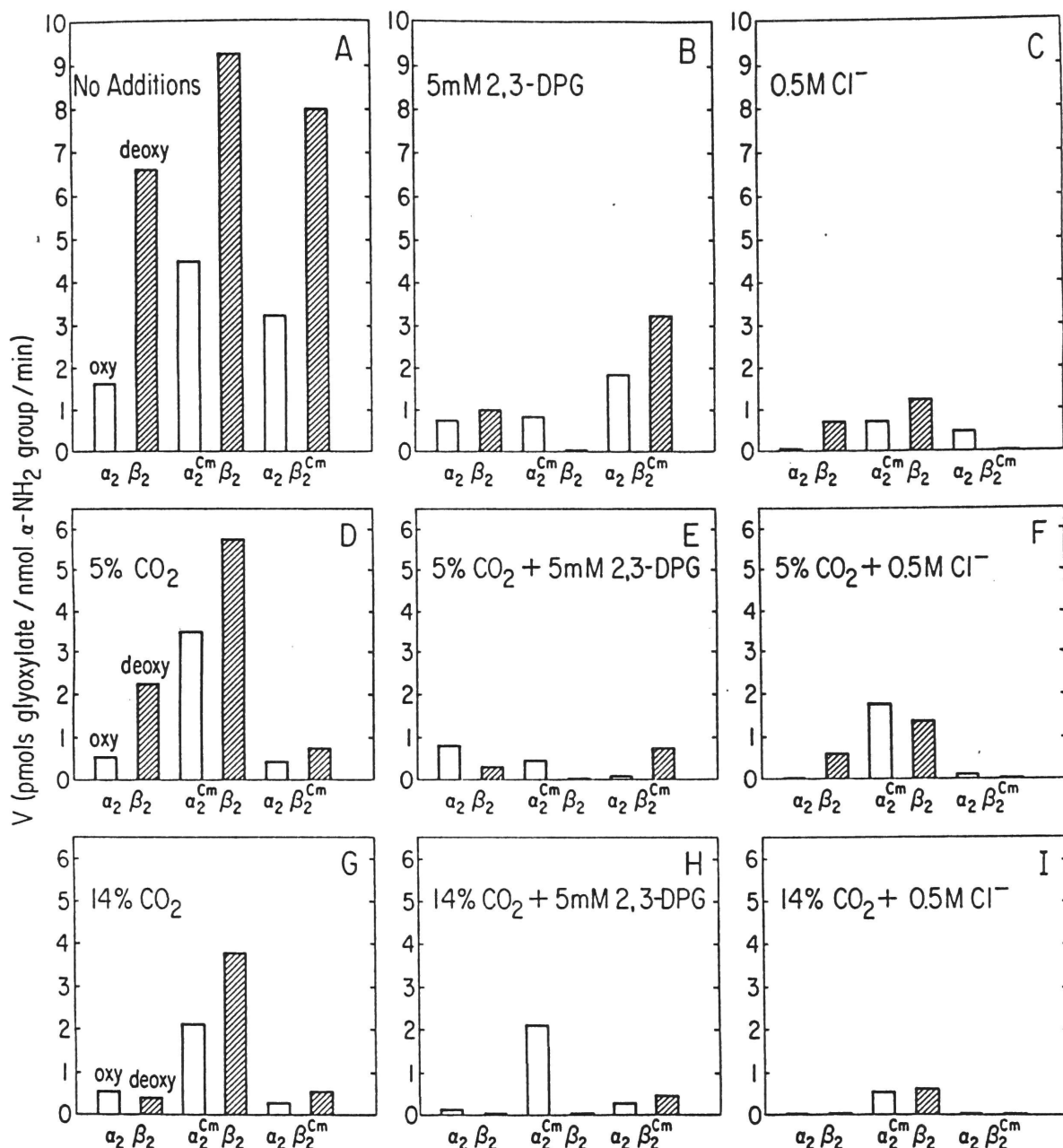


Figure 20 - Competitive binding experiments. Each sample (0.4 mM) was incubated in the presence of 0.1 mM $[^{14}\text{C}]/[^{12}\text{C}]$ -glyoxylate and 1 mM cyanoborohydride in 100 mM Hepes buffer at pH 7.2 for 9 minutes in the presence of various allosteric modulators as indicated on the Figure. Aliquots were withdrawn at certain times and precipitated into 5% TCA solution. After washing and drying on a filter disc, the radioactivity incorporated was measured in an LKB scintillation counter. The results are expressed as pmols glyoxylate incorporated per nmol free $\alpha\text{-NH}_2$ group.

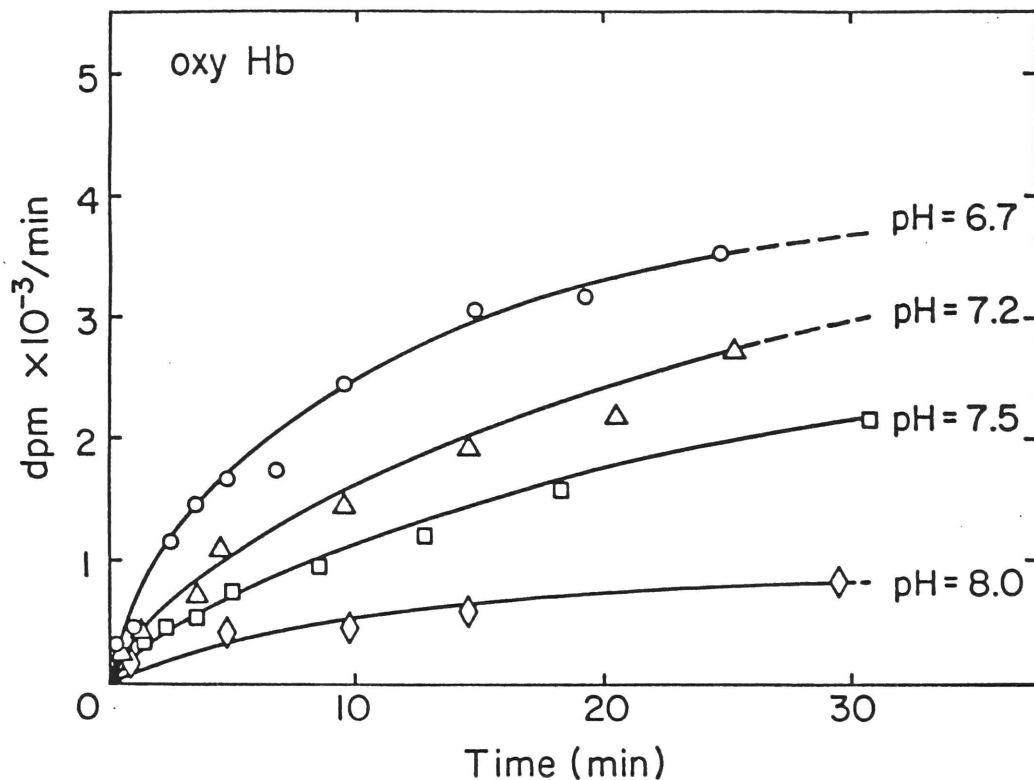


Figure 21 - Preliminary experiments that showed the rate of incorporation of $[^{14}\text{C}]$ -glyoxylate into a 1 mM solution of oxy Hb as a function of pH. Aliquots (5 μl) were precipitated into 5% TCA. The sample work-up was as described in the text.

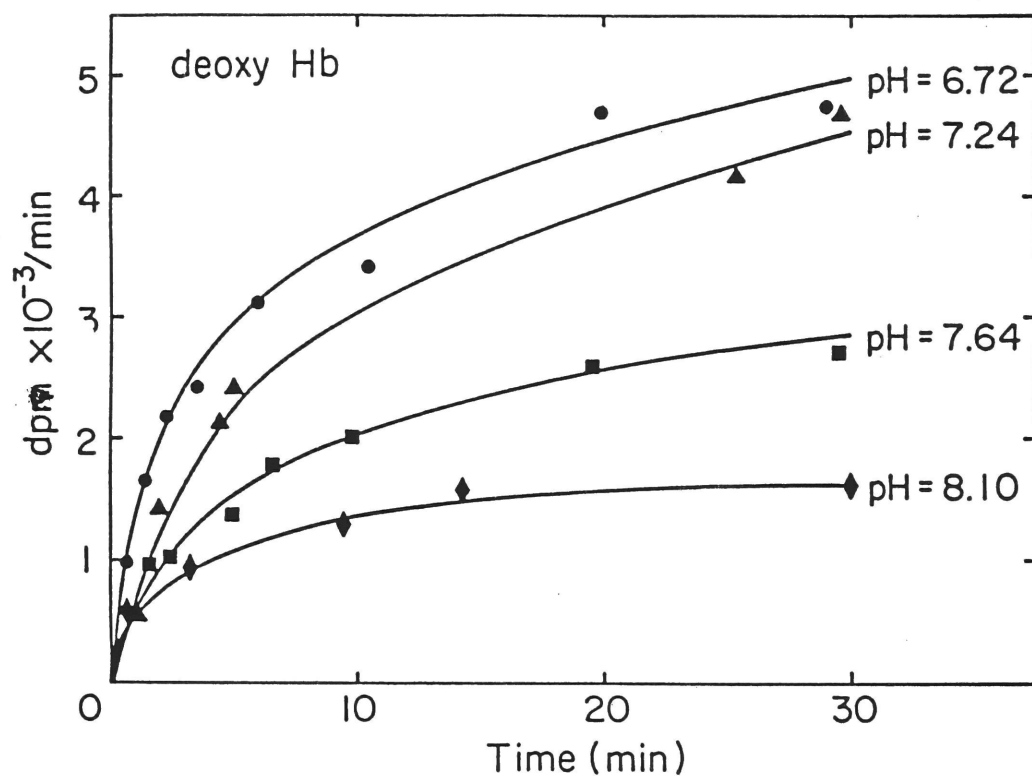


Figure 22 - Preliminary experiments that showed the rate of incorporation of [^{14}C]-glyoxylate into a 1 mM solution of deoxy Hb as a function of pH. Aliquots (5 μl) were precipitated into 5% TCA. The sample work-up was as described in the text. The solution at each pH value was deoxygenated.

Second Order Rate Constants for the Carboxymethylation of
Unmodified Hb $\alpha_2\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$ Under a Variety
of Experimental Conditions at pH = 7.2

a) No effector added

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	1.01
$\alpha_2\beta_2$ deoxy	4.50
$\alpha_2\beta_2^{\text{Cm}}$ oxy	2.80
$\alpha_2\beta_2^{\text{Cm}}$ deoxy	6.05
$\alpha_2\beta_2^{\text{Cm}}$ oxy	1.85
$\alpha_2\beta_2^{\text{Cm}}$ deoxy	5.13

Table 14a

The second order rate constants were calculated from the equation (see text) that Jensen et al (304) used to calculate the second order rate constants for the carbamylation of Val-1(α) and Val-1(β). This legend applies to Tables 14a to 14i.

b) + 5.0 mM 2,3-DPG

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0.46
$\alpha_2\beta_2$ deoxy	0.61
$\alpha_2^{Cm}\beta_2$ oxy	0.50
$\alpha_2^{Cm}\beta_2$ deoxy	0.00
$\alpha_2\beta_2^{Cm}$ oxy	1.12
$\alpha_2\beta_2^{Cm}$ deoxy	2.02

Table 14b

c) + 0.5 M Chloride

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0.02
$\alpha_2\beta_2$ deoxy	0.40
$\alpha_2^{Cm}\beta_2$ oxy	0.41
$\alpha_2^{Cm}\beta_2$ deoxy	0.73
$\alpha_2\beta_2^{Cm}$ oxy	0.26
$\alpha_2\beta_2^{Cm}$ deoxy	0.00

Table 14c

d) + 5% CO₂

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0.33
$\alpha_2\beta_2$ deoxy	1.40
$\alpha_2^{Cm}\beta_2$ oxy	2.20
$\alpha_2^{Cm}\beta_2$ deoxy	3.60
$\alpha_2\beta_2^{Cm}$ oxy	0.26
$\alpha_2\beta_2^{Cm}$ deoxy	0.50

Table 14d

e) + 14% CO₂

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0.33
$\alpha_2\beta_2$ deoxy	0.20
$\alpha_2^{Cm}\beta_2$ oxy	1.29
$\alpha_2^{Cm}\beta_2$ deoxy	2.32
$\alpha_2\beta_2^{Cm}$ oxy	0.15
$\alpha_2\beta_2^{Cm}$ deoxy	0.36

Table 14e

f) + 5% CO₂ and 5 mM 2,3-DPG

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0.48
$\alpha_2\beta_2$ deoxy	0.17
$\alpha_2^{Cm}\beta_2$ oxy	0.26
$\alpha_2^{Cm}\beta_2$ deoxy	0.00
$\alpha_2\beta_2^{Cm}$ oxy	0.05
$\alpha_2\beta_2^{Cm}$ deoxy	0.50

Table 14f

g) + 14% CO₂ and 5 mM 2,3-DPG

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	~ 0
$\alpha_2\beta_2$ deoxy	0
$\alpha_2^{Cm}\beta_2$ oxy	1.28
$\alpha_2^{Cm}\beta_2$ deoxy	0
$\alpha_2\beta_2^{Cm}$ oxy	0.15
$\alpha_2\beta_2^{Cm}$ deoxy	0.30

Table 14g

h) + 5% CO₂ and 0.5 M Chloride

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0
$\alpha_2\beta_2$ deoxy	0.32
$\alpha_2^{Cm}\beta_2$ oxy	1.07
$\alpha_2^{Cm}\beta_2$ deoxy	0.76
$\alpha_2\beta_2^{Cm}$ oxy	0.05
$\alpha_2\beta_2^{Cm}$ deoxy	0.00

Table 14h

i) +14% CO₂ and 0.5 M Chloride

Sample	k (mM ⁻¹ h ⁻¹)
$\alpha_2\beta_2$ oxy	0
$\alpha_2\beta_2$ deoxy	0
$\alpha_2^{Cm}\beta_2$ oxy	0.35
$\alpha_2^{Cm}\beta_2$ deoxy	0.30
$\alpha_2\beta_2^{Cm}$ oxy	0
$\alpha_2\beta_2^{Cm}$ deoxy	0

Table 14i

the concentration of free α -NH₂ groups at one minute and [glyoxylate] is the concentration of glyoxylate at one minute.

Carboxymethylation of Samples in the Absence of Allosteric Modulators - As seen in Figure 20 and Table 14a in all cases deoxygenation enhances the rate of carboxymethylation, since the deoxy samples react 2-4 times faster than their oxy counterparts. This result is also consistent with the findings in Table 4 where there was an enrichment of the components Hb₁, Hb₂, and Hb₃ that eluted from a DE52-cellulose column after the reaction between unmodified HbA and glyoxylate under deoxy conditions. Both Figure 20, panel A and Table 14a show that under both oxy and deoxy conditions the rate of reaction for the unmodified samples is less than the average for the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ in the oxy and deoxy form, respectively. The enhanced rate of reaction of the deoxy samples is reminiscent of the increased rate of carbamylation of deoxy HbA reported by Lee and Manning (305). However, this was mainly due to a 3-fold enhancement in the carbamylation of Val-1(α) (285). In the studies presented here (Table 14a), the increased reaction rate for unmodified HbA seems to be a composite of a similar (slightly greater for the α -chain amino terminus) enhancement at each chain. The factors that could contribute to this will be presented in the Discussion.

