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Studies On Subunit Interrelations In The Hemoglobin-haptoglobin Complex

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STUDIES ON SUBUNIT INTERRELATIONS IN THE HEMOGLOBIN-HAPTOGLOBIN COMPLEX

by

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ABBREVIATIONS

Hb	hemoglobin
Hp	haptoglobin
Hb-Hp	hemoglobin-haptoglobin complex
H	heavy chain of haptoglobin
L	light chain of haptoglobin
PMB	p-chloromercuribenzoate
DEAE	diethylaminoethyl
Tris	2-amino-2-hydroxymethylpropane-1,3 diol
BME	bis(N-maleimidomethyl)ether
DMA	dimethyl adipimidate
SDS	sodium dodecyl sulfate
FDNB	1-fluoro-2,4-dinitrobenzene
DNP-	dinitrophenyl-

ABSTRACT

A study has been made to select a bifunctional reagent for cross-linking complex formed between human hemoglobin and pig haptoglobin. Such a reagent provides a sensitive probe to obtain information on the subunit structure of complex and, ultimately, on interresidue distances.

Hemoglobin has been used as a model for testing reagents prior to their use with haptoglobin or hemoglobin-haptoglobin complex. Results obtained with hemoglobin following modification with maleic anhydride forced rejection of possible bifunctional anhydrides. Amidination, however, proved to be sufficiently mild to allow not only retention of haptoglobin-binding properties, but also of some 'heme-heme interaction', although the Bohr effect was lost.

The mild nature of amidination led to choice of dimethyl adipimidate as a cross-linking reagent. Conditions were selected for its introduction into hemoglobin, and these were then applied to haptoglobin and hemoglobin-haptoglobin complex. Cross-linked hemoglobin was analyzed by the recently developed technique of polyacrylamide gel electrophoresis in sodium dodecyl sulfate and products have

been tentatively identified from their mobilities in these gels. Cross-linked hemoglobin revealed bands characteristic of monomers, dimers, trimers, and tetramers. The region of dimers was poorly resolved into two bands, one of which has been tentatively identified as $\alpha_1\beta_1$. Of the cross-linked hemoglobin components, that which did not contain bridges between subunits displayed the best ability to bind with haptoglobin, although small fractions of dimers and other components were also bound.

Cross-linking of haptoglobin, followed by electrophoretic analysis, revealed cross-linked species consistent with a structure containing two heavy chains and two light chains. Cross-linking of hemoglobin-haptoglobin complex revealed a mixture of products, only three of which could be readily recognized. These corresponded to heavy chain of haptoglobin, dimer of hemoglobin, and monomers of hemoglobin. The presence of cross-linked hemoglobin dimer in cross-linked hemoglobin-haptoglobin complex has been the first direct evidence that two hemoglobin chains are in close proximity to each other when bound to haptoglobin.

Studies on the structure of pig haptoglobin were made to determine whether it could dissociate to half molecules in dilute solution. Analytical gel filtration has been used in conjunction with sedimentation velocity and it has been found that both elution volume and sedimentation rate failed

to change with decreasing concentration. In addition, polyacrylamide gel electrophoresis in sodium dodecyl sulfate with increasing amounts of 2-mercaptoethanol indicated a structure highly sensitive to reduction. Products of partial reduction indicated that haptoglobin light chains were bridged by disulfides to each other and to heavy chains. It was concluded from these studies that pig haptoglobin does not undergo dissociation in dilute solution.

Horse hemoglobin was reacted with a second bifunctional reagent, bis(N-maleimidomethyl)ether, because work in other laboratories had determined the effects of this modification on hemoglobin. Ability of modified hemoglobin to bind with haptoglobin was determined and a heretofore unreported interchain cross-link was discovered. A model for reaction of this reagent with hemoglobin has been proposed, and this has led to the suggestion that those sites on hemoglobin for binding haptoglobin are in the regions of the $\alpha_1\beta_2$ contacts.

INTRODUCTION

Haptoglobin is an α-2 glycoprotein normally present in plasma of humans and other species. Genetic heterogeneity of haptoglobins has been observed in our species only. Interest in the study of haptoglobin has been high for two reasons, first to elucidate the genetic basis for control of human haptoglobin phenotype, and second because of the functional homology of its reaction with hemoglobin to that of antibody with antigen. This homology has been extended to the level of amino acid sequences by Black and Dixon (1968) who reported similarities between the sequence of haptoglobin light chains and those of immunoglobulins. Availability of haptoglobin has therefore made it an attractive model for studies of protein-protein interaction in general, a subject of high current interest in clinical medicine. Its reaction with extracellular hemoglobin is particularly convenient for a model system because hemoglobin is the best studied of all oligomeric proteins, its precise three-dimensional structure having been established by x-ray analysis at 2.8 Å resolution.

In studies of protein structure, x-ray analysis of crystals far surpasses all other approaches both in quantity and quality of information gained. While crystals of hemoglobin-haptoglobin complex have been obtained (Waks et al., 1968), they have not yet proven suitable for this analysis. As an alternative, these investigations were undertaken to select a chemical probe suited to the hemoglobin-haptoglobin system. Human hemoglobin has been used because of its availability and pig haptoglobin again because large amounts of pig blood were available. Fraser and Smith (1971) have recently developed methods for isolation of pig haptoglobin from plasma.

A direct chemical approach to structural problems is afforded by bifunctional reagents capable of cross-linking two residues within a protein. The ultimate goal then is to cross-link residues and then to identify the joined residues. From known structural properties of the reagent, maximum interresidue distances can be calculated. Two fundamental premises must hold if the method is to be valid: first the reagent must not undergo changes such as polymerization before forming cross-links, and second the reagent must not induce structural changes in the protein to yield cross-links not possible in the native molecule. It is not expected that complete macromolecular structures can ever be elucidated by these procedures, but structural relations very difficult to show by any other chemical means can be

discovered. As a preliminary step in this type of analysis, these experiments sought to cross-link subunits of hemoglobin, haptoglobin, and complex and then to develop means of separating cross-linked subunits for further characterization.

For these studies, four types of bifunctional reagents have been considered and two have been used. Synthesis of a bifunctional anhydride was considered but reaction of hemoglobin with such a monofunctional compound, maleic anhydride, induced structural changes and anhydrides were therefore judged unsuitable. Several attempts were made to synthesize a bifunctional isocyanate derivative of azobenzene which has been used with myoglobin (Fasold, 1964) but synthesis always failed. Imidoesters were considered for amidination of amino groups without charge alteration (below pH 9) and necessary preliminary studies with ethyl acetimidate revealed that binding between hemoglobin and haptoglobin was unaltered by amidination. Hence a bifunctional analogue of this reagent, dimethyl adipimidate (Hartman and Wold, 1967), was synthesized and methods were selected for its introduction into hemoglobin, haptoglobin, and complex. Recent studies in other laboratories have shown important effects by the reagent bis(N-maleimidomethyl)ether on structural changes in horse hemoglobin (Perutz, 1970a; Arndt et al., 1971). This reagent therefore was also synthesized and introduced into horse hemoglobin in order to

ascertain its effects on haptoglobin binding properties.

A generally accepted model for the structure of human haptoglobin (Shim and Bearn, 1964) indicated two heavy and two light chains maintained by at least three interchain disulfide bridges. However, Waks and Alfsen (1968) gave evidence to show that haptoglobin was in reversible dissociation equilibrium with a promoter of approximately half the normal molecular weight. Since cross-linking experiments had to be performed in dilute solutions, it was desirable to know whether porcine haptoglobin existed as complete or as half molecules under these conditions. A study therefore was made to determine which model applied to these preparations of pig haptoglobin.

REVIEW OF THE LITERATURE

Haptoglobin structure

Haptoglobin was discovered in 1938 by Polonovski and Jayle (1938) during their studies on the peroxidase activity of hemoglobin. Upon combination with a non-dialyzable plasma factor, the pH optimum for peroxidase activity shifted from 5.6 to 4.2. This factor was named haptoglobin; early work has been reviewed by Jayle and Moretti (1962) and by Laurell and Gronvall (1962).

Assays for haptoglobin are based on the fact that combination with hemoglobin is stoichiometrically 1:1 (at excess Hb) and thus it can be titrated with hemoglobin until an excess appears. Hb-Hp complex can be detected by its increased peroxidase activity (Connell and Smithies, 1959), by electrophoresis (Bernier, 1967), by gel filtration (Killander, 1964; Ratcliff and Hardwicke, 1964), or by protection against change of heme spectral characteristics at pH 3.7 (Roy, Shaw and Connell, 1969).

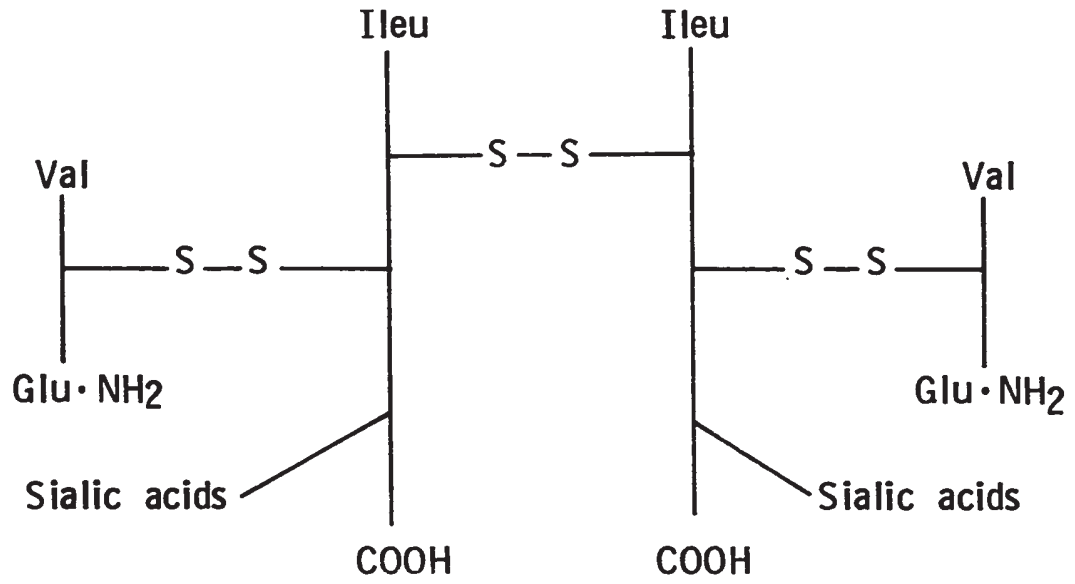
Haptoglobin is classified as a serum glycoprotein of the alpha-2 electrophoretic group. It is composed of two types of polypeptide chain, light and heavy, which may be

linked together by interchain disulfides, plus about 20 per cent carbohydrate (Cheftel et al., 1965). Shim and Bearn (1964) separated L and H chains on Sephadex following reduction and alkylation; carbohydrate was confined to heavy chain and they proposed a model, figure 1, which has been widely accepted, showing an L_2H_2 molecule containing three disulfide bonds.

Humans show genetic heterogeneity of haptoglobins but mammals other than primates have only that corresponding to human haptoglobin type 1-1 (Jayle and Moretti, 1962). The other human types are polymers of the basic haptoglobin 1-1 molecule containing additional genetic types of L chains, but they retain ability to bind hemoglobin in the same ratio as haptoglobin 1-1. That is, there is no detectable loss of binding sites which might be expected due to steric hindrance in a polymer (Hamaguchi, 1968). Common human genetic types have identical H chain peptide maps (trypsin) and identical H chain amino acid composition (Cleve, Gordon, Bowman, and Bearn, 1967). Smith, Edman, and Owen (1962) showed that each genetic type had equimolar amounts of valine and isoleucine as N-terminal amino acids and Smithies, Connell, and Dixon (1962) found valine N-terminal in the L chain, hence isoleucine must be N-terminal in the H chain. Cheftel and Moretti (1966) determined a molecular weight of 98,770 for human haptoglobin 1-1 by equilibrium centrifugation using a partial specific volume of 0.77 ml g^{-1}. They estimated the

Figure 1: Proposed model for the structure of human haptoglobin 1-1. (Shim and Bearn, 1964)

Figure 2: Amino acid sequence of light chain of human haptoglobin 1-1. (Black and Dixon, 1968)



10
H_2N-Val-Asn-Asp-Ser-Gly-Asn-Asp-Val-Thr-Asp-Ile-Ala-Asp-
20
Asp-Gly-Gln-Pro-Pro-Pro-Lys-Cys-Ile-Ala-His-Gly-Tyr-Val-
30 40
Glu-His-Ser-Val-Arg-Tyr-Gln-Cys-Lys-Asn-Tyr-Tyr-Lys-Leu-
50
Arg-Thr-Gln-Gly-Asp-Gly-Val-Thr-Thr-Leu-Asn-Asn-Lys-Lys-
60
Gln-Trp-Ile-Asn-Lys-Ala-Val-Gly-Asp-Lys-Leu-Pro-Glu-Cys-
70 80
Glu-Ala-Val-Gly-Lys-Pro-Lys-Asn-Pro-Ala-Asn-Pro-Val-Gln-

COOH

molecular weight of heavy chains at near 40,000 and this combined with a molecular weight of 8,900 for light chains (Connell, Smithies, and Dixon, 1966) fits the L_2H_2 model (figure 1) well.

In contrast to the model shown in figure 1, Waks and Alfsen (1968) have obtained evidence which suggests that haptoglobins dissociate reversibly. Elution volumes on Bio-Gel P-150 increased as protein concentrations decreased below 2.3 mg per ml. At higher protein concentrations elution profiles were asymmetric with leading edges sharper than trailing edges, which is characteristic of a reversible association equilibrium (Winzor and Scheraga, 1963). Moreover, sedimentation coefficients for haptoglobin 1-1 decreased from 4.1 at pH 9.5 to 2.9 at pH 11.5. Molecular weight at pH 11.5 was estimated at 42,000 by sedimentation equilibrium (Waks and Alfsen, 1968).

Information on the state of sulfhydryl groups is needed. There are 18 in fully reduced human haptoglobin 1-1 (Bernini and Borri-Voltattorni, 1970), the same as in pig haptoglobin (Fraser and Smith, 1971). None are reactive in the native haptoglobin 1-1 molecule (Lisowska and Dobryszycka, 1967). Tattrie and Connell (1967) found less than one sulfhydryl reactive to ^{14}C-iodoacetate or p-chloromercuribenzoate in 7.2 M guanidine hydrochloride. Similarly Bernini and Borri-Voltattorni (1970) found none

reactive to 5,5'-dithiobis-(2-nitrobenzoic acid) in either 6M guanidine hydrochloride or 33 mM sodium dodecyl sulfate. There is a discrepancy between these results and those obtained by Waks and Alfsen (1966) using amperometric titration. They found none reactive in the native molecule but 10 were titrated in 8M urea. It is of interest that Shim and Bearn (1964) subjected tryptic peptides of human haptoglobin 1-1 to electrophoresis, then exposed the strips to performic acid vapour and performed a second electrophoresis at right angles. Nine peptides were altered by the oxidation and of these only four yielded more than one new peptide. It is clear that no sulfhydryl groups can be reacted in denaturing solvents, but titration in urea and indications of dissociation leave the matter in some doubt.

There is considerably more information available on the light chains than on the intact molecule. Black and Dixon (1968) have determined the complete amino acid sequence of the L chains of human Hp 1-1. It contains 3 half-cystine residues at positions 21, 35, and 69 (figure 2). In a later publication Black and Dixon (1970) report a symmetrical bridge between light chains through a disulfide between half-cystines at position 21. The remaining two form intrachain loops between positions 35 and 69. This leaves none for disulfide links to heavy chains and casts doubt on the original model (figure 1).

Similarities between human haptoglobin 1-1 and porcine haptoglobin have been pointed out by Fraser and Smith (1971). Table 1 shows their amino acid composition for pig haptoglobin as compared with that obtained for humans by Black, Chan, Hew, and Dixon (1970). Both show typically high proportions of acidic amino acids and other similarities were noted in molecular weights and polypeptide chain compositions.

The site for binding hemoglobin is predominantly associated with the heavy chain; reduced alkylated haptoglobin 2-2 was separated on Sephadex G-100 in dilute propionic acid and the heavy chains retained binding ability but the light chains did not (Gordon and Bearn, 1966). This was supported by immunological experiments of Shim, Lee, and Kang (1965) who found that antiserum to light chains reacted with hemoglobin-haptoglobin complex but antiserum to heavy chains did not. A second method for preparing separated chains using gel filtration in 5 M guanidine HCl gave the same results (Gordon, Cleve, and Bearn, 1968).

Recently Ofosu, Campbell and Connell (1971) have obtained two fragments from haptoglobin 1-1 by digestion with plasmin. The larger, with molecular weight 78,000, had the same N-terminal amino acids--valine and isoleucine--as intact haptoglobin, while the smaller, with molecular weight 17,200, had only N-terminal serine. They have suggested that the smaller fragment consists of two identical segments

TABLE 1

Amino acid compositions of pig and human haptoglobins

Residue	Pig Haptoglobin	Human Haptoglobin 1-1
Lys	76	66
His	22	24
Arg	18	14
Cys/2	18	18
Asp	92	90
Met	12	8
Thr	58	46
Ser	36	40
Glu	78	84
Pro	40	44
Gly	54	58
Ala	50	56
Val	66	72
Ile	34	36
Leu	66	60
Tyr	28	38
Phe	14	20
Trp	12	12

Figures for pig haptoglobin are taken from Fraser and Smith (1971). Figures for human haptoglobin are taken from Black, Chan, Hew, and Dixon (1970) by adding their figures for light and heavy chains and doubling to fit an L_2H_2 model.

of heavy chain; preliminary studies have indicated that these segments are held together by non-covalent forces. These experiments suggest that heavy chains are not bridged to each other for at least one fifth of their chain length, probably from their carboxyl ends, and that both carbohydrate and light chains are excluded from these segments. Binding activity by either fragment has not yet been observed.

Haptoglobin function

Haptoglobin is made in the liver but its functions are poorly understood. It seems to be involved in the metabolism of hemoglobin and it is thought to determine the renal threshold for hemoglobin. Free hemoglobin does not appear in plasma until all circulating haptoglobin has been saturated; it then appears first in plasma then in urine (Jayle and Moretti, 1962). A probable mechanism for this is simply the larger size of hemoglobin-haptoglobin complex over free hemoglobin which makes it less subject to glomerular filtration (Laurell and Nyman, 1957; Allison and Rees, 1957). The metabolic fate of complex retained in the circulation is not well understood. Complex appears to be cleared much more rapidly than haptoglobin but somewhat more slowly than hemoglobin. Murray, Connell and Pert (1961) have shown that complex in rabbits is cleared from plasma

less rapidly than free hemoglobin and that this is also true of nephrectomized animals. Complex was found by Krauss and Sarcione (1966) to have a half time of 2 hours as compared with 19 hours for free haptoglobin. Complex appears to be catabolized in the liver (Engler et al., 1966; Murray et al., 1961), and Nakajima et al. (1963) have found a liver enzyme, heme α-methenyl oxygenase, to rapidly convert complex heme to a biliverdin precursor but to be inactive on hemoglobin.

In addition to its role in hemoglobin biochemistry, haptoglobin may have at least two other functions. Latner and Zaki (1957) found that isotopically labelled vitamin B_{12} was bound by alpha-2 plasma proteins and Jayle and Moretti (1962) have suggested that this was in fact due to haptoglobin and that it may function in transport of this vitamin. Also, from a clinical standpoint, there should be considerable interest in a brief report by Snellman and Sylven (1967) who found haptoglobin to be a potent inhibitor of cathepsin B.

Hemoglobin structure

Hemoglobin, the respiratory pigment of red blood cells, is a protein of molecular weight 64,500 which transports molecular oxygen from the lungs to body tissues and is also involved in return transport of carbon dioxide. It is probably the most studied and best understood of all

proteins. It consists of two pairs of polypeptides, α-chains with 141 amino acids each and β-chains with 146 amino acids each. Complete amino acid sequences of hemoglobins from man and several animals have been tabulated by Dayhoff (1969) and figure 3 shows sequences for human α and β subunits. The four chains are arranged tetrahedrally resulting in two axes of symmetry. Figure 4 shows a model of horse hemoglobin at 5.5 Å resolution--first along the axis showing contact between α_1 and β_1 subunits and second, perpendicular to that axis showing contact $\alpha_1\beta_2$ (Cullis et al., 1962; Perutz, 1969). Each subunit carries a heme group in a non-polar pocket near its surface and these may be seen in the model. An atom of ferrous iron forms part of each heme group and it is this atom which ligands with molecular oxygen.

Analysis of the three-dimensional structure of oxy-Hb has been extended to 2.8 Å by Perutz et al. (1968) and these data have permitted construction of an even more detailed molecular model (Perutz et al., 1968b). As a result of this work a list of atomic coordinates is available and figure 5 shows a model of the $\alpha_1\beta_1$ dimer constructed in this laboratory.

The four-chain structure (the tetramer) is maintained by non-covalent forces around a central water filled cavity lined with a variety of polar residues (Perutz, 1965). Polar residues are excluded from the interior of α and β

Figure 3: Amino acid sequences of α and β subunits of human hemoglobin. (Dayhoff, 1969) Drawing from Oda (1969)

```
                                  A-----------------------------------------
alpha  Val-    Leu-Ser-Pro-Ala-Asp-LYS-Thr-Asn-Val-LYS-Ala-Ala-Try-Gly -
         1              5                  10                  15
beta   Val-His-Leu-Thr-Pro-Glu-Glu -LYS-Ser-Ala-Val-Thr-Ala-Leu-Try-Gly -
         1              5                  10                  15

          ----------A          B-----------------------------------------
(a)-   LYS-Val-Gly-Ala-His-Ala-Gly-Glu-Tyr-Gly-Ala-Glu-Ala-Leu-Glu-ARG-
                        20                  25                  30
(b)-   LYS-Val-Asn-          Val-Asp-Glu-Val-Gly-Gly-Glu-Ala-Leu-Gly-ARG-
                        20                  25                  30

          ------------------B C-----------------------C
(a)-   Met-Phe-Leu-Ser-Phe-Pro-Thr-Thr-LYS-Thr-Tyr-Phe-Pro-His-Phe-
                        35                  40                  45
(b)-   Leu-Leu-Val- Val-Tyr-Pro-Try-Thr-Gln-ARG-Phe-Phe-Glu-Ser-Phe-
                        35                  40                  45

                                  D-----------------------D      E----------
(a)-       Asp-Leu-Ser-His-                                Gly-Ser-Ala-Gln-Val-LYS-
                        50                                                  55
(b)-   Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-LYS-Val-LYS-
                        50                  55                  60

          ------------------------------------------------------------
(a)-   Gly-His-Gly-LYS-LYS-Val-Ala-Asp-Ala-Leu-Thr-Asn-Ala-Val-Ala-His-
                  60                  65                  70
(b)-   Ala-His-Gly-LYS-LYS-Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-
                  65                  70                  75

          --E                       F----------------------  --------
(a)-   Val-Asp-Asp-Met-Pro-Asn-Ala-Leu-Ser-Ala-Leu-Ser-Asp-Leu-His-Ala-
                  75                  80                  85
(b)-   Leu-Asp-Asn-Leu-LYS-Gly-Thr-Phe-Ala-Thr-Leu-Ser-Glu-Leu-His-Cys-
                  80                  85                  90

          -F                  G-----------------------------------
(a)-   His-LYS-Leu-ARG-Val-Asp-Pro-Val-Asn-Phe-LYS-Leu-Leu-Ser-His-
            90                  95                  100
(b)-   Asp-LYS-Leu-His-Val- Asp-Pro-Glu-Asn-Phe-ARG-Leu-Leu-Gly-Asn-
            95                  100                 105

          ------------------------------G                        H---
(a)-   Cys-Leu-Leu-Val-Thr-Leu-Ala-Ala-His-Leu-Pro-Ala-Glu-Phe-Thr-Pro-
            105                 110                 115
(b)-   Val-Leu-Val-Cys-Val-Leu-Ala-His-His-Phe-Gly-LYS-Glu-Phe-Thr-Pro-
            110                 115                 120

          ------------------------------------------------------------
(a)-   Ala-Val-His-Ala-Ser-Leu-Asp-LYS-Phe-Leu-Ala-Ser-Val-Ser-Thr-Val-
       120                 125                 130                 135
(b)-   Pro-Val-Gln-Ala-Ala-Tyr-Gln-LYS- Val-Val-Ala-Gly-Val-Ala-Asn-Ala-
       125                 130                 135                 140

          ---------- H
(a)-   Leu-Thr-Ser-LYS-Tyr-ARG
                  140
(b)-   Leu-Ala-His-LYS-Tyr-His
                  145
```

Figure 4: Model of horse hemoglobin at 5.5 Å resolution. Above, view along the axis to show the contact $\alpha_1\beta_1$ Below, view perpendicular to that axis to show the contact $\alpha_1\beta_2$

Source: Cullis et al. (1962) and Perutz (1969)

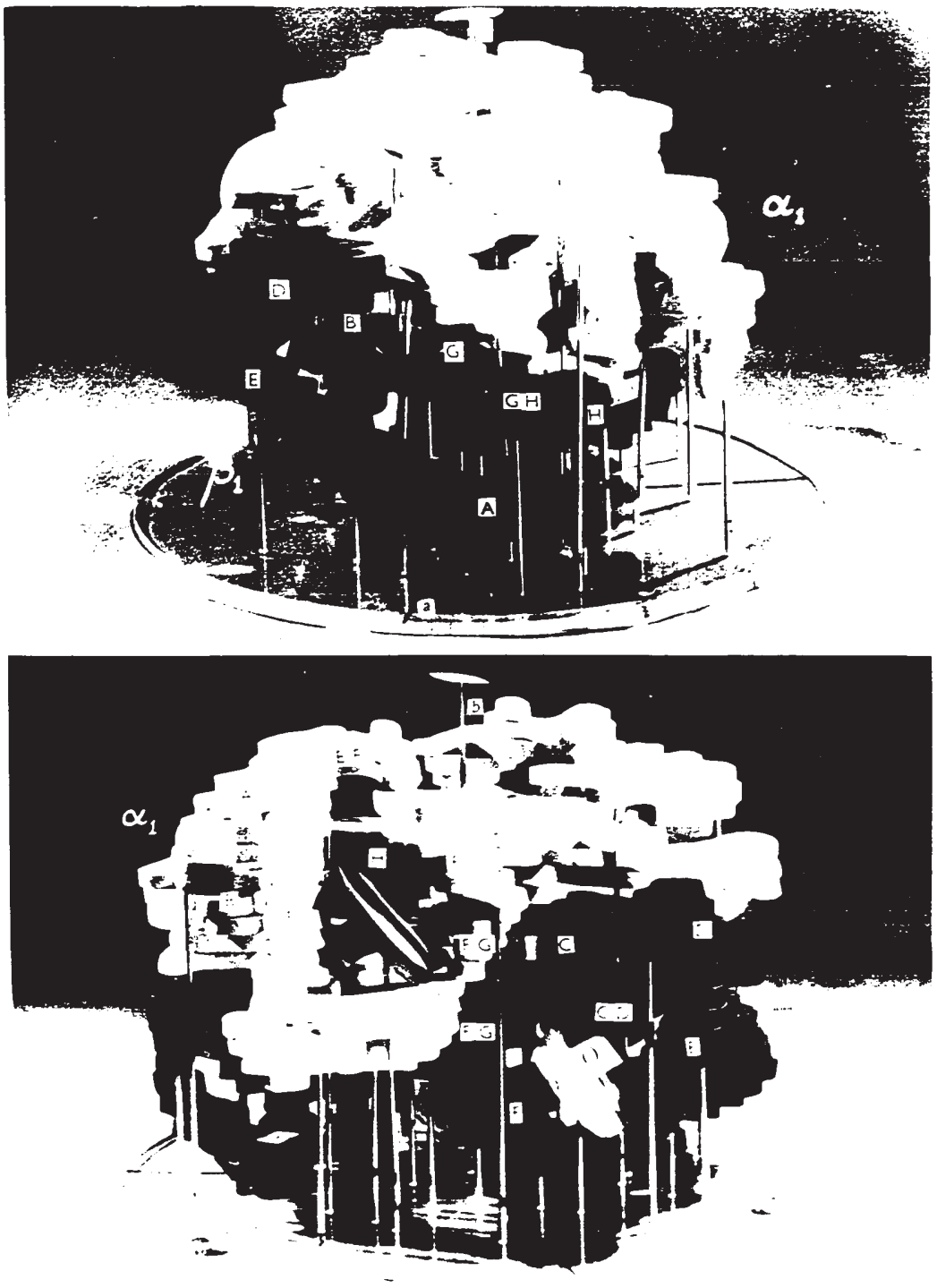
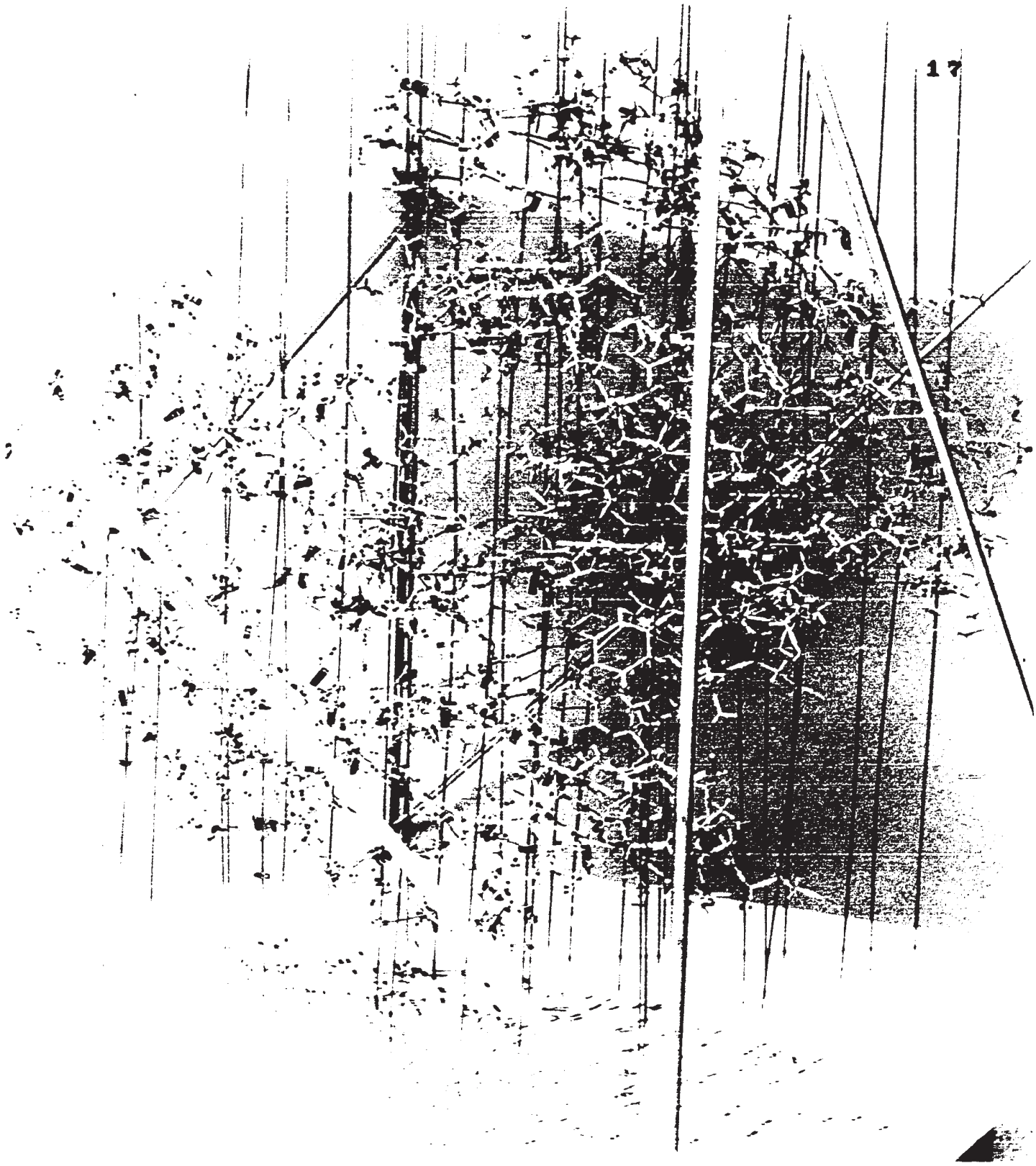

α_1
D
B
G
E
GH
H
A
a
α_1
b
H
E G
C
C
E G
B
E
F

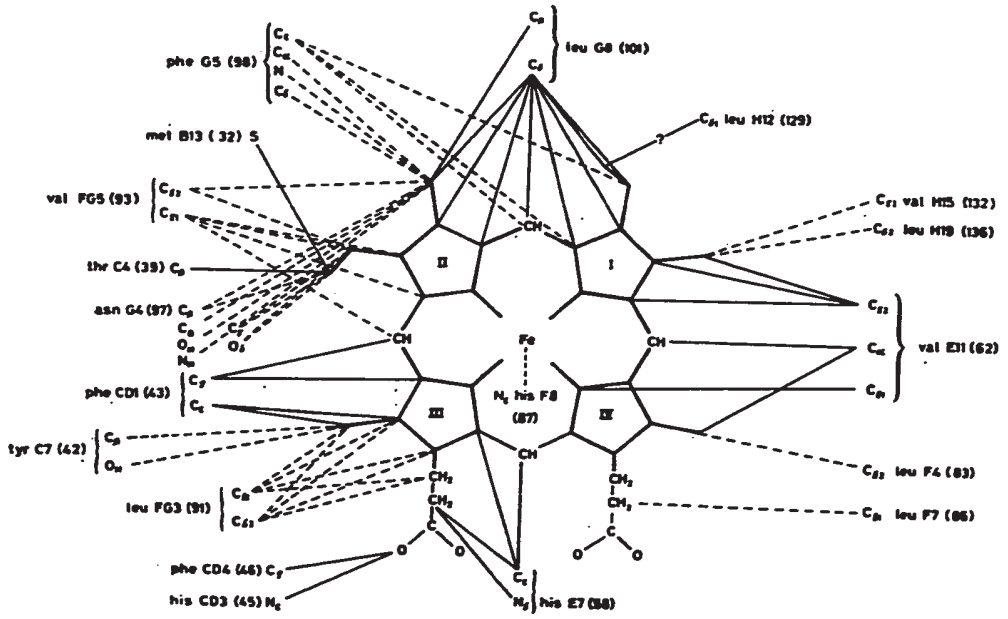
Figure 5: Model of $\alpha_1\beta_1$ dimer of horse hemoglobin at 2.8 Å resolution.
View is from the external tetramer surface showing α-subunit (upper right) and β-subunit (lower left)
Atomic coordinates supplied by Dr. M. F. Perutz.
Model built by Miss D. Jewett and Mr. D. L. B. Smith.



