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STUDIES ON THE COMPLEX OF
HAPTOGLOBIN AND HEMOGLOBIN

by

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Department of Biochemistry

Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Canada
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To my Parents
This study was supported by a grant from the Medical Research Council to Dr. D.B. Smith, Professor, Department of Biochemistry. The author is grateful for this assistance.
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ABSTRACT

Haptoglobin is a plasma protein which combines with the blood protein hemoglobin stoichiometrically in a 1:1 molar ratio. This union, although not covalent, has been reported to be irreversible. The identification of the type or types of interaction(s) involved in holding these two proteins together is the topic of study of this thesis.

For the project, porcine haptoglobin and human hemoglobin were purified. The hemoglobin was reacted with iodoacetamide-^{14}C, which produced a carbamide-^{14}C methylcysteine residue at the β-93 position of hemoglobin instead of the original cysteine. From this reacted-hemoglobin and normal hemoglobin two complexes were prepared with porcine haptoglobin (i.e., reacted-Hb-Hp complex and normal-Hb-Hp complex).

To investigate the nature of the interactions involved in the binding of hemoglobin and haptoglobin, mixtures of complex and hemoglobin were incubated (i.e., reacted-Hb with normal complex or reacted-HbHp complex with normal hemoglobin) in various dissociating media. The experimental conditions studied were pH 4.7, pH 7.4, pH 9.0, various salts, sodium dodecyl sulphate, acetate concentration, ethylene glycol, dioxane, alcohol, urea, guanidine hydrochloride, adenosine
triphosphate, diphosphoglycerate, heparin and glycine. After incubation for a predetermined period of time, the complex was separated from the excess hemoglobin by molecular sieving through monitored columns of Sephadex G-100. The eluted fractions were collected directly in scintillation vials and counted by means of liquid scintillation. Since one of the substrates is radioactive, even slight dissociation of the hemoglobin in the complex during incubation was detected by the appearance of radioactivity in the initially "cold" substrate (due to exchange of the radioactive hemoglobin by normal hemoglobin or visa versa depending on the complex which was used in the incubation).

By this method electrostatic and hydrophobic interactions were inferred to be involved in the stability of the hemoglobin-haptoglobin complex.

A problem with the above system was that the radioactive label was specific only for the $\beta$ chains of hemoglobin, and conclusions could not be drawn about movement of the complete functional dimer of hemoglobin. To study this aspect of the dissociation $\alpha$ chain-labeled hemoglobin was used. Alpha chains of llama hemoglobin have isoleucine present in their amino acid sequence while the $\beta$ chain does not. Thus, a dissociation of $\alpha$ chains was detected by the appearance of isoleucine in a human hemoglobin peak when the complex made from llama hemoglobin was incubated with human hemoglobin.

Another method for obtaining an $\alpha$ chain labeled hemoglobin involved the reacting of hemoglobin with the enzyme carboxypeptidase B, which is known to remove the C-terminal arginine of the $\alpha$ chain. After incubating this modified hemoglobin with normal
complex, analysis of the separated hemoglobin peak for liberated C-terminal arginine, by further CPB digestion, gave the amount of α subunits dissociated from the normal complex.

From these experiments, it has been concluded that both the α and β subunits of hemoglobin are dissociated from the complex under the conditions where electrostatic and/or hydrophobic interactions have been disrupted.

To detect whether amino group modification of hemoglobin had any effect on the binding of hemoglobin to haptoglobin, hemoglobin reacted with ethyl acetimidate-¹⁴C was prepared. From this hemoglobin a radioactive complex was prepared. This modification had no effect on the ability of hemoglobin to form complex, although there was a noticeable effect on the stability of the complex to dissociation.
A INTRODUCTION

Haptoglobin (Hp) is an $\alpha_2$ glycoprotein normally present in plasma of human and other mammalian species. It occurs in three main genetic forms which are electrophoretically distinct: Hp 1-1 migrates as a single band, whereas Hp 2-2 and Hp 2-1 exhibit a series of bands. With the exception of a recently discovered mutant all haptoglobins bind mammalian hemoglobins specifically to form a stable complex in a 1:1 molar ratio. Hp 2-1 and 2-2 are found only in humans.

The problem of interaction between haptoglobin and hemoglobin (Hb) has acquired renewed interest due to the availability of purified haptoglobin. This availability, plus the fact that haptoglobin binds specifically with hemoglobin, whose three-dimensional structure has been well established by x-ray analysis at 2.8 Å resolution, makes the complex an attractive model for studies of protein-protein interactions.

The elucidation of structural formulae and molecular shapes of common proteins is a problem currently occupying many laboratories. At the present time, no direct, reliable methods for determining the conformations in solution are available. X-ray crystallographic studies, however, give detailed information on their conformations in the crystalline state, which can be closely related to findings revealed by other physical and chemical methods. It is clear that no single
experimental approach can provide all, or many, of the answers to the important questions concerning the structure and functions of proteins.

Hemoglobin is one of the proteins which has had its molecular structure determined by x-ray crystallography. Although haptoglobin has yet to be crystallized, the haptoglobin-hemoglobin (HpHb) complex crystal has been obtained (Waks, Alfsen, Beuzard, Rosa, Lessin & Trautwein, 1968). This crystal has not yet successfully undergone x-ray analysis. There are, therefore, a multitude of newly exposed questions unanswered about this complex formation.

From the limited number of studies that have been made, it would appear that the strong binding that exists between haptoglobin and hemoglobin in the complex formed between these two proteins involves predominantly hydrogen and hydrophobic bonds. The hydrophobic area of the hemoglobin molecule involved has been concluded to be in some portion of the newly exposed area when hemoglobin tetramer dissociates to dimer. With this in mind, this investigation involved the use of dissociating media to attempt to discover the type of binding which holds the complex of haptoglobin and hemoglobin together in a rather stable manner.
B Historical Review

I Introduction

Haptoglobin (Hp) is a plasma protein which combines with the blood protein hemoglobin (Hb) stoichiometrically in a 1:1 molar ratio. This union, although not covalent, has been reported to be irreversible (Nyman, 1959). The identification of the type or types of interactions involved in holding these two proteins together is the topic of study of the thesis. With this in mind, the following review delves mainly into the knowledge surrounding these two proteins.

II Haptoglobin

In 1938 Polonovski and Jayle discovered that a component in plasma enhanced the weak peroxidase activity of hemoglobin. Later (Polonovski & Jayle, 1940), this plasma factor was determined to be a protein which bound specifically to hemoglobin and this was called haptoglobin (Greek: to attach + globin). Early work on haptoglobin has been reviewed by Jayle and Moretti (1962) and Laurell and Cronwall (1962) with more recent work by Sutton (1970).

Upon development of the technique of starch gel electrophoresis (Smithies, 1955), Smithies and Walker (1956) distinguished three types of haptoglobin in human sera: 1-1, 2-1 and 2-2. With the exception of humans, all mammals possess a haptoglobin similar
Table I: The Amino Acid Sequence of Hp α\textsuperscript{S} and Hp α\textsuperscript{2}.

Hp α\textsuperscript{P} is similar to α\textsuperscript{S} except that instead of glutamic acid in position 54, there is lysine. In the case of positions 54 and 113 of the Hp α\textsuperscript{2}, lysine is in one site and glutamic acid in the other. The assignments have been determined just recently along with a modification in the sequence, but as yet are unpublished (see text).
Haptoglobin α3

NH₂-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-
1 10
- Cys-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-
30 40
- Leu-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Glu-Lys-Gln-Trp-Ile-Asn-Lys-
50 60
- Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Val-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-
70 80
- Pro-Val-Gln-COOH

Haptoglobin α2

NH₂-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-
1 10
- Cys-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-
30 40
- Leu-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Glu-Lys-Gln-Trp-Ile-Asn-Lys-
50 60
- Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-Cys-
70 80
- Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-Leu-
90 100
- Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Lys-Lys-Gln-Trp-Ile-Asn-Lys-Ala-
110 120
- Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Val-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-Pro-
130 140
- Val-Gln-COOH
Figure 1: A Proposed Model for the Structure of Human Hp 1-1.

(Shim & Bearn, 1964; Barnett, Lee & Bowman, 1972)
to the 1-1 type (Jayle & Moretti, 1962). At the present moment Hp 1-1 is considered to be made up of two pairs of polypeptide chains: α or light (L) chains and β or heavy (H) chains. Contrary to the model of Shim and Bearn in 1964 (Figure 1), the L chains are linked together and to the H chains by disulphide bridges, with no such covalent bonds occurring between the H chains (Lockhart, 1971)(Figure 2). The smaller L chains have a molecular weight of 8,900, while the larger carbohydrate-containing H chains possess a molecular weight of 40,000-43,000 for a total molecular weight of 100,000-12,000 for the intact molecule. The other types of haptoglobin are considered to be polymers of the 1-1 type, with additional genetic types of L chains but with identical H chains (Smithies, Connell & Dixon, 1962).

The elucidation of the primary structure (amino acid sequence) of haptoglobin helped to clarify the picture concerning this protein. The presence of equal amounts of valine and isoleucine as amino-terminal residues in the various haptoglobin types supported the concept of two types of chains in equimolar ratios (Smith, Edman & Owen, 1962). The subtyping procedure of Smithies, Connell and Dixon (1962) led to the suggestion of at least three inherited forms of L chains in human haptoglobin. Using Smithies, Connell and Dixon's (1962) nomenclature for L chains of α^1F, α^1S and α^2, α^1F is similar to α^1S except at position 54, where α^1F has lysine, while α^1S has glutamic acid. The α^2 chain was made up both of α^1F and α^1S minus a peptide section (Smithies, Connell & Dixon, 1962; Black & Dixon, 1968)(Table I). Shim and Bearn (1964) proposed the structure (α^1β)_2 for Hp 1-1; (α^1β)_2(α^2β)_n, n=0,2,4,6,----- for Hp 2-1; and (α^2β)_n, n=4,6,8,----- for Hp 2-2. The problem was that there was no suggestion given for exactly how the 2-1 and 2-2 polymers were attached to each other. However, the present project uses
Hp 1-1 and it can be stated with some certainty that the molecule of Hp 1-1 is a single monomer consisting of a pair of L chains (either α₁F or β₁S) and a pair of H chains linked together by disulphide bridges.

Jayle, Boussier and Tonnellot (1956) indicated Hp to contain 11% carbohydrate, approximately half of that being hexosamine. Subsequent reports indicate 18.6% CHO (Schultze, Haupt, Heide & Heimburger, 1963; Cheftel, Cloarec, Moretti & Jayle, 1965), all of which appears to be attached to the H chains (Shim & Bearn, 1964; Cheftel & Moretti, 1966; Lisowska & Drobryszycza, 1967). Although efforts to establish the structure of the carbohydrate chains have not been successful, more than one kind of binding site in the polypeptide chain has been indicated. The proposal was eight side chains per 98,000 tetramer, consisting of interior residues of side chains of four galactose, two mannose and two glucosamine residues with two branches terminating in sialic acid residues (Cheftel, Cloarec, Moretti, Rafelson & Jayle, 1960; Cheftel, Cloarec, Moretti & Jayle, 1965). Thus, although the composition of the side carbohydrate chains is not certain, all the present evidence points to this moiety being attached to the H chains of haptoglobin.

Molecular weight determinations of Hp 1-1 have supported the proposal of Shim and Bearn (1964). Cheftel and Moretti (1966) determined a molecular weight of 98,770 for human Hp 1-1 by equilibrium centrifugation and estimated a molecular weight of H chains at 40,000. This, combined with a molecular weight of 8,900±400 for light chains (Connell, Smithies & Dixon, 1966), fitted the Shim and Bearn model (1964)(Figure 1).

The results of Waks and Alsen (1968), which suggested
that human haptoglobins dissociate reversibly, do not fit the
aforementioned model. Here, elution volumes (on Bio-Gel P150) of
haptoglobin near its isocionic point (pH 5.5, 0.1M KCl) were found to
increase as protein concentrations decreased below 2.0 mg/ml, but
remained stable at higher protein concentration. Sedimentation and
equilibrium ultracentrifugation indicated that at pH 11.5 Hp 1-1 was
dissociated into subunits of molecular weight 40,000±2,500. The whole
point of this was that they considered the Hp 1-1 to be reversibly
dissociated, which was contrary to the previous evidence.

Recently Lockhart (1971) investigated the possible
dissociation of porcine Hp 1-1 under the conditions of Waks and Alfsen.
Using their techniques, analysis of the shapes of the leading edge and
trailing edge of the elution profiles from Bio-Gel P150 led to the
conclusion that there was no dissociation. The elution volume and
sedimentation were essentially independent of protein concentration and
consistent with a molecular weight near 100,000. It can, therefore, be
concluded that porcine Hp 1-1 did not dissociate upon dilution.

Information on the state of sulphhydril groups has been
useful in clearing up the problem of dissociation. Both porcine
(Fraser & Smith, 1971) and human (Bernini & Borri-Voltattorni, 1970)
reduced Hp 1-1 have 16 sulphhydril groups, none of which occur as an
found less than one sulphhydril reactive to \( ^14 \)C-iodoacetate or para-
mercuribenzoate in 7.2M guanidine hydrochloride. Bernini and Borri-
Voltattorni (1970) found none reactive to 5,5'-dithiobis-(2-nitrobenzoic
acid) in either 6M guanidine hydrochloride or 33mM sodium dodecyl
sulphate. However, Waks and Alfsen (1966), using amperometric
titration, found no free -SH groups in native haptoglobin but 10
Figure 2: A Model of Hp 1-1 Based on Present Knowledge
available in 8M urea. An answer to this discrepancy could be the findings of Lockhart (1971), which showed porcine Hp 1-1 to be very susceptible to disulphide reduction. Waks and Alfsen may inadvertently have reduced some disulphide bridges. Thus, in light of the present evidence, it can be concluded that there are no free -SH groups in haptoglobin.

Considerably more information is available on the L or light chains than on the intact molecule of haptoglobin. Black and Dixon (1968) have sequenced the L chain of human Hp 1-1 (Table I). As can be seen, there are half cysteine residues at positions 21, 35 and 69. Black and Dixon (1970) have reported a symmetrical disulphide bridge between L chains at the half cysteines at position 21 and an intrachain loop between the residues at positions 35 and 69. This leaves no sulphhydril groups available to link to the H chains. However, a recent communication from the Dixon Laboratory (1972) has identified a half cysteine residue at position 73 of the L chain. This position then could be the bridge to the H chain. Barnett, Lee and Bowman (1972) have begun the sequencing of the H chain of human haptoglobin. This should be helpful in determining the positioning of the disulphide bridges between these and the L chains. From all the previous information, a model has been diagramed in Figure 2.

The site of hemoglobin binding is predominantly the H chain of haptoglobin. Gordon and Bearn (1966) separated the chains of reduced alkylated Hp 2-2 on Sephadex G-100 in dilute propionic acid and found that the H chains retained binding ability while the L chains retained only minimal activity. A second method for chain separation, using 5M guanidine hydrochloride on Sephadex G-200 by Gordon,
Clevé and Bearn (1968), further supported the evidence that the H chain makes the major contribution to the haptoglobin binding site of hemoglobin.

Ofose, Campbell and Connell (1971) have obtained, by digestion with plasmin, two fragments from Hp 1-1 with molecular weights of 78,000 and 17,000. The larger fragment probably consists of two intact L chains and most of the H chains still linked by disulphide bonds; the smaller fragment is the carboxyl-terminal fragment of the H chain. Evidence for this was that the larger fragment has the same amino-terminal amino acids (valine and isoleucine) as intact haptoglobin, while the smaller fragment has only amino-terminal serine. The suggestion is that the H chains are not bridged to themselves for at least one-fifth of their chain length, probably from their carboxyl-terminal ends, and that both carbohydrate and L chains are excluded from these fragments. More recently (Ofose & Connell, 1971), it has been shown that separately the fragments do not bind hemoglobin, but when they are combined, a product is formed which has binding activity very similar to that of native haptoglobin. It would seem that the binding of hemoglobin restored the fragments to a conformation closely approximating that of native haptoglobin.

Is the binding of hemoglobin one of the primary purposes for haptoglobin in the plasma? Javid and Fuhman (1971) studied sera from man and five other primate species for the antigenic structure of their haptoglobins and found at least eight antigenic determinants in human Hp 2-1 and seven in Hp 1-1. All nonhuman primate haptoglobins lacked the determinant which depended upon the polymeric configuration of haptoglobin although electrophoretically they resembled Hp 1-1.
The nonhuman primates, when ordered according to their evolutionary proximity to man, showed a progressive decrease in the number of determinants shared with man, possibly reflecting the cumulative effect of mutations on haptoglobin structure. The only determinants shared by all the tested species were located near the binding site of hemoglobin. The implication here may be that the ability of the haptoglobin molecule to bind hemoglobin conferred to it a selective advantage on the evolutionary scale.

In conclusion, Hp 1-1, the type used experimentally in the following thesis, can be considered a monomer made up of two pairs of like chains joined together by disulphide bridges. There are carbohydrate moieties associated with the heavy chains and the binding of hemoglobin is predominantly associated with these chains. A generalized diagram for the haptoglobin structure is illustrated in Figure 2.