Carboxymethylation of Samples in the Presence of 5 mM 2,3-DPG - The carboxymethylation of the deoxy $\alpha_2^{Cm}\beta_2$ hybrid was completely inhibited and that of deoxy unmodified HbA reduced by about 8-fold (Figure 20, Table 14b). These results are explained by the well-known binding of 2,3-DPG to the cleft between the two β -chains that includes Val-1(β). In all the oxy samples, as well as deoxy $\alpha_2\beta_2^{Cm}$, there is

also a significant reduction in the rate of carboxymethylation and this could be attributed to non-specific binding of 2,3-DPG (86-91).

Carboxymethylation of Samples in the Presence of 0.5 M Chloride -
Chloride at a concentration of 0.5 M (Figure 20, panel C, and Table 14c) causes a marked reduction in incorporation of glyoxylate for all the samples studied. The greatest inhibition is seen for unmodified HbA and the $\alpha_2\beta_2^{Cm}$ hybrid both in the oxy and deoxy conformation. Of note is the complete inhibition of carboxymethylation for deoxy $\alpha_2\beta_2^{Cm}$ and the 11-fold inhibition for deoxy unmodified Hb consistent with Val-1(α) as an anion binding site (44,57). Also the 8-fold inhibition of glyoxylate incorporation into the deoxy hybrid $\alpha_2^{Cm}\beta_2$ is consistent with the involvement of Lys-82(β) in anion binding (59, 61). Since chloride and glyoxylate compete for common sites, the drastic reduction in carboxymethylation observed in the oxy form of all the samples is probably due to the large excess of chloride which is able to inhibit incorporation of glyoxylate.

Carboxymethylation of Samples in the Presence of Either 5% or 14% CO_2 - Figure 20, panels D and G, Tables 14d and 14e show that both 5% (the concentration of CO_2 found in vivo) and 14% CO_2 , inhibit carboxymethylation to the greatest extent for unmodified HbA and for the $\alpha_2\beta_2^{Cm}$ hybrid in both their oxy and deoxy forms. A consideration of the rate constants, Tables 14a, 14b, and 14e shows that the presence of 5% CO_2 reduces carboxymethylation 3-fold for deoxy unmodified HbA, less than 2-fold for deoxy $\alpha_2^{Cm}\beta_2$ and 10-fold for deoxy $\alpha_2\beta_2^{Cm}$. Superficially, the results may indicate that CO_2 has a higher affinity for the α -chain amino terminus. These suggestions agree with the proposal put forward by Brenna et al (125) whereby Val-1(α) may be the

preferred site of carbamino formation. In the Discussion, however, an alternative hypothesis will be put forward. That CO_2 can bind to oxy Hb has been observed in other studies (248, 256, 258, 260). In vivo carbamino formation at Val-1(α) may reduce the reaction of CO_2 at Val-1(β), since carboxymethylation of $\alpha_2\beta_2^{\text{Cm}}$ is inhibited to a lesser extent than $\alpha_2\beta_2^{\text{Cm}}$.

Carboxymethylation of the Samples in the Presence of Combinations of the Allosteric Modulators - 5% or 14% CO_2 and 5 mM 2,3-DPG together (Figure 20, panels E and H, Tables 14f and 14g) completely inhibit carboxymethylation of deoxy unmodified HbA and deoxy $\alpha_2\beta_2^{\text{Cm}}$, an effect probably due predominantly to the 2,3-DPG rather than the 14% CO_2 . Panels F and I of Figure 20 show the almost complete inhibition of carboxymethylation of oxy and deoxy lysate and $\alpha_2\beta_2^{\text{Cm}}$ by 14% CO_2 in combination with 0.5 M Cl^- and a very marked reduction in the carboxymethylation of oxy and deoxy $\alpha_2\beta_2^{\text{Cm}}$. At these high concentrations CO_2 and Cl^- compete with glyoxylate for the same sites.

k) Probes of Conformational change for Specifically Carboxymethylated Hb

The spectral changes caused by the addition of IHP to met HbA are well documented (270, 57) and were indeed reproduced in these studies (data not shown). However, non-saturating titration data were also obtained for oxy HbA upon addition of IHP (data not shown). This is in agreement with the data of Adams and Schuster (306). In view of the possible artefactual nature of this effect (307), an alternative method was utilized to probe conformational changes.

The titration of either oxy or deoxy Hb with NEM according to the method of Riggs (162) is shown in Figure 23, and Table 15. The reaction profile measures how much NEM remains after reaction with Hb, i.e., the rate of disappearance of NEM with time. The reaction of NEM with oxy HbA produces a biphasic profile (Figure 23). At 30 minutes the initial reaction rate decreases after 1.8 -SH groups have been modified at a rate of 5 $\mu\text{mol}/\text{minute}$. Presumably this represents the modification of Cys-93(β). The slow-reacting phase represents a reaction rate of 1 μmol NEM with Hb per minute. This could represent reaction with the other two less accessible cysteines, namely, Cys-104(α) and Cys-112(β).

The reaction between NEM and deoxy Hb produces a monophasic reaction profile (Figure 23, top left hand panel) where the rate of modification is about 2 μmol NEM incorporated into Hb per minute. The rate can be explained by the decreased accessibility of Cys-93(β) which is shielded by the salt bridge between His-146(β) and Asp-94(β) or by the slow rate of reaction with Cys-104(α).

Table 15 shows the results of the treatment of unmodified HbA, met HbA, $\alpha_2\text{Cm}\beta_2$, and $\alpha_2\beta_2\text{Cm}$ with NEM under a variety of conditions at pH 6.8. Addition of IHP (in a 2-fold molar excess) to met HbA resulted in a titration profile with NEM that was 'in between' one that was fully oxy or deoxy. This possibly indicates the switch by IHP to some other intermediate conformation. Cys-93(β) in metHbA seems to have become less accessible to modification by NEM in the presence as opposed to the absence of IHP. The conformational change elicited by this organic anion is unclear.

The oxy hybrid $\alpha_2\text{Cm}\beta_2$ (Figure 23, lower right hand panel) exhibits a similar profile to oxy unmodified HbA. Of great interest is the

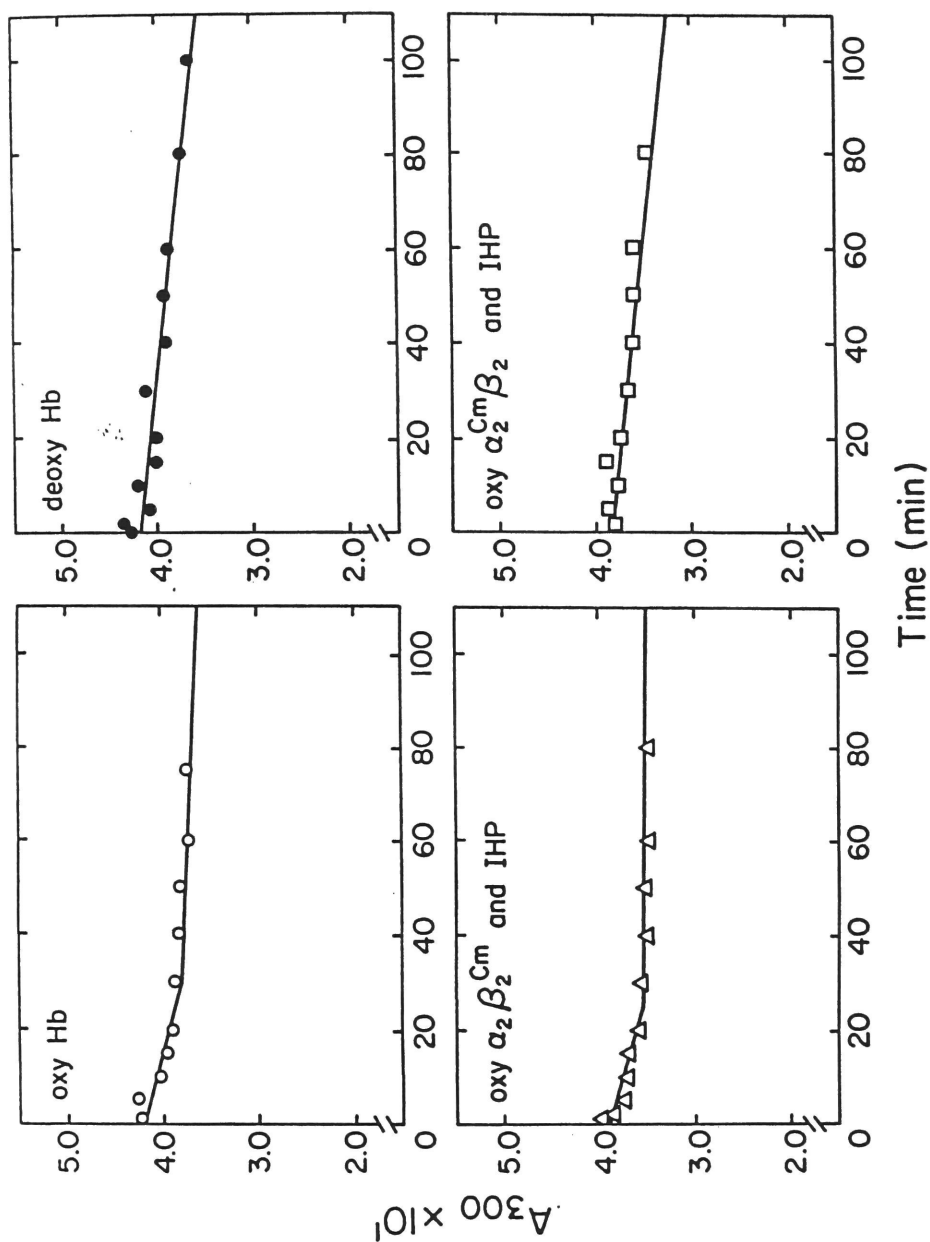


Figure 23 - Hb ($80 \mu\text{M}$) was incubated with N-ethylmaleimide (1.5 mM) at pH 6.8 in 100 mM potassium phosphate. Aliquots were precipitated into TCA as described in the Methods section. After standing at 4°C for a few hours, the samples were centrifuged and the absorbance at 300 nm measured. The decrease in absorbance indicates the incorporation of the NEM into the protein.

#-SH groups modified/Hb tetramer at 30 minutes

1. HbA (oxy)	1.8
2. HbA (oxy) + IHP (2-fold molar excess) + Cl ⁻ (0.1 M)	1.6
3. HbA (deoxy)	0.8
4. Met HbA	2.0
5. Met HbA + IHP (2-fold molar excess) + Cl ⁻ (0.1 M)	* (see below)
6. $\alpha_2\beta_2^{Cm}$ (oxy)	1.8
7. $\alpha_2\beta_2^{Cm}$ + Cl ⁻ (0.1 M)	1.7
8. $\alpha_2\beta_2^{Cm}$ + IHP (2-fold molar excess) + Cl ⁻ (0.1 M)	0.9
9. $\alpha_2\beta_2^{Cm}$ + IHP (5-fold molar excess) + Cl ⁻ (0.1 M)	0.4
10. $\alpha_2\beta_2^{Cm}$ + DHP (2-fold molar excess) + Cl ⁻ (0.1 M)	1.8
11. $\alpha_2\beta_2^{Cm}$ (oxy)	1.7
12. $\alpha_2\beta_2^{Cm}$ + IHP (2-fold molar excess) + Cl ⁻ (0.1 M)	1.9
13. $\alpha_2\beta_2^{Cm}$ + DPG (2-fold molar excess) + Cl ⁻ (0.1 M)	1.8
14. $\alpha_2\beta_2^{Cm}$ + Cl ⁻ (0.1 M)	1.9

* From the linear regressional analysis of the data for met HbA in the presence of IHP the curve could be plotted to give either a deoxy-like or oxy-like profile. This suggests that IHP may have elicited a change in conformation intermediate between R and T. With a correlation coefficient (r) of 0.97 when the profile was 'oxy-like' and a value of 0.99 when the profile was 'deoxy-like'.

Table 15

Data reporting the reaction of unmodified HbA and the hybrids $\alpha_2\beta_2^{Cm}$ and $\alpha_2\beta_2^{Cm}$ toward NEM. The reaction between each Hb sample (80 μ M) and NEM (1.5 mM) was carried out in potassium phosphate pH 6.8 as described in the text.

deoxy-like profile of the $\alpha_2^{Cm}\beta_2$ hybrid in the presence of a 2-fold molar excess of IHP (Figure 23, lower right hand panel). In contrast, the NEM reaction profile of the oxy hybrid, $\alpha_2\beta_2^{Cm}$ in the presence of IHP did not change (Figure 23, lower left hand panel), presumably due to the reduced binding of IHP.

The reaction profile for $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ with NEM under the various conditions described in Table 15 all produced oxy-like profiles. The modification was calculated to correspond to about 2 sulfhydryl groups modified per tetramer in each case.

From these data, therefore, the possibility arises that the carboxymethylation of Val-1(α) causes a slight shift in the R \rightarrow T equilibrium and consequently a change in L, the allosteric constant. If indeed carboxymethylation results in the formation of a carbamino analogue and since CO₂ binds to oxy Hb, then in vivo perhaps CO₂ binding to Val-1(α) also shifts the R \rightarrow T equilibrium.

DISCUSSION

a) Analysis of the Reaction

Hemoglobin is modified to form stable derivatives. This reaction by simple aldehydes such as glycolaldehyde (308), glyceraldehyde (309-312), and glucose (313-316) does not require the presence of a reducing agent because the C-2 hydroxyl in these carbohydrates permits the Amadori rearrangement, which results in a stable adduct. On the other hand, the glyoxylate moiety, which does not have a C-2 hydroxyl group, cannot undergo the Amadori rearrangement and, thus, a reducing agent is needed to stabilize the Schiff base. Indeed, it has been shown that in the absence of a reducing agent very little modification of the protein can be detected.

As reviewed in the Introduction, the relatively broad specificity of α -haloacids necessitated the choice of another reagent for the carboxymethylation of the α -amino groups of Hb. Means and Feeney (2, 13) demonstrated that the procedure of reductive alkylation is selective for the amino groups of proteins with no alkylation of cysteine, histidine, or methionine residues. The lack of reactivity of these latter residues is due to the fact that they do not form a Schiff base adduct with the aldehyde that could be subsequently reduced by sodium cyanoborohydride. Only amino groups are capable of such a reaction. Carboxymethylation of the lysine residues of antigen E from ragweed pollen was achieved by King et al (31) by reductive alkylation with glyoxylate and cyanoborohydride.