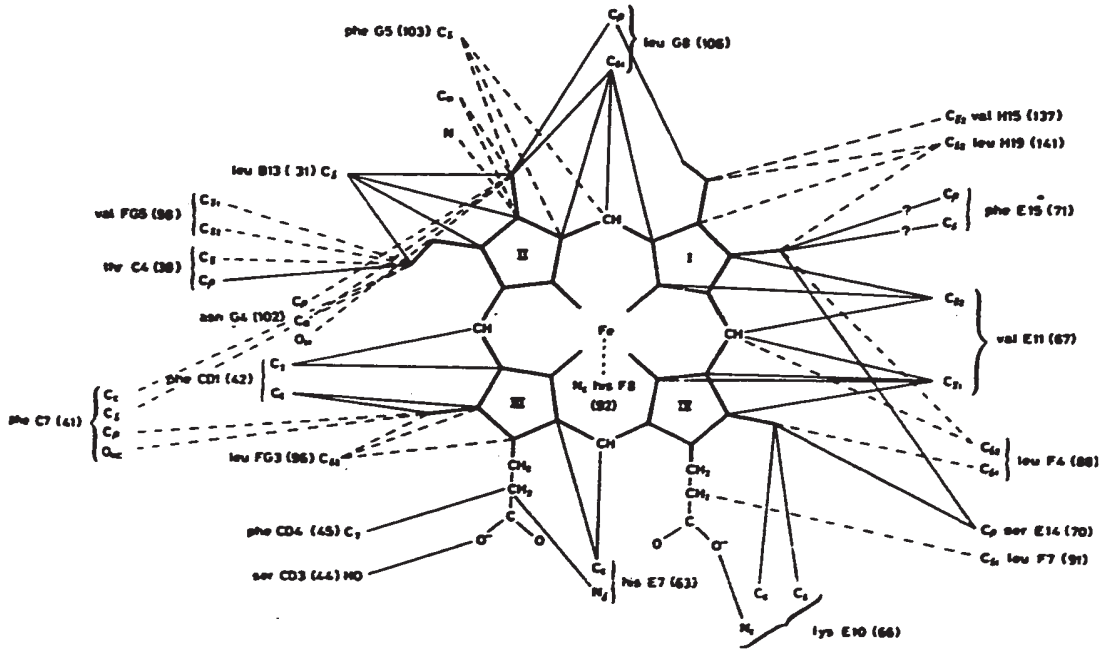
subunits except for serines and threonines participating in intrahelical hydrogen bonds to carbonyl groups. Large non-polar groups lie either in the interior of subunits, in surface crevices, or at boundaries between unlike subunits. Heme groups lie in non-polar pockets and figure 6 shows those atoms of each polypeptide approaching to within 4 Å of its heme group. In α-subunits all interactions with heme but one are non-polar in nature, while in β-subunits all but two are non-polar. All normal hemoglobins which have been sequenced show residues in correct positions to allow these same contacts except for one in α- and two in β-subunits. Thus it seems that nearly all heme contacts are essential to normal function.

Contacts between unlike chains are of two types as illustrated in figure 4, that is, the $\alpha_1\beta_1$ contact and the $\alpha_1\beta_2$ contact. Analysis at 2.8 Å has allowed identification of residues involved in each case and these are shown in figure 7. The $\alpha_1\beta_1$ contact is more extensive, including 110 atoms contributed by 34 amino acid residues; the $\alpha_1\beta_2$ contact consists of 80 atoms contributed by 19 residues. In both cases the great majority of interactions are non-polar in nature and no lysine residues are in either contact. Contacts between like chains are not visible on electron density maps but are thought to exist in the form of salt bridges or hydrogen bonds.

Figure 6: Atoms of each subunit approaching to within 4 Å or less of its respective heme group. Solid lines indicate contacts from side of the distal histidine and broken lines those from the side of the proximal histidine. Above, α-subunit; below, β-subunit. Source: Perutz, 1969.


phe G5 (98)
leu G8 (101)
met B13 (32) S
val FG5 (93)
thr C4 (39)
asn G4 (97)
val E11 (62)
phe CD1 (43)
tyr C7 (42)
leu FG3 (91)
leu F4 (83)
leu F7 (86)
phe CD4 (46)
his CD3 (45)
his E7 (58)
his F8 (87)
Fe
I
II
III
IV

(a)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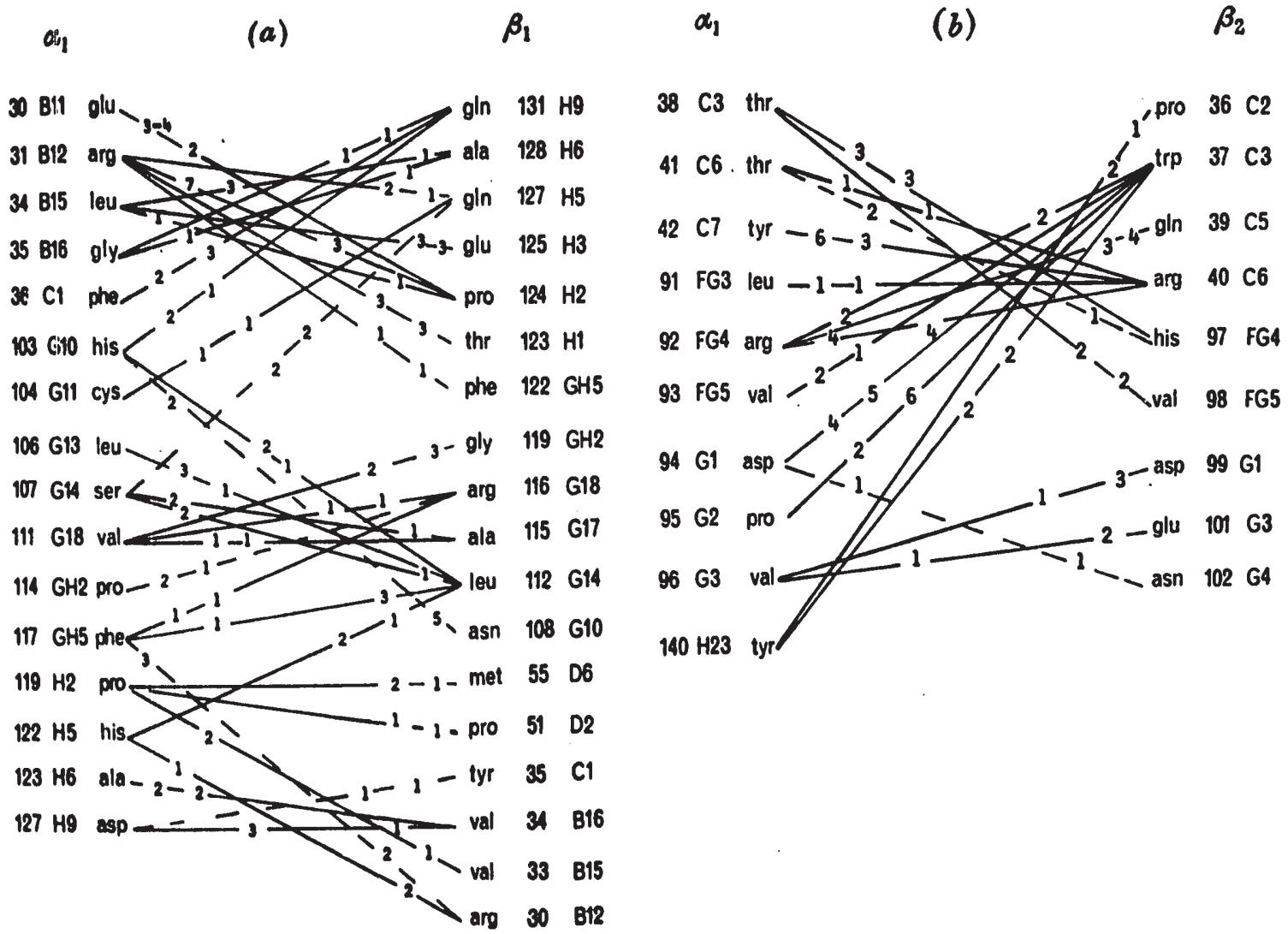
leu G8 (106)
leu B13 (31)
val FG5 (98)
thr C4 (38)
asn G4 (102)
phe CD1 (42)
phe C7 (41)
leu FG3 (96)
phe E15 (71)
val E11 (67)
leu F4 (88)
leu F7 (91)
phe CD4 (45)
ser CD3 (44)
his E7 (63)
lys E10 (66)
his F8 (92)
Fe
I
II
III
IV

Figure 7: Amino acids involved in the $\alpha_1\beta_1$ contact (a) and the $\alpha_1\beta_2$ contact (b).
Numbers on lines represent the number of atoms contributed to the contact by the nearer member of each pair joined by a line. A contact is defined as an approach to within 4 Å or less.
Source: Perutz, 1969.


α_1
(a)
β_1
30 B11 glu
31 B12 arg
34 B15 leu
35 B16 gly
36 C1 phe
103 G10 his
104 G11 cys
106 G13 leu
107 G14 ser
111 G18 val
114 GH2 pro
117 GH5 phe
119 H2 pro
122 H5 his
123 H6 ala
127 H9 asp
gln 131 H9
ala 128 H6
gln 127 H5
glu 125 H3
pro 124 H2
thr 123 H1
phe 122 GH5
gly 119 GH2
arg 116 G18
ala 115 G17
leu 112 G14
asn 108 G10
met 55 D6
pro 51 D2
tyr 35 C1
val 34 B16
val 33 B15
arg 30 B12
α_1
(b)
β_2
38 C3 thr
41 C6 thr
42 C7 tyr
91 FG3 leu
92 FG4 arg
93 FG5 val
94 G1 asp
95 G2 pro
96 G3 val
140 H23 tyr
pro 36 C2
trp 37 C3
gln 39 C5
arg 40 C6
his 97 FG4
val 98 FG5
asp 99 G1
glu 101 G3
asn 102 G4

The question of whether crystal structure represents accurately the structure in solution seems to have been answered, at least to the level of tertiary structure. Fasold (1965a, 1965b) has been able to isolate 4 cross-linked peptides from myoglobin reacted with 2,2'-dicarboxy-4,4'-azophenyldiisocyanate, all in exact agreement with the known crystal structure of that protein. For hemoglobin, MacLeod and Hill (1970) have obtained high yields of cross-linked N-terminal residues of α-chains after reaction with p,p'-difluoro-m,m'-dinitrodiphenylsulfone, again in good agreement with crystal structure. This would seem to extend the validity of crystal structures to quarternary structure in solution.

In solution hemoglobin tetramers are in equilibrium with dimers and possibly even monomers. The balance of the equilibrium depends on several factors including the nature of the ligand at the heme iron, the concentration of hemoglobin, of neutral electrolyte, and of hydrogen ion. Only symmetrical dissociation occurs as it requires breakage of both small polar contacts between like chains and the less extensive of the two non-polar contacts, namely the $\alpha_1\beta_2$ contact. This gives rise only to dimers of the type $\alpha_1\beta_1$ (Perutz, 1970a). This question of dissociation into subunits is of considerable importance in haptoglobin binding and has been the subject of recent debate.

Edelstein et al. (1970) have argued that the difference between 110 atoms in the $\alpha_1\beta_1$ contact and 80 atoms in the $\alpha_1\beta_2$ contact is not sufficient to completely rule out formation of an $\alpha_1\beta_2$ dimer by breakage of the more extensive contacts. They presented evidence from scanning ultracentrifuge studies to indicate that deoxyhemoglobin dissociates to form a dimer which reacts with carbon monoxide at a rate different from the dimer formed by dissociation of liganded hemoglobin. They suggested that the deoxy dimer was $\alpha_1\beta_2$ in contrast, as they pointed out, to the conclusion of Park (1970) who performed hybridization studies between human and dog hemoglobins. By assuming easy dissociation of deoxy tetramers to dimers in one plane, and by assuming easy dissociation of oxy tetramers to dimers (but not monomers) in one plane, her results indicated that the plane was the same in both cases. However, Perutz (1970a) quoted a private communication from Kellett, Midgarden and Schachmann who found that "the deoxy form remains tetrameric in all conditions formerly believed to produce dimers". Until better evidence to the contrary has been obtained, the conclusions of Perutz (1969, 1970a) may be considered valid; liganded hemoglobin dissociates to $\alpha_1\beta_1$ dimers only and unliganded hemoglobin does not dissociate.

Hemoglobin function

Hemoglobin binds reversibly with molecular oxygen and this binding is accompanied by a large change in crystal structure. The affinity for oxygen can be studied by plotting fractional saturation with oxygen against the partial pressure of oxygen required to produce that degree of saturation. This generates a sigmoid curve indicating that the four heme groups are not independent. Empirically this relation has been found to be satisfactorily described by the Hill equation (Hill, 1910). This equation may be written

$$Y = \frac{Kp^n}{1 + Kp^n}$$

where Y is the fraction of Hb in liganded form, p is the partial pressure of oxygen, and K and n are constants. The partial pressure required to half saturate a sample of hemoglobin, the $P_{\frac{1}{2}}$, is a commonly used measure of oxygen affinity. The value of the exponent, n, has been taken as a measure of the degree of interaction between binding sites, the heme-heme interaction. Physiologically heme-heme interaction is vital to make oxygen affinity fall as successive molecules dissociate resulting in increased release of oxygen to tissues. Recently Perutz (1970a) has given a detailed description of molecular events responsible for this property of hemoglobin.

As a means of carrying oxygen hemoglobin is made even more efficient by the Bohr effect. This effect may be observed either as a change in pH as ligands bind or as a change in ligand affinity as a function of pH. Molecular mechanisms underlying this effect have also been explained by Perutz (1970b).

Heme is rigidly attached to globin by a co-ordinate bond from iron to N_ε of the proximal histidine; on the distal side lie a histidine and a valine. In α chains there is sufficient room between heme and these distal residues to allow entry of a ligand but in β-chains there is not. It is thought that α-chains react first causing a movement of almost 1 Å by the proximal histidine which in turn causes many small changes including expulsion of the penultimate tyrosine from its pocket. This tyrosine pulls the carboxyl-terminal residue with it and thus breaks the salt bridges between α-chains. When both α-chains have reacted, four of the six salt bridges constraining the unliganded tetramer have been broken and the liganded tetramer conformation becomes favored. This involves a large change in tetramer structure, mainly in the region of $\alpha_1\beta_2$ contacts. Atoms in these contacts undergo relative displacements of as much as 7 Å, and this breaks the salt bridges through diphospho-glycerate which had linked β-chains together. The β-chains can now react with ligand more readily with accompanying

tyrosine expulsion, breakage of intrachain salt bridges and release of remaining Bohr protons. These changes in hemoglobin structure are of interest in a study of haptoglobin binding because binding only takes place with liganded hemoglobin. Furthermore, once bound to haptoglobin, hemoglobin no longer exhibits cooperative interactions.

Hemoglobin-Haptoglobin Complex

Binding between hemoglobin and haptoglobin takes place over a wide variety of conditions; Jayle and Moretti (1962) reported firm binding over a pH range between 3.5 and 11. It is apparent that the amino acid sequence of hemoglobin can be varied considerably without loss of binding ability because human haptoglobin will form complex with many mammalian hemoglobins. The quarternary structure of hemoglobin, however, is very important as unliganded hemoglobin does not bind (Nagel, Rothman, Bradley and Ranney, 1965). The nature of the ligand attached to heme is unimportant since carbonmonoxy, met-cyanide and oxyhemoglobin all bind equally well (Nagel and Gibson, 1967). These variously liganded hemoglobins all have identical globin structure (Perutz, 1970a) and it is therefore certain that binding is a property of the protein moity of hemoglobin. In addition, complex forms readily with globin alone (Laurell and Gronwall, 1962).

The mechanism by which binding occurs has been the subject of several studies but has not yet been completely elucidated. Nagel and Gibson (1967) studied complex formation by following Hp tryptophan fluorescence quenching by heme in a stopped-flow apparatus. Hp bound more rapidly to low concentrations of Hb than to high concentrations, suggesting that a Hb subunit might be involved. No such effect was found by varying the Hp concentration in the presence of excess Hb. It was not possible to identify the reactive species of Hb as either dimers or individual monomers, however, it was known (Antonini et al., 1962) that in 2M sodium chloride Hb exists largely as $\alpha_1\beta_1$ dimers, and binding kinetics were found to be unchanged under these conditions. Isolated α-chains with p-chloromercuribenzoate attached were able to bind to a limited extent but isolated β-chains, also with PMB attached, could not, unless α-chains were already bound.

Further experiments by the same authors (Nagel and Gibson, 1971) used similar techniques to follow the reaction between solutions of deoxy-Hb and carbon monoxide-saturated Hp 1-1. Conversion of deoxy-Hb to CO-Hb is much more rapid (half time about 10 msec) than is dissociation to Hb dimers (half time about 1 sec). The two solutions were mixed in a stopped-flow apparatus and a delay in fluorescence quenching was observed. Binding curves fitted

those predicted by a model which assumed that only dimers could bind. These experiments convincingly rule out Hb tetramers in favor of dimers but they have been unable to eliminate monomers.

Additional evidence in favor of the dimer hypothesis has been obtained by examining complex formed with sub-saturating amounts of Hb. It has been known for some time that this situation results in 3 bands on electrophoresis, one being complex, one being excess Hp, and a third intermediate band. Laurell and Gronvall (1962) speculated that this intermediate material represented Hp bound to one half molecule of Hb but made no attempt to determine its composition. Hamaguchi (1966a) observed similar intermediates when dog plasma was undersaturated with Hb and he was able to purify these by chromatography and determine their sedimentation coefficients (Hamaguchi, 1966b) to be intermediate between those of Hp and saturated complex. Comparable results with human material led Ogawa and Kawamura (1966) to postulate that a Hp 1-1 molecule had two binding sites, each for a half molecule of Hb. Hamaguchi and Sasazuki (1967) then observed that the intermediate complex disappeared when saturating amounts of Hb were added but did not reappear when Hp was added to saturated complex. The intermediate was found to have a molecular weight and heme content consistent with a structure of one Hp to one

half Hb (Hamaguchi, 1967). Ogawa, Kagiyama and Kawamura (1968) confirmed the molecular weight of the intermediate as 135,000 using a partial specific volume of 0.766 ml g^{-1} and suggested that the half molecule of Hb was the $\alpha\beta$ dimer rather than α_2 or β_2. This structure was confirmed immunologically by Kagiyama, Ogawa, and Kawamura (1968) who found that intermediate complex retained ability to bind antibodies specific for Hb α-chains as well as those specific for Hb β-chains. This finding carries with it the additional implication that binding sites on Hp are situated to allow access of antibody to bound Hb chains. There can be little doubt that a single Hb dimer can be bound to Hp but the binding mechanism, either through $\alpha\beta$ dimer or through independent α and β chains, has not been resolved.

Adams and Weiss (1969) have studied heats of complex formation by direct calorimetry. When haptoglobin was titrated with increasing amounts of hemoglobin, maximum heat of reaction was reached at a point of molar equivalence as was maximum peroxidase activity. However, when Hb was titrated with increasing amounts of Hp, maximum peroxidase activity was reached when only about half the maximum heat was evolved and more than a molar equivalent of haptoglobin was required to attain maximum heat. This was explained by assuming that haptoglobin first reacted with one then a second $\alpha\beta$ dimer of hemoglobin. They suggested that the

reason for high peroxidase activity in complex is that dimers are better peroxidases than intact hemoglobin tetramers and that haptoglobin functions by preventing their recombination. Structurally this implies that, when bound, Hb dimers are not associated with each other in the way they are in Hb tetramer. Measurements of heats of reaction at different temperatures allowed calculation of the change in heat capacity at constant pressure and this was found to be -2.3 kcal per mole per degree. This value is close in magnitude but opposite in sign to that obtained for changes in heat capacity on denaturation of β-lactoglobulin. They suggest that a change of this magnitude would result from removal of hydrocarbon moities from an aqueous environment. They concluded that hydrogen bonding reinforced by hydrophobic interactions were the probable forces involved in complex formation.

There seems little doubt that the $\alpha\beta$ dimer of Hb is the fundamental binding unit, and this suggests that each heavy chain of Hp contains one site for attachment of one Hb dimer. The question of the number and type of binding sites, however, is still in doubt. By assuming two equally available sites, and that binding was irreversible, Peacock et al. (1970) calculated the expected amounts of intermediate and saturated complex that should be found on mixing the proteins at various ratios. Their results

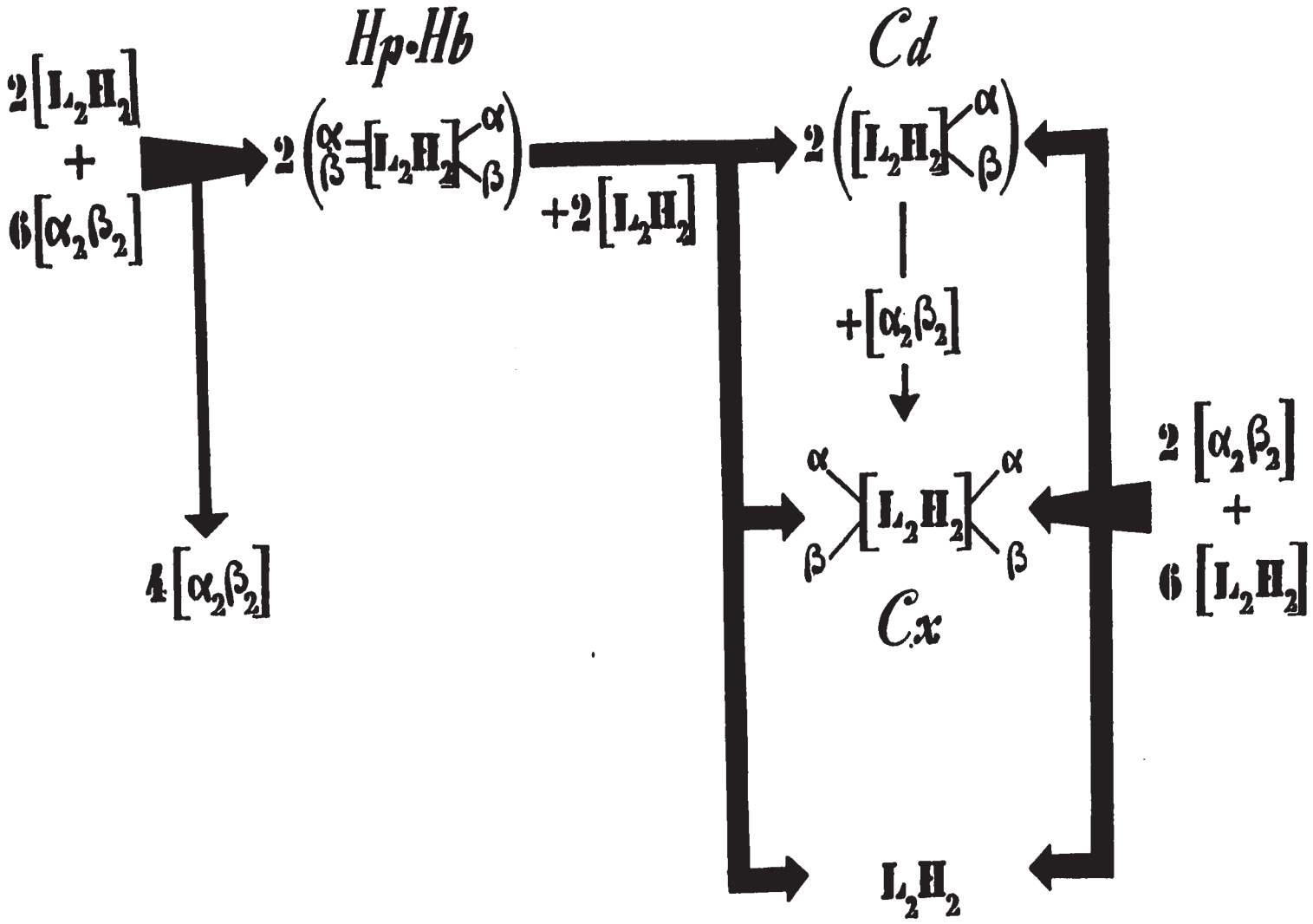
fitted the prediction almost perfectly and they concluded that two sites, each for an $\alpha\beta$ dimer must exist.

Studies on the binding behavior of isolated hemoglobin chains have yielded conflicting results. Nagel and Gibson (1967) found that β-chains did not bind unless α-chains had already bound. Chiancone et al. (1968) found binding by both types of chain, whether liganded or not. Since four α-chains would bind to Hp they concluded that Hp contained four non-specific binding sites, although complex with four β-chains was never observed. Similarly, Alfsen et al. (1970) have studied kinetics of binding by isolated chains and have concluded that four equivalent and independent sites of binding exist for α-chains. More recent studies by Boyd (1971) have indicated that the four α-chain binding sites are not independent but consist of two pairs, one pair of high affinity and one pair of low affinity. Furthermore, binding was strongly dependent upon Hp concentration and ionic strength. In any case, binding to form normal complex is the preferred pathway because four α-chains are not bound if β-chains are present and β-chains will displace two α-chains from complex saturated with α-chains (Chiancone et al., 1968).

There is evidence that more than one type of saturated complex can be formed. Brunori et al. (1968) reached this conclusion from their studies of oxidation-reduction potentials of complex. When the ratio of Hp to

Hb was 1:1, more than one type of complex was indicated. This question was investigated by Waks et al. (1969) using human haptoglobin and horse hemoglobin. In the presence of excess hemoglobin a 1:1 molar complex was formed, designated Hb•Hp in figure 8. In the presence of excess haptoglobin two types of complex were formed, one designated C_x which was saturated with Hb, and one designated C_d which was half saturated. The complex Hb•Hp produced C_x and C_d when exposed to excess haptoglobin and the half-saturated complex C_d formed C_x when exposed to Hb. Different reaction mechanisms were indicated in formation of different complexes. When Hb was added in small increments to Hp, the plot of protons released against protein concentration was linear with slope about 4; when Hp was added in small increments to Hb, the same plot was linear with slope about 1.5. Other evidence from kinetics of inhibition of peroxidase activity led them to postulate the scheme in figure 8. This scheme shows hemoglobin dimers bound to Hp by two different bonds in the case of Hp•Hb. One of these bonds was unstable in the presence of Hp. It is difficult to explain the failure of Hp•Hb to rearrange to the more stable C_x and it might also be expected to show exchange with other hemoglobin dimers, but Bunn (1967) found no exchange. It would seem that both bonds in Hp•Hb were formed irreversibly and this makes it even more difficult to understand any mechanism for its conversion to C_x and C_d in the presence

Figure 8: Scheme for mechanism of binding between hemoglobin and haptoglobin (Waks et al., 1969). Addition of excess hemoglobin to haptoglobin leads to the saturated complex Hp•Hb, which has two Hb dimers bound to Hp in different fashions. Exposure of this to Hp results in loss of one dimer to form C_d, and C_x when both dimers become bound in the stable manner. Once formed, the stable bond shown by diagonal lines is not altered by exposure either to Hb or Hp. The less stable bond shown by horizontal lines in Hp•Hb is not necessarily an intermediate because C_x and C_d were formed directly by mixing with Hp in excess.


Hp·Hb
Cd
2[L2H2]
+
6[α2β2]
4[α2β2]
+2[L2H2]
+[α2β2]
2 [α2β2]
+
6 [L2H2]
Cx
L2H2

of Hp. This scheme of Waks et al. (1969) will require confirmation.

Recent experiments by Pavlicek and Jaenicke (1971) on the binding of Hb by a mixture of Hp 2-1 and 2-2 have suggested that several intermediates are formed before saturation is reached, as indicated by levels of peroxidase activity in partially saturated complex. By adding small increments of Hb to a solution of Hp, saturated complex was formed in which 32 histidine residues had become unreactive to diazonium-1-H-tetrazole; by adding small increments of Hp to a solution of Hb, only 16 residues of histidine became unreactive at saturation. They have suggested that the former case is due to combination of Hp with dimers of Hb and that the latter case results from combination with intact tetramers.

The nature of the binding between Hb and Hp has therefore not been discovered, although a number of studies have attempted to explain it. It seems unlikely that binding will be fully understood until x-ray analysis becomes possible.

Early studies by van Royen (1950) suggested amino groups of hemoglobin might be involved since treatment with formaldehyde prevented formation complex with Hp. However, Fraenkel-Conrat (1957) has reported that reaction of HCHO with $-NH_2$ or -SH groups generates a reactive methylol derivative which can condense with amide, guanidyl, phenolic,

and heterocyclic groups to yield cross-linking methylene bridges. In view of this the effect of formaldehyde on binding cannot be attributed solely to steric effects at modified lysyl or valyl residues.

Shinoda (1965) partially trinitrophenylated amino groups of Hp with 2,4,6-trinitrobenzene-1-sulfonic acid and found that binding with hemoglobin was impaired. It was concluded that Hp amino groups play some essential role in the binding with Hb. However, this conclusion has not been supported by the extensive studies of Chan (1968). She was able to convert lysyl residues to homoarginine residues with the reagent 1-guanyl-3,5-dimethyl pyrazole nitrate. When this reaction was carried out on hemoglobin, haptoglobin and complex, the extent of reaction in complex agreed to within 3 per cent of that expected from reaction of the two proteins separately. She concluded that the area of contact between the two proteins must either be small or in regions lysine-deficient.

Kalous and Pavlicek (1965) have titrated Hb, Hp, and complex. Their results indicated no loss of carboxyl groups on complex formation but 26 histidine residues became buried. They were able to selectively destroy histidine or tyrosine by photooxidation and they found loss of peroxidase activity to parallel photooxidation of histidine in hemoglobin and tyrosine in haptoglobin. The validity of using peroxidase activity as a binding assay in the case of modified proteins

may, however, be questioned in view of the results of Dobryszycka et al. (1969). When tyrosine and tryptophan residues of Hp were modified with N-acetylimidazole and 2-hydroxy-5-nitrobenzyl bromide respectively, complex could be formed with hemoglobin as demonstrated by starch gel electrophoresis but no peroxidase activity was present. Taken together these results indicate that tyrosine residues of haptoglobin may not be important in linking the proteins together but rather have a role in the increased peroxidase activity of complex.

The role of reactive hemoglobin sulfhydryl groups in binding has been thoroughly studied by Bunn (1967). He reacted cysteine β-93 with several reagents specific for that residue and found no effect on binding. Although blocking the reactive sulfhydryl groups did not inhibit binding, binding has been found to inhibit reaction of these same groups with 2,2'-dithiopyridine, 4,4'-dithiopyridine, and ^{14}C-iodoacetamide (Malchy and Dixon, 1969).

Modifications with maleic anhydride

A severe modification for amino groups is their reaction with anhydrides. In the case of cyclic anhydrides the effect of this modification is to free a new carboxyl group and thus alter the charge in the region from positive to negative. Klotz (1967) has reviewed uses of succinic

anhydride and Butler et al. (1967) reported use of maleic anhydride for specific modification of amino groups in bovine chymotrypsinogen-A. Both Uyeda (1969) and Sia and Horecker (1968) reacted amino groups of oligomeric proteins with this reagent and reported dissociation.

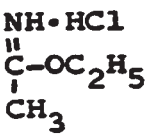
Cross-Linking reactions

The use of cross-linking reactions in the study of protein structure has been reviewed by Wold (1967) and early work has been discussed by Alexander et al. (1952). These reactions together with naturally occurring cross-links such as disulfides provide the only direct chemical means for determining distances between two residues. Reagents that have been discovered include maleimide derivatives, alkyl and aryl halides, isocyanates, imidoesters, glutaric dialdehyde, and others. Use of these reagents, particularly in experiments by Fasold (1965b), has largely confirmed the validity of crystal structure as a model for structure in solution. The azobenzene nucleus is a particularly desirable feature to include in a bifunctional reagent because of ease of cleavage to leave a label on each cross-linked residue.

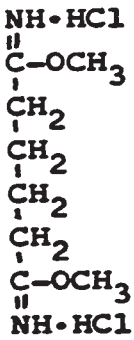
Amidination reactions

The search by Hunter and Ludwig (1962) for a group-specific reagent for protein modification without alteration in charge led them to investigate the formation of amidines by the reaction between imidoesters and amines.

The name imidoester refers to the class of compounds while specific compounds are called imidates, according to the extensive review by Roger and Nielson (1961). That is, compound I below is ethyl acetimidate HCl, the ethyl ester of the hypothetical imidic acid analogue of acetic acid, while compound II is dimethyl adipimidate dihydrochloride, the diester of the similar analogue of adipic acid.



Compound I



Compound II

Imidoesters are generally prepared by the condensation of a nitrile and an alcohol under anhydrous conditions in the presence of hydrogen chloride.

$$RCN + R'OH + HCl \longrightarrow RC\begin{matrix}=NH.HCl \\ \diagdown OR'\end{matrix}$$

In the presence of water these salts are readily hydrolyzed to the normal esters and ammonium chloride or they may decompose to the parent nitrile and alcohol. Hunter and Ludwig (1962) determined half-times for methyl acetimidate in the presence of several groups present in proteins as well as hydrolysis rates at various pH's at 25°C. It reacted rapidly with ϵ-aminocaproic acid and glycyl glycine in aqueous buffer at alkaline pH but did not react with N-acetylhistidine, sarcosine, p-hydroxyphenylacetic acid or hippurylarginine. Amidines formed are more basic than amines but less so than guanidines (Hunter and Ludwig, 1962); the chemistry of amidines has been reviewed by Shriner and Neumann (1944). The ϵ-NH_2 groups of lysine in insulin could be quantitatively amidinated as could the α-NH_2 group of glycine which is N-terminal in one chain, however phenylalanine which is N-terminal in the other chain was not completely reacted. Amidines do not react with 1-fluoro-2, 4-dinitrobenzene and the extent of modification can therefore be determined by reacting the amidinated protein with this reagent. Any dinitrophenyl amino acid so formed must represent a residue which failed to react with the imidoester. Amidination of lysyl groups of insulin rendered them completely resistant to the action of trypsin and negative to ninhydrin (Hunter and Ludwig, 1962).

Hand and Jencks (1962) investigated the mechanism of reactions of two aromatic imidates, ethylbenzimidate and ethyl m-nitrobenzimidate, with several amines and found that at alkaline pH, the rate-determining step was condensation of free amine with protonated imidoester to form a tetrahedral intermediate, followed by decomposition, mostly through a transition state with no net charge, to the amidine.

Ludwig and Byrne (1962) investigated regeneration of amino groups of acetamidino insulin by treatment with ammonia. They found that a solution of concentrated ammonia and ammonium acetate (ammonium hydroxide:glacial acetic acid, 15:1) completely removed the acetimidyl group so that the tryptic peptide map became indistinguishable from that given by unreacted insulin.