III Hemoglobin

Hemoglobin (Hb) refers to the main component in the red blood cells of vertebrates (e.g. 95% of the total protein in the red blood cells of man) and some heme-containing oxygen carriers of invertebrates. Its function is to combine reversibly with oxygen in the lungs and to release it in the tissues. Also, by alteration in hydrogen binding capacity, hemoglobin largely neutralizes the carbonic acid that arises from carbon dioxide.

Hemoglobin, as far as is known, exists under many conditions as a tetrameric molecule of molecular weight 65,000. The molecule consists of four subunits identical in pairs. The two
Figure 3: The Chemical Structure of Heme

(Fischer & Orth, 1937; Lehmann & Huntsman, 1966)
different polypeptide chains have been called α and β chains so that the full molecule can be indicated as $\alpha_2\beta_2$. To each polypeptide chain is bound a prosthetic group, the heme. The amino acid sequences of the chains of a number of hemoglobins is known. Since this work uses normal human adult hemoglobin A, the α and β chain sequences are given in Table II. Starting from the amino-terminal end of these chains, the letters A to H are assigned to the known helical segments, and AB, BC, etc., to the corners or non-helical segments.

X-ray analysis of horse (Cullis, Muirhead, Perutz, Rossmann & North, 1962) and human (Muirhead & Perutz, 1963; Muirhead, Cox, Mazzarella & Perutz, 1967) deoxyhemoglobin at 5.5 Å resolution showed it to consist of four chains, arranged in a tetrahedral conformation with the irregular parts meshing together so that there is little free space in the interior of the molecule. Although their primary amino acid sequences differ from each other in more than forty positions (Smith, 1964; Smith, 1968; Braunitzer & Matsuda, 1963; Braunitzer, Gehring-Muller, Hilschmann, Hobom, Rudloff & Wittmann-Liebold, 1961; Konigsberg & Hill, 1962; Konigsberg, Guidotti & Hill, 1961), Bolton, Cox and Perutz (1968) found that visual comparison of their superimposed density maps revealed no significant differences in their tertiary or quaternary structure. The assumption has been made that the quaternary structure is general for all mammalian hemoglobins.

The non-protein heme group of hemoglobin is an iron II complex of protoporphorin IX (Fischer & Orth, 1937) with the metal occupying the central position of a disc measuring 14 Å by 17 Å (Figure 3). Embedded in the polypeptide chains of globin (the protein
Table II: The α and β Chain Amino Acid Sequence of Hemoglobin A

(Data from Dayhoff & Eck, 1967-68)
<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
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<th>D</th>
<th>E</th>
<th>F</th>
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portion of hemoglobin), the heme is almost at the corners of the
globular, slightly tetrahedrally-shaped molecule. The x-ray data
show that one of the coordination bonds to the heme is directed to the
imidazole nitrogen of a histidine residue and that the second
coordination site on the other side of the plane can be bound to a
ligand (Kendrew, Watson, Strandberg, Dickerson, Phillips & Shore, 1961;
Cullis, Muirhead, Perutz, Rossmann & North, 1962). The imidazole
nitrogens come from the following residues in human adult hemoglobin A;
α-87 and β-92 (see Table II).

The 2.8 Å resolution study of horse hemoglobin by
Perutz, Muirhead, Cox and Goaman (1968) has revealed the following
features of the quaternary structure. The oxyhemoglobin resembles a
spheroid with a 64 Å length, a 64 Å width and a height of 50 Å. An
internal cavity which extends all the way along the molecular dyad axis
can be represented by two boxes, each about 20 Å length, 8-10 Å width
and 25 Å depth; one separating the α chains and the other the β chains
from each other. The internal cavity is lined with polar residues. For
example, a large number of serines and threonines are found facing the
cavity along the helix α-H. The suggestion has been made that these
polar groups protect the molecule from water by having hydroxyl groups
of these residues forming a hydrogen bond with the main carbonyl group
four residues back along the helix.

Polar groups are not found in the interior of the α and
β subunits except for the occasional serine and threonine (the hydroxyl
group is hydrogen-bonded to a carbonyl group within the same helix) and
the heme-associated imidazole groups. Larger non-polar side-chains
generally lie in the interior or in surface crevices of the subunits or
Figure 4: The Hemoglobin Molecule

(The subscripts 1 and 2 are used here to distinguish the two α and the two β chains from each other.
This positioning has been defined by Perutz (1969))
From Dickerson and Geis (1969)
Figure 5: The Amino Acids Involved in the $\alpha_1\beta_1$ Contact (a) and the $\alpha_1\beta_2$ Contact (b)

Numbers on lines represent the number of atoms contributed to the contact by the nearer member of each pair joined by a line. A contact is defined as an approach to within 4 A° or less. (Perutz, 1969)
at the boundaries between unlike units. Non-polar side-chains protruding into the surrounding water are rare, but occur occasionally; for example, leucine β-68.

Figure 4 shows the positioning of the four hemoglobin subunits (Dickerson & Geis, 1969) and Figure 5 the contacts between the individual chains (Perutz, 1969). The subscripts 1 and 2, used to distinguish the two α and β chains, have been defined by Perutz (1969). The α₁β₁ contact is more extensive, including 110 atoms contributed by 34 amino acid residues; the α₁β₂ contact consists of 80 atoms contributed by 19 residues. A great many of the interactions between the chains are non-polar, and no lysine residues are in either contact. Although Perutz, Muirhead, Cox and Goaman (1968) have been unable to distinguish the interactions between the unlike chains, no contacts between the like chains were visible on the x-ray maps. The authors assume that at low salt concentrations these exist as salt bridges involving terminal residues.

Perutz states that symmetrical dissociation occurs, since it requires less breakage of the contacts to form α₁β₁ dimers than α₁β₂ dimers. Edelstein, Rehmer, Olson and Gibson (1970) have argued that the difference between 110 atoms in α₁β₁ and 80 atoms in the α₁β₂ contact is not sufficient to completely rule out formation of the latter dimer. Other authors have supported Perutz’s theory (Kellett, Midgarden Schachmann, 1970), and until better evidence to the contrary can be obtained, the conclusion that liganded hemoglobin dissociates to α₁β₁ dimers is considered to be valid.

The quaternary structure seems to derive its coherence only from weak secondary forces, meaning that the stability of the
structure is largely entropic in origin. The full protein may be considered to be in a state of reversible equilibrium with its components, usually with its dimer component. The dissociation may not be evident under many conditions owing to the high value of the association constants.

Hemoglobin has been reacted with many reagents to study their effects on this protein's functional properties. This type of work has been reviewed by Rossi-Fanelli, Antonini and Caputo (1964), Antonini and Brunori (1970) and Buse (1971). For the purpose of this review only the modifications used experimentally in the following research will be discussed.

The reaction of hemoglobin's two "reactive" sulphydryl groups with various compounds is a means of altering the molecule which has provided considerable insight into the relationship between structure and function. Purified haptoglobin does not possess "reactive" -SH groups, and the complex is known to have the same number of "reactive" -SH groups as hemoglobin alone (Nyman, 1959; Tattrie & Connell, 1967). It is, therefore, unlikely that disulphide bridges are formed in the complex. This aspect will be discussed further in the following section of this historical review.

Treatment of hemoglobin with Hg++, Ag++, Cu+ or para-mercuribenzoate did not inhibit haptoglobin binding (Robert, Boussier & Jayle, 1957). Bunn (1967) produced data on human hemoglobin treated with iodoacetamide, N-ethyl maleimide, para-mercuribenzoate, cystine and cystamine to confirm that blocking of the "reactive" sulphydryl groups of hemoglobin per se has no effect on its ability to bind haptoglobin. Malchy (1970), studying the influence of haptoglobin on
the reactivity of the \(-\text{SH}\) groups, verified the fact that \(\beta-93\) cysteine was the only residue in hemoglobin which reacted with various reagents. He found the rate of reaction of iodoacetamide, 2,2'\'-dithiodipyridine and 4,4'\'-dithiodipyridine with the complex was markedly slower than with hemoglobin alone. The conclusion was that there was a conformational change induced in the environment about the \(\beta-93\) residues.

As far as is known now, all mammalian hemoglobins contain at least two \(-\text{SH}\) groups (generally called "reactive") that combine with various sulphhydryl reagents. In addition, many contain other \(-\text{SH}\) groups that do not react ("unreactive") unless the molecule is opened up in some way, as by acid, urea or detergents (Cecil, 1963). In human hemoglobin the "reactive" \(-\text{SH}\) is located at \(\beta-93\), which is adjacent to the \(\beta-92\) histidine residue. This residue is in close contact with the heme group of hemoglobin (Cullis, Muirhead, Perutz, Rossmann & North, 1962). Blocking the two \(\beta-93\) \(-\text{SH}\) groups affects the oxygen equilibrium of the hemoglobin. Since the nature and extent of the effects produced differed with the blocking reagent, it has been concluded that the "reactive" \(-\text{SH}\) groups, as such, do not play an essential role in ligand equilibrium (Rossi-Fanelli, Antonini & Caputo, 1964).

Taylor, Antonini, Brunori and Wyman (1966) have described the preparation of derivatives of hemoglobin reacted with cystine, cystamine, dimercaptoethanol and iodoacetamide. The important fact is that iodoacetamide was found to react at \(\beta-93\) cysteine to form a carbamidemethyl derivative of cysteine. This reagent forms a stable covalent bond with the cysteine and forms carboxymethylcysteine upon acid hydrolysis.
In conclusion, hemoglobin is made up of four subunits which are identical in pairs. The tertiary structure of the subunits is held together only by weak noncovalent forces, including salt bridges, hydrogen bonds and many non-polar interactions. The polar side-chains are mainly in contact with the solvent, while non-polar residues generally lie in the interior of the molecule or in regions of contact between subunits. The heme group is embedded in a hydrophobic pocket which is similar for both chains. Most of the residues in van der Waals contact with the hemes are "invariant" in different hemoglobins. Nearly all the bonds which are made with the non-polar part of the protoheme are hydrophobic, confirming the role of non-polar side chains in stabilizing the heme-globin complex.

IV Haptoglobin-Hemoglobin Complex

The most characteristic property of haptoglobin, although some consider its biological role to still be incompletely defined, is its ability to bind hemoglobin. Studies on the stoichiometry and the binding properties of haptoglobin-hemoglobin complexes have led to the conclusion that this binding is irreversible and characterized by a 1:1 molar ratio. This binding has tentatively been placed at the αβ dimer level of hemoglobin rather than at this protein's tetrameric level. It seems to follow that the haptoglobin molecule is bivalent towards the hemoglobin dimer.

Apparently, the primary sequence of hemoglobin can be varied considerably without the loss of binding ability because human haptoglobin has been shown to form complexes with many mammalian hemoglobins: e.g., human, monkey, pig, rabbit, horse and mouse. The
quaternary structure of hemoglobin is important in complex formation. Deoxyhemoglobin has been shown not to be bound to haptoglobin (Nagel, Rothman, Bradley & Ranney, 1965). Muirhead and Perutz (1965), using X-ray crystallographic techniques, have shown the conformation of hemoglobin to be changed on deoxygenation such that the β chains moved 7 Å apart. Apparently the association-dissociation equilibria of deoxyhemoglobin must differ from those of ligand-bound hemoglobin, and quantitatively deoxyhemoglobin must have a lower tendency to dissociate into subunits than ligand-bound hemoglobin (Antonini & Brunori, 1970).

The nature of the ligand attached to heme is unimportant, since carboxymethemoglobin, cyanmethemoglobin and oxyhemoglobin all form complex equally well (Nagel & Gibson, 1967). Complex formation takes place over a pH range of 4.6 to 11.0, a range in which it has been shown that changes in the environment about the heme group occur (Jayle & Moretti, 1962). The heme itself appears not to be necessary for complex formation, since the globin portion of hemoglobin will form a complex with haptoglobin (Laurell & Gronwall, 1962). Since the variously liganded hemoglobins have identical globin quaternary structure (Perutz, 1969), it is certain that binding is a property of the protein moiety of hemoglobin.

Oxyhemoglobin and deoxyhemoglobin, treated with carboxypeptidase A, which removes the two C-terminal residues of the β chains and consequently their conformational differences (Antonini, Wyman, Zito, Rossi-Fanelli & Caputo, 1961), have been shown to bind with Hp 2-2 (Chiancone, Wittenberg, Wittenberg, Antonini & Wyman, 1966). Deoxygenation had no effect on the stability of this complex. The fact that the complex did not split apart upon deoxygenation is an indication
either that the complex is very stable or that the hemoglobin upon complex formation is no longer a tetrameric structure. In the latter case, the hemoglobin would no longer be affected by deoxygenation, a condition which is known to retain the tetrameric structure of the protein (Perutz, 1969). Nagel and Ranney (1964) found that haptoglobin would not react with either Hb (β)₄ or Hb Barts (γ)₄, suggesting that the αβ subunit was essential for binding.

The mechanism by which binding occurs has been the subject of several studies but has yet to be completely elucidated. Nagel and Gibson (1967) followed tryptophan fluorescence quenching by heme in a stopped-flow apparatus and found that haptoglobin bound more rapidly to low concentrations of hemoglobin than to high, which suggests involvement of a hemoglobin subunit. This binding difference did not occur when an excess of hemoglobin over haptoglobin was tested. In 2M sodium chloride solution at neutral pH, hemoglobin, which exists largely as α₁β₁ dimer (Antonini, Wyman, Bucci, Fracicelli & Rossi-Fanelli, 1962), had the same binding kinetics, indicating that the dimer could be the reactive species.

Nagel and Gibson (1967) also found that separated α subunits of hemoglobin reacted with haptoglobin. Although separated β subunits did not complex with haptoglobin by themselves, they did react rapidly with a previously incubated mixture of haptoglobin and α subunits of hemoglobin. It was suggested that the haptoglobin site binds α subunits specifically and that the normal reaction between hemoglobin and haptoglobin proceeds either by consecutive binding of α and β monomers or by attachment of αβ dimers through the α subunits.

The stoichiometry and equilibrium of the reaction of
haptoglobin both with hemoglobin and with the individual α and β chains has been studied by Chiancone, Ioppolo, Vecchini, Finazzi Agro, Wyman and Antonini (1968) and Sugata (1971). In the case of the isolated α and β subunits, both liganded and unliganded forms reacted with haptoglobin equally well. However, the affinity of the β subunits for haptoglobin was much less than that of the α subunits, which in turn was much less than that of liganded hemoglobin. At saturation, four α subunits were bound per haptoglobin molecule, but these could be displaced by native hemoglobin. Sugata (1971) also found that isolated αβ dimer readily reacted with haptoglobin to yield a complex with similar affinity as has native hemoglobin.

Boyd, Smith and Andrews (1971) used complex saturated with human α subunits and showed that native mouse hemoglobin would displace the α chains from the complex. This does not support the assumption of Nagel and Gibson (1967), who studied the effects of adding hemoglobin to haptoglobin with α subunits, using the kinetic technique of fluorescent quenching in a stopped flow apparatus. They found that this reaction was slower than that between hemoglobin β subunits and haptoglobin-α subunit-saturated complex. They explained their finding on the assumption that time was required for dissociation of the hemoglobin tetramer to monomer whose β subunits subsequently bound to the haptoglobin-α subunit-saturated complex to form the normal complex. The results of Boyd, Smith and Andrews (1971), therefore, indicate the necessity for an alternative explanation for the kinetic fluorescent
Kinetic studies of binding of isolated chains have led to the conclusion that four equivalent and independent sites for binding of $\alpha$ chains exist on haptoglobin (Alfson, Chiácono, Wyman & Antonini, 1970). Boyd (1971) has shown that the number of $\alpha$ chains bound by haptoglobin was dependent on the molar excess of $\alpha$ chains, the concentration of haptoglobin and pH. The results of the dependence of binding on $\alpha$ chain excess indicated that haptoglobin possessed two distinct pairs of $\alpha$ chain binding sites. Boyd has suggested that the dependence of binding on concentration and pH could be interpreted on the basis of dissociation of the haptoglobin molecule.

In any case, the results, although sometimes conflicting, have indicated that the normal $(\alpha_\beta)_2$-Hp complex is the preferred complex because the $(\alpha)_4$-Hp complex could easily be changed to the $(\alpha_\beta)_2$-Hp complex in the presence of $\beta$ chains in any form.

The work of Nagel and Gibson (1971) has almost definitely ruled out the hemoglobin tetramer as the functional unit in the binding to haptoglobin. Using tryptophan fluorescence quenching, they followed the reaction of deoxyhemoglobin and carbon monoxide-saturated Hp 1-1. It had previously been shown that deoxyhemoglobin would not react with haptoglobin (Nagel, Rathman, Bradley & Hanney, 1965) and that the tendency was for deoxyhemoglobin to retain its tetrameric structure (Perutz, 1969). Conversion of deoxyhemoglobin to carbon monoxhemoglobin was much more rapid than was the dissociation to hemoglobin dimers. If dimers are required for complex formation to occur, one expects a delay in quenching when deoxyhemoglobin and CO-
saturated haptoglobin are combined. This indeed was observed, and therefore it was concluded that the functional unit in hemoglobin-
haptoglobin complex formation was probably the $\alpha_2\beta_2$ dimer.

