HAPTOGLOBINS AND HEMOGLOBIN-HAPTOGLOBIN COMPLEXES

by

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Abstract

Haptoglobins are serum glycoproteins which form complexes with hemoglobin. Three phenotypes of haptoglobin exist in serum (Hp 1-1, Hp 2-1, Hp 2-2). The latter two types exist as a series of polymers while the former type exists as a homogeneous protein. All three haptoglobin types consist of β (heavy) chains and α (light) chains which are attached by disulphides. The haptoglobin types differ in their α chains; Hp 1-1 contains only α^1 chains, while Hp 2-2 contains only α^2 chains and Hp 2-1 contains α^1 and α^2 chains. The hemoglobin-haptoglobin 1-1 complex consists of one molecule of hemoglobin attached to one molecule of haptoglobin.

The thesis has been divided into three parts. The first part (Section III) is concerned with the reaction of haptoglobin with an octameric (double) hemoglobin obtained from an inbred strain of mice. In this hemoglobin each of the hemoglobin dimers is joined together by a disulphide bond. The fact that haptoglobin binds αβ dimers indicates that it is a bivalent molecule like the antibody molecule, immunoglobulin G (IgG). This bivalence and resultant resemblance to IgG is examined by studying the reaction of haptoglobin with this mouse hemoglobin in which the αβ dimer is held together by a disulphide bond. The results of both precipitation studies and acrylamide gel electrophoresis confirm the postulated bivalence of haptoglobin and its resemblance to an antibody.

The second part (Section IV) of the thesis is concerned with confirming the results obtained in studying the disulphides of haptoglobin which were obtained by the cysteic acid diagonal technique. These results predicted a model in which the two halves of the haptoglobin molecule were held together by a disulphide bond at position 21α. Also the results predicted an intrachain loop disulphide between half-cystines at positions 35 and 69 in the haptoglobin α chain and an interchain disulphide between a half-cystine at position 73α and the β chain. This structure has been confirmed by studies on a cyanogen bromide fragment isolated from haptoglobin which contains the intact α chain. Also the structure has been confirmed by studies on a haptoglobin derivative in which the molecule has been split in half by the breaking of a disulphide bond.

The third part (Section V) of this thesis is an investigation into the nature of the β93 sulphydryl of hemoglobin when hemoglobin is bound by haptoglobin. The results demonstrate that there is a definite change in the environment of this sulphydryl upon formation of the hemoglobin-haptoglobin complex. Studies with ^{14}C-iodoacetamide demonstrate however that β93 can still react in the HbHp complex.

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I

INTRODUCTION

Haptoglobin Structure

Haptoglobin was discovered in 1938 (1) when it was found that serum had the property of increasing the peroxidase activity of hemoglobin. Utilizing the measurement of the oxidation of potassium iodide by ethyl hydroperoxide, Polonowski and Jayle found that, while hemoglobin has a low peroxidase activity with a pH optimum of 5.6, the addition of serum shifted the optimum to 4.2 with a considerable increase in peroxidase activity. The existence of haptoglobin was confirmed several years later by paper electrophoresis (2) which demonstrated that a hemoglobin-haptoglobin (HbHp) complex could be separated from hemoglobin. The early work on haptoglobin also demonstrated that this protein existed in more than one form (3). Haptoglobin 1 was found to precipitate between 54 to 64 per cent ammonium sulphate and to have a molecular weight around 100,000 while haptoglobin 2 precipitated between 40 and 51 per cent ammonium sulphate and had a molecular weight greater than 200,000 (4). Haptoglobin 1 passed the physico-chemical tests of homogeneity--solubility curve, electrophoretic migration, and ultra-centrifugation while haptoglobin 2 was found to be heterogeneous (5,6).

A great breakthrough in haptoglobin research came with the development of starch gel electrophoresis (7). Smithies and Walker

found that serum could be classified into three types (8). Type 1 produced α_1 and α_2 globulin bands of about equal intensity. Type 2A produced several more bands which migrated on the starch gels between α_2 and β globulin bands while the α_1 band decreased in intensity. In type 2B the pattern was similar to the 2A pattern except that the bands between the α_2 and β globulins moved more slowly. Using partially hemolyzed serum it was observed that these proteins were pink before staining and were in fact the hemoglobin-binding haptoblobins (9). The existence of these patterns in sera was explained by the results of family studies (10). It was postulated that there was a Hp^1 gene and a Hp^2 gene which were autosomal and exhibited incomplete dominance. Thus the phenotypes observed would be Hp 1-1, Hp 2-1 and Hp 2-2. These would correspond to the previously observed serum patterns type 1, type 2A and type 2B respectively. Purification of the haptoglobin components of serum confirmed these observations (9). Haptoglobin 1-1 ran on starch as a single hemoglobin binding component while haptoglobins 2-1 and 2-2 ran as a series of polymers with the 2-2 polymers running more slowly than the 2-1 polymers.

At this point the meaning of the multiple haptoglobin forms was a mystery. Although the results could be completely explained by postulating a Hp^1 and an Hp^2 gene, the presence of the Hp^2 gene in some unexplained way led to the formation of a series of polymers upon gel electrophoresis. Some authors (9,11) suggested that the polymer observation was an aggregation artifact but it was generally accepted to be a real phenomenon. Another break-through came when

haptoglobin was reduced with mercaptoethanol in 8 M urea and, after reduction, alkylated with iodoacetamide (12). The polypeptides produced were analysed by starch gel electrophoresis in 8 M urea using sodium formate buffer at pH 4.0. All three classes of haptoglobin produced a slow running very dark band which was called the β chain of haptoglobin and a faster running lighter band called the α chain. It was observed that the α chain band produced by haptoglobin 1-1 ran more quickly than the corresponding α chain band in haptoglobin 2-2 while haptoglobin 2-1 produced both bands. Thus the polymorphism of haptoglobin could now be seen to be associated with variations in α chains and the occurrence of multiple bands appeared to correlate with the presence of the more slowly running band (α^2 chain) observed upon gel electrophoresis. Also it was found that the haptoglobin 1-1's could be subdivided into two categories (α^{1F} and α^{1S}) on the basis of the mobility of their α^1 chains. Amino acid analyses of the purified α chains demonstrated the replacement of a lysine in Hp α^{1F} by an acidic amino acid in α^{1S}. The amino acid analyses of the α^1 and α^2 chains were also very similar but they could not be explained on the basis of a simple point mutation. Instead fingerprint analyses indicated the presence of a new peptide in the α^2 chain (13). This peptide had properties consistent with its resulting from the joining of the carboxyl terminal of the α^1 chain to the amino terminal of another α^1 chain. Amino acid analyses showed that this junction peptide is slightly smaller than one which would occur by joining the carboxyl terminal peptide to the amino terminal peptide. Since the α^2 chain was also shown to have about twice the

molecular weight of the α^1 the evidence strongly indicated that the polymeric haptoglobins had resulted from a genetic event in which a crossing-over had occurred at the DNA level with a resulting partial gene duplication.

This exciting result represented, for the first time, the detection at a molecular level of a partial gene duplication and also served to indicate how the haptoglobin polymers could be formed. The existence of gene duplications had been postulated before and was based upon cytological observations on the bar locus in Drosophila (14). Since the time of the haptoglobin discovery the presence of a partial gene duplication has been detected in many other proteins and in 5s RNA (15,16).

Fingerprint analyses of the α^2 chain also indicated that the chain resulted from the fusion of the genes for the α^{1S} and the α^{1F} chain. Thus once the duplication had occurred to form the $Hp^{2(F,S)}$ gene then a much more likely genetic event than this initial occurrence would be the formation of Hp^{2FF} and Hp^{2SS} genes by crossing over. Similarly the formation of a triple chain gene by mispairing should occur. Nance and Smithies have obtained evidence for the former prediction (18) while the discovery of haptoglobin Johnson appears to provide evidence for the latter (19).

Several other haptoglobin phenotypes have been identified. These include Hp Carlberg and Hp 2-1M in which it appears in the former case that there is an underproduction of α^1 chains and so 2-2 polymers appear along with the 2-1 polymers while in the latter case

there is an underproduction of α^2 chains so that the faster running polymers of smaller molecular weight are present in greater proportion (20). Other abnormal haptoglobin phenotypes are believed to be caused by the Hp^P, Hp^H, and Hp^L genes but the gene abnormalities have not been characterized (21). With the Hp^B gene a different mobility has been observed for the α chain in acidic urea starch gels (22). Both Hp Marburg and Hp Bellevue (23,24) are believed to result from mutations in the β chain of haptoglobin. Haptoglobin 2-1 Johnson, which migrates as a series of polymers moving more slowly than haptoglobin 2-2 polymers, when examined in the urea gels produces a normal α^1 chain and a new band which migrates more slowly than the α^2 band (25).

The complete sequence of both the α^1 and α^2 haptoglobin chains has now been established (26). The α^1 chain contains 84 amino acids and the α^2 chain has 143 (Figure 1). Both chains have an amino terminal valine and a carboxyl terminal glutamine and contain no methionine or phenylalanine. There are four half-cystines in the α^1 and seven in the α^2 chain. The sequence of the haptoglobin α chains has been compared with known sequences of some of the light chains of antibodies (Bence-Jones proteins) using a computer program developed by Fitch (27). The results indicated a homology between the region around half-cystine 86 in the Bence-Jones proteins and half-cystine 35 of the haptoglobin α chain. This result indicated that there was possibly a common evolutionary origin for haptoglobins and antibodies.

Haptoglobin α^1 84 residues α^1F=Lys at position 54
α^1S=Glu at position 54

```
    1                                   10                                  20
NH2-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-
                                        30                                  40
  -Cys-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-
                                        50                                  60
  -Leu-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Glu-Lys-Gln-Trp-Ile-Asn-Lys-
                                        70                                  80
  -Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Val-Cys-Gly-Lys-Pro-Lys-Asn-Pro-Ala-
              84
  -Asn-Pro-Val-Gln-COOH
```

Haptoglobin α^2 143 residues

```
    1                                   10                                  20
NH2-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-
                                        30                                  40
  -Cys-Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-
                                        50              -Lys-               60
  -Leu-Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Glu-Lys-Gln-Trp-Ile-Asn-Lys-
                                        70                                  80
  -Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Asp-Asp-Gly-Gln-Pro-Pro-Pro-Lys-Cys-
                                        90                                 100
  -Ile-Ala-His-Gly-Tyr-Val-Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-Leu-
                                      110         -Lys-                     120
  -Arg-Thr-Gln-Gly-Asp-Gly-Val-Tyr-Thr-Leu-Asn-Asn-Glu-Lys-Gln-Trp-Ile-Asn-Lys-Ala-
                                      130                                 140
  -Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-Glu-Ala-Val-Cys-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-
          143
  -Pro-Val-Gln-COOH
```

FIGURE 1

CORRECTED SEQUENCES OF THE α^1 AND α^2 CHAINS OF HAPTOGLOBINS

The heavier β chains of haptoglobin have a molecular weight of 40,000 to 42,000 (28,29,30) and contain all of the carbohydrate attached to the molecule. Haptoglobin contains 14 to 16 per cent carbohydrate which has the composition, 4.6 per cent sialic acid, 4.2 per cent glucosamine, 0.2 per cent fucose, and 5.6 per cent hexose (31). The β chain has an amino terminal isoleucine (32,33) and this chain appears to have a very similar structure in all three major classes of haptoglobins (34).

The disulphide bonds of haptoglobin (35) have been investigated using the cysteic acid diagonal technique (36). When these studies were performed after haptoglobin was digested with pepsin only one disulphide peptide could be isolated in good yield. This peptide corresponded to a region of the α chain of haptoglobin which contained the half-cystine at position 21 (Figure 2). A peptide also running off the diagonal in a corresponding position was not observed and so it appeared that this peptide was linked to itself and thus a symmetrical linkage occurred between the α chains of haptoglobin. By performing cysteic acid diagonals on haptoglobin peptides after thermolysin digests of haptoglobin 1-1 it was possible to account for all of the disulphides in haptoglobin (Figure 2). Peptide Th1 represents a sequence of the α chain from position 11 to 21 and again confirms that the half-cystinyl group at position 21 is joined in a symmetrical interchain linkage in the haptoglobin molecule. Peptide Th2 A corresponds to residues 61 to 71 in the α chain while Th2 B corresponds to residues 31 to 38 in the α chain. Thus this disulphide

PEPSIN DISULPHIDE

P1

Ile Ala Asp Asp Gly Glu Pro Pro Pro Lys Cys Ile Ala His Gly

Ile Ala Asp Asp Gly Glu Pro Pro Pro Lys Cys Ile Ala His Gly

THERMOLYSIN DISULPHIDES

TH1

Ile Ala Asp Asp Gly Glu Pro Pro Pro Lys Cys

Ile Ala Asp Asp Gly Glu Pro Pro Pro Lys Cys

TH2

Ala Val Gly Asp Lys Leu Pro Glu Cys Glu Ala Th2a

Val Arg Tyr Gln Cys Lys Asn Tyr Th2b

TH3

Val Cys Gly Lys Pro (Pro Lys Asp) Th3a

Ile Cys Pro Leu Ser (Asp Lys) Th3b

TH4

Tyr Gln Glu Asp Thr Cys

Phe Asp Lys Cys(Ser Ala)

TH5

Val Ala Asp Gln Asp Glu Cys

Phe Cys

Figure 2 STRUCTURES OF THE HAPTOGLOBIN DISULPHIDE PEPTIDES

peptide demonstrates that the half-cystinyl group at position 35 in the α chain is joined to a half-cystinyl group at position 69 in the α chain. Peptide Th3 A is identical to an α chain peptide corresponding to residues 72 and 78 if an additional half-cystinyl group is placed at position 73 in the α chain sequence. Reinvestigation of the α chain sequence has shown that in fact a half cystinyl group is present at this position. Thus the β chain is joined to the α chain by a half-cystinyl group at position 73 in the α chain. Peptides Th3 B, Th4, and Th5 cannot be assigned to the α chain and so must be present in the β chain of haptoglobin. The disulphides of haptoglobin will be discussed further in Section IV of the thesis.

Hemoglobin Structure

Hemoglobin without doubt has been the most assiduously studied protein. This is primarily because it could be easily obtained in large quantities in a high state of purity (3). Hemoglobin consists of an apoprotein part, globin, and an oxygen carrying chromophore, heme. It consists of 4 polypeptide chains, two α chains and two β chains each with a molecular weight of about 16,500 resulting in a hemoglobin molecular weight of 65,000 (38). It was demonstrated by sequencing the hemoglobin chains (39,40) that the two types of chains had homologous structures (41).

In comparing various globin chains it has been shown that the chains have related sequences in all regions and that 64 residues are identical in the α and β hemoglobin chains. However these chains

only share 21 identical residues with myoglobin.

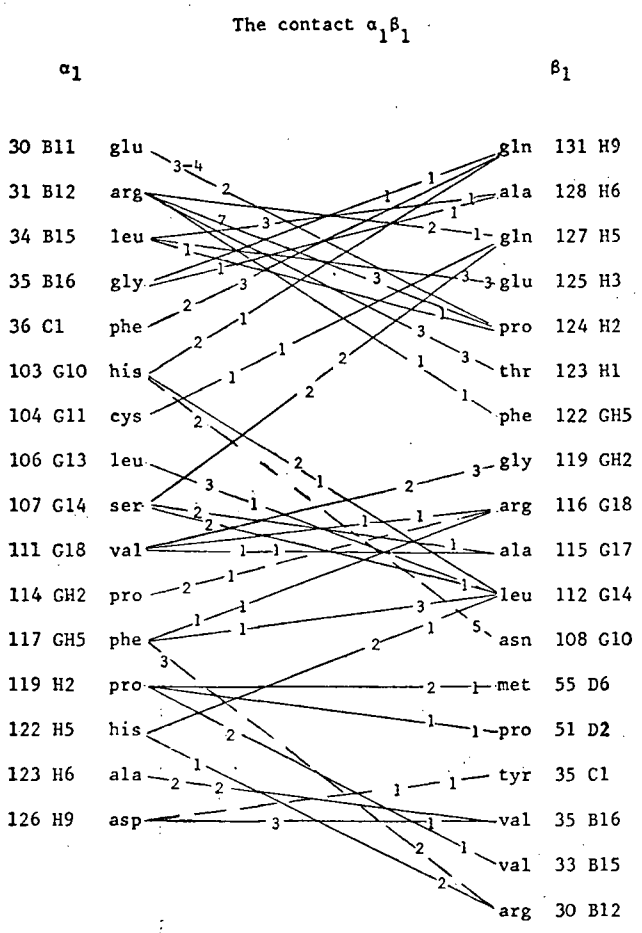
The three dimensional structure of hemoglobin is now known at a resolution of 2.8A^{o} (42). The four chains are arranged tetrahedrally around an axis of two-fold symmetry and the conformation of the chains closely resembles the conformation of myoglobin (43). In general terms, the nonpolar residues reside in the interior of the molecule forming Van der Waals contacts and the polar residues are at the surface. Glycine residues and alanine residues also appear to reside at the surface of the molecule. Each chain consists of eight helical regions (A-H) with seven corners and some nonhelical areas.

When the three dimensional structure of hemoglobin was determined (43) it was found that the structure of each of the hemoglobin chains was remarkably like the three dimensional structure of myoglobin. In fact more recent results indicate that the three dimensional structure of myoglobin provides a model around which all hemoglobin and myoglobin conformations fit (44). However in comparing the primary structures of all of the known vertebrate globin chains only nine residues remain identical. All of the three dimensional structures appear to be similar because of the maintenance of a pattern of nonpolar and polar residues (44) of which the nonpolar residues appear to be the most invariant. The evidence is based on the results of x-ray studies and an analysis of the sequences of many hemoglobin chains of the vertebrates.

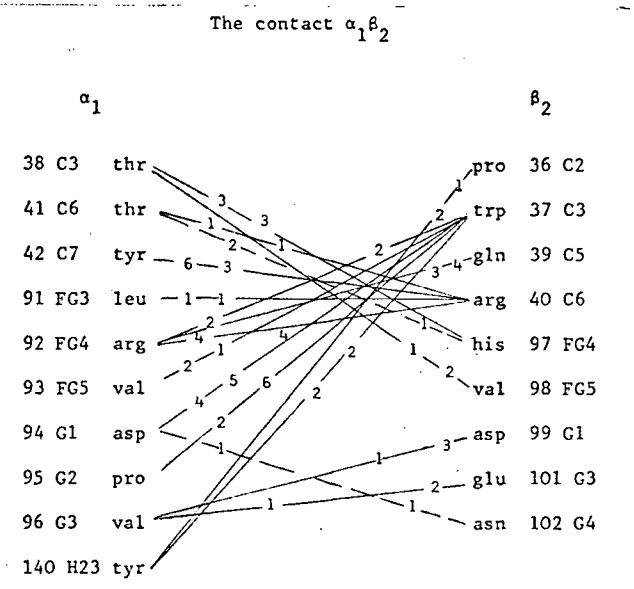
Characteristic amino acids are usually found between the helical regions in the hemoglobin chains. For example, in the β chain

each of the five prolines occurs in position two of a helix. Model building demonstrates that, because of its imino group, in any α helix containing N residues proline can only exist at positions 1, 2, 3 or N+1 (44). Position 1 is often occupied by a serine, threonine, aspartic acid or asparagine. The hemes are located in hydrophobic pockets and are attached by coordinate covalent bonds to histidines at position F8. There are sixty interactions between globin chain atoms and heme atoms which are within 4A^{o} and all but three are nonpolar. The similarities in the structures of globin chains in the regions corresponding to these interactions is striking.

Each α chain of hemoglobin is in contact with two β chains and with the other α chain. The converse is true for each β chain. The contact $\alpha_1\beta_1$ is more extensive than the contact $\alpha_1\beta_2$. It consists of 110 atomic interactions which are mainly nonpolar. The contact $\alpha_1\beta_2$ has only 80 interactions (Figure 3). Again they are mainly nonpolar but there is one clear hydrogen bond between aspartic 94 and asparagine 102. Upon deoxygenation of hemoglobin there is a large movement in the $\alpha_1\beta_2$ area with a displacement by as much as 5.7A^{o} for some contact atoms while movement in the region of the $\alpha_1\beta_1$ contact is slight with the contact becoming more extensive. The $\alpha_1\beta_2$ contact is such that it allows the two subunits to slide past each other with a resultant effect on the environment of the hemes. The electron density maps do not show definite contacts between like chains although they probably exist.



Thirty-four residues, including about 110 atoms in contact.



Nineteen residues, including about 80 atoms in contact.

Plain connecting lines indicate van der Waals contacts; broken ones indicate that the contact includes a hydrogen bond. The numbers on the lines give the number of atoms contributed to the contact by the two residues on each side.

The contact $\alpha_1\beta_1$ comprises thirty-four residues. Twenty-one of these are common to all the normal mammalian haemoglobin sequences analysed so far. The contact $\alpha_1\beta_2$ comprises nineteen residues. All but one of these are common. The one replacement [C5(39)β gln→arg], reported for llama, would not affect the stereochemistry of the contact.

Figure 3 Interatomic contacts between residues in unlike hemoglobin chains

Hemoglobin has three sulphydryl groups per dimer which are located at positions β93, β112 and α104. Of these only one, β93, will react with iodoacetamide under normal conditions (45,46). In deoxyhemoglobin this cysteine becomes unreactive (47). This effect upon the reactivity of β93 also appears related to the dissociation of hemoglobin from tetramers to dimers which occurs to a small extent under physiological conditions (48) and can be increased by increasing the salt concentration or by extremes of pH (48). Dissociation occurs to a much smaller extent in deoxyhemoglobin than in oxyhemoglobin (49). X-ray crystallography has revealed that the β93 SH interacts with histidyl β97 and that this histidyl residue is involved in the contact which is broken upon dissociation from tetramers to dimers (50,42). The decreased reactivity of β93 has now been explained as resulting from restricted access to the exposed SH. This restriction is caused by the interaction of histidyl β146 with the β carboxyl of aspartyl β94 (51).

Over 100 varients of the normal hemoglobin structure are now known (52). Most of these have been detected by abnormal electrophoretic mobility and the modified structure can be explained by single base changes in the genetic code. In a European population, a study showed the frequency of mutant hemoglobins to occur with a frequency of about one in two hundred (52). Several abnormal hemoglobins have been observed which affect the subunit contacts in hemoglobin (53). Those affecting the $\alpha_1\beta_2$ contact, which is broken when hemoglobin dissociates in solution, are Hb J Capetown $\alpha 92^{(arg \rightarrow gln)}$,

Hb Chesapeake $\alpha 92^{(arg \rightarrow leu)}$, Hb Yakima $\beta 99^{(asp \rightarrow his)}$, Hb Kempsey $\beta 99^{(asp \rightarrow asn)}$, Hb Kansas $\beta 102^{(asn \rightarrow thr)}$.

Hemoglobin-Haptoglobin Structure

The reaction of haptoglobin with hemoglobin can be detected by starch, paper, acrylamide or cellulose acetate electrophoresis, by ultracentrifugation or by Sephadex chromatography (54). All of these techniques produce a separation of the HbHp complex from hemoglobin or haptoglobin and the complex can be detected by its absorbance at 407 or 540 nm or by its peroxidose activity. The complex is extremely stable and does not dissociate at all under normal conditions as there appears to be no exchange between isotopically labelled hemoglobin and the complex (56). Also the complex will form under conditions of pH from 4 to 9 and in 2M sodium chloride (54). Human haptoglobin will react with hemoglobins from a series of related animals and will bind globin but will not bind myoglobin (54). It can be observed upon starch gel electrophoresis that all of the haptoglobin polymers bind hemoglobin (9). There was a report that the large polymers bound less hemoglobin per gram (56) but this has been disproven and it now appears that all polymers bind 0.7 grams of hemoglobin per gram of haptoglobin (54).

In 1964, Nagel and Ranney reacted a variety of hemoglobins with haptoglobin and tested the reaction by starch gel electrophoresis (57). They found that hemoglobins A_2, F, I, and Lepore bound haptoglobin while H and Bart's failed to show binding. These last two hemoglobins

are tetramers of the β chain and the α chain of hemoglobin respectively. Since oxyhemoglobin H resembled deoxyhemoglobin A in crystal structure (58) they went on to test this protein for reaction with haptoglobin (59). If hemoglobin were completely deoxygenated with a small amount of dithionite, no binding to haptoglobin was observed. This lack of binding of deoxyhemoglobin was confirmed with haptoglobin 2-2 by analysis in the ultracentrifuge (60). Deoxygenation of the oxygenated HbHp complex did not reverse the combination and release deoxyhemoglobin and haptoglobin. Carboxypeptidase A (CpA) treated hemoglobin reacted with haptoglobin whether in the deoxy or oxy form (60). In CpA-treated Hb the C-terminal histidine is removed and conformational changes accompanying deoxygenation cannot occur (51).

Two physiologically significant properties of hemoglobin are its reduced oxygen-carrying ability under acidic conditions, "Bohr effect", and the sigmoidal nature of its oxygen binding as a function of oxygen tension, usually ascribed to "heme-heme interaction" (61). The hemoglobin-haptoglobin complex has radically altered oxygen-binding properties when compared with free hemoglobin (62) including a 30-fold increase in oxygen affinity and a nonsigmoidal oxygen binding curve. Further studies showed the absence of a Bohr effect, no change in the carbon monoxide combination rate with pH, and a decrease in the molar extinction coefficient (ε) at 430 nm (63). These findings all suggest that in the complex the hemoglobin can no longer undergo conformational changes. Interestingly, it was also

found that HbHp 2-2 combines with carbon monoxide faster than HbHp 1-1.

Recently hemoglobins which show an impaired binding to haptoglobin have been reported (64). The first of these was a hemoglobin obtained after reaction with bis (N-maleimidomethyl) ether (65). This bifunctional maleimide* reagent first adds to the β93 SH and then reacts with histidyl β97 (66). When prepared from human hemoglobin this derivative shows reduced dissociation into dimers in a similar manner to deoxyhemoglobin. However it has been shown that horse bis (N-maleimidomethyl) ether-Hb is crystallographically similar to oxyhemoglobin and has similar dissociation properties whether in the oxy or deoxy form (67).

In addition to the standard methods for studying the hemoglobin-haptoglobin reaction, Bunn (64) has devised an interesting new method. In this method haptoglobin is added to a solution containing equal amounts of radioactive and nonradioactive hemoglobin and then the complex is separated from excess hemoglobin by gel filtration and the relative specific activities in the two proteins determined. Since the hemoglobin to be tested for binding is nonradioactive, if the specific activity in the complex peak is greater than in the hemoglobin peak, then the labelled hemoglobin bound more rapidly to haptoglobin.

Bunn (64) has tested a series of hemoglobins, all modified at β93 by treatment with either iodoacetamide, p-hydroxymercuribenzoate

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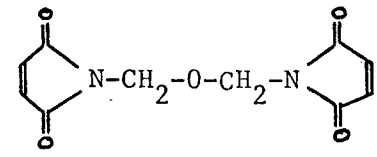
*Its structure is



(pHMB), cystine, cystamine, or N-ethylmaleimide. In all cases he found that their combination with haptoglobin was essentially unchanged except for a slightly faster rate of reaction with pHMB-hemoglobin. However both less complete and less rapid binding for bis (N-maleimidomethyl) ether-hemoglobin (BME-Hb) to haptoglobin were detected by gel filtration, peroxidase assays, and by the radioactive hemoglobin method. When the BME-HbHp was rechromatographed on Sephadex G-100 no dissociation of the complex was observed. Bunn suggests that this BME-hemoglobin shows decreased binding because it is less dissociated and haptoglobin only reacts with the dissociated $\alpha\beta$ dimer of hemoglobin. Since it has not been demonstrated that human BME-hemoglobin has the same conformation as human oxyhemoglobin this conclusion cannot be drawn definitely. However it should be possible to test this interpretation by using human BME-hemoglobin or deoxyhemoglobin under conditions of pH and ionic strength in which they are as dissociated as oxyhemoglobin. Since the hemoglobin-haptoglobin binding is so very strong it seems likely that even a small degree of dissociation of hemoglobin would lead to a complete reaction and so BME-hemoglobin and deoxyhemoglobin must bind less tightly to haptoglobin because of an altered conformation. Thus if hemoglobin dissociates less readily, this could explain an effect on the rate of reaction with haptoglobin but not on the equilibrium.

In a further study, Bunn has provided more evidence for the relationship between hemoglobin dissociation and binding to haptoglobin (68). He found that Hb Kansas, which is more highly dissociated

than hemoglobin A, bound haptoglobin more rapidly and that for Hb Chesapeake, which is less highly dissociated, the reverse is true.

Since the hemoglobin-haptoglobin complex has a molecular weight of 170,000 (4) it was felt that 1 molecule of haptoglobin combines with 1 molecule of hemoglobin. The first indication that haptoglobin might bind "half-hemoglobin", the αβ dimer, came from Laurell's laboratory when he observed that, if excess haptoglobin was mixed with hemoglobin, a new species was observed after starch gel electrophoresis at pH 7 (69). The new peroxidase positive band migrated between the HbHp complex and free haptoglobin and Laurell suggested that it was a complex of one haptoglobin and one αβ hemoglobin dimer. Several years later, Hamaguchi (70) purified this HbHp intermediate and showed that its molecular weight of 134,000 and heme content were consistent with Laurell's postulate.