Wofsy and Singer (1963) used ethyl acetimidate HCl to amidinate bovine serum albumin and several antibodies. They found that antigenic activity of BSA, as indicated by precipitin determinations, was not altered significantly even by greater than 90 per cent amidination. They detected no major changes in physical properties and concluded that lysine residues were not vital components of reactive sites of the antibodies studied nor of the antigenic sites of BSA.

A detailed review of amidination giving procedural details has been published by Ludwig and Hunter (1967). The versatility of imidoesters is considerably enhanced by

the wide variety of nitriles which can be used to synthesize them. The nitriles in turn are conveniently made from corresponding alkyl chlorides by reaction with cyanide ion which is readily available with the ^{14}C isotopic label.

Several bifunctional imidoesters have been used. Dutton, Adams, and Singer (1966) prepared the first one, diethyl malonimidate dihydrochloride, and showed that extensive modification of both BSA and human gamma-globulin resulted in no loss of ability to combine with antibodies to the unmodified proteins and no change in electrophoretic mobility. Aggregates were formed by intermolecular cross-linking and this was enhanced by carrying out the reaction at higher protein concentration. A 20 per cent solution of BSA formed a solid gel after one hour reaction time.

The most comprehensive study involving a bifunctional imidoester has been that of Hartman and Wold (1967). They obtained an enzymatically hyperactive cross-linked derivative of bovine pancreatic ribonuclease A after reaction with dimethyl adipimidate. Direct analysis for cross-linked lysines was possible using the short column of an amino acid analyzer with a borate buffer pH 9.7. Analysis of the tryptic peptides allowed identification of two specific cross links, one from lysine 31 to lysine 37 and one from lysine 7 to lysine 37. Since the maximum distance the reagent can span is 8.6 Å, then it follows that lysine 37 is within 8.6 Å of both lysine 7 and lysine 31. Both these

cross-links were consistent with the interresidue distances shown by x-ray studies on RNase crystals.

More recently Davies and Stark (1970) have used dimethyl suberimidate to cross link subunits of several oligomeric proteins. Cross-linking was followed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In each case the number of major bands was equal to the number of subunits. When the mobility was used as a measure of molecular weight it was clear that cross-linked species had molecular weights that were integral multiples of the protomer molecular weight. They noted a small error in molecular weights, in the direction of higher weights than expected, but attributed this either to reduced binding of SDS by cross-linked species or to added molecular weight due to incorporation of reagent. Nevertheless their plot of log molecular weight against electrophoretic mobility was almost perfectly linear, in excellent agreement with the results of Weber and Osborn (1969).

Since the combination of cross-linking and SDS acrylamide gel electrophoresis is the major technique used in this investigation, it deserves some additional comments. SDS gel electrophoresis as developed by Shapiro et al. (1967), and by Weber and Osborn (1969) yields molecular weights within 10 per cent by simply plotting electrophoretic mobility against log M for known proteins and fitting the unknown protein to the standard curve so

obtained. It is an empirical technique without firm theoretical basis but Weber and Osborn (1969) showed it to be highly accurate for some 40 well characterized proteins in their study. Fish, Reynolds, and Tanford (1970) have begun studies on the physical properties of protein-SDS complexes and have concluded that they form rod-like particles in solution. The hydrodynamic properties of protein-SDS complexes are a unique function of the polypeptide chain length (Reynolds and Tanford, 1970). As a rod-like particle becomes shorter it begins to approximate a sphere when the length approaches the diameter in magnitude. Fish, Reyholds and Tanford (1970) have stated that "this phenomenon sets a lower limit of about 15,000 to molecular weights that may be estimated reliably by gel filtration and related methods in SDS solution". Swank and Munkres (1971), however, have obtained molecular weights for peptides as low in molecular weight as 1,225 by SDS gel electrophoresis although accuracy was reduced and there was a marked inflection in their plots in the 10,000-12,000 range. The presence of cross-links such as disulfides might be expected to reduce ability to form a rod-like particle and Pitt-Rivers and Impiombato (1968) have observed large reductions in the amount of SDS bound by unreduced proteins. Regarding this, Fish, Reynolds and Tanford (1970) have stated, without performing any gel electrophoresis, that "gel chromatography or gel electrophoresis of unreduced proteins in SDS solution cannot, in

general, be used to obtain estimates of molecular weight". The plots obtained by Davies and Stark (1970) after cross-linking with dimethyl suberimidate tend to conflict with this. This generalization has been even more stringently tested by Dunker and Rueckert (1969) who compared several reduced and unreduced proteins and found excellent agreement. Even bovine serum albumin with 17 disulfides had an error of only 6 per cent when it was not reduced, as compared with an error of 2 per cent when it was reduced.

Modifications with bis(N-maleimidomethyl)ether (BME)

Simon and Konigsberg (1966) first used this reagent for modification of hemoglobin. A reaction product containing 2 moles of reagent per hemoglobin tetramer ($Hb\text{-}BME_2$) was obtained but none containing 1 mole of reagent was found. Sedimentation velocity experiments in 2.5 M guanidine hydrochloride indicated that it could still dissociate into dimers. Treatment of Hb-BME with PMB and chromatography on carboxymethyl cellulose yielded a peak containing 83 per cent β-chains having 3.47 moles BME per 66,000 molecular weight, plus poor yield of a second peak having no substituent. They concluded that the reagent had been bound specifically to the reactive sulfhydryl at position β-93 and that no interchain cross-links had been formed. Further studies by Simon et al. (1967) have characterized this derivative

crystallographically and have shown for horse Hb-BME_2 that it remains locked in the liganded configuration regardless of the presence of ligand. It has been this lock which allowed Perutz (1970a) to determine the mechanism of hemoglobin action in exact molecular detail. Simon et al. (1967) also studied the ability of human and horse hemoglobins, treated with BME, to dissociate into dimers in a dissociating medium, 0.25 M $MgCl_2$. They found that human Hb-BME_2 could no longer dissociate but that horse Hb-BME_2 did dissociate to the same extent as unreacted horse Hb.

In his study of the effects of sulfhydryl group modifications of hemoglobin on haptoglobin binding, Bunn (1967) determined the binding properties of human Hb-BME_2. He found that they were reduced to about one quarter of normal values and he concluded that this was probably due to reduced dissociation into dimers. In addition, human Hb-BME_2 was readily displaced from Hp by unreacted Hb.

It has been established that BME causes conformation restraint in tetramers of horse Hb-BME_2 (Perutz, 1970a) but they still dissociate into dimers. There has been no explanation for the failure of human Hb-BME_2 to dissociate in 0.25 M $MgCl_2$; in both hemoglobins reaction of one maleimide ring of BME is with sulfhydryls at position 93 of β-chains (Simon and Konigsberg, 1966; Perutz, 1970a). It is unlikely that human Hb-BME_2 contained a large

proportion of interchain bridges because it did dissociate in 2.5 M guanidine HCl (Simon and Konigsberg, 1966).

Chemical studies of Hb-βME_2 have been reported by Arndt et al. (1971). They obtained peptides containing a cross-link between residues cysteine β-93 and histidine β-97 and these could account for 70 per cent of the total radioactivity. They concluded that other cross links were unlikely although they were unable to conclusively rule them out.

MATERIALS AND METHODS

Chemicals

The following chemicals were obtained from Eastman Organic Chemicals: ethyl acetimidate HCl, 1,4-dichlorobutane, maleimide, N,N'-methylenebisacrylamide, N,N,N'N'-tetramethylethylenediamine, naphthalene, and 2-mercaptoethanol. Labelled compounds, ^{14}C-sodium cyanide and ^{14}C-formaldehyde, were from New England Nuclear. Sodium dodecyl sulfate was purchased from Matheson, Coleman and Bell; scintillation counting reagents were from Packard. For reacting amino groups, 1-fluoro-2,4-dinitrobenzene was obtained from British Drug Houses and 1-dimethylamino-naphthalene-5-sulfonyl chloride (dansyl chloride) from Mann Research Laboratories. Nutritional Biochemicals Corp. supplied ε-N-dinitrophenyl-L-lysine, and Sigma Chemical Co. supplied p-chloromercuribenzoate (sodium).

All other chemicals were reagent grade.

Chromatography supplies

Sephadex gels were obtained from Pharmacia (Canada); Bio-Gel P-150 and Bio-Rex 70 were from Bio-Rad Laboratories;

DEAE-cellulose (DE-52) was from Whatman.

For thin layer chromatography of dansyl derivatives, polyamide sheets were obtained from the Cheng Chin Trading Co., Taiwan.

Elemental analysis

Elemental analyses were performed by Microanalysis Laboratories Ltd., Toronto, Ontario.

Preparation of haptoglobin

Fresh whole pig blood was collected using as anti-coagulant either 50 ml saturated sodium oxalate or 150 ml standard acid citrate dextrose per liter. (22.0 g trisodium citrate dihydrate, 8.0 g citric acid monohydrate, 24.5 g dextrose monohydrate, made to 1 liter with water) (Damm and King, 1965). After standing at 4°C for several hours the upper plasma layer was decanted and centrifuged at 6,000 rpm for 10 minutes at 0°C to clear it of cells. Plasma pH was then adjusted to 4.7 with acetic acid and it was recentri-fuged to remove precipitated material.

Plasma was deionized in 500 ml lots on 8.5 x 60 cm columns of Sephadex G-25 equilibrated in distilled water saturated with toluene. Haptoglobin in salt-free plasma was then adsorbed on DEAE-cellulose by either of two pro-cedures. For some preparations an 8.5 x 20 cm column of DEAE-cellulose was equilibrated with 5 mM sodium acetate

pH 4.7 at 4°C. After application of up to 1.3 liters of desalted plasma, this column was washed with equilibration buffer made to 0.01 M with NaCl until effluent absorbance had fallen below 0.1. Haptoglobin was then eluted by making the buffer to 0.1 M with NaCl or with a linear gradient from 0.01 M to 0.2 M in NaCl. This procedure gave satisfactory purification but yields were low. For most preparations a batch procedure was used at room temperature for reasons of speed, convenience, and yield. DEAE-cellulose, equilibrated in 5 mM sodium acetate at pH 4.7, was suspended in desalted plasma at the same pH and allowed to adsorb protein for 10 minutes. Adsorbent was removed by vacuum filtration and washed with two 2-liter changes of starting buffer made to 0.05 M with NaCl. Haptoglobin was then eluted by stirring the DEAE-cellulose with the same buffer made to 0.5 M in NaCl. This procedure allowed time of exposure to DEAE-cellulose to be kept under 1 hour and resulted in recovery of 80-90 per cent of starting activity.

Haptoglobin solution was cooled to 0°C and sufficient solid ammonium sulfate was added to give 55 per cent saturation (Di Jeso, 1968). After centrifugation, haptoglobin present in the supernatant was precipitated by increasing ammonium sulfate to 70 per cent saturation. This precipitate was redissolved, dialyzed against several 6-liter changes of distilled water, and concentrated by ultrafiltration through

a Diaflo XM-50 membrane. This solution, in 60-ml lots, was passed down a 5 x 100 cm column of Sephadex G-200 superfine equilibrated in 0.1 M Tris-HCl, 0.1 M NaCl, and 0.02% sodium azide, pH 7.4, with a flow rate of 8-10 ml per hour at room temperature. Following this, haptoglobin was dialyzed against distilled water and lyophilized. These procedures are essentially those of Fraser and Smith (1971) as adapted from methods for preparing human haptoglobin (Connell and Shaw, 1961).

For some experiments a final purification step of preparative polyacrylamide gel electrophoresis was performed using a Canalco Prep Disc apparatus and the methods of Davis (1964). This removed the last traces of impurities.

Determination of haptoglobin concentration

Haptoglobin in solutions was measured by its ability to bind a known amount of hemoglobin. This was routinely determined by upward flow gel filtration on a 1 x 30 cm or a 1 x 100 cm column of Sephadex G-100 equilibrated in 0.15 M NaCl. Column effluent was continuously monitored for heme at 412 or 416 nm with a Beckman DB spectrophotometer equipped with a flow cell and a Sargent recorder. Two peaks were obtained, hemoglobin-haptoglobin complex and excess hemoglobin, and these were integrated graphically to give the proportion of hemoglobin bound. Accurate determination of

original hemoglobin concentration and use of a constant volume Chromatronix sample injecting valve allowed calculation of amounts of complex and hence of haptoglobin concentration, assuming 1:1 stoichiometry. For some determinations column effluent was monitored simultaneously for absorbance at 280 nm as well as for heme by including use of a Beckman DB programmer.

Preparation of hemoglobin

Citrated whole human blood was obtained from the Red Cross blood bank, within 21-30 days after collection. After centrifuging at 5,000 rpm for 10 minutes at 0°C, plasma was discarded and cells were resuspended in approximately 5 volumes of cold 1 per cent NaCl. Several such washes were performed before cells were taken up in approximately 1 volume of distilled water. A few drops of toluene were added to hasten hemolysis and the suspension was allowed to stand at 4°C for 2 hours with periodic shaking. It was then filtered through several layers of cheese cloth and centrifuged twice, first at 5,000 rpm for 10 minutes at 0°C to remove most particulate matter and then in fresh tubes at 20,000 rpm for 30 minutes at 0°C to remove finest particles. These procedures were modified from those of Drabkin (1946) and resulted in solutions approximately 10 per cent in hemoglobin.

Carboxyhemoglobin was prepared by bubbling carbon monoxide gas through fresh preparations of oxyhemoglobin.

Similar procedures were used for preparation of horse hemoglobin except that blood was freshly drawn.

Determination of hemoglobin concentration

Hemoglobin concentrations were determined as oxyhemoglobin using an extinction coefficient of 1.53×10^4 M^{-1} cm^{-1} per heme group at 540 nm (Benesch, MacDuff, and Benesch, 1965). Concentrations were more often determined as methemoglobin cyanide (converted with Hycel Cyanmethemoglobin Reagent) using a commercial methemoglobin cyanide preparation (Hycel) as standard.

Preparation of hemoglobin-haptoglobin complex

Hemoglobin-haptoglobin complex was readily prepared by adding a 3-fold molar excess of hemoglobin to purified haptoglobin and passing this through the same 5 x 100 cm column of Sephadex G-200 superfine used in the final stage of haptoglobin isolation.

Synthesis of dimethyl adipimidate dihydrochloride (DMA)

Labelled dimethyl adipimidate dihydrochloride was synthesized essentially by the methods of Hartman and Wold (1967).

One millicurie of ^{14}C-sodium cyanide was dissolved in 5 ml dimethyl sulfoxide and then heated to 90°C with stirring. Then 540 mg of 1,4-dichlorobutane were added; a small rise in temperature was noted. After a few minutes the solution had cooled to 90°C but it was not allowed to cool further. After half an hour 500 mg unlabelled sodium cyanide were added; there was an increase in temperature to 130-140°C and it was not allowed to fall below 130°C for one half hour. Heating was then stopped and the solution was allowed to cool to room temperature. It was then extracted with three washes each consisting of 20 ml chloroform and 30 ml half-saturated sodium chloride. The combined chloroform fractions were dried over sodium sulfate and evaporated to constant volume under reduced pressure at 50°C to yield 550-600 mg crude adiponitrile. Infrared spectra revealed a strong characteristic nitrile peak, with contamination by chlorinated hydrocarbon and another material, thought to be dimethyl sulfoxide.

The entire yield of crude adiponitrile was dissolved in a solution of 8 ml dioxane and 1 ml methanol at 0°C. Anhydrous hydrogen chloride gas was bubbled through this solution for 15 minutes and it was then allowed to stand tightly closed overnight at 0°C. Product was precipitated by addition of 15 ml cold diethyl ether and collected by vacuum filtration on a Buchner funnel. It was washed

immediately with 200 ml cold methanol:ether (1:3) with minimum exposure to air. Crystals were then washed through the filter with cold methanol and precipitated with 3 volumes of cold ether. This was repeated until a constant melting point of 218-220°C (adipamide) was reached. This procedure always yielded 650-700 mg DMA of specific activity near 1.4×10^6 dpm per mg.

Elemental analysis: (per cent)

	Calculated	Found
C	39.19	38.98
H	7.40	7.14
N	11.43	11.10
O	13.05	13.48
Cl	28.93	29.22

Synthesis of bis(N-maleimidomethyl)ether (BME)

Bis(N-maleimidomethyl)ether was synthesized by the method of Tawney et al. (1961).

To 8.1 ml 37 per cent aqueous formaldehyde were added 9.8 g maleimide and 0.3 ml 5 per cent sodium hydroxide at room temperature. The maleimide dissolved in a few minutes with a small temperature rise to 32°C. After standing three hours at room temperature, product was collected by filtration and recrystallized from ethyl acetate to yield 4.07 g N-methylolmaleimide, mp 97-99°C. This product was

heated under reflux for 7 hours with 20 ml toluene and 100 mg p-toluenesulfonic acid to yield an insoluble product which was collected by filtration. Three recrystallizations from ethanol yielded 800-850 mg BME, mp 127-129°C.

Elemental analysis: (per cent)

	Calculated	Found
C	50.86	52.09
H	3.41	3.27
N	11.86	11.69
O	33.87	33.16

For preparation of radioactive BME, the formaldehyde was supplemented with 250 microcuries ^{14}C-formaldehyde. This gave BME of specific activity near 47,800 dpm per mg.

Reaction of hemoglobin with maleic anhydride

Maleic anhydride was reacted with hemoglobin essentially by the method of Uyeda (1969).

Solid maleic anhydride (1.586 g) was reacted with 500 mg hemoglobin in 50 ml 0.1 M borate buffer pH 9.2 with stirring at 0°C. The reagent was added in several small lots with addition of 50 per cent sodium hydroxide as required to maintain the pH. One half hour after addition of the last lot of reagent, the solution was dialyzed against three 6-liter changes of 0.1 M phosphate buffer pH 7.1 and then concentrated by ultrafiltration through a Diaflo UM-2 membrane.

Reaction of hemoglobin with ethyl acetimidate HCl

Ethyl acetimidate HCl was reacted with hemoglobin using procedures modified slightly from those of Wofsy and Singer (1963).

Ethyl acetimidate HCl (380 mg) was added in small lots to a solution of 0.2 per cent hemoglobin in either 0.1 M phosphate or 0.1 M borate at pH 9.5 at room temperature. With each addition of reagent sufficient 0.5 N NaOH was added to maintain the pH. One half hour after addition of the last lot of reagent, the solution was dialyzed and concentrated as with the maleyl derivative.

For those preparations to be used in studies of oxygen equilibria, amidination was carried out in the same manner except that temperature was maintained at 0°C in order to minimize formation of methemoglobin.

Reaction of hemoglobin with dimethyl adipimidate (DMA)

Procedures for cross-linking hemoglobin were adapted from those of Hartman and Wold (1967).

Hemoglobin was reacted with DMA at a variety of molar ratios, reaction times, and hemoglobin concentrations. This led to selection of a hemoglobin concentration of 0.1 per cent for reaction with an amount of reagent equal to ten times the lysine content. To react 50 mg of hemoglobin, dilution was made to 1 mg per ml with 0.1 M borate pH 9.5.

This amount of hemoglobin required 84 mg DMA, which was added in several portions at 10-minute intervals. Addition of base was seldom necessary because buffering capacity accommodated this amount of reagent without change in pH. One hour after addition of the last lot of reagent the solution was either dialyzed or passed through a column of Sephadex G-25. Even when dialysis was used, a small portion was passed through the column so that free and bound reagent could be determined readily from radioactivity levels.

Reaction of haptoglobin and hemoglobin-haptoglobin complex with DMA

Identical conditions were used for these reactions as for those with hemoglobin except that protein concentrations were adjusted to keep them the same on a molar basis as hemoglobin at 1 mg per ml. That is, haptoglobin, with molecular weight near 98,000, was reacted at 1.5 mg per ml with sufficient reagent for 66 lysine residues per molecule (Black et al., 1970). Complex, with molecular weight near 165,000, was reacted at 2.5 mg per ml with sufficient reagent for 110 lysine residues per molecule (44 from Hb and 66 from Hp). Following reaction treatment was as with hemoglobin.

Reaction of hemoglobin with bis(N-maleimidomethyl)ether (BME)

Hemoglobin was reacted with BME by the methods of Simon and Konigsberg (1966).

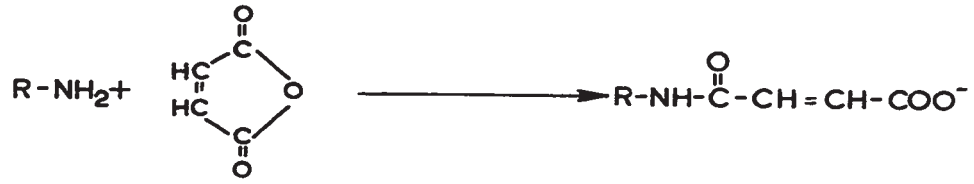
Solid BME was added to solutions 4 per cent in horse oxyhemoglobin or horse carboxyhemoglobin with stirring at 4°C for 24 hours. The amount of reagent was twice the molar quantity of hemoglobin tetramer. Following reaction, the mixture was chromatographed on a 2 x 50 cm column of Bio-Rex 70 using a discontinuous sodium phosphate buffer gradient at pH 6.8 (Simon and Konigsberg, 1966). These same procedures were used for reacting human carboxyhemoglobin with BME.

The various chemical modifications used in these studies have been summarized in figure 9.

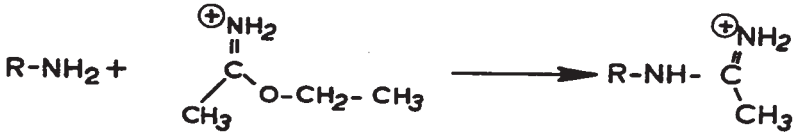
Amino acid analysis

Dinitrophenylation with 1-fluoro-2,4-dinitrobenzene (FDNB) was performed in bicarbonate buffer. Following acidification and extraction with ether, proteins were hydrolyzed in 6N HCl in sealed evacuated tubes for 16-18 hours at 110°C. Amino acid analyses were performed on a Beckman 120-C amino acid analyzer using recommended procedures. For basic amino acids an 18-cm column eluted with citrate buffer pH 5.26 was used to obtain better separation between arginine and ϵ-DNP-lysine.

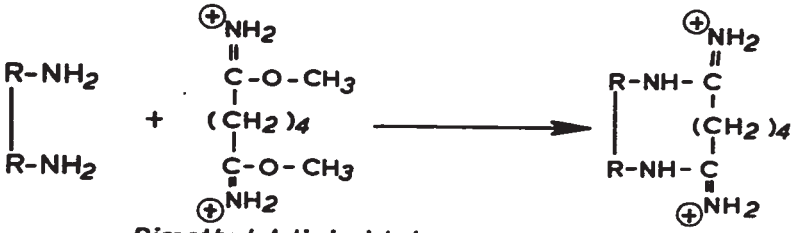
Figure 9: Summary of reactions used to modify proteins for these studies.



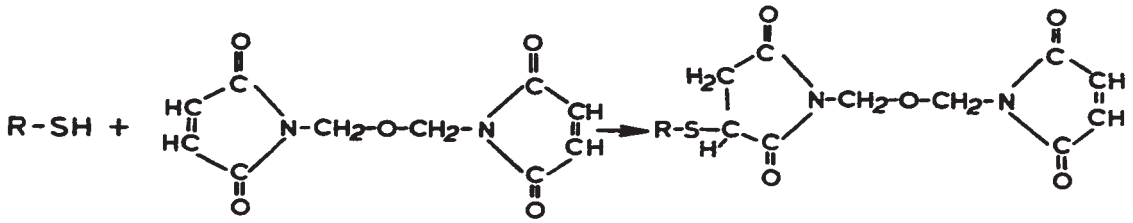
Maleic Anhydride



Ethyl Acetimidate



Dimethyl Adipimidate



bis (Maleimidomethyl) Ether

Determination of sulfhydryl groups with p-chloromercuribenzoate (PMB)

Sulfhydryl groups were titrated with PMB by the method of Boyer (1954). PMB reagent was prepared by dissolving p-chloromercuribenzoate in water made alkaline with NaOH. When this was acidified with HCl, the reagent precipitated and was collected by centrifugation. After three such precipitations an alkaline solution of reagent was diluted with phosphate buffer pH 7.0 and its concentration was determined using a molar extinction coefficient of 1.69×10^4 M^{-1} cm^{-1} at 232 nm.

Hemoglobin solutions in phosphate buffer pH 7.0 were placed in both cuvettes of a Beckman DB spectrophotometer. To the sample cuvette was added PMB reagent in several small increments. By determining the absorbance at 250 nm after each addition, two straight lines were obtained when absorbance (corrected for dilution) was plotted against volume added, that of larger slope resulting from mercaptide formation before saturation of sulfhydryl groups, and that of smaller slope resulting from addition of excess reagent. The point of intersection of these lines was taken as the volume of reagent required to titrate all available sulfhydryl groups and this allowed calculation of the number of such groups reacted in hemoglobin.

Determination of oxygen equilibria

The pH dependence of the oxygen equilibrium of amidinated hemoglobin was compared with unreacted hemoglobin. For studies at pH's 6.0, 6.8, and 7.5, 0.1 M phosphate buffers were used; for pH 9.2, 0.05 M borate was used. Concentrated protein solutions were diluted in a tonometer similar to that of Rossi-Fanelli and Antonini (1958) (except for a second side arm containing a serum stopper) with the required amount of buffer to yield 1.1 ml of solution. To this 0.1 ml of 0.05 M 2,3-diphospho-D-glyceric acid was added. Deoxygenation was accomplished by evacuation and equilibration in a 30°C water bath until a criterion for deoxygenation of $A_{555}/A_{540} \geq 1.24$ was satisfied (Benesch, MacDuff, and Benesch, 1965). After recording its spectrum from 480 nm to 640 nm with a Spectronic 505 spectrophotometer, air was added from a gas tight Hamilton syringe through the serum stopper. After 5 minutes equilibration by mechanical rotation in the water bath, the mixture was assayed spectrophotometrically for relative amounts of oxyhemoglobin and deoxyhemoglobin, using the equations of Benesch, MacDuff, and Benesch (1965).

$$\% \text{ oxyhemoglobin} = \frac{(A_{540} - A_{540}^{deoxy}) + (A_{560}^{deoxy} - A_{560})}{(A_{540}^{oxy} - A_{540}^{deoxy}) + (A_{560}^{deoxy} - A_{560}^{oxy})} \times 100$$

$$\% \text{ oxyhemoglobin} = \frac{(A_{576} - A_{576}^{deoxy}) + (A_{560}^{deoxy} - A_{560})}{(A_{576}^{oxy} - A_{576}^{deoxy}) + (A_{560}^{deoxy} - A_{560}^{oxy})} \times 100$$

Several additions of air permitted determination of fractional saturation with hemoglobin as a function of partial pressure of oxygen. Partial pressures of oxygen were determined from the barometric pressure, the volume of air admitted, and the gas volume in the tonometer. Tonometer volumes were determined from weights empty and completely filled with water (density corrected to ambient temperature). When oxygen affinities were high, pO_2 values were also corrected for oxygen already bound by oxyhemoglobin. Logs of fractional saturations were plotted against logs of partial pressures of oxygen, and that pressure corresponding to a mixture half oxy- and half deoxyhemoglobin, log $P_{\frac{1}{2}}$ (the usual measure of the oxygen affinity of hemoglobin), was read from these plots. Log $P_{\frac{1}{2}}$ values could then be plotted as a function of pH.

Analytical gel filtration

Analytical gel filtration was carried out using Bio-Gel P-150 polyacrylamide beads, 100-200 mesh size, which had been swollen 72 hours in 0.05 M phosphate buffer pH 5.5 made to 0.1 M in KCl. Fine particles were removed by allowing swollen gel suspension to settle until about 90 per cent formed a layer at the bottom; the remainder containing fine particles was removed by suction. After thorough removal of fines, deaeration was accomplished by vacuum and gel was

poured into a 0.9 x 50 cm jacketed Chromatronix column which had previously been coated with Fisher Dricote. By adding a reservoir to the column top all gel suspension needed could be poured in one operation. After settling with a flow rate of approximately 3 ml per hour, a flow adaptor was fitted to the inlet, the column was inverted, and samples were injected by means of a Chromatronix injecting device so that volumes were always the same. Flow rate was maintained constant by an LKB ReCyChrom peristaltic pump and effluents were monitored with a Beckman DB spectrophotometer equipped with a flow cell and a Photovolt recorder. All tubing was fine teflon with the exception of a short piece of Technicon tygon used in the pump. Effluents were collected in a burette tube and volumes were marked on the chart paper from time to time. This allowed reproducibility within about 1 ml on duplicate runs of the same sample.

Liquid scintillation counting

Radioactivity counts were made with a Nuclear Chicago Mark I Liquid Scintillation Counter equipped with 3 channels and an external standard. A correction curve of efficiency against channel ratio for the external standard was prepared using a series of ^{14}C quenched standards in toluene-based scintillation fluid. This allowed determination on dpm in samples of DMA or BME. For samples containing protein,

Brays fluid was used with hyamine hydroxide as solubilizing agent. A similar curve was obtained for efficiency corrections by quenching known amounts of DMA or BME in Brays fluid with water.

Ultrafiltration

Samples were concentrated in Amicon ultrafiltration cells using XM-50 membranes for haptoglobin and hemoglobin-haptoglobin complex; for hemoglobin, UM-2 membranes were used.

Polyacrylamide gel electrophoresis

Analytical scale electrophoresis was performed by the method of Davis (1964). Gels were stained with 1 per cent Napthol blue black in 7% acetic acid and destained by transverse electrophoresis. On a preparative scale the same procedures were followed except that a Canalco Prep Disc apparatus was used and protein in the eluate was detected by absorbance at 280 nm.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS)

SDS acrylamide gel electrophoresis was performed by the methods of Weber and Osborn (1969). For some separations the amount of N,N'-methylenebisacrylamide was reduced

to 3/4 or 1/2 that used by these authors, and for separation of cross-linked complex, both acrylamide and cross-linker were reduced by half. Gels were stained with Coomassie blue and destained by transverse electrophoresis.

Ultracentrifugation

Sedimentation velocity studies were carried out in a Spinco model E analytical ultracentrifuge at 20°C, and 59,780 rpm. Photographs of schlieren patterns were measured from reference hole to maximum ordinate using a Nikon shadowgraph. For studies on dilute haptoglobin solutions a 30 mm cell was used for scanning by ultraviolet absorption optics. With this rotor, maximum speed was 50,740 rpm and distances were measured directly from the chart.

Dansyl chloride procedure

To approximately 300 micrograms of haptoglobin in 20 microliters 0.2 M sodium bicarbonate was added 20 microliters of a dansyl chloride solution (2.5 mg per ml in acetone). This was covered and allowed to stand at 37°C for 1 hour, then evaporated to dryness and hydrolyzed with 6 N HCl for 18 hours at 110°C. The hydrolysate was evaporated to dryness, taken up in 20 microliters 50% pyridine and spotted on one surface of a polyamide sheet. To the reverse surface were spotted standards of dansyl amino acids.

Chromatography in the first dimension was in a solvent containing 200 ml water and 3 ml 90% formic acid. Chromatography at right angles was in a solvent containing 180 ml benzene and 20 ml glacial acetic acid. After drying, spots were visible under ultraviolet light.

Freeze drying

Dialyzed protein solutions were frozen in freeze-drying flasks and lyophilized in a Thermovac model F.D. 6 Lyophilizer at -50°C under vacuum of 40-60 microns of Hg for 24 hours.

Performic acid oxidation

Performic acid oxidation of haptoglobin was carried out by the method of Hirs (1967).

RESULTS AND DISCUSSION

Purification of haptoglobin

Haptoglobin was purified by the methods of Fraser and Smith (1971). Yields were near quantitative until the DEAE-cellulose chromatography step, shown in figure 10. Yield from this particular column was only 20.5 per cent of starting activity although good enrichment was obtained. Using a batch procedure, purification was less satisfactory but yields of binding activity were greater when time of exposure to the adsorbent was kept to a minimum. Following chromatography or adsorption, haptoglobin-rich fractions were pooled and precipitated between 55 and 70 per cent saturation with ammonium sulfate at 0°C. The precipitate was redissolved, dialyzed, and concentrated before gel filtration on Sephadex G-200 superfine. Figure 11 shows a sample elution profile with haptoglobin obtained in good yield, quite well separated from a large trailing peak, thought to be albumin. The purity of the preparation at this stage was assayed by polyacrylamide gel electrophoresis. Figure 12 shows a tris-glycine gel of Hp, and SDS gel of Hp,

Figure 10: Elution profile of 1.3 liters desalted pig plasma from 8.5 x 20 cm column of DEAE-cellulose at 4°C.
Flow rate was approximately 200 ml per hour and fractions of 15 ml were collected.
Open circles, absorbance at 280 nm.
Filled circles, micrograms Hb bound by 0.5 ml of fraction.
Triangles, conductivity in reciprocal ohms x 10^{-3}.