In the present study, conditions were defined to limit carboxymethylation to the N-terminal amino groups with minimal

modification of lysine residues. The pK_a values of the N-terminal valines are about 7 in liganded Hb (159). The pK_a of the ϵ -NH₂ group of lysine is about 9.5. Incorporation of this residue within a protein can, however, change this pK_a value depending upon the microenvironment. The reaction between HbA and glyoxylate in the presence of cyanoborohydride was carried out at pH 7.2 to minimize modification of lysine side-chains. Separation of Hb derivatives, carboxymethylated to varying degrees, was possible by ion exchange chromatography due to the incorporation of extra negative charge into the molecule.

A comparison between the extent of reaction of oxy and deoxy Hb on DEAE cellulose provides evidence that the rate of carboxymethylation of deoxy Hb occurred at a faster rate than oxy Hb (Table 4). This enhanced reactivity of deoxy versus oxy Hb toward carboxymethylation will be considered in more detail later in the Discussion.

Carboxymethylated chains were obtained from the Hb₁ and Hb₂ derivatives (Figure 5), where incorporation of [¹⁴C]-glyoxylate was 2 and 4 mols per mol Hb respectively. The results with the Hb₁ derivative showed that about 20% to 30% of the α -chains were carboxymethylated, and that 70-80% of the total β -chains were carboxymethylated. In the Hb₂ derivative, separation of the chains showed that about 60-70% of the α -chains were carboxymethylated and the β -chains were carboxymethylated to an extent of about 100% (Table 5). It appears from these results that the β -chains are modified preferentially to the α -chains when the Hb is in the carbonmonoxy state.

The carboxymethylation was predominantly on the amino terminus as ascertained from the HPLC analysis. At this stage it might be of interest to compare the preferred site of modification by other reagents

with HbA. In the absence of a reducing agent, the preferred N-terminus to be modified by both glyceraldehyde and glucose is Val-1(β) (309, 311, 313-316). (Lys-16(α) is, however, the site most readily modified by these latter two reagents). It may be argued that the low pK_a of the α -amino groups makes this site more prone to modification than the ϵ -amino groups of lysine. Therefore, why is Val-1(α) refractory to modification? It was shown (311) that the environment around Val-1(α) is such that, although Schiff base formation can occur, the Amadori rearrangement occurs at a very slow rate. The idea was proposed that since the Amadori rearrangement is an acid-catalyzed process, a nearby histidine residue could facilitate the reaction at Val-1(β) (311). On the other hand, Shapiro et al (317) proposed a role for the carboxyl groups in the vicinity of the amino groups for catalysis of the subsequent Amadori rearrangement.

Another aldehyde, pyridoxal phosphate also exhibits high selectivity toward the α -amino groups of Hb (318). In oxy Hb, the preferred site of reaction is Val-1(α), whereas in deoxy Hb it is Val-1(β). This pattern of reactivity can be interpreted if pyridoxal phosphate is considered an analogue of 2,3-DPG.

As previously mentioned in the Introduction, Val-1(α) of deoxy Hb is carbamylated at a rate 3 times greater than it is in oxy Hb (57, 285, 304, 305). The mechanism proposed for this enhancement considered the isocyanate anion to have a high affinity for the anion binding site of deoxy HbA (57) (latter corroborated by the X-ray data of deoxy Hb by Arnone (43, 44)) between Val-1(α_1) and Arg-141(α_2). This would serve to increase the effective local concentration of this anion (isocyanate) in close vicinity to Val-1(α) facilitating the reaction at this site.

How does the above discussion relate to the reactivity of glyoxylate toward Val-1(α) or Val-1(β)? When unmodified carbonmonoxy HbA was reacted with glyoxylate and separated on DE-cellulose (Figure 5), carboxymethylation of the β -chain was higher, 74% and 100%, respectively for Hb₁ and Hb₂, whereas only 20% to 30% of the α -chains were carboxymethylated in Hb₁ and 60% to 70% in Hb₂. This was taken to indicate that in carbonmonoxy HbA (and therefore also in oxy HbA), Val-1(β) was carboxymethylated at a greater rate than Val-1(α). Indeed, the kinetic data shown in Figure 20, panel A, and Table 14a support this view, as reaction at Val-1(β) occurs to a greater extent (30%) than at Val-1(α). In oxy Hb perhaps the positive charge at His-2(β), or indeed other positive charges close to Val-1(β) could allow glyoxylate to interact with these residues, thereby increasing the effective local concentration of glyoxylate which would enhance the reaction with Val-1(β). It is noteworthy that in oxy HbA, the residues under discussion form the 2,3-DPG binding cleft which closes up upon the oxygenation of HbA to expel 2,3-DPG. The distance between these positively charged residues could be less in oxy than in deoxy HbA. Indeed it would be of interest to compare the relative rates of carboxymethylation of the α - and β -chains in mutants where substitution of these positively-charged residues by neutral or anionic residues has occurred.

In deoxy HbA, the elution pattern from the DE cellulose chromatography (Table 4) shows that the Hb₁ and, in particular, Hb₂ components have been enriched by about 30% and 43%, respectively. Moreover, the kinetic profile (Figure 20 panel A, and Table 14a) shows that the rate of reaction for deoxy HbA is enhanced by a factor of 4,

and by a factor of 2 and 2.5 for deoxy $\alpha_2^{Cm}\beta_2$ and deoxy $\alpha_2\beta_2^{Cm}$, respectively (although the absolute value for $\alpha_2^{Cm}\beta_2$ is higher). These data can be interpreted with respect to enhanced reactivity of similar magnitude both at Val-1(α) and Val-1(β) in deoxy HbA. These similarities observed could be a function of two factors. First, Val-1(α) participates in an anion binding site with Arg-141(α) (43, 44) and the enhanced reaction rate at this site in deoxy $\alpha_2\beta_2^{Cm}$ may be mechanistically similar to that observed for carbamylation at Val-1(α) in deoxy HbA (57). Second, the enhanced reaction rate observed for deoxy $\alpha_2^{Cm}\beta_2$, i.e., carboxymethylation at Val-1(β), could perhaps be due to either the participation of Lys-82(β) as an anion binding site under 'stripped' conditions in deoxy HbA or else an even greater interaction between His-2(β) with glyoxylate in the deoxy form. Thus, the 4-fold enhancement of the rate of reaction of unmodified deoxy HbA compared with unmodified oxy HbA, is a composite of the enhanced rate of reaction of the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$. These kinetic results will be presented in greater detail later in this section.

b) Identification of Carboxymethylated Residues Within α - and β -chains

In principle the lower pK_a 's of the amino termini would favor carboxymethylation at this site. Even so, certain lysine residues could have an unexpectedly low pK_a and react with glyoxylate. It was, therefore, necessary to establish whether the nature of the N-terminal carboxymethylation was limited to the mono- or di-substituted derivative. In the case of reductive formylation, the dimethylated product is formed preferentially, with the second step proceeding more rapidly than the first (15, 17). A larger size of the aldehyde-

containing compound could reduce the second step on the basis of steric factors. However, King et al (31) under their experimental conditions found that the carboxymethylation of antigen E resulted in a derivative with 10, 6 and 2 residues of monocarboxymethylated, dicarboxymethylated, and unmodified lysyl residues respectively per molecule of protein. In view of their results a system was established in these studies to identify mono- and dicarboxymethylvaline and monocarboxymethyllysine. Moreover, although HPLC analysis of the $[^{14}\text{C}]\text{-}\alpha^{\text{Cm}}$ -chain and $[^{14}\text{C}]\text{-}\beta^{\text{Cm}}$ -chain localized the radioactivity in peptides $\alpha\text{T-1}$ and $\beta\text{T-1}$, it was not possible to decipher from this result whether carboxymethylation was mono- or di-. It was also not possible to quantitate the amount of carboxymethyllysine. Thus, after acid hydrolysis of $[^{14}\text{C}]\text{-}$ carboxymethylated HbA two peaks of radioactivity were detected by amino acid analysis. One of these was in the void volume of the analyzer column and the second corresponded to carboxymethyllysine. Table 4 shows the quantitation of these data. The material in the breakthrough peak was found by Dowex-2 chromatography to represent the monocarboxymethyl derivative which was usually the only product found. Only when non-radioactive $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ was carboxymethylated with $[^{14}\text{C}]\text{-}$ glyoxylate (relevant to the competitive binding experiments) was 14% dicarboxymethylvaline detected (Table 9).

Thus, the purity of the carboxymethylated hybrids $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$, $\alpha_2\beta_2^{\text{Cm}}$, and $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ after rechromatography on CM-52 cellulose (see Results) was established by the following criteria: a) HPLC analysis showed that the $[^{14}\text{C}]\text{-}$ glyoxylate was incorporated into the $\alpha\text{T-1}$ and/or $\beta\text{T-1}$ tryptic peptides (Figure 11), b) after performic acid oxidation of the hybrids, <3% carboxymethyllysine was found (Table 7), c) the absence of

dicarboxymethylvaline was determined from Dowex-2 chromatography (Table 4), d) a non-denaturing gel (Figure 12) showed each hybrid to be a single band after staining with Coomassie Blue.

c) X-Ray Diffraction Data

The rationale behind the specific N-terminal carboxymethylation of Hb was to prepare a stable analogue of CO₂-hemoglobin. The results presented have clearly demonstrated the purity of these analogues. A study of the functional behavior of such analogues has established that at least part of the functional behavior parallels that observed for carbamino adducts. However, before a discussion will be presented with respect to the functional studies, it may be useful to discuss the data from the electron density maps. The X-ray data, performed by Dr. A. Arnone in his laboratory at the University of Iowa, has aided greatly in the interpretation of the data derived from the functional studies.

A comparison between the X-ray data of carbamino HbA and the X-ray data from carboxymethylated HbA makes it possible to see how close the identity of the latter adduct is to a carbamino analogue. As described earlier, in the first X-ray study that was conducted with deoxy Hb CO₂ (223), carbamino formation occurred only with the N-terminal β -chain residues. In this study (223), there was also an unexpected positive difference peak behind the β -haem group. The reason for this observation as well as the absence of a carbamate moiety in the region of Val-1(α) was unexplained and, therefore, further study was necessitated. This initial X-ray study used high CO₂ pressure (1 atm), high organic solvent concentration, and an undetermined amount of

sulfate. In a later study (254) the conditions were modified. The Hb was crystallized out from solutions of polyethylene glycol. This solvent avoids the use of high salt solutions which can perturb local conformations (319). In addition, high salt concentrations represent conditions which are far removed from physiological. The deoxy crystals that formed from the polyethylene glycol solutions with 0.1 M chloride were exposed to a lower pressure of CO₂ (254) (about two and a half times physiological concentration) than in the previous study (223).

In contrast with the former study (223), the latter (254) did show that carbamate formation occurred with Val-1(α), although the positive electron density peaks were less intense than those at the β -chain termini. The carbamate moiety at Val-1(α) seemed to stabilize the deoxy conformation by intramolecular hydrogen bonding to the hydroxyl group of Ser-131. This site did not appear to be an oxygen-linked anion binding site (44). Chloride was present at a concentration of 0.1 M in the polyethylene glycol from which the deoxy Hb crystals were grown. This anion binding site (Val-1(α) to Ser-131(α)) probably has a low affinity for anions because carbamate was able to displace an undetermined amount of the chloride. How does the aforementioned data for carbamino deoxy Hb compare with carboxymethylated deoxy Hb?

Difference electron density maps were plotted for the regions around the α - and β -chain termini respectively from deoxy crystals of the $\alpha_2^{Cm} \beta_2^{Cm}$ hybrid. At the region around the β -chain termini (Figure 13), positive electron density contiguous with Val-1(β) was observed. In addition, it appeared that the anion normally bound between Val-1(β) and Lys-82(β) had been displaced by the carboxymethyl group. This result is analogous to carbamate formation at the same

locus. Also, there is a pair of negative/positive peaks which indicated that the side-chain of His-2(β) rotates approximately 180° about its $C_\alpha-C_\beta$ bond so that it can also interact with the carboxymethylated β -chain terminus.

At the α -chain termini the carboxymethyl group appears as a region of positive density contiguous with Val-1(α) and appears to interact with Ser-131(α) (Figure 14). In addition, an anion seems to have been displaced from the region between Val-1(α_1) and Arg-141(α_2). The interaction with Ser-131(α) is analogous to the interaction observed with the CO_2 adduct at Val-1(α). Thus, by the criterion of X-ray crystallography, carboxymethylated Hb at the amino termini could be considered as a carbamino analogue.

Although not directly relevant to the thesis presented, more information still, can be gleaned from the electron density profiles in the region of the DPG binding cavity. Three alternating negative/positive pairs of difference electron density peaks show that a movement of Val-1(β) towards Leu-3(β) shifts that residue and the beginning of the A-helix resulting in a perturbation of Glu-6(β). A larger shift of the β -chain N-terminal residue in the same direction occurs in deoxy Hb when the α -amino group is carboxymethylated and this could cause the increased solubility of carboxymethylated HbS (320) as suggested by Dr. A. Arnone. It is therefore of interest and completely consistent with this suggestion that the minimum gelling concentration of carboxymethylated HbS is increased from 16 g/l to 25 g/l (320). In contrast, carbamate formation at Val-1(β) does not effect the environment of Glu-6(β) and therefore would not be expected to influence gelation of HbS (223). In this respect, therefore, carboxymethylation

differs from carbamate formation. Yet another feature present in the electron density map of carboxymethylated Hb is the absence of any perturbation of residues Phe-85(β) and Leu-88(β). Since these residues interact with Val-6(β) in deoxy HbS fibers, the increase in the minimum gelling concentration of carboxymethylated HbS (223) is probably due solely to the perturbation of Glu-6(β) as described above.

d) Effect of Carboxymethylation on the Functional Properties of Hb

Oxygen Affinity - The oxygen affinity of the unmodified and carboxymethylated hybrids decreases in the order $\alpha_2\beta_2$ (7 mm Hg), $\alpha_2^{Cm}\beta_2$ (12 mm Hg), $\alpha_2\beta_2^{Cm}$ (17 mm Hg), and $\alpha_2^{Cm}\beta_2^{Cm}$ (37 mm Hg). Thus, the decrease in oxygen affinity observed upon carboxymethylation is similar to the observed effect whereby increased amounts of CO_2 also cause a decrease in oxygen affinity of unmodified HbA (classical Bohr effect (136)). Although the effect of CO_2 on decreasing the oxygen affinity was at first thought to be a pH effect (136), later studies confirmed that carbamino formation with the N-terminal amino groups occurred, and caused oxygen affinity to decrease (237, 238). Thus, CO_2 per se can decrease the oxygen affinity. In this case, the lowest oxygen affinity is associated with the greatest amount of carboxymethylation.