Previous evidence in favour of the dimer hypothesis has been obtained by examining complex formed with subsaturating amounts of hemoglobin. Under these conditions, electrophoresis produces three bands: one complex, one excess haptoglobin and a third intermediate band. Laurell and Gronwall (1962) speculated that the third band represented haptoglobin bound to a half molecule of hemoglobin. Finding the sedimentation coefficient of this intermediate in dog plasma (Hamaguchi, 1966) and human plasma (Ogawa & Kawamura, 1966) to be intermediate between haptoglobin and complex, led Ogawa and Kawamura to the postulation that Hp 1-1 had two binding sites, each for a half molecule of hemoglobin. Hamaguchi and Sasazuki (1967) observed addition of saturating amounts of hemoglobin to cause disappearance of the intermediate complex, which did not reappear upon further addition of haptoglobin. Hamaguchi (1967) determined its molecular weight and heme content to be consistent with the half saturation theory. This was confirmed by Ogawa, Kagiyama and Kawamura (1968), who further suggested that the half molecule of hemoglobin was $\alpha_3\beta_2$ dimer rather than a pair of like chains. Immunological confirmation of this occurred in 1968 (Kagiyama, Ogawa & Kawamura, 1968), when the intermediate complex was shown to bind both $\alpha$-specific and $\beta$-specific antibodies.

There is evidence that it is possible to form more than one type of saturated complex. A study of oxidation-reduction potentials of complexes when the ratio of haptoglobin to hemoglobin
Figure 6: Scheme for Mechanism of Binding of Hemoglobin to Haptoglobin

(Waks, Alfsen, Schwaiger & Meyer, 1969)

Addition of excess hemoglobin to haptoglobin leads to the saturated complex HpHb, which has two dimers bound to Hp in different fashions. Exposure of this to Hp results in loss of one dimer to form Cd, and Cx when both dimers become bound in the stable manner. Once formed, the stable bond shown by diagonal lines is not altered by exposure either to Hb or Hp. The less stable bond shown by horizontal lines in HpHb is not necessarily an intermediate because Cx and Cd were formed directly by mixing with Hp in excess.
was one to one indicated more than one of these to be formed (Brunori, Alfsen, Sagesse, Antonini & Wyman, 1968). Weka, Alfsen, Schweiger and Meyer (1969) have investigated this further. They isolated and characterized two different complexes of identical stoichiometry (1:1): HpHb and Cx (Figure 6). A third complex, Cd, of stoichiometry 2:1 was also described. As illustrated, the nature of the resulting complex depended on the mole ratio of the haptoglobin and the hemoglobin in the starting mixture. HpHb and Cx are similar except that one of the dimers of the former complex can be displaced by the addition of Hp. Cx and Cd complexes are bound reversibly to haptoglobin. It is difficult to explain the failure of HpHb to rearrange to the more stable Cx. It might also be expected to show exchange with other hemoglobin dimers, but Bunn (1967) found no such exchange. Since it seems that both bonds in HpHb are irreversible, one finds it difficult to suggest any mechanism for its conversion to Cx and Cd in the presence of haptoglobin.

The binding of haptoglobin and hemoglobin results in marked changes in the properties of hemoglobin, one being the marked increase in the weak hemoglobin peroxidase activity upon complex formation (Polonovski & Jayle, 1938). The fact that haptoglobin titrated with hemoglobin gave a maximum heat of reaction at the point of molar equivalence and maximum peroxidase activity, but that hemoglobin titrated with haptoglobin gave half the maximum heat of reaction at maximum peroxidase activity and that more than a molar equivalent of haptoglobin was required to attain maximum heat of reaction has led Adams and Weiss (1969) to suggest that haptoglobin reacts sequentially with dimers of hemoglobin. The reasoning for the high
peroxidase activity in complex is that dimers are better peroxidases than tetramers, and haptoglobin prevents reassociation to the tetramer form of hemoglobin. This implies that bound hemoglobin does not have the same quaternary structure as unbound. Measurements of heats of reaction at different temperatures yielded a value of the change in heat capacity at constant pressure similar in magnitude but opposite in sign to that obtained for like changes in β-lactoglobulin. Suggesting that this resulted from a loss of hydrocarbon moieties, Adams and Weiss (1969) concluded that hydrogen bonding reinforced by hydrophobic interactions was the probable force involved in complex formation. Peacock, Paslewka, Real and Ness (1970) calculated the expected amounts of intermediate and saturated complex that should be found on mixing the proteins at various ratios. Assuming two equally available sites along with irreversible binding, results were obtained to support the existence of two sites, each of which could bind the αβ dimer. This work was carried out under neutral conditions, and the question arises as to whether a slight change in the environment would or could affect the binding property.

Levels of peroxidase activity in partially saturated complexes of hemoglobin and a mixture of Hp 2-1 and 2-2 have suggested that several intermediates are formed before saturation is reached (Favilick & Jaenicke, 1971). Adding hemoglobin to haptoglobin gave the complex Hb→Hp. The inverse sequence of mixing led to a complex of identical composition but different arrangement of constituents (Hp→Hb), as shown by differences in the accessibility of histidine in both types of complex. The suggestion here is that Hb→Hp is the combination of αβ dimers of hemoglobin with haptoglobin, while Hp→Hb
corresponds to the complex with intact hemoglobin tetramers. This seems contrary to most of the evidence which has become available.

The nature of the binding between hemoglobin and haptoglobin has thus not yet been elucidated although a number of studies have been attempted to explain it. Amino groups had been thought to be involved in complex formation because treatment of hemoglobin with formaldehyde prevented complex formation (van Royen, 1950). Frankel-Conrat (1957) has shown that this reagent reacts with -NH₂ or -SH groups to produce methylol, which can condense with amine, guanidyl, phenolic and heterocyclic groups to yield cross-linking methylene bridges. This reactivity of formaldehyde shows that the loss of complex-forming ability cannot be due only to modification of the lysine and valine (N-terminal residues of the hemoglobin chains) residues.

Shinoda's (1965) finding that complexing was impaired when the amino groups of haptoglobin were partially trinitrophenylated by reaction with 2,4,5-trinitrobenzene-1-sulphonic acid led him to conclude haptoglobin amino groups play some role in the complexing with hemoglobin. This, however, was not supported by Chan (1968). After the lysyl residues were converted to homoarginine residues in hemoglobin, haptoglobin and complex with 1-guanyl-3,5-dimethyl pyrazole nitrate, the extent of reaction in complex was found to agree to within 3% of that of the two proteins separately. The area of contact between the two proteins was concluded to be either small or in lysine-deficient regions. Lockhart (1971) quantitatively modified the lysyl residues of hemoglobin with ethyl acetimidate-HCl and found no change in the ability to bind with haptoglobin. He concluded that lysyl residues of hemoglobin play no significant role in the binding to haptoglobin.
Furthermore, x-ray analysis of horse hemoglobin (Perutz, 1969) revealed that lysyl residues were well-distributed over the external surface of the hemoglobin molecule, leading Lockhart to propose that complexing occurred on the interior of the hemoglobin tetramer.

Kalous and Pavliceck (1965) have titrated hemoglobin, haptoglobin and their complex and found that there was no apparent $pK_a$ change of carboxyl groups between the individual proteins and the complex. This was verified by measuring the amount of glycine introduced into haptoglobin by reacting the haptoglobin carboxyl groups with glycine methyl ester (Chiao & Bezkorovainy, 1972). Although these authors found modification to be only moderate (50%), optical rotations indicated that a considerable randomization of the haptoglobin structure had taken place. Even so, there was no decrease in binding ability. The carboxyl groups of haptoglobin do not seem to participate directly in the haptoglobin-hemoglobin interaction. The carboxyl groups of the hemoglobin $\beta$ chains have been shown to have no effect on binding (Antonini, Wyman, Zito, Rossi-Panelli & Caputo, 1961), but no such work has been done concerning the $\alpha$ chain carboxyl groups. By observing the scale model available in the Smith laboratory, one can see that this terminal-carboxyl position is near the external surface of the model. If the interior surface of the dimer is involved in the complex formation, then one would expect that the $\alpha$ chain terminal-carboxyl group would have little effect on binding.

Upon titrating hemoglobin, haptoglobin and complex, Kalous and Pavliceck (1965) found that 26 residues of histidine had become buried upon complex formation. With peroxidase activity as a measure of binding, photooxidation showed binding to be dependent on the
histidyl residues of hemoglobin and the tyrosyl residues of haptoglobin. However, when tyrosine and tryptophan residues of haptoglobin were modified with N-acetylimidazole and 2-hydroxy-5-nitrobenzyl bromide, respectively, the complex could be formed but no peroxidase activity was present (Dobryszyc, Pusztai & Kukral, 1969). These results together indicate that tyrosine residues of haptoglobin may not be important in the complexing but have a role in the peroxidase activity.

A rather extensive investigation of the role of tyrosyl residues of haptoglobin in the hemoglobin-binding reaction was carried out by Dobryszyc, Pusztai & Kukral (1969) on canine haptoglobin. The canine haptoglobin was modified by acetylation of the phenolic groups of tyrosine, and as a result, it was observed that the modified complex had not acquired an increased peroxidase activity although complexing occurred. Chiao and Bazkorovainy (1972), using human haptoglobin, observed that nitration abolished both its complexing ability and its ability to enhance the peroxidase activity of hemoglobin. In addition, all the tyrosyl residues of human haptoglobin were accessible to nitration, whereas canine haptoglobin tyrosyl residues were not and were assumed to be buried. This difference should be followed up in various species before any conclusions are made about tyrosine.

Jaenicke and Pavlicek (1970) have used diazonium-1-tetrazole to modify the histidyl residues of hemoglobin and concluded that these are involved in binding to haptoglobin. Chiao and Bazkorovainy (1972) found that carboxymethylating hemoglobin results in dissociation into subunits together with dehemeing. They, therefore, suggest that the histidyl residues are involved in maintenance of the
quaternary structure of hemoglobin rather than in the binding process.

Jaenicke and Pavlicek (1970) have studied the interactions between both Hp 2-1 and Hp 2-2 and bovine hemoglobin, taking inhibition of complex formation and complex dissociation in various solvent media as criteria. As shown by relative peroxidase activity and gel chromatography, complex dissociation occurred at high concentrations of guanidine-HCl, urea, sodium chloride, dioxane, and formaldehyde, while in the case of sodium dodecyl sulphate a low molar ratio was sufficient to dissociate the complex. It was suggested from these results, that ion-pairs and hydrogen bonds were the prevalent interactions involved in the stabilization of the complex and that hydrophobic interactions were of little importance. The latter conclusion concerning hydrophobic interactions was contrary to the evidence of Adams and Weiss (1969). This conflict leaves the effect of hydrophobic interactions in complex formation still in question.

From the evidence of this review the following pertinent conclusions can be drawn. The modification of β-93 cysteine in hemoglobin caused no noticeable effect on the formation of complex with haptoglobin. The binding of hemoglobin occurs predominantly on the H chain of haptoglobin, while the interior surface of the dimer of hemoglobin binds to haptoglobin. The amino groups of hemoglobin and haptoglobin have been shown not to be involved in the formation of complex. The carboxyl-terminal of the β chain of hemoglobin has also been shown not to be involved although there is still question concerning the α chain carboxyl-terminal. The tyrosine groups of haptoglobin have not definitely been eliminated, but the histidine groups have in haptoglobin. The important conclusion for this work is that
it would seem that ion-pairs and hydrogen bonding reinforced by 
hydrophobic interactions are the probable forces involved in the 
complex formation.

V. Modes of Dissociation of Proteins

When considering proteins, it is convenient to divide 
the structure into four levels of organization. The primary 
structure of a protein refers to the number, configuration and sequence 
of amino acid residues that are linked by repeating peptide bonds. 
Secondary structure refers to any regular and periodic folding that 
occurs in the backbone of a polymer molecule. Usually this folding 
results from formation of hydrogen bonds in a regular and systematic 
fashion. Helix formation which occurs in the subunits of hemoglobin 
is an example of secondary structure. Proteins often undergo folding 
due to forces which are neither regular nor systematic. Folding due 
to these irregular causes yields the tertiary structure. This folding 
usually involves interactions between side chains in proteins but 
may involve backbone interactions. Quaternary structure refers to the 
organization resulting from interactions of subunits, as in hemoglobin.

Having described a set of divisions into which it is 
convenient to classify levels of organization in protein structure, 
the types and principles of bonding that are involved will be 
described.

1. Covalent bonds occur in the primary structure as bonds 
between the amino acids and in disulphide bonds between cysteine 
residues. It is not always expedient to classify disulphide bonds as 
part of the primary structure. Depending upon the particular case,
these may be considered as a part of the primary structure or the tertiary structure or as a separate component of protein structure.

2. Hydrogen bonds are due primarily to electrostatic interactions in a system consisting of two electronegative atoms, such as F, O, N or S separated by a hydrogen atom, usually unequally distant from the two electronegative atoms, so that it is considered covalently bonded to one and electrostatically bonded to the other. They are important in aqueous systems, in which they largely account for the high dielectric constant and high boiling point and other unusual properties of water. When hydrogen bonds repeat systematically, as in an\( \alpha \)-helix, their total contribution to stabilization of conformation makes this type of bonding primarily responsible for the specificity of secondary structure in proteins.

3. Salt linkages such as that between\( \text{\textsuperscript{5}}\text{O}^{\text{-}} \), and \( \text{\textsuperscript{8}}\text{H}^{\text{+}} \) are likely to occur in low dielectric media; i.e., the hydrophobic interiors of globular molecules. That they can contribute to stability is indicated by the partial breakdown of the quaternary structure of hemoglobin at high salt concentrations.

4. Van der Waal's bonding is a short range polarization interaction. This is a relatively weak force and arises through synchronization of the motions of the electrons in the separate atoms as they approach one another. The attractive force that results is rapidly overcome by repulsive forces when the atoms get very close to one another.

5. Hydrophobic bonds result when the apolar side chains found in the amino acids alanine, leucine, isoleucine, valine, phenylalanine, proline, tyrosine and methionine come together and
and exclude water to form a hydrophobic region, as in micelle formation. This can alternatively be considered as water molecules coming together with the exclusion of the nonpolar material, which accounts for the endothermic nature of the hydrophobic bond. In general, a protein might be expected to assume a conformation which maximizes the number of hydrophobic side chains in the interior and spreads hydrophilic groups over the surface in contact with water. The formation of relatively nonspecific hydrophobic bonds may provide a great deal of the driving force for tertiary folding in proteins, while specificity is a result of hydrogen bonds and salt linkages.

After this short introduction into the nature of forces involved in the determination of the conformation of proteins, the discussion will now turn to the modes of dissociation of proteins. Although there are many ways of dissociating proteins (reviewed by Tanford, 1968), this review will consider those which are dealt with experimentally and, if possible, specifically with their effects on hemoglobin, haptoglobin and their complex.

a) Effects of pH: Swedberg and Nichols (1927) were the first to establish that the "stability zone" of hemoglobin was between pH 6 and pH 10. Sedimentation studies between pH 6 and 4.5 have shown hemoglobin to undergo a dissociation into subunits where the molecular weight of the protein approached 1/2 its normal value at about pH 4.5 (Field and O'Brien, 1955). The dissociation appeared to be completely reversible upon neutralization of the solution.

Below pH 4.5 hemoglobin undergoes a further dissociation into quarter molecules (Reichmann and Colvin, 1956; Smith, Haug, and Wilson, 1957), but in marked contrast with the situation above
pH 4.5, here Hb is denatured and the reaction is not rapidly reversible. The driving force is the exposure of histidyl residues that are buried in their uncharged form in the native state (Steinhart & Zaiser, 1951). At very low pH values dissociation of the prosthetic group heme is also very pronounced.

The dissociation of human Hb from pH 9.5 to 11 is similar in many respects to the acid dissociation. The presence of one symmetrical peak in the ultracentrifuge at all pH values, the rapidity of the splitting, its reversibility and the unaltered oxygen capacity and spectrum of Hb indicate the presence of a rapid association-dissociation equilibrium in the native protein in this pH range (Rossi-Fanelli, Antonini & Caputo, 1964).

A careful study of the dissociation of human oxyhemoglobin at neutral pH and moderate ionic strength has been carried out by Chiancone, Gilbert, Gilbert and Kellett (1968), employing a method based on gel filtration. Their results lead to an accurate value of the equilibrium constant for the tetramer-dimer dissociation. Over the concentration range examined, however, the amount of monomer was so small that only an approximate value for the dimer-monomer dissociation constant could be given. Kellett and Schachman (1971), using sedimentation equilibrium, indicated absence of monomers at Hb concentrations as low as $10^{-6}$ to $10^{-7}$ M (in heme). In summary, this indicates that at neutral pH and moderate ionic strength there is a lack of monomer, and the prevalent state of conformation of Hb is the tetrameric form.
b) Effects of Salts: The pronounced effect of neutral salts on dissociation of Hb, notably on the tetramer-dimer dissociation, has been reviewed by Rossi-Fanelli, Antonini and Caputo (1964). In the salt solutions, in contrast to the situation in solutions of extreme pH, Hb shows no signs of denaturation. Molecular weight studies have shown the dissociation to approach a value half that at neutral pH in 2M NaCl. The dissociation was readily and completely reversible once the salt was removed. Salts are known to accentuate the antagonism between water and hydrophobic groups; however, in salt-denatured ribonuclease, regions consisting entirely of hydrophobic groups were found. These were either retained on denaturation or were newly formed as a part of the denaturation process. The evidence points to the hydrophobic bond regions being so stable between the α and β chains in the dimers of Hb as to retain this conformation at least to the 2M NaCl concentration.