The fact that haptoglobin can react with first one αβ hemoglobin dimer and then a second indicates that haptoglobin is bivalent and that the full complex consists of haptoglobin plus a pair of hemoglobin dimers. Further confirmation of this model comes from the work of Nagel and Gibson (71). They were able to measure the rate of reaction of hemoglobin with haptoglobin by measuring quenching of the tryptophane fluorescence of haptoglobin by the heme groups. Their results showed that the rate of reaction did not increase linearly with hemoglobin concentration and thus a dissociation appeared to precede reaction. They also tested the reaction of haptoglobin with the isolated α and β hemoglobin chains and observed

a reaction with isolated α but not with β chains. When haptoglobin was incubated with α chains and then β chains added, an initial rapid reaction was observed, but when the incubation was with β chains and then α chains were added, the rate was similar to that with α chains alone. This indicated that the α chains form a complex with haptoglobin which can then react rapidly with β chains.

More recently another detailed study of the reaction of hemoglobin chains with haptoglobin has also provided interesting results (72). Both α and β hemoglobin chains were found to bind to haptoglobin but to a much smaller extent than hemoglobin itself. The α chains had a higher affinity than the β chains and four α chains could be bound per haptoglobin (Figure 4). The reaction with the chains appears to be reversible and they can be displaced by adding hemoglobin.

Outline of the Present Study

The present study represents attempts to further understand both the nature of hemoglobin-haptoglobin binding and some aspects of haptoglobin structure. This study has been divided into three parts, one pertaining to studies on haptoglobin bivalence, one to studies on the disulphides of haptoglobin, and the last to studies on the sulphydryls of the hemoglobin-haptoglobin complex.

The first part of the thesis (Section III) is concerned with the reaction of haptoglobin with an octameric (double) hemoglobin obtained from an inbred strain of mice. In this hemoglobin each of

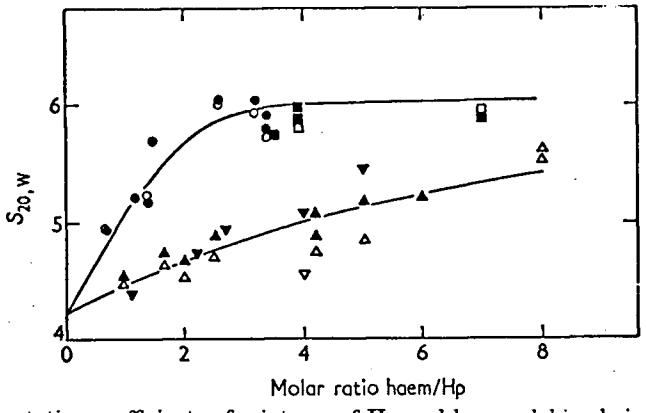


FIG. 4. Sedimentation coefficients of mixtures of Hp and haemoglobin chains as a function of the molar ratio haem/Hp. Hp concentration 2 to 4×10^{-5} M.
α^{PMB} (○) oxy or carboxy; (●) deoxy.
α^{SH} (□) oxy or carboxy; (■) deoxy.
β^{PMB} (△) oxy or carboxy; (▲) deoxy.
β^{SH} (▽) oxy or carboxy; (▼) deoxy.

the hemoglobin dimers is joined together by a disulphide bond. The fact that haptoglobin binds αβ dimers indicates that it is a bivalent molecule like the antibody molecule, immunoglobulin G (IgG). This bivalence and resultant resemblance to IgG is examined by studying the reaction of haptoglobin with this mouse hemoglobin in which the αβ dimer is held together by a disulphide bond. The results of both precipitation studies and acrylamide gel electrophoresis confirm the postulated bivalence of haptoglobin and its resemblance to an antibody.

The second part (Section IV) of the thesis is concerned with confirming the results obtained in studying the disulphides of haptoglobin which were obtained by the cysteic acid diagonal technique. These results predicted a model in which the two halves of the haptoglobin molecule were held together by a disulphide bond at position 21α. Also the results predicted an intrachain loop disulphide between half-cystines at positions 35 and 69 in the haptoglobin α chain and an interchain disulphide between a half-cystine at position 73α and the β chain. This structure has been confirmed by studies on a cyanogen bromide fragment isolated from haptoglobin which contains the intact α chain. Also the structure has been confirmed by studies on a haptoglobin derivative in which the molecule has been split in half by the breaking of a disulphide bond.

The third part (Section V) of this thesis is an investigation into the nature of the β93 sulphydryl of hemoglobin when hemoglobin is bound by haptoglobin. The results demonstrate that there is a

definite change in the environment of this sulphydryl upon formation of the hemoglobin-haptoglobin complex. Studies with ^{14}C-iodoacetamide demonstrate however that β93 can still react in the HbHp complex.

II

MATERIALS AND METHODS

Starch-Urea Gel Electrophoresis

The vertical method of Smithies was employed (73). Either 80 to 83 g of starch (Connaught Medical Research Laboratories, Toronto, Canada) or 67 to 70 g (Otto Hiller, Electrostarch Company, Madison, Wisconsin, lot 682) were weighed into a one litre beaker. Two hundred and forty grams of urea were added and the two powders thoroughly mixed. Three hundred milliliters of 0.083M sodium formate buffer pH 3.0 (prepared from 50 ml of solution containing 0.5M formic acid and 0.1M sodium hydroxide) or 300 ml of aluminum lactate buffer pH 3.7 (stock solution of Sung and Smithies (74) diluted four fold) were added to the mixture of starch and urea. Subsequent steps in the preparation of the gels containing urea followed the previously described method (75) except that degassing is omitted (74).

Bridge solutions for the formate gels were the same as those previously described (75) and for the aluminum lactate gels they were the four fold diluted stock aluminum lactate pH 3.7. The gels were sliced and stained by the method of Smithies, Connell and Dixon (75). Both the Amido Black stain and the Wool Fast Blue Stain were used. The Wool Fast Blue stain was not as sensitive as the Amido Black stain but the gel could be destained more quickly. Thus, except when a preliminary analysis was required, the Amido Black stain was used in

preference to the Wool Fast Blue stain.

Two dimensional starch urea gel electrophoresis was performed according to the method of Smithies, Connell and Dixon (76). However a 2-bladed cutting tool was used in which the blades were 0.7 cm apart instead of 0.4 cm. In the second dimension, gel markers of reduced and alkylated haptoglobins were inserted at both ends of the gel at the starting line by means of a small piece of Whatmann 3MM paper (73).

Polyacrylamide Disc Gel Electrophoresis

Polyacrylamide gels were prepared following conditions described by Davis (77). Although the concentration of acrylamide was altered to suit particular experiments, the preparation of a five per cent polyacrylamide gel serves as an example of the experimental technique.

Glass tubes approximately 10 cm in length and 5 mm diameter were used as moulds for the disc gels. The bottom of the tubes was sealed with a rubber plug and the tubes were clamped in a rack for filling. Then 1.3 g acrylamide and 63 mg N, N^1-methylenebisacrylamide were added to 25 ml of 0.1M sodium phosphate buffer. Immediately before casting of the gels 10 µl of tetramethylethylenediamine (TEMED) and 15 mg of ammonium persulphate were added to the acrylamide solution. The gelling solution was added to the glass tubes with a Pasteur pipette, taking care that in each tube the levels of the gel solutions were equal. Water was carefully layered on top of the acrylamide

solution surface with a micropipette in order that the polyacrylamide gel would have a flat meniscus. The gelling time varied considerably with the composition of the buffer but by varying the ammonium sulphate concentration the gelling time can be adjusted to about one-half hour. The polymerized gel was washed on the surface with distilled water to remove any ungelled material.

Electrophoresis was performed in either of two sets of apparatus which both were similar to that described by Davis (77) and which could hold either 8 or 20 gels. The gels in the glass tubes made electrical contact with the upper and lower containers which held the electrophoresis buffer. Rubber plugs sealed the openings through which the gels penetrated the bottom of the upper container. Usually sucrose was dissolved in the electrophoresis sample to increase its density. The surface of the gel was first covered with the electrophoresis buffer and then 5 to 20 µl of sample were applied onto the gel surface by a micropipette. The compartments of the apparatus were filled with sodium phosphate buffer until the platinum electrodes were covered. Electrical contact was made to a Heathkit IP-32 power source and electrophoresis was carried out at a maximum of 200 to 300 volts. A fine wire or, when available, the inner shaft of a 22-gauge hypodermic needle was used to remove the gel from the glass tube.

Gels were stained by keeping them for approximately 45 minutes in a 0.1 per cent Amido Black (w/v) in 10 per cent acetic acid (v/v) solution. After the staining period the dye solution was removed and the gels were destained in 10 per cent acetic acid.

Three different acrylamide gel buffers; 0.05M glycine, 0.01M Tris, pH 8.5; 0.1M sodium phosphate, pH 7.0; and 0.11M Tris, 0.062M boric acid, 2.5mM disodium [ethylenedinitrilo]tetraacetate (EDTA), and a variety of gel lengths were employed in the experiments described in this thesis. However, where appropriate the particular modifications are discussed along with the experiment. A more detailed description of the technique of polyacrylamide gel electrophoresis can be found in the article by Davis (77).

Eight molar urea-polyacrylamide gels were prepared in a similar manner to that described above. However, because urea increases the volume of aqueous solutions the gel solution was made up to volume after the urea was dissolved. Also, since urea decreased the polymerization time for the gels, the concentration of ammonium persulphate and TEMED used was one-half that used for making gels that contained no urea.

High Voltage Electrophoresis

High voltage electrophoresis was performed in a vertical apparatus using four buffers; pyridinium acetate pH 6.5 (100 ml pyridine--4 ml acetic acid--900 ml water), pyridinium acetate pH 3.6 (10 ml pyridine--100 ml acetic acid--890 ml water), formic-acetic acid solution pH 1.9 (20 ml formic acid--80 ml acetic acid--990 ml water), and pyridinium formate pH 4.0 (48 ml pyridine--35 ml formic acid--3920 ml water) on Whatmann 3MM paper. The apparatus is described by Ryle (78) and the technique is discussed in detail by J. Legget

Baily (79). A series of coloured markers, xylene cyanol FF (XCFF), methyl green (MG), orange G (OG), and crystal violet (CV) were used in order to help monitor the progress of the electrophoresis.

The electrophoretograms were stained for peptides or amino acids with a 0.5 per cent ninhydrin in acetone solution. For a permanent stain a cadmium acetate ninhydrin dipping reagent was used (80). Amino-terminal proline was detected by dipping the electrophoretogram in an isatin solution (0.2 g isatin, 100 ml acetone, and 4 ml acetic acid). Histidine was detected by spraying with Pauly reagent (79). It was possible to stain first with isatin, then with ninhydrin and then Pauly reagent. However, in order to remove most of the isatin from the 3MM paper before ninhydrin staining the 3MM paper was dipped twice in ethanol.

Acid Hydrolysis (82) and Amino Acid Analysis (83)

For a protein hydrolysis 100 to 200 µl of 6N HCl (1:1 dilution of reagent concentrated HCl) was added to 0.5 to 2 mg of lyophilized protein in a 10 by 70 mm Pyrex test tube. After heating in an oxygen flame a section of the tube 2 to 3 cm from the top was pulled to a bore of about 2 mm. The sample was then cooled in an alcohol dry ice bath and the tube was connected by means of an adaptor to an oil pump. The sample was evacuated and allowed to warm to room temperature. When bubbles of air ceased to form in the sample the Pyrex tube was sealed under vacuum with the oxygen flame. The sample was hydrolyzed for 15 to 20 hours in a oven at 110° C. After hydrolysis the

evacuated tube was cooled, opened, and then dried in a vacuum desiccator over sodium hydroxide.

Partial acid hydrolyses were performed in 100 µl of 6N HCl (1:1 dilution of reagent concentrated HCl) at 110° C. The hydrolyses were performed in Pyrex tubes (10 by 70 mm). The samples were heated to 100° C for 1 minute in a boiling water bath and then hydrolyzed for 19 minutes in a 110° C oven. Then the samples were cooled, diluted five-fold with water, and lyophilized.

Peptide hydrolysis was performed after elution of the peptide with water (0.3 to 0.4 ml) from a paper electrophoretogram. The peptide was eluted into a test tube (84) and then dried at 50° C in a Buchler Rotary Evapomix. One hundred microliters of 6N HCl was added to the dried peptide and hydrolysis was performed in the same manner as used for proteins.

Amino acid analyses were performed on a Beckmann 120 C amino acid analyzer according to the method of Spackman, Stein and Moore (83). The dried hydrolysates were dissolved in 0.2 ml to 0.4 ml of 0.2N sodium citrate buffer, pH 2.2. For protein hydrolysates the precipitate resulting from the degradation of tryptophane by HCl was removed by centrifugation or filtration through a Millipore filter. Then a 50 to 75 per cent aliquot of the sample was used for analysis.

Amino acid analysis of peptides was performed using a single column procedure developed by Devenyi (85). Amino acid analyses of homoserine peptides were performed following the method of Tang and Hartley (87). Dry hydrolysates were dissolved in 100 µl of 2N NH_4OH

and incubated at 37° C for one hour to convert homoserine lactone to homoserine. The samples were then dried on a Buchler Rotary Evapomix and analyzed as described above.

Amino-Terminal Amino Acid and Carboxy-Terminal Amino Acid Analyses

Amino-terminal analysis were performed following the dansyl chloride method of Gray (88). After reaction of the polypeptides with dansyl chloride (1-Dimethylaminonaphthalene-5-sulphonyl chloride) the sample was dried on a rotary evaporator and hydrolyzed in 6N HCl as described in the procedure for amino acid analysis. In order to identify dansyl-proline the sample was only allowed to hydrolyze for 6 hours instead of the normal 15 to 20 hour hydrolysis time. Dansyl-amino acids were identified by the thin layer chromatographic method of Black and Dixon (89).

Carboxy-terminal analysis was determined after digestion with carboxypeptidase A. The enzyme was prepared by the method of Potts, Berger, Cooke and Anfinsen (90). Four milligrams of fragment PC III were dissolved in 200 µl of performic acid at 0° C and oxidized for 90 minutes at 0° C (91). Then 1.0 ml of water at 2° C was added and the resulting solution was then frozen and lyophilized. Performic-acid oxidation converted the fragment into a denatured form which was suitable for digestion with carboxypeptidase. Carboxypeptidase digestion of non-oxidized fragment resulted in only a very low yield of amino acids. Two milligrams of performic-oxidized PC III were dissolved

in 100 µl 2N NH_3 for 1 hour at 37° C to convert any homoserine lactone to homoserine and subsequently the solution was dried. The fragment was then redissolved in 0.5 ml of 0.2N ammonium bicarbonate to which 10 µl of carboxypeptidase A solution (44 µg) were added. Digestion was allowed to proceed for 6 hours at 37° C. After digestion the amino acids which were released were absorbed on Dowex 50 and subsequently eluted with 5N NH_3 (92). The amino acid containing solution was then dried and analyzed on the amino acid analyzer as described previously.

Enzymatic Digestions of Fragment PC III*

One per cent solutions of fragment PC III and pepsin (3 x crystallized, Nutritional Biochemicals Corporation) were prepared by dissolving the proteins in an appropriate volume of 5 per cent (v/v) formic acid. To a given volume of PC III solution was added a 1/10 volume of pepsin solution. Digestion was allowed to proceed for 16 to 18 hours at 37° C. After this time the sample was dried on a rotary evaporator and then redissolved in pH 6.5 buffer (100 ml pyridine--4 ml acetic acid--900 ml water) equal in volume to the volume of 5 per cent (v/v) formic acid used in initially dissolving the fragment. To this solution was added a 1/20 volume of 1 per cent (w/v) porcine trypsin (Novo Industri) solution (weight ratio enzyme to fragment = 1 to 20). Then digestion was allowed to proceed for from 5 to 7 hours at 37° C. After the

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*See Section IV for a description of PC III.

digestion period was over the sample was again dried and then dissolved in a small volume suitable for electrophoresis.

Preparation of Hemoglobins

The hemoglobin used in this thesis (except where indicated) was prepared by Chan (54) following the method of Drabkin (37). Although the hemoglobin had been prepared as carbonmonoxyhemoglobin it had been stored as a powder at -20° C for several years and when redissolved produced a spectrum identical with methemoglobin.

Double molecules of mouse hemoglobin were prepared following the method of Riggs (93). Mouse blood of inbred strain DBA/2J was obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbour, Maine. The cells were washed 3 times in 0.9 per cent NaCl and then were lysed in an approximately equal volume of distilled water. The lysate was then dialysed against 0.2M sodium chloride for 24 hours with four changes of saline solution. This solution was then stored for 1 to 2 weeks at 4° C. Double hemoglobin (HbHb) was separated from single hemoglobin by chromatography on a Sephadex G-100 column (2.2 by 90 cm) using a sample volume of 3 ml (88). The column fractions containing HbHb were dialyzed, lyophilized, and stored at -20° C. Upon redissolving, this material produced the spectrum of methemoglobin. In order to prepare larger amounts of HbHb, 15 ml of dialyzed lysate were fractionated on a Sephadex G-100 column (5 by 90 cm).

For the study of the reaction of C^{14}-iodoacetamide with oxyhemoglobin the oxyhemoglobin solutions were prepared from human red

blood cells (37), dialysed against 0.1M sodium phosphate, 0.2M sodium chloride, pH 7.2, stored at 4^{o} C and used within three weeks of preparation.

Reactions with Hemoglobin and the Hemoglobin-Haptoglobin Complex

Dithiodipyridines

The dithiodipyridines (2-PDS and 4-PDS) were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. The reaction between 2-PDS or 4-PDS and hemoglobin (Hb) or the hemoglobin-haptoglobin (Hb-Hp) complex was followed by the absorbance of the solutions at 343 and 324 nm, respectively, with a Model 15 Cary spectrophotometer (94). An aliquot of stock dithiodipyridine solution was mixed with the hemoglobin solution in a spectrophotometer cuvette and the change in A_{343} or A_{324} as a function of time was determined using the hemoglobin solution as blank. All reactants were dissolved in 0.05M sodium phosphate, 0.05 sodium chloride, pH 6.0. The final concentration of reactants were Hb, 7.0×10^{-6}M (in heme); 4-PDS, 3.3×10^{-5}M, for the reaction of Hb and 4-PDS; and Hb 2.4×10^{-5}M and 2-PDS, 2.5×10^{-4}M with the reaction with 2-PDS.

^{14}C-iodoacetamide

^{14}C-iodoacetamide (1.53 mCi/mMole) was obtained from Volk Radiochemical Company and reacted at room temperature in 0.05M sodium phosphate, 0.1M sodium chloride, pH 7.3, with Hb (5.7×10^{-4}M in heme) or Hb-Hp (Hb 5.7×10^{-4}M in heme to which an excess of Hp is added) using

a concentration of 4 x 10^{-3}M ^{14}C-iodoacetamide. Control reactions with free haptoglobin were performed at 1.2 and 2.2 x 10^{-4}M haptoglobin. The reactions were terminated at various times by diluting a 15 or 25 µl aliquot into 0.2 ml of 0.07M β-mercaptoethanol. The excess iodoacetamide-mercapthoethanol adduct was removed on a Sephadex G-25 column (0.7 cm by 70 cm) using 0.05M Tris-HCl, pH 8.0, or 0.01M NH_4HCO_3 as buffer and the amount of radioactivity in the protein determined by mixing a 1.2 ml aliquot with Bray's solution (95) and counting in a Unilux 1 liquid scintillation counter. The amount of the iodoacetamide attached to the hemoglobin was determined by the ratio of the c.p.m. to the absorbance at 407 nm.

Reactions with Haptoglobins

Cyanogen Bromide Cleavage of Haptoglobins

Cyanogen bromide (CNBr) cleavages were attempted in three media--70 per cent formic acid or 0.1 or 0.01N sodium acetate buffer, pH 4.7. For the formic acid reaction a 3.3 per cent aqueous solution of haptoglobin (Hp) was prepared and 3 parts of this solution was mixed with 7 parts of a 14 mg/ml cyanogen bromide in 98 per cent formic acid solution. The final concentrations of Hp and CNBr were 1 per cent and the molar ratio of CNBr to Hp was 1000 to 1. In general, the reactions were allowed to proceed for greater than 15 hours and then the solutions were diluted with water at least 4-fold so that they could be easily frozen and lyophilized. A similar protocol was followed for the acetate reactions.

Reaction of Haptoglobin with a Mixture of
Sodium Sulphite and p-chloromercurisulphonate (pCMS)

The 'half-molecule' of haptoglobin was prepared by the method of Rorstad and Dixon (96) which used sodium sulphite and parachloromercurisulphonate. The reaction was allowed to proceed for from 30 to 60 minutes and the solution was then desalted. In the preparation of labelled half-haptoglobin the specific activity of the ^{35}S-sulphite was 11 mCi/mMole.

Haptoglobin Preparation

Haptoglobin was prepared following the method of Chan (54). The starting material was ascites fluid, a rich source of haptoglobin, to which ammonium sulphate was added to give 55 per cent saturation. The precipitate was dissolved in 0.01M sodium acetate buffer, pH 4.7, and dialyzed against this buffer to remove the sulphate. Any precipitate forming during dialysis was removed by centrifugation and the supernatant applied to a DEAE-cellulose column equilibrated with 0.01M sodium acetate buffer at pH 4.7. The column was then washed with a large volume of buffer and then eluted with a gradient of 0.01M NaCl to 0.3M NaCl in the same acetate buffer. The protein peak obtained was then dialyzed against distilled water and lypohilized. The protein was then dissolved 0.05M ammonium acetate at pH 8.6 and run on a Sephadex G-200 column. One major peak of haptoglobin was usually obtained with a minor faster-running peak of caeruloplasmin and a minor slower-running peak of albumin and postalbumin. The

haptoglobin was then characterized for purity and hemoglobin binding by starch gel electrophoresis (54) or by polyacrylamide disc gel electrophoresis.

III

HAPTOGLOBIN DOUBLE HEMOGLOBIN (Hb.Hb) REACTION

Introduction

Human haptoglobin of phenotype 1-1 is constructed of two dissimilar pairs of polypeptide chains held together by disulphide bonds (12). The smaller α-chains have a molecular weight of 8,800 (92) while the larger, carbohydrate-containing β-chains, possess a molecular weight of 40,000 to 43,000 (28,29,30) for a total of 98,000 ± 1,000 for the intact haptoglobin 1-1 molecule (28,30). The overall structure of haptoglobin 1-1 thus bears a strong resemblance to that of immunoglobin G. Since the complete amino acid sequence of haptoglobin α-chains has been determined and a detailed comparison between this sequence and those of a series of Bence-Jones proteins indicated homology between portions of the haptoglobin α-chains and Bence-Jones sequences (26), it is reasonable to examine whether haptoglobin and the immunoglobins possess any functional similarities. The major function of haptoglobin appears related to its remarkable property of binding hemoglobin extremely tightly and specifically giving rise to a complex of M.W. 163,000 with a stoichiometry of 65,000 gm of hemoglobin to 98,000 gm of haptoglobin 1-1 (4). Since hemoglobin is normally contained within the red blood cells it can be considered that when it is released by hemolysis into the plasma, the location of haptoglobin, it becomes a protein foreign to that particular compartment of the body.

Thus haptoglobin in complexing with it acts in a manner analogous to that of an antibody binding to a foreign protein. Thus, in some ways, haptoglobin can be considered functionally as a constitutive hemoglobin antibody although there are a number of differences between haptoglobin 1-1 and immunoglobulin G (IgG). These include different sites of synthesis (liver and lymphoid tissue respectively), isoelectric points, and molecular weights of the light chains (97,98) as well as the absence of complement fixation by the hemoglobin-haptoglobin complexes (99). The 1:1 stoichiometry of the hemoglobin-haptoglobin complex would, at first sight, suggest that haptoglobin might possess only a single binding site for hemoglobin, a clear difference from antibody molecules such as IgG which are bivalent towards antigens. However, Laurell (69) found upon adding less than stoichometric amounts of hemoglobin to haptoglobin that a distinct intermediate could be observed upon electrophoresis and postulated that this complex consisted of a half molecule of hemoglobin bound to one molecule of haptoglobin (100). In more recent studies Hamaguchi (70) has purified this intermediate and has found that its molecular weight is 140,000 and its stoichiometry indeed 1/2 hemoglobin to 1 haptoglobin. In addition, Nagel and Gibson (71), in studies of the kinetics of the hemoglobin-haptoglobin reaction have found evidence that the combination of haptoglobin is not with intact hemoglobin tetramers but with either ($\alpha\beta$) dimers or with first α and then β hemoglobin chain monomers.

In order to gain further insight into the number of binding sites in haptoglobin, the reaction between covalently-linked double

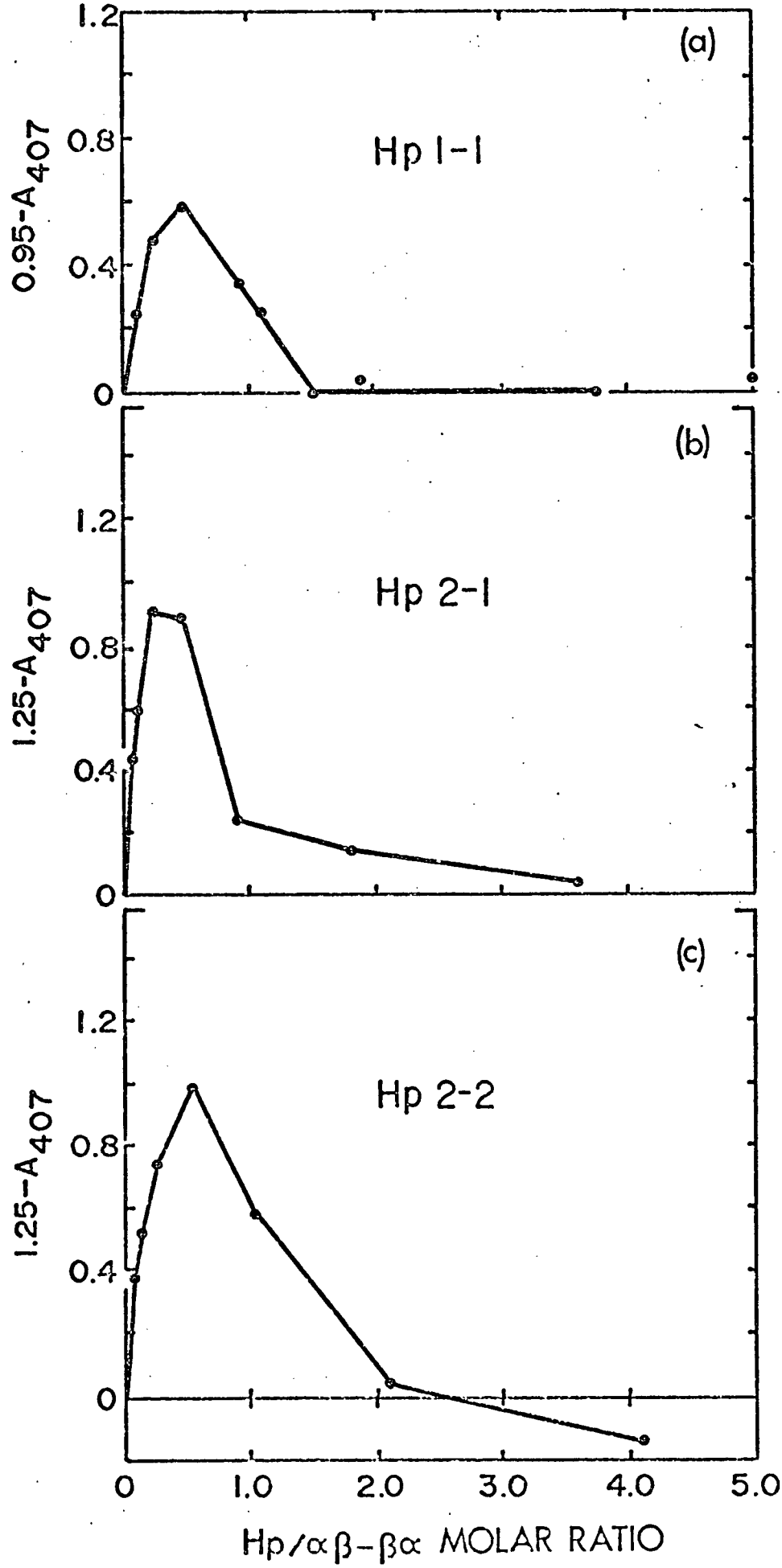
hemoglobin molecues (having eight chains) and haptoglobin has been studied. Riggs (93) has shown that when the hemoglobin of certain strains of mice is allowed to stand in air, disulphide bonds can form between free sulphydryl groups of cysteine residues in the β chains giving rise to hemoglobin octamers (α_4 β_4) in which each pair of β chains is connected by a single, intermolecular symmetrical disulphide bond. In this section, such hemoglobin octamers have been found to combine readily with haptoglobin to produce a series of aggregates of increasing size which precitipate at low ionic strength in a manner analogous to the precipitation of antigen-antibody complexes. The formation of these complexes can most easily be explained if haptoglobin is bivalent in its combination with hemoglobin. Treatment of these molecular aggregates of haptoglobin and octameric hemoglobin with mercaptoethanol converts them to the usual single hemoglobin-haptoglobin complexes.