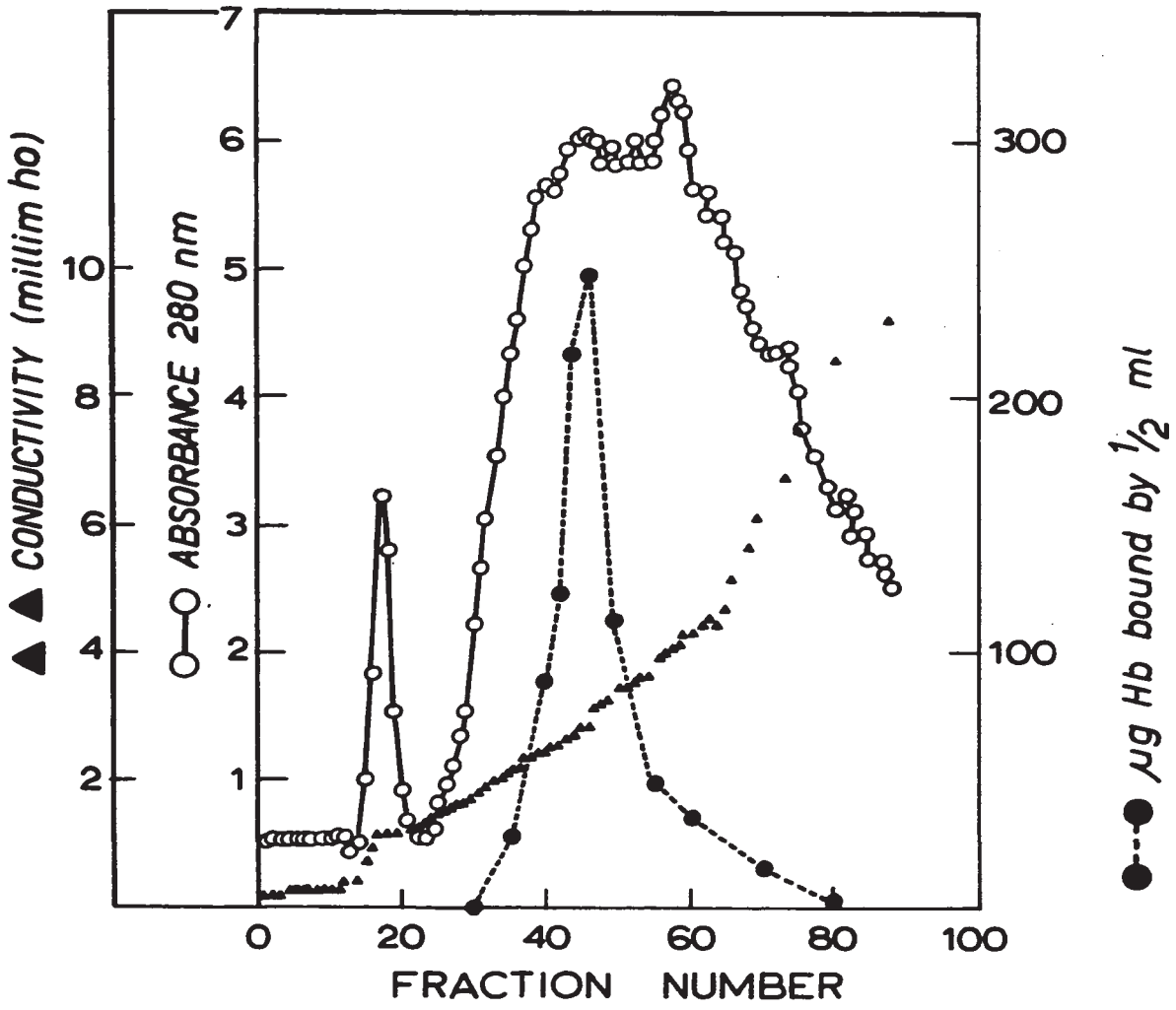

▲ ▲ CONDUCTIVITY (millim ho)
ABSORBANCE 280 nm
μg Hb bound by 1/2 ml
FRACTION NUMBER
0
20
40
60
80
100
1
2
3
4
5
6
7
2
4
6
8
10
100
200
300

Figure 11: Elution of haptoglobin from 5 x 100 cm column of Sephadex G-200 superfine equilibrated in 0.1 M Tris-HCl, 0.1 M NaCl, pH 7.4. Flow rate was 8-10 ml per hour and fractions of 4 ml were collected.
Open circles, absorbance at 280 nm.
Closed circles, haptoglobin concentration as determined by hemoglobin binding capacity.

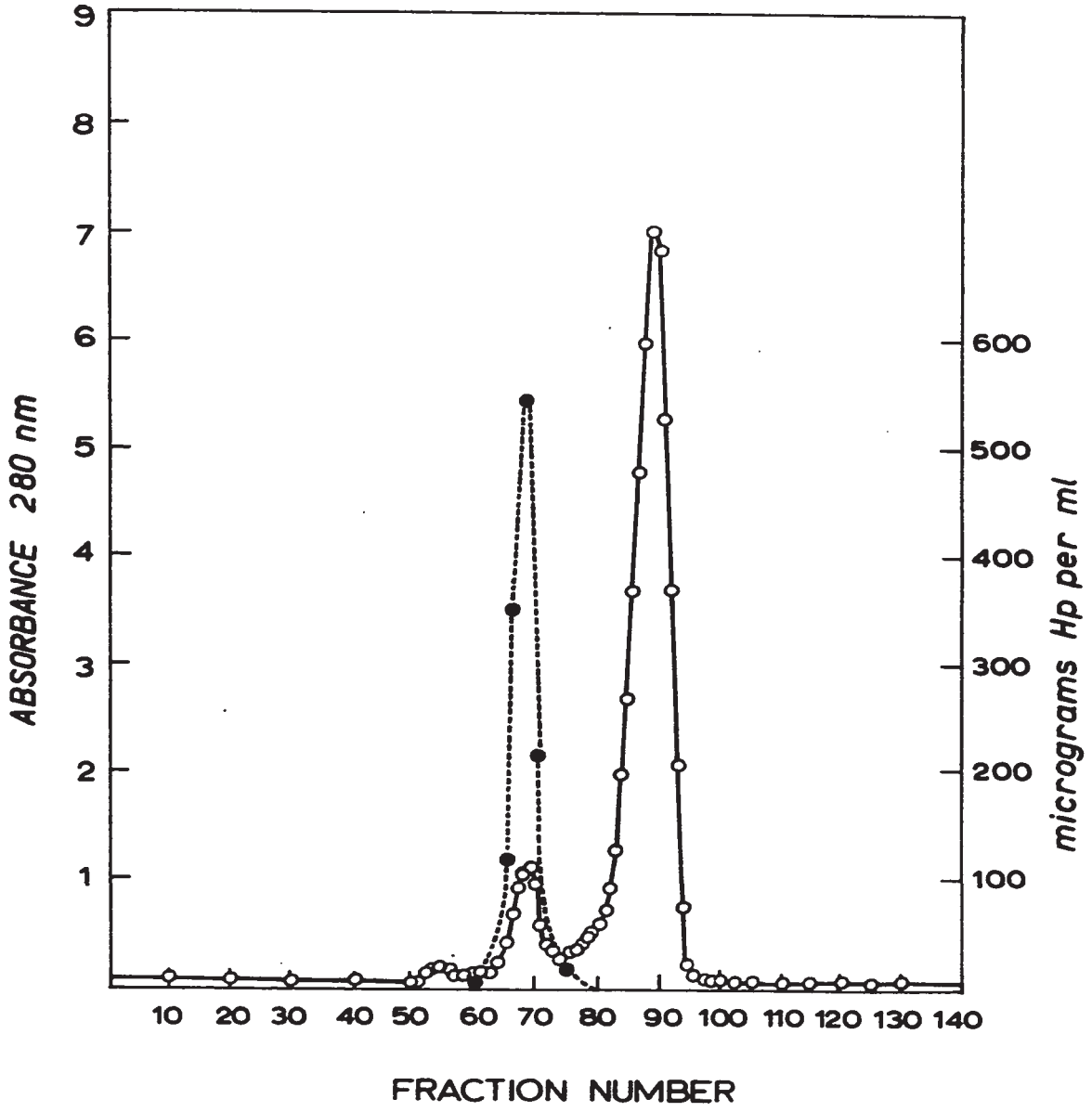
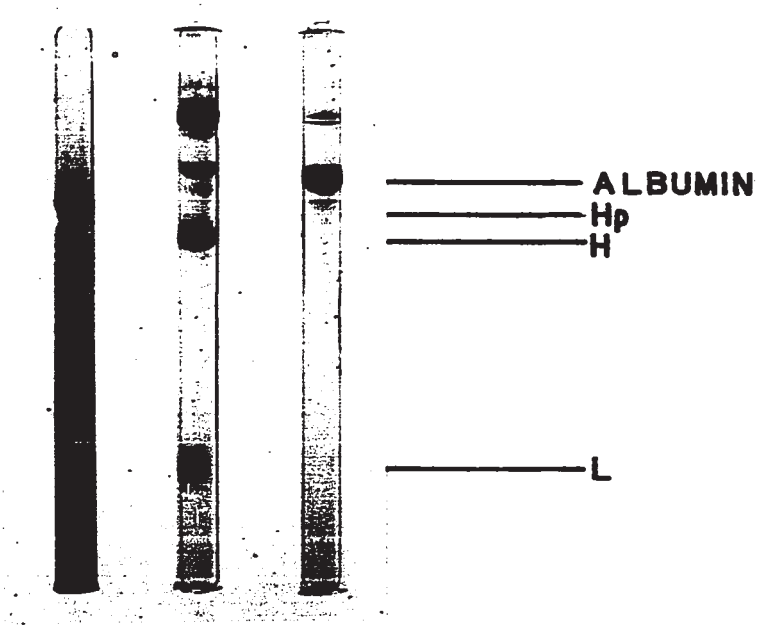
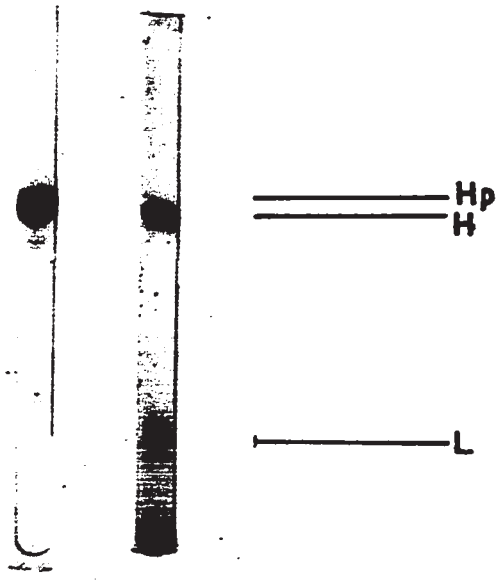

ABSORBANCE 280 nm
micrograms Hp per ml
FRACTION NUMBER
1
2
3
4
5
6
7
8
9
100
200
300
400
500
600
10 20 30 40 50 60 70 80 90 100 110 120 130 140

Figure 12: Polyacrylamide gel analysis of semi-pure haptoglobin.
Left, Tris-glycine gel of haptoglobin.
Center, SDS gel of haptoglobin.
Right, SDS gel of bovine serum albumin.

Figure 13: Polyacrylamide gel analysis of pure haptoglobin.
Left, Tris-glycine gel of haptoglobin.
Right, SDS gel of haptoglobin.


ALBUMIN
Hp
H
L


Hp
H
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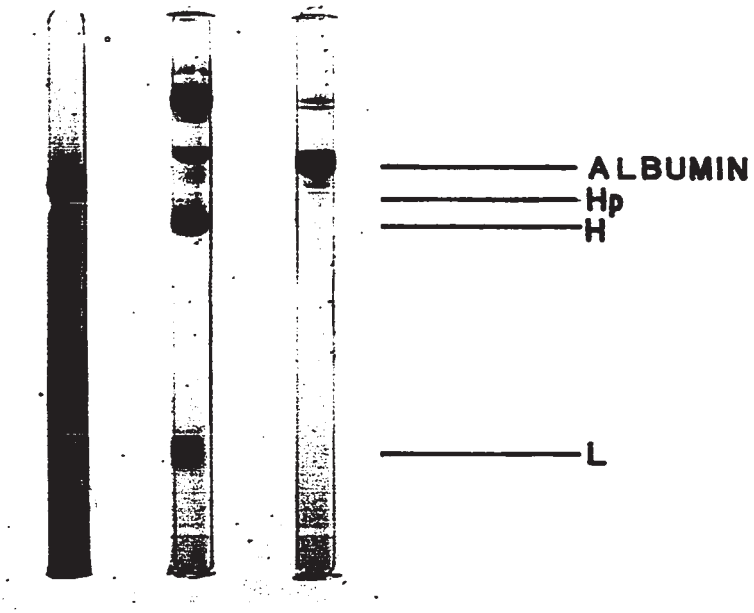
ALBUMIN
Hp
H
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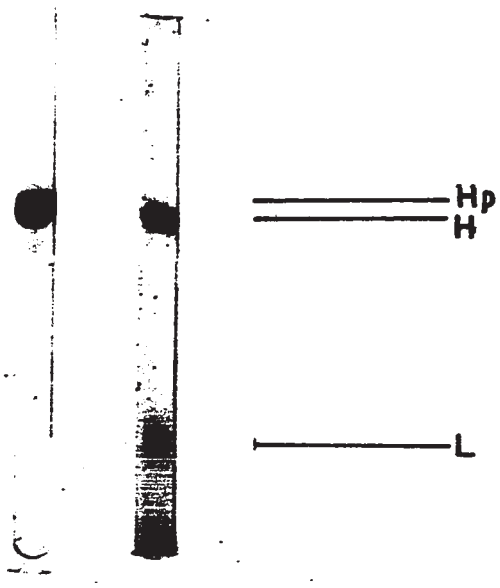

Hp
H
L

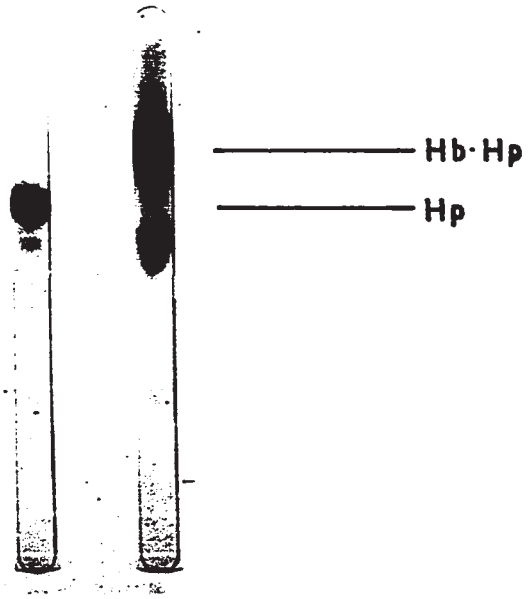
and an SDS gel of bovine serum albumin. A variety of impurities were still present; the major one was tentatively identified as albumin because of similar mobilities in SDS gels, and it will be designated thus subsequently although there has been no positive identification. For most experiments this preparation was satisfactory and it was routinely used for assaying binding properties of modified hemoglobin derivatives. For some experiments, however, higher purity was required and this was obtained by preparative gel electrophoresis using a Canalco Prep Disc apparatus. The high purity of this preparation is shown in figure 13; a tris-glycine gel showed faint impurities only when a very large amount of sample was applied, and an SDS gel with a normal amount of sample showed only heavy and light chains. That this material was in fact haptoglobin was shown by exposure to hemoglobin; the haptoglobin band was quantitatively removed to the region of hemoglobin-haptoglobin complex (figure 14).

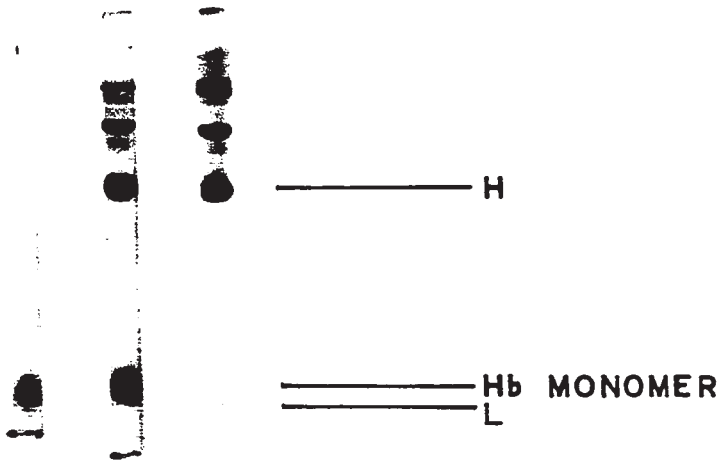
Purification of hemoglobin-haptoglobin complex

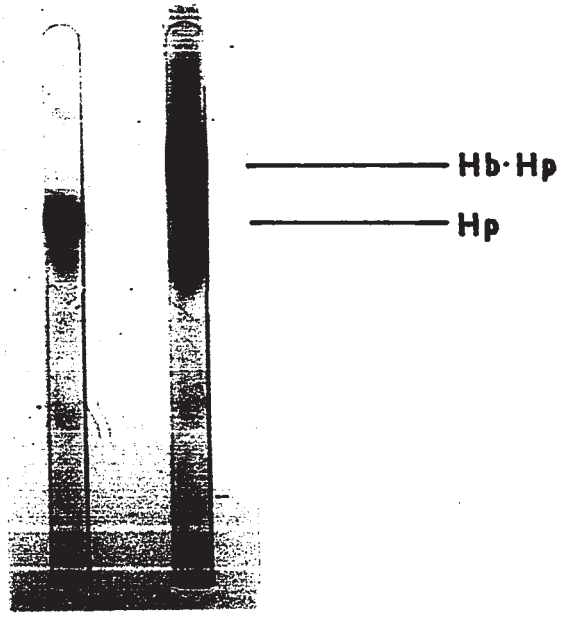
Semi-pure haptoglobin from the Sephadex G-200 column was exposed to an excess of hemoglobin and this mixture was passed through a 5 x 100 cm column of Sephadex G-200 superfine. Complex emerged well separated from the slowly moving band of excess hemoglobin. Figure 15 shows SDS gels of complex, as compared with hemoglobin and haptoglobin.

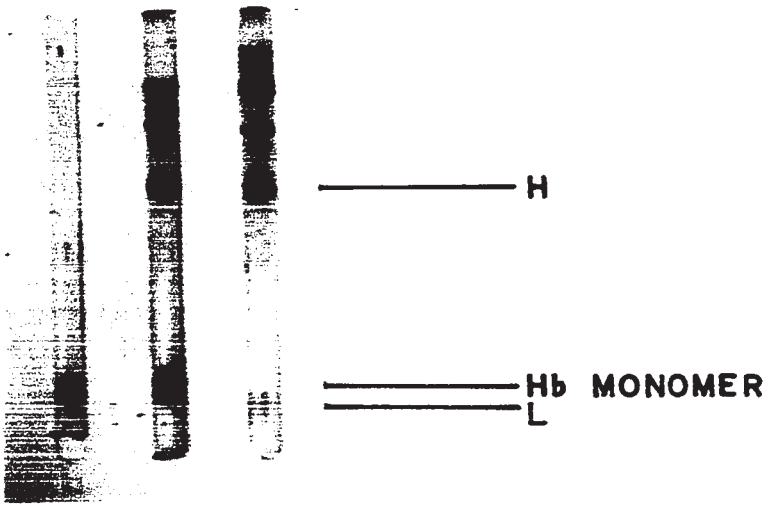
Figure 14: Polyacrylamide gel analysis of pure haptoglobin before and after exposure to excess hemoglobin.
Left, Hp not yet exposed to hemoglobin.
Right, Complex formed on exposure to Hb.

Figure 15: SDS polyacrylamide gel analysis of hemoglobin-haptoglobin complex made using excess of hemoglobin.
Left, hemoglobin.
Center, hemoglobin-haptoglobin complex.
Right, haptoglobin.


Hb·Hp
Hp


H
Hb MONOMER
L


Hb·Hp
Hp


H
Hb MONOMER
L

Monofunctional modifications of hemoglobin

The search for a satisfactory bifunctional reagent was begun by considering anhydrides because these derivatives have desirable solubility properties (Butler et al., 1967). Hemoglobin was reacted with maleic anhydride and evidence was obtained that extensive maleylation altered physical properties of Hb and destroyed its ability to bind Hp. Since these alterations admitted the possibility that a bifunctional anhydride might produce cross-links not possible in the native molecule, studies with anhydrides were not continued.

The second modification carried out was amidination with ethyl acetimidate HCl. In this case little alteration of physical properties was indicated and haptoglobin binding was retained. This led to the choice of a bifunctional imidoester.

Analysis of maleyl hemoglobin

Hemoglobin was reacted with maleic anhydride as described in 'methods'. It was necessary to obtain evidence that a reaction had in fact taken place and 1-fluoro-2,4-dinitrobenzene (FDNB) was chosen to achieve this. Both reagents would be expected to react with $-NH_2$ groups of lysyl residues and maleylation might therefore be expected to protect these groups from reaction with FDNB. Preliminary

studies with unreacted hemoglobin indicated that reaction with FDNB completely removed lysine, histidine, and tyrosine. Figure 16 shows an analysis for basic amino acids of DNP-hemoglobin, performed on an 18-cm column of a Beckman 120-C amino acid analyzer. Both lysine and histidine were absent and appeared instead as their DNP derivatives (Wofsy and Singer, 1963). The broad ninhydrin positive peak which eluted after arginine was compared with an authentic sample of ε-DNP-lysine and elution volumes were the same. Imidazolyl-DNP-histidine was identified only from its elution volume (Wofsy and Singer, 1963) and this procedure has recently been confirmed by Henkart (1971).

Since products of dinitrophenylation were so readily analyzed, maleyl hemoglobin was reacted with FDNB and analyses were performed. To 5 mg maleyl hemoglobin in 2 ml water was added an equal weight of sodium bicarbonate and 1 ml FDNB solution (5% FDNB in 95% ethanol). After stirring for 1 hour, an additional 1 ml FDNB solution was added and stirring was continued for another hour. Exposure to light was kept to a minimum during and after reaction with FDNB. After acidification with HCl and extraction with ether the DNP-protein was hydrolyzed with 6 N HCl and the hydrolysate analyzed for basic amino acids. Figure 17 shows the analyzer trace obtained; imidazolyl-DNP-histidine was still produced near quantitatively but no ε-DNP-lysine was found.

Figure 16: Amino acid analysis for basic amino acids of hemoglobin treated only with FDNB.

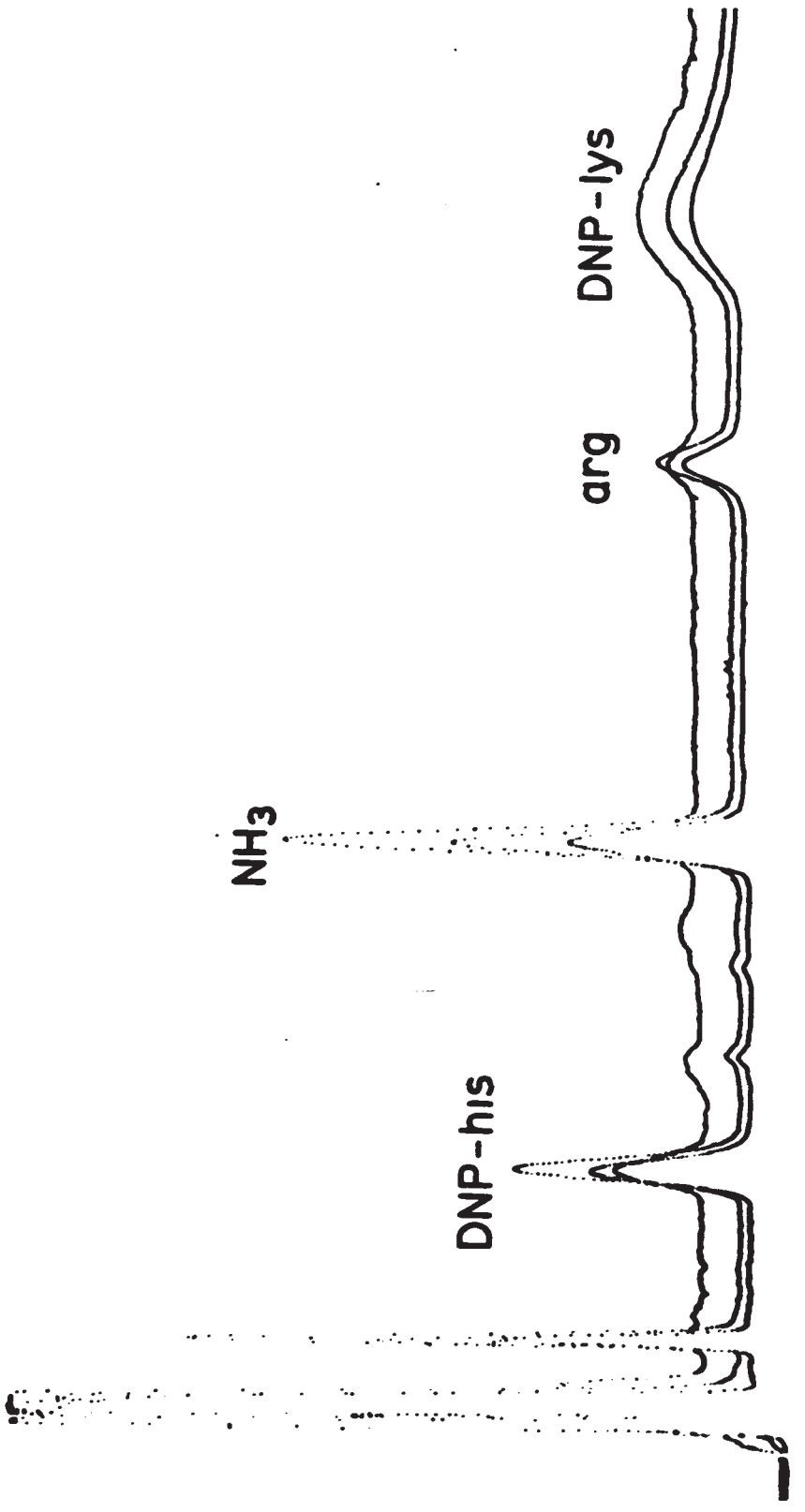
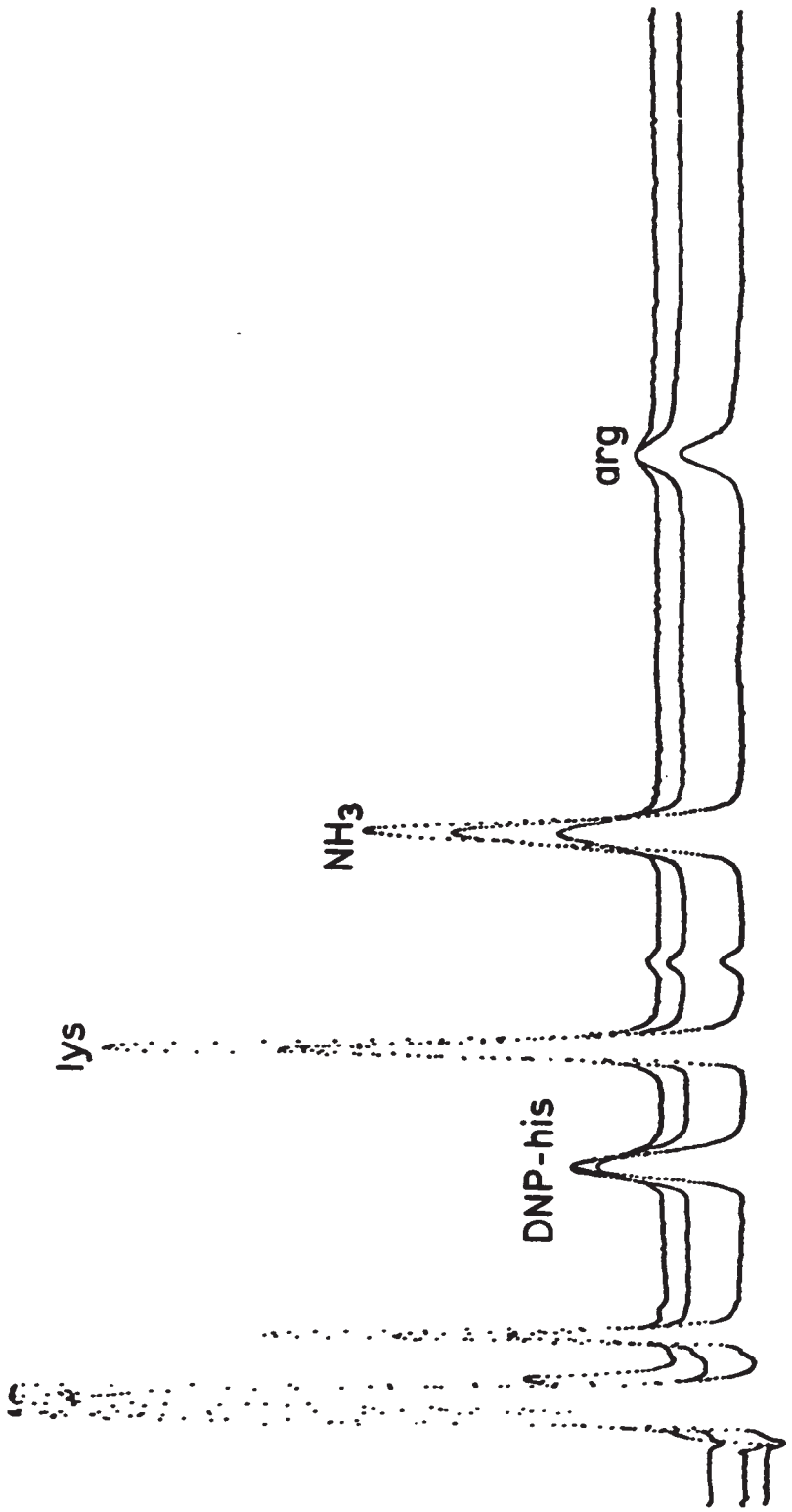

DNP-hemoglobin
NH3
DNP-his
arg
DNP-lys

Figure 17: Amino acid analysis for basic amino acids of maleyl hemoglobin treated with FDNB.


DNP- maleyl hemoglobin
lys
NH_3
DNP-his
arg

The amide formed upon maleylation of lysine would be expected to hydrolyze in HCl to regenerate lysine and this was observed. Since no DNP-lysine was present it was concluded that maleylation had protected all lysine residues from reaction with FDNB. Dinitrophenylation of histidine served to show that reaction with FDNB was successful and indicated that lysyl residues would have been reacted with this reagent had they been available. There was no indication of significant loss of any amino acids except histidine and tyrosine which were both dinitrophenylated as in the control (Table 2).

Electrophoresis at pH 8.6 on gelatinized cellulose acetate (figure 18) shows that maleyl hemoglobin migrated to the anode more rapidly than the control; the extra carboxyl groups set free when the anhydride ring opened would be expected to cause this. Although amounts of protein were comparable, maleyl hemoglobin had a low affinity for the dye, Procion Brilliant Blue, and thus appeared as a band of low intensity. Before staining, a band of heme (methanol-soluble material) was evident at the origin. There was no evidence of separation into α and β monomers; they would not be expected to separate as charge differences would be small at this pH.

Sedimentation velocity analysis of 0.2 per cent maleyl hemoglobin in 0.1 M phosphate pH 7.0 yielded an

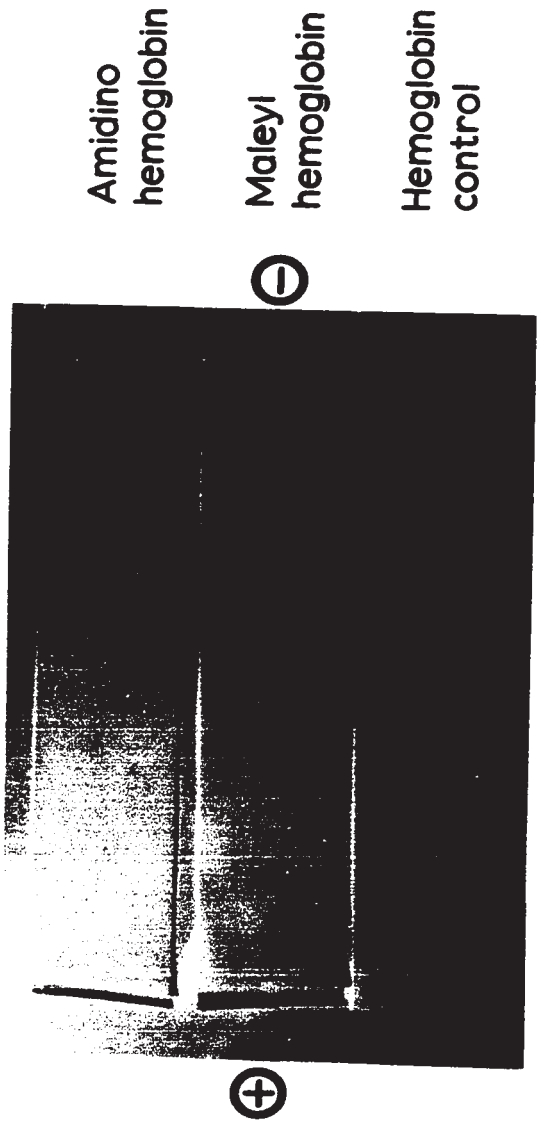
TABLE 2

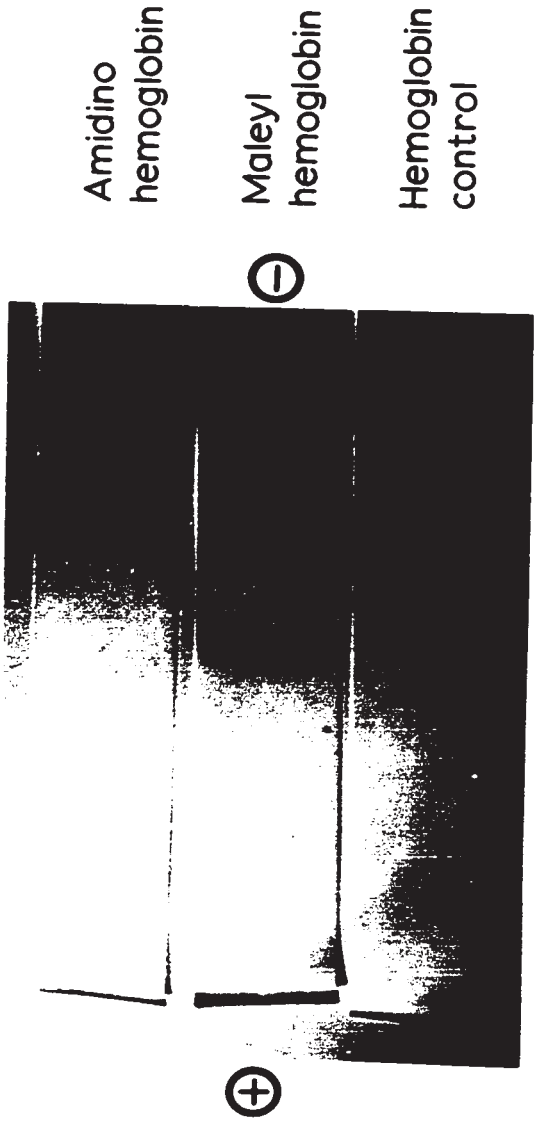
AMINO ACID ANALYSES

	Unreacted Hemoglobin	Maleyl-Hemoglobin	Amidino-Hemoglobin	Accepted Value
Lys	0.0	19.6	3.0	22.0
His	0.0	0.0	0.0	19.0
Arg	4.3	3.7	4.2	6.0
Asp	25.0	25.0	25.0	25.0
Thr	12.5	12.0	12.5	16.0
Ser	12.9	10.8	11.4	16.0
Glu	15.2	14.3	15.4	16.0
Pro	10.3	10.8	11.0	14.0
Gly	18.6	16.9	19.8	20.0
Ala	32.4	30.8	32.3	36.0
Val	26.7	28.6	27.8	31.0
Met	1.0	1.4	1.8	3.0
Leu	34.9	34.4	36.3	36.0
Tyr	0.0	0.0	0.0	6.0
Phe	11.2	9.4	12.1	15.0

Amino Acid Analyses (molar ratios standardized to ASP = 25) of hemoglobin and derivatives after dinitrophenylation. Figures are averages of either two or three analyses. No corrections for hydrolytic losses or incomplete hydrolysis have been made. Accepted values are those per hemoglobin dimer (Dayhoff, 1969).