Guidotti (1967) studied the ability of several salt solutions to dissociate tetrameric Hb into dimers by measuring osmotic pressure of the solutions. In the solvents studied, the sequence of decreasing stability of the tetramer was deoxyhemoglobin > carbonmonoxymoglobin > oxyhemoglobin ≠ cyanmethemoglobin. The order of effectiveness of the salts in dissociating the Hb tetramers was NaI> guanidinium Cl > NaClO₄ > MgCl₂ > NaCl ≠ CH₃COONa. It would seem from these studies that oxyhemoglobin and cyanmethemoglobin could be considered to be similar.

c) Effect of Alcohols and Dioxan: The addition of alcohols or dioxan to aqueous protein solutions reduces the dielectric
constant and therefore favors the aggregation or precipitation of many proteins if they are at or near their isoelectric points. The denatured product usually possesses an ordered conformation, which, however, is usually quite different from the native structure. The tendency is to form an α-helix whether the protein is randomly coiled or globular (Tanford, 1968).

The addition of 10-20% alcohol or dioxan to water significantly alters its solvent properties (Herskovits, Gadegbeku & Jeillet, 1970). Even though this may not suffice to alter the conformation of typical globular proteins at room temperature, it may be expected to affect the stability of the native protein relative to other possible conformations.

Ethylene glycol, glycerol and sucrose, which have a higher content of -OH groups, are far less effective denaturing agents than the simple aliphatic alcohols when mixed with water (Herskovits et al, 1970). The native conformations of many proteins remain stable to high concentrations of these reagents. The stability of native proteins in the presence of these reagents is what makes them suitable as conformational probes.

d) Effect of Detergents: Detergents are able to produce a drastic cooperative conformational change at remarkably low reagent concentrations. This can be explained only by the existence of strong binding forces between the denaturant and the protein molecule which exist in the denatured conformation but not in the native state.

Weber and Osborn (1967) have used the property of
denaturation by low concentrations of sodium dodecyl sulphate (SDS) to
determine the molecular weights of forty different polypeptide chains
by means of polyacrylamide gel electrophoresis. The dehemed hemoglobin
was found to have a molecular weight of 15,500, which means the
prevalent form here was the monomer. One must assume in SDS the
individual charge pattern of each protein is totally changed by the
binding of SDS anions, thereby rendering all molecules negatively
charged. Thus, extensive disruption of hydrogen bonds within the
molecule must occur.

e) Effect of Guanidinium Hydrochloride (GuHCl): Most
proteins with an ordered native structure undergo a marked transition
in GuHCl. The transition is usually complete at a concentration of
6-8M at room temperature, and the transition is found to be to random
coils. The action of GuHCl is to disrupt hydrogen bonds by competi-
tively vying for the hydrogen bonds. Because GuHCl is a strong
electrolyte, electrostatic interactions have little or no importance
in concentrated solution and therefore pH changes have no effect on the
properties of the randomly coiled hemoglobin.

Tanford, Kawahara and Lapanje (1967), varying the GuHCl
concentrations from 5M to 7.5M, determined molecular weights of various
proteins. A molecular weight of 15,500 was found for hemoglobin,
indicating monomer predominance in GuHCl. Thus, it would seem that
GuHCl, a reagent which leads to loss of all noncovalent structure,
has disrupted all the noncovalent bonds between polypeptide chains
of hemoglobin.
Bernini and Borri-Voltattorni (1970) have subjected purified human haptoglobin to reductive cleavage by β-mercaptoethanol in 8M urea and 6M guanidine hydrochloride. Removal of the denaturing reagents and reactivation in the presence of O₂ and a catalytic amount of thiol was found to cause a regain in the ability of the protein to bind hemoglobin. Tanford, Kawahara and Lapenge (1967) have shown 6M guanidine hydrochloride was able to disrupt the three-dimensional structure of many proteins. Because of this, Bernini and Borri-Voltattorni suggest that their treatment of haptoglobin has led to the generation of randomly coiled chains. They further suggest that the primary sequences of the light (L) and heavy (H) haptoglobin chains are sufficient to direct the refolding of the single chains and their reassembly to polymers of high molecular weight having chemical and immunological characteristics identical with those of the native protein.

f) Effect of Urea: Intramolecular hydrogen bonds may be disrupted by the addition of competitive hydrogen bond-formers such as urea and guanidinium salts. Urea also interacts favorably with polar side chains as well as being a mild hydrophobic bond breaker.

Randomly coiled proteins in urea are subject to effects of pH and ionic strength which are not observed in guanidine hydrochloride solutions: the electrostatic effects of interactions between the charged groups of the protein must be considered (Tanford, 1968). At extremes of pH the charged groups are alike and the random coil dimensions expand, while at the isocionic point a
a contraction of the coil occurs.

The only work on hemoglobin conformation in urea which has been reported was based on carbomonoxyhemoglobin (Kawahara, Kirshner & Tanford, 1965). The protein was dissociated to the half molecule, that is, to the dimer rather than to the expected monomer. The product retained its native structure, at 25°C, to the limit of urea solubility.

Fraser (1969) has tried to use 8M urea and 5M guanidine hydrochloride to separate the hemoglobin-haptoglobin complex. It was only after reduction and aminoethylation of the disulphide bonds in this complex that separation into its individual components by column chromatography could be attained.

Smithies, Connell and Dixon (1966) reported that long-term exposure (for several days) of haptoglobin to urea in alkaline solution produced irreversible changes in the protein. The authors do not speculate on the cause of this irreversible denaturation.

The spontaneous refolding of haptoglobin after extensive reduction and dissociation in 8M urea has been discussed in the previous section (Bernini & Borri-Voltattorni, 1970).

g) Effect of Acetate: Chiancone and Gilbert (1965), using sedimentation velocity, have shown hemoglobin to be dissociated to the αα dimer due to the effect of acetate* rather than the low pH. Cann and Goad (1965), studying the reaction of proteins with small uncharged molecules such as undissociated buffer acid, concluded that the effect is a net positive electrical charge on the protein. The difference in net *(acetate+= undissociated acetic acid)
charge between the native and acid-complexed protein presumably resided in the differences in the pK values of carboxyl groups which were exposed upon addition of acid.

h) Effect of Organic Phosphates: Erythrocytes of man and several other mammals contain considerable amounts of organic polyphosphates, such as diphosphoglycerate (DPG). Diphosphoglycerate is bound to deoxyhemoglobin but not to oxyhemoglobin under physiological conditions in a ratio of one mole per mole of hemoglobin tetramer (Benesch, Benesch & Yu, 1968). Benesch, Benesch and Enoki (1968) showed by work with isolated chains of hemoglobin that the diphosphoglycerate binds at the two β chains in deoxyhemoglobin.

Klinger, Zahn, Brox and Freunder (1971) confirmed the effect of diphosphoglycerate using the organic phosphate ATP. However, they found that this organic phosphate had two further binding sites on hemoglobin. One site to which DPG also binds has been demonstrated only in the presence of an excess of complexing anions; the second is a site which binds ATP much more strongly than DPG and does not depend on the oxygenation state of the hemoglobin.

In general, the addition of organic phosphates tends to maintain the hemoglobin in its tetrameric deoxy-form.

i) Effect of Heparin: Heparin has been used for many years as an anticoagulant in blood analysis. Its action is to inhibit both factor VIII and factor IV and the action of thrombin upon fibrinogen (Davie & Ratnoff, 1965).

Recently, Chiao and Bezkorovainy (1971) have shown the inhibition of the hemoglobin-haptoglobin interaction by various
polyelectrolytes. Sodium heparin was one of these. Using increase in peroxidase activity of hemoglobin alone as the standard, they concluded that the negatively charged polyelectrolytes were capable of inhibiting interaction between hemoglobin and haptoglobin and that the basic amino acid residues of either the hemoglobin or haptoglobin both were probably involved in the formation of the complex between the two proteins. The question arises as to whether heparin will help dissociate the complex.

j) Effect of Temperature: Since an increase in temperature facilitates rotation about single bonds of the polypeptide backbone, the properties of a randomly coiled polypeptide chain should approach more closely those of a random coil with free rotation as the temperature is raised (Tanford, 1968). This type of action would lead one to believe that a mild temperature elevation might have an effect on the dissociation of the hemoglobin-haptoglobin complex.

At lower temperature, the effect is to lessen hydrophobic interactions (Tanford, 1968).

From this section of the review one can recognize the fact that little study of the dissociation of the complex of haptoglobin and hemoglobin has been done. This aspect will be discussed in the following section of the thesis.
C EXPERIMENTAL APPROACH

I Introduction

The covalent bonds in proteins permit the various atoms of the backbone of the polypeptide chain to assume a number of positions relative to each other. Even though the interatomic distances and bond angles in the chain are fixed, the possibility of rotation about each of the two single bonds per amino acid residue (bonds in the peptide backbone) makes the number of conformations an astronomical one. It is clear that other noncovalent interactions must be present if the conformation of the protein is to be unique.

Considerable attention has been devoted to the nature of the bond between haptoglobin and hemoglobin. It would seem that the "irreversible" bonding that exists between these two proteins involves predominantly hydrogen and hydrophobic ones along with ion-pair interactions. The purpose of this investigation was to attempt to discover what type or types of interaction(s) are involved in this complex formation by using media which are known to dissociate proteins.

For the project, porcine haptoglobin and human hemoglobin were purified. Human hemoglobin was reacted with $^{14}$C-iodoacetamide, which produced a $^{14}$C-carbamide methylcysteine residue at the β-93 position of hemoglobin instead of the original
cysteine. From these various combinations of substrate for reaction could be prepared and subsequently used for incubations in dissociating media. Examples of incubation mixtures are: hemoglobin + $^{14}$C-complex, $^{14}$C-hemoglobin + complex, only $^{14}$C-hemoglobin and only $^{14}$C-complex ($^{14}$C-complex is made from porcine haptoglobin and $^{14}$C-reacted hemoglobin).

After incubation in a dissociating medium, the substrates were separated by molecular sieving through monitored columns. The eluted fractions were collected directly in scintillation vials and counted by means of liquid scintillation. If, in the reaction of the mixture of complex and $^{14}$C-hemoglobin, an exchange took place between the hemoglobin in the complex and the free hemoglobin, one would expect to find instead of one radioactive peak coinciding with the hemoglobin peak another radioactive peak at the position of the complex. This, of course, would occur in the opposite direction where the complex was radioactive and the hemoglobin unlabeled. If the cysteine modification did not affect the exchange reaction, one would expect the percent radioactivity which is dissociated to be equal whether the complex or the hemoglobin was the radioactive protein.

A problem with the above system was that the radioactive label was specific only for the $\beta$ chain of hemoglobin, and conclusions could not be drawn about complete movement of the functional dimer of hemoglobin. To study this aspect of the dissociation an $\alpha$ chain-labeled hemoglobin had to be found. Such a hemoglobin was isolated from the llama. Alpha chains of this mammal have isoleucine present in their amino acid sequence while the $\beta$ chain does not (Braunitzer, Hilsa, Rudloff & Hilschmann, 1964). Thus, a dissociation of $\alpha$ chains could be detected by appearance of isoleucine in a "human" hemoglobin peak if the complex
made from llama hemoglobin was incubated with human hemoglobin*.

Llama hemoglobin was obtained and characterized for its isoleucine content. The complex was prepared from this and used in various incubation reactions.

Another method for obtaining an $\alpha$ chain-labeled hemoglobin involved the reacting of hemoglobin with the enzyme carboxypeptidase B, which is known to remove the C-terminal arginine residue from the $\alpha$ chain (Zito, Antonini & Wyman, 1964). Upon incubating this modified hemoglobin with normal complex, one could analyse the separated hemoglobin peak for liberated C-terminal arginine, which would have come from the complex, after further carboxypeptidase B digestion.

To detect whether amino group modification of hemoglobin had any effect on the binding of hemoglobin to haptoglobin, hemoglobin reacted with $^{14}C$-ethyl acetimidate was prepared. Similar experiments to the $^{14}C$-iodoacetamide-reacted hemoglobin were considered.

* Human hemoglobin contains no isoleucine (Table II).
II Materials

1. Human hemoglobin was purified from freshly drawn blood of the author. Llama hemoglobin was obtained from freshly drawn blood of the younger female llama at Storybrook Gardens, London. The purification is discussed in the methods section.

2. Haptoglobin was purified from porcine plasma obtained from the Coleman Packing Co. Ltd.

3. Carboxypeptidase B (CPB) was used from the stock solution which was obtained from Worthington Biochemical Corporation, Winley-Morris Co. Ltd., Montreal (Canada).

4. Iodoacetamide-1\(^{14}\)C was obtained from Amersham/Searle Corporation, Des Plaines, Illinois, U.S.A., in 50-microcurie batches.

5. Acetonitrile-2\(^{14}\)C was obtained from Amersham/Searle Corporation, Des Plaines, Illinois, U.S.A., in 250-microcurie batches.

6. Chemicals were reagent grade unless otherwise noted.

7. Sephadex gels were obtained from Pharmacia (Canada) Ltd., Montreal.

8. Carboxymethyl cellulose (Whatman CM 23) was obtained from Mandel Scientific Co., Toronto.

9. Beckman Spinco Amino Acid Analyser, Beckman Spinco, Palo Alto, Calif., was used for amino acid analyses.
III Methods

a) Preparation of Hemoglobin

i. Preparation

A modified Drabkin's (1946) technique has been used. Freshly drawn blood was mixed with several volumes of cold, 1.5% sodium chloride and centrifuged at 0.5°C (Sorvall, type GSA, 8,000 rpm) for twenty minutes. The packed cells were washed repeatedly with saline until the supernatant gave a negative protein test.

The approximate volume of the packed cells was noted. Twice this volume of cold water and half this volume of toluene was added to the packed cells and the mixture shaken vigorously for five minutes.

After standing for one hour, to allow the stroma to coagulate and settle, the mixture was centrifuged at 0-5°C (Sorvall, type-34, 16,000 rpm) for twenty minutes. The hemoglobin solution was decanted through filter paper (H. Reeve Angel & Co., #202) to remove the coagulated stroma.

The solution was concentrated by the addition of enough dry Sephadex G-25 to obtain a final hemoglobin solution of about 30 gram per 100 ml.

ii. Cyanmethemoglobin

Because of its stability and its similar properties to oxyhemoglobin in binding to haptoglobin, the above hemoglobin was converted to cyanmethemoglobin. For each ml of hemoglobin solution, approximately 40 mg K$_3$Fe(CN)$_6$ and 10 mg KCN was added. After slight agitation for several minutes, the solution was either chromatographed through a Sephadex G-25 column (2.0x50 cm)
using 0.15M sodium chloride as eluting buffer or dialysed overnight against a similar buffer to remove excess reagents (Cameron & George, 1969). The hemoglobin used experimentally was the cyanmet-form.

iii. $^{14}$C-1-Iodoacetamide-Reacted Hemoglobin

Essentially, the method of Taylor, Antonini, Brunori and Wyman (1966) was used to prepare this derivative. This involves the transfer of the carbamide methyl group from iodoacetamide to the $\beta$-93 cysteine residue of hemoglobin.

A volume of 20 ml hemoglobin solution was adjusted to pH 7.0 with dilute NaOH. One ml of this was added directly to a 50-microcurie vial (tin-foil covered) of $^{14}$C-1-iodoacetamide and gently agitated for one and a half hours. A further 10 mg of "cold" iodoacetamide was added after the first reaction, and the vial was further reacted for two hours. These reactions were all carried out at room temperature.

The resulting solution was desalted, either by dialysis or Sephadex G-25 column chromatography, and refrigerated.

iv. $^{14}$C-Ethyl Acetimidate-Reacted Hemoglobin

The synthesis of $^{14}$C-ethyl acetimidate (McElvain & Nelson, 1942) was the first step in the preparation of $^{14}$C-acetimidate hemoglobin. Dry HCl was bubbled through a mixture of 0.1 ml acetonitrile (99%) (precooled to 0°C) and 0.153 ml absolute alcohol in a vial containing 250 microcuries of $^{14}$C-acetonitrile. After several minutes, to insure that the product was saturated with HCl, the vial was sealed and incubated for two hours at 0°C. Then 0.127 ml anhydrous ether was added, the tube resealed and subsequently supercooled to $-30^\circ$C until the $^{14}$C-ethyl acetimidate-HCl crystallized

* c.a. 10% solution
out. The purity of the product was confirmed by comparing its melting point with the commercial product which was used as carrier.