Precipitation Studies

When solutions of mouse double hemoglobin (Hb.Hb) were mixed with each of the three major haptoglobin phenotypes (Hp 1-1, Hp 2-1, Hp 2-2) precipitates formed when the input ratios of the two proteins were within certain limits, a clear difference from the reaction of haptoglobin with single hemoglobin molecules which yields only soluble complexes (55). Two series of experiments were conducted; in the first, (series A), the concentration of Hb.Hb remained constant and the haptoglobin concentration was varied, while in the second, (series B),

increasing concentrations of Hb.Hb were added to a constant amount of haptoglobin. These mixtures were allowed to stand for several hours at 4^{o} C. The extent of precipitation in series A was followed by measuring the decrease of absorbance due to Hb.Hb at 407 nm in the supernatant after centrifugation of the precipitate. The extent of precipitation was dependent upon ionic strength, there being little precipitate in 0.2 M NaCl. However in the buffer that was routinely employed, 4 mM sodium phosphate, pH 6.2 there was extensive precipitation. In Figure 5, the precipitation curves in series A of the three common haptoglobin phenotypes with Hb.Hb show a close resemblance to classical antibody-antigen precipitation curves. In calculating the molar input ratios of the three haptoglobin phenotypes and Hb.Hb it is necessary to take account of the fact that while Hp 1-1 is a monomeric protein of molecular weight (98,000), Hp 2-1 and Hp 2-2 both exist as a series of stable polymers of increasing size (98). However it has been shown that the binding capacity per gram of each phenotype of haptoglobin is virtually identical (4) and that 1 mole of hemoglobin is bound per 98,000 gms of haptoglobin of any of the three phenotypes. In the case of Hb.Hb, whose molecular weight is 130,000, it appears that the species combining with haptoglobin is not the full double molecule but rather, as with the single molecules of human hemoglobin, there is first cleavage. For Hb.Hb this would produce disulphide linked half hemoglobin molecules ($\alpha\beta$-$\beta\alpha$) as shown in Figure 6. In accordance with this scheme of cleavage the haptoglobin-hemoglobin ratios are calculated in every case on the basis of the 'monomeric unit' of haptoglobin and the ($\alpha\beta$-$\beta\alpha$) molecules

Figure 5 Precipitation curves with the concentration of Hb.Hb maintained constant. The ordinate is one-half the absorbance of the original Hb.Hb solution minus the absorbance of the supernatant solution and therefore represents the amount of Hb.Hb precipitated. Haptoglobin solutions were prepared by dilutions of a concentrated haptoglobin solution to final volumes of 0.5 ml; to each of these solutions 0.5 ml of the Hb.Hb solution was added. In curve (a) the final concentrations are as follows: Hb.Hb 0.16 mg/ml, Hp 1-1 varied from a high concentration of 1.7 mg/ml down to 0.027 mg/ml. In curve (b) Hb.Hb 0.20 mg/ml, Hp 2-1 varied from 1.1 mg/ml to 0.018 mg/ml. In curve (c) Hb.Hb 0.20 mg/ml, Hp 2-2 1.4 mg/ml to 0.022 mg/ml.


(a)
Hp 1-1
0.95-A407
1.2
0.8
0.4
0
(b)
Hp 2-1
1.25-A407
1.2
0.8
0.4
0
(c)
Hp 2-2
1.25-A407
1.2
0.8
0.4
0
0
1.0
2.0
3.0
4.0
5.0
Hp/αβ-βα MOLAR RATIO

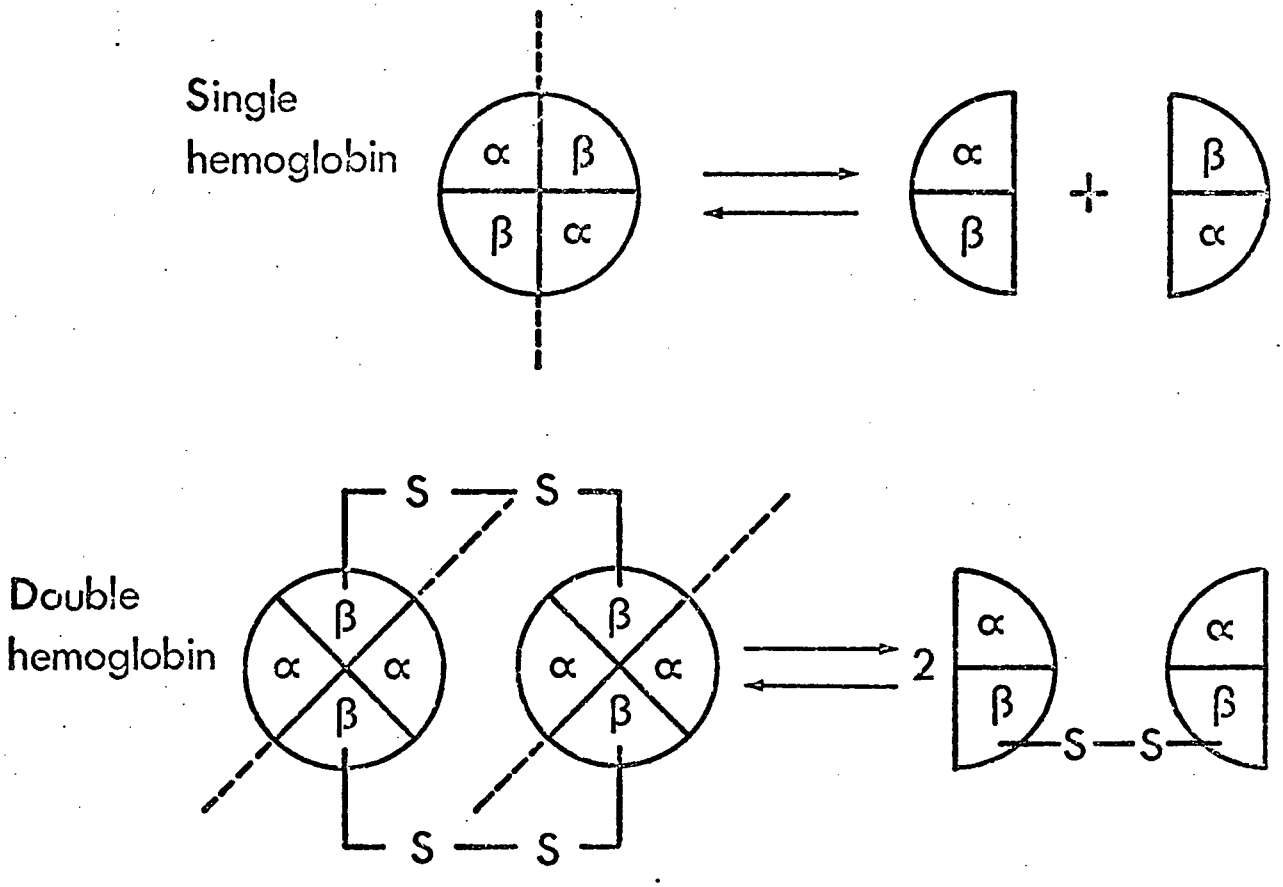


Figure 6 A scheme for the cleavage of Hb and Hb.Hb into halves; the disulphide bond is between cysteines at position 13 in the β-chain in BALB/cJ mice.

having molecular weights of 98,000 and 65,000 respectively. The ratios are also calculated on the basis of an $E^{1\%}_{280nm}$ of 12.0 for the haptoglobins and 17.5 for the mouse hemoglobin.

In the series B experiments, the haptoglobin concentration was kept constant and increasing concentrations of Hb.Hb added. Precipitation was again observed and, as may be seen in the photograph, (Figure 7), was dependent on the input ratios of the combining proteins. At both high (0.96) and low (0.015) ratios of haptoglobin to αβ-βα, there was little precipitate but in the range 0.06-0.48, precipitation was extensive.

Acrylamide Gel Electrophoresis

When solutions of Hb.Hb and haptoglobin 1-1 in 0.112 M Tris, 0.062 M boric acid and 2.5 mM disodium EDTA at pH 8.6 were mixed no precipitation was observed. These soluble complexes were then examined by acrylamide disc gel electrophoresis in the above buffer. In Figure 8a, it may be seen that a series of hemoglobin-haptoglobin complexes appears with the relative concentration of each complex within the series depending upon the input ratio of the two proteins. At high ratios of haptoglobin/αβ-βα (3.0-6.0) the major complexes migrated into the gels but as the ratio approaches that at which maximal precipitation occurred at lower ionic strength, an increasing proportion of the complexes barely entered the gels, thus behaving as if they were very large. In Figure 8b, an enlarged photograph of the first six gels shows that up to six separate complexes of decreasing mobility are resolved.

Figure 7 A photograph (by reflected light) of the turbidity observed shortly after mixing Hb.Hb and Hp. To obtain the precipitates a concentrated Hb.Hb solution was diluted to a final volume of 0.5 ml and added to 0.5 ml of a haptoglobin solution. The final concentrations of Hb.Hb varied from 2.3 mg/ml to 0.036 mg/ml while haptoglobin 1-1 was maintained constant at 0.05 mg/ml. The white areas at the bottom of the tubes are caused by reflection from the glass and are not indicative of precipitation.

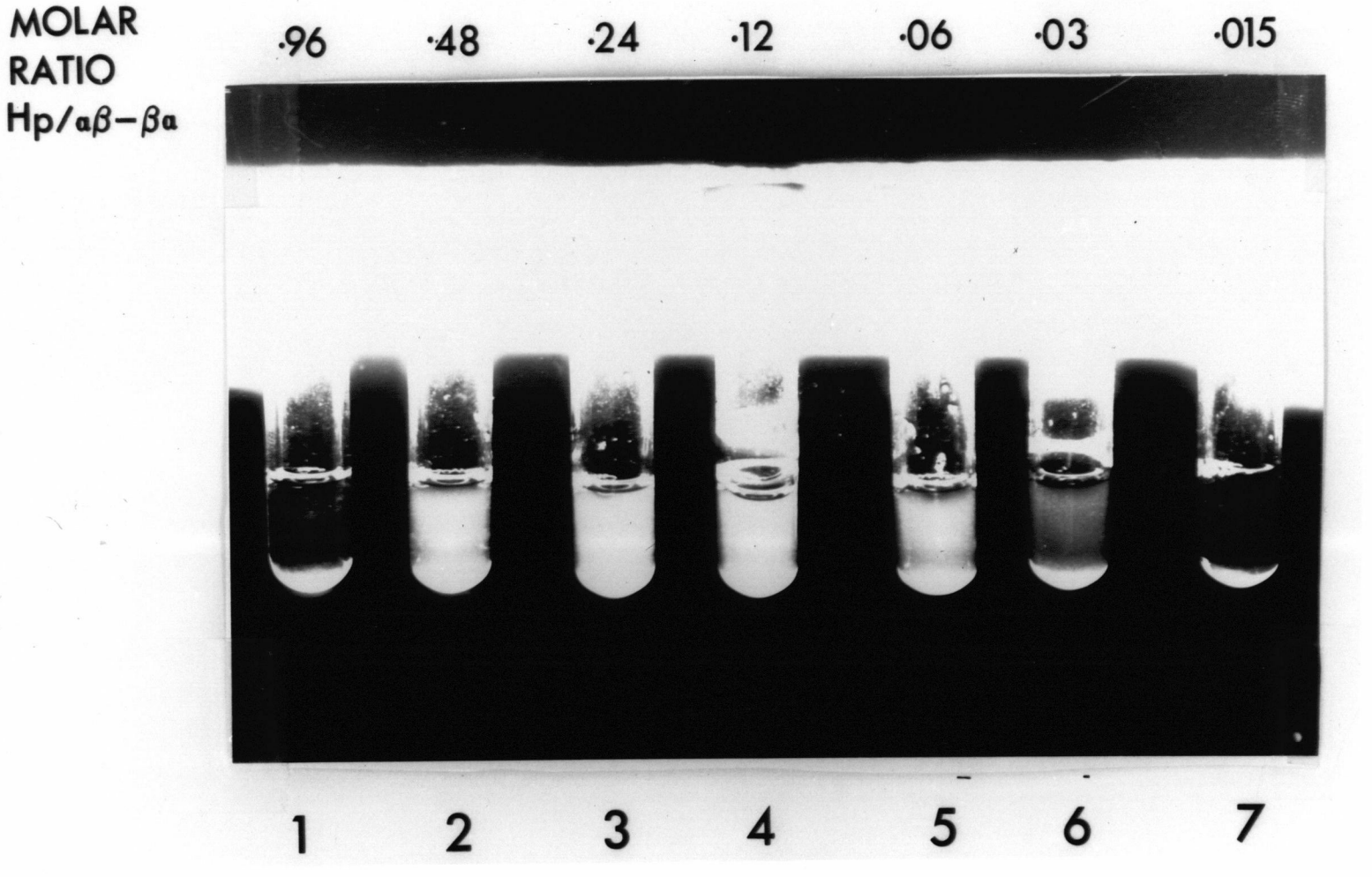

MOLAR
RATIO
Hp/αβ−βα
·96
·48
·24
·12
·06
·03
·015
1
2
3
4
5
6
7

Figure 8a Disc gel electrophoresis of mixtures of Hp and Hb.Hb. Solutions were prepared by mixing varying proportions of an 11.5 mg/ml solution of Hb.Hb and a 10 mg/ml solution of Hp. The gels contained 5% acrylamide and 0.25% N,N' methylene-bisacrylamide and were 0.5 x 7.0 cm. Electrophoresis was performed for 1.5 hours at 200 volts at 4° C.

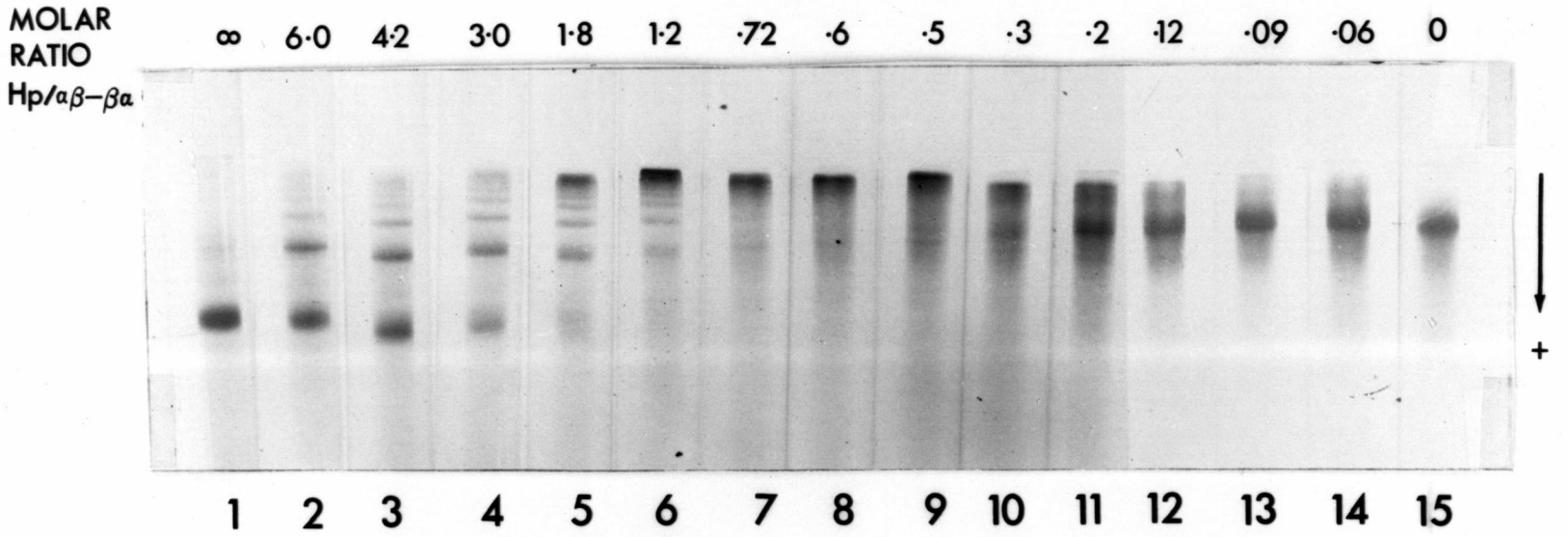
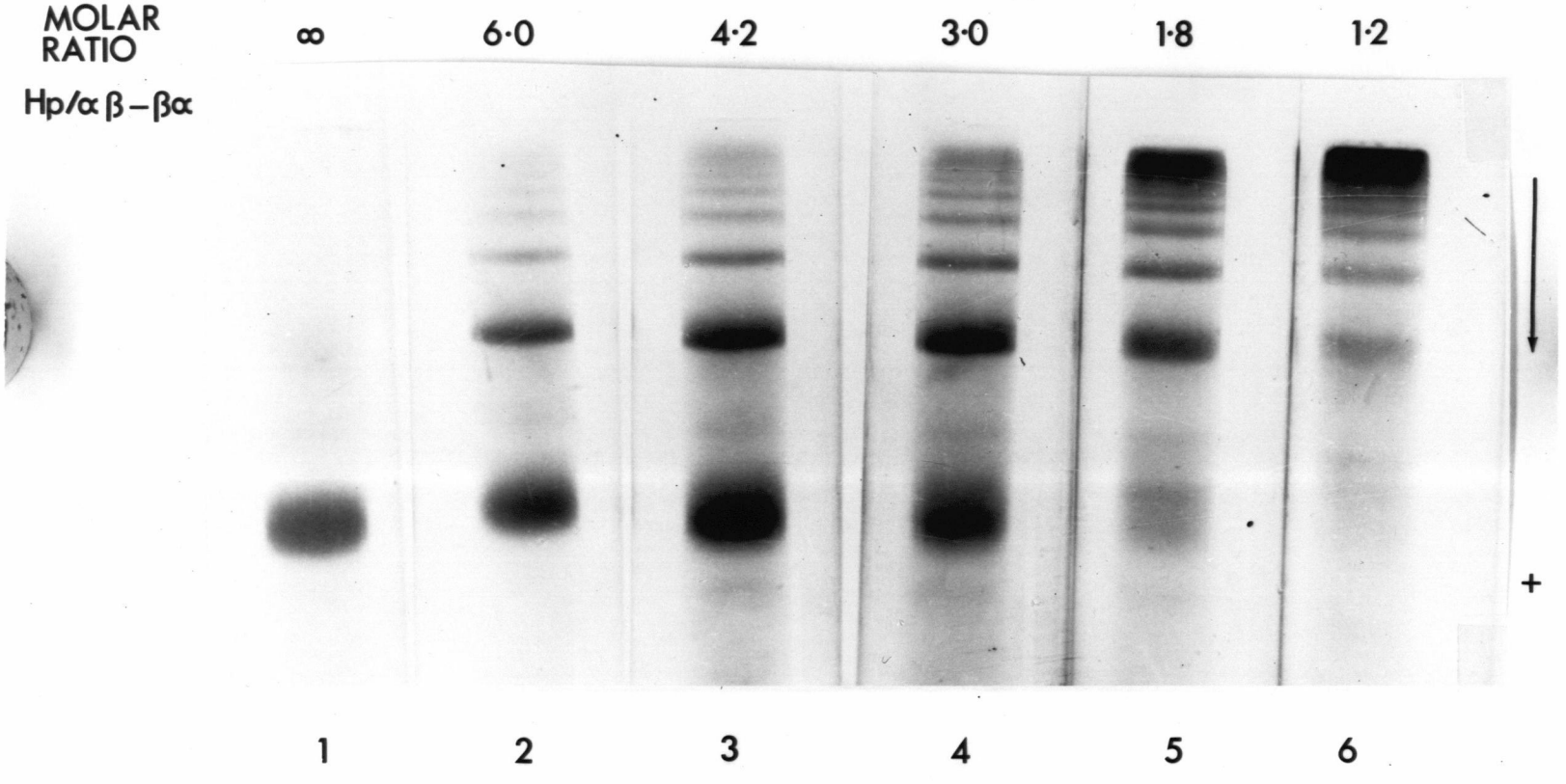

MOLAR RATIO
Hp/αβ–βα
∞ 6·0 4·2 3·0 1·8 1·2 ·72 ·6 ·5 ·3 ·2 ·12 ·09 ·06 0
+
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

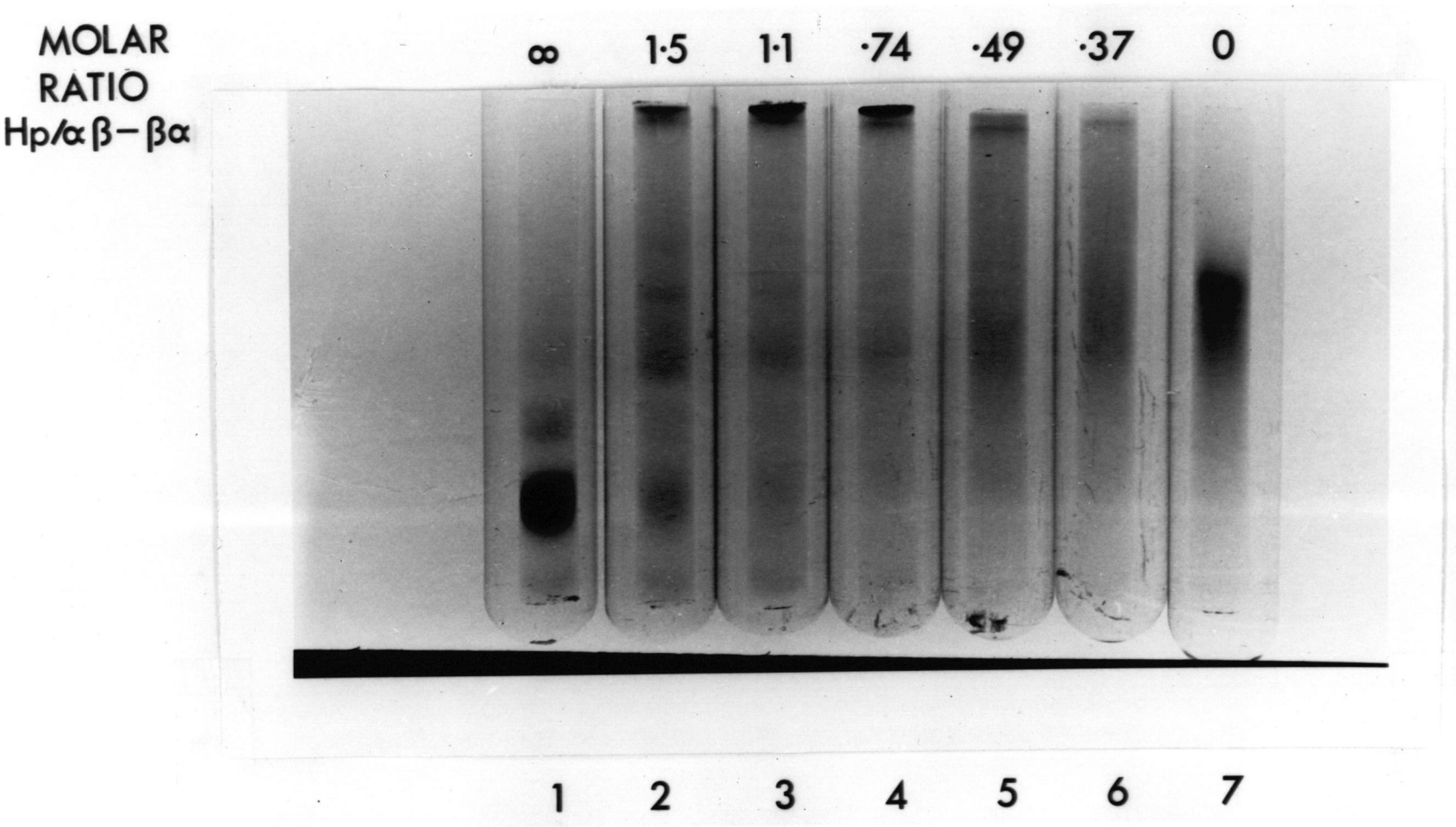
Figure 8b Enlarged photograph of the first 6 gels in Figure 8a.


MOLAR RATIO
Hp/αβ–βα
∞
6·0
4·2
3·0
1·8
1·2
+
1
2
3
4
5
6

Qualitatively, it may be seen that as the proportion of Hb.Hb is increased each complex reaches a maximum concentration and then is replaced by a more slowly running complex until the majority of the complexes are too large to migrate appreciably into the gel. A series of complexes characterized by increasing sedimentation coefficients has also been observed by ultracentrifugation of mixtures of haptoglobin and Hb.Hb. The best evidence that the largest species are found at ratios of haptoglobin to $\alpha\beta$-$\beta\alpha$ near 1.0 was obtained from 3.5% acrylamide disc gels of larger pore size. Figure 9 shows that at a haptoglobin to $\alpha\beta$-$\beta\alpha$ ratio of 0.74 to 1.1 the major protein band is at the origin while at ratios of 0.50 and 1.5 a greater proportion of the complexes runs well into the gels. Using 2.5% gels in plexiglass tubes (101) similar results have also been obtained. Although the slow-running material at the origin now enters the gel, a series of discrete bands proceeding from the origin of the gels out to the position of haptoglobin or Hb.Hb cannot be observed because of streaking. This streaking also prevents the observation of a series of bands in the gels where Hb.Hb is in excess. However at ratios near 1.0 there is a definite increase in staining of complexes, which although streaky, run much closer to the origin of the gel and hence behave as if they are much larger.

It would be expected that in any complex formation between two proteins each possessing two binding sites that the largest complexes would be formed when an equal number of moles of each bivalent reacting species is present. In the present case it has been assumed that the

Figure 9 Disc gel electrophoresis of solutions prepared by mixing a 12.5 mg/ml solution of Hb.Hb with a 14 mg/ml solution of Hp 1-1. The gels contained 3.5% acrylamide and 0.18% N,N' methylene-bisacrylamide and electrophoresis was performed at 4^{o} C for 1.3 hours at 200 volts and 3.5 ma per gel.


MOLAR
RATIO
Hp/αβ−βα
∞
1·5
1·1
·74
·49
·37
0
1
2
3
4
5
6
7

reacting species from Hb.Hb is a pair of disulphide linked αβ units, αβ-βα, of molecular weight 65,000. Thus the largest complexes should occur at a ratio of 1.0 and this appears consistent with the fact that the complexes formed at this ratio cannot enter even the large pored gels. In contrast to these findings, maximal precipitation was seen at ratios of 0.2-0.6. Thus it appears that complexes richer in αβ-βα tetramers are more insoluble in 4 mM phosphate at pH 6.2 than those larger complexes in which the amount of haptoglobin and αβ-βα is more nearly equal.

The behaviour observed upon mixing haptoglobin with Hb.Hb is in strong contrast to its reaction with normal hemoglobin where only two soluble complexes are formed, an intermediate of stoichiometry 1/2 Hb/1 Hp and the full complex 1 Hb/1 Hp. Since the intermolecular disulphides of Hb.Hb can be cleaved under mild conditions by mercaptoethanol (93), a 20 µl aliquot of haptoglobin/Hb.Hb mixtures in Tris-borate-EDTA, pH 8.6, was reacted with 2 µl of 0.5 M mercaptoethanol for 1 hour at 25° C followed by the addition of 2 µl of 0.6 M iodocetamide for 30 minutes at 25° C.

In Figure 10, the series of haptoglobin (αβ-βα) complexes formed at ratios of 3.4 to 1.0 (Gel 6) and 1.1 to 1.0 (Gel 5) gave rise upon treatment with mercaptoethanol to the patterns seen in Gels 2 and 1 respectively. At the higher input ratio of 3.4 (Gel 6) there is still excess uncombined haptoglobin as well as a series of complexes which move well into the gel. It is likely that these complexes are of the type depicted in Figure 11a as Complex 1 and Complex 2. As indicated

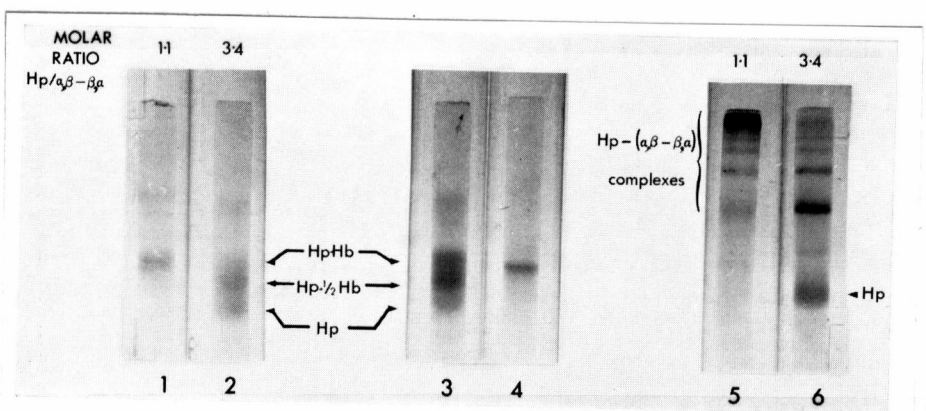


Figure 10 Disc gels (5%) showing the effect of mercaptoethanol on Hp-(αβ-βα) complexes. Solutions were prepared by mixing an 8 mg/ml solution of Hb.Hb and a 14 mg/ml solution of Hp. Aliquots were removed and then mercaptoethanol and iodoacetamide were added (see text). Electrophoresis was performed for 2.3 hours at 200 volts. Gel 3 shows the bands produced by mixing single mouse hemoglobin molecules with excess haptoglobin. Gel 4 shows single mouse hemoglobin.