That carboxymethylation at Val-1(α) elevates the P_{50} value from 7 mm Hg in $\alpha_2\beta_2$ to 12 mm Hg in $\alpha_2^{Cm}\beta_2$ can be interpreted from the X-ray data. The difference electron density map of the $\alpha_2^{Cm}\beta_2^{Cm}$ hybrid in the deoxy state, suggests that there is an intramolecular hydrogen bond between the carboxymethyl group and the hydroxyl group of Ser-131(α). Thus, by analogy with carbamate formation at Val-1(α) (254), carboxymethylation at Val-1(α) could stabilize the deoxy conformation and

thereby shift the $R \rightarrow T$ equilibrium towards a lower oxygen affinity T state. This result is in contrast to carbamylation at Val-1(α) where an increase in oxygen affinity is observed (265). The removal of positive charge by carbamylation at Val-1(α) was seen to preclude the formation of a salt bridge between Val-1(α_1) and Arg-141(α_2) in deoxy Hb (43). As a consequence, the R conformation should be favored relative to the T, i.e., a decrease in L, the allosteric constant resulting in an increased oxygen affinity (265).

The introduction of the $-\text{CH}_2\text{COO}^-$ moiety at Val-1(β) decreases the net positive charge of the 2,3-DPG binding pocket. In addition, the interaction of this negative charge with Lys-82(β), as seen from the deoxy difference electron density map of carboxymethylated Hb, reduces the positive charge further. This decrease in oxygen affinity could be explained by the hypothesis of Bonaventura and Bonaventura (61), and Perutz et al (111). The hypothesis suggested that the allosteric constant L is lowered by modifications or mutations which serve to reduce the repulsive energy between the cationic groups of the 2,3-DPG binding cleft. Thus, as previously discussed in the Introduction, the replacement of a cationic residue for one that is neutral or negatively charged should decrease this repulsive energy and stabilize the T conformation with a resultant decrease in oxygen affinity. By this criterion, the introduction of positive charge into this central cavity should destabilize the T state and increase the oxygen affinity. Examples of the former class of Hb's, where the oxygen affinity is decreased include Hb Helsinki (Lys-82(β) \rightarrow Met) (115), Hb Providence (Lys-82(β) \rightarrow Asn/Asp) (61, 71), Hb Raleigh (Val-1(β) \rightarrow Acetyl Ala) (68), and Hb carbamylated at Val-1(β) (69). On the basis of the above

argument, Hb carboxymethylated at Val-1(β), could be predicted to have a lowered oxygen affinity due to a stabilization of the T state by removal of positive charge from the 2,3-DPG binding cleft. Moreover, as previously discussed, binding of anions within this cleft decreases the oxygen affinity by decreasing the repulsive interactions between the positively charged residues and thus stabilizes the T conformation.

In light of the above discussion, the carboxymethyl moiety at Val-1(β) may be considered as a covalently bound anion. Such an attachment should be more effective in lowering the oxygen affinity than the presence of an anion which can interact only electrostatically. This increase in efficiency by carboxymethylated Val-1(β) in lowering the oxygen affinity may be considered semi-quantitatively. As reported in the Methods section, the concentration of Hb for functional studies was in the range of 0.32 mM to 0.48 mM. Suppose the $\alpha_2\beta_2^{\text{Cm}}$ sample was at a concentration of 0.4 mM in tetramer, then the concentration of the carboxymethyl anion covalently attached would be 0.8 mM. The oxygen affinity of this sample is 17 mm Hg. From Figure 17, it is possible to see very readily that the P_{50} of $\alpha_2\beta_2$ (which can be assumed to also be 0.4 mM) does not change (or else the technique employed is not sufficiently sensitive to detect a very small change in P_{50}) in the presence of 0.8 mM Cl^- (assuming, of course, that all of the chloride bound to hemoglobin). In fact from Figure 17, a chloride concentration of about 0.2 M is required to elevate the P_{50} of $\alpha_2\beta_2$ from 7 mm Hg to 16 mm Hg. Thus, the covalent attachment of the carboxymethyl moiety at Val-1(β) is 25 times more effective than a comparable concentration of chloride in the stabilization of the T conformation and as a consequence causes a decrease in the oxygen affinity.

The aforementioned mechanisms as to how the carboxymethyl moiety at the α - and β -chain termini may lower the oxygen affinity could also be applied to carbamate formation in vivo. However, in vivo not all the Val-1(β) sites would have carbamate attached to them. In the erythrocyte Hb is 5 mM and only 30% (estimates vary from 10% to 60%) of the total CO_2 produced by respiring tissues reacts with the N-terminal amino groups (136). Thus, within the erythrocyte the concentration of N-terminal groups available for carbamate formation is 20 mM. Carbamino formation in arterial blood occurs at a concentration of 0.81 milliequivalents of CO_2 per liter of whole blood, whereas for venous blood the value is 1.39 milliequivalents of CO_2 per liter of whole blood (136). This corresponds to a value of a 4% and 7% modification by CO_2 of the N-terminal amino groups for arterial and venous blood, respectively. The P_{50} of unmodified stripped HbA (in 0.1 M Cl^- at pH 7.2) in the absence and presence of physiological concentrations of CO_2 , is 12 mm Hg and 19 mm Hg respectively (321). Thus, the fractional modification is very small and yet the change in P_{50} relatively large. This seems to indicate that non-saturating carbamate formation at the N-terminal amino groups of both α - and β -chains can cause considerable stabilization of the T state, although the presence of the other allosteric modulators should not be neglected.

The extremely low oxygen affinity of $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$ (37 mm Hg) is greater than the sum of the lowered oxygen affinities observed for $\alpha_2^{\text{Cm}}\beta_2$ (12 mm Hg) and $\alpha_2\beta_2^{\text{Cm}}$ (17 mm Hg) compared with $\alpha_2\beta_2$ (7 mm Hg). This is in contrast to a hybrid where all four terminals are carbamylated and the P_{50} value of 2.6 mm Hg is an average of the value for $\alpha_2^{\text{Cb}}\beta_2$ (2.2 mm Hg) and $\alpha_2\beta_2^{\text{Cb}}$ (4.8 mm Hg) (266). The very low

oxygen affinity of $\alpha_2^{Cm}\beta_2^{Cm}$ could be related to the biphasic nature of the Hill plots. At low oxygen tensions between pO_2 10 mm Hg to 30 mm Hg the value for Hill's constant is 1.6. Possibly the first oxygen molecule binds to the $\alpha_2^{Cm}\beta_2^{Cm}$ derivative with great difficulty so that k_1 , the Adair constant for the binding of the first oxygen molecule, is lowered. At this stage, however, this hypothesis remains conjectural. It would have to be tested by extremely accurate oxygen binding measurements made at low oxygen tension such as were carried out by Kilmartin et al (322).

Thus, from the above discussion it is readily apparent that carboxymethylation at either of the two α -chain termini or β -chain termini or at all four resulted in a diminished oxygen affinity. The lowered oxygen affinity is consistent with the same effect observed for unmodified HbA in the presence of increased amounts of CO_2 . From this observation, in addition to the similar electron density profiles plotted for carbamino and the carboxymethylated hemoglobin hybrids, $\alpha_2^{Cm}\beta_2$, $\alpha_2\beta_2^{Cm}$, and $\alpha_2^{Cm}\beta_2^{Cm}$ these two hemoglobin derivatives could be considered as analogues of one another.

Thus, the next question to ask is, if a CO_2 analogue ($-CH_2COO^-$) is bound at either Val-1(α), Val-1(β), or at both, what would be the effect of other allosteric modulators (2,3-DPG, chloride, and protons) on the functional properties of these adducts?

Effect of 2,3-DPG on Oxygen Affinity - From the 2,3-DPG titration curves Figure 16, the availability of binding sites for this organic phosphate is comparable for $\alpha_2\beta_2$ and $\alpha_2^{Cm}\beta_2$. Addition of 2,3-DPG to $\alpha_2^{Cm}\beta_2$ lowers its oxygen affinity about 4-fold (from 12 mm Hg to 48 mm Hg), which is about the same degree to which addition of 2,3-DPG

lowers the oxygen affinity of unmodified Hb (from 7 mm Hg to about 33 mm Hg). The equivalence point of the titrations for both $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2$ is greater than one, most likely because the measurements were carried out in the absence of chloride, so there may be some non-specific binding of 2,3-DPG (83, 89-91), as discussed in the Introduction and the Results sections. The absolute P_{50} values are, however, greater for $\alpha_2^{Cm}\beta_2$. This indicates that the presence of the carboxymethyl moiety at Val-1(α) causes an additional decrease in oxygen affinity in the presence of 2,3-DPG compared to the value for unmodified Hb in the presence of 2,3-DPG.

The reduced inability of 2,3-DPG to lower the oxygen affinity of $\alpha_2\beta_2^{Cm}$ or $\alpha_2^{Cm}\beta_2^{Cm}$ is in accord with the fact that Val-1(β) is a site for binding of this organic phosphate (62). This result is also consistent with the diminished response toward 2,3-DPG of other hemoglobins where Val-1(β) is blocked (85), such as HbA_{1c}, HbF and Hb Raleigh (Val-1(β) \rightarrow Acetyl-ala). It seems as if the presence of the carboxymethyl group at Val-1(β) precludes access of 2,3-DPG toward the other residues within the 2,3-DPG binding cleft. The binding of 2,3-DPG to $\alpha_2^{Cm}\beta_2^{Cm}$ saturates at a P_{50} value of 50 mm Hg, which is the same as for $\alpha_2^{Cm}\beta_2$.

Effect of Chloride on Oxygen Affinity of Hybrids - The chloride titration curves (Figure 17) show the effect of increasing concentrations of this anion on the oxygen affinity. As seen from Figure 17, the effect of chloride on the $\alpha_2^{Cm}\beta_2$ hybrid is diminished by about 13%, an indication that binding of this anion is slightly impaired. This value is derived by a comparison between the $\Delta \log P_{50}$ (the ratio of P_{50} measured in chloride-free buffer to that measured in

the presence of 1 M chloride) for $\alpha_2^{Cm}\beta_2$ compared with $\alpha_2\beta_2$. This result may be interpreted with respect to the X-ray data seen for the carboxymethylation at the α -amino terminus. As stated above, the carboxymethyl moiety attached to Val-1(α) interacts with Ser-131(α). This would still allow chloride to bind between Val-1(α) and the guanidinium side-chain of Arg-141(α_2) (43, 44). This latter anion binding site is oxygen-linked (43, 44). There could be three possible interpretations as to why the binding of chloride to $\alpha_2^{Cm}\beta_2$ is diminished by about 13%. As discussed in the Results section, the X-ray data represents a static state of affairs and shows an interaction between Val-1(α_1) and Ser-131(α_1). Perhaps in solution there is an equilibrium between the interaction of the carboxymethyl group with both Arg-141(α_2) or Ser-131(α_1) and only the latter interaction was seen from the X-ray data. A second interpretation is that the binding of chloride is impaired at the Val-1(α_1) to Arg-141(α_2) site because of repulsive energy between chloride and the larger $-\text{CH}_2\text{COO}^-$ moiety, covalently attached to Val-1(α). A third possible interpretation is that chloride could be accommodated between Val-1(α_1) and Arg-141(α_2), but somehow the overall binding is weaker. Previous estimates have attributed a contribution by Val-1(α) to chloride binding of about 20% (60). Carboxymethylation seems to have reduced this contribution by about one half. It may be of interest to discuss from where this estimate of a 20% contribution by Val-1(α) is derived. Poyart et al (60) compared chloride titration data of both unmodified HbA and $\alpha_2^{Cbm}\beta_2$, where Val-1(α) is carbamylated. The data from these chloride titrations were then submitted to a computer analysis to obtain a plot of $\Delta[\text{Cl}^-]$ per heme versus chloride concentration. This analysis showed that chloride

binding had been diminished by 20% in $\alpha_2^{Cb\beta_2}$ compared with $\alpha_2\beta_2$ (60). Poyart et al (60) also showed that the oxygen affinity of $\alpha_2^{Cb\beta_2}$ was still dependent on chloride concentration even at concentrations below 10 mM of this anion. This result is at variance with the data of O'Donnell et al (44). In their study (44), the oxygen affinity of $\alpha_2^{Cb\beta_2}$ did not change when chloride was less than 10 mM. This may be interpreted to indicate that carbamylation of Val-1(α) eliminates a high affinity chloride binding site (44). In other words low chloride concentration and carbamylation of Val-1(α) could be considered as functionally equivalent. In the same study (44) at a chloride concentration greater than 10 mM, the $\Delta \log P_{50}$ was the same for both $\alpha_2\beta_2$ and $\alpha_2^{Cb\beta_2}$. The data of Manning et al (59) compared $\log P_{50}$ versus chloride concentration for $\alpha_2\beta_2$ and $\alpha_2^{Cb\beta_2}$. $\Delta \log P_{50}$ (between 10 mM and 1000 mM) for $\alpha_2\beta_2$ and $\alpha_2^{Cb\beta_2}$ indicated that chloride binding had been reduced by about 25% for $\alpha_2^{Cb\beta_2}$. At a chloride concentration below 10 mM the difference between the P_{50} of $\alpha_2\beta_2$ and the P_{50} of $\alpha_2^{Cb\beta_2}$ was reduced compared to the difference above this chloride concentration and this latter result is in partial agreement with the result of O'Donnell et al (44). X-ray data of Hb crystals grown at low ionic strength could perhaps help to elucidate these discrepancies. (The crystals in the study by O'Donnell et al (44) were grown from concentrated ammonium sulfate solutions.) In another estimate of the contribution of Val-1(α) to chloride binding, Adachi et al (74) found that when the Lys-82(β) residues of Hb York (His-146(β) \rightarrow Pro) were cross-linked, the chloride binding that remained was consistent with a contribution from Val-1(α). Supposedly, in this derivative, Lys-82(β) and His-146(β), which are estimated in the study to make a contribution

of 80% to oxygen-linked chloride binding, are prevented from doing so. The only contribution to chloride binding can come from Val-1(α) (which is unmodified.)

In this thesis it appears that the carboxymethylated hybrid $\alpha_2^{Cm}\beta_2$ has a partially inhibited or a slightly weaker binding for chloride. This situation is different from carbamylation of Val-1(α) which inhibits chloride binding at this site completely (44). Possibly, under physiological conditions carbamate formation at Val-1(α) could result in a similar inhibition or weakening of chloride binding as observed for carboxymethylation at the same locus. In vivo, however, only a small fraction of the total available Val-1(α) sites would react with CO_2 . Also the contribution of Val-1(α) to the alkaline Bohr effect (to be discussed later) must not be neglected. In the studies reported in this thesis the P_{50} values for $\alpha_2\beta_2$ and $\alpha_2^{Cm}\beta_2$, in the absence or the presence of 10 mM chloride, were virtually the same. Possibly the Hem-O-Scan apparatus employed for these measurements was not precise enough to produce the differences observed by other experiments (60).