The acetamidino derivative of hemoglobin was prepared by the method of Lockhart and Smith (1971). To a 50-ml volume of 2% hemoglobin, preadjusted to pH 9.5, were added small amounts of $^{14}$C-ethyl acetimidate-HCl. The pH was adjusted to 9.5 after each addition. After all the prepared $^{14}$C-ethyl acetimidate-HCl was added, the mixture was incubated with slow agitation for thirty minutes. A further 300 mg of ethyl acetimidate-HCl was added in small amounts and incubated for thirty more minutes. The resulting solution was concentrated to approximately 10% (i.e. 10 ml) by ultrafiltration through a Diaflo Ultrafilter system. The reagents were then completely removed by Sephadex G-25 column chromatography (similar to the method used to purify cyanmethemoglobin).

v. Carboxypeptidase B-Reacted Hemoglobin

To produce a hemoglobin which had its arginine residue at the α-141 carboxyl-terminal removed, 20 ml of 7% hemoglobin was reacted with carboxypeptidase B for three hours at 37°C (Oda, 1969). The activity of preparations of carboxypeptidase B was variable (Worthington's assay), so the amount of enzyme added was relative to the activity of each batch. Experimentally, 1300 units of enzyme per gram of hemoglobin was used.

This whole solution was eluted from a 2.5x90 cm Sephadex G-150 superfine column, using 0.15M sodium chloride as the eluant. This operation separated the hemoglobin from carboxypeptidase B and the arginine which was released from the reacted hemoglobin.
vi. Determination of Hemoglobin Concentration

Hemoglobin concentrations were determined as
cyarmethemoglobin (converted with Hycel Cyarmethemoglobin Reagent)
using a commercial methemoglobin preparation as standard.

b) Preparation of Haptoglobin

i. Preparation

Fresh whole pig blood was collected, using as
anticoagulant 150 ml standard citrate-dextrose per liter of blood
(22.0 g sodium citrate:2H₂O, 8.0 g citric acid:2H₂O, 22.3 g anhydrous
dextrose, made up to 1 liter with water) (Damm & King, 1965). After
standing at 4°C for several hours, the upper plasma layer was decanted
and centrifuged at 6,800 rpm (Sorvall, type GS-3) for ten minutes at
0-5°C to clear it of cells. Plasma pH was then adjusted to 4.7 with
acetic acid and recentrifuged to remove precipitated material.

The plasma was deionized in 500-ml lots on an 8.5x60 cm
column of Sephadex G-25, equilibrated in distilled water saturated with
toluene. Haptoglobin in the salt-free plasma was then absorbed on a
DEAE-cellulose column (8.5x20 cm) which had been equilibrated with
pH 4.7, 5mM sodium acetate at room temperature. After application of up
to 1.3 liters of desalted plasma, this column was washed with an
equilibrium buffer made up to 0.02M in sodium chloride until the
effluent absorbance had fallen below 0.1 (280 nm). The column was then
washed with a 0.15M sodium chloride solution and the first 200 ml
collected. This was blue in colour (due to its caeruloplasmin content)
and was the fraction which contained haptoglobin.

The haptoglobin solution was cooled to 0°C, and sufficient
solid ammonium sulphate was added to produce 55% saturation (Dijesco,
1968). After centrifugation, haptoglobin present in the supernatant was precipitated by increasing the ammonium sulphate to 65% saturation. This precipitate was redissolved, dialysed against several 6-liter changes of distilled water and lyophilized.

A solution of 1.5-2 gram per cent of crude haptoglobin was prepared. This solution, in 50-ml lots, was passed through a 5x90 cm column of Sephadex G-200 superfine. The eluting buffer, 0.1M tris-HCl, 0.1M NaCl and 0.02% sodium azide, pH 7.4, was pumped through the column at 15 ml per hour (run at room temperature). The fractions were collected every half hour and analysed for haptoglobin. Following this, the haptoglobin peak was pooled, dialysed against distilled water, lyophilized and, after several such runs had been pooled together, rechromatographed through the same Sephadex G-200 column. These procedures are essentially those of Fraser and Smith (1971) as adapted from methods for preparing human haptoglobin (Connell & Shaw, 1961).

ii. Determination of Haptoglobin Concentration

Haptoglobin in solution was measured by its ability to bind a known amount of hemoglobin. This was routinely determined by upward flow gel filtration on a 0.9x12 cm Sephadex G-100 superfine column equilibrated in 0.15M sodium chloride. Column effluent was continuously monitored for heme at 418 mp with a Beckman DB Spectrophotometer equipped with a flow cell and a 6-inch recorder. Two peaks were obtained when hemoglobin was added to haptoglobin; hemoglobin-haptoglobin complex and excess hemoglobin. These could be integrated graphically to give the proportion of hemoglobin bound.

Accurate original hemoglobin concentration and use of a constant volume
Chromatronix sample injecting valve allowed calculation of amounts of complex and hence of haptoglobin concentration, assuming 1:1 stoichiometry.

c) Preparation of Hemoglobin-Haptoglobin Complex

i. Preparation

The various derivatives of hemoglobin were complexed with porcine haptoglobin by adding an excess of hemoglobin to purified haptoglobin and allowing the mixture to stand for several minutes. The complex was purified by passing the resulting solution through a 5x90cm Sphadex G-150 superfine column with the same eluting buffer used in the G-200 Sephadex step of the haptoglobin isolation. The resulting complex peak was pooled and refrigerated.

ii. Affinity of Haptoglobin for Hemoglobin

To determine whether there was any preference of haptoglobin for native hemoglobin over modified hemoglobin, a solution of a combination of the two hemoglobins was prepared (native and reacted). The hemoglobin mixture was several times in excess of the haptoglobin to insure random complex formation between the haptoglobin and the two types of hemoglobin. The complex was separated from the hemoglobin on a Sphadex G-100 superfine column (0.9x12 cm) using 0.15M NaCl as the eluting buffer (12 ml/hr). Fractions were collected every 2.5 minutes and the radioactivity determined. From this and the elution profile obtained from monitoring the effluent at 418 mp with a Beckman DB Spectrophotometer the \textsuperscript{14}C to heme ratio for both the complex and the hemoglobin can be calculated.
d) Separation of Subunits of Hemoglobin

i. Separation

The separation of hemoglobin chains was carried out using a modified Clegg, Naughton and Weatherall (1968) procedure. CM-cellulose was supplied in the Na\(^+\) form, and the slurry for pouring the column was made simply by suspending the cellulose (5 g) in starting buffer. After a few minutes to equilibrate, the cellulose was poured to make a 1x10 cm column.

Buffers of pH 6.8 were used throughout, and the linear Na\(^+\) ion gradient was obtained by mixing 150 ml of starting buffer (0.005M Na\(_2\)HPO\(_4\), 0.05M 2-mercaptoethanol, 8M urea, pH 6.8) with 150 ml limit buffer (0.04M Na\(_2\)HPO\(_4\), 0.05M 2-mercaptoethanol, 8M urea, pH 6.8).

The hemoglobin (30 mg or 0.5 ml) was dialysed against two changes of 5-fold excess of starting buffer for a period of 3 hours at room temperature before washing into the CM-cellulose column with 2 ml of starting buffer. The column was then washed with starting buffer for 30-60 minutes, during which the hemoglobin bound at the top of the column turned brown and the heme slowly washed away down the column. When no more colour was eluted (as judged by eye), the heme-free α and β chains were eluted by application of the Na\(^+\) ion gradient. Monitoring the eluant was accomplished by means of a Beckman DB Spectrophotometer set at 280 nm. Buffer was pumped through the column at 1 ml/minute and 10-ml fractions were collected.

ii. Analysis of the Subunits

The β chains were made radioactive by reacting with \(^{14}\)C-iodoacetamide and could be determined by \(^{14}\)C incorporation.
For this analysis 0.5 ml of each 10-ml fraction was used. For a chain
determination of llama hemoglobin, the protein peaks (after removal
of aliquots for radioactive counting) were hydrolysed and analysed
for isoleucine content.

e) Amino Acid Analysis

i. Sample Preparation

(1) Solution Deproteinization: After
reaction of hemoglobin with the enzyme carboxypeptidase B, release of the
amino acid arginine would be expected. Before analysis for this amino
acid, the hemoglobin solution was deproteinized using sulphosalicylic
acid (DeWolfe, 1966).

An equivalent of 40-50 mg hemoglobin was made up to a
volume of 10 ml with water. One hundred mg sulphosalicylic acid was
added and the mixture shaken for several minutes with care being taken to
avoid frothing. After centrifugation, the supernatant, which was at
about pH 2, could be applied directly to the analyser.

(2) Protein Hydrolysis: Hemoglobin was
hydrolysed in 5.5N HCl in evacuated, sealed tubes at 110°C for 18 hours.

ii. Amino Acid Analysis

The Beckman 121 Amino Acid Analyser was used
for amino acid analyses. The method of operation is outlined in the
manual supplied by the manufacturer.
f) Liquid Scintillation Counting

Counting was done on a Nuclear Chicago Mark I Scintillation Counter equipped with a teletype and an Olivetti-Underwood Programma computer. A correction curve of efficiency against channel ratio was prepared using a series of $^{14}$C quenched standards (picric acid in methanol) in Bray's scintillation fluid. This solution consists of 60 g naphthalene, 20 ml ethylene glycol, 100 ml methanol, 4 g PPO (2,5-diphenyloxazole) and 0.2 g POPOP (p-bis-$[2-(5$-phenyl-oxazolyl)]-benzene in dioxane to make one liter. This formula was developed for $-8^\circ C$, at which temperature 3 ml of aqueous solution is dissolved by 10 ml of Bray's solution.

With the correction curve, the computer can be used to directly calculate the disintegrations per minute in each vial.

IV This Project

Hemoglobin was incubated with complex in media being tested for complex dissociation. The amount of hemoglobin and complex used was such that a reasonable peak for each would appear on the Beckman 6-inch recorder when the separating column was monitored at 418 mp with a Beckman DB Spectrophotometer. Volumes were made up to allow duplicate separations for each incubation. An example of an incubation vial is as follows: 0.5 ml buffer, 0.5 ml complex and 20 lambda's hemoglobin.

Generally, four incubation vials were prepared for each experiment: one which had $^{14}$C-complex and hemoglobin, one with complex and $^{14}$C-hemoglobin, one with $^{14}$C-hemoglobin only and one with $^{14}$C-complex alone. The latter two samples were blanks to insure that there was no permanent dissociation or association upon incubation in the dissociating
media.

After the predetermined incubation time sufficient for the dissociation of the complex to have occurred, the sample was injected into a Sephadex G-100 superfine column (0.9x12 cm) with an automatic Chromatronics Injector Valve (0.5 ml) and the eluant monitored directly through a flow cell in a Beckman DB Spectrophotometer equipped with a 6-inch recorder. The hemoglobin and complex were separated by eluting 0.15M NaCl pumped at 12 ml/hr. Fractions were collected every 2.5 minutes in a fraction collector which had been adapted to hold counting vials. After Bray's scintillation fluid was added, these vials were counted. The radioactivity and the heme peaks were superimposed, and the per cent dissociation after incubation was calculated.

In the case of the isoleucine determinations or carboxypeptidase B-treated hemoglobin incubations, a larger volume was incubated (2.0 ml), and this was separated on a 2.5x30 cm Sephadex G-150 column. The fractions which were eluted at 12 ml/hr were collected every 15 minutes. Of each fraction 0.5 ml was counted while the remainder was kept to be pooled for amino acid analysis. The reason for the larger volumes and the longer column was to insure complete separation of the two proteins and for the amino acid analyses, which required far more sample than the radioactive technique.

Carboxypeptidase B-reacted hemoglobin was incubated with radioactive complex. After separation, the hemoglobin was pooled and further treated with carboxypeptidase B. The protein was then precipitated with sulphosalicylic acid and analysed for exchanged arginine.
For the isoleucine determinations, $^{14}$C-iodoacetamide-reacted llama hemoglobin was prepared. Complex, which was prepared from this hemoglobin was incubated with excess human hemoglobin. The hemoglobin separated after incubation was hydrolysed and analysed for isoleucine content.

The conditions to be studied experimentally were the effects of pH, salts, urea, guanidine hydrochloride, sodium dodecyl sulphate, heparin, organic phosphates, dioxane, ethanol, ethylene glycol and glycine.

The stability of the $^{14}$C-ethyl aceticamide-reacted reactants was also tested.
Figure 7: Summary of Reactions Used to Modify Hemoglobin
\[
R-NH_2 + \text{CH}_3\text{-C-OC}_2\text{H}_5 \rightarrow R-NH\text{-C-CH}_3
\]
ethylic acetimidate

\[
R-SH + \text{ICH}_2\text{CONH}_2 \rightarrow R-S\text{-CH}_2\text{CONH}_2
\]
iodoacetamide

\[
\text{CPB}
R-\text{Arg-COOH} \rightarrow R-\text{COOH} + \text{Arginine}
\]
Figure 8: The Purification of Hemoglobin (Iodoacetamide-[14C]Reacted) by Dialysis

After dialysis, a small amount of the dialysate was separated by column chromatography (Sephadex G-25, 2 ml/fraction). One ml of each fraction was used for counting.
Figure 9: Removal of Excess Ethyl Acetimidate-$^{14}C$ from Reacted-Hemoglobin by Sephadex G-25 Column Chromatography

Each fraction is two ml and one ml was used for counting.
RESULTS AND DISCUSSION

I. PREPARATIONS

a) Iodoacetamide- \(^{14}C\)-Reacted Hemoglobin

The derivative produced by the reaction with iodoacetamide was carbamide methyl cysteine hemoglobin (Figure 7). The \(\beta\)-93 cysteine of hemoglobin is the only cysteine which has been reported to be reacted with this reagent under the experimental conditions (Taylor, Antonini, Brunori & Wyman, 1963).

Figure 8 illustrates the purification of 0.5 ml hemoglobin from excess radioactive iodoacetamide after dialysis. A little potassium ferricyanide was added to the hemoglobin solution to show where the small molecular weight substances were eluted from the column. It seems that dialysis was successful in removing unbound reagent from the reaction mixture.

b) Ethyl Acetimidate- \(^{14}C\)-Reacted Hemoglobin

The reaction proceeds as illustrated in Figure 7. According to Lockhart (1971), all the lysines and N-terminal valines were reacted in this manner in hemoglobin. Figure 9 shows the results of 3 ml reacted-hemoglobin separated on a Sephadex G-25 column.

There is an adequate separation of the excess radioactive reagent from the reacted-hemoglobin by column chromatography.
Figure 10: Amino Acids Released by the Action of Trypsin and Carboxypeptidase B on Native Hemoglobin

A stoichiometric yield of 1 residue per dimer was equivalent to 0.29 micromoles of liberated amino acid.
TRYSIN DIGESTION

- 0 hours
- + 1 hour
- □ 2 hours
- △ 5 hours
- ○ 10 hours

Liberated Amino Acid (µmole)

CPB Digestion (hours)

--- LYSINE
--- ARGinine
Figure 11: Purification of Haptoglobin

I. Profile of first elution through a Sephadex G-200 column (5x90 cm).

II. Sephadex G-200 column chromatography separation after the pooling of several of the first elutions (I).
Figure 12: Preparation of Radioactive Complex from
Iodoacetamide-1-\(^{14}\)C-Reacted Hemoglobin

Complex is in fractions 35-43 and hemoglobin in 46-54
(5 ml fractions).

Note: After insuring that the haptoglobin was
completely complexed with radioactive hemoglobin
(i.e. a slight excess of \(^{14}\)C-Hb was recorded when
the mixture was analysed on a Sephadex G-100
column), more "cold" iodoacetamide-reacted hemoglobin
to insure an observable separation on the preparation
column. This give rise to the difference in the
\(^{14}\)C/heme ratios for the hemoglobin and complex peak.
Figure 13: Preparation of Complex from Ethyl Acetimidate-\textsuperscript{14}C-Reacted Hemoglobin

The upper figure is the normal complex: excess-hemoglobin profile; the lower uses the \textsuperscript{14}C-reacted hemoglobin as precursor. The complex is eluted before the excess hemoglobin in both cases.
c) Carboxypeptidase B-Reacted Hemoglobin

The reaction of carboxypeptidase B to remove the C-terminal arginine of the α chain is shown in Figure 7. Figure 10, from the author's previous work (Oda, 1969), involves the action of trypsin and carboxypeptidase B on hemoglobin. The figure shows that within one hour all the susceptible C-terminal arginines from the α chain of hemoglobin have been removed by carboxypeptidase B. A three-hour incubation was, therefore, considered adequate to hydrolyse all the susceptible C-terminal arginines.

d) Haptoglobin

Upon separating the crude extract on a Sephadex G-200 column, three protein peaks were obtained (Figure 11-I). These peaks overlapped, and the middle haptoglobin-containing peak was impure. After pooling several haptoglobin peaks and reapplying to a similar column, a relatively homogeneous haptoglobin solution was obtained (Figure 11-II).

e) Hemoglobin-Haptoglobin Complex

The elution profile for the separation of iodoacetamide-\(^{14}\)C-reacted hemoglobin and its complex is illustrated in Figure 12. For the radioactive counting 20 lambda of each 5-ml fraction was used. Similarly, 20 lambda of each fraction was diluted to 2 ml with water for the absorbance readings. This was the complex (fractions 36-41) used in the experimental incubations.