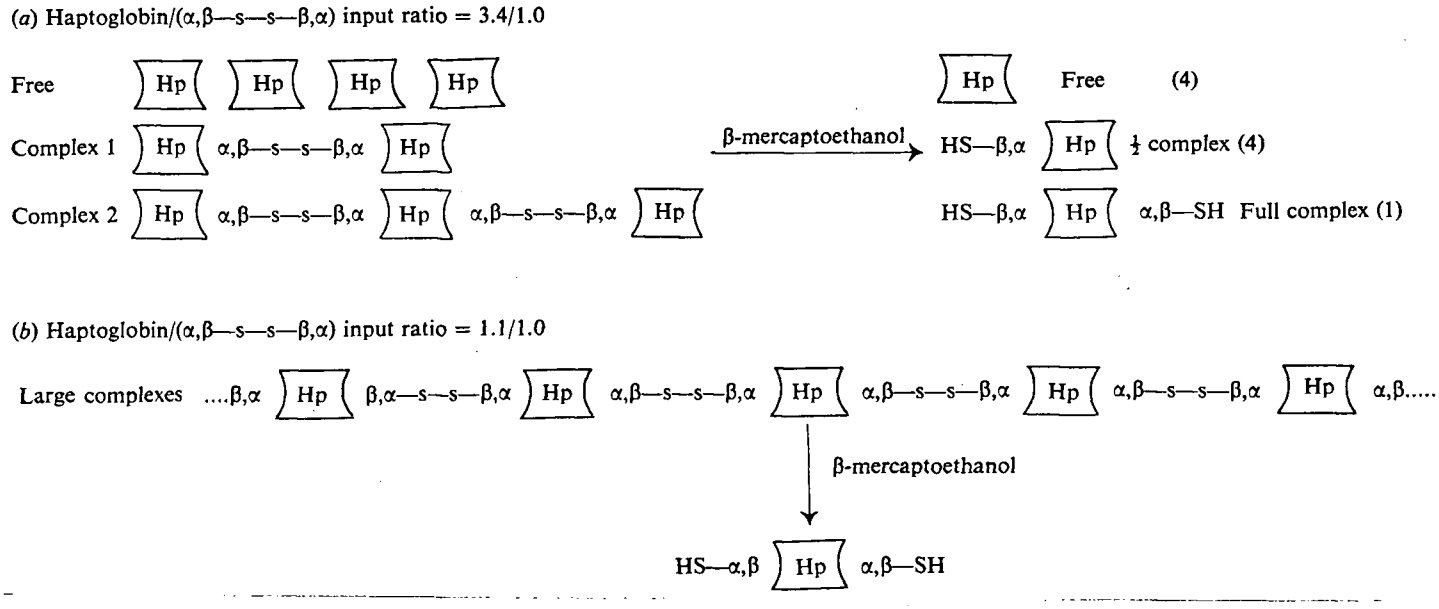


Figure 11 A scheme to illustrate the possible complexes of haptoglobin with double hemoglobin at (a). High input ratio (b). Equimolar input ratio.

in Figure 11a, a mixture of free haptoglobin, Complex 1 and Complex 2 would give rise, upon treatment with β-mercaptoethanol predominantly to the 1/2 Hb:1Hp complex with a smaller amount of the full complex. This prediction is consistent with the pattern observed in Figure 10, Gel 2. In contrast, at the input ratio of 1.1/1.0, the complexes are very much larger (Figure 10, Gel 5) and upon thiol treatment (Figure 10, Gel 1) give rise almost quantitatively to the full Hb.Hp complex. Since the 1/2 Hb:Hp complex comes only from the ends of polymeric chains of Hb.Hb/Hp complexes, the absence of this species in Figure 10, Gel 1 and the predominance of the full Hb:Hp complex indicates that the slow-running polymeric complexes of Hb.Hb/Hp formed at equimolar input ratios of Hb.Hb and Hp are either very long, as depicted in Figure 11b or alternatively these complexes could be circular in which case, the full Hb:Hp complex would be the sole product. At the moment it is not possible to choose between these alternatives.

In most antigen-antibody reactions, both antigen and antibody possess at least two binding sites. This leads to the formation of large, three-dimensional complexes which are often insoluble. The interaction of hemoglobin with haptoglobin at first sight appears not to be of this type since the complex is soluble and its overall stoichiometry is consistent with a single binding site on each protein. However, the fact that at ratios of Hb to Hp of less than one an intermediate complex of stoichiometry 1/2 Hb/1 Hp is seen suggests that only one of the two sites is occupied by a half molecule of hemoglobin and that the full complex would comprise two half molecules of

hemoglobin combined at two separate sites. Thus hemoglobin, a symmetrical and bivalent molecule, dissociates into two monovalent halves upon combination with haptoglobin. There is, therefore, no possibility of forming large complexes as in the case of an antigen-antibody reaction.

In the present study, a hemoglobin has been examined in which the two halves of the molecule are covalently linked by a disulphide bond so that it behaves as a bivalent molecule. When haptoglobin combines with the bivalent hemoglobin a noticeably different behavior is seen. Large complexes are formed which precipitate at low ionic strength in a manner very similar to that of antigen-antibody reactions. As soon as the covalent linkage between the β chains of Hb.Hb is broken the large complexes disappear and are replaced by the simple ones.

IV

THE DISULPHIDES OF HAPTOGLOBINS - STUDIES ON CYANOGEN BROMIDE REACTIONS WITH HAPTOGLOBIN AND ON HALF-HAPTOGLOBIN

Introduction

The α and β chains of the human haptoglobins are held together by disulphide bonds (12). This fact was based on the observations that haptoglobin 1-1 migrated as a single band on gel electrophoresis in 8M urea but when very low concentrations of β-mercaptoethanol, a reagent which is known to break disulphide bonds, were added the haptoglobin was split into its α and β chains. Similarly the polymeric haptoglobins (2-1 and 2-2) maintained their structure in 8M urea but were split into α and β chains by mercaptoethanol.

As discussed previously Kauffman and Dixon isolated an α-α' disulphide peptide after pepsin digests of haptoglobin 2-1. The structure of this peptide is shown in Figure 2 (page 8). In addition, Smithies, Connell and Dixon (76) have presented evidence for the existence of an intrachain disulphide in the α chain of haptoglobin. After reduction of haptoglobin 1-1 with very low concentrations of mercaptoethanol, they detected a band after electrophoresis which migrated slightly faster than the completely-reduced α chain. Thus this polypeptide appeared to migrate faster than the fully-reduced α chain of haptoglobin because it contained an intrachain disulphide and thus had a more compact

conformation. However, since in these experiments no mercaptoethanol was included in the gel buffer, such a loop disulphide might have been formed by oxidation during electrophoresis and so need not necessarily be present in native haptoglobin.

The binding of the haptoglobin chains solely by noncovalent forces appears very unlikely because of the stability of the molecule under a variety of denaturing conditions. In addition to its migration as a single band in 8M urea, haptoglobin 1-1 maintains its molecular weight after complete succinylation (54), a very powerful method for disrupting non-covalently bonded subunits (102), and it is not dissociated in 0.1% sodium dodecyl sulphate (SDS) (96). Also the haptoglobin polymers maintain their polymeric structure in 8M urea, after succinylation (54) or in 0.1% SDS.

In 1961 (103) Dixon and Connell showed that when haptoglobin was treated with sulphite and parahydroxymercuribenzoate (pHMB) a limited cleavage of disulphide bonds occurred. The product migrated identically with haptoglobin in borate starch gels but in the acidic 8M urea gels it moved slightly more rapidly and exhibited a broader band than haptoglobin. Since there was only one product formed it appeared that haptoglobin was being split into symmetrical halves. This interpretation has been confirmed by molecular weight studies on the "half-haptoglobin molecule" using gel electrophoresis in sodium dodecyl sulphate (104,96). Also the very interesting observation was made that if the multiple haptoglobin polymers were treated with sulphite they were converted to a single product which also appeared to contain a haptoglobin light and heavy chain (103).

Since the bond cleaved by sulphite and p-chloromercurisulphonate (pCMS) must be symmetrical to give rise to symmetrical halves, it seemed logical that the sulphite must be splitting the α-α' disulphide and thus breaking haptoglobin into halves. It also appeared that this particular disulphide was responsible for haptoglobin polymerization. Thus it was possible to investigate the disulphides of haptoglobin further by using ^{35}S-sulphite for cleavage and then determining in which part of the molecule the radioactivity was located (96). This approach was attempted by treating the half-haptoglobin molecule, which had been prepared with ^{35}S-sulphite, under more rigorous conditions with 8M urea and unlabelled sulphite. In this way, haptoglobin was split into its heavy and light chains which were subsequently separated on Sephadex G-75 (28). The rigorous conditions left only a small amount of the ^{35}S-sulphite in the haptoglobin and this was found in the β chain fraction. Thus at first sight it seemed that the α-α' disulphide was not being split by ^{35}S-sulphite.

It appeared possible to analyze the nature of the α-β linkages and α-α' linkages further because the α chain of haptoglobin contained no methionine (28). Thus if one cleaved the haptoglobin molecule with a reagent which split peptide bonds at methionine residues the β chain of haptoglobin would be cleaved into a series of smaller fragments but the α chain would remain intact. Also, since the disulphide bonds in haptoglobin would still be intact, one could study the β chain peptide fragment(s) to which the α chain was attached since the α chain could be characterized after the fragment containing it was reduced with

mercaptoethanol and alkylated with iodoacetamide (12). In fact, a well characterized reagent for cleavage of methionyl peptides, cyanogen bromide, appears to be well suited for these studies (105).

Cyanogen bromide is a useful reagent for protein studies. Although it reacts with basic groups in proteins in alkali, in acid it reacts only with cysteine and methionine. The reaction with cysteine is a slow oxidation to cysteic acid and the reagent will not react with carboxymethylcysteine or S-benzylcysteine (106). The reaction with methionine in a methionyl peptide results in the cleavage of a peptide bond (Figure 12) and the methionyl residue is converted into an equilibrium mixture of homoseryl and homoseryl lactone residues at the carboxyl-terminal portion of the cleaved peptide. The mixture can be converted to homoserine lactone by heating in acid or can be opened to homoserine by treatment with alkali at room temperature. The reagent has now been used with success on over 20 proteins and frequently is the reagent of choice for limited cleavage of peptide bonds.

During this investigation of the reaction of cyanogen bromide with haptoglobins it was not known that the peptide Th3A isolated by Kauffman and Dixon was in fact a peptide from the α chain of haptoglobin (see Introduction, page 8), and that this peptide was linked to the β chain of haptoglobin. However, the studies to be described are in complete agreement with this linkage and, as will be shown, do confirm the disulphides presented in the Introduction.

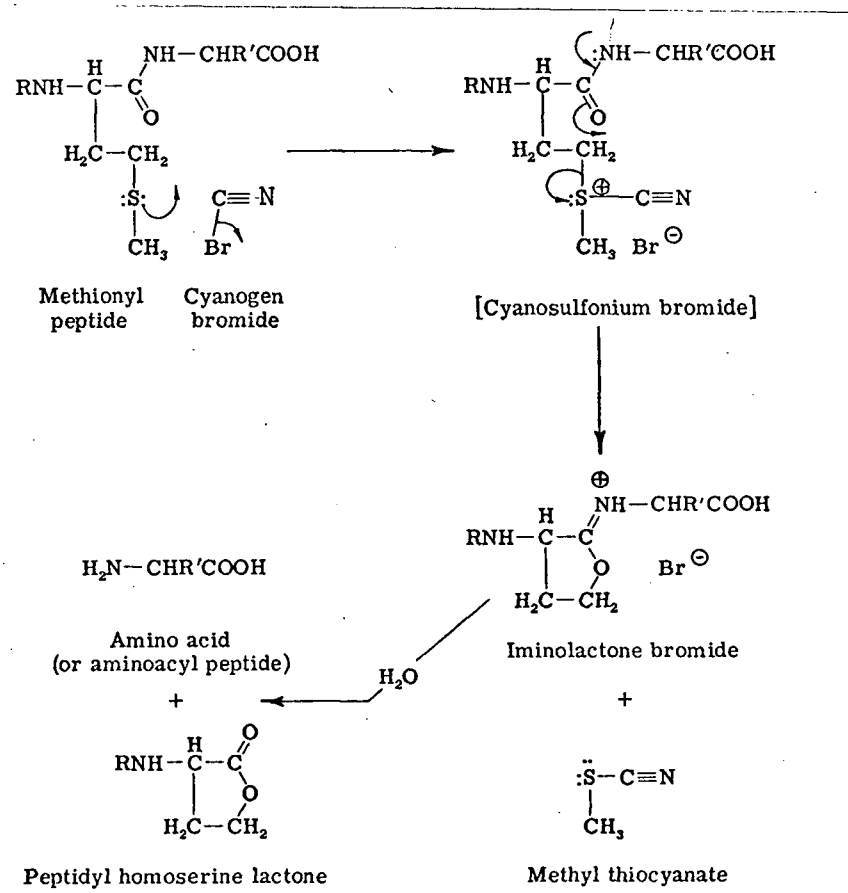


Figure 12 The reaction of cyanogen bromide with a methionyl peptide.

Nature of the Reaction as Examined by Disc Gels

Cyanogen bromide cleavages were attempted in 70% formic acid and in 0.01 or 0.1M sodium acetate buffers pH 4.7. The former has been used successfully on IgG by Edelman (107) and the latter was attempted because it was thought possible to get a more limited cleavage under conditions where the protein maintains a more compact three-dimensional structure. Also haptoglobin retains hemoglobin-binding ability at pH 4.7 and so it might be possible to obtain a fragment with hemoglobin-binding ability.

The reactions at pH 4.7 were done with 0.01M or 0.1M sodium acetate, using Hp concentrations of 1% and CNBr concentrations of 0.5 or 5%. In both cases the reaction solution developed a precipitate after a short time and was not studied further.

Reactions in 70% formic acid were analyzed by disc gel electrophoresis as described earlier. Samples were dissolved in Tris-glycine 4 to 5 times more concentrated than the gel buffer. The results shown in Figure 13 demonstrate that one main slow-running band and two fast-running bands are formed by the reaction and the pattern of bands appears similar in different haptoglobin types (Fig. 13a Gels 1 and 2). When electrophoresis is carried out for a longer time the slow-running band resolves into a series of multiple bands (Figure 13b Gels 3,4,5).

These multiple bands form a characteristic pattern with darker bands at the center and fainter bands at the outside. Because of this variation in intensity of the bands they form a gaussian distribution and for simplicity will subsequently be referred to as 'gaussian' bands.

Figure 13 Acrylamide disc gel electrophoresis (7.5%) of the reaction products of cyanogen bromide with haptoglobins.

Figure 13a shows the results after electrophoresis for 10 minutes at 300 volts in 0.05M glycine, 0.01M Tris pH 8.5.

Figure 13b shows the results when electrophoresis is carried out for 75 minutes.

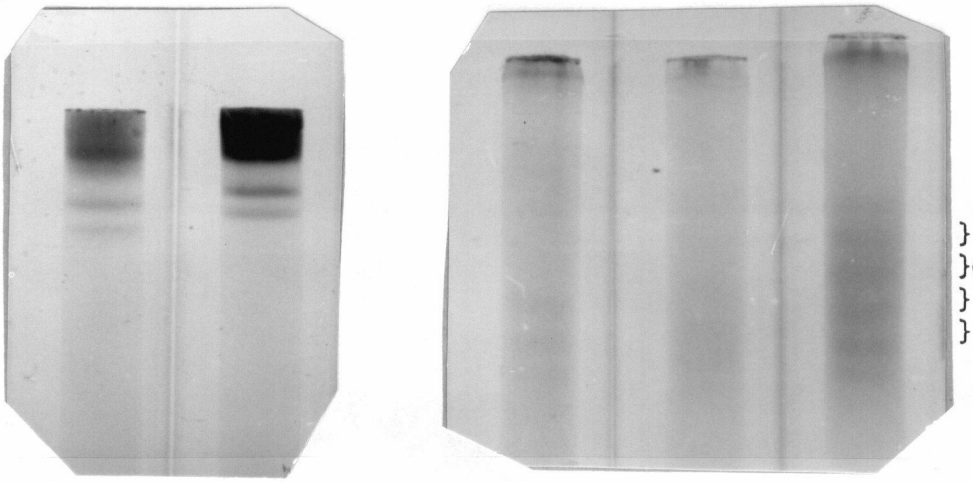
Figure 13c - the acrylamide gels contain 8M urea and electrophoresis is for 40 minutes at 300 volts. Gels 1, 5, and 6 show CNBr Hp 1-1; 2, 3, and 8 show CNBr Hp 2-2; and 4 and 7 show CNBr Hp 2-1. Gel length 5 cm.

13a

1 2

13b

3 4 5



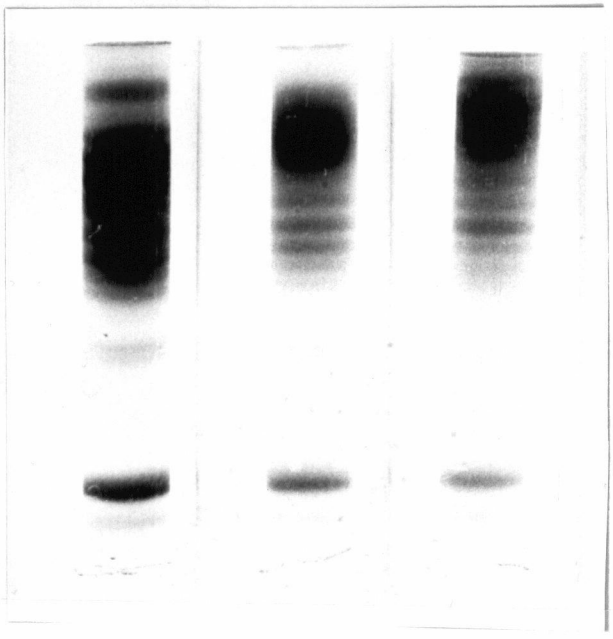
}
}Gaussian
} bands
}

13c

6 7 8

Maximum staining region

2-2 →
2-1 →
1-1 →



}
}Gaussian
} bands
}

A series of bands with a similar distribution has been obtained after starch gel electrophoresis of the Hp β chain (76) and it appears that a similar phenomenon is responsible for the pattern of banding in both cases. Other studies in this laboratory suggest that the pattern may be caused by a variable degree of attachment of sialic acid residues to the haptoglobin β chain (32).

The CNBr reaction products were also analyzed by disc gel electrophoresis in Tris-glycine buffer containing 8M urea (Figure 13c). Ten microliters of 2% (v/w) solutions of the reaction mixture were applied to the gels. The resolution of bands in the 8M urea gels appeared to be better than in the gels which contained no urea.

In all of the disc gels observed the patterns obtained from Hp 1-1, 2-1, and 2-2 were similar, but the region of maximum staining, although composed of a complex of bands, was in different positions in the three haptoglobin types (Figure 13c). However much clearer differences were seen in later studies using starch gels to resolve the cyanogen bromide fragments (as discussed below).

Since the α haptoglobin chains should not be cleaved by cyanogen bromide and since there are only 4 methionines in the β chain of haptoglobin (28), it was logical to investigate more closely the reaction of cyanogen bromide with the haptoglobin polymers to see whether or not a polymeric series was present after the reaction of the haptoglobin polymers with cyanogen bromide.

In order to examine the effect of cyanogen bromide on haptoglobin polymers more closely and to characterize the reaction further, a time

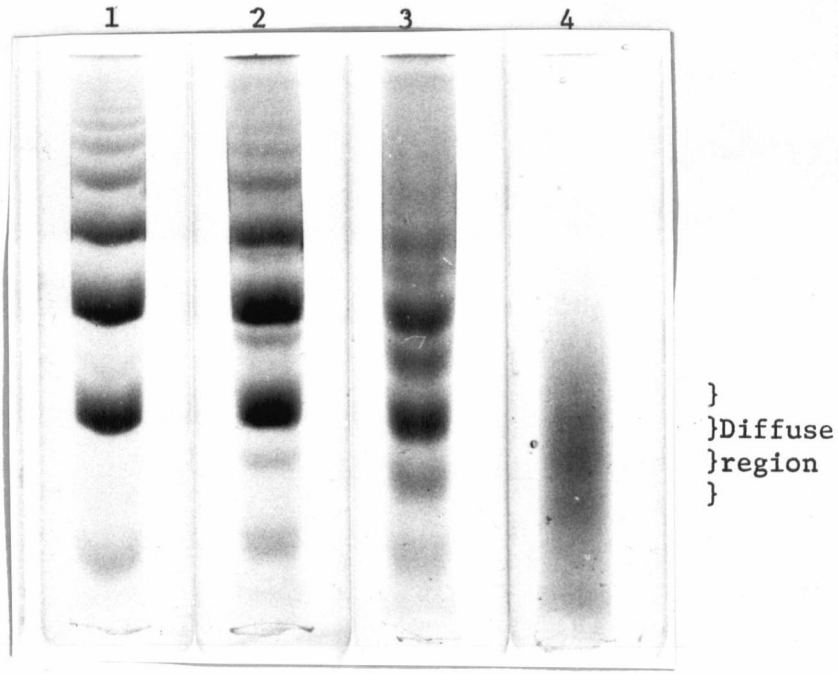
study of the reaction with haptoglobin 2-1 was performed (Figure 14). The reaction was stopped at various times by a 10-fold dilution of the reaction mixture and subsequent freezing and lyophilization. With the 5 minute sample a small amount of reaction could be observed (Figure 14 Gel 2). New bands appeared running slightly ahead of each of the polymers and these appeared also to form a polymeric series. Also a small amount of a fast-running band appeared. At 20 minutes (Figure 14 Gel 3) the appearance of this new series and also of the fast-running band was more pronounced. Also the multiple "gaussian" bands began to appear. After 24 hours this "new series" was no longer present while the fast-running band remained and a new slow-running diffuse region was present (Figure 14 Gel 4).

Starch-Urea Gel Analyses of the Reaction Products

Improved resolution of the CNBr fragments in pH4 starch gels relative to the separation in acrylamde gels was obtained, partly because of the greater length of the slab gels and partly because of the different relative mobilities of the polypeptides at the lower pH. CNBr Hp 1-1 (Figure 15 slot 3) showed a dark-staining fast-running band not present in the other haptoglobins (Figure 15 slots 4,5). The latter appeared to have a greater proportion of slower-running bands. After reduction and alkylation, the CNBr Hp 1-1 produced the α^1 haptoglobin chain (Figure 15 slot 6) while the CNBr Hp 2-1 produced both α^1 and α^2 chains (Figure 15 slot 7), and CNBr Hp 2-2 produced the α^2 chain (Figure 15 slot 8). The prediction that CNBr would not attack the α chains of haptoglobin was thus confirmed.

Figure 14 Acrylamide disc gel analysis of the reaction of CNBr with Hp 2-1 as a function of time. Figure 14a shows the results of electrophoresis in a 4% gel at 300 v for 150 minutes using Tris-glycine buffer. Figure 14b shows an analysis of the same samples using 7.5% gels at about 300 v for 85 minutes. For the 7.5% gels a good separation was achieved by running the electrophoresis twice as long as it takes a marker of bromophenol blue to migrate from the top to the bottom of the gel. Gels 1a 1b, 2a 2b, 3a 3b, 4a 4b, show the results after reaction times of 0 minutes, 5 minutes, 20 minutes, and 24 hours respectively. Gel length 7 cm.

14a - 4% gel



14b - 7.5% gel

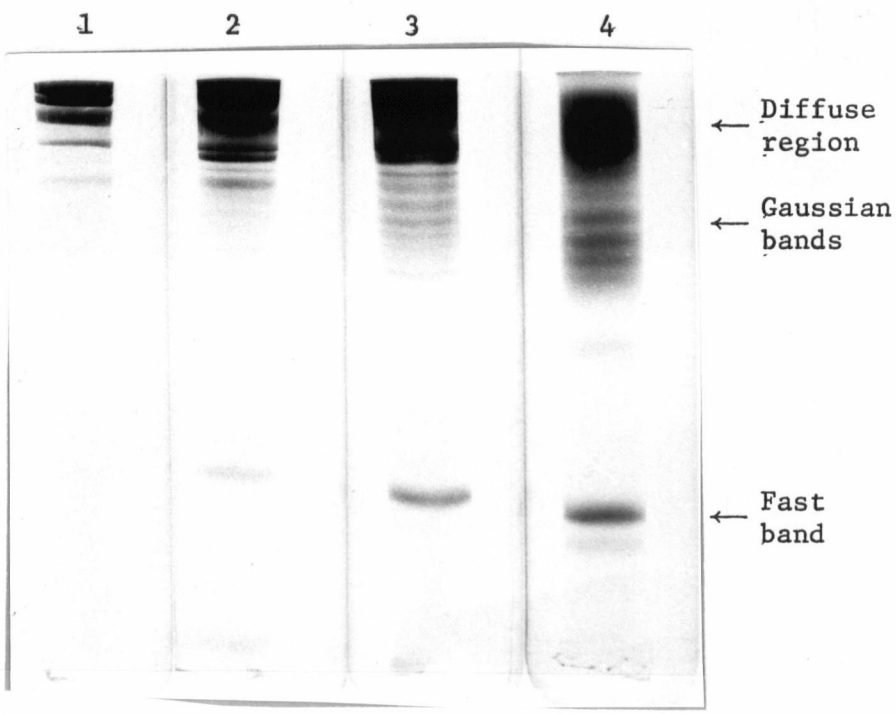
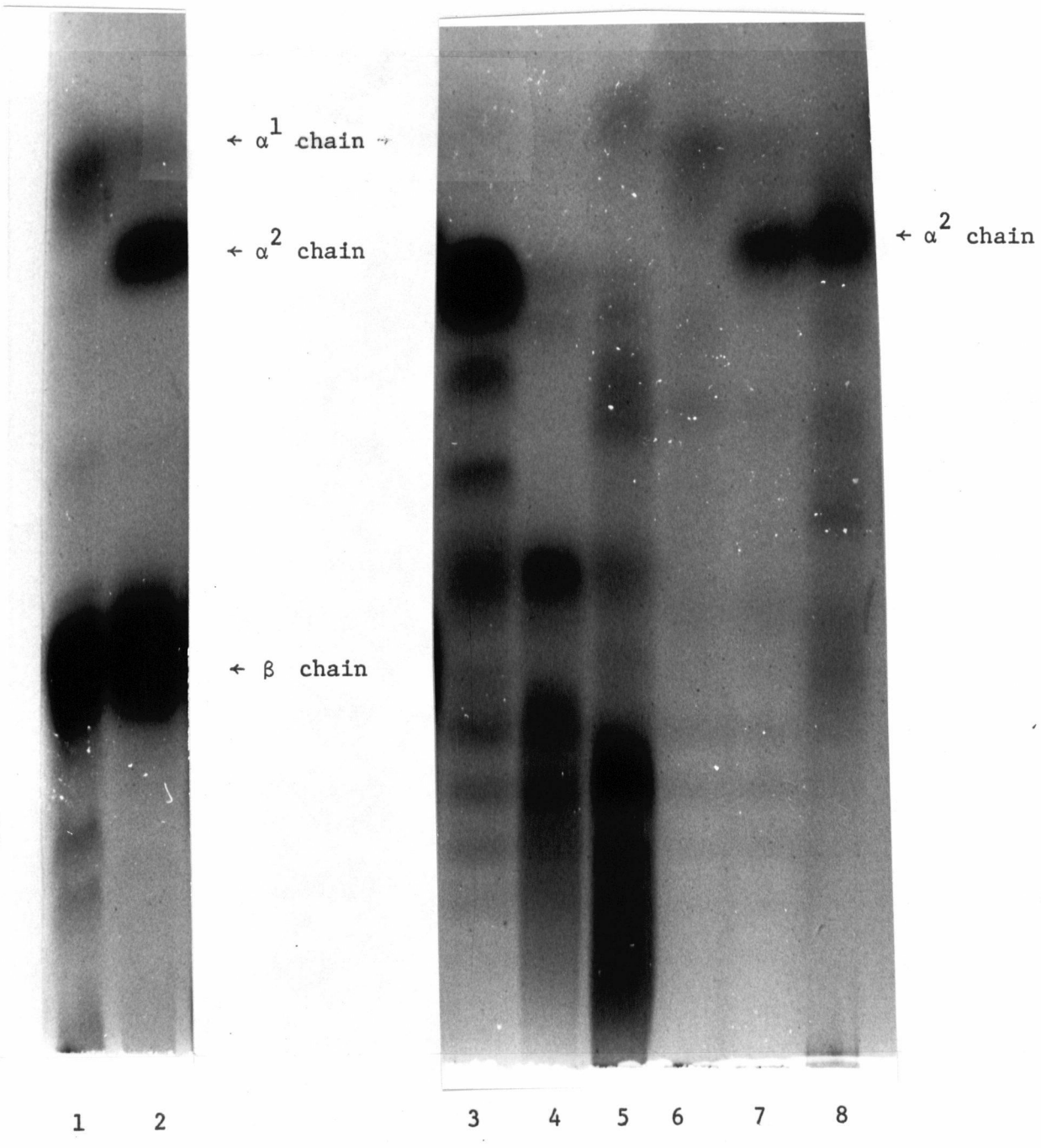


Figure 15 Analysis of the CNBr Hp reactions using starch gel electrophoresis in 8M urea-formate buffer pH 4.0. Gels 1 and 2 show haptoglobins 1-1, and 2-1 respectively which have been reduced and alkylated. Gels 3, 4, and 5 show the results of cyanogen bromide reaction with haptoglobins 1-1, 2-1, and 2-2 respectively. Gels 6, 7, and 8 show the CNBr reaction products of 1-1, 2-1, and 2-2 respectively after they have been reduced and alkylated. Protein concentrations were;

samples 1 and 3, 6 to 8, 2%

samples 3 to 5, 3%

Samples 6 to 8 were reduced with 0.04M β-mercaptoethanol for 30 minutes and alkylated with 0.1M iodoacetamide.