Figure 18: Sample electrophoresis of hemoglobin, maleyl hemoglobin, and acetamidino hemoglobin on gelatinized cellulose acetate at pH 8.6, 250 V, 40 min, 2 ma per strip.


Amidino hemoglobin
Maleyl hemoglobin
Hemoglobin control
⊖
⊕


Amidino hemoglobin
Maleyl hemoglobin
Hemoglobin control
⊖
⊕

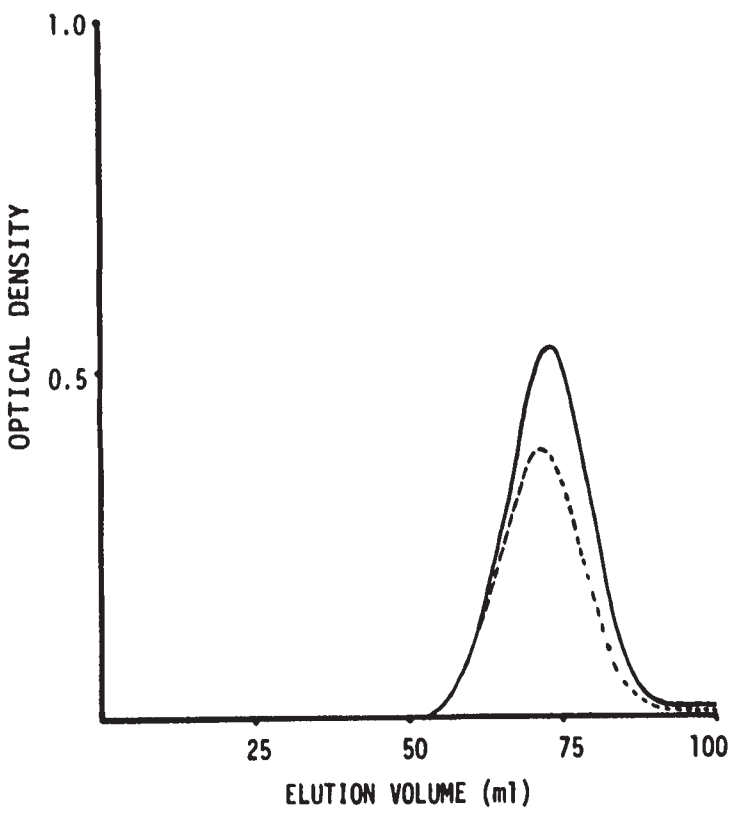
uncorrected S value of only 1.6 as compared with 3.9 for unreacted hemoglobin. The analysis was repeated on a 0.1 per cent solution of maleyl hemoglobin in 1 M KCl to ensure nullification of charge effects and the $S_{20,w}$ obtained was 2.5 which may be compared with an $S_{20,w}$ of 3.75 for hemoglobin in 1 M NaCl (Chiancone et al., 1966).

Maleyl hemoglobin was assayed for ability to bind haptoglobin but no complex was detected (figure 19) in the elution from Sephadex G-100. A band of heme separated early on the column as in electrophoresis and this caused a low ratio of absorbance at 416 nm to that at 280 nm. Figure 20 shows control runs with unreacted hemoglobin; complex preceded excess hemoglobin when haptoglobin was present.

In view of alteration of physical properties, as indicated by electrophoresis, sedimentation rate, and gel filtration, it was not possible to decide whether binding was destroyed by modification at lysyl residues or by structural changes accompanying the modification. Uyeda (1969) and Sia and Horecker (1968) have reported that maleylation disrupts the structure of oligomeric proteins and it is now apparent that it also has this effect on hemoglobin. This, however, was not known for certain before these experiments. The possibility of using a bifunctional anhydride was therefore abandoned because of these difficulties with the monofunctional anhydride and studies of maleyl hemoglobin were not continued.

Figure 19: Elution profiles from 1 x 100 cm column of Sephadex G-100 of maleyl hemoglobin (left) and maleyl hemoglobin plus haptoglobin (right).
Solid line, absorbance 416 nm.
Broken line, absorbance 280 nm.

Maleyl hemoglobin



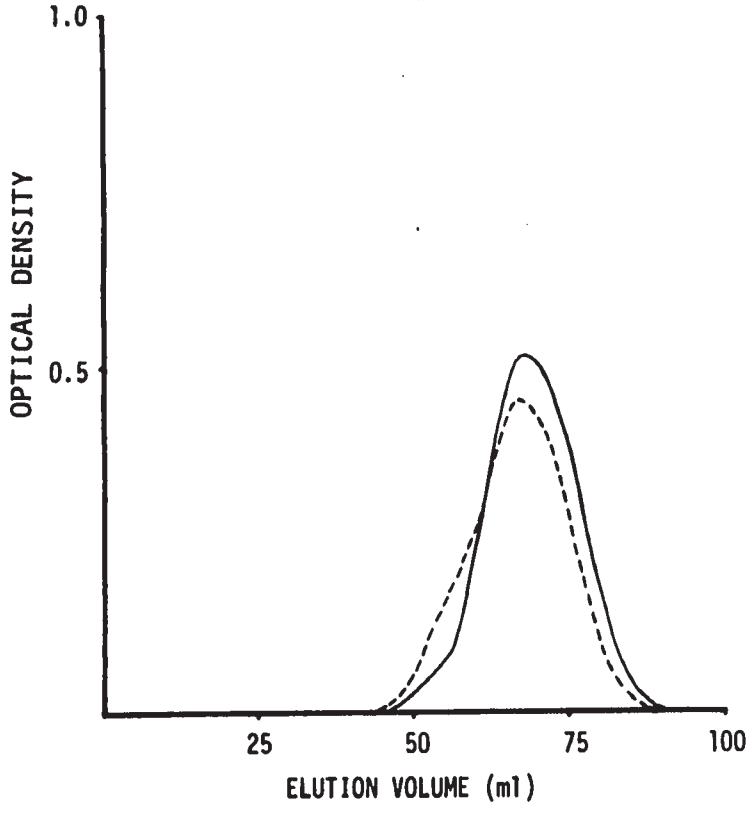
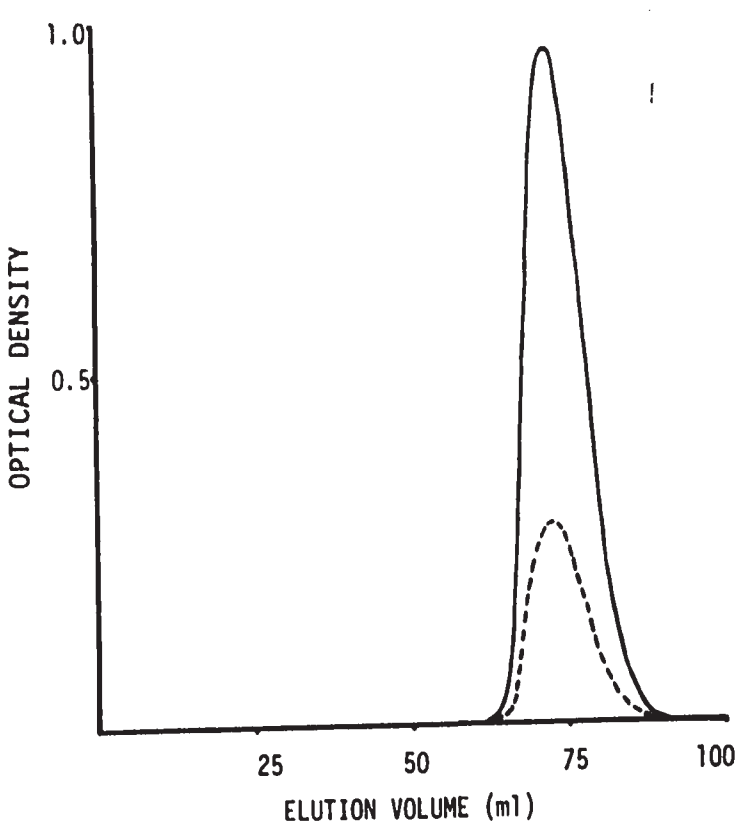
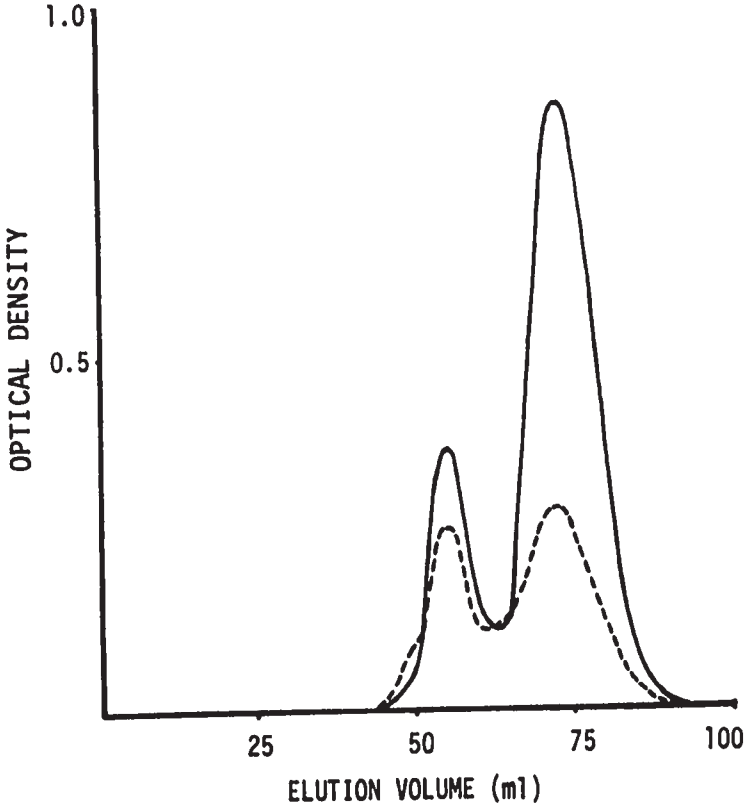


Figure 20: Elution profiles from 1 x 100 cm column of Sephadex G-100 of hemoglobin (left) and hemoglobin plus haptoglobin (right).
Solid line, absorbance 416 nm.
Broken line, absorbance 280 nm.

Hb - Control


1.0
0.5
OPTICAL DENSITY
25
50
75
100
ELUTION VOLUME (ml)

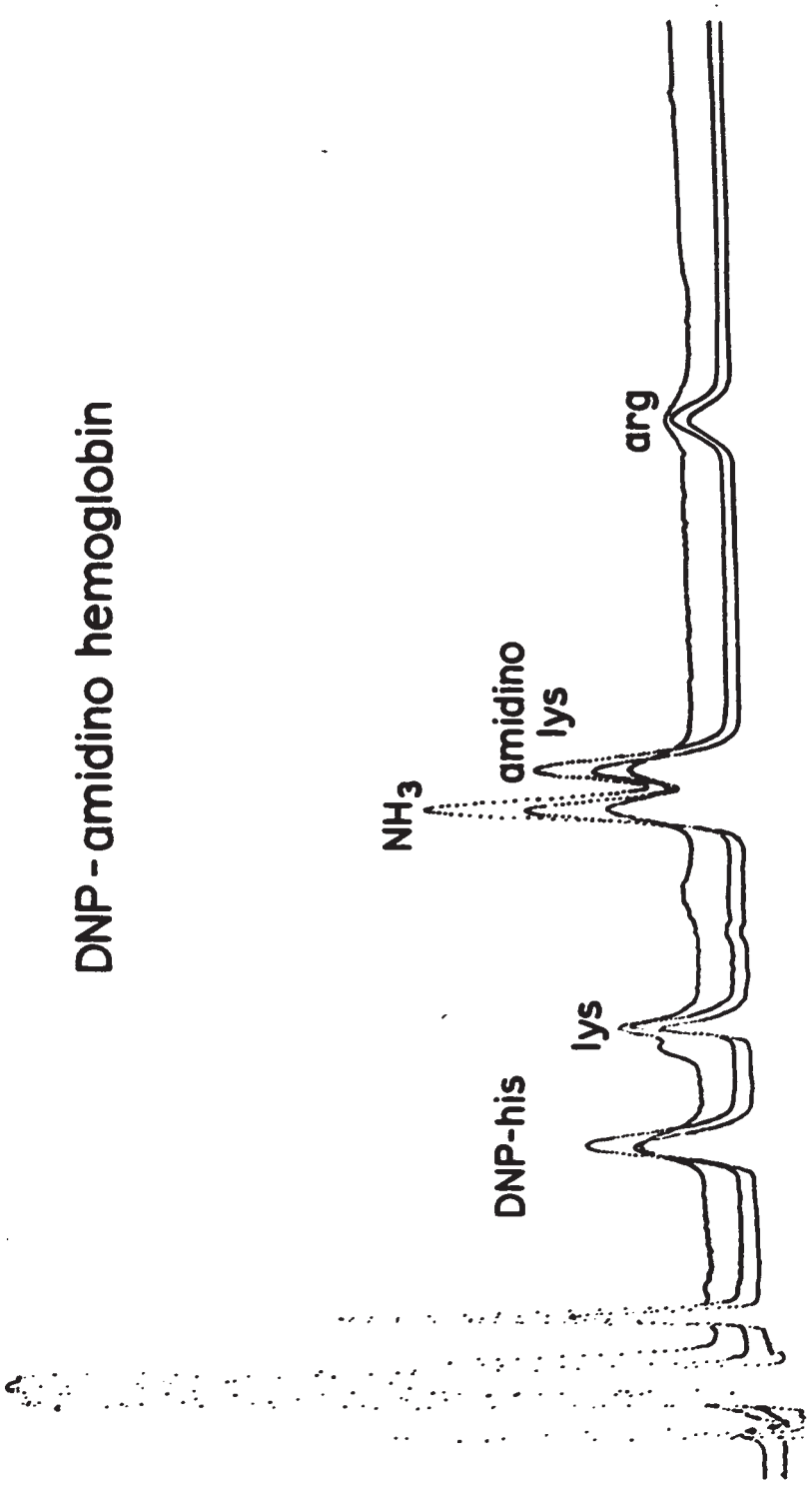

1.0
0.5
OPTICAL DENSITY
25
50
75
100
ELUTION VOLUME (ml)

Analysis of acetamidino hemoglobin

Amidination of lysyl residues offered a much milder modification with retention of positive charges at amino sites and studies were undertaken with ethyl acetimidate hydrochloride as a preliminary to bifunctional amidination.

Following reaction of hemoglobin with a large excess of ethyl acetimidate HCl, and following removal of excess reagent by dialysis, dinitropnenylation was carried out as in the case of maleyl hemoglobin. Amino acid analysis of DNP-acetamidino hemoglobin is shown in figure 21. Again no ε-DNP-lysine was found indicating that complete amidination had occurred. Complete loss of histidine and tyrosine (Table 2) indicates that these groups had been available for dinitrophenylation; imidazolyl-DNP-histidine can be seen in figure 21 and O-DNP-tyrosine ran as a very broad low peak well after the position of ε-DNP-lysine (figure 16). Most lysine was accounted for just after ammonia as ε-acetamidino lysine (Wofsy and Singer, 1963), however, some was re-generated by acid hydrolysis. The fact that no ε-DNP-lysine was detected indicates that this free lysine was regenerated rather than a few residues unreactive to both reagents, especially since histidine had fully reacted with FDNB. Again no significant losses of other amino acids were evident (Table 2).

Figure 21: Amino acid analysis for basic amino acids of acetamidino hemoglobin treated with FDNB.


DNP-amidino hemoglobin
DNP-his
lys
NH3
amidino lys
arg

Electrophoretically, amidino hemoglobin was similar to unreacted hemoglobin (figure 18) at pH below 9. The higher pKa of amidines over amines could be seen in similar electrophoresis at higher pH. At pH 10.0 acetamidino hemoglobin migrated slightly slower than the control and at pH 10.9 it moved 2.6 cm as compared with 3.6 cm for unreacted hemoglobin. Sedimentation velocity analysis of a 0.2 per cent solution of acetamidine hemoglobin yielded an uncorrected S value of 3.7, close to the value of 3.9 obtained for control hemoglobin.

Since physical properties of this derivative seemed little altered, it was desirable to determine whether the modification was so mild as to allow highly sensitive physiological properties to have escaped modification. Therefore, the oxygen affinity of amidino hemoglobin was determined as a function of pH. Figure 22 shows a sample set of spectra obtained and the equations used to calculate fractional saturations are given in 'methods'. Similar curves were obtained for acetamidino hemoglobin except that smaller additions of air were required. Plots of log partial pressure of oxygen against log fractional saturation were made (figure 23) and the log $P_{\frac{1}{2}}$ could be read from them. The plots in figure 23 were determined by the Hill equation, (Hill, 1910) which may be written

$$\log \frac{Y}{1 - Y} = \log K + n \log pO_2$$

Figure 22: Sample spectra of hemoglobin in 0.1 M phosphate pH 6.8. Volumes of air admitted to produce each mixture are shown at right.

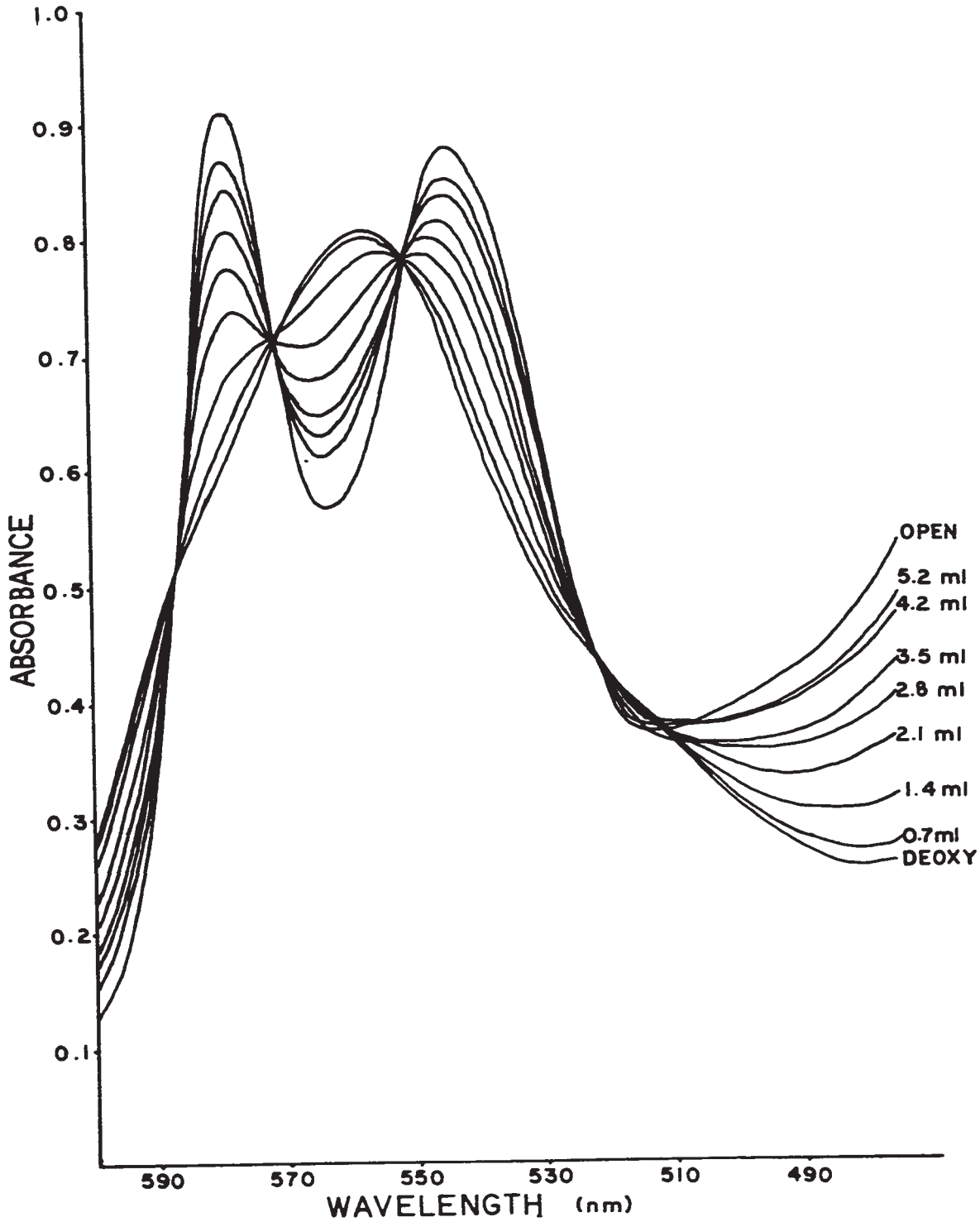
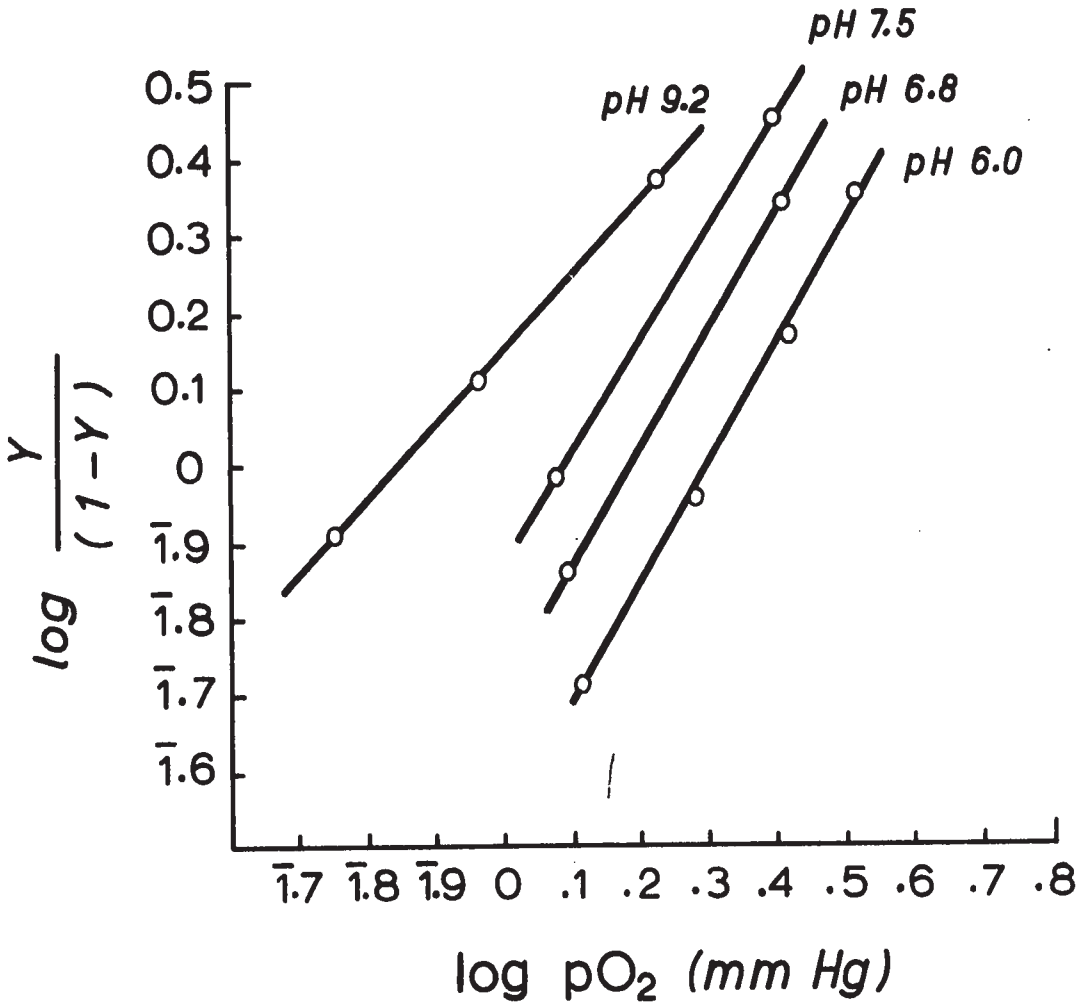

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
590
570
550
530
510
490
WAVELENGTH (nm)
OPEN
5.2 ml
4.2 ml
3.5 ml
2.8 ml
2.1 ml
1.4 ml
0.7ml
DEOXY

Figure 23: Hill equation plots for acetamidino hemoglobin in buffers of several different pH's.


pH 7.5
pH 9.2
pH 6.8
pH 6.0
0.5
0.4
0.3
0.2
0.1
0
$\bar{1}$.9
$\bar{1}$.8
$\bar{1}$.7
$\bar{1}$.6
log $\frac{Y}{(1-Y)}$
$\bar{1}$.7 $\bar{1}$.8 $\bar{1}$.9 0 .1 .2 .3 .4 .5 .6 .7 .8
log pO_2 (mm Hg)

where Y is the fraction of oxyhemoglobin, pO_2 is the partial pressure of oxygen, and K and n are constants. Slopes of lines in figure 23 give values of 'n' (Table 3) and have been taken as measures of 'heme-heme interaction'. For unaltered hemoglobin this value has been found to be 2.7 (Perutz, 1970a), whereas in complete absence of 'heme-heme interaction' n becomes 1 (Antonini, 1965). It is evident from table 3 that some degree of interaction has been retained because slopes at 3 pH's have not been reduced to one. Lysine α-40 and the N-terminal valines of α-chains are involved in this interaction (Perutz, 1970a) and these would be expected to be amidinated. Nonetheless, even partial retention of cooperative effects testifies to the mild nature of this modification.

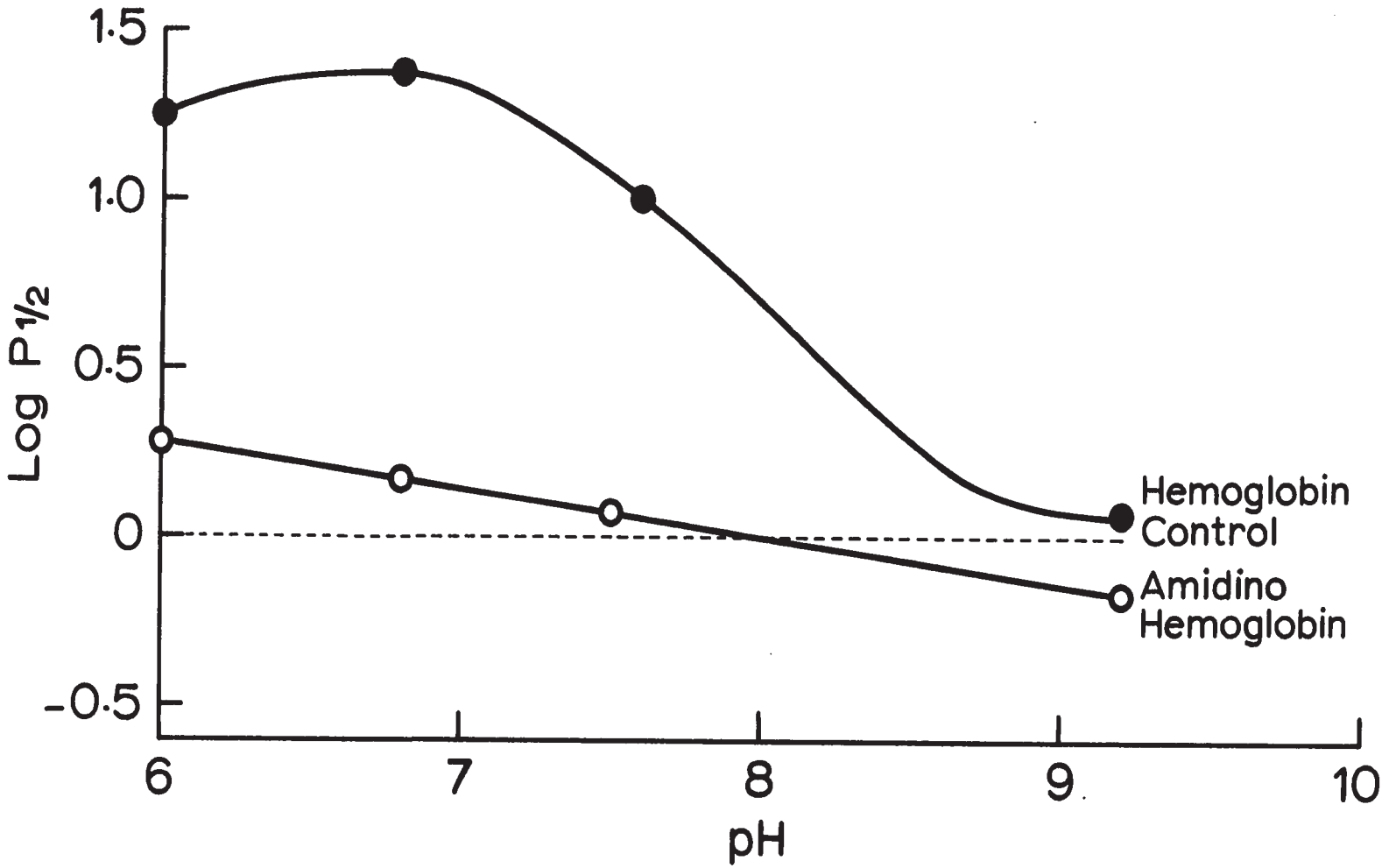
Figure 24 shows log $P_{\frac{1}{2}}$ as a function of pH. The dependence of oxygen affinity on pH as shown in unreacted Hb is known as the Bohr effect and these results are in good agreement with those of Antonini et al. (1965). It is evident that Bohr effect is lacking or very much reduced in amidinated hemoglobin. This could also be anticipated for amino groups of N-terminal valines of α-chains and of lysine α-127 are involved (Perutz, 1970b) and they are thought to have been amidinated. It is of interest that Kilmartin and Rossi-Bernardi (1969) achieved specific modification of N-terminal amino groups of α-chains with cyanate and found

TABLE 3

Oxygen equilibrium data for acetamidino hemoglobin

pH	log $P_{\frac{1}{2}}$	Hill constant (n)
6.0	0.296	1.54
6.8	0.182	1.53
7.5	0.088	1.47
9.2	$\bar{1}$.845	0.97

Figure 24: Log of partial pressure of oxygen required to half saturate a sample of hemoglobin (filled circles) and amidino hemoglobin (open circles) as a function of pH.


1.5
1.0
0.5
0
-0.5
Log P1/2
6
7
8
9
10
pH
Hemoglobin
Control
Amidino
Hemoglobin

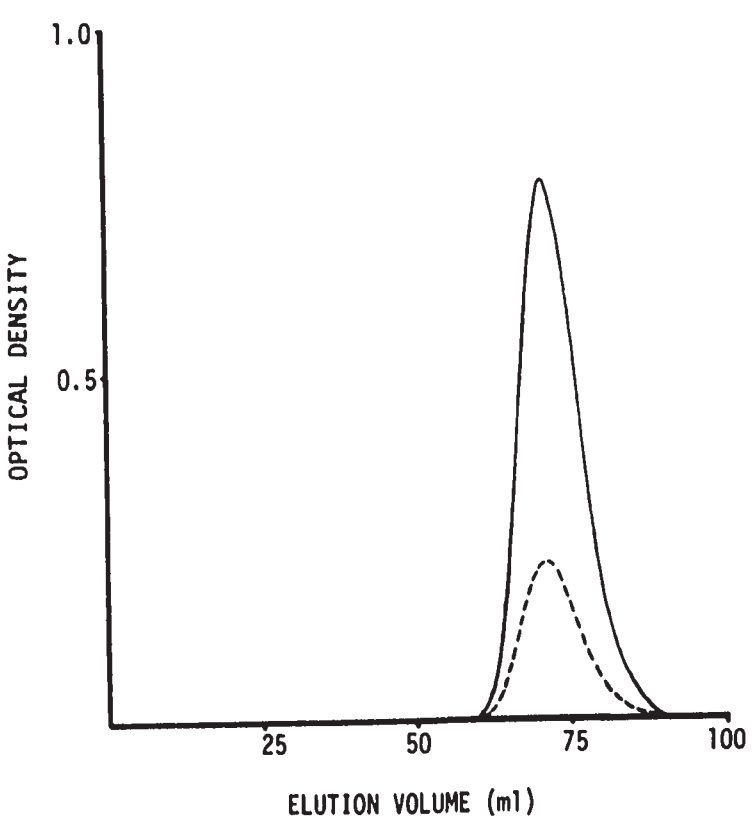
25 per cent reduction of Bohr effect but little or no change in the Hill constant. However, several modified hemoglobins show changes in Bohr effect without accompanying large structural changes (Antonini, 1965).

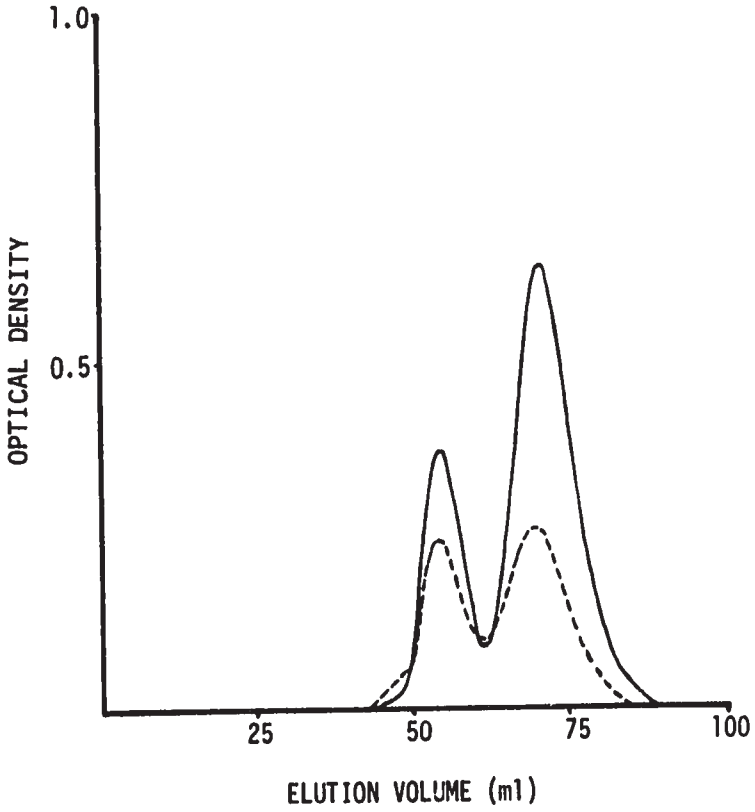
Acetamidino hemoglobin was assayed for ability to bind haptoglobin and elution profiles from Sephadex G-100 are shown in figure 25. These may be compared with curves for unreacted hemoglobin (figure 20) in which complex emerged at 55 ml, followed by excess hemoglobin at 73 ml. This same pattern was obtained with acetamidino hemoglobin, giving clear evidence that binding ability was retained. Ratios of absorbances at 416 nm to those at 280 nm for the complex peak were the same as in the control and indicate binding in the same molar ratio. The apparent homogeneity of acetamidino hemoglobin and the quantitative amidination of all lysyl residues made it unnecessary to titrate haptoglobin with increasing amounts of hemoglobin.