A similar profile for ethyl acetimide-\(^{14}\)C-reacted hemoglobin prepared complex is illustrated in Figure 13 (lower profile). The upper figure of Figure 13 shows the preparation of normal
Figure 14: Affinity of Haptoglobin for Types of Hemoglobin

Upper Profile: Complex formation using iodoacetamide-\(^{14}\)C-reacted human hemoglobin, native hemoglobin (human) and porcine haptoglobin.

Lower Profile: Complex formation using iodoacetamide-\(^{14}\)C-reacted llama hemoglobin, native llama hemoglobin and porcine haptoglobin.
**TABLE III**

**AFFINITY OF HAPTOGLOBIN FOR TYPES OF HEMOGLOBIN**

<table>
<thead>
<tr>
<th>SUBSTRATES</th>
<th>$^{14}$C/HEME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>complex</td>
</tr>
<tr>
<td>$H_p$ (porcine) + $^{14}$C-Hb (human) + Hb (human)</td>
<td>2.98</td>
</tr>
<tr>
<td>$H_p$ (porcine) + $^{14}$C-Hb (llama) + Hb (llama)</td>
<td>1.56</td>
</tr>
</tbody>
</table>
hemoglobin-haptoglobin complex. In both cases, the complex is in fractions 30-40. It can be noted that the modification has made the $^{14}C$-hemoglobin elute slightly before normal hemoglobin. This could be an indication that the $\delta_2 \rightleftharpoons 2\delta_1$ equilibrium has been shifted to the left upon modification.

II Affinity of Haptoglobin for Types of Hemoglobin

Figure 14 shows the results obtained for this experiment. Table III presents ratios of $^{14}C$/HEME calculated by integration of the various peaks in the elution profiles (Figure 14). An example of this calculation, using complex made from a mixture of human hemoglobins (Figure 14 upper) normal human hemoglobin (Hb) + iodoacetamide-$^{14}$C-reacted human hemoglobin ($^{14}$C-Hb) and porcine haptoglobin (Hp), was as follows: (the hemoglobins, Hb and $^{14}$C-Hb, were approximately in equal molar amounts) a. for the complex: area of radioactivity peak ($^{14}$C) = 821 units, area of heme absorption peak (HEME) = 274 units, ratio $^{14}$C/HEME = 821/274 = 2.98.

b. for the hemoglobin: area of radioactivity peak ($^{14}$C) = 2070, area of heme absorption peak (HEME) = 703, ratio $^{14}$C/HEME = 2070/703 = 2.95.

The results show constant $^{14}$C/HEME ratios for the complex and its respective mixture of hemoglobin (i.e., the mixture of llama hemoglobin gave constant values). If there had been specific preference of haptoglobin for either Hb of $^{14}$C-Hb, these ratios would not have been the same (within experimental error) in each analysis. One can conclude, therefore, that there is no different affinity of the haptoglobin for the reacted hemoglobin than for the normal hemoglobin in the formation of complex.
Figure 15: Separation of Subunits of Hemoglobin

Upper figure: radioactive profile
Center figure: isoleucine content profile
Lower figure: protein profile
III Separation of Subunits of Hemoglobin

This procedure was used to illustrate the specificity of iodoacetamide-$^{14}$C for the $\beta$ chain of llama hemoglobin and to demonstrate the presence of isoleucine residues in only the $\alpha$ chains. This can be seen in Figure 15, where 0.5 ml of radioactive llama hemoglobin was separated. The lowest profile represents the protein (absorbance 280 mp) as it was eluted from the CM-cellulose column after the heme had been washed out and the gradient started. The top profile was obtained by counting 0.5 ml of each 10-ml fraction to detect where the iodoacetamide-$^{14}$C had been incorporated. The center profile shows the amount of isoleucine in each fraction after hydrolysis and analysis. The analyses were performed on the protein remaining after the aliquot had been removed for radioactive counting.

There is a definite relationship between the three profiles. The radioactivity peak aligns with the first protein peak and the isoleucine peak aligns with the second protein peak. On the basis of previous work on llama hemoglobin and the action of iodoacetamide (Braunitzer, Hilse, Rudloff & Hilschmann, 1964; Taylor, Antonini, Brunori & Wyman, 1966), it can be concluded that the first protein peak (to be eluted after the start of the gradient) is the $\beta$ chain and the second is the $\alpha$ chain of hemoglobin. This is in agreement with the work of Clegg, Naughton and Weatherall (1968) on the separation of the $\alpha$ and $\beta$ chains of human hemoglobin.
Experimental conditions used were: pH 4.7, 0.04M acetate + 50% ethylene glycol, 30°C, 96 hours.

I. $^{14}$C-complex + hemoglobin ($^{14}$C-HpHb + Hb)
II. Complex + $^{14}$C-hemoglobin (HpHb + $^{14}$C-Hb)
III. $^{14}$C-complex ($^{14}$C-HpHb)
IV. $^{14}$C-hemoglobin ($^{14}$C-Hb)
IV. Experiments in Dissociating Media

These experiments used the iodoacetamide-\(^{14}\)C-reacted human hemoglobin and its resulting complex as radioactive reactants for the incubation mixtures. These are denoted \(^{14}\)C-Hb for the hemoglobin and \(^{14}\)C-HpHb for the complex. Normal hemoglobin and normal complex are denoted Hb and HpHb, respectively.

The elution profiles of the various combinations of complex and hemoglobin are illustrated in Figure 16. These results were obtained when the medium was 0.04M acetate + 50\% ethylene glycol, pH 4.7. The incubation was for 96 hours at 30\(^{\circ}\)C.

There has been a displacement or exchange of some \(^{14}\)C-Hb from the \(^{14}\)C-HpHb to Hb and also from \(^{14}\)C-Hb to the HpHb. Both the \(^{14}\)C-HpHb and \(^{14}\)C-Hb were incubated alone, and their elution profiles show single peaks (Figure 16), indicating no dissociation or aggregation of these reactants.

By integrating the areas of the heme and radioactivity peaks, one can calculate the degree of dissociation which has occurred during the incubation in the dissociating medium. An example of the calculation is shown in Table IV.

Let us consider the incubation where \(^{14}\)C-HpHb was the radioactive reactant and the Hb the excess acceptor substrate (i.e. acceptor of \(^{14}\)C-Hb). The assumption has been made that all the radioactivity in the incubation mixture is initially from the radioactive substrate. If this is the case, then after the incubation, the total initial radioactivity would be given by the counts found in both complex and hemoglobin peaks (i.e., 5245 units). If one considers the possibility of equilibrium occurring in the
### TABLE IV

**CALCULATIONS**

(Using pH 4.7, 0.04M acetate + 50% ethylene glycol, 30°C, 96 hours)

<table>
<thead>
<tr>
<th>INTEGRATED AREAS</th>
<th>using $^{14}$C-HpHb</th>
<th>using $^{14}$C-Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Radioactivity</td>
<td>5245</td>
<td>3054</td>
</tr>
<tr>
<td>Exchanged Radioactivity</td>
<td>3315</td>
<td>2158</td>
</tr>
<tr>
<td>Total Heme Content</td>
<td>2412</td>
<td>1937</td>
</tr>
<tr>
<td>Heme Content of Acceptor Substrate</td>
<td>1669</td>
<td>1509</td>
</tr>
</tbody>
</table>

(A) Theoretical Ratio ($^{14}$C/Heme)

(if complete equilibrium occurs) = \( \frac{\text{Total Radioactivity}}{\text{Total Heme}} \)

\[
= \frac{5245}{2412} = \frac{3054}{1937}
\]

(B) Experimental Ratio ($^{14}$C/Heme)

of the acceptor substrate after incubation = \( \frac{\text{Exchanged Radioactivity}}{\text{Acceptor Heme Content}} \)

\[
= \frac{3315}{1669} = \frac{2158}{1509}
\]

Per Cent of Theoretical Equilibrium = \( \left( \frac{B}{A} \right) \times 100 \)

\[
91.2\% = 90.7\%
\]
incubation mixture, then the $^{14}$C-Hb would be distributed randomly between the complex and the hemoglobin in the mixture. Thus, the theoretical ratio for complete equilibrium of $^{14}$C/Heme is (total radioactivity/heme content in both complex and hemoglobin) (i.e., 5245/2412). This assumes that the Hb has the same affinity for haptoglobin as does the $^{14}$C-Hb and that these two types of hemoglobin are not preferentially dissociated from the haptoglobin.

The value of the ratio $^{14}$C/Heme for the acceptor substrate (in this case, Hb) is the actual experimental ratio obtained after incubation. This value is calculated by dividing the exchanged radioactivity (the radioactivity found in the acceptor substrate peak) (3315) by the heme content of the acceptor substrate peak (1669) (i.e., 3315/1669).

If the experimental ratio is equal to the theoretical ratio, complete equilibrium has occurred. The percentage of the experimental value as compared to the theoretical ratio shows how far the incubation has proceeded towards equilibrium. In the case being considered, it was 91.2%. That means under the experimental conditions of pH 4.7, 0.04M acetate + 50% ethylene glycol, 30°C, 96 hours incubation, the $^{14}$C-Hb in $^{14}$C-HpHb was almost completely equilibrated between the complex and hemoglobin.

The question of why the complex was not incubated alone and then separated on a column equilibrated with the incubation buffer may be posed here. Under these experimental conditions, one would expect any dissociated hemoglobin to be separated from the complex on column chromatography. However, the rationale of using hemoglobin as an excess substrate was that the dissociation of complex might be only
<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using 14C-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl, 37°C, 1 hour</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl, 30°C, 96 hours</td>
<td>0</td>
</tr>
</tbody>
</table>
momentary, and unless there was a molecule of hemoglobin to substitute for the one molecule removed, the latter quickly would reassociate with haptoglobin to form the complex again.

If excess hemoglobin is used, it is unnecessary to equilibrate the column with each different solution which is being tested for its ability to dissociate complex. The problem of the dissociating media causing permanent changes in the flow properties of the Sephadex G-100 gels would also be eliminated. Complex and hemoglobin, even if disrupted in a dissociating medium, are expected to stabilize their structures as the solvent is separated from these reactants on the column.

In a medium containing 0.04M Tris-HCl, pH 7.4 (pH 7.4 is the normal pH of blood in humans), it was noted that there appeared to be exchange of radioactivity to the complex but not from the complex (Table V) in the incubation mixtures. This difference could be due to aggregation of the hemoglobin to produce a radioactive polymer of hemoglobin which would elute at the position of complex. However, this is unlikely. Under these experimental conditions hemoglobin is not known to aggregate, and the hemoglobin blank profile shows single radioactivity and protein profiles. This does not exclude the possibility that in the complete reaction mixture a complex, \( \alpha^\text{Hb}_\alpha \beta^\text{Hb}_\beta \), is formed. The absorbance ratio of 418/280 were compared for the blank HpHb and the HpHb after incubation with the \(^{14}\text{C}-\text{Hb}\) and the was no evidence for this polymer formation. The experimental technique used here was not sensitive enough to detect a slight change in molecular weight, since the complex already elutes close to the excluded volume of the column.

Another possibility could be that excess \(^{14}\text{C}-\text{reagent}\) had not been completely removed by gel chromatography or dialysis after reaction with hemoglobin. This lightly absorbed or lightly bound
reagent would then react with -SH groups on the normal complex.
However, when one makes complex from $^{14}\text{C-Hb}$, it might be expected that this "loosely bound" reagent would be dissociated from the complexed hemoglobin and appear as an excess radioactive peak after the hemoglobin on separation on Sephadex G-150 gel chromatography.
Although this is not illustrated in Figure 12, further analyses after fraction 57 have shown no such extra radioactive peak.

The possibility that at pH>7 the carbamide methyl group can be removed and reattached to another -SH group can be discounted by evidence of Taylor, Antonini, Brunori and Wyman (1968), who have shown that the carbamide methyl derivative of hemoglobin was stable at high pH values.

Another possibility is that normal complex is actually dissociating while the radioactive complex is more stable. However, it can be seen that there is a leveling off of the "dissociation" after 36 hours. If there was even a slow dissociation occurring, the equilibrium point would not be expected at such a low "dissociation". Thus, it has been assumed that a level of approximately 14% of the theoretical equilibration, when using radioactive hemoglobin as a precursor, was not sufficient to indicate a dissociation of the complex. Where radioactive complex was the precursor, any level of equilibration was considered to indicate a dissociation.

Since this experiment showed that the point of equilibration of this unknown "dissociation" occurred at about 36 hours, 96-hour incubations were used experimentally in a majority of cases in order to be completely confident of observing any dissociation.
### TABLE VI
EFFECT OF SALTS IN SOLUTION

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
<th>using $^{14}$C-HpHb</th>
<th>using $^{14}$C-Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 1M NaCl, 22°C,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hours</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>48 hours</td>
<td>0</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>72 hours</td>
<td>0</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>96 hours</td>
<td>0</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 2M NaCl, 22°C,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72 hours</td>
<td>0</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>96 hours</td>
<td>0</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 1M LiBr, 22°C,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96 hours</td>
<td>0</td>
<td>24.1</td>
<td></td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 4M LiCl, 26°C,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96 hours</td>
<td>39.9</td>
<td>84.2</td>
<td></td>
</tr>
</tbody>
</table>
a) Effect of Salt Solutions

The results are illustrated in Table VI. There was no dissociating effect in 1M and 2M NaCl at neutral pH. One molar lithium bromide, reported to have a greater perturbation activity than 2M NaCl (Bigelow & Geschwind, 1961), showed slight dissociation of HpHb when incubated with $^{14}$C-Hb but not when $^{14}$C-HpHb was incubated with Hb.

Four molar lithium chloride, used by Gerahman and Dreisen (1970) to dissociate peptide chains from myosin, had some effect on the dissociation of the complex (although it still seemed to be precursor-dependent).

Salts are known to accentuate the antagonism between water and hydrophobic groups (Tanford, 1968). Electrostatic forces also should be eliminated by the shielding effect of salt (Taylor, Antonini, Brunori & Wyman, 1966). In salt-denatured ribonuclease, ordered regions consisting of hydrophobic groups were found. There was no way of deciding whether they were newly formed or retained from the native structure. It might be inferred from the knowledge that hemoglobin in 2M NaCl at neutral pH is dissociated only to the dimer that there is a retention of such ordered regions of the hemoglobin-haptoglobin complex. The complex was not disrupted in 2M NaCl, which indicates a maintenance of the protein complex's conformational structure. However, increased salt concentration (4M LiCl) caused noticeable dissociation of the complex. It has been suggested that in very concentrated LiBr solutions hydrogen bond stabilization occurs in proteins, and changes in proteins were a result of an increase in the amount of hydrogen bonding (Harrington & Schellman, 1956). However, Bigelow and Geschwind (1961) have postulated that the denaturation found
### TABLE VII

**EFFECT OF SODIUM DODECYL SULPHATE**

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM using $^{14}C$-HpHb</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM using $^{14}C$-Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 0.002% SDS, 22°C, 96 hours</td>
<td>0</td>
<td>8.4</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 0.02% SDS, 22°C, 96 hours</td>
<td>0</td>
<td>slight</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 0.1% SDS, 22°C, 96 hours</td>
<td>99.1 *</td>
<td>83.8 *</td>
</tr>
</tbody>
</table>

*Complex alone dissociated 40.5% to a smaller molecular weight, heme-containing protein.

Hemoglobin alone dissociated 81.6% to a smaller molecular weight, heme-containing protein."
with LiBr was a result of specific interactions which cause the lithium ion to act directly as a hydrogen bond breaking agent. Hence, just what the effect of high concentrations of salt in the denaturation of the complex is still in question.

b) **Effect of Sodium Dodecyl Sulphate (SDS)**

The experiments using SDS in the medium are found in Table VII.

There was a dissociating effect here in 0.1% SDS at neutral pH (7.4). This detergent is known to disrupt protein structure extensively. The disruption was indicated by the blank analyses, where the $^{14}$C-precursors were shown to dissociate to heme-containing products of lower molecular weight. However, the complex seemed more resistant to dissociation than hemoglobin. This could be due to the stability of the haptoglobin molecule itself toward SDS or possibly to the fact that the hemoglobin when complexed with haptoglobin produces a more stable compound than either of the two proteins alone.

SDS binds to proteins as the anion (Tanford, 1968), causing the complex to become negatively charged. The binding between hemoglobin and haptoglobin possibly makes this region more resistant to SDS than if the two proteins were separate. Since this detergent disrupts the protein structure almost completely, it cannot be definitely determined which interactions are involved in the complex formation.

An additional problem was that the column chromatographic method used to separate the complex from the excess hemoglobin was not able to remove the bound SDS from these proteins. This makes the assay
TABLE VIII
EFFECT OF pH

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using  $^{14}$C-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl, 37°C,</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>6 hours</td>
</tr>
<tr>
<td></td>
<td>12 hours</td>
</tr>
<tr>
<td></td>
<td>36 hours</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
</tr>
<tr>
<td>pH 9.0, 0.04M veronal-HCl, 37°C</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate*, 37°C,</td>
<td>24 hours</td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate, 22°C,</td>
<td>24 hours</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
</tr>
<tr>
<td>pH 4.7, 0.04M NaCl adjusted</td>
<td>96 hours</td>
</tr>
<tr>
<td>with HCl, 30°C</td>
<td></td>
</tr>
</tbody>
</table>

* acetate = sodium acetate-acetic acid buffer
procedure an ineffectual method for detecting the degree of equilibration.