← α1 chain →
← α2 chain
← β chain
← α2 chain
1
2
3
4
5
6
7
8

In the hope of locating which band(s) contained the α chains, two-dimensional starch gel electrophoresis was performed. As shown in Figure 15 (page 70) a series of bands was resolved in the CNBr haptoglobins and, after reduction and alkylation, haptoglobin α chains were obtained. If a slice of gel containing these CNBr-produced bands was removed and transferred to another gel containing mercaptoethanol with subsequent electrophoresis at right angles to the first direction, then any bands which contained the α chains should be reduced by the mercaptoethanol in the second gel and α chains should appear. By the use of a haptoglobin marker in the second dimension it should be possible to ascertain which bands were α chain bands and thus which band in the first dimension contained the haptoglobin α chains. All of the polypeptides which contain no disulphides should retain the same mobility in the second dimension as in the first. The method is a diagonal technique in which disulphide-containing polypeptides will run off the diagonal.

In the case of CNBr haptoglobin 1-1, a peptide running off the diagonal with the same mobility as the α^1 chain (Figure 16) was observed. This α^1 chain was produced by the fast-running band in CNBr Hp 1-1 which did not appear in the other haptoglobins. Using haptoglobin 2-2 (Figure 17) a series of α^2 chains running off the diagonal was obtained. Thus a polymeric series of bands, with each band in the series containing an α chain, appeared to be present in haptoglobin 2-2 after cyanogen bromide cleavage of this polymeric haptoglobin.

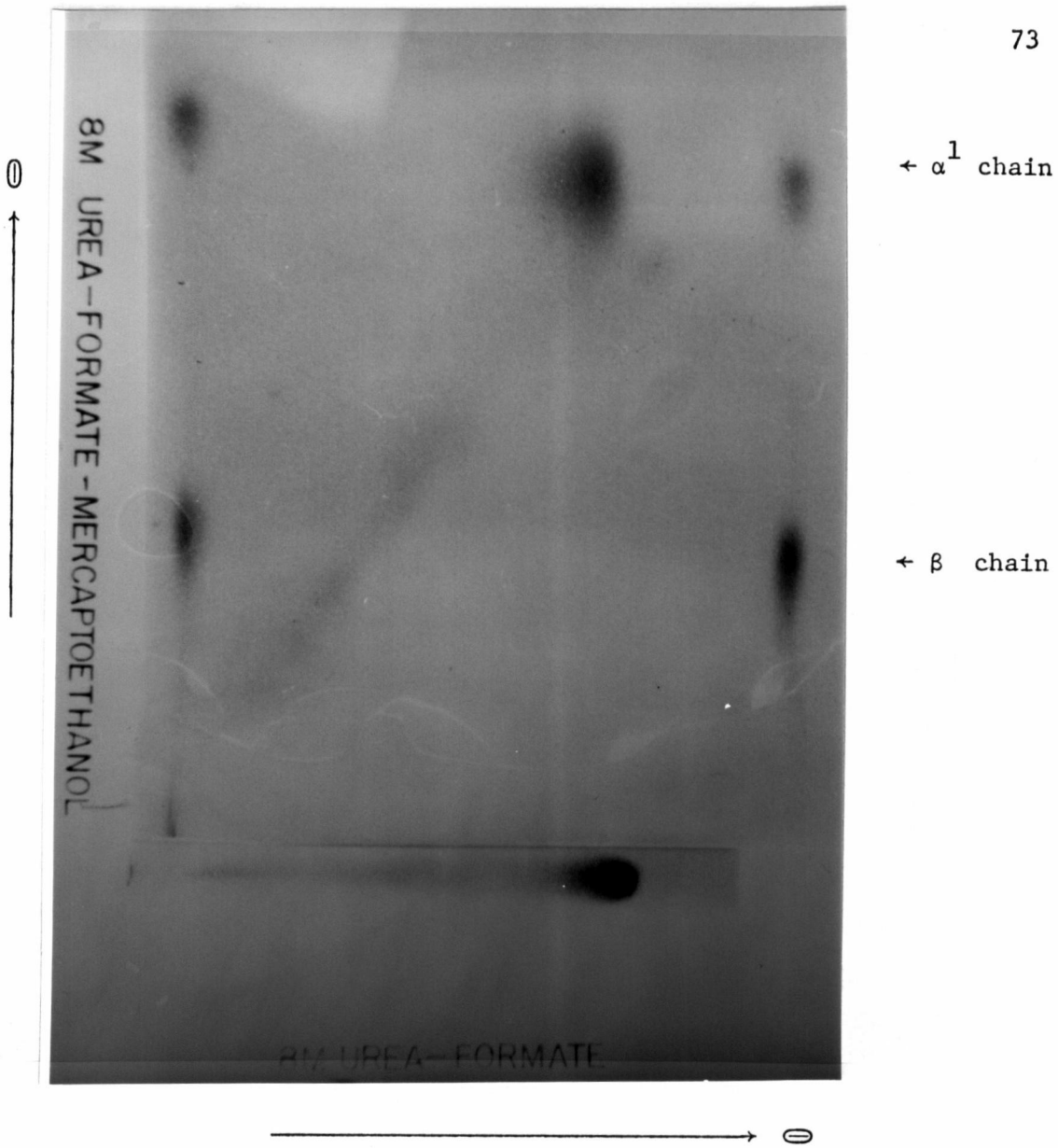


Figure 16 Two dimensional urea-formate electrophoresis of CNBr Hp 1-1. In the second dimension the gel contains 0.1M mercaptoethanol.

Figure 17 Two dimensional starch gel analysis of CNBr Hp 2-2. In the first dimension the gel contains aluminum lactate buffer and 8M urea. In the second dimension the gel contains formate buffer, 8M urea and 0.2M mercaptoethanol.

8M Urea-Formate-Mercaptoethanol

⊖

← α^2 chain

← β chain

⊕

8M Urea-Aluminum Lactate

Studies of the Reaction of CNBr with the Half-Haptoglobin Molecule

As discussed previously, half-haptoglobin (1/2 Hp) can be formed by limited cleavage of haptoglobin with sodium sulphite and p-chloromercurisulphonate and appears to result from the scission of a limited number of disulphide bonds as sulphite is known to cleave disulphide bonds. Thus the half-haptoglobin is a useful derivative for the study of the particular disulphide(s) binding the two halves of the haptoglobin molecule.

Figure 18 shows the results of a starch-urea analysis of the products obtained after haptoglobin was cleaved with sulphite and p-chloromercurisulphonate (pCMS) according to the method of Rorstad and Dixon (96). In agreement with the previous results, the product of the reaction (half-haptoglobin) is seen to migrate more rapidly in the gels than haptoglobin (Figure 18a).

The nature of the splitting of haptoglobin by sodium sulphite and pCMS has been examined by further cleaving the half-haptoglobin with cyanogen bromide in 70% formic acid. Half-haptoglobin produced by the sulphite-pCMS reaction with haptoglobin must have either a sulphite group or a p-mercurisulphonate group attached to one of its cysteines. The stability of these two groups when attached to cysteines in proteins has not been studied extensively. However, the S-sulphocysteine group in S-sulphokeratin is stable from pH 1 to pH 9 (108).

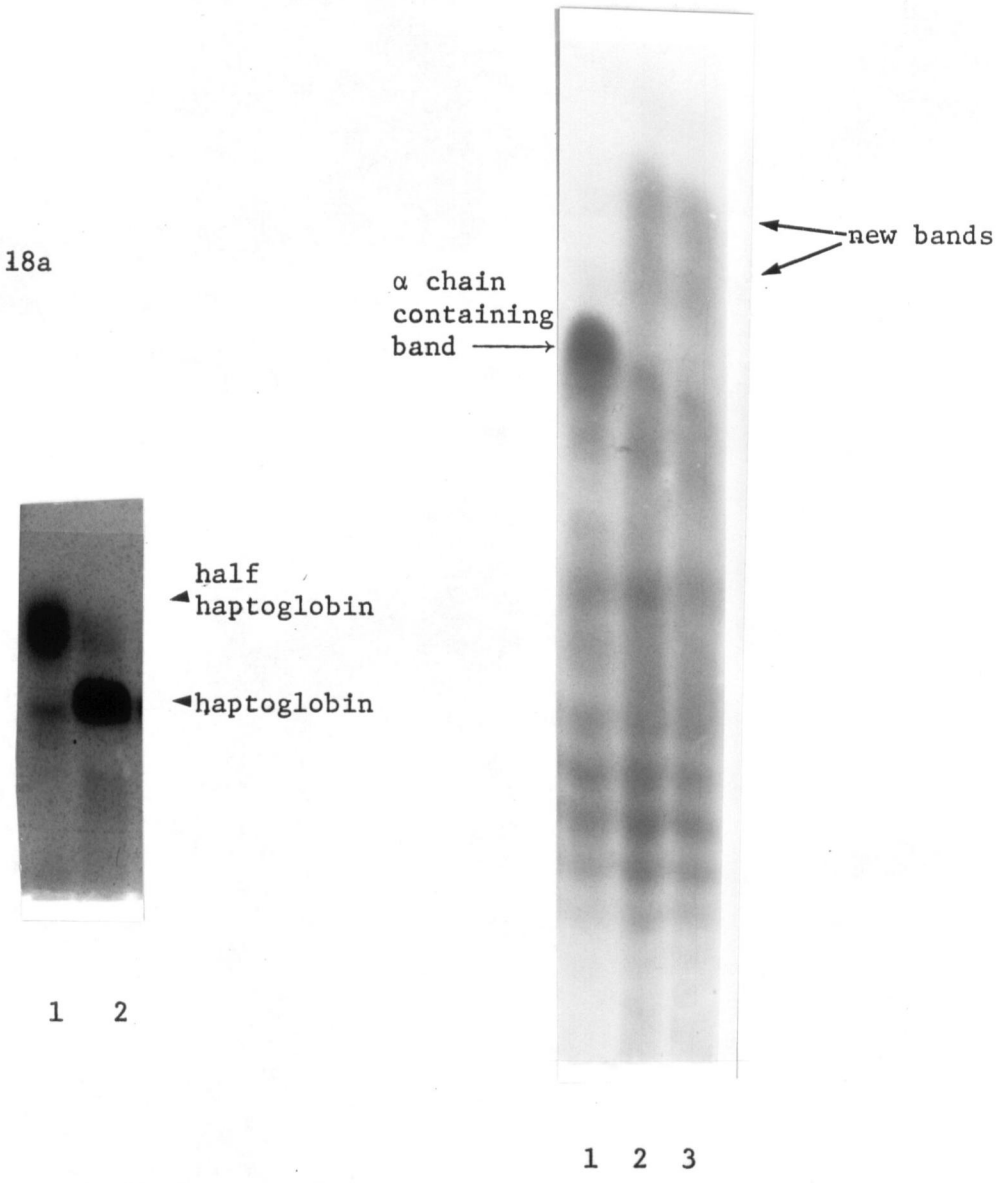


Figure 18 a) Demonstration of the formation of Hp/2 by starch-urea gel electrophoresis. Slot 1, half-haptoglobin; slot 2, haptoglobin.

b) Comparison of the reaction products of Hp 1-1 and Hp /2 with CNBr by starch gel electrophoresis in formate-urea. Slot 1, CNBr haptoglobin; slots 2 and 3, CNBr half-haptoglobin.

Figure 18b compares the peptides produced by the reaction of cyanogen bromide with half-Hp 1-1 (slots 2 and 3) with the peptides produced by the reaction with Hp 1-1 (slot 1). All of the fragments in the haptoglobin 1-1 reaction mixture appeared to be present in the half-haptoglobin mixture. However, the fast-running, dark-staining band appeared to be much fainter in the half-haptoglobin reaction mixture than in the Hp 1-1 reaction mixture. In addition two new very fast-running bands appeared in the CNBr-split half-haptoglobin slot which were not present in the CNBr-split Hp 1-1 slot. The reaction of CNBr with half-haptoglobin was terminated after 10 hours whereas that with haptoglobin 1-1 was terminated after 18 hours. However, it is not likely that this difference in the duration of the reactions would account for the differences in gel patterns. The difference in patterns between CNBr-split Hp 1-1 and CNBr-split half-haptoglobin must have been caused by the previous splitting of haptoglobin 1-1 by sodium sulphite and pCMS. This prediction has been confirmed by gel analysis of a 20-hour CNBr half-haptoglobin reaction.

The fast-running dark-staining peptide, virtually absent in CNBr-split half-haptoglobin, corresponded to the CNBr fragment shown by two-dimensional gel electrophoresis to contain the α chain of haptoglobin. Also, studies of the purified CNBr fragment PC III (see section on purification and properties of fragments, p. 80) confirmed that this fast-running dark-staining peptide did contain the α chain. Thus the α chain-containing peptide in CNBr-split Hp 1-1 was almost absent in CNBr-split half-haptoglobin. However, since the half-molecule of

haptoglobin still contained the α chains and since only two new bands appeared in the CNBr-split half-haptoglobin, it follows that these new fast-running bands in the half-haptoglobin must contain the α chains.

Half-haptoglobin consists of two essentially identical species differing by only the presence of either a S-sulpho-cysteine or p-mercurisulphonate mercaptide in the molecule. Treatment of the two essentially identical half-haptoglobins with CNBr, should produce two altered peptides, one containing a S-sulpho-cysteine and the other a p-mercurisulphonate mercaptide, which previously had formed a symmetrical bond. These new CNBr fragments would be expected to have molecular weights one-half that of the corresponding fragment from native haptoglobin.

In CNBr-split half-haptoglobin two new bands appeared, one which ran slightly faster than the α chain of haptoglobin and one slightly slower. A possible explanation for the existence of the two new bands in CNBr-split half-haptoglobin is that under the conditions of cyanogen bromide cleavage, the sulphite was hydrolyzed from the S-sulpho-cysteinyl peptide thus removing a negative charge from this peptide. This postulate has been confirmed by Rostad and Dixon (96) using ^{35}S-sulphite-labelled half-haptoglobin. They have shown that 70 to 80% of the radioactivity was released from the half-haptoglobin under the conditions of CNBr cleavage. The slower-running of the two new bands probably has the mercurisulphonate mercaptide group. Since the two new bands derived from half-haptoglobin ran faster than the α chain-containing band derived from haptoglobin, they must be of smaller size because

the introduction of a negative charge by sulphite or mercurisulphonate would decrease rather than increase the mobility of these cationic polypeptides.

Purficiation and Properties of Cyanogen Bromide Fragments

In order to prepare sufficient material for further characterization of the α chain containing fragments from the CNBr-treated haptoglobins, the CNBr reaction mixture was fractionated by ion exchange chromotography on phosphocellulose. Ion exchange was used in preference to gel filtration because the two-dimentional starch gel (Figure 17, p.75) indicated that the alpha chains from Hp 2-1 and 2-2 were still present as a polymeric series and so would not appear as a single peak upon gel filtration but would be eluted as a broad peak or series of peaks. However, it was known that the isoelectric points of the polymers were all similar (4) so that a single peak for these α chain-containing fragments might be obtained upon ion exchange chromatography. Chromatography was performed at pH 4.0 in 8M deionized urea since it was known from the starch gels that all the CNBr fragments were positively charged under these conditions. Phosphocellulose was chosen in preference to carboxymethylcellulose since each phosphoryl group would possess a full negative charge at this pH and would retain high capacity for absorbing cationic proteins.

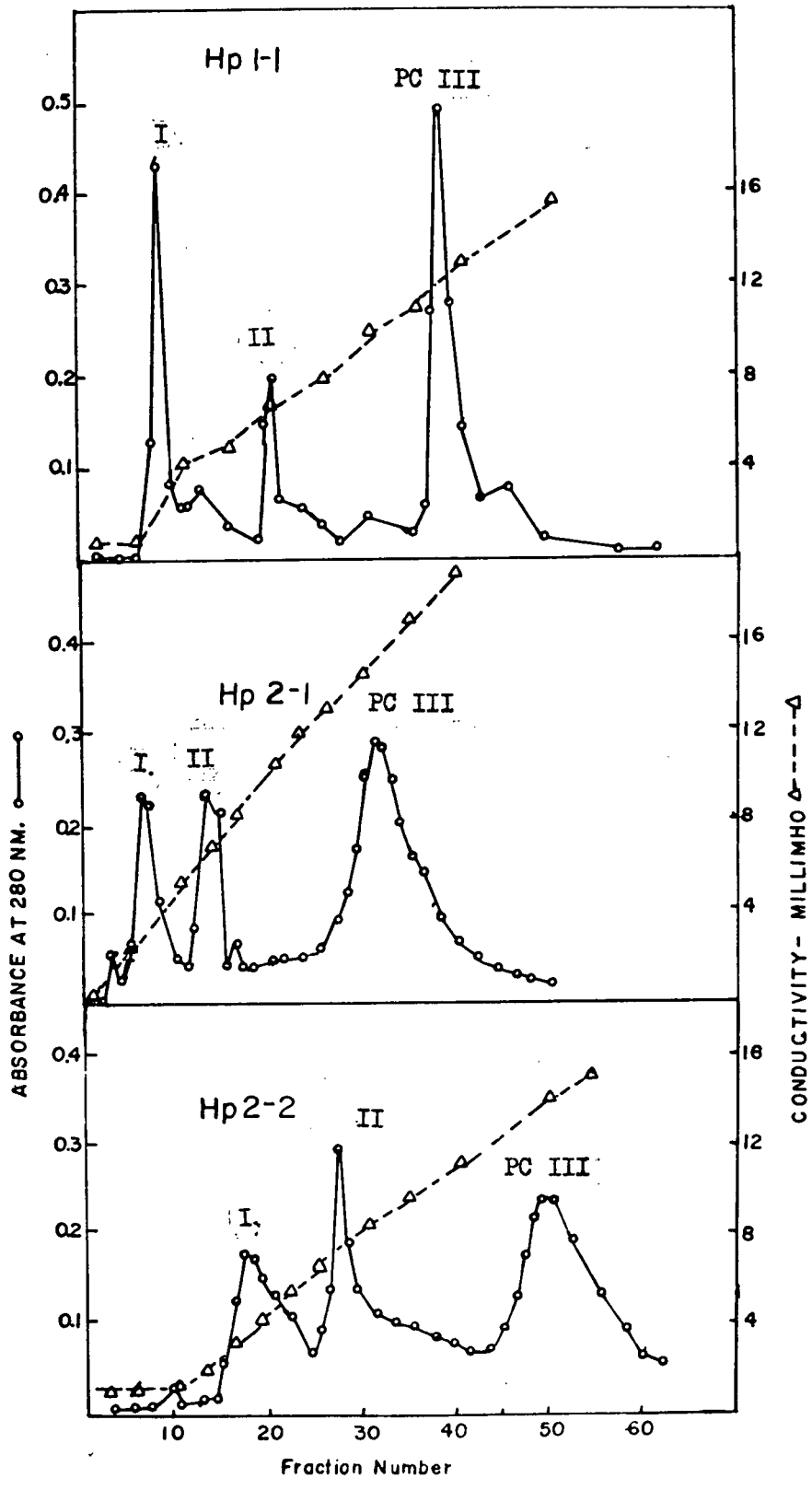
As shown in Figure 19, three peaks were obtained after chromatography of all three haptoglobin types. In the case of haptoglobin 1-1 and haptoglobin 2-2 there was a salt and a pH gradient (Figure 19a,c)

Figure 19 Phosphocellulose (Bio-Rad Laboratories Lot #6049) chromatography of the fragments obtained after the reaction of haptoglobin with cyanogen bromide.

Top Figure — CNBr Hp 1-1; column 0.9 cm; gradient, 500 ml 0.05M formic acid, 0.01N NaOH, 8M deionized urea, pH 4.0 to 500 ml 0.03M formic acid, 0.016M NaOH, 8M urea, 2M NaCl pH 4.6, (3.5 ml fractions); 20 mg of sample were dissolved in 1.0 ml of starting buffer and then applied to the column.

Middle Figure — CNBr Hp 2-1; column 0.9 by 50 cm; gradient 500 ml 0.05M formic acid, 0.01M NaOH, 7.2M urea pH 3.8 to 500 ml 0.05M formic acid, 0.01M NaOH, 7.2M urea 0.8M NaCl. The sample contained 120 mg and 12 to 15 ml fractions were collected.

Bottom Figure — CNBr Hp 2-2; column 0.9 by 25 cm; gradient, 200 ml 0.05M formic acid, 0.01M NaOH, 8M urea, pH 4.0 to 200 ml 0.03M formic acid, 0.016M NaOH, 8M urea, 0.8M NaCl pH 4.6; sample 40 mg. For large scale preparations of cyanogen bromide fragments 600 mg of CNBr 1-1 was chromatographed on a phosphocellulose column 2 cm by 40 cm with a gradient of 1 litre of 0.05M NaCl, 0.05M formic acid, 0.01M NaOH, 7.2M deionized urea pH 3.9 to 1 litre of 0.4M NaCl, 0.05M formic acid, 0.01M NaOH, 7.2M urea.

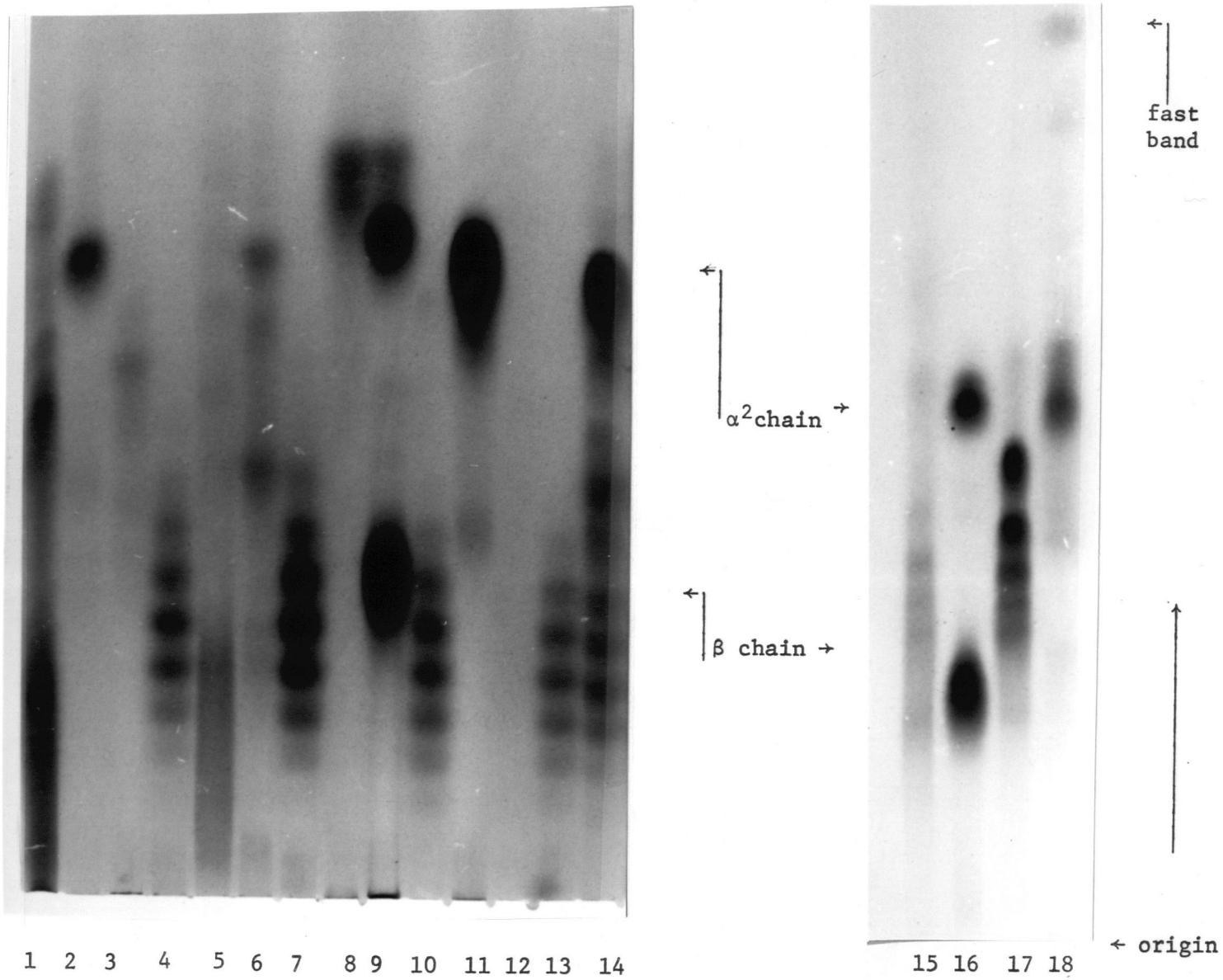

Hp 1-1
PC III
I
II
Hp 2-1
PC III
I
II
Hp2-2
II
PC III
I
ABSORBANCE AT 280 NM.
CONDUCTIVITY- MILLIMHO
Fraction Number
0.5
0.4
0.3
0.2
0.1
16
12
8
4
10
20
30
40
50
60

but it was subsequently found that an equally good separation with Hp 2-1 could be obtained using only a salt gradient. The tubes corresponding to the optical density peaks were pooled, and desalted by chromatography on Sephadex G-25 coarse using 0.2N acetic acid, and then lyophilized.

The purified peptides were then analysed by starch-urea gel electrophoresis in formate buffer. The gel (Figure 20) showed that the first peak eluted from phosphocellulose represented the part of the haptoglobin molecule which ran electrophoretically as a series of bands with a gaussian distribution (Figure 20 slot 13). The gel also showed that these bands were very similar in Hp 1-1 and Hp 2-2 (Figure 20, slots 13 and 7 respectively) and that the mobility of the bands within this series did not change after reduction and alkylation (Figure 20, compare slot 4 with slot 7). A similar series of bands was present in the CNBr-split β chain of haptoglobin as shown by Hew (32). When examined electrophoretically the second peak (PC II) from phosphocellulose showed stained material only in the case of haptoglobin 2-2 (Figure 20, slot 6). The third peak (PC III) in the case of haptoglobin 1-1 consisted of a major band which had a mobility almost the same as the α^2 chain of haptoglobin and a faint minor band (Figure 20, slot 11). After reduction and alkylation this major peptide has a mobility identical with that of the α^1 chain (slot 8). In the case of CNBr Hp 2-2 this same peak (PC III) was a slow-running streaky band (slot 5) which upon reduction and alkylation gave rise to the α^2 chain of haptoglobin (slot 2).

Figure 20 Starch urea gel electrophoresis in formate and aluminum lactate buffers of CNBr peptides after purification on phosphocellulose.

Key:
1. CNBr Hp 2-2
2. CNBr Hp 2-2, PC III, reduced and alkylated
3. CNBr Hp 2-2, PC II, reduced and alkylated
4. CNBr Hp 2-2, PC I, reduced and alkylated
5. CNBr Hp 2-2, PC III
6. CNBr Hp 2-2, PC II
7. CNBr Hp 2-2, PC I
8. CNBr Hp 1-1, PC III, reduced and alkylated
9. Hp 2-1, reduced and alkylated
10. CNBr Hp 1-1, PC I reduced and alkylated
11. CNBr Hp 1-1, PC III
12. CNBr Hp 1-1, PC II
13. CNBr Hp 1-1, PC I
14. CNBr Hp 1-1
15. CNBr Hp 2-2, PC III
16. Hp 2-1, reduced and alkylated
17. CNBr Hp 2-1, PC III
18. CNBr Hp 2-1, PC III, reduced and alkylated


Formate
Aluminum Lactate
fast band
α2chain
β chain
origin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
15 16 17 18

It was expected that another polypeptide in addition to the α chain would be observed after reduction and alkylation of peak III. None was observed. However another polypeptide could have escaped detection if it migrated so rapidly as to move completely through the gel under the electrophoretic conditions or if it washed out of the gel during staining and destaining.

The results of the two-dimensional electrophoresis using aluminum lactate buffer in the first dimension and sodium formate buffer containing mercaptoethanol in the second dimension have shown that the α chains were present in a polymeric series in CNBr Hp 2-2. After fractionation of CNBr 2-2 on phosphocellulose the third peak (PC III) ran as a broad band during electrophoresis. However when the PC III fragments from CNBr Hp 2-1 and CNBr Hp 2-2 were run using aluminum lactate buffer (74) in 8M urea the bands which previously streaked were now resolvable into a polymeric series (compare slots 15 and 17 in the aluminum lactate gel with slot 5 in the formate gel). In fact, the series bears a strong resemblance to the polymeric series of Hp 2-1 and Hp 2-2 except that the bands run faster. This result was confirmed by disc gel electrophoresis in 0.1% SDS. Again a faint series of polymers was observed with phosphocellulose peak III from 2-1 and 2-2 and a single band was observed from 1-1. The polymers were seen to run considerably faster than Hp 2-1 in these disc gels. After reduction and alkylation of PC III from CNBr 2-1 (Figure 20, slot 18) a faint fast-running band appeared which may represent a piece of the β chain attached to the α chain by a disulphide bond.