The major conclusion reached from these studies with monofunctional reagents is that lysyl residues are unlikely to be part of the hemoglobin site for haptoglobin binding. Ethyl acetimidate is a mild reagent inducing little change in observed physical properties and no change in haptoglobin binding. Although positive charges at lysyl sites are maintained after amidination (at pH below 9) the bulk of this substituent must have steric effects which would be expected

Figure 25: Elution profiles from 1 x 100 cm column of Sephadex G-100 of acetamidino hemoglobin (left) and acetamidino hemoglobin plus haptoglobin (right).
Solid line, absorbance 416 nm.
Broken line, absorbance 280 nm.

Amidino hemoglobin





to alter haptoglobin binding if hemoglobin amino groups were involved. Several lines of evidence indicate that haptoglobin binds dimers of hemoglobin rather than intact tetramers. Nagel and Gibson (1967, 1971) reached this conclusion from the kinetics of complex formation, Adams and Weiss (1969) from calorimetry, and Hamaguchi (1967) by isolation. Examination of the three-dimensional model (figure 5) of hemoglobin $\alpha_1\beta_1$ dimer reveals that lysyl residues are distributed over the surface of the tetramer which suggests that the external surface may not contain the binding site. Moreover, the area of contact between the two proteins is probably in lysine deficient regions of both proteins because Chan (1968) found that guanidination of lysyl groups in complex almost exactly totalled that for the two proteins separately. These considerations, together with the finding by Malchy and Dixon (1970) that cross-linked mouse hemoglobin can form several polymeric complexes, all suggest that haptoglobin binding site is in the interior of the hemoglobin tetramer.

Of greater importance for the purposes of this study, however, is the finding that amidination of lysyl residues does not, in itself, alter binding in the hemoglobin-haptoglobin system. It is therefore with some confidence that a bifunctional imidoester can be used to cross link residues in a study of the subunit structure of complex.

It seems most unlikely that a progressive structural change accompanies amidination and it can be assumed that any subunits cross-linked were sufficiently close together to allow cross linking in the native molecule.

Studies on haptoglobin structure

Recent studies in this laboratory on the binding of isolated hemoglobin chains and on difference spectra of dilute haptoglobin solutions (Boyd, 1971) indicated that changes occurred in haptoglobin structure as a function of concentration. Although the model shown in figure 1 (Shim and Bearn, 1964) has been widely accepted, Waks and Alfsen (1968) have suggested that haptoglobin dissociates reversibly to a protomer of molecular weight near 42,000. Since cross-linking reactions must necessarily be carried out in dilute protein solutions to avoid extensive polymer formation, it was desirable to know whether dilute solutions of porcine haptoglobin existed as L_2H_2 units of molecular weight near 100,000 or as smaller units of near half that molecular weight. A study was therefore made of dilute solutions of porcine haptoglobin by analytical gel filtration with Bio-Gel P-150 equilibrated with 0.05 M phosphate pH 5.5 made to 0.1 M with KCl.

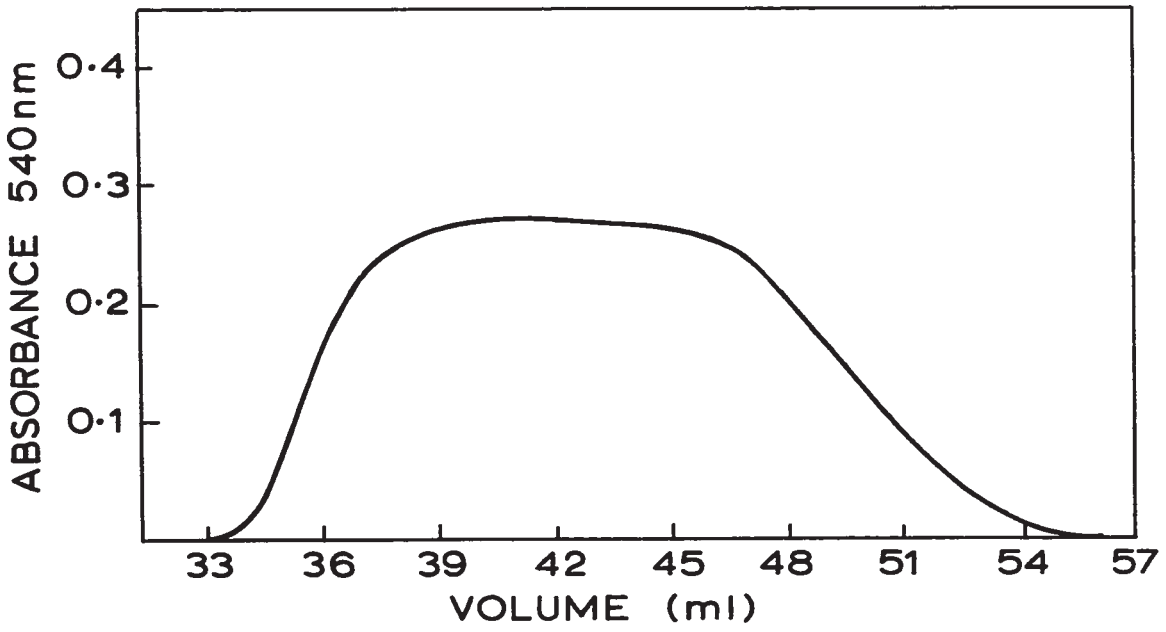
In each case control experiments were performed with hemoglobin which is known to undergo reversible dissociation

(Chiancone et al., 1968). Figure 26 shows an elution profile for hemoglobin at 0.548 mg per ml; this shape is typical of a system in reversible association equilibrium since the leading edge is much sharper than the trailing edge. This is best shown by considering the positive value of the first derivative at points along the elution profile (figure 27). It is readily apparent that a major difference exists between leading and trailing slopes and this is very similar to results obtained by Winzor and Scheraga (1963) with α-chymotrypsin which undergoes reversible association equilibrium. These results with hemoglobin are included only as a control to prove that the column system was sufficiently sensitive to detect a reversible association-dissociation equilibrium when it was known to occur.

An elution profile for haptoglobin at 0.312 mg per ml is shown in figure 28. In this case there was no indication that the leading edge was sharper than the trailing edge and consideration of the two peaks in the derivative plot reveals virtual superimposability (figure 29). While this can be taken as evidence against dissociation by haptoglobin, it does not conclusively rule dissociation out. Studies on non-dissociating proteins such as ovalbumin (Winzor and Scheraga, 1963) or serum albumin (Waks and Alfsen, 1968) consistently show a sharpening of the trailing edge. Since no such sharpening was observed, further experiments were undertaken.

Figure 26: Elution of hemoglobin at 0.548 mg per ml from a 0.9 x 50 cm column of Bio-Gel P-150 equilibrated with 0.05 M phosphate buffer pH 5.5 made to 0.1 M with potassium chloride.

Figure 27: Positive values of first derivative at points along the hemoglobin elution profile.


ABSORBANCE 540nm
0·4
0·3
0·2
0·1
33
36
39
42
45
48
51
54
57
VOLUME (ml)

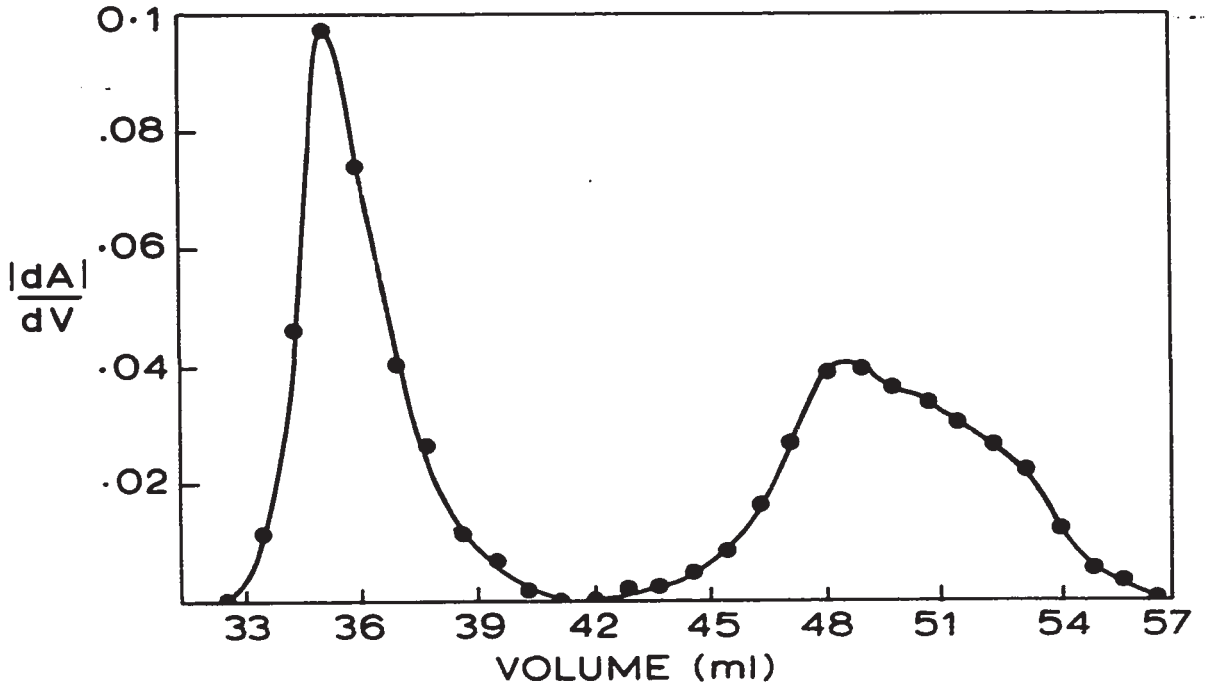
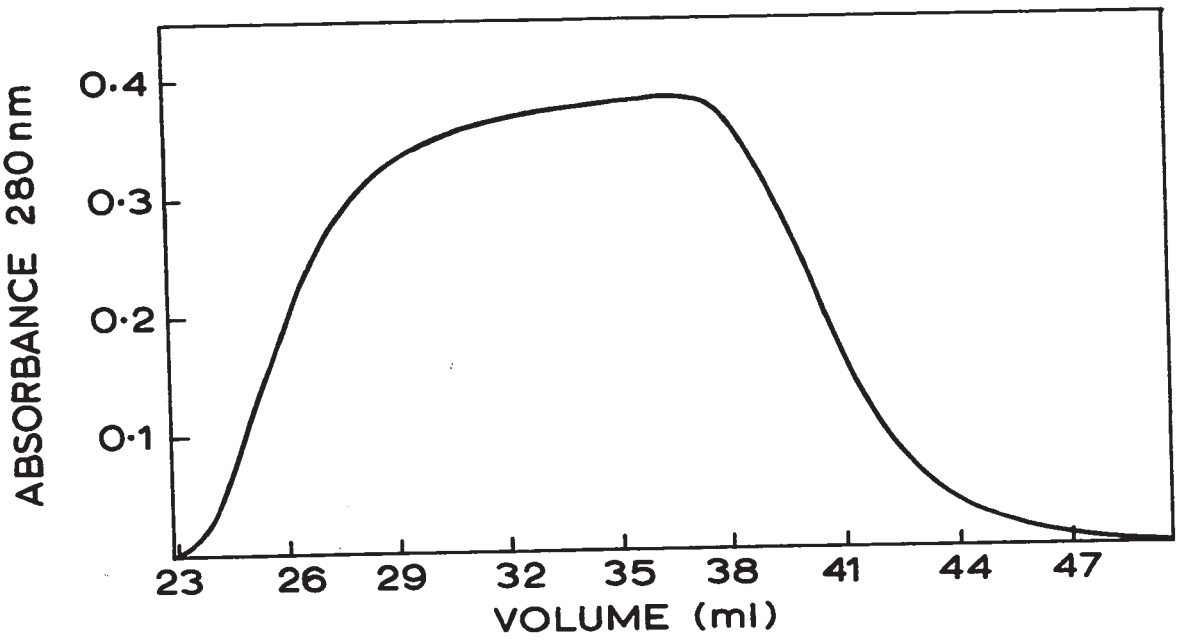
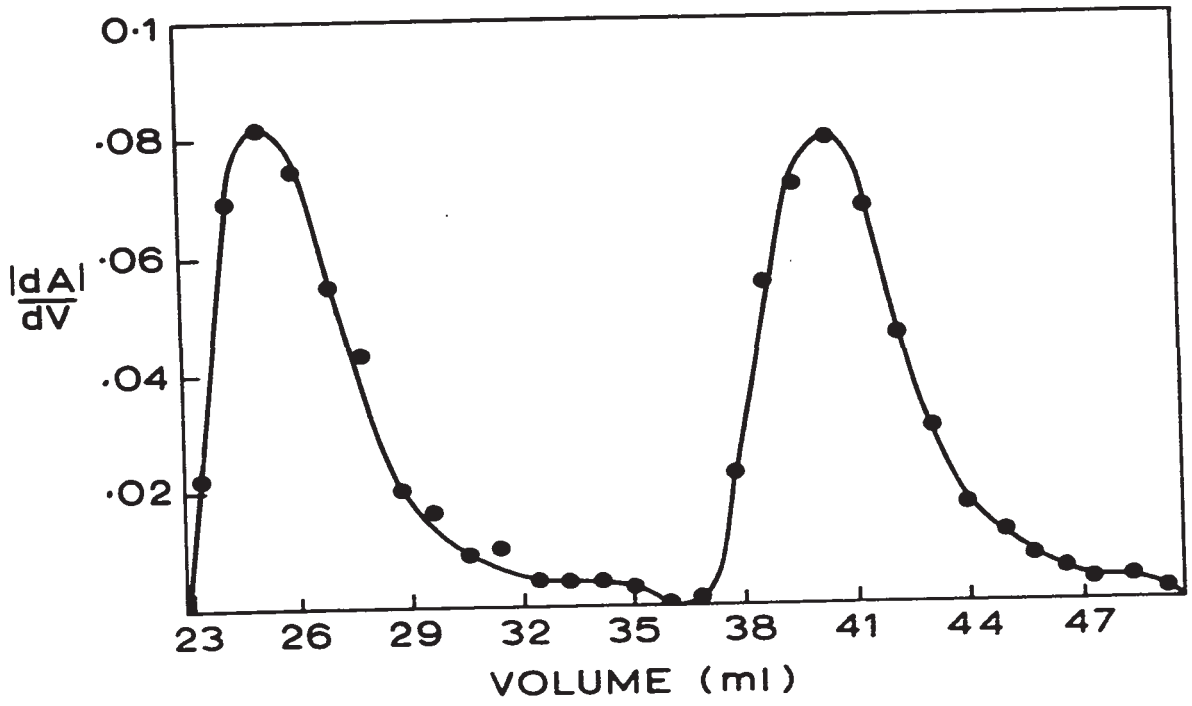

|dA|/dV
0·1
.08
·06
·04
·02
33
36
39
42
45
48
51
54
57
VOLUME (ml)

Figure 28: Elution of haptoglobin at 0.312 mg per ml from a 0.9 x 50 cm column of Bio-Gel P-150 equilibrated with 0.05 M phosphate buffer pH 5.5 made to 0.1 M with potassium chloride.

Figure 29: Positive values of first derivative at points along the haptoglobin elution profile.


ABSORBANCE 280 nm
0·4
0·3
0·2
0·1
23
26
29
32
35
38
41
44
47
VOLUME (ml)


|dA|/dV
0·1
·08
·06
·04
·02
23
26
29
32
35
38
41
44
47
VOLUME (ml)

The concentration dependence of the rate of the elution of leading edges was studied. If a protein is in reversible dissociation equilibrium, dilution favors smaller species and elution volumes are expected to increase as concentration decreases. Figure 30 shows elution volumes of hemoglobin and haptoglobin plotted as a function of concentration. Again hemoglobin data is included only to show that sensitivity was sufficient to detect dissociation in the known case. Lines were not fitted statistically since an obvious upward trend was evident for hemoglobin as it was diluted, in agreement with Chiancone *et al.* (1968), but no such trend was observed for haptoglobin. This led to the conclusion that haptoglobin was not undergoing dissociation to half molecules in the concentration range studied.

This conclusion was supported by sedimentation velocity studies on the same solutions used for gel filtration. Ultraviolet absorption optics had to be used since concentrations were too low to give satisfactory peaks with the schlieren system. Table 4 shows sedimentation coefficients obtained and again suggests failure to dissociate.

Since these concentrations were all well below the 2.3 mg per ml at which Waks and Alfsen (1968) observed dissociation, it might be argued that haptoglobin already existed mostly as the dissociated protomer. This was tested by comparing its elution volume with those of some other

Figure 30: Elution volumes of hemoglobin (filled circles) and haptoglobin (open circles) as a function of protein concentration.

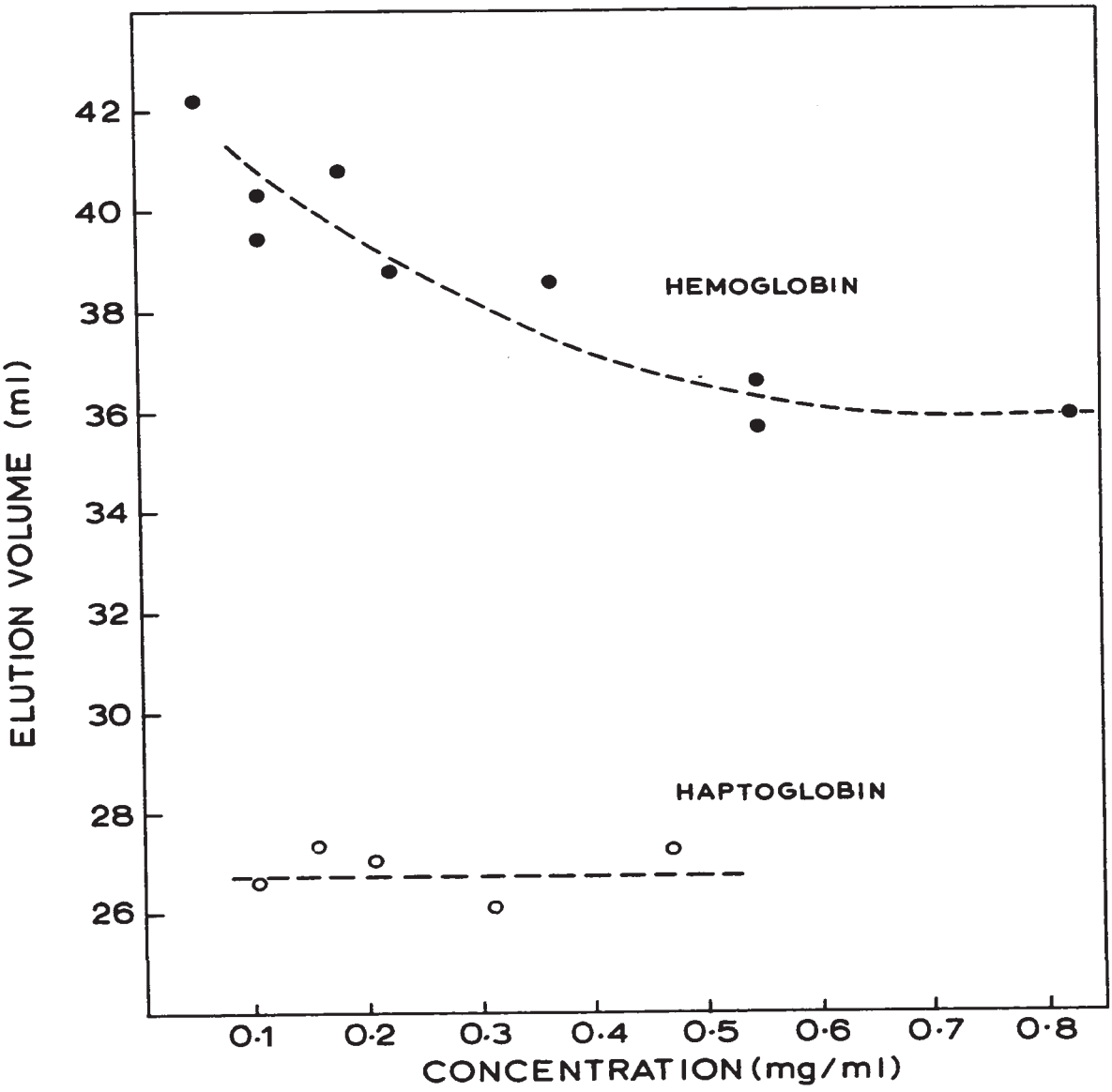

ELUTION VOLUME (ml)
42
40
38
36
34
32
30
28
26
HEMOGLOBIN
HAPTOGLOBIN
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
CONCENTRATION (mg/ml)

TABLE 4

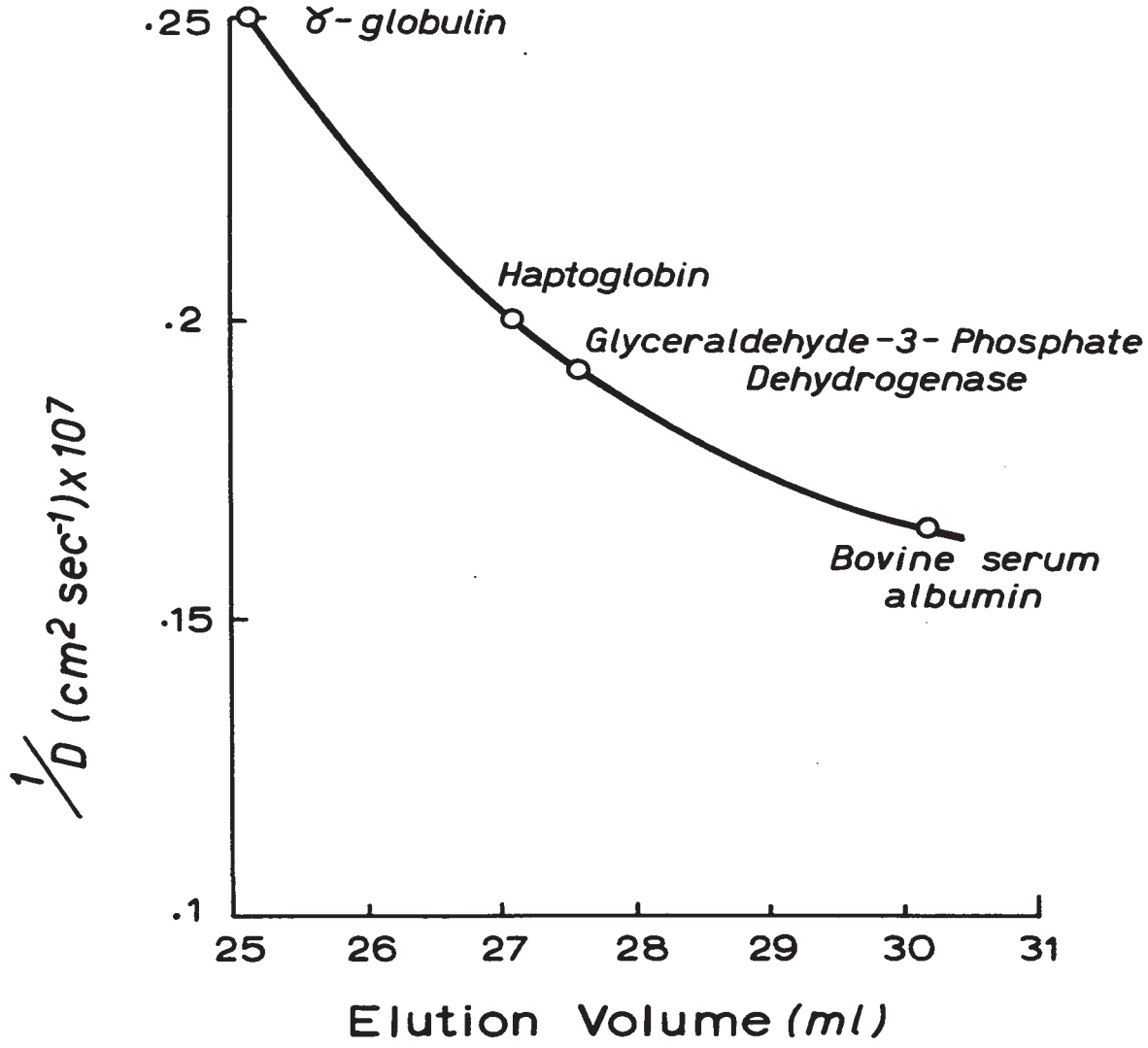
Sedimentation coefficients for haptoglobin in solutions at several concentrations. (0.1 M KCl buffered at pH 5.5 with 0.05 M phosphate)

Haptoglobin concentration mg per ml	Uncorrected sedimentation coefficient
0.312	4.16
0.208	4.38
0.156	4.64
0.104	4.33

proteins. Standards chosen were bovine serum albumin, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and human gamma globulin. Andrews (1965) observed deviations of up to 150 per cent when molecular weights were obtained by plotting elution volume against log M for several glycoproteins, but these deviations were eliminated by plotting elution volume against the reciprocal of the diffusion coefficient. Winzor (1969) has discussed the theory of this approach and has pointed out that the parameter governing elution volume is not molecular weight but rather the radius of an equivalent hydrodynamic sphere, the Stokes radius. This is inversely proportional to the diffusion coefficient and therefore plots of elution volume against the reciprocal of the diffusion coefficient avoid deviations found in plotting log M directly, and allow evaluation of the diffusion coefficient which can then be used in combination with the sedimentation coefficient to solve the Svedberg equation. The data have been plotted in figure 31 using tabulated values of diffusion coefficients (Sober, 1968) of standards. A smooth curve was obtained with haptoglobin having a diffusion coefficient of $D = 5.0 \times 10^{-7}$ cm^2 sec^{-1}.

The sedimentation coefficients were extrapolated to zero concentration and then corrected for buffer density to give $S^{o}_{20,w} = 4.46$. These values were then substituted into the Svedberg equation

Figure 31: Relation between elution volume and the reciprocal of the diffusion coefficient for haptoglobin and other proteins.


.25
.2
.15
.1
γ-globulin
Haptoglobin
Glyceraldehyde-3-Phosphate Dehydrogenase
Bovine serum albumin
1/D (cm² sec⁻¹) x 10⁷
25
26
27
28
29
30
31
Elution Volume (ml)

$$M = \frac{s\ R\ T}{D\ (1 - \bar{v}\rho)}$$

where $s = 4.46 \times 10^{-13}$ sec

$R = 8.314 \times 10^{7}$ g cm^2 sec^{-2} mole^{-1} $^oK^{-1}$

$T = 298.1\ ^oK$

$D = 5.0 \times 10^{-7}$ cm^2 sec^{-1}

$\bar{v} = 0.766$ cm^3 g^{-1} (Jayle and Moretti, 1962)

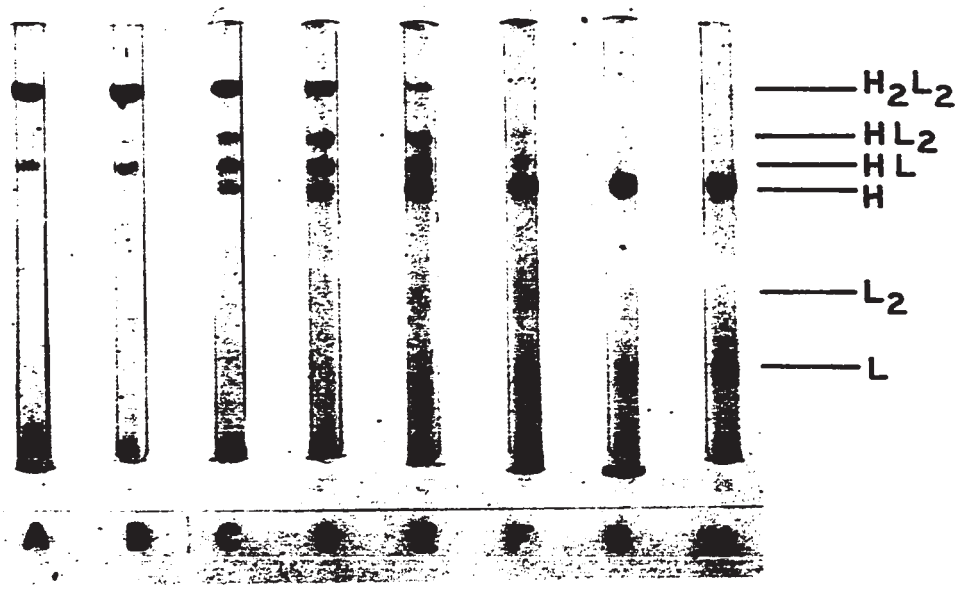
$\rho = 1.012$ g cm^{-3}

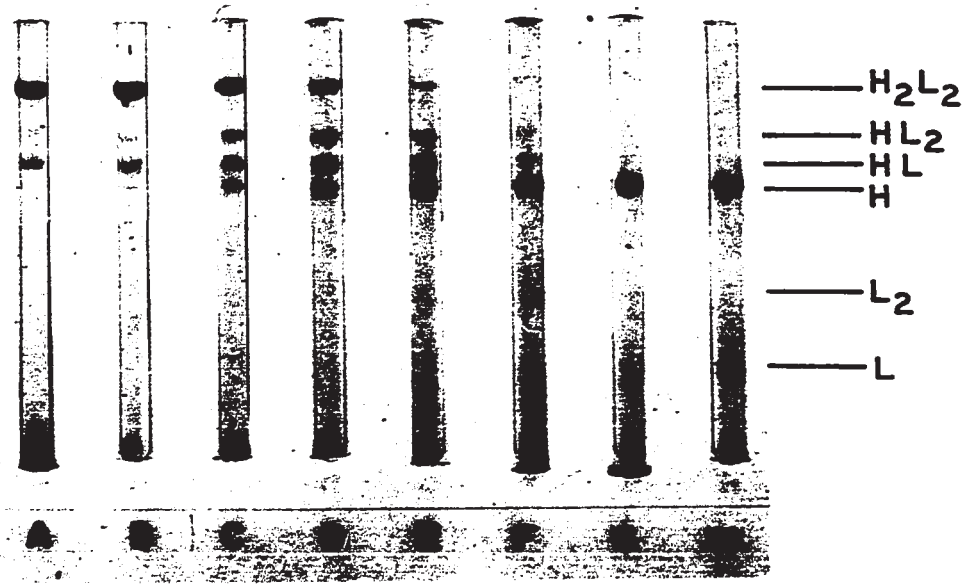
This allowed calculation of M = 96,500 g per mole without interference by deviations typical of glycoproteins and indicates failure to dissociate.

Since no evidence to support dissociation was obtained, it was assumed that forces maintaining the intact structure were disulfide bridges as indicated in the model (figure 1). These would be expected to be broken by either oxidizing or reducing agents but not by denaturants which fail to break covalent bonds. Samples of electrophoretically purified haptoglobin were subjected to SDS gel electrophoresis using the conditions of Weber and Osborn (1969) except that the amount of N,N'-methylenebisacrylamide was reduced to 450 mg and different amounts of mercaptoethanol were added to each sample. Figure 32 shows the series of gels obtained when amounts of mercaptoethanol ranged from zero to a very high excess. It is evident that haptoglobin was highly sensitive to mercaptoethanol and that it can be completely broken down

Figure 32: SDS polyacrylamide gel analysis of pure haptoglobin with increasing amounts of 2-mercaptoethanol added.

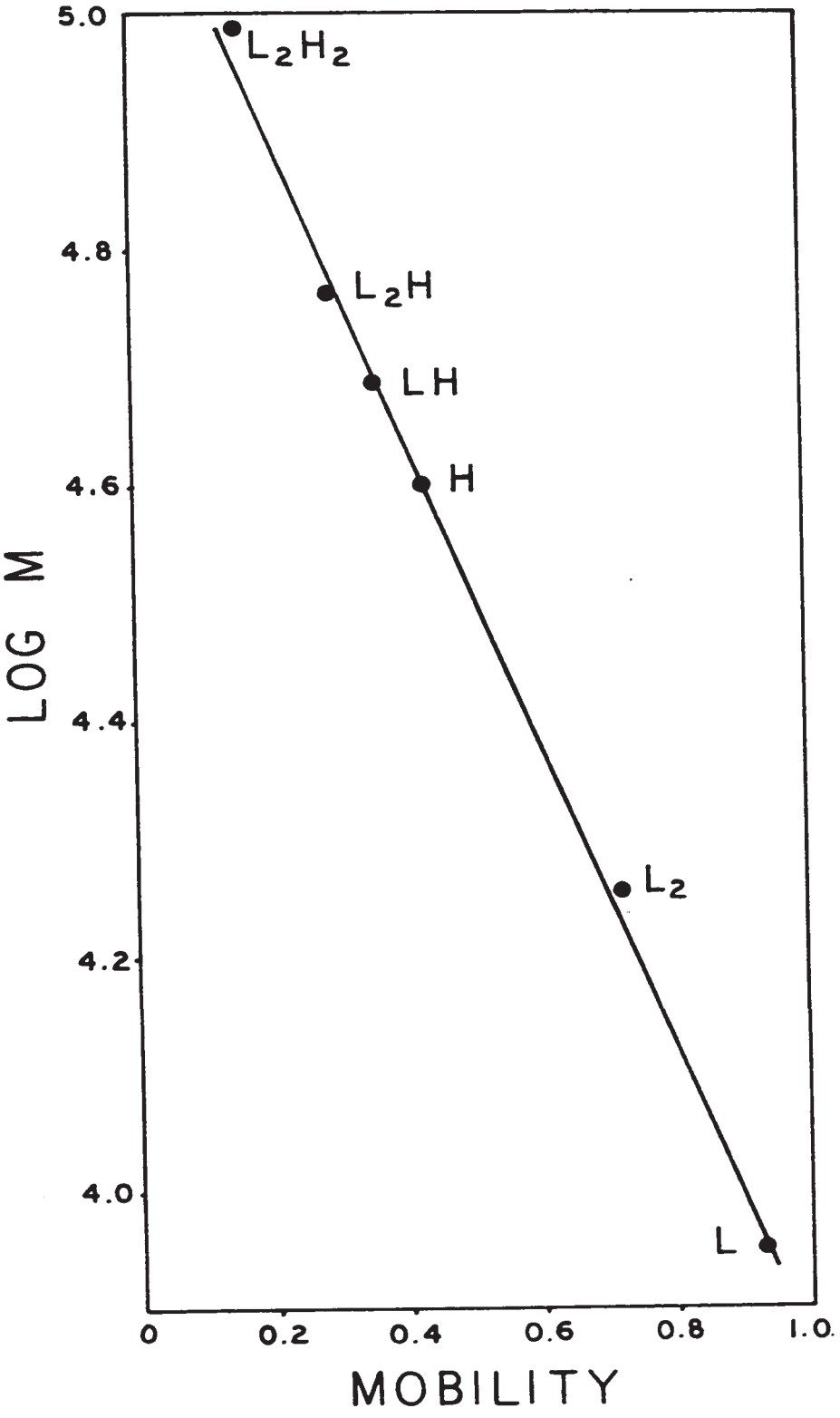
Gel	Approximate molar excess of 2-mercaptoethanol over Hp
a	0
b	15
c	38
d	77
e	155
f	310
g	770
h	770,000


H_2L_2
HL_2
HL
H
L_2
L


H_2L_2
HL_2
HL
H
L_2
L

to two products, but that it does this through a series of isolatable intermediates. By comparison of their mobilities with standards, the two bands in fully reduced haptoglobin were identified as light chains (L_1) with a mobility corresponding to a molecular weight of near 9,000, and heavy chains (H_1) with a mobility characteristic of 40,000. Using these two values as internal standards within a gel all other bands could be tentatively identified from their mobilities. By this means the best identifications were, from the top, L_2H_2, L_2H, LH, H_1, L_2, and L_1. No material corresponding to a mobility of H_2 was observed, and agreement with this identification was quite good (figure 33). It might be argued that two other effects have combined to produce this banding pattern as an artifact. Fish, Reynolds and Tanford (1970) have suggested that protein-SDS complexes form rod-like particles and that these migrate at a rate proportional to the length of the rod. On this basis it would be expected that an internally cross-linked molecule would be unable to form a fully extended rod and would migrate faster than the reduced molecule. However, opposing this is the observation that unreduced proteins bind less SDS than the same reduced proteins (Pitt-Rivers and Impiombato, 1968) and this would be expected to reduce the negative charge and hence reduce the migration rate. Figure 34 shows a test of these effects in which

Figure 33: Mobilities of bands of partially reduced haptoglobin as a function of the log of molecular weight.
Bands are identified as to chain composition on the basis of mobility.