Jaenicke and Pavlicek (1970) have suggested that the SDS anions most likely react with lysyl and histidyl residues of the substrates. Lysyl groups of hemoglobin have essentially been excluded from the binding site by the work of Lookhart (1971), using acetimidated-hemoglobin. Thus, lysyl groups, if involved in complex formation, must come from the haptoglobin molecule. Perutz (1969) reported few histidine residues found in the contact surface between the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers of hemoglobin. This is the surface where some believe hemoglobin binds to haptoglobin. It is likely, in the hemoglobin molecule, that histidine residues are not directly involved in the binding site but are involved in maintaining the conformation of the molecule. The addition of the anionic SDS molecules causes a disruption of the interactions holding the hemoglobin and haptoglobin molecules together sufficiently to bring about dissociation.

c) **Effect of pH**

The effects of pH variation are tabulated in Table VIII. The pH does have some effect on the dissociation of complex. Appreciable concentrations of monomers of hemoglobin appear only in the pH range below 5 or above 11 (Perutz, 1969). In addition the oxygen-binding properties are altered markedly outside the 5-9.5 pH range (Antonini & Brunori, 1970), indicating physical changes in the hemoglobin molecule at extremes of pH. Haptoglobin is denatured below pH 4.5, and for this reason pH 4.7 was the pH used experimentally
<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
<th>( ^{14}\text{C-HpHb} )</th>
<th>( ^{14}\text{C-Hb} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.7, 0.04M acetate, 22°C,</td>
<td>24 hours</td>
<td>9.9</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>15.7</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
<td>31.6</td>
<td>47.0</td>
</tr>
<tr>
<td>pH 4.7, 0.5M acetate, 22°C,</td>
<td>96 hours</td>
<td>69.0</td>
<td>71.9</td>
</tr>
<tr>
<td>pH 4.7, 1.0M acetate, 22°C,</td>
<td>96 hours</td>
<td>precipitation</td>
<td></td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate, 37°C,</td>
<td>24 hours</td>
<td>precipitation</td>
<td></td>
</tr>
</tbody>
</table>
to study the effect of low pH on the dissociation of complex. Solutions of pH 7.4 and 9.0, which are intermediate between the extremes of hemoglobin stability, were studied.

As discussed previously, at pH 7.4 there was some "dissociation" when $^{14}$C-Hb and HpHb are incubated together but not when $^{14}$C-HpHb and Hb are incubated together. At pH 9.0 there was no change in the dissociation when compared to the effect at neutral pH. It would seem that a slight change in the electrostatic interactions within the complex molecule has little effect on the stability of the complex.

Use of pH 4.7 has resulted in an appreciable equilibration, using $^{14}$C-Hb or $^{14}$C-HpHb as the radioactive precursor. This equilibrium also seems to be independent of the radioactive substrate in the mixture (i.e., the per cent of the theoretical equilibrium values are nearly equal if one considers the "dissociation" at pH 7.4). The lowering of pH, which causes an increase in positive charges on the protein (or a loss of negative charges) has, thus, some effect on the dissociation of the complex which implies that electrostatic interactions (ion-pairs, salt linkages and hydrogen bonds) are involved in the binding of hemoglobin to haptoglobin.

d) **Effect of Acetate (Sodium Acetate-Acetic Acid)**

Table IX lists experiments illustrating the effect of acetate concentration on the dissociation of complex. There was a dissociation due to acetate concentration. In fact, 1M acetate caused an observable precipitation of the four precursors ($^{14}$C-Hb,
$^{14}$C-HpHb, Hb & HpHb) after 96 hours of incubation, suggesting that these conditions were too severe.

By sedimentation velocity studies, Chiancone and Gilbert (1965) showed that dissociation of oxyhemoglobin to the αβ dimer was promoted by the presence of molecular acetic acid rather than by low pH. However, if it was the formation of the dimer of hemoglobin which caused dissociation of the complex, then dissociation should have occurred when the incubation contained 2M NaCl. If the effect was solely due to low pH, then the degree of exchange should have been the same for 0.04M and 0.5M acetic acid (note: although the buffers were prepared using sodium acetate acid, the pK of acetic acid is 4.7 and therefore the acid can be considered to be in its undissociated form). Cann and Goad (1965) state that the effect is a net positive electrical charge on a protein due to the addition of undissociated buffer acid to the protein. The difference in net charge between the native-protein and the protein-acid complex must lie in differences in the pK values of the carboxyl groups which are exposed upon addition of the undissociated acid. This change in the net charge must make the hemoglobin-haptoglobin complex more susceptible to dissociation, indicating that the binding depends on a degree on electrostatic interactions. An alteration of pK also argues for a conformational change which could be the key feature of binding rather than electrostatic interactions.

At low ionic strength the acetic acid effect has the same degree of equilibration as that of the 0.04M NaCl solution adjusted to pH 4.7 by hydrochloric acid (0.04M NaCl was used to insure the same Na⁺ concentration in both incubation mixtures) (Table VIII).
Thus it is not the binding of the undissociated acetic acid, but the effect of pH which was responsible for the dissociation of the complex at low acetic acid concentrations.

If the effect of dissociation is due to the disruption of the hydrophobic interactions by the addition of the undissociated acetic acid at higher concentration (0.5M), then the effect would be similar to the effect caused by ethylene glycol (discussed in the following section).
<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using $^{14}$C-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 12.5% ethylene glycol, 22°C, 96 hours</td>
<td>0</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 25% ethylene glycol, 22°C, 96 hours</td>
<td>0</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol, 22°C, 96 hours</td>
<td>37.4</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol, 26°C, 24 hours</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
</tr>
<tr>
<td></td>
<td>96 hours</td>
</tr>
<tr>
<td></td>
<td>120 hours</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol, 30°C, 96 hours</td>
<td>43.4</td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate + 50% ethylene glycol, 30°C, 96 hours</td>
<td>91.2</td>
</tr>
</tbody>
</table>
e) Effect of Ethylene Glycol

Definite dissociation due to ethylene glycol is tabulated in Table X. As the concentration of ethylene glycol was increased the dissociation increased. Higher concentrations of ethylene glycol (≥50%) could not be investigated due to the collapse of the Sephadex G-100 gel in the presence of 50% ethylene glycol. A different method of monitoring dissociation, such as binding haptoglobin-hemoglobin complex to a Sepharose-Concanavalin A column,* might permit higher concentrations of ethylene glycol to be used.

Changes in dielectric constant of the medium due to addition of ethylene glycol to a protein solution may be expected to cause some destabilization of the native, folded structure of the protein. This can be attributed to increased electrostatic repulsion of like charges on the protein surface as well as increased attraction between unlike charges, which are exposed upon protein unfolding. There could be a change in the arrangement of the molecules of water surrounding the protein due to the addition of the ethylene glycol. However, such effects are relatively unimportant at low net charge near the isoelectric point of proteins (hemoglobin pI = 6.8, haptoglobin pI = 4.2-4.25). Because of this Herskovits, Gadebsku and Jalllelt (1970) have concluded that the effect of ethylene glycol is one of denaturation due to hydrophobic changes within the molecule.

The combination experiment of low pH and 50% ethylene glycol (Table X) provides some insight into the dissociation of haptoglobin and hemoglobin. Comparison of the degree of dissociation found under these conditions to that obtained at pH 7.4 in 50% * This gel binds glycoproteins
ethylene glycol, 96 hours incubation, shows lowering the pH has increased the degree of dissociation by a factor of two (i.e., averaging both $^{14}$C-substrates, 91% at pH 4.7 as compared to 44% at pH 7.4). However, if one considers the effect of pH 4.7, 0.04M acetate, 96 hours incubation without ethylene glycol, an average value of the degree of equilibration would be 40% (Table VIII). If the separate effects of ethylene glycol and low pH are combined additively, they give about 84% as compared to the 91% value obtained for degree of dissociation in the combined medium. This would seem to indicate that low pH and ethylene glycol did not act in the same way to promote dissociation of the complex. The low pH effect at low acetate concentration has previously been attributed to disruption of electrostatic interactions.

Raising the temperature has the effect of loosening hydrogen bonds and is said to tend to strengthen hydrophobic interactions (Tanford, 1968). In the case of reversible thermal denaturation, the products, while highly disordered, are not random coils but have retained regions of ordered structure. The temperatures used here, although not high enough to cause denaturation (excepting 37°C at pH 4.7, Table IX), seemed to cause a slight increase in the degree of equilibration attained at 26°C as compared to 22°C. This was not a large increase in temperature, and the dissociating effect may be due to experimental error. However, since 26°C and 30°C provided consistent values of equilibration, these temperatures were used in most experiments.
### TABLE XI

**EFFECT OF DISSOCIATING MEDIA**

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using $^{14}$C-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 25% dioxane, 22°C, 96 hours</td>
<td>precipitation</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 10% ethanol, 30°C, 96 hours</td>
<td>25.1</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 20% ethanol, 30°C, 96 hours</td>
<td>23.7</td>
</tr>
</tbody>
</table>
f) Effect of Alcohol and Dioxane

Experiments using ethyl alcohol and dioxane as dissociants are shown in Table XI. Dioxane at 25% concentration caused a precipitation of the proteins. The concentrations of ethyl alcohol studied indicated a dissociation of complex. This was expected after the results obtained from the ethylene glycol experiments.

Alcohols and dioxane, like ethylene glycol, disrupt the hydrophobic interior of proteins. The addition of these solvents to aqueous solutions reduces the dielectric constant of the solution and therefore favours the aggregation or precipitation of proteins if they are at or near their isoelectric points (Tanford, 1968). Dioxane has shown this effect. Since it has been reported that dioxane has the same effect as alcohol and ethylene glycol, experiments using lower concentrations of dioxane were not attempted (Herskovits, Gadgebeku & Jaillot, 1970).

Although there was a dissociation with ethyl alcohol up to the 20% concentration level, no further experiments at > 20% ethyl alcohol concentration could be attempted because the Sephadex G-100 gel which was used to monitor the dissociation changed its flow characteristics with increase in concentration of ethanol. An additional complication was the difference in rate of dissociation of the HpHb as compared to $^{14}$C-HpHb. That is, in 20% ethyl alcohol with HpHb the degree of equilibration after 96 hours was 94% of the expected value, while using $^{14}$C-HpHb only 24% of the expected equilibration value was attained after 96 hours of incubation. The substitution of the unionized carbamid methyl group for the weakly ionizable hydrogen
<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using $^{14}C$-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 8M urea</td>
<td>precipitation</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 1M urea</td>
<td>22.3</td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate + 1M urea</td>
<td>76.5</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + saturated guanidine-HCl</td>
<td>precipitation</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 1M guanidine-HCl</td>
<td>38.5</td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate + 1M guanidine-HCl</td>
<td>precipitation</td>
</tr>
</tbody>
</table>
of the thiol group of cysteine seems to make the complex more resistant
to dissociation. It is possible that the conformation of complex is
such that the β-93 cysteine of hemoglobin is preferentially unionized.
Then, unless the replacement hemoglobin is also unionized, the
preference would be for the original dimer in the complex, and the
rate of dissociation using Hb and $^{14}C$-HpHb would be slower than that
starting with $^{14}C$-Hb and HpHb (assuming that the acceptor substrate,
that is, the unreacted substrate in each incubation is in sufficient
excess over the radioactivity donor).

The effects of these solvents, however, further support:
the proposal that hydrophobic interactions are involved in the complexing
of hemoglobin to haptoglobin.

g) Effect of Guanidine Hydrochloride and Urea

The experimental results are in Table XII. The
higher concentrations of urea and guanidine-hydrochloride (GuHCl)
(8M and saturated, respectively) showed that there was definite disruption
of the protein. Precipitation occurs, possibly due to "salting out"
effects on the proteins. This did not occur over a short period of
time. However, it was evident after 96 Hours of incubation (there was
no heme colour left in the supernatant and the protein precipitated).

With the 1M guanidine hydrochloride, there was also a
precipitation, but there was still some protein left in solution, and
this was analyzed. Guanidine hydrochloride is usually reported to
keep proteins soluble even if the protein structures are disrupted.
The precipitation may be related to impurities in the technical grade
guanidine hydrochloride which was used directly without the further
purification according to the directions of Gordon, Cleve and Bearn (1968).

Because guanidine hydrochloride is a strong electrolyte, electrostatic interactions should be minimized in concentrated solutions of this reagent. Therefore, the influence of low pH (i.e. electrostatic interactions) should be negligible in the presence of guanidine hydrochloride.

Guanidine hydrochloride (1M) at pH 7.4 produced a dissociation of the complex in the supernatant which was not precipitated. This indicates a disruption of the interactions involved in the binding of hemoglobin to haptoglobin. The low pH experiment resulted in precipitation, making analysis impossible.

A comparison cannot be made between the guanidine hydrochloride experiments at 1M concentration and the urea experiments at 1M concentrations because of this protein precipitation problem.

Urea, although a mild hydrophobic bond breaker (organic solvents weaken hydrophobic bonds by diluting water and provide an environment not incompatible with apolar groups), acts primarily as a hydrogen-bond breaker in producing the unfolding of secondary and tertiary structures to more random conformations. At pH 7.4 in 1M urea, there was dissociation of complex. At pH 4.7 the degree of equilibration was slightly increased over that at pH 7.4 for the mixture using \(^{14}\text{C-}\text{Hb}\) but greatly increased for the mixture using \(^{14}\text{C-HpHb}\). Evidently the electrostatic interactions of the \(^{14}\text{C-HpHb}\) were disrupted more than those of the HpHb by the effect of low pH.
Just why one complex is affected more than the other is unknown. The lowering of the pH from 7.4 to 4.7 changes the β-93 cysteine of hemoglobin from the sulphide form, predominant at pH 7.4, to the sulphydryl form. This increase in degree of equilibration of the $^{14}C$-HbHb at the lower pH could then be further evidence for the stability of the complex being governed by the ionization state of the cysteine residue. This, once again would be dependent on the relative amounts of the substrates involved but care was taken to insure excess amounts of acceptor substrate.

It is evident from comparing the results at low pH without urea (Table IX) and at low pH with urea (Table XII) that the effect of these two conditions on the stability of the complex is different.

The 8M urea experiment at pH 7.4 started to show precipitation after 48 hours of incubation, and it was almost complete after 96 hours (judging from loss of heme colour in the supernatant). Both the $^{14}C$-substrates were found to precipitate. When attempts were made to separate the components of the supernatants of these incubation mixtures, the Sephadex G-100 columns shrank and the separations were unsuccessful. This was probably due to loading the column with urea, which caused an alteration in the physical properties of the Sephadex gel.

Smithies, Connell and Dixon (1966) reported that long-term exposure of haptoglobin to urea in alkaline solution produced irreversible changes in the protein. It is possible that 96 hours of incubation at slightly alkaline pH has also caused this irreversible
<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using $^{14}$C-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 0.01M ATP</td>
<td>0</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + $10^{-8}$M DPG</td>
<td>0</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 0.3% heparin</td>
<td>0</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 1M glycine</td>
<td>0</td>
</tr>
</tbody>
</table>
change.

The hemoglobin molecule also precipitated due to the long period of incubation at 30°C. A disadvantage in the use of urea as a denaturant, especially at elevated temperatures, is the tendency of urea to undergo decomposition, with the formation of cyanate ions (Tanford, 1968). The cyanate reacts with the free amino groups to form carbamyl derivatives. This chemical alteration of the protein would be relatively slow, and probably unimportant at low pH. However, it may be the cause of the formation of precipitates of hemoglobin at pH 7.4 during the long incubation period.

h) Effect of Some Other Compounds

Table XIII contains some miscellaneous experiments using iodoacetamide-¹⁴C-reacted substrates which were unsuccessful in dissociating the complex.

In hemoglobin there is a binding system with great affinity for ATP (and other anions) which is saturable at physiological concentrations. Because the technique used in preparation of hemoglobin had removed the ATP bound to it, the question arose as to whether a saturating amount of ATP on hemoglobin would have an effect on the dissociation of complex. No experimental evidence for this effect could be found.

The binding site for diphosphoglycerate in the deoxyhemoglobin molecule has been elucidated. Diphosphoglycerate fits into the cavity of deoxyhemoglobin in such a way that its charged groups are within hydrogen bonding distance of the side chains of β subunit residues. Therefore, diphosphoglycerate stabilizes the
conformation of deoxyhemoglobin. Experimentally, cyanmethemoglobin has been used. In this derivative, hemoglobin is "locked" into the oxy-conformation, and thus diphosphoglycerate should have no effect on the hemoglobin binding. There was a slight possibility that diphosphoglycerate would bind to the hemoglobin on the complex and aid in its dissociation. It was found that diphosphoglycerate at physiological concentration and neutral pH had no effect on the dissociation of the complex of hemoglobin and haptoglobin.