The $\Delta \log P_{50}$ value (defined earlier in this section) for the hybrid $\alpha_2\beta_2^{Cm}$ compared to that value for $\alpha_2\beta_2$ shows that the effect of chloride on P_{50} for this derivative has been reduced by about 28%. Examination of the X-ray crystallography map for deoxy $\alpha_2^{Cm}\beta_2^{Cm}$ at the region of the β -chain terminus can help to interpret this observed decrease in anion binding. There seems to be an interaction between the negative charge of the carboxymethyl group attached to Val-1(β) with Lys-82(β), that would preclude or decrease chloride binding to the latter site. The chloride titration data for a Hb tetramer carbamylated at Val-1(β) showed that since there was no difference in the effect of

chloride on the P_{50} for $\alpha_2\beta_2$ and $\alpha_2\beta_2^{Cbm}$, Val-1(β) was not an anion binding site (69). By inference a tentative identity for a site involved in chloride binding was assigned to Lys-82(β) (69). Direct evidence for this suggestion came from studies on Hb Providence (70) and Rahere (71) as well as NMR studies (73). Moreover, Nigen et al (72) abolished 80% of chloride binding in a hybrid, where Val-1(α) was carbamylated and the β -chain was derived from Hb Providence Asn, where lysine-82(β) is substituted by asparagine. Since as discussed above Val-1(α) makes a 20% contribution to chloride binding, the remaining 60% must be derived from Lys-82(β) from this study (72). Adachi et al (74) calculated a contribution of 40% by Lys-82(β) to chloride binding. The value of 28% reported in this study for $\alpha_2\beta_2^{Cm}$ could be interpreted with respect to binding of fewer chloride ions or with respect to a weaker binding of chloride at Lys-82(β). If $\alpha_2\beta_2^{Cm}$ could be considered as an analogue of $\alpha_2\beta_2^{carbamate}$, then a similar reduction in chloride binding at Lys-82(β) may be observed if CO_2 was bound to Val-1(β) in the absence of 2,3-DPG. Moreover, mutant Hb's where Lys-82(β) has been substituted for by another residue exhibit decreased chloride binding (61). Chloride binding of Hb Providence Asn or Asp (Lys-82(β) \rightarrow Asn/Asp) exhibit a 40% and 80% diminution respectively (61). The difference observed for the inhibition in Hb Providence Asn and Hb Providence Asp maybe related to the nature of the substitution, a neutral residue in the former mutant and one that is negatively charged in the latter.

There is probably no physiological relevance with regard to chloride binding at Lys-82(β). In vivo Lys-82(β) will be occupied by 2,3-DPG in deoxy Hb. Thus, oxygen-linked anion binding to this residue will be precluded under physiological conditions. Whether chloride

binding occurs to any significant extent at Lys-82(β) in oxy Hb after 2,3-DPG has been discharged from its binding cleft remains to be determined, especially with regard to a change in L, the allosteric constant.

The effect of chloride on the P_{50} value of the tetracarboxymethylated hybrid $\alpha_2^{Cm}\beta_2^{Cm}$ is reduced by 80%, which is considerably greater than addition of the 14% and 28% reduction observed for $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$, respectively. The reason for this remains conjectural. However, it may be related to the biphasic Hill plots for the $\alpha_2^{Cm}\beta_2^{Cm}$ hybrid which perhaps is an indication of reduced heme-heme interaction. Since the oxygen affinity of $\alpha_2^{Cm}\beta_2^{Cm}$ is so low (37 mm Hg) compared with $\alpha_2\beta_2$ (7 mm Hg), the T structure could be stabilized to an extent such that chloride confers very little extra stability on this hybrid. Indeed, the stabilization seems to be greater than the sum of the 'individual' increases in stability of the T state as observed from the P_{50} values of $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$.

Alkaline and Acid Bohr Studies

Alkaline Bohr Effect Under Conditions of Low Salt - In the studies presented, the Bohr effect was measured by two techniques, proton titration and/or change in oxygen affinity with respect to pH measured on an Aminco Hem-O-Scan. We chose to place greater emphasis upon data obtained from the former technique. With the latter technique the P_{50} value of a sample is measured to ± 1 mm Hg, and this can greatly influence the value of the slope $\Delta \log P_{50} / \Delta pH$. The fall in oxygen affinity with respect to an increase in pH is the Bohr effect and the uptake of protons with respect to pH is the Haldane effect. However

under physiological conditions both measurements may be considered functionally equivalent (111). The first part of this Discussion on the Bohr effect will be focused on results measured for the alkaline Bohr effect. This measures the uptake of protons or the change in P_{50} with pH at pH values above 6. Below pH 6, protons are absorbed upon oxygenation and the oxygen affinity rises with a fall in pH. This is the acid or reverse Bohr effect.

The limitations arising from the leakage of chloride by the electrode that were used made it virtually impossible to measure the alkaline Bohr effect in the complete absence of chloride as discussed in the Results section. The lowest possible concentration of chloride that could be achieved in these studies was between 1.2 and 1.6 mM chloride. In the studies of others (56, 296), the Bohr effect measured under conditions of 'low salt' usually means 5 mM chloride, but it is not apparent whether chloride leakage was taken into account in proton titration experiments (no mention of this problem was made).

At the chloride concentrations of 1.2 to 1.6 mM in these studies the alkaline Bohr effect for $\alpha_2\beta_2$ and $\alpha_2^{Cm}\beta_2$ was -0.39 and -0.40 protons released per heme, respectively (at 5 mM chloride, O'Donnell et al (44) reported a value of -0.36 protons released per heme (44)). From the X-ray data of the carboxymethylated $\alpha_2^{Cm}\beta_2^{Cm}$ hybrid in the deoxy form, an inspection of the region around Val-1(α) suggests that the carboxyl group of the covalently attached carboxymethyl moiety may interact intramolecularly with Ser-131(α) as stated previously. This would leave the anion binding site between Val-1(α_1), and Arg-141(α_2) vacant. It is the binding of chloride at this locus that elevates the pK of Val-1(α) in unmodified HbA making it an alkaline Bohr group (44). Thus, under

conditions of low ionic strength, $\alpha_2\beta_2$ and $\alpha_2^{Cm}\beta_2$ could both be considered to be functionally equivalent with regard to the alkaline Bohr effect. The value reported for the alkaline Bohr coefficient for each sample seems to support this hypothesis.

The determination of the alkaline Bohr coefficient for $\alpha_2\beta_2^{Cm}$ yields a value of -0.26 protons released per heme. This represents a reduction of about 30% compared with the value obtained for unmodified Hb. Again the X-ray data of deoxy $\alpha_2^{Cm}\beta_2^{Cm}$ in the region of the two β -chain termini are helpful in the interpretation of the lowered value. The covalent attachment of $-\text{CH}_2\text{COO}^-$ to Val-1(β) results in an electrostatic interaction between the negative charge of the carboxyl group with the positively charged Lys-82(β). In other words the net positive charge within the 2,3-DPG binding crevice has been reduced. In the Hb mutants, Providence Asn and Providence Asp, the positive charge within the cavity has been reduced in both cases. Bonaventura et al (61, 66) measured the alkaline Bohr coefficient for both mutants. Their determinations were made in 50 mM bis tris buffers containing low concentrations of chloride. Under these conditions the alkaline Bohr coefficient was reduced by 30% for Hb Providence Asn and 50% for Hb Providence Asp. A comparison can be made between $\alpha_2\beta_2^{Cm}$ and Hb Providence Asn and/or Hb Providence Asp. Thus, under conditions of low ionic strength, a reduction in the positive charge in the 2,3-DPG binding cavity reduces the intrinsic Bohr effect. How is this manifested? The hypothesis formulated by Bonaventura et al (66) suggested that in the absence of a positively charged group at position 82(β), the pK_a value for His-143(β) could be lowered in the deoxy compared to the oxy conformation. This effect would act in opposition

to the normal alkaline Bohr effect and cause protons to be absorbed on oxygenation. Perhaps, then in the absence of chloride, the interaction between the carboxymethylated β -chain and Lys-82(β) reduces repulsive energy between the positively charged residues of the 2,3-DPG binding cleft with the result that the pK_a of His-143(β) is lowered and absorbs protons upon oxygenation. Since the 2,3-DPG binding cleft closes up upon oxygenation, His-143(β) is perhaps at a closer distance to the Lys-82(β) residue and perhaps also to the carboxymethyl moiety that is attached to Val-1(β) in $\alpha_2\beta_2^{Cm}$. X-ray crystallography of oxy $\alpha_2\beta_2^{CmCm}$ would perhaps verify this. Possibly carbamate formation at Val-1(β) has a similar effect on the pK_a value of His-143(β). Thus, under conditions of low salt, CO_2 could lower the Bohr effect of unmodified HbA due to carbamate formation at Val-1(β), which could be analogous with the carboxymethyl moiety at Val-1(β).

Under conditions of low salt $\alpha_2\beta_2^{CmCm}$ exhibits an alkaline Bohr effect that is reduced by 30% compared with unmodified HbA. As already discussed the hybrid $\alpha_2\beta_2^{Cm}$ can be considered to be functionally equivalent to $\alpha_2\beta_2$ with regard to the Bohr effect. Carboxymethylation of Val-1(α) in the $\alpha_2\beta_2^{CmCm}$ hybrid should not be expected to exert effects any different to those observed for $\alpha_2\beta_2^{Cm}$ under conditions of low salt. For this reason $\alpha_2\beta_2^{CmCm}$ maybe considered to be equivalent to $\alpha_2\beta_2^{Cm}$ with respect to the alkaline Bohr effect under conditions of low salt.

Alkaline Bohr Effect Under Conditions of Physiological Salt Concentration - The change in the alkaline Bohr effect under the conditions of 0.1 M chloride is considered for unmodified HbA as well as for the hybrids $\alpha_2\beta_2^{Cm}$, $\alpha_2\beta_2^{Cm}$, and $\alpha_2\beta_2^{CmCm}$. The 25% increase in the

alkaline Bohr effect for unmodified HbA in the presence of 0.1 M chloride is consistent with reports from other laboratories (44, 56). Indeed Rollema et al (56) observed that the Bohr effect increased to a maximum value as the chloride concentration was raised from 5 mM to 100 mM and then declined as the chloride concentration continued to be raised to 2 M. Why such a vast excess of chloride (0.1 M, Hb 60 μ M in these studies) is required for the 'full' Bohr effect to be manifested seems hard to comprehend. Perutz et al (111) gave evidence to suggest that under such conditions and in the absence of organic phosphate, chloride binding to Lys-82(β) is thought to contribute to about 20% to 30% of the alkaline Bohr effect. The conclusion was reached primarily from studies on Hb mutants with an alternative residue at Lys-82(β). In a particularly striking example, a comparison was made between the response of the alkaline Bohr effect to chloride concentrations between 0 and 0.1 M for unmodified HbA and Hb Helsinki (Lys-82(β) \longrightarrow Met) (111). The resultant curves are biphasic, consistent with two kinds of binding sites. The high affinity binding sites most probably lie between Val-1(α_1) and Arg-141(α_2) (111). Hb Helsinki retained the high affinity binding sites which produced the steep part of the curve below 0.1 M chloride, but most of the weak chloride binding sites, whose contribution is dominant above 0.01 M chloride were lost (111) and were attributed to Lys-82(β) which is missing in Hb Helsinki. Moreover, Manning et al (59) showed that when both high and low affinity chloride binding sites were blocked, as in the hybrid tetramer $\alpha_2^{Cbm} \beta_2^{Hb Providence Asn}$, there was only a very weak dependence of oxygen affinity on the concentration of chloride. What seems hard to consolidate at first, however, is if there is a 20-30% contribution made

by Val-1(α) and a similar contribution by Lys-82 why is the Bohr effect not reduced by 40-60% at low salt? The answer may be due to an increased contribution to the alkaline Bohr effect made by His-146(β) at low salt (197). Thus, depending on the concentration of salt and ionic conditions, the percentage contribution from specific residues changes.

A 20% enhancement is observed for the alkaline Bohr effect of $\alpha_2^{Cm}\beta_2$ when the chloride concentration is increased from about 1.6 mM to 100 mM. This is very similar to the 26% increase observed for unmodified HbA under similar conditions. This necessarily implies that the differential pK_a value of the secondary amino function of the carboxymethylated Val-1(α) in deoxy and oxy $\alpha_2^{Cm}\beta_2$ is the same as the primary amine of Val-1(α) in unmodified HbA. Any change in the pK_a value of the secondary amine that might have occurred, presumably did not preclude chloride binding, which accounts for the 20% enhancement of the alkaline Bohr effect observed for $\alpha_2^{Cm}\beta_2$ in the presence of 0.1 M chloride.

In light of the above result what predictions could be made with regard to the pK_a of the resultant secondary amine with a carboxymethyl moiety attached? A change in pK_a value of an α -amino group upon reductive methylation is seen in the values given for a series of amino acids and small peptides (17). What is apparent is that any changes in pK_a are small, 0.2 to 0.5 units. However, this change can represent an elevation or decrease in pK_a depending on the compound. In a protein the situation is even more complex. Presumably, the difference in pK_a between an amino or methylamino moiety reflects differences in the extent to which the corresponding cations are stabilized by hydration and this, in turn, must be dependent upon the microenvironment that

surrounds the residue in question. This last statement is also relevant to an α -amino function within a protein that is carboxymethylated. Since the alkaline Bohr coefficient of $\alpha_2^{Cm}\beta_2$ is virtually the same as for $\alpha_2\beta_2$ the pK_a value of the secondary amine at this residue is probably changed to only a small extent. Whether this represents an elevation or decrease in pK_a compared to the pK_a of a primary amine can only be ascertained directly by titration. This was not performed in these studies.

In the presence of 0.1 M chloride, the alkaline Bohr coefficient of $\alpha_2\beta_2^{Cm}$ is elevated from +0.26 to +0.38 which corresponds to an increase of about 30%. The binding of chloride between Val-1(α_1) and Arg-141(α_2) with the subsequent increase in pK_a of Val-1(α) in the deoxy compared with the oxy conformation could account for this elevation of the alkaline Bohr coefficient. However, the value of the Bohr coefficient for $\alpha_2\beta_2^{Cm}$ of +0.38 protons released per heme in 0.1 M chloride is still lower by about 28% than the value for unmodified HbA under similar conditions.

As stated previously chloride binding to Lys-82(β) can contribute to the alkaline Bohr effect. Another question that needs to be considered therefore is by what mechanism this occurs. The suggestion was made by Perutz et al (111) that either Lys-82(β_1) and Lys-82(β_2) are bridged by a hydrated chloride ion or else that hydrated chloride ions take up various positions between the hydrated ϵ -amino groups of the lysines. Furthermore, as more chloride ions are accommodated between the two Lys-82 residues in the deoxy conformation, so more water molecules could bind after their protonation. As a consequence the suggestion was made that the increased pK_a observed for the water

molecules that bind chloride within the 2,3-DPG cleft in the T state contribute to the alkaline Bohr effect (111).