5.0
4.8
4.6
4.4
4.2
4.0
LOG M
L_2H_2
L_2H
LH
H
L_2
L
0
0.2
0.4
0.6
0.8
1.0
MOBILITY

reduced and unreduced lysozyme have been compared. It is clear that reduction has had very little influence on the migration rate and it is likewise possible that effects are comparably small in haptoglobin. It also seems unlikely that any combination of such effects would lead to discrete bands which change only in intensity with additional reducing agent, rather they would be expected to yield a poorly resolved smear of high complexity. The light chain bands were poorly visible with the amount of protein used in figure 32 and they are more evident in figure 35 in which more sample was used. In this case less highly purified haptoglobin was used but it contained no impurities which migrated faster than heavy chains and so light chain behavior could be studied. A band can be seen between light and heavy chains but only in incompletely reduced samples. When complete reduction had occurred, no trace of this band could be found. This behavior, together with its mobility which corresponded to 17,000, support the conclusion that it must be two light chains bridged by a disulfide.

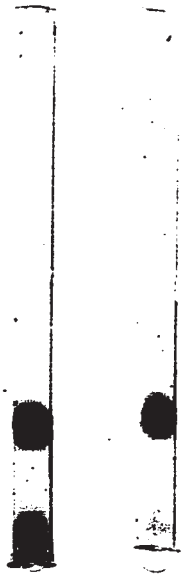
The possibility that these results were due to oxidation or reduction of disulfides by SDS solutions alone was investigated by analysis for sulfhydryl groups in phosphate buffer to which increasing amounts of SDS were added using the PMB method of Boyer (1954). After incubation at 37°C for two hours no -SH groups were detected in any of these

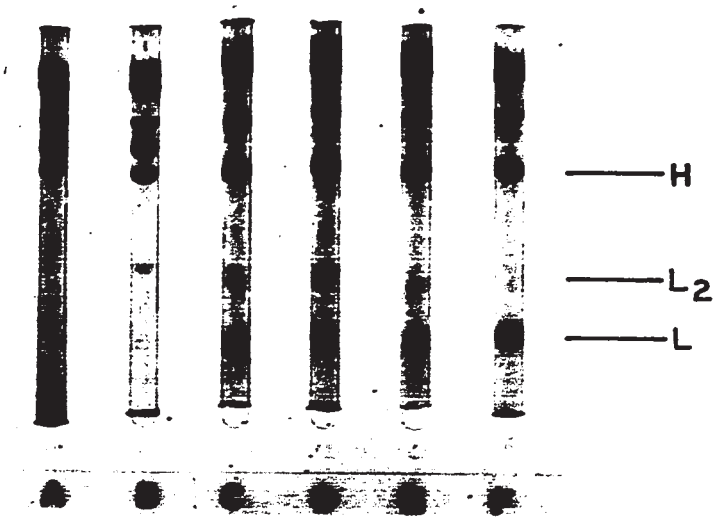
Figure 34: SDS polyacrylamide gel of reduced and unreduced lysozyme.

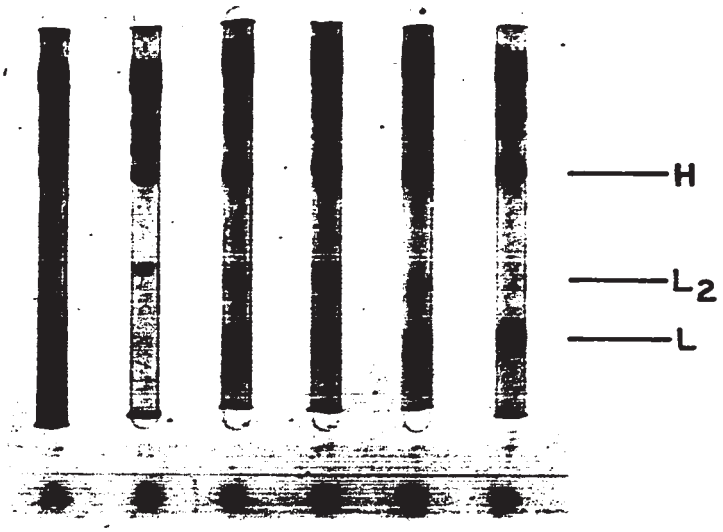
a unreduced

b reduced

Figure 35: SDS polyacrylamide gels of partially reduced semi-pure haptoglobin showing labile band corresponding in mobility to two light chains. Amounts of 2-mercaptoethanol increase from gels A to F.



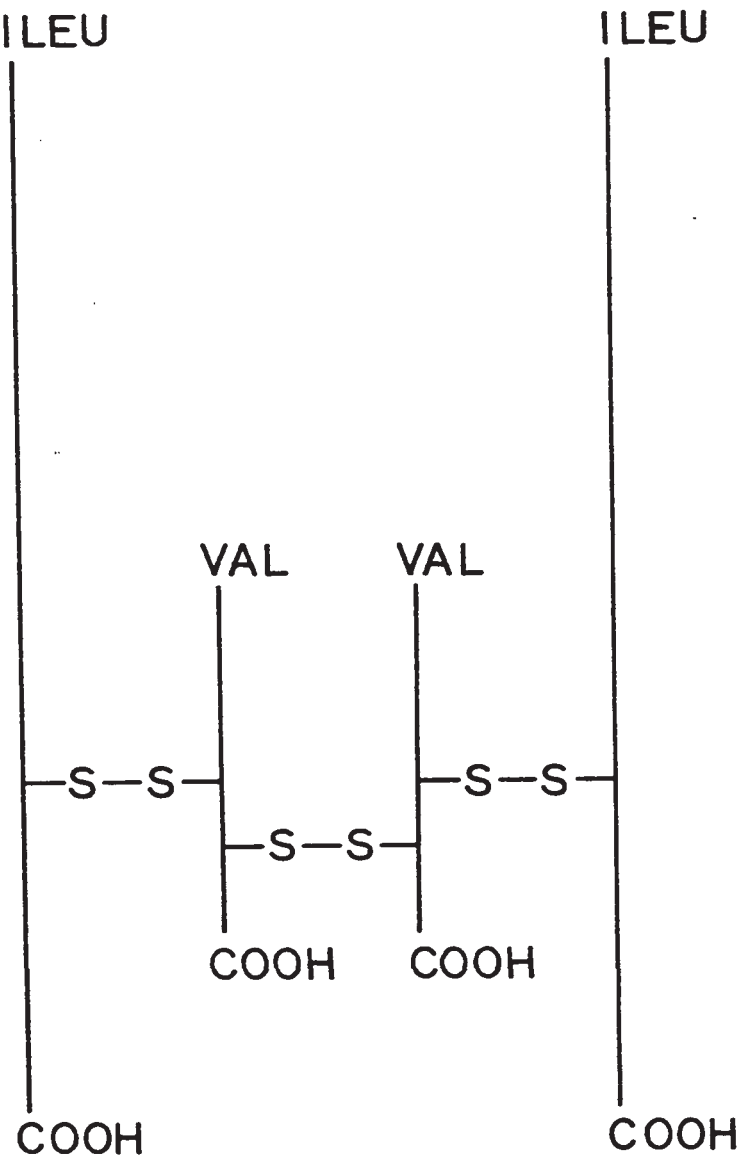

H
L2
L


H
L2
L

samples, an indication that porcine haptoglobin, like human haptoglobin, possesses no reactive sulfhydryl groups. Duplicate samples, oxidized with performic acid by the method of Hirs (1967), were analysed on the long column of the amino acid analyzer and there was no consistent increase of cysteic acid with increasing SDS concentration. Although no one has observed random recombination of proteins in SDS, samples were allowed to stand in air for several days and bands found were identical. The small yield of partial molecules in the case when no mercaptoethanol was added (gel a, figure 32) is thought to be due to trace contamination at some point because of the extreme small amounts required and because of the volatile nature of the compound.

From these structural studies it is concluded that porcine haptoglobin does not dissociate into half molecules on dilution. On the basis of the SDS gel electrophoresis results, a minor rearrangement in the haptoglobin model (figure 1) is proposed to accommodate a species consisting of two light chains. This new model (figure 36) would be expected to yield all the bands observed here, whereas the original model would be expected to show a band for H_2 but none for L_2.

Figure 36: Model proposed for the structure of porcine haptoglobin.
This arrangement of disulfides would represent a minimum number of disulfides. Assignments of N-terminal amino acids to H and L chains is by analogy to human haptoglobin.


ILEU
ILEU
VAL
VAL
S—S
S—S
S—S
COOH
COOH
COOH
COOH

N-terminal amino acid determination

N-terminal amino acids were shown to be the same as in human haptoglobin by chromatography of dansyl derivatives on polyamide sheets. The only amino acid found other than valine and isoleucine was glycine which probably remained from buffers used in preparative gel electrophoresis. The assignment to H and L chains is in analogy with human haptoglobin (Smith et al., 1962; Smithies et al., 1962).

Studies with bifunctional reagents

Hemoglobin, haptoglobin, and hemoglobin-haptoglobin complex were modified with dimethyl adipimidate (DMA) and analyzed by SDS gel electrophoresis. Hemoglobin was used as a model to establish conditions for use of this reagent. Hemoglobin was also reacted with bis(N-maleimidomethyl)ether and the effects of this modification on haptoglobin binding were determined.

Modification of hemoglobin with dimethyl adipimidate (DMA)

The first problem to be encountered in application of a reagent to a new system is that of detecting its presence. This was conveniently accomplished by use of a ^{14}C label during synthesis which yielded activities near 1.4×10^6 dpm per mg.

Since the hemoglobin-haptoglobin system is much more complex than RNase, the only other protein in which this reagent has been used (Hartman and Wold, 1967), reaction conditions were varied in order to discover suitable methods for cross-linking in this system. Hemoglobin was used for these studies, as a model of Hb-Hp complex. As in Hb-Hp complex the possible cross-links are:

between chains between molecules
between chains within molecules
within chains.

Not all molecules bound by protein would be expected to cross-link, however, since imidoesters have short half-lives in aqueous solution (Hunter and Ludwig, 1962). Reagent molecules would often therefore be expected to hydrolyze at one end before or after the other end had reacted with protein.

A buffer of 0.1 M sodium borate made to pH 9.5 with NaOH was used in most cases although phosphate was used from time to time. An initial experiment was performed by dissolving solid reagent in 0.2 per cent hemoglobin and then taking aliquots at time intervals to determine free and bound reagent. Samples were passed through a 2 x 25 cm column of Sephadex G-25 and radioactivity was determined in protein and salt fractions by liquid scintillation counting. Knowing the hemoglobin concentration and the specific

activity of the reagent, amounts bound were calculated and plotted in figure 37. One hour was chosen as a reaction time for further reactions as little further change occurred after that time.

A very similar experiment was performed in which the variable studied was the relative excess of reagent over protein. These results, figure 38, indicated that a ten-fold excess of reagent over lysine was satisfactory since larger amounts gave little further uptake for the additional reagent consumed. For most other experiments a reaction time of one hour with a ten-fold excess of reagent over lysine was chosen.

A third variable which required study was that of protein concentration. With cross-linking reagents inter-molecular cross links would be expected to be sensitive to protein dilution while those within molecules would not. Hemoglobin solutions cross-linked at several concentrations were therefore analysed by gel filtration on a 1 x 100 cm column of Sephadex G-100 equilibrated in 0.15 M sodium chloride and polymers were eluted before hemoglobin. Table 5 shows amounts of soluble polymer detected; at 0.2 per cent no polymer was detected, hence 0.1 per cent was selected for experiments in which only intramolecular cross-links were desired.

Figure 37: Micromoles DMA bound by one micromole hemoglobin at time intervals after mixing.
DMA was equimolar with hemoglobin lysyl residues.

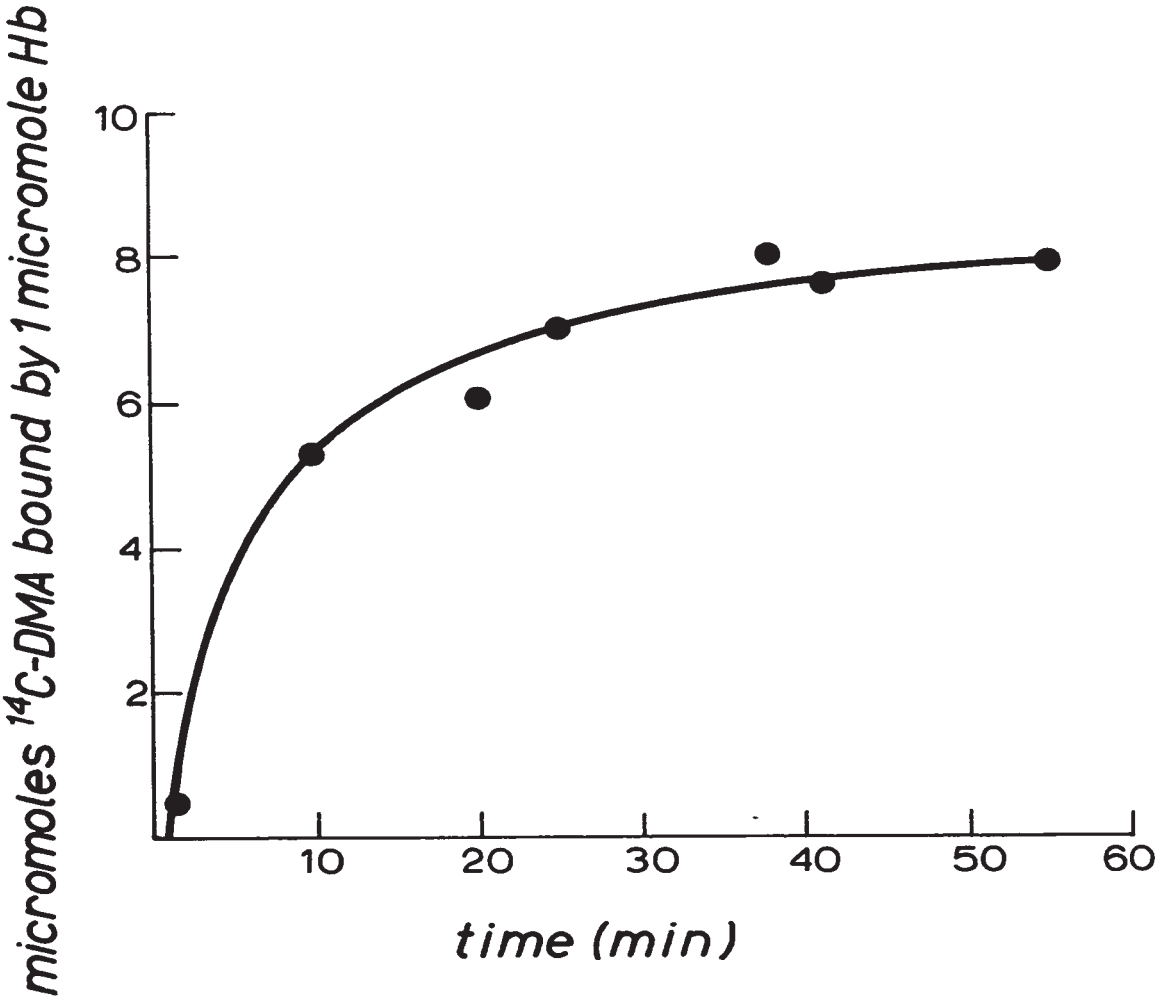

micromoles ^{14}C-DMA bound by 1 micromole Hb
10
8
6
4
2
10
20
30
40
50
60
time (min)

Figure 38: Micromoles DMA bound by hemoglobin one hour after mixing, at several different ratios of DMA to hemoglobin lysyl residues.

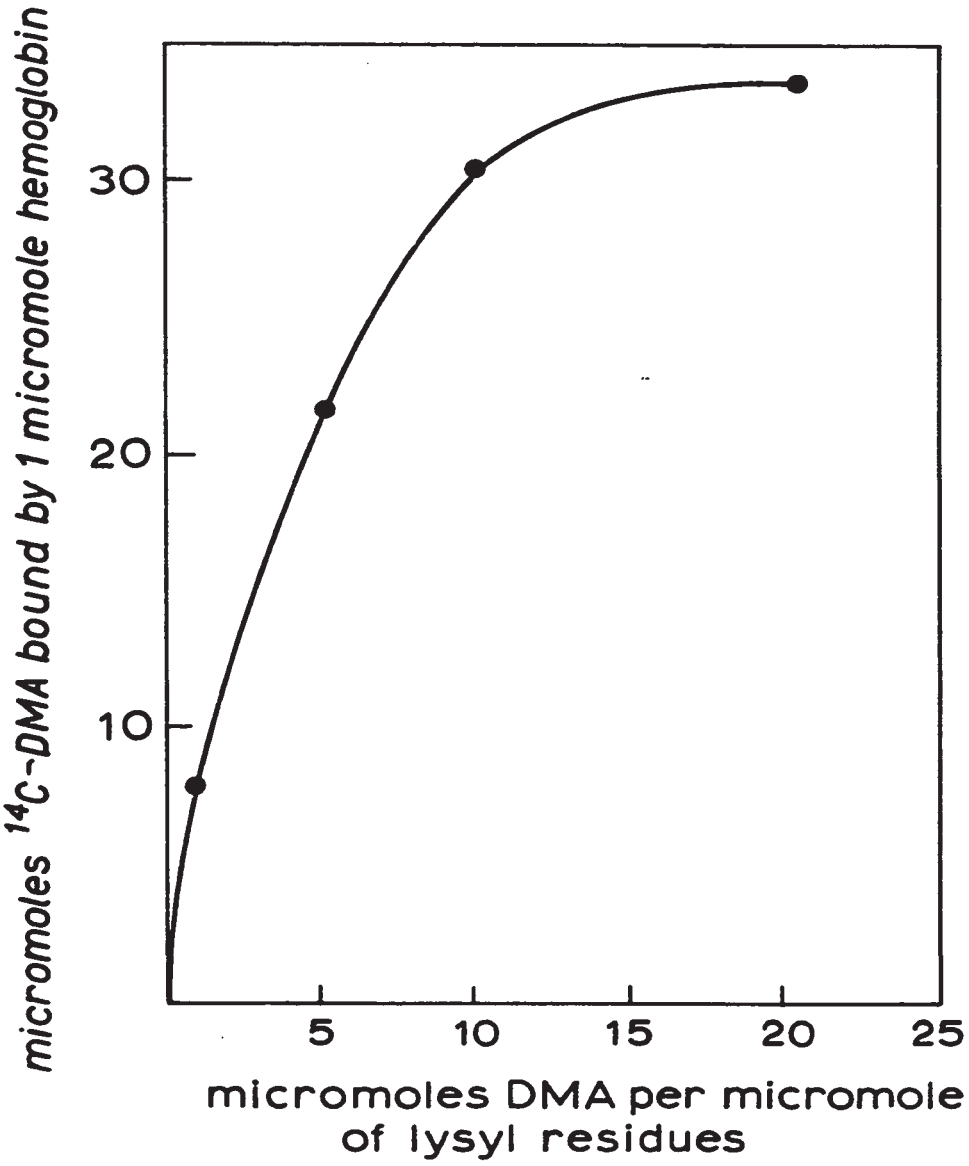

micromoles ^{14}C-DMA bound by 1 micromole hemoglobin
30
20
10
5
10
15
20
25
micromoles DMA per micromole
of lysyl residues

TABLE 5

Polymer formed in hemoglobin solutions cross-linked with ^{14}C-DMA at different hemoglobin concentrations

Hemoglobin concentration (per cent)	Soluble polymer formed (per cent of total)
2.0	22.0
1.0	13.5
0.5	5.3
0.2	0.0

It was desirable to determine haptoglobin binding properties of cross-linked hemoglobin polymer since any retention of binding ability would serve to confirm the conclusion reached earlier that binding sites are not located on the external tetramer surface. Figure 39 shows the elution from Sephadex G-200 superfine of hemoglobin cross-linked at 5.26 per cent using an amount of reagent calculated to be equimolar with hemoglobin lysine. Two polymer peaks were obtained (fractions 12 and 16) as well as a trailing peak characteristic of hemoglobin tetramer (fraction 19). A fraction, tube 16, of the polymer nearest hemoglobin in size was purified by recycling twice on the same column until it contained neither hemoglobin nor the larger polymers. A sample of it was then run alone, with haptoglobin added, and with hemoglobin added (figure 40). The amount of Hp added was sufficient to have bound all of the Hb polymer present, but clearly some remained unbound because a second peak characteristic of Hb polymer was obtained. Hb was also added to polymer and the mixture was separated (figure 40, bottom) in order to show that the polymer had remained cross-linked during the time between its formation and the assay (approximately 1 month). That the Hb polymer failed to bind quantitatively with Hp was not surprising since some molecules would be expected to cross-link internally as well and be unable to dissociate.

Figure 39: Elution of hemoglobin cross-linked at 5.26 per cent from Sephadex G-200 superfine. Column was 1 x 100 cm equilibrated in 0.15 M NaCl.
Hemoglobin was cross-linked with DMA equimolar to hemoglobin lysine.

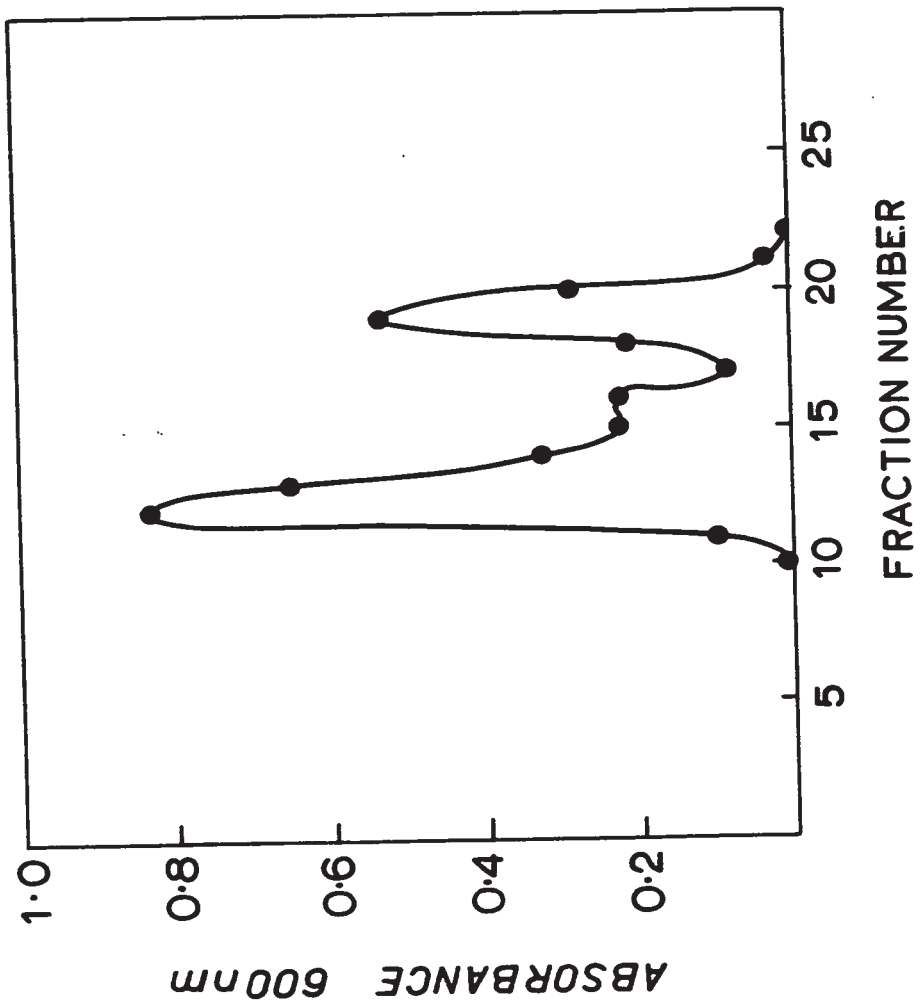
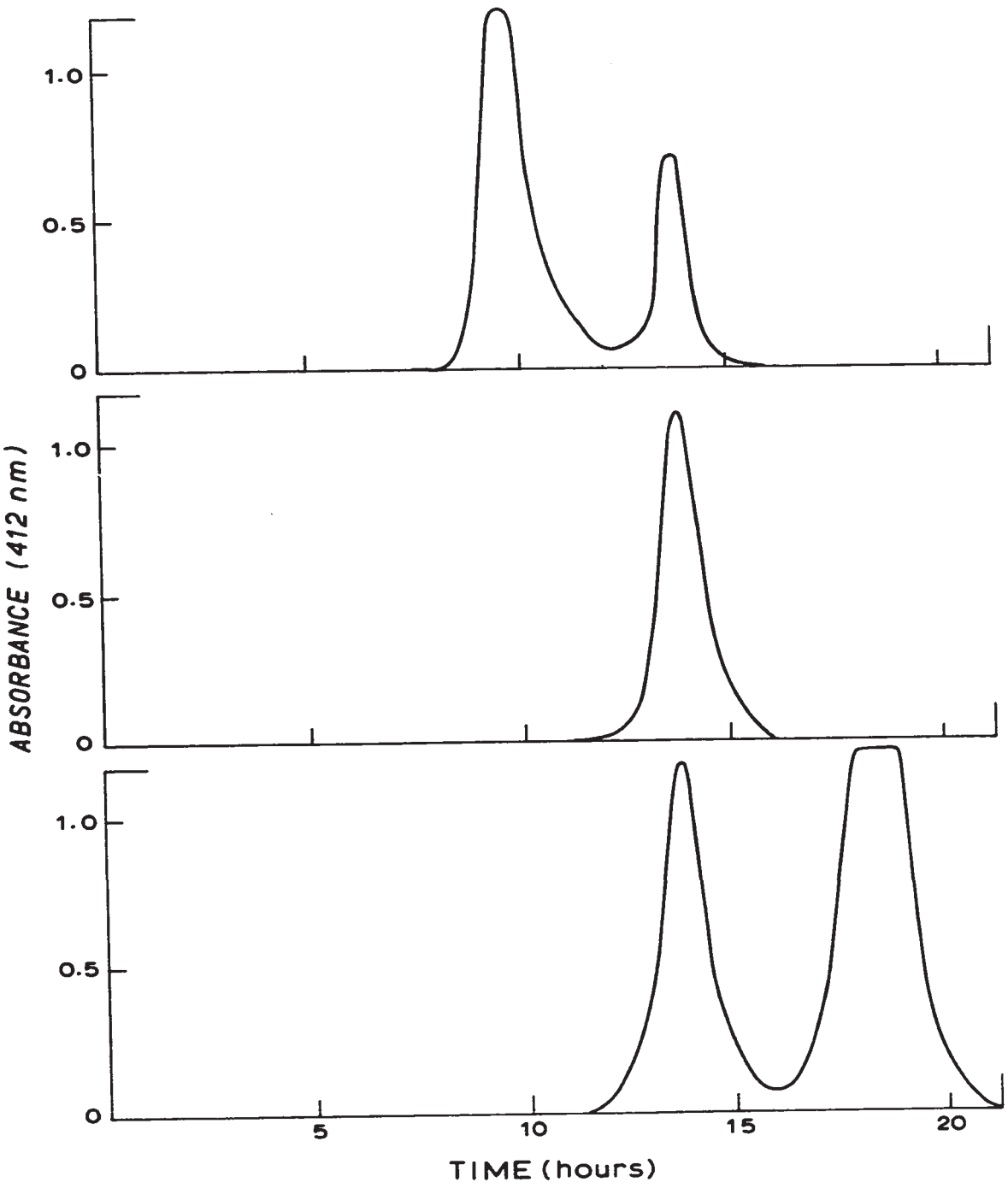

ABSORBANCE 600nm
1·0
0·8
0·6
0·4
0·2
5
10
15
20
25
FRACTION NUMBER

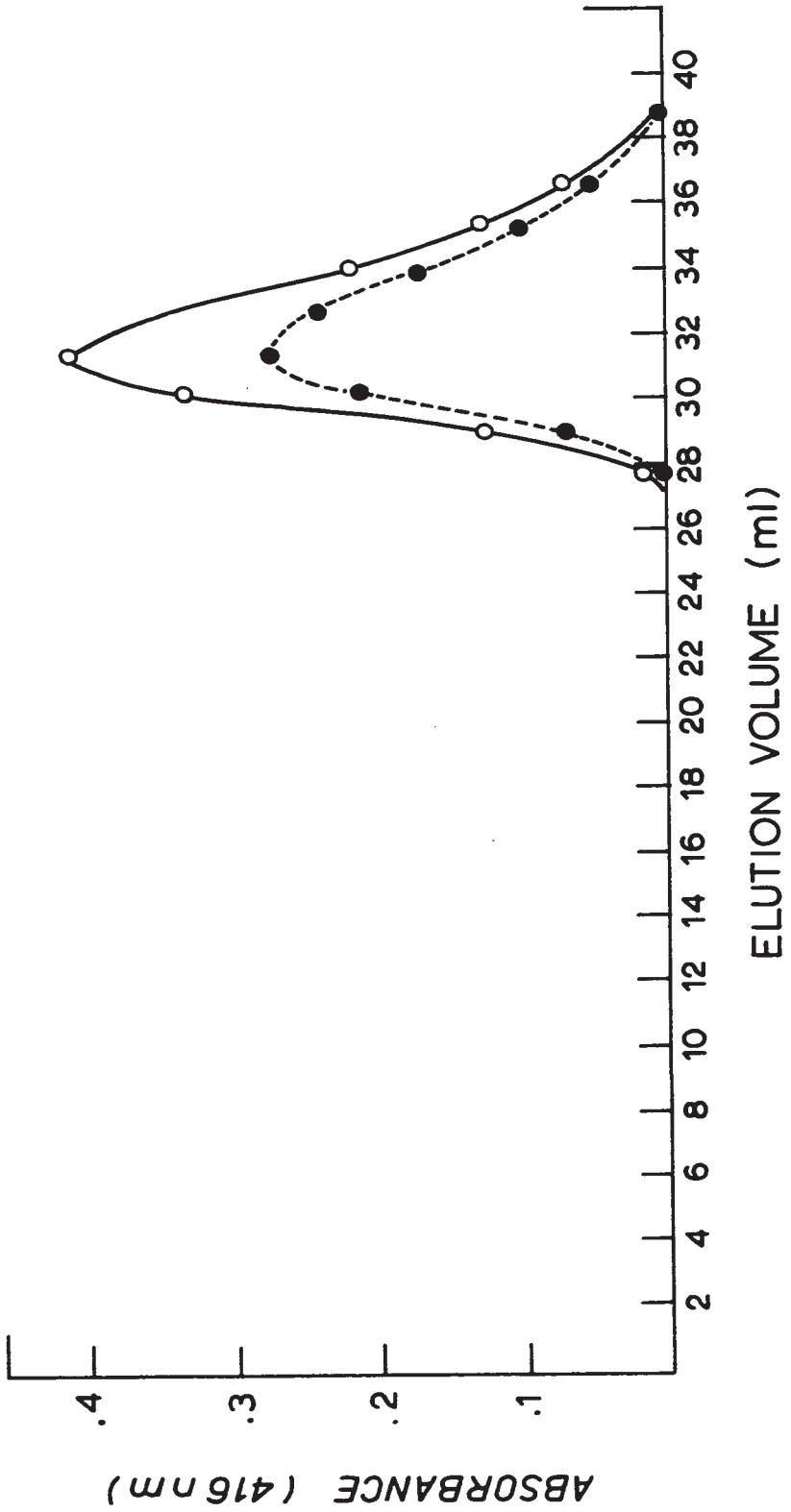
Figure 40: Haptoglobin binding ability of cross-linked hemoglobin polymer.
Elution profiles are from 1 x 100 cm column of Sephadex G-200 superfine.
Above, hemoglobin polymer plus haptoglobin.
Center, hemoglobin polymer alone.
Below, hemoglobin polymer plus hemoglobin.


1.0
0.5
0
1.0
0.5
0
1.0
0.5
0
ABSORBANCE (412 nm)
5
10
15
20
TIME (hours)

Hp-binding by any of the Hb polymer serves to confirm the conclusion reached earlier that sites on the external tetramer surface of Hb are not involved. Since amino groups are on the external tetramer surface (Perutz, 1969), these polymers must represent molecular species which have been cross-linked by various points on this surface. This would lead to a high degree of steric hindrance to the approach of a third macromolecule and would be expected to eliminate binding if the external surface were involved.

For more detailed structural analysis hemoglobin was cross-linked under conditions which yielded only intramolecular cross-links; that is, 0.1 per cent Hb with DMA at ten-fold excess over lysine. Figure 41 shows Sephadex G-100 elution characteristics of this internally cross-linked Hb as compared with unreacted Hb. There was no detectable change in elution volume in spite of incorporation of over 30 moles of reagent per mole of tetramer. From this it was concluded that cross-linking was entirely with the hemoglobin molecule rather than between different molecules. Sedimentation velocity analyses of 0.2 per cent solutions in 0.1 M NaCl gave uncorrected S values of 4.11, 4.15, and 4.12 for three separate preparations, as compared with 3.9 for unreacted hemoglobin. This increase can be attributed to additional weight due to uptake of reagent and to decreased dissociation of Hb tetramers.