It had been reported that haptoglobin pretreated with heparin would not form complex (Chiao & Bezkorovainy, 1971). The question posed was whether heparin would act on the preformed complex to promote dissociation of the hemoglobin from the haptoglobin. There was no such effect evident at the concentration of heparin which was reported to inhibit the binding of the two proteins.

Glycine, which increases the dielectric constant of the solvent, had little effect on the dissociation of complex. Introduction of this dipolar molecule into the complex would be expected to reduce electrostatic effects within the molecule. No interactions seem to be disrupted in the incubation using \(^{14}\text{C-HpHb}\), although glycine seems to affect the dissociation of the incubation using \(^{14}\text{C-Hb}\). However, it is not a very high degree of equilibration and could be attributed to increased affinity of the haptoglobin for \(^{14}\text{C-Hb}\) rather than HB under the slight dissociating effect of glycine. In the case of \(^{14}\text{C-HpHb}\), perhaps the glycine molecule was unable to penetrate the regions involved in the interaction of the hemoglobin and haptoglobin because of the carbamyl methyl group and thus had no effect.
TABLE XIV

INCUBATIONS USING $^{14}$C-HpHb AND CPB-REAETED Hb

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{14}$C exchanged from HpHb to Hb</td>
<td>Arginine exchanged from HpHb to Hb</td>
<td></td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate, 30°C, 96 hours</td>
<td>37.1</td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol, 30°C, 96 hours</td>
<td>42.6</td>
<td>43.7</td>
<td></td>
</tr>
</tbody>
</table>

a ($^{14}$C in Hb peak/$^{14}$C in HpHb + Hb peaks) x 100.

b Per cent C-terminal arginine in Hb peak as compared to the total hemoglobin C-terminal arginine available in the initial incubation mixture (i.e., C-terminal arginine from the Hb in the HpHb).
These results are contrary to the results obtained by Jaenicke and Pavlicek (1970). However, these authors used Hp 2-1 and Hp 2-2, which are polymers of the Hp 1-1 which was used in this work. The effect of glycine on Hp 2-1 and Hp 2-2 might be to dissociate the polymer, which in turn might be enough to loosen the hemoglobin from haptoglobin (whereas the Hp 1-1 complex is stable under these conditions).

V Experiments Using α Chain-Labeled Hemoglobins.

All the previous experiments have used the β chain-labeled hemoglobin. The following experiments were carried out to discover whether it was only the β chain which was being dissociated or whether both chains were being dissociated. In these experiments the hemoglobins employed were "labeled" in the α chain.

Carboxypeptidase-B-reacted hemoglobin (CPB-Hb) is hemoglobin in which the C-terminal arginine residue of the α chain has been removed by the action of the enzyme carboxypeptidase B. The llama hemoglobin was "labeled" because its α chain has the amino acid isoleucine in its primary sequence while its β chain does not.

a) Experiment Using CPB-Reacted Hemoglobin

The conditions used experimentally were those which indicated definite dissociation without the problems of precipitation and Sephadex G-100 shrinkage (pH 4.7, 0.04M acetate and pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol). The incubations were for 96 hours at 30°C. The difference between these experiments
and the preceding ones was the use of CHB-Hb as the excess hemoglobin instead of normal hemoglobin (Hb). There was definite exchange of the radioactive component from the complex to the excess hemoglobin (Table XIV). Since the radioactivity is on the β-93 cysteine residue, this exchange would only be an indication of the movement of the β chain of hemoglobin from the complex to the excess hemoglobin peak.

There was also an exchange of C-terminal arginine from the hemoglobin in the complex to the excess hemoglobin peak. This appearance of arginine indicated that the α chains as well as the β chains were dissociated under the experimental conditions.

Theoretically the two values of radioactivity and arginine obtained should be the same if there is a movement of the dimer of hemoglobin from complex. If however β chains come off first and α chains are retained for a short period of time before being dissociated (and replaced by dimers of excess hemoglobin) then the value should be lower than the radioactivity value and conversely if the α chains came off the complex before the β chains.

There seems to be this difference in the values obtained for the pH 4.7 experiment. However, this might be attributed to experimental error. The amino acid analyses are probably only accurate to within 10%. There was also an appearance of CPA activity in the CHB because there was some other amino acids present in the analyses for arginine. There is also the possibility that some of the arginine was hydrolysed to ornithine by the action of the enzyme arginase because there was an appearance of lysine in the analyses. It is possible that this enzyme (arginase) was inactivated in 50% ethylene glycol and
<table>
<thead>
<tr>
<th>HEMOGLOBIN SOURCE</th>
<th>ISELEUCINE/LYSINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>Human</td>
<td>0</td>
</tr>
<tr>
<td>Llama</td>
<td>0.090</td>
</tr>
</tbody>
</table>
not at pH 4.7. This would account for the lower value for arginine obtained at pH 4.7 than at pH 7.4 + 50% ethylene glycol.

b) Experiments Using Llama Hemoglobin

The llama hemoglobin contains isoleucine in the $\alpha$ chains, which "labels" it compared to its $\beta$ chain. The human hemoglobin $\alpha$ and $\beta$ chains contain no isoleucine. The isoleucine/lysine ratio of 0.091 reported by Braunitzer, Hilse, Rudloff and Hilschmann (1964) for llama hemoglobin was confirmed by hydrolysis (Table XV).

The reaction media used were pH 4.7, 0.04M acetate and pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol. The results have been tabulated in Table XVI. The per cent of the total radioactivity of the incubation mixture (counts in Hb + counts in complex) of the incubation mixture which was found in the hemoglobin after incubation is an indication of the extent of exchange of $\beta$ chain that has occurred. The per cent isoleucine found in the hemoglobin peak after incubation when compared to the total isoleucine available from the hemoglobin in complex is an indication of the amount of exchange of $\alpha$ chains that has occurred upon incubation. These two values should be the same for each experiment only if the $\alpha\beta$ dimer was dissociated from the complex as a unit. Each set of values cannot be compared directly with any other set because the per cent of dissociation will be dependent on the amount of excess hemoglobin available for the hemoglobin in the complex to become randomly equilibrated with. That is, the more hemoglobin there is in excess, the higher the exchange there could be.
## TABLE XVI

INCUBATIONS USING H\textsubscript{H}H\textsubscript{b}(llama) AND H\textsubscript{b}(human) (30\textdegree{}C, 96 hours)

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITIONS</th>
<th>PER CENT (^{14}\text{C}) IN HEMOGLOBIN, PEAK (OF TOTAL (^{13}\text{C})) AFTER INCUBATION</th>
<th>PER CENT OF EXPECTED ISOLEUCINE IN THE HEMOGLOBIN PEAK AFTER INCUBATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4, 0.04M Tris-HCl + 50% ethylene glycol</td>
<td>41.8</td>
<td>34.8</td>
</tr>
<tr>
<td></td>
<td>53.3</td>
<td>50.2</td>
</tr>
<tr>
<td></td>
<td>47.9</td>
<td>40.2</td>
</tr>
<tr>
<td></td>
<td>73.2</td>
<td>35.9</td>
</tr>
<tr>
<td>pH 4.7, 0.04M acetate</td>
<td>57.8</td>
<td>56.9</td>
</tr>
<tr>
<td></td>
<td>30.5</td>
<td>38.8</td>
</tr>
<tr>
<td></td>
<td>20.9</td>
<td>28.9</td>
</tr>
<tr>
<td></td>
<td>27.8</td>
<td>23.0</td>
</tr>
</tbody>
</table>
These results show that there has been a dissociation of α chains as well as β chains under both experimental conditions being considered. One can state with relative certainty that at low pH, where the electrostatic interactions have been disrupted, there has been an exchange of both α and β subunits in equal amounts. However, at pH 7.4 + 50% ethylene glycol there is a tendency for all the values of isoleucine exchanged to be lower than those for radioactivity exchanged, and in one case it is over 50% lower (i.e., 35.9% isoleucine to 73.2% radioactivity). This may be an indication that disruption of the hydrophobic interactions has caused the exchange of the β chains of hemoglobin to occur before the exchange of α chains. This evidence supports the work of Nagel and Gibson (1971), who showed that although there are separate binding sites for the α and β subunits on haptoglobin, the binding site for the β subunit is induced by the binding of the α subunit. The hydrophobic interactions which are disrupted could be between the α and β chains of hemoglobin or between the β and H chains of the hemoglobin and haptoglobin or both or even within the β chain itself. The removal of the β chain then could facilitate the removal of the α chains.

There also could be the formation of the \((\alpha_{llama}\beta_{human})_2^-\) Hp hybrid complex, which would provide an answer to the differences in the two experimental values found (i.e., radioactivity vs. isoleucine). The great difference in the one set of values obtained (73.5 and 35.9) is probably an indication that there was some poorly understood change in experimental conditions which slowed the dissociation of the α chain from the complex. However, this does support the idea that the β chain is dissociated before the α chain.
**TABLE XVII**

INCUBATION USING $^{14}$C-ETHYL ACETIMIDATE-REACTION SUBSTRATES

<table>
<thead>
<tr>
<th>EXPERIMENTAL CONDITION</th>
<th>PER CENT OF THEORETICAL EQUILIBRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>using $^{14}$C-HpHb</td>
</tr>
<tr>
<td>pH 7.4, 0.04M Tris-HCl, 37°C, 96 hours</td>
<td>13.9</td>
</tr>
</tbody>
</table>
The pH 4.7, 0.04M acetate experiments do not contradict the supposition that the β subunits are removed before the α subunits. However, it may be that the disruption of the electrostatic interactions causes the α subunits to dissociate more readily after the removal of the β subunits than if only the hydrophobic interactions (predominant effect of ethylene glycol) were affected. At low pH there is a predominance of monomers of hemoglobin (Perutz, 1969). This effect may assist in the dissociation of the subunits of hemoglobin from the complex.

VI Experiments Using Ethyl Acetimidate-Reacted Hemoglobin

Lockhart (1971) has shown that all lysyl residues of hemoglobin are modified by ethyl acetimidate. The introduction of so many large groups into the molecule of hemoglobin would be expected to have some effect on the binding of the hemoglobin. The results of the study of the degree of equilibration are tabulated in Table XVII, and a slight increase in the degree of equilibration was observed when compared to the experiments using iodoacetamide-¹⁴C-reacted precursors.

The experiment shows that this modification has some effect on the binding of hemoglobin to haptoglobin. Since both complexes (normal complex and ethyl acetimidate-reacted complex made from ethyl acetimidate-reacted hemoglobin and porcine haptoglobin) were dissociated, it cannot be due to disruption of the conformation of the hemoglobin molecule by the modification. That is, the reacted hemoglobin can displace the hemoglobin in normal complex just as normal hemoglobin can displace the reacted-hemoglobin in the reacted-hemoglobin-haptoglobin
complex. Thus, although the modification does not inhibit binding, there was some change in the hemoglobin which made it easier for dissociation to occur. The modification would not be useful for the studies of the effects of the various dissociating media because the dissociation could be attributed to the modification rather than to the dissociant. Thus further experiments were not pursued with the ethyl acetimidate-reacted precursors.


E. CONCLUSIONS

Hemoglobin and haptoglobin form a rather stable complex, without the formation of a covalent bond between them. Attempts have been made to elucidate the types of interactions between these two proteins.

Electrostatic and hydrophobic interactions have been implicated in the formation of complex by the effects obtained by increasing the concentration of salts. Because 2M NaCl had little effect on the dissociation, and because it is known to maintain dimers of hemoglobin, the dissociation due to 4M LiCl would seem to indicate that the dissociation of the complex involves the physical disruption of the dimer of hemoglobin.

Sodium dodecyl sulphate has been shown to cause almost complete dissociation. This detergent is known to extensively disrupt the secondary and tertiary structure of protein molecules. It is impossible to determine from the data which interactions are involved in the complex formation.

The effect of lowering the pH to 4.7 had some effect in dissociating the complex. The effect of extremes of pH would be to break ion-pairs within the molecule by changing the protein charges to those of like sign. This would seem to indicate that electrostatic interactions are involved in the formation of the hemoglobin-haptoglobin complex.
Cann and Goad (1965) have stated that the effect of the addition of undissociated acetic acid to protein is a net positive electrical charge on the protein. Any absorption of the polar end of the undissociated acid to polar groups on the complex would aid in dissociation or any interaction with the hydrophobic areas may cause dissociation. These effects were probably the reason for the occurrence of dissociation of the complex at 0.5M acetic acid concentration. However, it has been shown that at pH 4.7, 0.04M acetic acid that the majority of the dissociation effect was probably due to the electrostatic interactions which were disrupted in the protein-complex.

It is commonly observed that solvents such as ethylene glycol, dioxane and ethanol, which are much less polar than water, act as strong denaturing agents towards proteins. These solvents which were shown to cause dissociation of the hemoglobin-haptoglobin complex would seem to show that hydrophobic bonds are important in maintaining the stability of this protein.

Both urea and guanidine hydrochloride are known to cause denaturation in proteins. All proteins that have undergone a complete transition by the addition of guanidine hydrochloride have so far been found to be random coils, without residual noncovalent structure (Tanford, 1968). Because of precipitation at neutral pH, even at 1M guanidine hydrochloride, definite conclusions can not be made for this reagent.

Although urea is perhaps the most commonly used denaturing agent for proteins, there are many proteins which do not undergo a complete transition, at least at room temperature, to the limit of
urea solubility. Carbonmonoxyhemoglobin is known to be dissociated to half molecules under these conditions. Experimentally, it has been shown that the hemoglobin-haptoglobin complex undergoes dissociation. This has been attributed to the effect of disruption of hydrophobic interactions. Tanford (1968) has found that randomly coiled proteins in urea are subject to effects of pH and ionic strength which are not observed in guanidine hydrochloride solutions, these being the effects of electrostatic interactions between the charged groups of the protein. The experiment using pH 4.7 and 1 M urea illustrated that electrostatic interactions as well as hydrophobic interactions were involved in the binding of hemoglobin and haptoglobin.

The ability of a protein to absorb small molecules depends on the existence of suitable adsorption sites in the regions of the protein that are accessible to the molecules. When the protein changes its conformation some or all these sites may be destroyed and new sites may also become available. The sites of ATP binding to hemoglobin have been discussed. The saturation of these sites by ATP appeared to have no effect on the stability of the hemoglobin-haptoglobin complex. DPG, which also has had its binding site to hemoglobin well characterized, had no noticeable effect in disrupting the hemoglobin-haptoglobin complex.

Heparin was reported to inhibit complex formation (Chiao & Bezkorovainy, 1971), but was found to have no effect once hemoglobin was bound to haptoglobin.

Glycine, which would be expected to reduce electrostatic effects within the molecule seemed to have little effect on the
stability of the hemoglobin-haptoglobin complex.

All the previous experiments were carried out using the iodoacetamide-$^{14}$C-reacted precursors. When equilibration had occurred under dissociating conditions, this only indicated the involvement of the $\beta$ chain of hemoglobin. Hemoglobin treated with carboxypeptidase B and llama hemoglobin were two $\alpha$ chain"labeled" hemoglobins. Using these, it has been shown that both the $\alpha$ and $\beta$ chains are involved in dissociation.

The preparation of ethyl acetimidate-reacted hemoglobin and its complex has shown that this modification has caused some changes in the hemoglobin molecule which causes dissociation of the complex.

Thus, in conclusion, electrostatic (ion-pairs and hydrogen bonds being included in this) interactions and hydrophobic interactions seem to be involved in the formation of hemoglobin-haptoglobin complex. Under these dissociating conditions both the $\alpha$ and $\beta$ subunits of hemoglobin have been dissociated.
F EXPERIMENTAL SUGGESTIONS

1) A time study, using complex prepared from llama hemoglobin and excess hemoglobin, incubated together could be attempted to determine whether the \( \alpha \) and \( \beta \) chains are being dissociated as a unit or one of these chains is being dissociated preferentially. The \( \beta \) chain movement could be followed by \(^{14}C\) exchange and the \( \alpha \) chain dissociation by the isoleucine exchange. For this experiment larger quantities of substrates would have to be prepared and larger columns would be necessary for the separation process.

2) Another method of determining the authenticity of the possibility that the \( \alpha \) and \( \beta \) chains were moving at different rates in the 50% ethylene glycol experiments would be to determine whether the llama and human hemoglobins can actually form hybrids. If they cannot then the results are due to experimental error rather than a true representation of the movement of the \( \beta \) chain before the \( \alpha \) chain. However, it would seem more than likely that hybridization does occur, even though this may be difficult to detect if one starts with complex. One would have to incubate the hemoglobins under the dissociating conditions and then separate them and analyse for isoleucine content.

3) The effect of lysyl groups in the complex formation could be pursued further. Acetimidate-reacted hemoglobin might be reacted with similarly treated haptoglobin to confirm or deny the conflicting results reported by several groups on the influence of these groups in the association and dissociation of the complex.
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ABBREVIATIONS

Hb - Normal hemoglobin
Hp - haptoglobin
HpHb - hemoglobin-haptoglobin complex
CPA - carboxypeptidase A
CPB - carboxypeptidase B
GuHCl - guanidine hydrochloride
DPG - diphosphoglycerate
Tris-HCl - Tris(hydroxymethyl) Aminomethane-hydrochloric acid
N-terminal - amino-terminal
C-terminal - carboxyl-terminal