One problem in the study of disulphide bonds in proteins is the possibility of disulphide interchange which can result in the incorrect assignment of disulphides. The disulphide interchange reaction was first characterized by Ryle and Sanger (109) who found an interchange in strong acid (6N HCl and 10N H_2SO_4) and in weak base pH 8.0 and above. These studies were subsequently extended by Spackman, Stein and Moore who confirmed that the ideal pH for studying disulphides was around pH 2(110). The occurrence of disulphide interchange in actual protein disulphide studies was first observed for insulin (111) and may have contributed to incorrect disulphide assignments for ribonuclease (110) and carboxypeptidase A (112).

The conditions used for the preparation of fragment PC III were reaction in 70% formic acid and chromatography at pH 4.0. Both of these conditions are unlikely to cause disulphide interchange. Also, Edelman (107) has studied the disulphides of a myeloma protein after reaction in 70% formic acid and has obtained no evidence for disulphide interchange. Figure 15 slot 3 and Figure 20 slot 14 show gels of Hp 1-1 after reaction with cyanogen bromide and in both cases that the PC III fragment is one of the major bands. If disulphide interchange had occurred during the reaction with cyanogen bromide it might be expected that the α chain would not be present solely in a major band in PC III but in a series of minor bands. Similarly the PC III fragment produced a major peak after ion exchange chromatography indicating that disulphide exchange had not occurred.

Further Studies on Fragment PC III

Further characterization of fragment PC III was carried out by molecular weight determinations using 7.5% acrylamide gels which contain SDS. The results (Figure 21) showed that fragment PC III (gel 5) definitely had a molecular weight not only greater than the α^2 chain of haptoglobin (gel 3) but also greater than chymotrypsinogen (gel 4). The molecular weight of this PC III fragment from haptoglobin 1-1 was calculated to be near 30,000 by the dodecylsulphate gel technique (104). Fragment PC III must still have two α^1 chains and two pieces of β chain since no disulphides have been split. One can deduce that the molecular weight of the β chain piece is approximately 6,000, i.e.,

$$\frac{(\text{M.W. PC III} - 2 \times \text{M.W. of } \alpha^1)}{2} = \frac{(30{,}000 - 2 \times 9{,}000)}{2} = 6{,}000.$$

Gel 7 shows that in addition to the α^1 chain of haptoglobin a band with approximately the same mobility as insulin (see arrow) is obtained after reduction and alkylation of PC III, thus confirming the approximation of 6,000 for the molecular weight for the β chain piece.

The fragment of the β chain of haptoglobin which is attached to the α chain in the PC III cyanogen bromide fragment has been isolated by gel exclusion chromatography on Sephadex G-75 after reduction and alkylation of PC III. Figure 22 shows that after reduction and alkylation with ^{14}C-iodacetamide two main bands of optical density and radioactivity were seen (peaks 1 and 2) in addition to the peak corresponding to the reagents. A small amount of other material running near the void volume was also observed. A similar separation of peaks 1 and

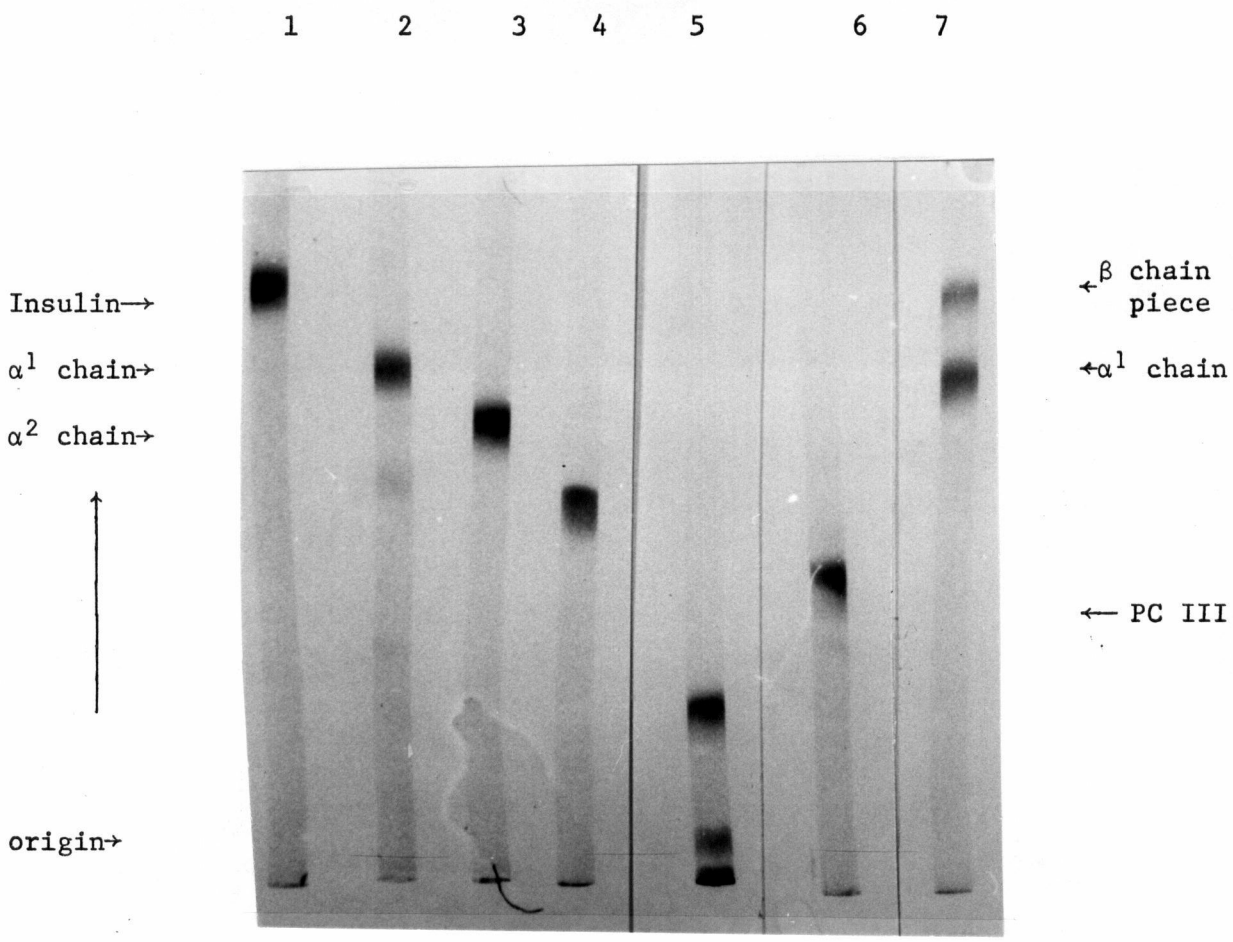


Figure 21 Electrophoresis in 0.1M sodium phosphate pH 7.0, 0.1% SDS. The gels were 10 cm long and contained 7.5% acrylamide, and 0.38% bismethylene acrylamide. The samples were dissolved in 8M urea before electrophoresis. Gels were stained overnight with 1% Amido black in 10% acetic acid. Gel 1, insulin; gel 2, α^1 chain; gel 3, α^2 chain; gel 4, chymotrypsinogen; gel 5, bovine serum albumin; gel 6, CNBr Hp 1-1 PC III; gel 7, gel 6 sample after reduction and alkylation. Electrophoresis was performed at approximately 50 volts until a marker of hemoglobin moved 7.5 cm.

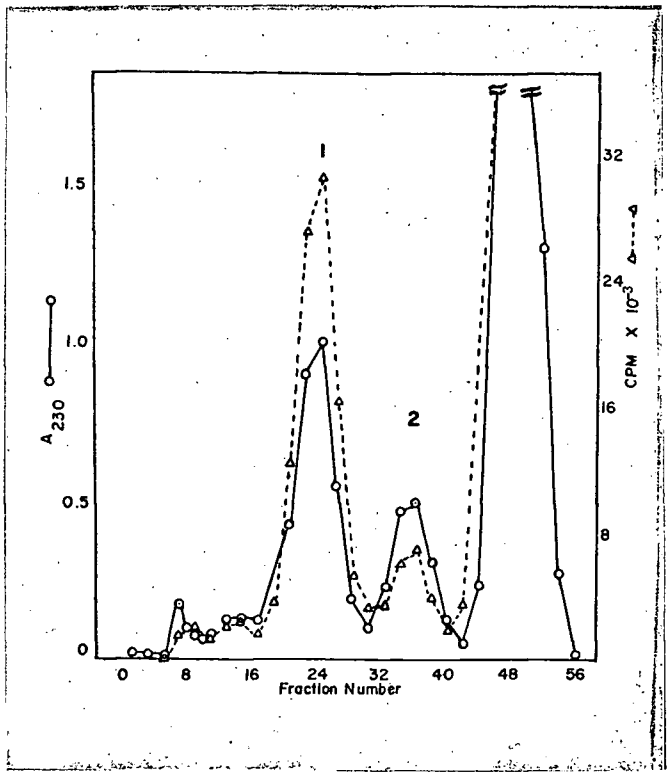


Figure 22 Separation of Fragment III Polypeptides after Reduction and Alkylation

Fragment III (7.0 mg) was dissolved in 100 µl 0.16M boric acid, 0.06M NaOH, 8.0M urea, 0.029M β-mercaptoethanol pH 8.8. After 15 to 20 minutes 20 µl of 0.6M ^{14}C-iodoacetamide (.34 mci/mMole) were added. The pH of the solution was maintained with 0.5M NH_4OH and after 30 to 40 minutes 20 µl of 1.4M β-mercaptoethanol was added and again the pH was maintained for 5 to 10 minutes. The pH was brought down to about pH 2 using 1.0M HCl and a ml of 0.01M HCl was added to the solution. The solution was chromatographed on Sephadex G-75 (2 cm by 65 cm) using 0.01M HCl. Fractions of 3 ml were collected and from these fractions 100 µl aliquots were taken for scintillation counting (see methods).
The PC III sample had been chromatographed 2 times on phosphocellulose.

2 was also obtained by chromatography on Sephadex G-50. The separation on G-50 showed a third peak of optical density which appeared in the void volume. However, gel electrophoresis in 0.1% SDS showed that this peak consisted of three minor bands which were impurities in the PC III preparation.

Gel electrophoresis of peaks 1 and 2 in 0.1% SDS confirmed that these peaks corresponded to the polypeptides which had been observed previously upon gel electrophoresis. The separations were performed in 10% acrylamide gels and it was found more suitable to use 0.25% Coomassie Blue to stain the gels instead of 1% Amido Black (113). As can be seen in Figure 23, peak 1 corresponds to the α chain of haptoglobin and peak 2 corresponds to the fragment of the β chain linked by disulphides to the α chain.

Amino acid analysis of the β chain fragment is shown in Table I. When the number of amino acid residues in the peptide were determined on the basis of the value of carboxymethylcysteine being .95 the values for the other amino acids were close to whole number values with the only two exceptions being those values for homoserine and valine. However in the case of homoserine it is known that hydrolytic losses amount to about 20% (113) and in the case of valine there is incomplete recovery during short times of hydrolysis.

From studies on the reaction of cyanogen bromide with the isolated β chain of haptoglobin, a peptide has been obtained which has a very similar amino acid composition to the β chain fragment obtained from the PC III fragment (Table I) (32). Also, tryptic fingerprints of the two

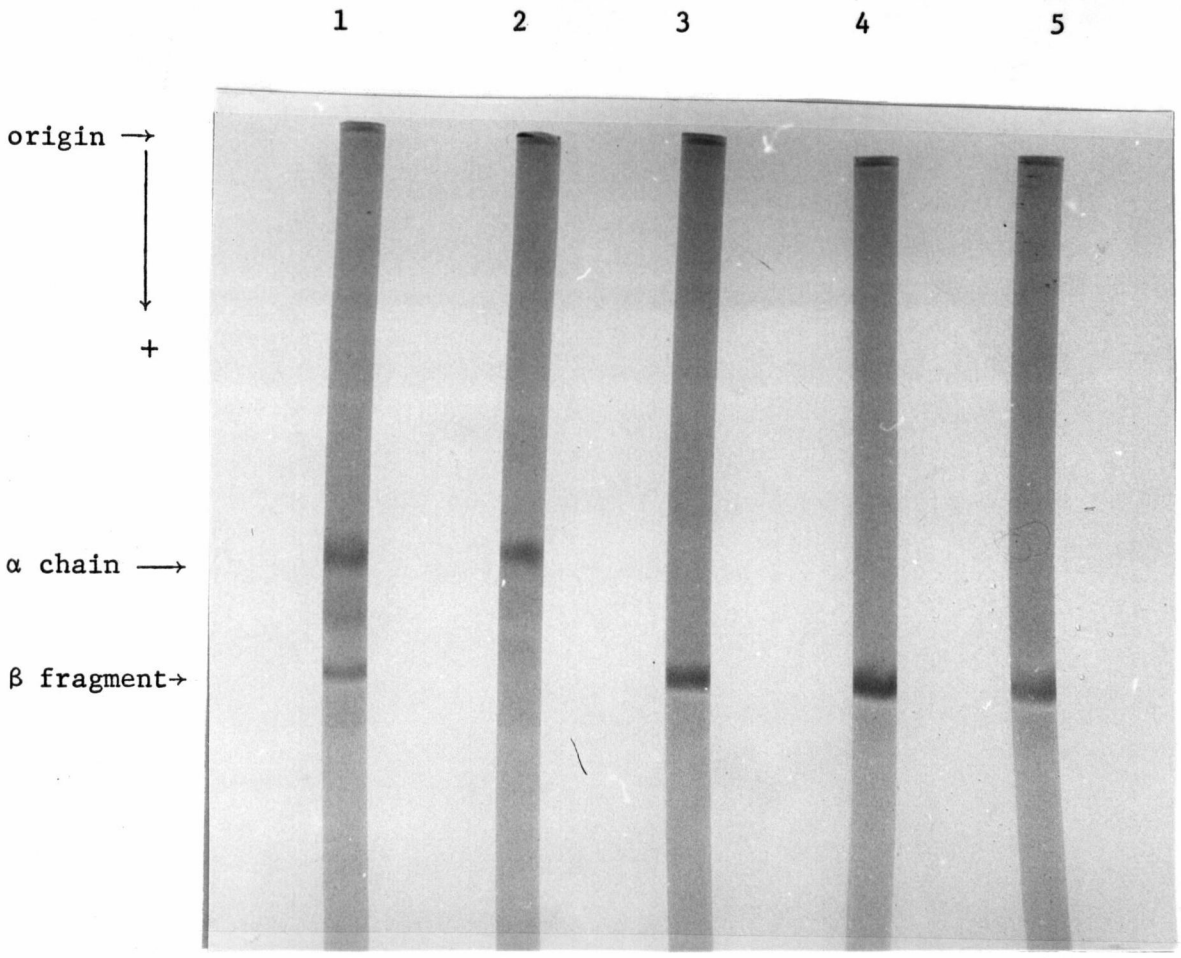


Figure 23 Electrophoresis in 0.1M sodium phosphate pH 7.0, 0.1% SDS. Gels contained 10% acrylamide, 0.5% bismethylene acrylamide and were 10 cm in length. Samples were dissolved in 0.1M borate NaOH buffer pH 8.8 containing 8M urea. Electrophoresis was performed until a marker of bromophenol blue moved approximately 9/10 of the length of the gel. Gel 1, 20 µg PC III reduced and alkylated; gel 2, G50 peak 1 10 µg; gel 3, G50 peak 2, 10 µg; gel 4, G50 peak 2, 20 µg; gel 5 insulin, 10 µg.

TABLE I

AMINO ACID ANALYSIS OF β CHAIN FRAGMENT ISOLATED FROM FRAGMENT PC III AFTER REDUCTION AND ALKYLATION
The results presented are an average of two determinations.

	μM	Residues	Fragment Isolated from β chain by Hew and Dixon
Carboxymethylcysteine	0.0232	.95	-
Aspartic Acid	0.105	4.3	3.5
Threonine	.0253	1.0	.85
Serine	.0492	2.0	1.6
Glutamic Acid	.0305	1.25	1.0
Proline	.0525	2.1	1.8
Glycine	.0985	4.0	3.8
Alanine	.0520	2.1	1.8
Valine	.0828	3.4	3.8
Methionine	.00		
Isoleucine	.024	.97	.85
Leucine	.051	2.1	2.0
Tyrosine	.0726	3.0	2.6
Phenylalanine	.0463	1.9	1.8
Homoserine	.0183	.75	.8
Lysine	-	-	2.5
Histidine	.0292	1.2	.8
Arginine	.0480	1.95	1.6

β chain fragments indicate that these fragments have identical amino acid sequences. The only apparent difference between the amino acid compositions of the fragment obtained from PC III and that obtained from the β chain is the low amount of carboxymethylcysteine present in the fragment obtained from the β chain. However, subsequent analyses of peptides obtained from the β chain fragment (fragment E) showed that, instead of carboxymethylcysteine, cystine was present (32). In fact, the sequence of this fragment has now been completed by Hew, Kauffman and Dixon and is shown in Figure 24. The sequence demonstrates that the fragment contains 4 valines and thus confirms that the hydrolytic conditions used in determining the amino acid analysis resulted in incomplete release of valine.

The sequence and amino acid analysis of fragment E both demonstrate that there is only 1 half-cystine in this peptide. Since this fragment is joined to the α chain by a disulphide bond, fragment E must be joined to the α chain by this particular half-cystine. From the results of Kauffman and Dixon it can be seen that the sequence of the β chain cysteic acid peptide (Th3B) which is joined to the α chain is identical to a sequence present in fragment E. Therefore the fact that peptide Th3B contains a sequence identical to that present in fragment E demonstrates independently that the half-cystine present in Th3B is attached to the α chain.

The presence of a β chain polypeptide in the PC III fragment which was identical in sequence to the fragment isolated by Hew and Dixon (fragment E) was further confirmed by amino-terminal and

Pro-Ile-Cys-Pro-Leu-Ser-Lys-Asp-Tyr-Ala-Glu-Val-Gly-Arg-Val-Gly-Tyr-Val-Ser-Gly-Try-Gly-Arg-Asp-Ala-Asn-Phe-Lys-Phe-Thr-Asp-His-Leu-Lys-Tyr-Val-Hsr

Figure 24 The sequence of fragment E

carboxy-terminal analysis. The results of amino-terminal analysis are shown in Figure 25. In Figure 25a analysis of the dansyl-amino acids shows that from PC III a fluorescent spot with a mobility corresponding to that of either dansyl-valine or dansyl-proline is obtained. However only a small separation of dansyl-valine and dansyl-proline is obtained in this system. A better separation of these two dansyl-amino acids was obtained after thin layer chromatography in a solvent system containing chloroform 90 mls, isoamyl alcohol 10 mls, and acetic acid 0.5 mls (Figure 25b). Figure 25b demonstrates the separation of dansyl-valine from dansyl-proline in this system and shows that fragment PC III contains both proline and valine as amino-terminal amino acids. Since the amino-terminal of the α haptoglobin chain is known to be valine and the amino-terminal of fragment E is known to be proline the result confirms the assignment of the β chain and fragment E as constituents of fragment PC III.

The results of carboxypeptidase A digestion of performic-oxidized PC III are shown in Table II. These results showed that equimolar amounts of glutamine, homoserine, valine, tyrosine, and leucine are released from fragment PC III. Lysine was not determined in this analysis because of the large amount of ammonia which still remained after the ammonia extraction of the released amino acids from Dowex 50. Previous studies on the carboxy-terminal of the α chain of haptoglobin have shown that only glutamine was released by carboxypeptidase A. Thus the remaining amino acids must have been released from the fragment attached to the α chain. The other four amino acids are found at four

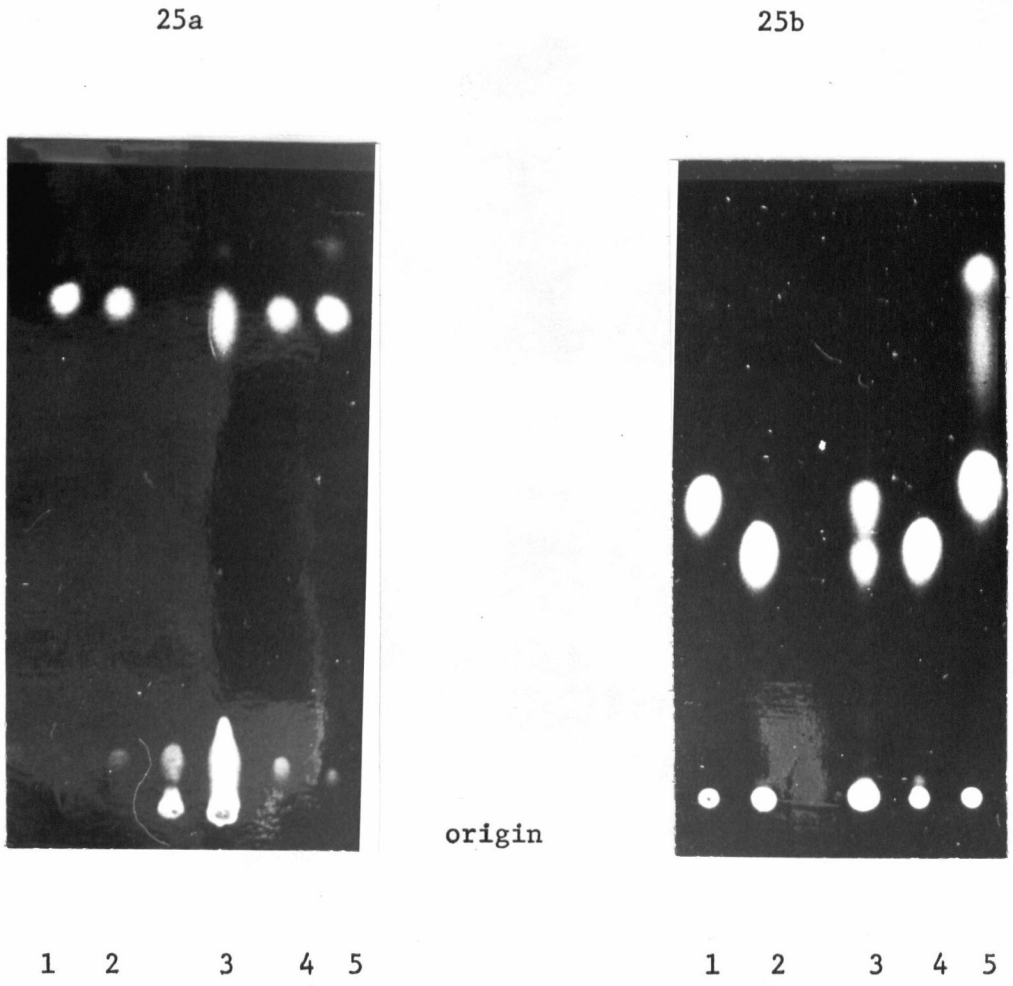


Figure 25 Analysis by thin layer chromatography of the dansyl-amino acids obtained from the amino-terminal residues of fragment PCIII. 1.0 mg of fragment was reacted, hydrolysed, dried and then redissolved in 25 µl of 2N ammonia. 1/5 to 1/8 of this sample was used per spot for analysis.

25a Analysis using the system of Black and Dixon (89). Samples 1 and 5, dansyl-valine; samples 2 and 4, dansyl-proline; sample 3, fragment PC III.

25b Analysis using chloroform 90 ml, isoamyl alcohol 10 ml, and acetic acid 0.5 ml as solvent. Samples are the same as in 25a.

TABLE II

AMINO ACID ANALYSIS OF THE AMINO ACIDS RELEASED BY CARBOXYPEPTIDASE A DIGESTION OF FRAGMENT PC III AFTER THE FRAGMENT HAD BEEN OXIDIZED USING PERFORMIC ACID

	μM	Residues
Homoserine	0.021	1.0
Glutamine	0.023	1.1
Valine	0.018	0.88
Leucine	0.017	0.84
Tyrosine	0.017	0.84

of the five carboxy-terminal positions of fragment E, the other amino acid being lysine which was not determined. Thus the data are consistent with carboxypeptidase removing the first five amino acids from the carboxy-terminal of fragment E and not removing the histidine at the sixth position. This histidine does not appear to be removed because it is next to an aspartic acid (a residue which carboxypeptidase A removes only very slowly) and it is known that the penultimate residue affects the removal of the carboxy-terminal residue (114). Also, although lysine is not normally considered to be released by carboxypeptidase A, it is one of the residues which is released slowly by this enzyme (114).

Diagonal Analyses on Fragment PC III

The disulphides of fragment PC III were investigated following the diagonal technique described by Brown and Hartley (115). Using this technique the polypeptide under investigation is digested with an enzyme and the resulting peptides are separated by high voltage electrophoresis. Then a strip of paper containing the separated peptides is performic-oxidized, sewn onto another sheet of high voltage paper, and then, as described by Brown and Hartley (115), electrophoresis is again performed at the same pH as the first electrophoresis but at right angles to the direction of the first electrophoresis. Using this technique the peptides which do not contain disulphides form a diagonal on the paper and disulphide peptides usually migrate off the diagonal and so can be identified. In addition Brown and

Hartley (115) have shown that this method can be used for isolating and identifying disulphide peptides.

In analysing disulphide-containing proteins or polypeptides the enzyme or enzymes used for digestion is somewhat critical because of two phenomena. On one hand many proteins cannot be digested by some enzymes when their disulphides are intact (110) and on the other hand when digestion is allowed to proceed at slightly alkaline pH disulphide interchange can occur (109). As a result of these problems most proteins are initially digested with pepsin both because it attacks native proteins and because it attacks at low pH where disulphide interchange is not favoured. Then, if necessary, a further digestion with another enzyme has often been used (110,115).

In the present study on the disulphides of fragment PC III diagonal analyses have been performed after pepsin digests, pepsin-trypsin digests, pepsin-trypsin-chymotrypsin digests, pepsin-subtilisin digests, pepsin-thermolysin digests, partial acid hydrolyses, and pepsin-partial acid digests. The digest which appeared to be most suitable for further study was the pepsin-trypsin digest.

The result of a pepsin-trypsin diagonal analysis of PC III from haptoglobin 1-1 is shown in Figure 26. The peptide AA had identical mobilities in the first dimension to the 21 α disulphide peptide obtained by Kauffman and Dixon from a pepsin digest of haptoglobin 1-1 and in the second dimension to the 21 α cysteic acid peptide obtained from haptoglobin 1-1. Also, its identity with the 21 α peptide was confirmed by the fact that it gave a positive histidine reaction with

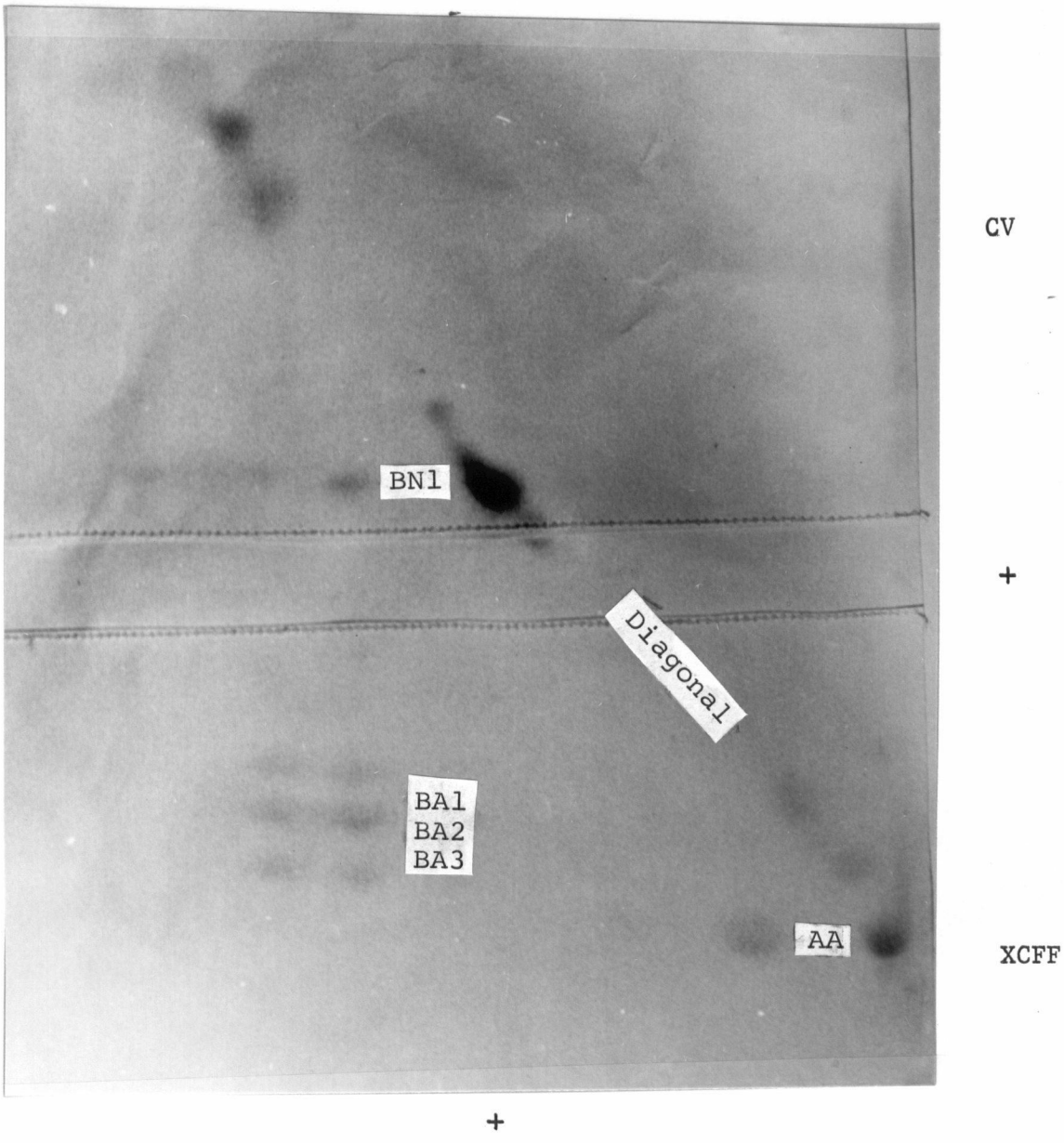


Figure 26 pH 6.5 diagonal map of 1.5 mg of a peptic-tryptic digest of fragment PC III. BA1 is the isatin positive spot. Electrophoresis was performed in the first dimension until a marker of crystal violet moved 13 cm.

the Pauly stain and that it reacted slowly with ninhydrin. Peptide BA1 was found to stain blue with isatin reagent thus demonstrating that it contained proline as amino-terminal amino acid. It was suspected that peptide BA1 came from the amino-terminal of fragment E which does have proline at its amino-terminal. Also it was reasoned that this proline-amino-terminal peptide would contain half-cystine and thus would run off the diagonal since it is only two residues removed from the only half-cystine residue which is present in fragment E.