How can the 28% reduction in the alkaline Bohr effect of $\alpha_2\beta_2^{Cm}$ compared to unmodified Hb in 0.1 M chloride be interpreted in light of the above discussion? The X-ray data indicate that the carboxymethyl group covalently attached to Val-1(β) may interact electrostatically with Lys-82(β). Possibly, then the binding of chloride with the subsequent hydration reaction described above are precluded or weakened on both steric and electrostatic grounds. Thus, to briefly compare the alkaline Bohr coefficient for $\alpha_2\beta_2^{Cm}$ in low or physiological concentrations of chloride, the influence of His-143(β) in opposing the alkaline Bohr effect could be responsible for the observed decreases under both sets of conditions. Preclusion of chloride binding to Lys-82(β) under conditions of 0.1 M chloride would not allow compensation for the negative contribution of His-143(β) to the alkaline Bohr effect. In addition, carboxymethylation has not resulted in any changes in conformation that might impair chloride binding to the unmodified Val-1(α).

As discussed earlier the parallel Bohr effects observed for unmodified HbA and $\alpha_2^{Cm}\beta_2$ could perhaps allow $\alpha_2\beta_2^{Cm}$ and $\alpha_2^{Cm}\beta_2^{Cm}$ to be considered as functionally equivalent with regard to the Bohr effect. The 26% increase in the alkaline Bohr effect observed for $\alpha_2^{Cm}\beta_2^{Cm}$ in 0.1 M chloride is in close agreement with the increase observed for $\alpha_2\beta_2^{Cm}$ under identical conditions.

Alkaline Bohr Effect in the Presence of 0.5 M Chloride - The decrease in the alkaline Bohr effect observed as the concentration of chloride is raised from 0.1 M to the unphysiological conditions of 2.0 M

(56) may be interpreted with respect to an increase in anion binding to oxy Hb at high chloride concentrations. This necessarily means that at high chloride concentrations the protons absorbed by oxy Hb will serve to lower the difference in protons absorbed or released by oxy and deoxy Hb. Rollema et al (56) measured a 14% decrease in the alkaline Bohr effect when the chloride concentration was elevated from 0.1 M to 0.5 M. In the studies reported here this decrease in the alkaline Bohr coefficient could not be reproduced. Only a small (4%) decrease was recorded, which is most likely not significant. The reasons for this discrepancy are unclear.

The Bohr coefficient determined for $\alpha_2^{Cm}\beta_2$ did not exhibit a decreased value in 0.5 M chloride as observed by Rollema et al (56) for unmodified Hb. This does not differ for the value of $\alpha_2\beta_2$ (discussed above) and is consistent with the proposal that considers $\alpha_2^{Cm}\beta_2$ as functionally equivalent to $\alpha_2\beta_2$ with respect to the alkaline Bohr effect.

The Bohr coefficient of the hybrid $\alpha_2\beta_2^{Cm}$ does not change from its value of +0.38 protons released per heme when the chloride concentration is elevated from 0.1 M to 0.5 M. In addition, this value is still about 26% lower than the value measured for unmodified Hb under identical conditions. Whether the more dilute Hb solutions here (60 μ M) compared to those used by Rollema et al (150 μ M) was responsible for the absence of a decreased Bohr effect is unclear. Calculations show that under the conditions presented in this thesis the amount of dimer was present as 13% of the total Hb. In the presence of 0.5 M chloride the alkaline Bohr coefficient for $\alpha_2^{Cm}\beta_2^{Cm}$ has a similar value to $\alpha_2\beta_2^{Cm}$ as

determined by proton titration again suggesting the functional similarities between these two hybrids.

Relevance of the Alkaline Bohr Effect Results to Carbamino Hb - It may be useful to summarize the data discussed so far for measurements of the alkaline Bohr coefficient in various conditions of ionic strength and extrapolate to a situation in which the carboxymethyl moiety is substituted for by carbamate. Unmodified HbA and the $\alpha_2^{Cm}\beta_2$ hybrid both exhibit a similar Bohr effect under conditions of 1 mM, 100 mM, and 500 mM chloride respectively. Modification at Val-1(α) by cyanate (52) or pyridoxal phosphate (323, 324) reduces the alkaline Bohr effect for different reasons. The alkaline Bohr coefficient for $\alpha_2\beta_2^{Cm}$ on the other hand is decreased by about 30% in 1 mM, 100 mM, and 500 mM chloride compared to both unmodified Hb and $\alpha_2^{Cm}\beta_2$. Results analogous to $\alpha_2\beta_2^{Cm}$ are obtained for $\alpha_2^{Cm}\beta_2^{Cm}$ in 1 mM, 100 mM, and 500 mM chloride. Under conditions of low ionic strength both unmodified Hb, as well as a tetramer where Val-1(α) has a carbamate group attached, would be equivalent. In conditions of 0.1 M chloride even though carbamate formation at Val-1(α) may change the pK_a of the secondary amine it does not appear to have changed the differential pK_a between this residue in the deoxy compared to the oxy conformation. Under conditions of 1 mM chloride or 0.1 M chloride, and in the absence of 2,3-DPG, the attachment of a carbamate to Val-1(β) may be expected to decrease the alkaline Bohr effect. In low chloride, extrapolation from the X-ray data of carbamino formation of unmodified HbA in the presence of 0.1 M chloride (254) is suggestive of an interaction between the carbamate anion at Val-1(β) and Lys-82(β). This would reduce the positive charge within the 2,3-DPG binding pocket. By analogy with the reduced Bohr

effect for Hb Providence Asp (70), discussed previously, this could possibly increase the pK_a of His-143(β) in oxy compared to deoxy Hb. The normal Bohr effect would then be opposed, by the absorption of proteins upon oxygenation. When the chloride concentration is raised to 0.1 M the contribution of Val-1(α) should serve to increase the Bohr effect of a Hb tetramer where Val-1(β) has a carbamate attached but Val-1(α) is unmodified. Thus, based upon the discussion above, a lowered Bohr effect for a Hb tetramer in which all four termini have carbamate attached should be due to the carbamate formation at Val-1(β) at 1 mM or 100 mM chloride. Thus, in vivo the decrease in the alkaline Bohr effect observed for unmodified Hb in the presence of CO_2 could be due to carbamate formation at Val-1(β) and its resultant influence on His-143(β). However, this would depend upon the binding constants of 2,3-DPG and CO_2 for Val-1(β).

Effect of 2,3-DPG on the Alkaline Bohr Effect - At physiological pH the alkaline Bohr effect is increased by 2,3-DPG (36, 126-129). Dahms et al (321) showed that a slightly greater than equimolar concentration of 2,3-DPG elevated the alkaline Bohr effect from -0.50 to -0.67 as determined from the slope of the plot of $\log P_{50}$ versus pH. This result may be explained on the basis of the proposal of Riggs (127). He suggested that the alkaline Bohr effect may be composed of two parts, one is independent of the presence of 2,3-DPG and the second part is dependent on the presence of 2,3-DPG. Thus, based on the data of Dahms et al (321), 0.5 protons are released per heme in the absence of 2,3-DPG. The presence of this organic anion raises the alkaline Bohr coefficient by 0.17 protons per heme. A mechanism for the enhancement of the alkaline Bohr effect by 2,3-DPG was proposed by Riggs (321).

Since 2,3-DPG binds to the protonated residues in the cleft between the two β -chain termini (62), the binding of this organic phosphate will shift the ionization equilibrium reaction in favor of protonated deoxy Hb. Thus, when 2,3-DPG is expelled upon oxygenation, protons will be released. In other words, the residues involved in binding 2,3-DPG exhibit oxygen-linked protonation in the presence but not in the absence of the organic phosphate. Moreover, the additional Bohr effect due to the presence of 2,3-DPG will be pH dependent and, as shown by Kilmartin (325), this pH dependency differs with respect to the conformation of the Hb.

The additional Bohr effect due to the presence of 2,3-DPG and 0.1 M chloride is clearly seen for unmodified HbA in Table 10. From a value of +0.39 protons per heme in 1 mM chloride and +0.50 protons per heme in 0.1 M chloride, the alkaline Bohr coefficient increases to +0.62 protons per heme. For the hybrid $\alpha_2^{Cm}\beta_2$ the value in 1 mM chloride, of +0.40 increases to +0.49 which is close to the value of +0.50 observed for this hybrid in 0.1 M chloride. At first glance this result is a little inconsistent, since the DPG titration data (Figure 16), shows that 2,3-DPG lowers the oxygen affinity of $\alpha_2^{Cm}\beta_2$ about 4-fold, which is about the same degree to which addition of this effector lowers the affinity of unmodified HbA. These results were measured in the absence of chloride. In the presence of chloride (data not shown), about a 2-fold increase in oxygen affinity is observed for both tetramers. In light of these data, therefore, carboxymethylation of Val-1(α) appears not to have changed the binding properties of this derivative toward 2,3-DPG. Since a large molar excess of DPG over Hb was used, it is not readily apparent from the above data to what extent 2,3-DPG binds to the

oxy conformation of the $\alpha_2^{Cm}\beta_2$ hybrid. This would reduce the differential between the protons absorbed or bound between the two conformational states, thereby decreasing the value for the Bohr coefficient. It is certainly of interest to interrelate this result, for a diminished additional Bohr effect, with the preliminary results obtained for the reactivity of $\alpha_2^{Cm}\beta_2$ toward NEM (refer back to Results section). The deoxy-like NEM profile for $\alpha_2^{Cm}\beta_2$ in the presence of IHP is suggestive of a shift in the $R \rightarrow T$ equilibrium toward the right. Thus, the binding of 2,3-DPG to the oxy form of $\alpha_2^{Cm}\beta_2$ may be increased due to a shift in the $R \rightarrow T$ equilibrium, and could exert an effect on the $R \rightarrow T$ equilibrium similar to IHP. This would serve to lower the alkaline Bohr effect.

The alkaline Bohr effect of $\alpha_2\beta_2^{Cm}$ does not respond at all to the presence of 2,3-DPG and 0.1 M chloride and in fact the coefficient is reduced compared to the value in 0.1 M chloride. This is consistent with the very low level or absent binding of 2,3-DPG observed in the absence or presence of 0.1 M chloride respectively (Figure 16). It is, however, in contrast to the 30% increase in the alkaline Bohr effect for $\alpha_2\beta_2^{Cm}$ in 0.1 M chloride. Possibly there could be a large portion of non-specific binding by 2,3-DPG to the oxy form of $\alpha_2\beta_2^{Cm}$, which would tend to reduce ΔH^+ and also caused the contribution made by oxygen-linked chloride binding to Val-1(α). The carboxymethylation of Val-1(β) and what appears from X-ray crystallography to be the consequent electrostatic interaction with Lys-82(β) precludes binding of 2,3-DPG to any of the other residues within the organic phosphate binding cleft. Since both CO_2 and 2,3-DPG bind to Val-1(β) there must be an interaction between these two effectors for this site (250). 2,3-DPG increases the

alkaline Bohr effect of unmodified Hb, while CO_2 diminishes it (257). In the presence of both effectors, CO_2 diminishes the enhanced Bohr effect due to 2,3-DPG and 2,3-DPG increases the reduced Bohr effect due to CO_2 (257). Direct CO_2 binding experiments (256) showed that in the presence of 2,3-DPG, the amount of CO_2 bound to Val-1(β) was decreased by a third in deoxy Hb. If the assumption is made that the deoxy form is fully saturated with 2,3-DPG, then either, CO_2 displaces some 2,3-DPG or else populations of Hb exist with both CO_2 and 2,3-DPG bound to Val-1(β) (HbCO_2DPG and $\text{Hb}(\text{CO}_2)_2\text{DPG}$) (258). In the case of the hybrid, $\alpha_2\beta_2^{\text{Cm}}$, 2,3-DPG did not have any additional effect on the alkaline Bohr coefficient, in contrast to the effect of CO_2 and 2,3-DPG on unmodified Val-1(β) (256). This would tend to argue against the occurrence of species of the type $\text{Hb}(\text{CH}_2\text{CO}_2^-)_2\text{DPG}$ in $\alpha_2\beta_2^{\text{Cm}}$. The explanation for this may be due to the unfavorable steric interactions due to the covalent attachment of a carboxymethyl moiety of each β -chain terminus and also to the fact that the $-\text{CH}_2\text{COO}^-$ moiety is covalently bound.

The diminished reactivity toward 2,3-DPG by $\alpha_2\beta_2^{\text{Cm}}$ is also reflected by the absence of any further increment in the alkaline Bohr effect for the hybrid $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$. For $\alpha_2\beta_2^{\text{Cm}}$ and $\alpha_2^{\text{Cm}}\beta_2^{\text{Cm}}$, although the inability of 2,3-DPG to bind to these tetramers causes no change in the alkaline Bohr coefficient, compared to the value at low salt, there could also be equal binding of 2,3-DPG to both the oxy and deoxy forms of these samples. This too could cause the observed minimal response of the alkaline Bohr effect toward 2,3-DPG.

How relevant to carbamino Hb are the above findings that measure the influence of 2,3-DPG on the alkaline Bohr effect of carboxymethylated Hb? Carbamate formation at Val-1(α) should not

influence the binding of 2,3-DPG to deoxy Hb. However, if carbamate formation at Val-1(α) does indeed slightly shift the allosteric equilibrium, there might be an increased tendency for 2,3-DPG to bind to the oxy form of this tetramer. Under physiological conditions, therefore, carbamate formation at Val-1(α) might decrease the alkaline Bohr effect because of an increase in the binding of 2,3-DPG to oxy Hb. This is, however, highly speculative.

At first glance the absence of any response of the alkaline Bohr effect of the hybrid $\alpha_2\beta_2$ ^{Cm} toward 2,3-DPG indicates that 2,3-DPG cannot bind when the terminus of each β -chain is carboxymethylated. On extrapolation to carbamino Hb the same interpretation may be put forward. At any pH the amount of 2,3-DPG bound depends upon the amount of protonated amine, whereas CO₂ binds to the unprotonated amine. In view of this, it seems hard to decipher how both modulators may be bound simultaneously (HbCO₂DPG and/or Hb(CO₂)₂DPG) as proposed by Kilmartin (257), unless CO₂ somehow increases the pK_a of the secondary amine when it binds and 2,3-DPG binding decreases the pK_a of the same group. As stated earlier in this Discussion one estimate was that 30% of the total CO₂ produced by actively respiring tissues is converted to carbamino Hb under physiological conditions (136). Calculations show this to represent a 7% modification of the total N-terminal amino groups. Even if this modification was exclusive to the β -amino termini, the 86% of the remaining β -chain terminals would be available for combination with 2,3-DPG. Thus, the proposal made in this thesis is that at physiological pH in deoxy Hb, the high DPG-deoxy Hb association constant $6.7 \times 10^4 \text{ M}^{-1}$ (46) (there are a number of values for this constant in the literature which differ depending on the experimental conditions)

favors complex formation with 2,3-DPG. Since the pK_a of Val-1(β) is about 7 (159), a certain number of unprotonated amino groups should allow CO_2 to combine with Val-1(β), where the pH dependent association constant for CO_2 at Val-1(β) is 579 M^{-1} (256). Thus, most of the Hb would have 2,3-DPG bound, and no more than 7% would be present as carbamino Hb. In other words there could be two populations of Hb, having either 2,3-DPG or CO_2 bound.

Change in pH values of the Maximum Amplitude and When $\Delta H^+ = 0$ - Is there any change in the pH at which the maximum amplitude occurs? Table 11 was compiled from measurements of pH at which this maximum was achieved under the conditions stated. What seems readily apparent is that an increase in the ionic strength increases the pH at which the maximum amplitude is manifested and this is consistent with the results of Rollema et al (56). This could be interpreted to indicate that there is considerable binding of anion to both the oxy and deoxy conformations at pH values of about neutrality. (The pK_a value of Val-1(α) in oxy Hb is 7 (159).) As the ionic concentration increases there is less binding to oxy Hb, since there is an increase in the number of unprotonated groups. This could bring about a decrease in anion binding to the oxy form compared with the deoxy state increasing the differential anion binding and pK_a values of certain residues between the two states. Thus, increased ionic concentration serves to shift the maximal differential toward a higher pH. Alternatively, in the presence of high salt concentrations the cumulative contributions from a large number of residues becomes dominant (182, 183) and a maximal difference between oxy and deoxy proton absorption or release is shifted to a higher pH. The pH shift in maximum amplitude for $\alpha_2^{Cm}\beta_2$ from 7.0, in 1 mM chloride,

to 7.8 in 100 mM chloride and 2 mM 2,3-DPG is noteworthy and could indicate that there is significant binding of 2,3-DPG to the oxy form of this hybrid. Thus, although entirely speculative and with reference to the NEM data (see Results) which indicate an R \rightarrow T shift of the hybrid $\alpha_2^{\text{Cm}}\beta_2$ in the presence of IHP, carbamate formation to Val-1(α) followed by small but significant binding of 2,3-DPG could be early events in a shift in the R \rightarrow T equilibrium.

A shift to higher pH is also observed, under conditions of increased salt, for $\Delta\text{H}^+ = 0$ (Table 12). Perhaps similar reasoning could be applied here. In other words the pH where the number of protons absorbed and/or released by deoxy and oxy Hb is equal, could be elevated as a consequence of elevations in pK_a value due to increased concentrations of salt. Other interpretations are not readily apparent.

Acid Bohr Effect

The uptake of protons upon oxygenation below pH 6 is the acid or reverse Bohr effect (111). This necessarily means that the group or groups involved should have a pK_a that is higher in oxy Hb than in deoxy Hb so that protons will be absorbed upon the binding of ligand. The pK_a of such a group or groups has been proposed to increase either from 5.25 to 6.25 (150) or from 4.9 to 5.5 (111). The acid Bohr effect is most likely not of physiological importance, but as stated previously could be important from a protein chemistry point of view.

The possibility exists that a carboxyl group may be an acid Bohr group and indeed a reduced acid Bohr effect has been observed for des-(Arg-141(α)) HbA (230), when compared to that of unmodified HbA in 0.1 M chloride. In the present study the acid Bohr effect for des-(Arg-141(α)) HbA was found to be -0.08 in acceptable agreement with the value of -0.12 reported by Kilmartin et al (230). This represents a reduction of ~70% and 50%, respectively. In addition, the alkaline Bohr effect is also diminished by ~50% (326) and 60% in the measurements reported here. IHP is able to restore both the alkaline and acid Bohr effects of des-(Arg-141(α)) to a normal value (230). Perutz et al (270) provided evidence to show that IHP could stabilize the T structure of deoxy (NES-des-(Arg-141(α)) HbA. Perhaps then, the reduced Bohr effect of des-(Arg-141(α)) Hb is due to the presence of R-like conformations in the unliganded form which has only a small Bohr effect upon ligation (230). However, a calculation of the amount of R structure showed that the difference in the concentration of R structure in the absence or presence of organic phosphate was too small to account for the observed

decrease and increase respectively of the alkaline Bohr effect. An alternative suggestion was made (230) based on a finding reported by Perutz and Ten Eyck (47), whereby there appeared to be an alteration in the T-structure of des-(Arg-141(α)) Hb. This alteration may weaken the alkaline Bohr group salt bridges and decrease the differential pK_a of these groups in oxy compared to deoxy des-(Arg-141(α)) HbA. IHP is perhaps able to strengthen the salt bridges in the T-state of this Hb derivative. In the same report the data are shown but not discussed for an increase in the acid Bohr effect of des-(Arg-141(α)) Hb (230). Thus, perhaps the same mechanisms apply to the acid Bohr effect as those presented above regarding the alkaline Bohr effect. Van Beek et al (200), also measured the Bohr effect of des-(Arg-141(α)) Hb with and without the addition of IHP. In contrast to the results of Kilmartin et al (230) they were not able to restore the alkaline Bohr effect fully in the presence of IHP. Although not discussed, inspection of the titration curve below pH 6.5 for both unmodified HbA and des-(Arg-141(α)) Hb in the presence of IHP show an acid Bohr effect of similar magnitude for both samples (200). With respect to the study of Van Beek et al (200), therefore, in des-(Arg-141(α)) Hb, there may be some alterations in structure which are responsible for the decrease in the acid and alkaline Bohr effects. However, other structural alterations may be unique to only the alkaline Bohr effect. This latter alteration is most likely due to the absence of a pK_a shift of Val-1(α) upon ligation of des-(Arg-141(α)) Hb.

Could the arguments presented above for the diminished Bohr effects of des-(Arg-141(α)) Hb, namely, either a weakening of the Bohr group salt bridges and/or the absence of a pK_a shift of Val-1(α) upon

ligation, be applicable to the greater than 50% reduction of the acid Bohr coefficient measured for the carboxymethylated hybrid $\alpha_2^{Cm}\beta_2$ (Figure 18, Table 13)? Probably not since all the salt bridges are intact as observed by X-ray crystallography of $\alpha_2^{Cm}\beta_2^{Cm}$ compared with unmodified HbA.

The possibility exists that the close proximity of the carboxymethyl moiety at Val-1(α) with the carboxylate anion of Arg-141(α) serves to raise the pK_a of this group to a greater extent in deoxy compared with oxy Hb. This effect could be caused by increased repulsive energy due to the negative charges in close proximity. The elevated pK_a of the carboxylate group of Arg-141(α) in deoxy Hb could perhaps reduce the difference in the pK_a value between the oxy and deoxy states and decrease the acid Bohr effect. (The pK_a of this carboxyl moiety should be greater in the oxy conformation in order for it to be an acid Bohr group.) If this is the case, it appears that increased ionic strength does not restore the acid Bohr effect to normal in the studies of this thesis. This observation appears to be in contrast to the increase in the acid Bohr effect that Van Beek et al (200) observed in the presence of 0.1 N chloride. Thus, below pH 6, carboxymethylation of Val-1(α) seems to have precluded the enhancement of the acid Bohr effect in the presence of 0.1 N chloride. Thus, the acid Bohr effect measured for $\alpha_2^{Cm}\beta_2$ could be due solely to the contribution of His-143(β).

The involvement of His-143(β) in the acid Bohr effect came from hydrogen-deuterium exchange reaction followed by mass spectral determinations (193). From these experiments, His-143(β) (CO) had a pK_a value of 6.1 and His-143(β) (deoxy) had a pK_a value of 5.6. Also the

decreased acid Bohr effect of HbF (111, 327) could be due to the replacement of His-143(β) by serine at this position in the γ chain. Matsukawa et al (203) suggested that apart from His-143(β) another residue involved in the acid Bohr effect could be one with a pK_a of 4.6 and 5.4 for deoxy and CO HbA respectively. Perhaps, in light of the reduced acid Bohr effect of des-(Arg-141(α)) HbA and $\alpha_2^{Cm}\beta_2$ respectively, the carboxylate of Arg-141(α) could be this 'missing' residue. This is entirely speculative and would have to await specific studies such as a specific modification of the carboxyl of Arg-141(α) and perhaps X-ray crystallography of oxy and deoxy Hb crystals grown at low pH. It is of interest that $\alpha_2^{Cb\beta_2}$, where the α -chain amino terminus is carbamylated does not show a diminished acid Bohr effect (255). The pK_a differential of the carboxylate of Arg-141(α) appears not to have been altered by the presence of carbamylated Val-1(α) nearby.

In 0.1 M chloride both $\alpha_2\beta_2^{Cb\beta_2}$ (253) and $\alpha_2\beta_2^{Cm}$ show an enhanced acid Bohr effect of 60% and about 25% respectively compared to the acid Bohr effect of unmodified HbA. It is of interest to compare the 25% increase in the acid Bohr effect of $\alpha_2\beta_2^{Cm}$ with the 30% decrease for the alkaline Bohr effect of this hybrid, since the mechanism proposed for the diminished alkaline Bohr effect is directly relevant to the increase for the acid Bohr effect. Briefly, the mechanism that was proposed earlier to account for the decreased alkaline Bohr effect of $\alpha_2\beta_2^{Cm}$ in low and physiological concentrations of chloride, proposed that the interaction observed between the carboxymethyl moiety covalently attached to Val-1(β), could reduce the net positive charge within the 2,3-DPG binding cavity through an electrostatic interaction with

Lys-82(β). The reduction of positive charge at Lys-82(β) might lower the pK_a values for His-143(β) in the deoxy compared with the oxy conformation. This could be responsible for a greater uptake of protons upon oxygenation which would oppose the normal alkaline Bohr effect. This mechanism was first described by Bonaventura et al (66) to explain the decreased alkaline Bohr effect observed for Hb Providence Asn and Hb Providence Asp. Thus, if the differential between the protons absorbed by His-143(β) is increased between the oxy and deoxy state of Hb Providence Asn and Asp, as well as in $\alpha_2\beta_2^{Cm}$ (such that more protons are absorbed upon oxygenation) this might increase the acid Bohr effect. Indeed, for $\alpha_2\beta_2^{Cm}$ in the presence of 0.1 N chloride there was a 25% increase in the acid Bohr effect compared with unmodified oxy Hb. In contrast to this, however, Perutz et al (111) showed a titration of Hb Providence Asp where the acid Bohr effect of this mutant was diminished by about 30%, in conditions of low salt. The hybrid $\alpha_2\beta_2^{Cbm}$ exhibits an increased acid Bohr effect of under very similar conditions (253), whereas this modified tetramer exhibits an alkaline Bohr effect of the same magnitude as unmodified HbA. Thus, below pH 6 certain residue(s) absorb more protons upon oxygenation and this is a result of carbamylation of Val-1(β).

The acid Bohr effect of $\alpha_2^{Cm}\beta_2^{Cm}$ in 0.1 N chloride reflects the reduction observed for $\alpha_2^{Cm}\beta_2$ rather than an average of the coefficients determined both for $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$.

To summarize briefly, therefore, the reduced acid Bohr effect of the hybrid $\alpha_2^{Cm}\beta_2$, under conditions of 0.1 N chloride, has provided further evidence implicating the involvement of the carboxylate of Arg-141(α) in the acid Bohr effect. Moreover, in $\alpha_2\beta_2^{Cm}$ the close

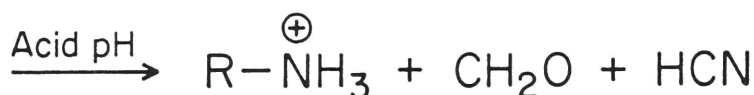
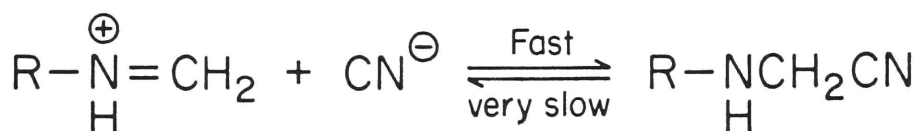
proximity of the carboxymethyl moiety to His-143(β) could somehow allow the imidazole side-chain to absorb more protons upon oxygenation and indeed this proposal is also consistent for the diminished alkaline Bohr effect observed in the studies reported here and the increased acid Bohr effect for the hybrid $\alpha_2\beta_2$ C_m. Furthermore, it would be of great interest to determine the pK_a values of His-143(β) in Hb Providence Asp or Asn as well as in $\alpha_2\beta_2$ C_m for both the oxy and deoxy forms of these Hb's. The hydrogen-deuterium exchange-mass-spectral technique of Ohe and Kajita (193) could perhaps be an interesting application.

e) Competitive Binding Studies

Conditions were selected for the competitive binding experiments such that a) initial rates of reaction could be calculated and b) the carboxymethylation was directed predominantly to the amino termini as opposed to the ϵ -amino groups of lysines. The results of Figure 9 show that glyoxylate incorporation is linear up to a concentration of 1 mM. Since a glyoxylate concentration of 0.1 mM minimized but did not eliminate formation of carboxymethyllysine, this glyoxylate concentration was utilized for the experiments. In addition, in contrast to reductive methylation where the dimethyl derivative predominates (15, 17) in these studies monocarboxymethylvaline was the predominant species. As stated earlier formation of the dicarboxymethyl derivative could be precluded on steric and/or electrostatic grounds.

Figures 21 and 22 show the response of the rate of the reaction with respect to increased pH. It is readily apparent that in both oxy and deoxy unmodified Hb the rate of reaction is diminished as the pH is elevated. There are three possible interpretations of this result.

First, it could be due to a decrease in the concentration of glyoxylate due to reduction by cyanoborohydride at higher pH values. However, measurement of the 2,4-dinitrophenylhydrazone derivative (279) of glyoxylate with respect to increased pH showed no evidence for such a diminution. A second possibility as to why high pH might reduce the rate of carboxymethylation could be due to increased formation of the N-cyanomethyl adduct which could arise from contaminating quantities of cyanide present in the cyanoborohydride (as described below).



Since cyanide should be absent in recrystallized cyanoborohydride, and there was no difference in the rate of carboxymethylation whether the cyanoborohydride used was recrystallized or not, it was concluded that the decrease in reaction rate observed at high pH was not due to the presence of this contaminant. The third, and most likely interpretation of the decreased reaction rate observed at high pH, can be explained with reference to the mechanism by which the carboxymethylation reaction occurs (Figure 3 in the Introduction and part j of Results) in addition to environmental factors within the protein. The unprotonated amino function forms a Schiff base which must be protonated before it can be reduced by cyanoborohydride (18). If the pH of the reaction is increased, then the concentration of unprotonated amine should increase but the concentration of the protonated Schiff base intermediate should

diminish. The resultant reactivity as the pH increases should then be a compromise between the two facts stated above. From the observed decrease in the rate of reaction as the pH is increased (Figures 21 and 22) the properties of the protein are such that N-terminal carboxymethylation is decreased due to a diminished quantity of the protonated Schiff base intermediate.