Peptides BA1, BA2, and BA3 have been isolated from peptic-tryptic digests of fragment PC III in sufficient amount and purity for their position in the haptoglobin sequence to be established. The amino acid analyses and amino-terminal amino acids of these peptides are shown in Table III. Peptide BA1 as expected corresponded to the amino-terminal peptide from fragment E. This peptide would have a net charge of -1 at pH 6.5 and since it contains one cysteic acid, its charge must have been zero before oxidation. Peptides BA2 and BA3 are derived from a part of the α chain corresponding to residues 61 to 77 and 64 to 77 respectively. These two peptides have a net charge of -2 at pH 6.5 and since they contain two cysteic acids their charge must also have been zero before oxidation.

The diagonal map shown in Figure 26 indicates that peptides BA2 and BA3 are both attached by a disulphide to BA1 before performic oxidation because all three peptides had the same electrophoretic mobility before oxidation. To further confirm this point diagonal analysis of peptic-tryptic peptides from PC III was performed at pH 4.0. As shown

TABLE III

AMINO ACID ANALYSES AND AMINO-TERMINAL ANALYSES
OF PEPTIDES BA1, BA2, AND BA3

The peptides were obtained from 25 mg of a peptic-tryptic digest of fragment PC III as described in methods. The peptides were eluted from pre-washed Whatmann 3 MM paper with 30% acetic acid. 22% of each peptide was dried and hydrolyzed for amino acid analysis. Then 2/3 of the hydrolyzate was used for analysis.

	BA1			BA2		BA3	
	μM	μM* corr.	Residues	μM	Residues	μM	Residues
Lysine	.0703	.052	0.9	.113	3.0	.063	3.0
Cysteic	.0534	.046	0.8	.0631	1.7	.0349	1.6
Aspartic	.0800	.073	1.25	.0466	1.25	.0268	1.25
Serine	.0639	.063	1.1	0	0	0	
Glutamic	.0181	.004	0	.0845	2.25	.0502	2.3
Proline	.132	.118	2.0	.0840	2.25	.0470	2.15
Glycine	.0147	0	0	.0808	2.2	.0274	1.25
Alanine	.0129	0	0	.0748	2.0	.0253	1.2
Valine	.0115	0	0	.0726	2.0	.0228	1.05
Isoleucine	.0613	.061	1.0	0	0	0	
Leucine	.0710	.064	1.0	.0416	1.1	.0239	1.1
Amino Terminal	Proline			Alanine		Aspartic Acid	

*The micromolar values of peptide BA1 are corrected for a 10% contamination by peptide BA2.

in Figure 27 the isatin-positive peptide was seen to be mated to three other peptides. The question remained as to whether two of these three peptides were identical with peptides BA2 and BA3. To answer this question a performic-oxidized pH 4.0 electrophorogram of a peptic-tryptic hydrolysate of fragment PC III was run in the second dimension at pH 6.5. Peptides BA1, BA2, and BA3 could be identified and so confirmed that the β chain must be attached either to a half-cystine at position 69 or 73 in the α chain of haptoglobin.

Since peptides BA1, BA2, and BA3 are each present as doublets in the pH 6.5 diagonals they must arise from two disulphide peptides which are separated by electrophoresis in the first dimension. Thus there must be two αβ disulphide peptides present in peptic-tryptic digests of PC III. The diagonal analysis of PC III shows that peptide BN1 is also mated to BA1, BA2, and BA3. If peptide BN1 contains 1 cysteic acid it would have a net charge of +1 before oxidation and since BA1, BA2, and BA3 all have charges of zero before oxidation, therefore the net charge on the disulphide peptide would be +1. This is consistent with the slightly basic mobility of the disulphide peptide containing BN1, BA1 and BA2 or BA3.

Kauffman and Dixon have shown that the β chain of haptoglobin is joined to a half-cystinyl group at position 73 in the α chain of haptoglobin and that the half-cystinyl group at position 69 is joined to position 35. The results of the peptic-tryptic diagonals of fragment PC III are consistent with this structure. Proposed structures for the two αβ disulphide peptides isolated from the peptic-tryptic digests of

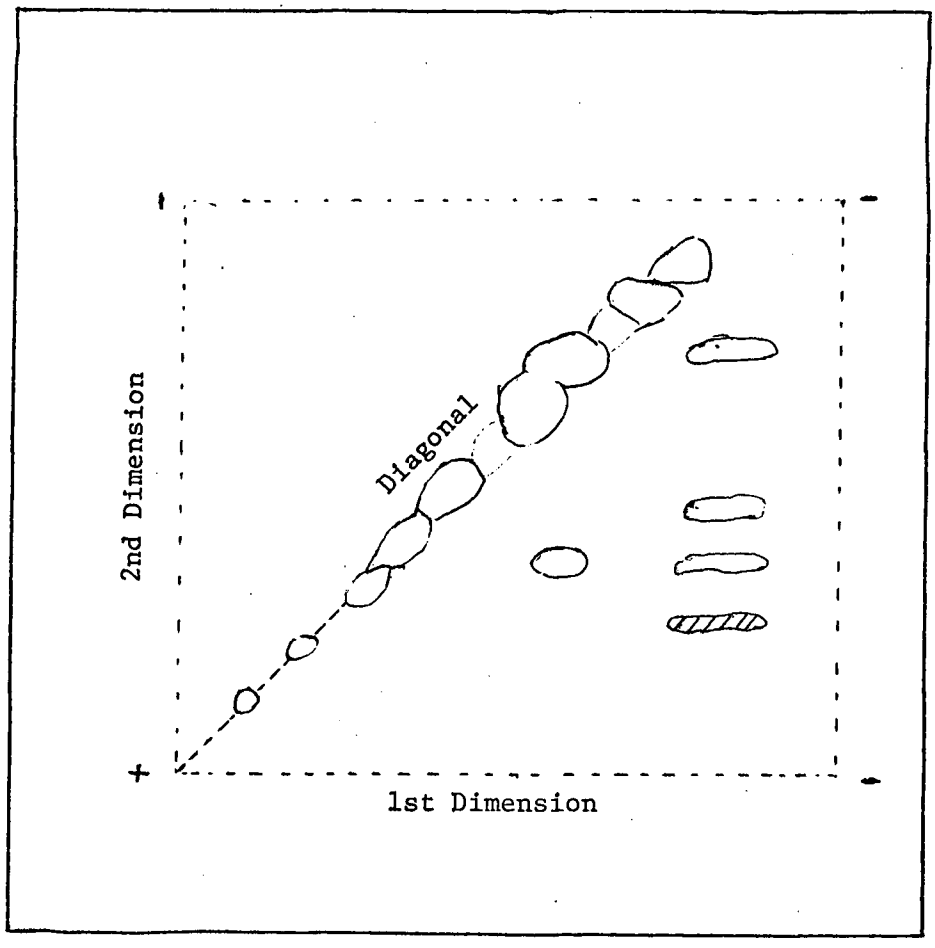


Figure 27 pH 4.0 diagonal map of 2.0 mg of a peptic-tryptic digest of fragment PCIII. The hatched spot is isatin positive. Electrophoresis was performed in the first dimension until a marker of crystal violet moved 13 cm.

fragment PC III are shown in Figure 28. These two disulphide peptides differ only in the structure of one of their component half-cystine peptides (from the α35 region), the other two peptides (from the β chain and from the α69 region) being identical. As the half-cystine peptides from the α35 region are presumed to have different charges the presence of two disulphide peptides before oxidation is explained.

Further Studies on Half Haptoglobin

The results of Kauffman and Dixon (35) suggested that the two halves of the haptoglobin molecule are linked together by a symmetrical disulphide bond located at the half-cystinyl residue 21 of the haptoglobin α chain. Thus one would predict that when haptoglobin is split into symmetrical halves by sodium sulphite and pCMS that the α21 disulphide would be split and thus the α21 half-cystinyl group would be present in the half-haptoglobin as a mixture of S-sulfocysteine and the p-mercurisulphonate mercaptide derivative. This prediction has been examined by analysing the peptic peptides produced when haptoglobin which had been split by ^{35}S-sodium sulphite and pCMS, was digested with pepsin.

Figure 29 shows the results of an autoradiogram of a peptic digest of ^{35}S-labelled half-haptoglobin 1-1 after high voltage electrophoresis at pH 6.5. One major acidic radioactive band is obtained from the peptic digest. Previous results (35) had shown that (among the disulphide peptides) under the conditions of peptic digestion only the α-α' disulphide peptide was obtained in good yield. Thus this result

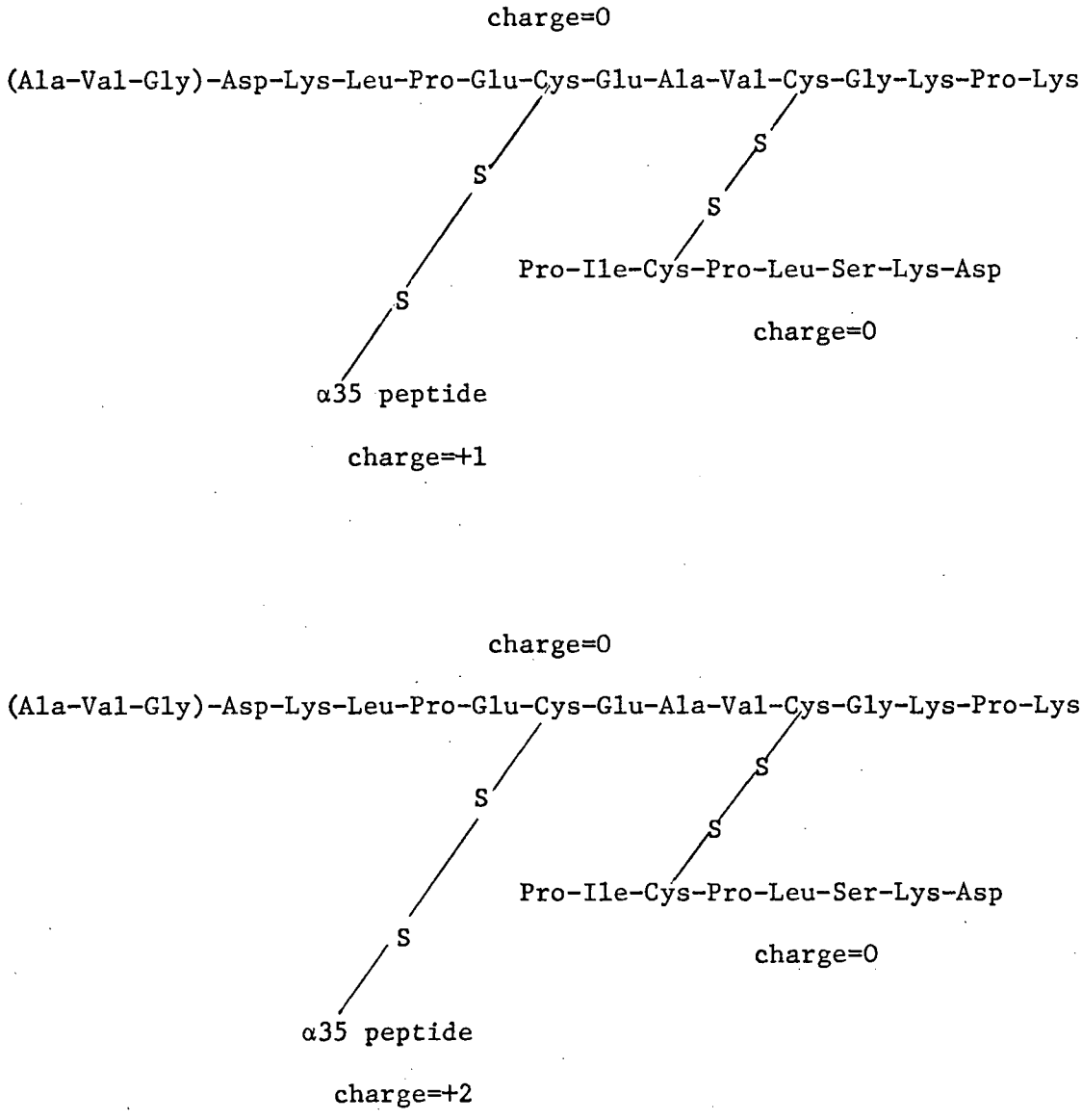


Figure 28 Structure of αβ disulphide peptides obtained from peptic tryptic digests of fragment PCIII.

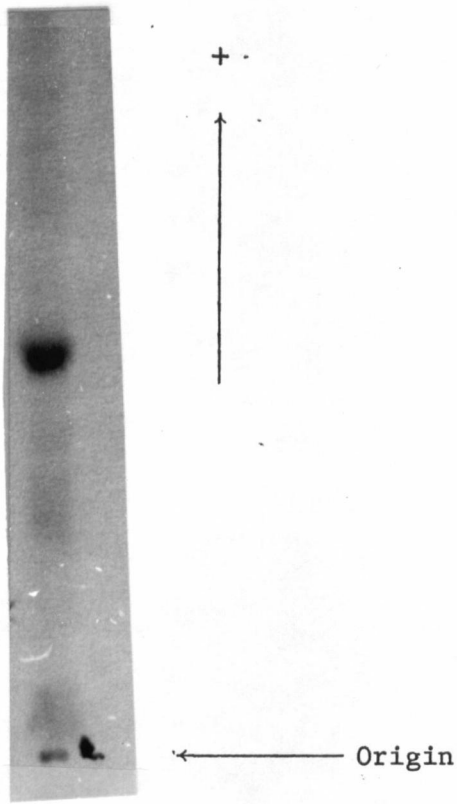


Figure 29 Autoradiogram of the acidic peptides after high voltage electrophoresis at pH 6.5 of a peptic digest of ^{35}S-half-haptoglobin.

suggested that the ^{35}S-peptide was the α chain peptide.

To further characterize this ^{35}S-labelled peptide 40 mgs. of the peptic digest of labelled half-haptoglobin was applied to 20 cm of Whatmann 3 MM paper and electrophoresed at pH 6.5. The position of the radioactive peptide was determined by autoradiography and then the area corresponding to the radioactivity was cut out and sewed onto another piece of 3 MM paper for pH 3.6 high voltage electrophoresis. In order to obtain pure peptide, electrophoresis was performed first at pH 6.5 then at pH 3.6 and finally at pH 1.9.

This peptide at pH 6.5 had exactly the same mobility as the dye XCFF and thus XCFF was a useful marker for locating the peptide at this pH. At pH 3.6 the peptide ran faster than ε-DNP lysine but slower than crystal violet and at pH 1.9 it had about 2/3 the mobility of crystal violet. In order to purify the peptide it was electrophoresed until it had run 27 cm at pH 6.5. Some minor slower running radioactive bands were observed but were very faint. At pH 3.6 two peptides were observed. The minor peptide which consisted of about 1/4 to 1/3 of the amount of the major peptide was not studied further. The major peptide was run until it migrated 16 cm relative to a migration of 10 cm for ε-DNP lysine. At pH 1.9 a single radioactive band was observed which ran 20 cm relative to a mobility of 29 cm for crystal violet. Ten cm of the strip was then cut out and the radioactive peptide eluted chromatographically from the paper with 0.5 ml distilled water. After drying, the peptide was hydrolysed in vacuo for 15 hours at 110^{o} C and 3/4 of the sample was used for amino acid analysis.

Table IV shows a comparison between the amino acid analysis of the ^{35}S-peptide and the amino acid composition of the cysteic acid peptide derived from the peptic 21α-21α' disulphide peptide. The fact that these peptides had the same amino acid composition showed that the ^{35}S-peptide and the 21α-cysteic acid peptide represented the same sequence.

As mentioned previously, when ^{35}S-labelled half-haptoglobin was further cleaved using unlabelled sodium sulphite in the presence of 8M urea and the haptoglobin chains were subsequently separated, the majority of the radioactivity was found in the β chain. This result is not contradictory to the results obtained here since it has been shown that under the conditions of further cleavage with unlabelled sulphite more than 80 per cent of the ^{35}S-sulphite was lost from the half-haptoglobin molecule (96). Thus it can be argued that the ^{35}S label on the α chain was very labile and all of the sulphite was lost from it under the above conditions. The reason why counts were found in the heavy chain region is not known at present but could have been due to exchange of the ^{35}S-sulphite, cleaved from the 21α position, with the five half-cystinyl residues of the β chain.

The fact that the 21α-21α' disulphide was split in the formation of half-haptoglobin has also been demonstrated by diagonal analyses. Figure 30a shows a Pauly-stained, performic-acid-diagonal analysis of haptoglobin which has been digested with pepsin. Only one Pauly-positive spot was off the diagonal. The mobility of this spot in the

TABLE IV

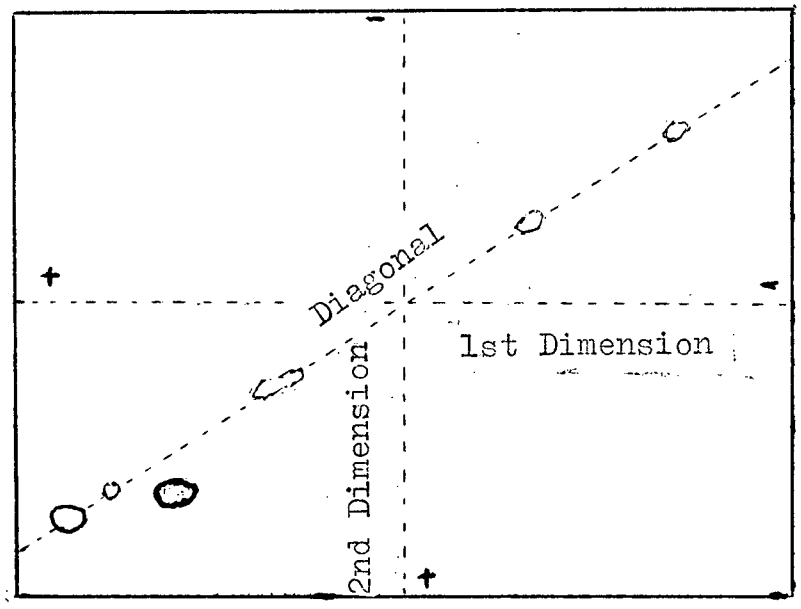
COMPARISON OF THE AMINO ACID COMPOSITION OF THE α-α' DISULPHIDE PEPTIDE OBTAINED AFTER PEPSIN DIGESTION OF Hp 1-1 WITH THE ^{35}S-PEPTIDE OBTAINED AFTER PEPSIN DIGESTION OF ^{35}S-Hp/2

	μ M	^{35}S-Peptide from Half-Haptoglobin	Amino Acid Composition of Cysteic Acid Peptide from 21 -21 ' Disulphide Peptide.* (Kauffman and Dixon).
Aspartic Acid	0.023	2.2	2
Threonine	0.0	0.0	0
Serine	0.001	0.1	0
Glutamic Acid	0.013	1.2	1
Proline	0.031	3.0	3
Glycine	0.023	2.1	2
Alanine	0.021	2.0	2
Valine	0.0	0.0	0
Methionine	0.0	0.0	0
Isoleucine	0.020	1.9	2
Leucine	0.0	0.0	0
Tyrosine	0.0	0.0	0
Phenylalanine	0.0	0.0	0
Lysine	0.0093	0.9	1
Histidine	0.0094	0.9	1
Arginine	0.0	0.0	0

* The amino acid composition was deduced from the sequence of the peptide.

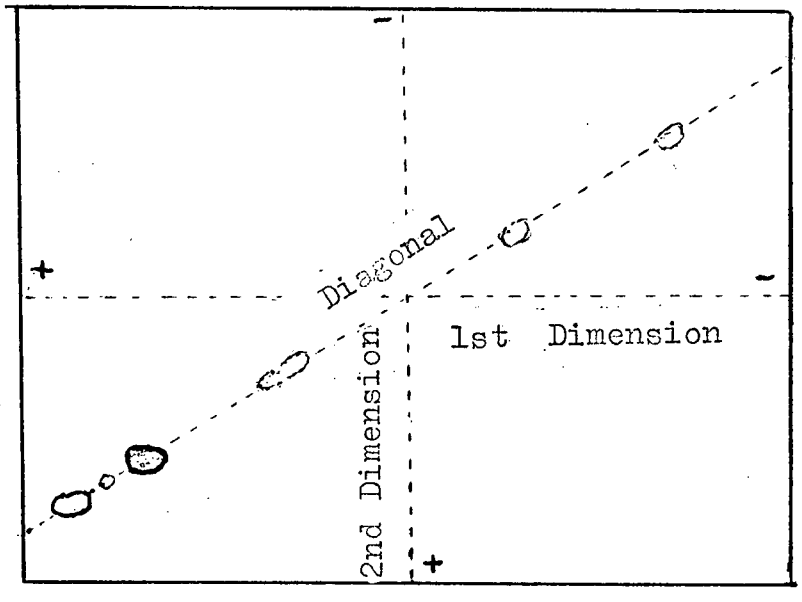
Figure 30 Diagonal map of peptic digests of haptoglobin and half-haptoglobin. Haptoglobin or half-haptoglobin (4 mg) were dissolved in 0.4 mls of 5% formic acid. To this solution 40 µl of a 1% pepsin solution were added and digestion proceeded for 17 hours at 37° C. Then the samples were dried, redissolved in a small volume of pH 6.5 pyridine-acetate buffer, applied to 2.5 cm of Whatmann 3 mM paper, and diagonal analysis was performed as described by Hartley (115). After diagonal analysis the paper was sprayed with Pauly reagent.

HAPTOGLOBIN



a

HALF-HAPTOGLOBIN



b

first dimension was about 1/2 that of the marker XCFF and was equal to the marker in the second dimension. In the second dimension this spot corresponded in mobility to the 21α cysteic-acid-containing peptide isolated by Kauffman and Dixon. It also had the same mobility as the ^{35}S-sulphite-labelled peptide obtained from sulphite-labelled half-haptoglobin. After performic-acid oxidation of the 21α-21α' disulphide peptide the cystinyl moiety which was present in the peptide would be converted to cysteic acid and, after splitting with sulphite, the moiety would be converted to S-sulphocysteine. In both of these cases this sulphur containing amino acid would be negatively charged and so the peptide after oxidation or after splitting with sulphite would have the same change as well as essentially the same size. In the Pauly-stained diagonal of half-haptoglobin (Figure 30) the Pauly-positive band which was off the diagonal in the case of Hp 1-1 is not seen and instead a new Pauly positive spot is seen on the diagonal which has the same mobility as XCFF in both the first and second dimensions. Again the result is consistent with the prediction that the S-sulpho-peptide and the cysteic peptide have the same mobilities. A schematic representation of the comparative diagonals is shown in Figure 31.

The splitting of the 21α disulphide in haptoglobin with the resulting formation of half-haptoglobin can be seen in two different ways. In one case using ^{35}S-labelled sulphite, the peptide corresponding to the sequence around the 21α position has been isolated, and in the other case using comparative diagonals it is seen that the 21α

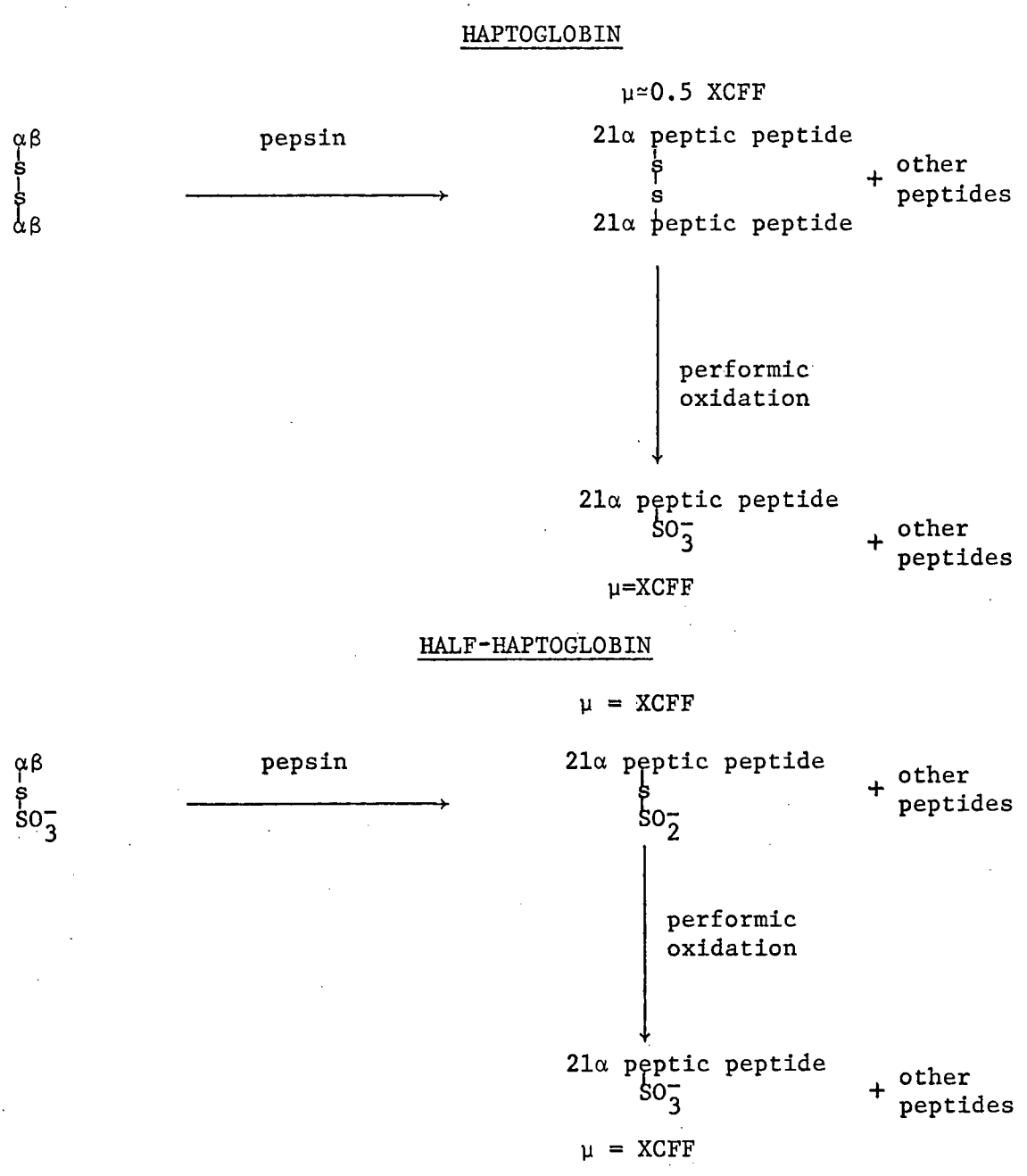


Figure 31 Scheme to explain the comparitive diagonals of haptoglobin and half-haptoglobin.

disulphide peptide is missing in half-haptoglobin but is replaced by another peptide which has the properties of a 21α-peptide in the S-sulphocysteinyl form. In addition it was also shown previously that a comparison of the cyanogen bromide fragments of haptoglobin and half-haptoglobin showed that the α chain containing fragment (PC III) was altered in the half-haptoglobin. Since PC III contains a part of the β chain linked by a disulphide to the α chain, and this part of the β chain (fragment E) contains only 1 half-cystine it is apparent that only a α-α' disulphide could have been broken in the conversion of haptoglobin to half-haptoglobin.

Discussion and Conclusions

The haptoglobin 1-1 molecule contains nine disulphide bonds as shown in Figure 32. Six of these disulphides are intrachain disulphides and the other three are interchain. Four of the intrachain disulphides are in the β chains and two are in the α chains connecting the half-cystinyl 35 residues with the half-cystinyl residues at position 69. Of the three interchain disulphides one is a symmetrical interchain disulphide between the half-cystinyl residues at position 21 in the α chain and the others are the αβ linkage disulphides which join the β chains to the α73 half-cystinyl residues.

The existence of only one unique αβ disulphide in haptoglobin has been confirmed by studies on the PC III fragment. From this PC III fragment another fragment (E) has been isolated which is a part of the β chain of haptoglobin. This fragment E which is the only β chain

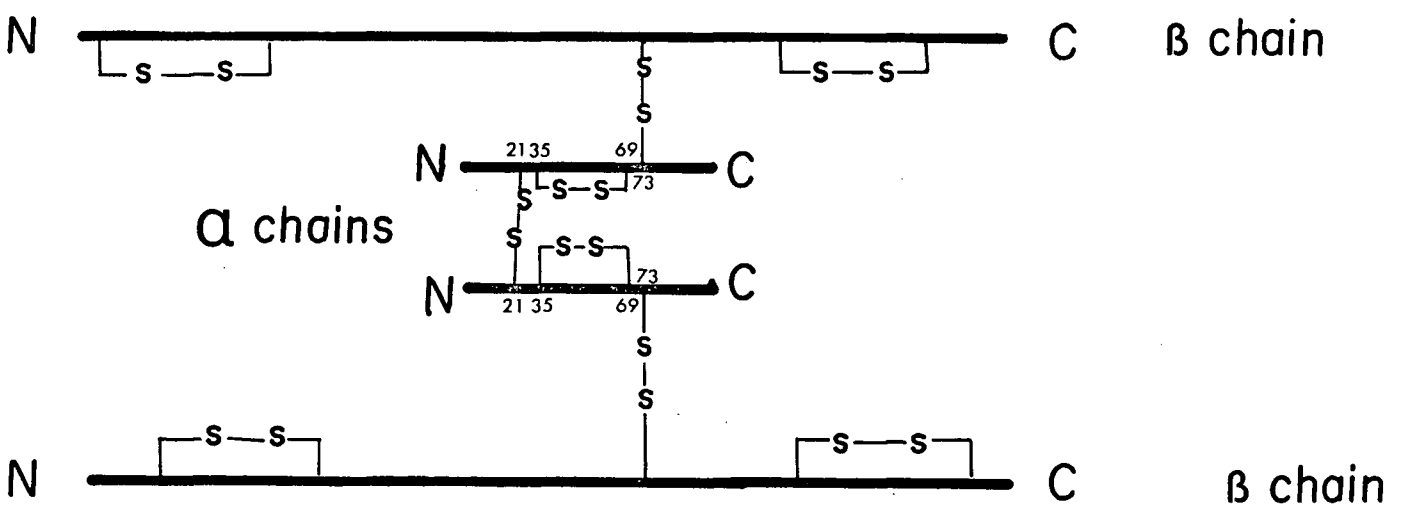


Figure 32 Structure of haptoglobin 1-1 showing the disulphide bonds. The position of all five half-cystines in the ß chain is unknown.

fragment linked to the α chain contains only one half-cystinyl group and so there can only be one disulphide between the α and β chains in haptoglobin. The results of peptic-tryptic digests are consistent with a structure in which there are two half-cystinyl groups at positions 69 and 73 and in which one of these half-cystinyl groups is attached to the β chain.

It is interesting to observe that as a result of the postulated partial gene duplication which gives rise to the 2α chain of haptoglobin, the region of the DNA corresponding to a sequence containing the half-cystine at position 73α has been deleted. Although the half-cystine which would be at 73α if the α chain were completely duplicated has been deleted in the α^2 chain of haptoglobin there is still a linkage of the α chain to the β chain by the half-cystine at position 132 α^2. However, because one of the linkage half-cystines which would be present if the α chain were completely duplicated is missing in α^2, in the case of both the α^1 and α^2 haptoglobin chains, each α chain can be joined to only 1 β chain.

The gammaglobulins are tetrachain molecules with two pairs of identical chains and so structurally resemble the haptoglobins. The disulphides of the γG subclass γG1 have been studied in detail (107, 116,117). It has been shown that γG1 myeloma proteins have two intrachain disulphides in each of the light chains and four in each of the heavy chains. There is only one unique light-heavy disulphide and there are two symmetrical disulphides linking the heavy chains. The disulphides of the gammaglobulins differ markedly between the

subclasses (117) and it has been shown that in subclass γG3 there are five symmetrical interchain disulphides between the heavy chains. Also it has been shown in one antibody subclass γA2 that there is no disulphide linking the light and heavy chains (118).

In comparing the disulphides of γG1 and haptoglobin 1-1 many differences are apparent. In both the light and heavy chains (α and β chains respectively for haptoglobins) there are twice as many intrachain disulphides in γG1 as in Hp 1-1. In addition there are twice as many symmetrical interchain disulphides in γG1 as there are in Hp 1-1 and the symmetrical interchain disulphides attach the heavy chains together in γG1 while they attach the α (light) chains together in Hp 1-1. In both molecules there is one unique interchain disulphide which is located towards the carboxy-terminal end of each of the light chains.

Recently a very interesting study on the refolding of haptoglobin has been performed by Bernini and Borri-Voltattorni (119). These workers have been able to completely reduce haptoglobin with mercaptoethanol in the presence of 8M urea and then by carefully reoxidizing a mixture of α and β chains in the absence of urea they have been to reform haptoglobin. In the case of haptoglobin 1-1 they have been able to obtain a recovery of 85 to 90 per cent. In the case of haptoglobin 2-2 they have been able to reform the haptoglobin polymers with a yield of 60 to 70 per cent. Also these workers have been able to separate the α and β chains of haptoglobin, remix them, and oxidize the remixed chains to form haptoglobin. In this way they have been able to make Hp 2-1 from the chains derived from Hp 1-1 and Hp

2-2. Studies on the reoxidation of the individual chains showed that α^1 chains formed dimers while α^2 formed polymers and β chains did not polymerize.

The results of the Italian workers are completely consistent with those presented here. In the first instance the only symmetrical interchain disulphides that we have found is between α chains. This explains why only α chains form dimers. Similarly the cleavage of this α-α' disulphide breaks down the haptoglobin polymers and it can be seen that the α-α' disulphide alone can be responsible for haptoglobin polymerization. Thus the α^2 chain by itself should be able to polymerize and this has been found by Bernini and Borri-Voltattorni. Finally since there are no β-β' disulphides the β chain should remain monomeric after oxidation as found by Bernini and Borri-Voltattorni.

V

THE INFLUENCE OF HAPTOGLOBIN ON THE REACTIVITY ON THE -SH GROUPS OF HEMOGLOBIN

This section of the thesis examines the reactivity of the β93 cysteinyl residue in free hemoglobin and in the hemoglobin-haptoglobin complex toward three sulphydryl reagents, iodoacetamide, 2,2' dithiodipyridine (2-PDS) and 4,4' dithiodipyridine (4-PDS) (46,94).

Iodoacetamide is one of a group of compounds containing active halogen atoms which are used as reagents for sulphydryl groups. Generally the reaction takes place with the mercaptide ion. The reagents will also react with amino groups but the rate of reaction with sulphydryl groups is much faster. Pyridine disulphides react with sulphydryl groups by the disulphide interchange reaction. The thiopyridone products of the reaction are almost exclusively in the tautomeric thio form and as a result the ultraviolet absorption spectra of the thiopyridones is quite different from the corresponding disulphides.

Pyridine Disulphide and Iodoacetamide Reactions

The dithiodipyridines are especially suited for reaction with hemoglobin since the products of the reaction absorb at 324 or 343 nm (94) where hemoglobin has little absorbance. In contrast, other reagents such as para-hydroxymercuribenzoate (PMB) and Ellman's reagent (121) absorb in regions where hemoglobin also absorbs strongly.

Figure 33 shows a comparison between the reaction of 4-PDS with hemoglobin and its reaction with the Hb-Hp complex in which it can be seen that after complex formation with haptoglobin there was a greater than 90 per cent inhibition of the reaction rate of hemoglobin. As shown in Figure 34, the inhibition, in the case of both 4-PDS and 2-PDS was proportional to the amount of complex formed with a maximum inhibition at a 1 to 1 molar ratio of hemoglobin to haptoglobin. Although the reaction with the Hp-Hb complex was much slower than with free Hb, at a higher concentration of reagents, i.e., 8×10^{-4}M 4-PDS and 9.2×10^{-6}M Hb, reaction with Hp-Hb complex occurred and was essentially complete in 10 minutes.

Similarly, the reaction of iodoacetamide with the complex was slower than that with hemoglobin (Figure 35). The inhibition of the reaction of ^{14}C-iodoacetamide with Hb by Hp was 70 to 80 per cent whether the Hb was in the form of methemoglobin or oxyhemoglobin whereas with the dithiodipyridines the inhibition was greater than 90 per cent. An effect of haptoglobin on the environment of cysteinyl β93 has also been seen (122) by comparing the electron spin resonance (E.S.R.) spectrum of N-(1-oxyl-2,2,5,5,-tetramethyl-3-pyrolidonyl) iodoacetamide-labelled hemoglobin with the labelled Hb-Hp complex

Since the rate of reaction of sulphydryl reagents with the Hb-Hp complex was markedly different from that of Hb, it was necessary to determine the site of reaction on the Hp-Hb complex to be sure that, in fact, the reaction was still with the β93 sulphydryl group. Autoradiography (Figure 36), after complete acid hydrolysis and high

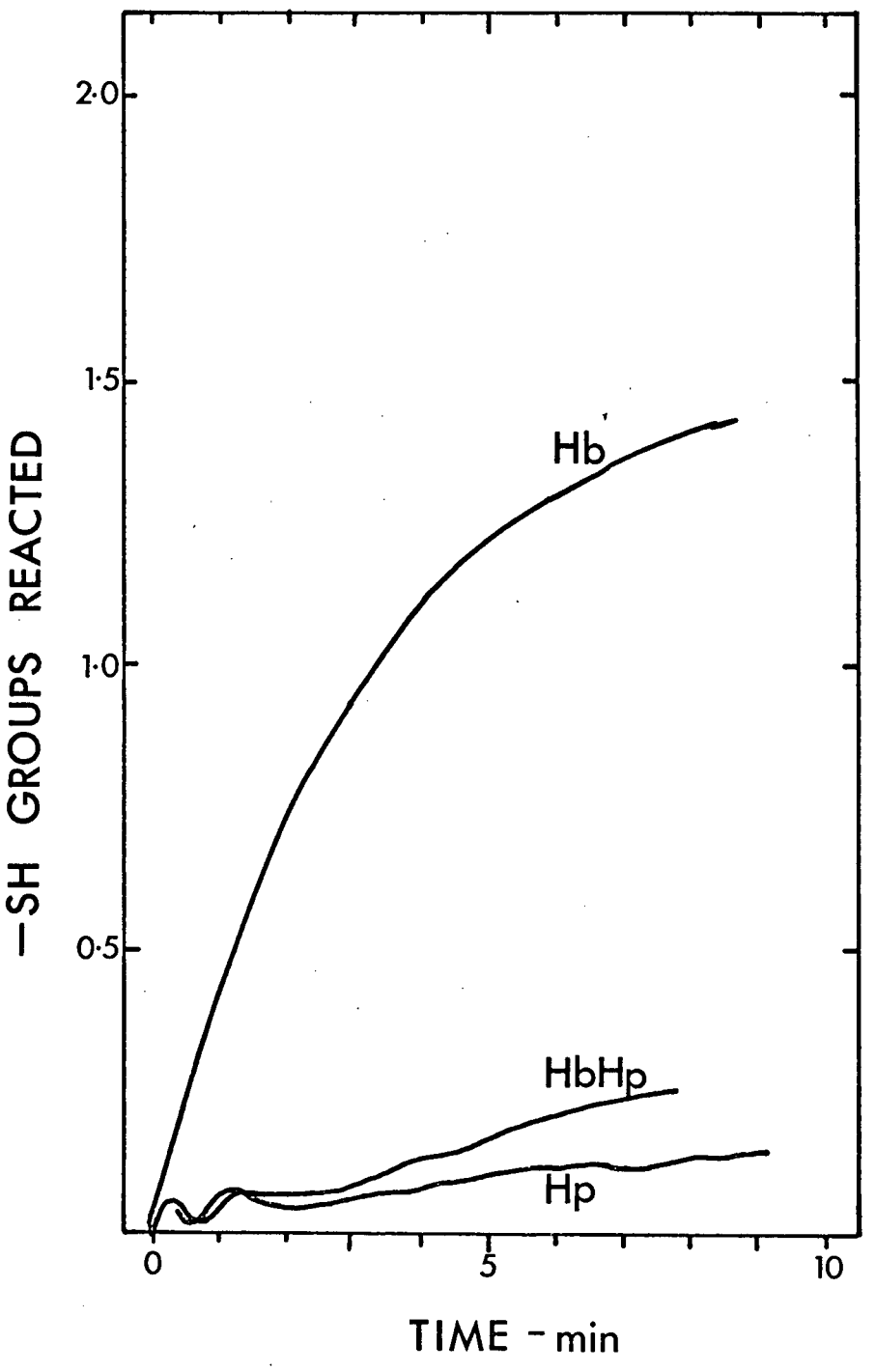


Figure 33 The reaction of 4-PDS with hemoglobin, the hemoglobin-haptoglobin (Hb-Hp) complex, and free haptoglobin. The ordinate represents -SH groups reacted per mole of hemoglobin tetramer. Thus complete reaction would generate 2 moles of thiopyridone per mole of hemoglobin tetramer.

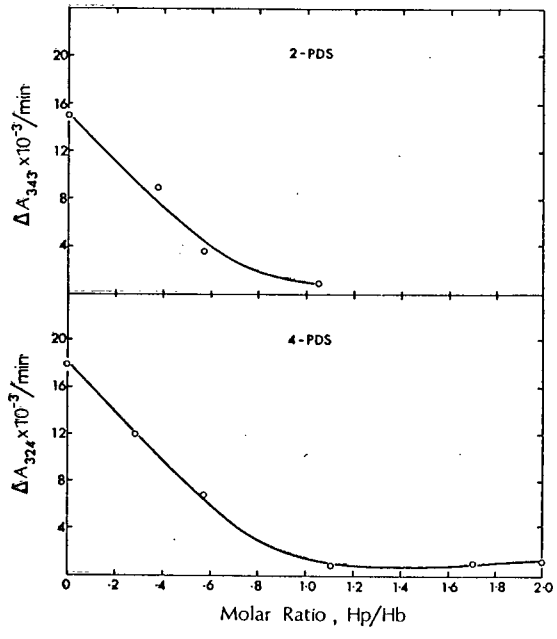


Figure 34 The reaction of 4-PDS and 2-PDS with methemoglobin in the presence of increasing amounts of haptoglobin. The initial velocity of the reaction is plotted against the molar ratio of haptoglobin to hemoglobin.

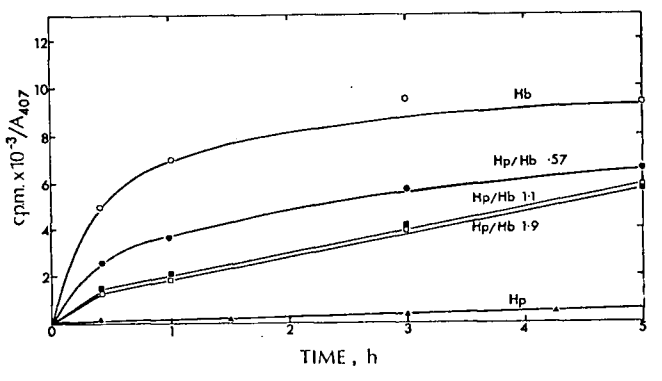


Figure 35 The reaction of ^{14}C-iodoacetamide with hemoglobin and hemoglobin haptoglobin mixtures. For the reaction of ^{14}C-iodoacetamide with free haptoglobin, the rate is plotted per 0.19 absorbance at 280 nm since the absorbance of hemoglobin at 407 nm is 5.2 times that of haptoglobin at 280 nm.

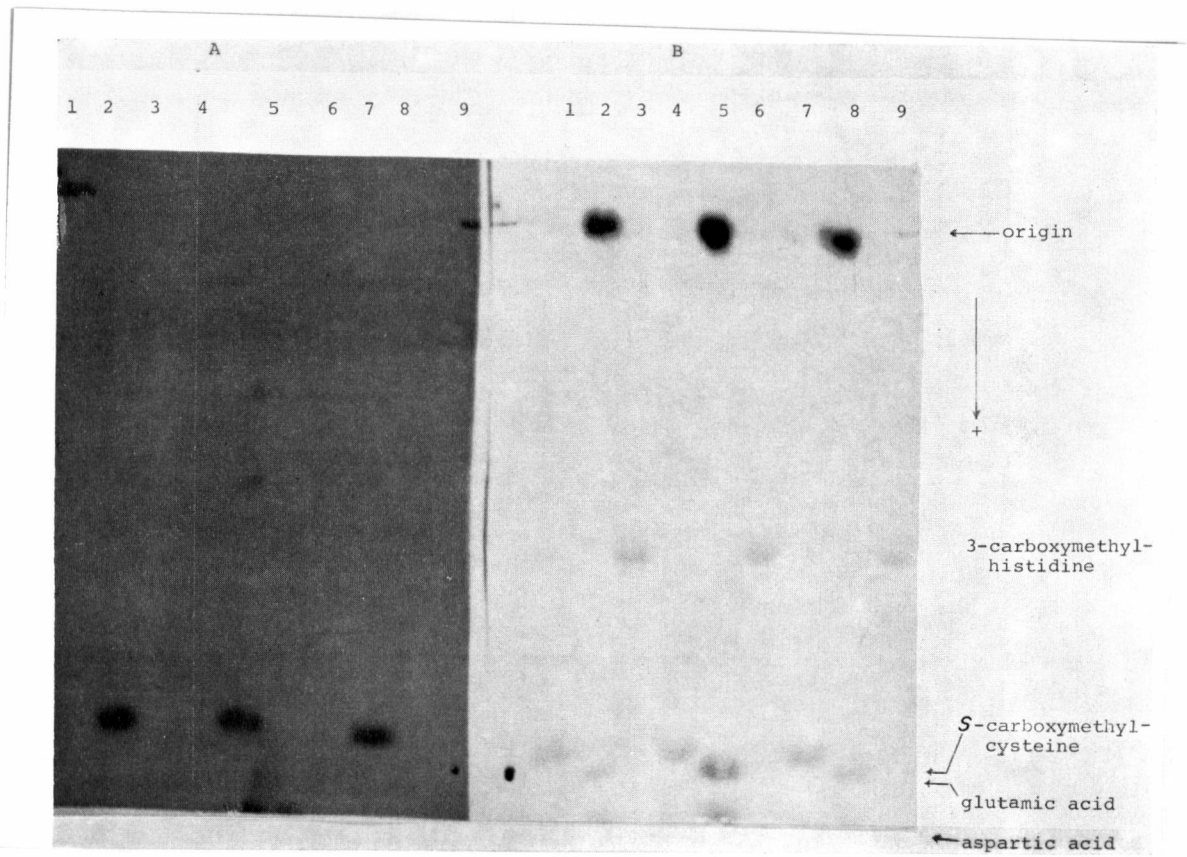


Figure 36 High voltage electrophoresis at pH 6.5 after 16 hour acid hydrolysis of ^{14}C-carboxymethyl-Hb and ^{14}C-carboxymethyl-Hb-Hp. 36B is a ninhydrin stain and 36A is an autoradiograph of the high voltage paper. Numbers 1, 4, and 7 show markers of S-carboxymethylcysteine and 3, 6, and 9 are 3-carboxymethylhistidine. Numbers 2 and 8 show acid hydrolysates of carboxymethyl-Hb and number 5 is the hydrolysate of carboxymethyl-Hb-Hp.

voltage electrophoresis at pH 6.5 showed that in the Hb-Hp complex the major radioactive spot was the same as that with hemoglobin and had the same mobility as authentic S-carboxymethylcysteine. Two minor spots which ran more slowly toward the anode than 3-carboxymethylhistidine were also seen. Products with similar mobilities to these minor spots have also been described in a previous analysis of the reaction of haptoglobin with iodoacetamide following acid hydrolysis (123).

Comparison of Residues Reacting in Hb-Hp Complex With Those in Hb

In order to identify which cysteinyl residue in hemoglobin was reacting, the technique of comparative partial acid hydrolysis, developed for comparison of the active sites of serine proteases, was used (124). Partial hydrolysates of ^{14}C-carboxymethyl-labelled Hb and Hb-Hp complex were separated by high voltage electrophoresis at pH 3.6 and an autoradiograph of the electrophoretogram is shown in Figure 37. At least eight radioactive bands show identical mobilities and appear in approximately similar proportions in sample 1 (free Hb) and sample 2 (the Hb-Hp complex). This demonstrates conclusively that the site of reaction in the Hp-Hb complex is still at the β93 cysteinyl residue of hemoglobin. Sample 3 shows the products of partial acid hydrolysis of free haptoglobin after treatment with ^{14}C-iodoacetamide. The site(s) of modification, which leads to the appearance of the two slightly cathodic radioactive bands, has not been characterized but

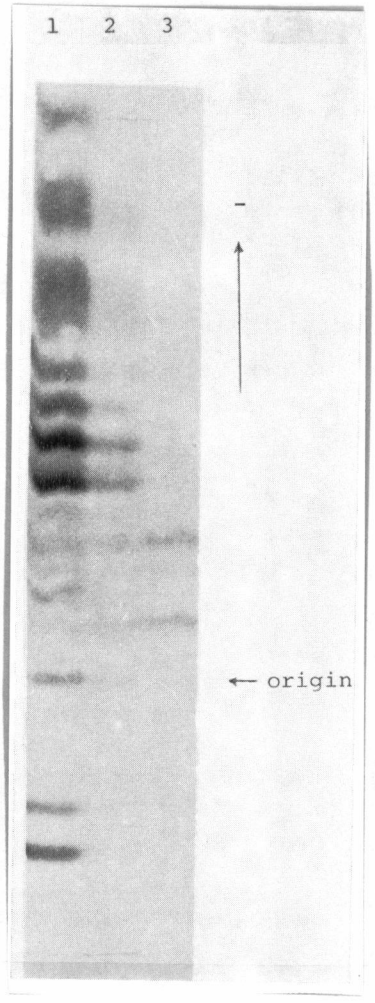


Figure 37 High voltage electrophoresis at pH 3.6 after 20 minute acid hydrolysis of ^{14}C-carboxymethyl-Hb, ^{14}C-carboxymethyl Hb-Hp and ^{14}C-carboxymethyl Hp. Electrophoresis was performed at approximately 3,500 volts until a marker of crystal violet had moved 22 cm.

faint corresponding bands in sample 2 indicate that a similar reaction is probably occurring with the Hb-Hp complex.

The explanation for the decreased reactivity of cysteinyl β93 in the Hb-Hp complex cannot, at the moment, be unequivocal. Three possibilities exist,

(a) There is a covalent interaction between the sulfhydryl group of the cysteinyl residue at β93 and a group in haptoglobin, for example, a reactive disulfide. Two lines of evidence make this unlikely; first, β93 can react completely, albeit more slowly, with iodoacetamide or the dithiodipyridines so that the decreased reactivity is essentially a rate phenomenon. Secondly, the Hb-Hp complex can be completely dissociated by succinylation of amino groups (54), a procedure which does not affect -SH or -S-S- groups but is known to cause an extensive physical unfolding of haptoglobin and presumably destruction of the conformation of the hemoglobin binding site on haptoglobin.

(b) β93 is a "contact" amino acid (125) in the hemoglobin site bound by haptoglobin. This possibility has been explored by Bunn (64) who prepared several derivatives of hemoglobin in which β93 was modified by groups varying in size from carboxymethylamido- (from iodoacetamide) to p-mercuribenzoate (from p-HMB) and found that there was no effect on the binding of hemoglobin by haptoglobin. This would argue against the decrease in reactivity of β93 in the complex being the result of a direct shielding by haptoglobin. Moreover, HbH (β_4) is not bound by haptoglobin (57) and isolated β-chain is bound only weakly (72), so that it seems unlikely that a major portion of the

binding site is on the β-chain.

(c) The third and perhaps most likely possibility is that a conformational change is induced in the environment of β93 as a result of the reaction with haptoglobin. The effect of haptoglobin on β93 is very similar to the decrease in reactivity of β93 toward iodoacetamide upon deoxygenation of hemoglobin (47). Since this effect has been interpreted (47) as resulting from the known conformational differences between oxy- and deoxy-hemoglobin (42,50), the same type of conformational change upon haptoglobin binding seems reasonable. Detailed studies of the immediate environment of β93 (42,50) have shown that cysteinyl β93 is close to histidyl β97, a residue intimately involved in the (α_1,β_2) contact area between the dissociating halves of the hemoglobin molecule (50). A more recent X-ray crystallographic study indicates that upon formation of deoxyhemoglobin, histidyl β146 forms a hydrogen bond with the β-carboxyl group of aspartyl β94 and restricts access to the sulphydryl group of β93 (51) thus accounting for the decreased reactivity of β93 (47). Since the combination of haptoglobin appears to be with dissociated αβ dimers of hemoglobin (70,71) it is possible that haptoglobin may react with some portion of the exposed α_1,β_2 contact area thus indirectly affecting the reactivity of β93 toward sulphydryl reagents.

Thus the evidence indicates that haptoglobin induces a conformational change in the vicinity of the β93 sulphydryl of hemoglobin. It is known that haptoglobin combines with a number of hemoglobins modified at β93 (64) but it is not known if haptoglobin induces a

similar conformational change in these modified hemoglobins.

The question also arises as to how hemoglobins with a modified conformation at β93 react with haptoglobin. Deoxyhemoglobin, known to have a different conformation at β93, does not combine with haptoglobin. Human bis(N-maleimidomethyl)ether hemoglobin, which is modified at β93 and has different dissociation properties than human oxyhemoglobin, reacts less completely with haptoglobin than oxyhemoglobin does. In fact this βME-hemoglobin has recently been shown to have a different conformation in the FG corner of the hemoglobin β chain (the β93 region) (126). Thus two hemoglobins both with different conformations in the cysteinyl β93 region both have different affinities for haptoglobin than oxyhemoglobin has. As a result this region of the hemoglobin molecule or an area near this region appears to be the likely site of haptoglobin attack. Since the $\alpha_1\beta_2$ contact region is near the β93 area a conformational change in the β93 area could change the conformation in the $\alpha_1\beta_2$ contact area. The fact that both human deoxyhemoglobin and human BME-hemoglobin have different dissociation properties than oxyhemoglobin (49,65) confirms the prediction that these hemoglobins have different conformations in the $\alpha_1\beta_2$ contact area than oxyhemoglobin. In the case of deoxyhemoglobin the different conformation in the $\alpha_1\beta_2$ contact area has been demonstrated directly by X-ray crystallography (43). As mentioned previously, this change in conformation in the $\alpha_1\beta_2$ contact area may be the reason that haptoglobin reacts differently with human BME-hemoglobin and human deoxyhemoglobin than with human oxyhemoglobin. Since haptoglobin does

combine with αβ hemoglobin dimers and does react more rapidly with hemoglobins which are dissociated into dimers to a greater extent (68), haptoglobin appears to be combining with an area of the hemoglobin molecule which is exposed after hemoglobin dissociation. This area appears to be the $\alpha_1\beta_2$ contact area.

The following observations are consistent with the prediction that haptoglobin combines with the $\alpha_1\beta_2$ contact area of hemoglobin; haptoglobin combines with the αβ dimer of hemoglobin; haptoglobin causes a conformational change in a region of the hemoglobin molecule (β93 region) which is in contact with the $\alpha_1\beta_2$ contact region; two hemoglobins with modified conformations in the $\alpha_1\beta_2$ contact area combine less completely or not at all with haptoglobin; and haptoglobin combines most rapidly with those hemoglobins which are dissociated to the greatest extent.

In recent years nitroxide derivatives have been discovered which contain unpaired electrons and are stable in aqueous solutions (127). The attachment of these compounds to macromolecules has provided new and exciting information about the nature of protein structure because the electron spin resonance spectra of these modified proteins has been studied.

Because the β93 cysteinyl group of hemoglobin can readily be modified and because it is sensitive to the conformation of hemoglobin this group has been modified with several spin-label reagents and studied in detail in H. M. McConnells laboratory (127,128).

One very interesting aspect of this work was the demonstration of a new component in the spectra of spin-label compounds after the spin-label reagents were attached to the β93 sulphydryl in hemoglobins. In fact the spectra of horse carbonmonoxyhemoglobin and horse methemoglobin show different amounts of this component (129). The explanation for the varying amounts of the new component in the spin resonance spectrum of modified carbonmonoxyhemoglobin and methemoglobin was that the conformation of these two proteins in the area of the spin-labels was different. X-ray crystallography of horse methemoglobin and horse carbonmonoxyhemoglobin had shown that these proteins had identical structures (130). However crystallization of these two spin-labelled hemoglobins demonstrated that the difference in spectra between the two proteins remained (131) and thus the two proteins appear to have a very small conformational difference which cannot be demonstrated by X-ray crystallography. The discovery that haptoglobin has an effect on human hemoglobin, which is spin-labelled at β93, opens up the possibility of investigating aspects of the nature of the hemoglobin-haptoglobin complex with this new and powerful technique (122).

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