What is also readily apparent from the results presented in Table 14a and Figure 20 is the enhancement of the rate of the reaction in deoxy as compared to oxy Hb in all cases in the absence of allosteric modulators. Tables 14a-14i shows the second order rate constants calculated (as described in the Results) for each sample under various conditions. Figure 20 shows the velocity of the reaction expressed as nmols of glyoxylate incorporated per free α -amino group per minute. Of note, however, is the fact that the sum of the rate constants for oxy $\alpha_2\beta_2$ and deoxy $\alpha_2\beta_2$ does not represent an average of the rate constants for oxy $\alpha_2^{Cm}\beta_2$ and oxy $\alpha_2\beta_2^{Cm}$ and of deoxy $\alpha_2^{Cm}\beta_2$ and oxy $\alpha_2\beta_2^{Cm}$ respectively, unlike similar experiments that measured the rate of carbamylation at Val-1(α) or Val-1(β) (57). In unmodified oxy and deoxy $\alpha_2\beta_2$ the rates are less than the calculated average. Perhaps then, carboxymethylation of the amino terminus of one chain enhances the rate of reaction at the opposing chain. In particular the value of V (Figure 20) is 6.5 for deoxy $\alpha_2\beta_2$, close to the calculated average of 8.5. In oxy $\alpha_2\beta_2$ the value of V is 1.6 compared to the calculated average of 4.5. It is conceivable that the oxy hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ have a more deoxy-like structure than oxy $\alpha_2\beta_2$ which would enhance the rate of carboxymethylation at the unmodified termini. Of the two α -amino termini, that of the β -chain appears to react faster

than the terminus of the α -chain in oxy Hb. Indeed this result is consistent with the separation of the pHMB chains (Figure 6 and Table 5) which shows a 70% modification at Val-1(β) but only 30% at Val-1(α). From the tables of rate constants (Tables 14a-14i), the rate of deoxy unmodified Hb is enhanced by 4.5-fold compared to its oxy counterpart (Table 14a). There is, however, only a 2-fold and 3-fold enhancement for deoxy $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ respectively compared to their oxy counterparts.

It is of interest to compare carboxymethylation at the amino terminus of each chain with carbamylation. The rate of carbamylation of deoxy HbA or HbS is about two and a half times greater than the rate of carbamylation for the oxy samples (57, 285, 304, 305). Val-1(β) is carbamylated about 50% faster in the deoxy state, whereas for Val-1(α) the enhancement in reactivity towards carbamylation is about 3-fold (57, 304). The mechanism proposed by Nigen et al (57) to account for the enhanced reactivity of Val-1(α) towards cyanate suggested that the cyanate anion formed a complex with protonated Val-1(α) and other positively charged residues in the vicinity, i.e., Arg-141(α) and Lys-127(α). Proton transfer from the protonated amine to the cyanate anion produces the isocyanate anion which had been shown by Stark (58) to be the reactive species in the carbamylation reaction. The binding of the cyanate anion to Val-1(α) is consistent with the identification of this locus as an anion binding site in deoxy Hb (43, 44). As stated above the rate of carboxymethylation for deoxy $\alpha_2^{Cm}\beta_2$ was enhanced 2-fold compared to the oxy samples. The analogous rate for $\alpha_2\beta_2^{Cm}$ was enhanced 3-fold compared to its oxy counterpart. The latter result could be interpreted in a similar manner to that described above (57)

for the enhanced carbamylation of Val-1(α). The greater reactivity of oxy Val-1(β) toward carboxymethylation as well as the enhancement in reactivity of deoxy $\alpha_2^{Cm}\beta_2$ could be explained either on steric and/or electrostatic grounds. Perhaps His-2(β) can interact with the glyoxylate anion and participate in the protonation of the resultant Schiff base. This hypothesis is conjectural and should await further study, perhaps with horse Hb, where glutamine replaces His-2(β).

Carboxymethylation in the Presence of 2,3-DPG - Figure 20, panel B and Table 14b show the reaction rates and second order rate constants respectively for the samples in the presence of 5 mM 2,3-DPG. Immediately apparent is the 86% and 100% inhibition of carboxymethylation for deoxy unmodified HbA and deoxy $\alpha_2^{Cm}\beta_2$. This is entirely consistent with Val-1(β) as a residue that contributes to the binding of organic phosphates. Non-specific binding due to the rather large molar excess of organic phosphate (5 mM compared to 0.4 mM Hb) and the absence of chloride could have contributed to the 55% inhibition observed for the carboxymethylation of oxy unmodified $\alpha_2\beta_2$. These two factors could also have contributed to the large (88%) reduction observed for oxy $\alpha_2^{Cm}\beta_2$ (see Results).

Carboxymethylation in the Presence of 0.5 M Chloride - From Table 14c and Figure 20 there is considerable inhibition of carboxymethylation for all the samples in the presence of 0.5 M chloride. The inhibition of carboxymethylation of deoxy $\alpha_2\beta_2$ and $\alpha_2\beta_2^{Cm}$ is consistent with Val-1(α) as an anion binding residue (43, 44). In oxy unmodified Hb, a large percentage of non-specific binding was probably responsible for the near abolition of carboxymethylation. The similarity in the magnitude of inhibition observed for oxy and deoxy

$\alpha_2^{Cm}\beta_2$ in the presence of 2,3-DPG or chloride could be likened to the fact that 0.5 M chloride can exert effects similar to a much lower concentration of 2,3-DPG (36).

Carboxymethylation in the Presence of 5% CO₂ - Physiological concentrations of CO₂ (5%) lower the second order rate constant for carboxymethylation of all the samples studied both in the oxy and deoxy states (Table 14d). (The same relative rates of lowered carboxymethylation are also observed in the presence of 14% CO₂.) The interpretation of this observation indicates the ability of CO₂ to bind to both the oxy and deoxy conformations of Hb, a result analogous to that reported by others (248, 256, 261, 264). Of note here is the greater inhibition of carboxymethylation at oxy and deoxy Val-1(α) compared to oxy and deoxy Val-1(β). This could indicate that under these conditions, 5% CO₂, Val-1(α) is the preferred site for carbamino formation. This is in agreement with the results of Brenna et al (125). However, although a carboxymethylated hybrid of the type $\alpha_2^{Cm}\beta_2$ or $\alpha_2\beta_2^{Cm}$ may be considered as a carbamino analogue, the mechanisms by which carbamino formation and carboxymethylation are achieved could be entirely different. Therefore, the fact that carboxymethylation of oxy and deoxy $\alpha_2\beta_2^{Cm}$ is not inhibited by CO₂ to the same extent as oxy and deoxy $\alpha_2^{Cm}\beta_2$ may reflect a preference for carboxymethylation as opposed to carbamino formation at Val-1(β). Jensen et al (304) measured the rate of carbamylation of deoxy HbA in the presence of CO₂ and found that carbamylation was inhibited to a similar extent in both the α - and β -chains. The carboxymethylation studies presented here showed that the extent of inhibition by CO₂ at each chain differed in the deoxy hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ and are in contrast to the observations of Jensen et al (304).

These results may be a reflection of mechanistic differences for carbamate formation, carboxymethylation and carbamylation at Val-1(α) and/or Val-1(β). Steric and electrostatic factors most likely are of considerable importance.

Carboxymethylation in the Presence of Combinations of the Allosteric Modulators - 2,3-DPG in the presence of 5% CO₂ reduces greatly the carboxymethylation of unmodified HbA and both oxy and deoxy $\alpha_2^{Cm}\beta_2$ consistent with the binding of 2,3-DPG to Val-1(β) (Figure 20, panel D, Table 14f). On the other hand, unmodified HbA and $\alpha_2\beta_2^{Cm}$ under oxy and deoxy conditions have similar rate constants in the presence of 5% CO₂ and 5% CO₂ with 5 mM 2,3-DPG. It may be concluded that 2,3-DPG is a much more potent inhibitor of carboxymethylation at Val-1(β) than is 5% CO₂. 5% inhibits carboxymethylation at Val-1(α) The preferential binding of 2,3-DPG and CO₂ to separate sites is best illustrated in going from panel B to E, where 5% CO₂ does not change the extent of inhibition shown for $\alpha_2^{Cm}\beta_2$. However, the inhibition is increased for $\alpha_2^{Cm}\beta_2$ when the values are compared for 2,3-DPG (panel b) to 2,3-DPG and 5% CO₂ (panel e).

The combination of 5% CO₂ and 0.5 M chloride (Figure 20, Panel f, Table 14g) inhibits carboxymethylation in all cases to a greater extent than 0.5 M chloride alone. Carboxymethylation of oxy and deoxy $\alpha_2\beta_2^{Cm}$ is inhibited to a greater extent than that of $\alpha_2^{Cm}\beta_2$ under corresponding conditions. This indicates that under the conditions employed for this study CO₂ and chloride bind preferentially to Val-1(α). This is consistent with previous studies that reported Val-1(α) as a site for carbamino formation (254, 256, 258) and for anion binding (43, 44).

Similar patterns of reactivity are observed for carboxymethylation in the presence of 14% CO₂ and 0.5 M chloride.

In summary, therefore, the second order rate constants have been calculated for the competitive binding experiments carried out under a variety of conditions. The key results to emerge are first, an enhancement in the rate of carboxymethylation of all the samples in the deoxy state compared with their oxy counterparts. The calculated average rate constants for the hybrids $\alpha_2^{Cm}\beta_2$ and $\alpha_2\beta_2^{Cm}$ in both oxy and deoxy forms are greater than the rate constants for oxy and deoxy unmodified HbA. This is perhaps an indication that the modification of one terminus can enhance the modification of the other perhaps through a shift in the R \rightarrow T equilibrium. That such a shift may occur could also be an interpretation of the decreased enhancement observed in the deoxy hybrids compared to unmodified HbA (2 to 3-fold enhancement of the former and 4.5 for the latter).

2,3-DPG is a potent inhibitor when the rate of carboxymethylation is measured in samples where Val-1(β) is unmodified especially in the deoxy conformation. Chloride is a most effective inhibitor when there is an unmodified Val-1(α) present. Both 5% CO₂ (physiological conditions) and 14% CO₂ appear to be more effective in their inhibition of the carboxymethylation at Val-1(α) than at Val-1(β). This could be interpreted as either a preference of carbamino formation at Val-1(α) or due to an enhanced reactivity of glyoxylate compared to CO₂ toward Val-1(β). Addition of 2,3-DPG and either 5% or 14% CO₂ effectively abolishes or reduces greatly carboxymethylation of deoxy $\alpha_2^{Cm}\beta_2$ and deoxy unmodified HbA.

f) Relevance of Data to the R \rightleftharpoons T Equilibrium

In light of the data obtained from the Bohr effect and the kinetic binding experiments, as well as the NEM data (see Results), it may be possible to tentatively suggest a possible sequence in the events that cause the shift between the R and T conformation. An early event could be carbamino formation at the amino termini. A number of studies (256, 260) as well as the data reported here give evidence for CO₂ binding to oxy HbA. From the kinetic data (Figure 20), CO₂ binds preferentially to the α -NH₂ group of the α -chains. This resultant Hb, where the two α -chain α -amino groups are modified, could have its R \rightleftharpoons T equilibrium shifted toward the right hand side based upon two pieces of experimental evidence found in these studies. First, the 3-fold increase in the second order rate constant for oxy $\alpha_2^{Cm}\beta_2$ compared to oxy unmodified HbA could be a reflection of more deoxy-like characteristics of this hybrid. Second, from the NEM data, the presence of IHP caused a reduction in the reaction of $\alpha_2^{Cm}\beta_2$ toward this reagent (see Results). With regard to $\alpha_2\beta_2^{Cm}$, the second order kinetic rate constant for this oxy hybrid was about two orders of magnitude higher than for oxy unmodified $\alpha_2\beta_2$. Moreover, IHP did not appear to induce a conformational change in the $\alpha_2\beta_2^{Cm}$ hybrid (see Table 15). Thus, perhaps carbamino formation at Val-1(α) is an early event in the sequence that changes the conformation from the R to the T state. This would be of particular importance in the tissues where CO₂ is the product of metabolism. The Hb molecule with carbamate attached at Val-1(α) could have a conformation intermediate between R and T. Since both 2,3-DPG and CO₂ compete for Val-1(β) in an oxygen-linked manner, the next event in the sequence could be the binding of either of these modulators to Val-1(β). As

discussed earlier the binding constant for 2,3-DPG to Hb is several orders of magnitude higher than CO_2 to Hb is, in deoxy Hb. Whether this still holds for a tetramer of structure intermediate between R and T forms is unknown. Thus, depending on this latter value, CO_2 and/or 2,3-DPG binding to Val-1(β) should tilt the allosteric equilibrium further toward the T state. Of interest from the results presented in this thesis is the fact that 2,3-DPG appears to bind significantly to oxy $\alpha_2^{\text{Cm}}\beta_2$ (Figure 20, panel b). This organic phosphate reduces the second order rate constant for $\alpha_2^{\text{Cm}}\beta_2$ about 6-fold from 2.8 mM h^{-1} to $0.5 \text{ mM}^{-1} \text{ h}^{-1}$ in the absence and presence of this effector, respectively. This should be compared with the 2-fold decrease in the rate constant of oxy unmodified HbA in the presence of 2,3-DPG. Whether this has some significance toward the $\text{R} \rightarrow \text{T}$ equilibrium is unclear. Since the affinity of 2,3-DPG is much higher for the deoxy compared to the oxy conformation this modulator may be able to shift the equilibrium further toward the T-state. Whether the Bohr group salt bridges form after or simultaneously with 2,3-DPG binding is difficult to ascertain from these experiments.

While the proposed scheme is extremely speculative it is felt that the structural similarity of carboxymethylated Hb hybrids $\alpha_2^{\text{Cm}}\beta_2$ and $\alpha_2\beta_2^{\text{Cm}}$ to carbamino Hb may have shed some further light on the function of CO_2 binding in vivo. Thus, not only would carbamino formation be a means to transport a portion of CO_2 back to the lungs, but it may also be involved in a shift of the $\text{R} \rightarrow \text{T}$ equilibrium at an early stage.

General Conclusions

The aim of this project, namely the formation of a carbamino analogue has been achieved, as judged by a number of criteria. The decreased oxygen affinity of Hb hybrids carboxymethylated N-terminally is analogous with the effect that CO_2 has on lowering the oxygen affinity of unmodified HbA. Moreover, the X-ray crystallography data carried out by Dr. A. Arnone at the University of Iowa also provided strong evidence that Hb, carboxymethylated at the α -amino termini could be considered as a carbamino analogue. Despite the vast quantity of literature that exists on Hb, one of the central issues is still not known with certainty, namely the exact sequence of events, controlled by the allosteric modulators, that causes one conformation to change into the other. Of interest from this study, despite its preliminary nature is the possibility that the $\text{R} \rightarrow \text{T}$ equilibrium of the carboxymethylated hybrid $\alpha_2^{\text{Cm}}\beta_2$ may be shifted toward the T state. We were also able to show that CO_2 was able to inhibit carboxymethylation of $\alpha_2\beta_2^{\text{Cm}}$ to a greater extent than it was for $\alpha_2^{\text{Cm}}\beta_2$. Thus, although entirely speculative perhaps CO_2 binding to oxy Val-1(α) is an event that occurs early within the whole sequence involving the $\text{R} \rightarrow \text{T}$ transformation.

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