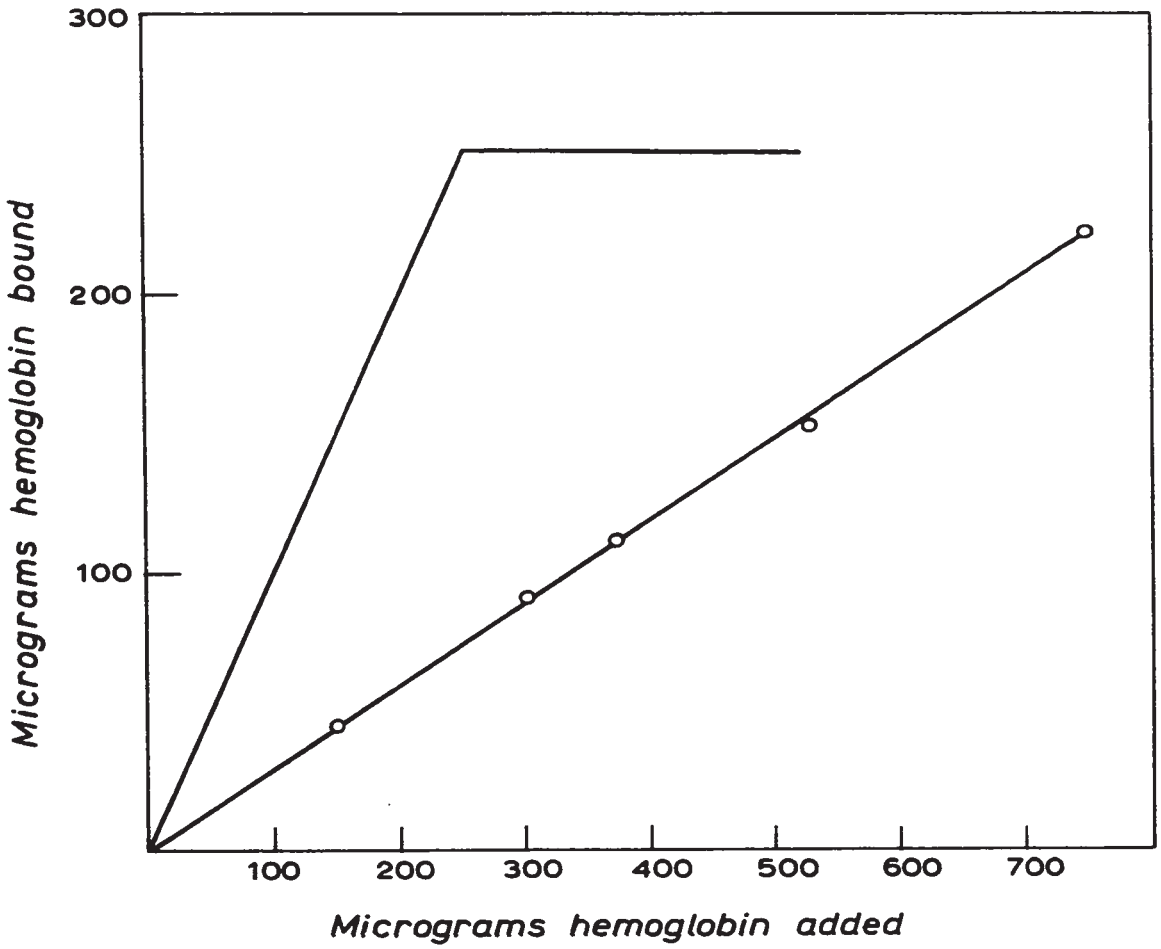
Figure 41: Elution profiles of hemoglobin (filled circles) and hemoglobin internally cross-linked with DMA (open circles) from a 0.9 x 50 cm column of Sephadex G-100.


ABSORBANCE (416 nm)
.4
.3
.2
.1
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
ELUTION VOLUME (ml)

Haptoglobin binding properties of this material were determined by gel filtration of mixtures of haptoglobin and cross-linked hemoglobin. For this derivative it was necessary to titrate haptoglobin with increasing amounts of hemoglobin-DMA because it was thought to be highly heterogeneous in regard to positions of cross-links. Figure 42 shows amounts bound by haptoglobin when various amounts of Hb-DMA were added. If binding were normal, a linear relation with slope 1 would be expected up to saturation of all available haptoglobin. Rather a linear relation with reduced slope was obtained indicating that only about one third of the Hb-DMA was capable of binding with haptoglobin. Since amidination per se did not interfere with binding, the restrictions observed here can reasonably be attributed to the cross-linking function of this reagent.

Direct analysis for cross-linked lysine residues was possible using the amino acid analyzer and the conditions developed by Hartman and Wold (1967). A normal short column analysis was performed until arginine had emerged, at which point the buffer was changed to borate pH 9.7; a peak of radioactivity emerged about 40 minutes later at the position expected for cross-linked lysines. All fractions were collected and counted for radioactivity which revealed that the majority of label (70%) eluted at the front with neutral and acidic amino acids. An additional 11% eluted as

Figure 42: Titration of haptoglobin (390 micrograms) with increasing amounts of hemoglobin cross-linked with DMA (circles).
Solid line is that expected if binding is not restricted.

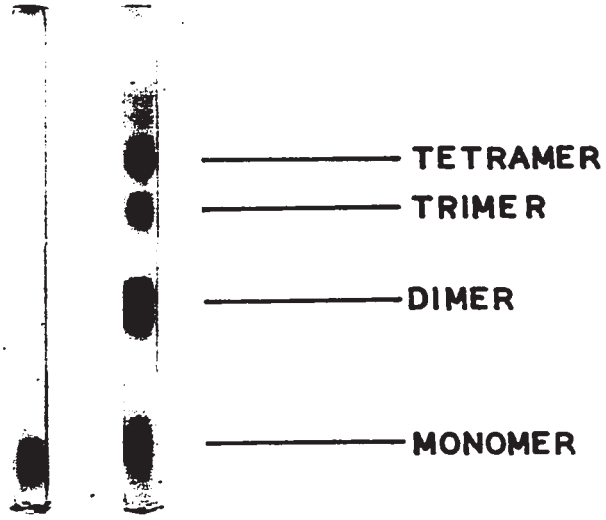

300
200
100
Micrograms hemoglobin bound
100
200
300
400
500
600
700
Micrograms hemoglobin added

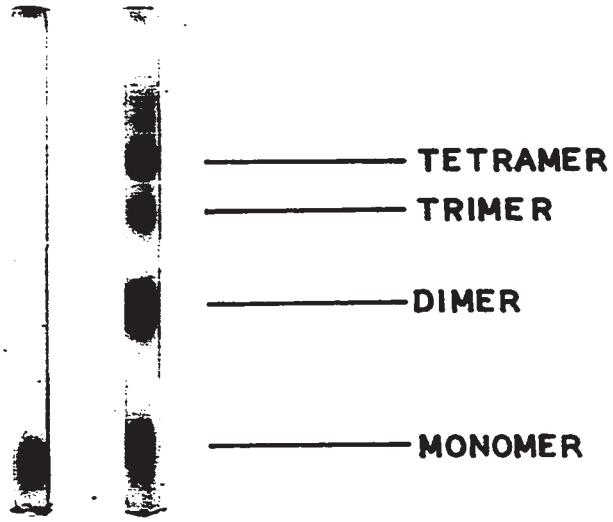
ninhydrin-negative material 13 minutes after arginine; the remaining 19% eluted in the position of cross-linked lysines and was ninhydrin positive. Hartman and Wold (1967) found cross-linked lysine (N_{ε},N_{ε}-adipamidino-bis-L-lysine) to be 83-86% stable to acid hydrolysis, and this would indicate a maximum of 8 cross-links per hemoglobin tetramer, since 34 moles of DMA were bound per mole of tetramer.

The nature of the other radioactive components has not been investigated. Acidic components would be expected from the 26 moles of DMA which were bound but did not cross-link since the free end would form either the normal ester or the nitrile (Hunter and Ludwig, 1962), either of which would produce the acid during hydrolysis. Even still bound to lysine, such a compound would be neutral.

Figure 43 shows a representative SDS gel of hemoglobin and hemoglobin cross-linked with DMA. The mobility of hemoglobin was characteristic of quantitative dissociation to monomers but hemoglobin-DMA showed 3 major regions in addition to monomers. Davies and Stark (1970) have cross-linked several oligomeric proteins with dimethyl suberimidate and then analyzed cross-linked proteins by SDS-gel electrophoresis. They found bands formed with mobilities typical of molecular weights which were integral multiples of the fastest moving monomer. The number of major bands could be used to count the number of subunits by assuming bands represented monomers, dimers, trimers, etc., up to the

Figure 43: SDS-gel electrophoresis of hemoglobin (left) and of hemoglobin cross-linked with DMA (right).


TETRAMER
TRIMER
DIMER
MONOMER


TETRAMER
TRIMER
DIMER
MONOMER

number of subunits per molecule. In hemoglobin then the four major regions can be designated, from the top, tetramer, trimer, dimer, and monomer. The expectation that these products should be formed and their relative mobilities support this interpretation. Since Weber and Osborn (1969) found SDS treatment to cause complete dissociation of a large number of oligomeric proteins, it can be concluded that forces maintaining Hb-DMA in dimers or higher aggregates are covalent bridges introduced between subunits by reaction with DMA.

Unfortunately the resolution achieved on an analytical scale has not yet been achieved by conventional preparative methods. Several attempts were made to factionate Hb-DMA on gel filtration columns equilibrated with such denaturing solvents as 8M urea, 5M guanidine HCl, or 0.1 M SDS, but none of these gave satisfactory resolution.

The broad region designated hemoglobin dimer consisted of two poorly resolved bands. This was expected from knowledge of hemoglobin quarternary structure; at least two dimers would be expected to form on cross-linking, namely $\alpha_1\beta_1$ and $\alpha_1\beta_2$. Similar heterogeneity would be expected in trimer and tetramer bands but, if present, these different products have not been resolved.

It was of interest to determine whether binding with haptoglobin was a property of any single one of these

electrophoretic bands. A three-fold molar excess of Hp was therefore added to Hb-DMA and the mixture was separated into complex and non-binding Hb-DMA by gel filtration on Sephadex G-100. Figure 44 shows the SDS-gel analysis of these fractions as compared with Hb-DMA and Hp. All of the bands present in Hb-DMA can be recognized in the fraction which bound to haptoglobin and in the fraction which failed to bind. There are obvious quantitative differences, however, for the monomer fraction has almost disappeared from non-binding Hb-DMA (gel c) and very little dimer, trimer, or tetramer have bound to Hp (gel d).

In solution, before denaturation with SDS, Hb-DMA would presumably resemble native Hb except that cross-links would impose new conformational restraints. It is evident that cross-linking has severely restricted ability to bind with Hp (figure 42). It is also evident from figure 44 that Hp has selected for binding mostly those molecules or sub-units of Hb-DMA which did not contain interchain cross-links. If Hb binds as dimers (Nagel and Gibson, 1967, 1971; Adams and Weiss, 1969), then it is difficult to explain failure of at least one cross-linked dimer band of Hb-DMA to bind near quantitatively with Hp. It would seem that either the dimer hypothesis is incorrect or that interchain cross-linking has disallowed a binding conformation of Hb. The latter explanation seems the more likely because cross-linked

Figure 44: SDS-gel electrophoresis of Hb-DMA and its Hp-binding and non-binding components.

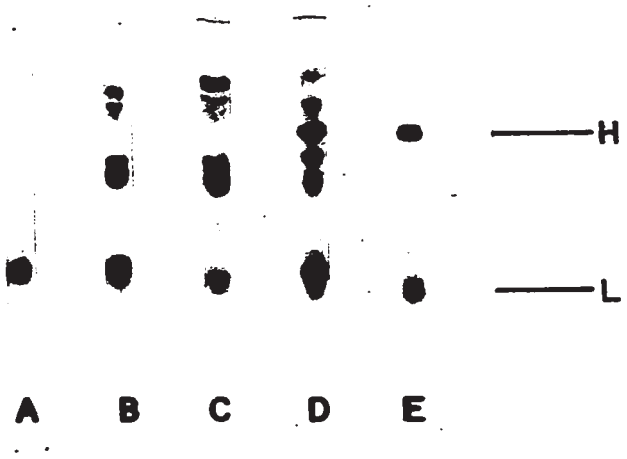
a hemoglobin

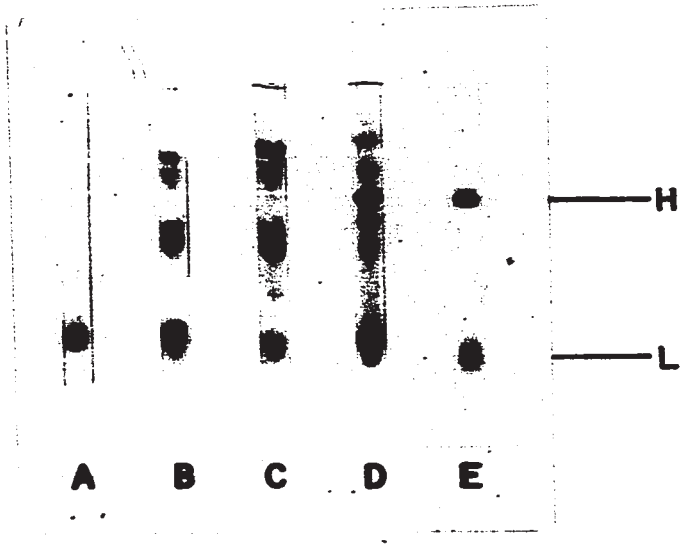
b hemoglobin-DMA

c hemoglobin-DMA which did not bind

d hemoglobin-DMA-haptoglobin complex

e haptoglobin


H
L
A
B
C
D
E


H
L
A
B
C
D
E

dimers did bind to some extent, indicating that dissociation to monomers is not a prerequisite.

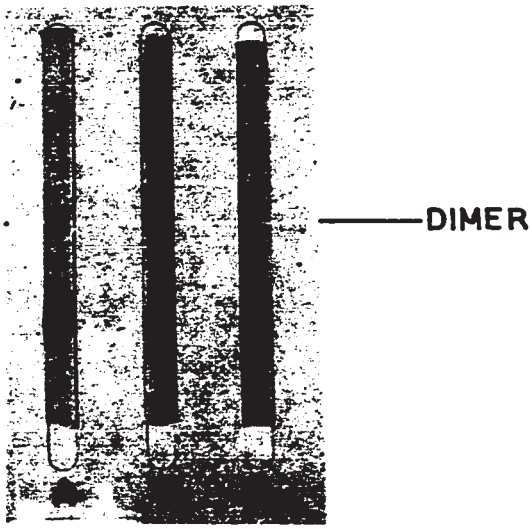
A tentative identification of the more rapidly migrating of the two dimer species has been made. As was shown earlier, (figure 30), hemoglobin undergoes dissociation in dilute solution. Perutz (1969) has reported that the $\alpha_1\beta_2$ contacts break giving rise to $\alpha_1\beta_1$ dimers. Therefore a solution of hemoglobin was cross-linked at a concentration of 0.001 per cent, in which the predominant species present would be this dimer (Chiancone et al., 1968). Under these conditions only one dimer band was formed (figure 45) and it corresponded exactly to the more rapidly migrating one present in other preparations.

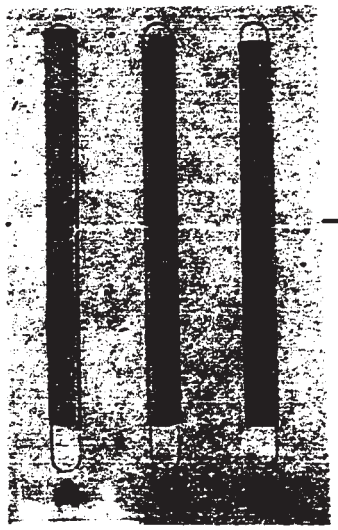
These experiments on cross-linking indicated that DMA was a useful reagent for studying the hemoglobin-haptoglobin system because cross-linked subunits could be obtained.

Modification of haptoglobin with dimethyl adipimidate (DMA)

Haptoglobin was modified with DMA in the same fashion as was hemoglobin except the protein concentration was 0.15 per cent to keep comparable molarity and the amount of reagent was increased in accordance with a lysine content of 66 residues per molecule (Black et al., 1970). Again no indication of intermolecular cross-links was obtained by gel

Figure 45: Comparison of hemoglobin cross-linked at 0.001 per cent (left) and at 0.1 per cent (center. Hemoglobin is shown at right for comparison.


DIMER


DIMER

filtration and analysis was carried out by SDS gel electrophoresis.

Figure 46 shows an SDS gel obtained of Hp-DMA. Mobilities of bands were calculated as (from the top) 0.193, 0.287, 0.420, 0.467, 0.533, and 0.960. The most rapidly moving band was clearly some free light chain but no band corresponding to 2 light chains was observed. Figure 47 shows a plot of mobility against log molecular weight. Molecular weights were estimated on the assumption that certain combinations of chains would be present and the fit was so close that it seems probable that these assignments are correct. The band with mobility 0.380 has been omitted because it corresponds to an impurity known to have been present in this preparation.

From this experiment it may be concluded with some certainty that haptoglobin in solution at 1.5 mg per ml exists as an H_2L_2 molecule rather than a dissociated half molecule. Failure to obtain a band corresponding to L_2 was unexpected; it seems likely that cross-links form between light chains because a band with the mobility of L_2H was found. Apparently light chains cross-link to heavy chains more readily than to each other. Most material was cross-linked into a high molecular weight species thought to be L_2H_2 but no conclusion can be reached about structures present there.

Figure 46: SDS-gel electrophoresis of haptoglobin cross linked with DMA.
A faint band of free light chains was present but was difficult to photograph.

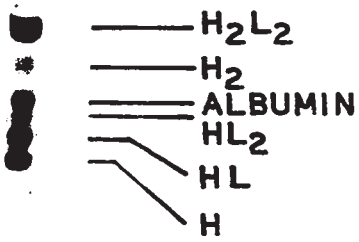
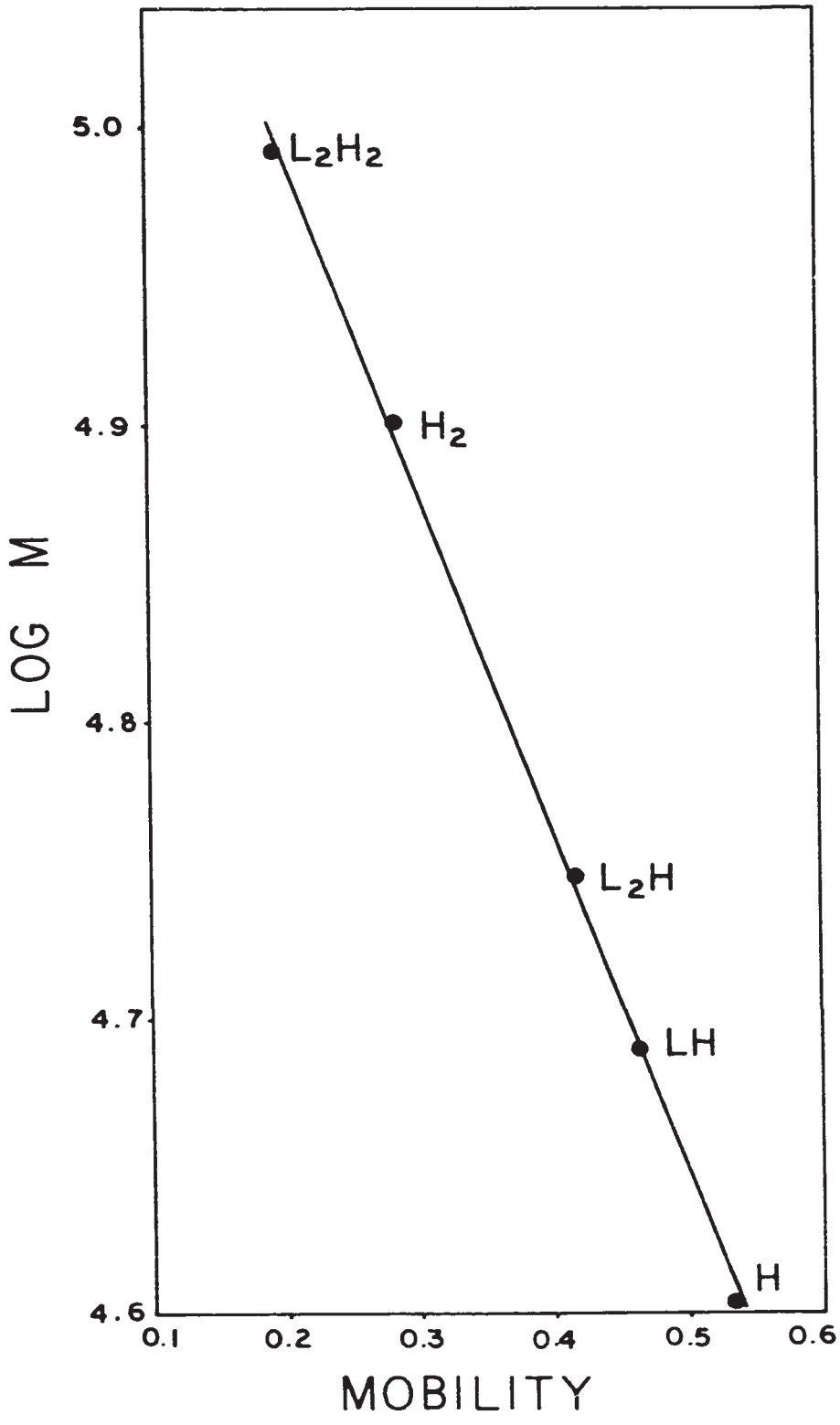

H_2L_2
H_2
ALBUMIN
HL_2
HL
H

Figure 47: Mobilities of bands of cross-linked haptoglobin as a function of the log of expected molecular weight.
Bands are identified as to chain composition on the basis of mobility.


5.0
4.9
4.8
4.7
4.6
LOG M
L_2H_2
H_2
L_2H
LH
H
0.1
0.2
0.3
0.4
0.5
0.6
MOBILITY

Modification of hemoglobin-haptoglobin complex with dimethyl adipimidate (DMA)

A similar cross-linking experiment was carried out on hemoglobin-haptoglobin complex, again with the exceptions that protein concentration was increased to 0.25 per cent to maintain the molarity used with hemoglobin and the amount of reagent was increased to keep it at 10 per cent lysine (assuming 110 lysine residues). Under these conditions 74 moles of reagent were taken up but no attempt was made to locate sites of incorporation because of the high complexity of the products as revealed by SDS gel electrophoresis (figure 48). In order to achieve this separation it was necessary to reduce the N,N'-methylenebisacrylamide by half and also to halve the acrylamide concentration, which results in extremely fragile gels. The properties of such loosely cross-linked gels have not been investigated in SDS electrophoresis and it has not been established whether they still separate on the basis of molecular weight. It would seem however that they do because similar banding patterns were observed with normal gels except that resolution was not as good. Three bands can be readily identified with certainty, namely the fast moving monomer band of Hb chains and possibly L chains (resolution of small molecules was very poor with such porous gels), the H chain band, and one between these characteristic of hemoglobin dimer. This yield

Figure 48: SDS-gel electrophoresis of hemoglobin-haptoglobin complex cross-linked with DMA.

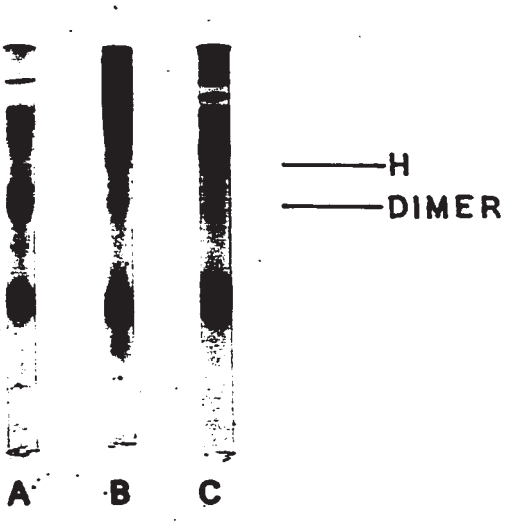
Above: Gel with normal cross-linking and acrylamide

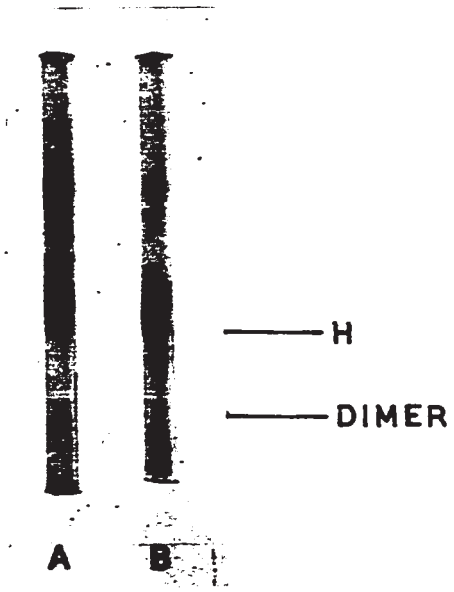
- a Hb-DMA
- b Complex-DMA
- c Complex

Below: Gel with half cross-linking and half acrylamide

- a Complex-DMA
- b Complex

Hb monomers and L chains have been run off the end in an effort to separate slow components.


H
DIMER
A
B
C


H
DIMER
A
B

of cross-linked hemoglobin dimer is highly significant because it is the first indication that, when bound, hemoglobin chains are sufficiently close together to be cross-linked by a reagent which can span only 8.6 Å. The fact that this dimer corresponds closely the more rapidly migrating one which is also formed when hemoglobin exists as $\alpha_1\beta_1$ dimers suggests that this may in fact represent an $\alpha_1\beta_1$ dimer which has been cross-linked while bound to haptoglobin. It is very much more difficult to identify bands moving more slowly than H chain in this case because of the additional possibilities due to various combinations of hemoglobin chains. While such an attempt can be made it would be highly speculative and may better await preparation of these materials.

This experiment has shown that a very complex mixture of interchain bridges has resulted from cross-linking Hb-Hp complex. The most significant finding has been a product tentatively identified as hemoglobin dimer obtained by cross-linking Hb-Hp complex. No such band was obtained from unreacted hemoglobin, haptoglobin, or complex; a band of that mobility was present only in cross-linked complex. It was well separated from all other bands except the other Hb dimer and this tends to support an identification based on mobility. Since the more rapidly moving dimer is thought to be $\alpha_1\beta_1$, the dimer formed by cross-linking Hb-Hp complex may

well represent that dimer. This would support the hypothesis that Hp binds $\alpha_1\beta_1$ dimers and that these chains maintain a close spatial relationship when bound to Hp.

The potential of cross-linking reagents for elucidating structural features of the Hb-Hp complex has been established, and thus the purpose of these studies has been fulfilled. The difficulty in this approach has been in separating cross-linked subunits. It is necessary to use denaturants in order to separate cross-linked subunits from those same subunits that have not become cross-linked. It seems likely that preparative SDS-gel electrophoresis will succeed when a method has been developed to give greater mechanical strength to highly porous gels. Successful preparation of cross-linked subunits will allow their positive identification and lead to peptide analysis to identify cross-linked residues. Knowing the structure of hemoglobin this would help to locate sites for Hp-binding. Analysis of the cross-linked Hb dimer obtained from complex would determine whether the same cross-links have been formed as in Hb and hence answer questions of conformation. There can be little doubt that cross-linking with DMA offers an opportunity to elucidate several important structural features of Hb-Hp complex.

Modification of hemoglobin with bis(N-maleimidomethyl) ether (BME)

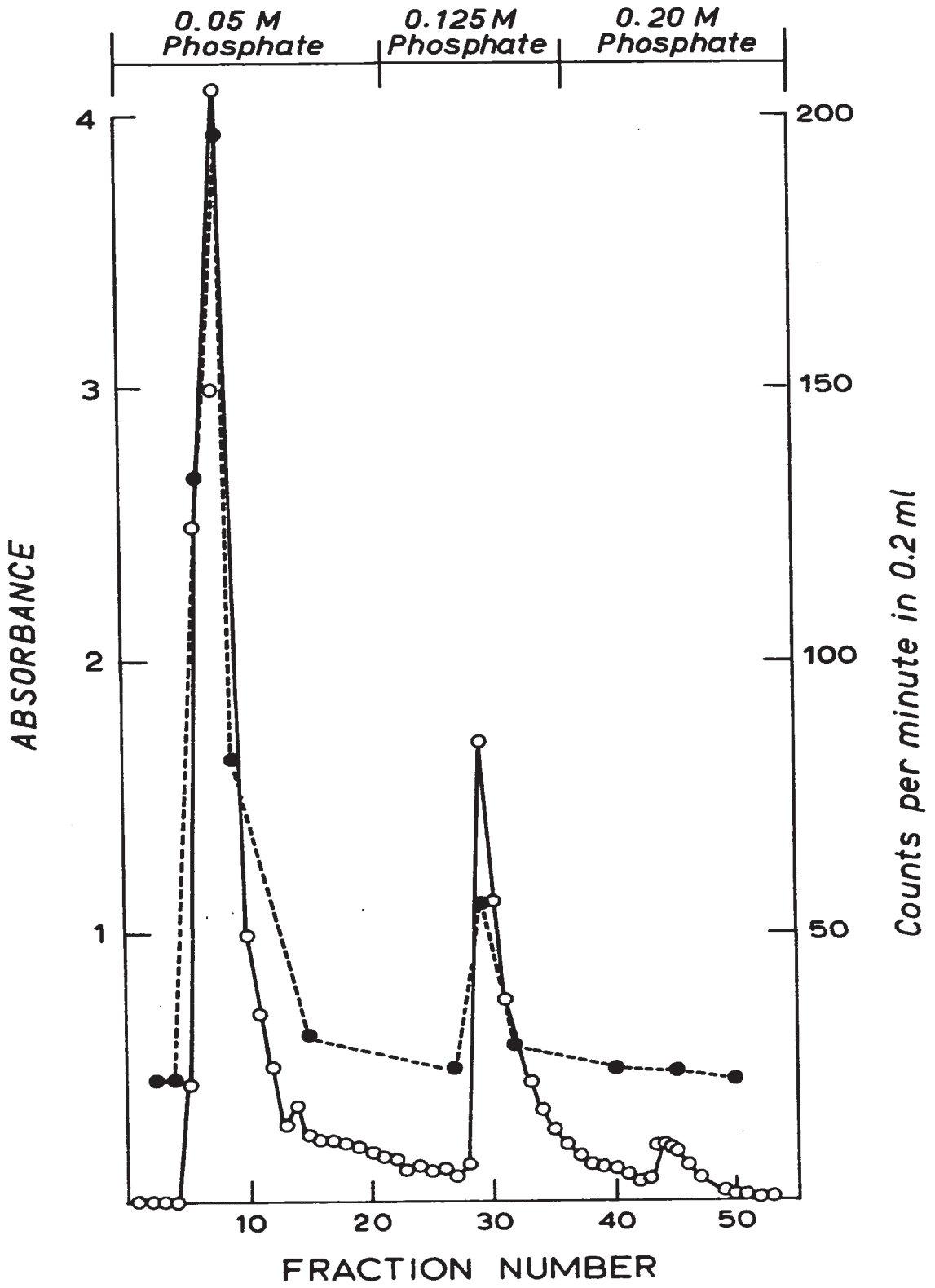
BME was first introduced as a protein reagent by Simon and Konigsberg (1966) for modification of reactive sulfhydryl groups of hemoglobin (figure 9). Following reaction with a 2-fold molar excess of ^{14}C-BME, they separated the mixture on Bio-Rex 70 and obtained three fractions containing 3, 2, and 0 molecules of BME respectively per tetramer (determined by planchet counting of radioactivity and yield of modified cysteine in hydrolysates). They found that human Hb-BME_2 failed to dissociate into dimers in 0.25 M $MgCl_2$ but did so in 2.5 M guanidine HCl. Exposure to excess cysteine did not increase the yield of modified cysteine and they concluded that the second maleimide ring had reacted to form an acid-labile product with some group within the β-chain, that is, it had formed a bridge between cysteine β-93 and some other residue in the same β-chain.

The effect of this modification on dissociation was of interest and Simon et al. (1967) made osmotic pressure determinations of human and horse Hb-BME_2 in 0.25 M $MgCl_2$, a solvent which normally dissociates hemoglobin into dimers. They found this solvent to dissociate horse Hb-BME_2 to the same extent as unreacted horse Hb; human Hb-BME_2, however, did not dissociate in this medium. Furthermore, crystallographic analysis revealed horse Hb-BME_2 to be locked in its liganded configuration even as deoxyhemoglobin.

Bunn (1967) determined the haptoglobin-binding properties of human Hb-BME_2 and found it to bind only one quarter as well as unreacted Hb. In view of restricted dissociation of human Hb-BME_2, these results appeared to be a test of the hypothesis that hemoglobin dimers are the units which bind to haptoglobin. Failure to dissociate would thus be expected to cause failure to bind. The observations of Simon et al. (1967) on horse Hb-BME_2 appeared to offer an opportunity to confirm the hypothesis of binding by hemoglobin dimers because it dissociated into dimers to the same extent as unmodified Hb in 0.25 M $MgCl_2$. Horse Hb-BME_2 would therefore be expected to bind haptoglobin stoichiometrically. We sought therefore to perform experiments to determine the binding properties of horse Hb-BME_2.

BME was synthesized by the methods of Tawney et al. (1961) and reacted with human and horse hemoglobin so that binding properties could be determined. To a 4 per cent solution of human or horse hemoglobin was added sufficient solid BME to give a ratio of 2 moles BME per mole of hemoglobin tetramer. The mixture was then chromatographed on Bio-Rex 70 and figure 49 shows a separation of horse hemoglobin into 2 major components. Fractions 9 and 29 were selected and all further analyses were performed on them. Accurate radioactivity counts revealed that fraction 9 contained 2 molecules of BME per tetramer but that fraction 29 contained only 1; hemoglobin tetramers containing only

Figure 49: Chromatography of horse hemoglobin reacted with BME (at a BME:tetramer ratio of 2:1) on a 2 x 50 cm column of Bio-Rex 70 in phosphate buffer pH 6.8. Buffer concentrations refer to sodium content. Fractions of approximately 12 ml were collected at a flow rate of 70-80 ml per hour.
Solid line, absorbance 570 nm.
Broken line, counts per minute in 0.2 ml.


0.05 M Phosphate
0.125M Phosphate
0.20 M Phosphate
ABSORBANCE
Counts per minute in 0.2 ml
FRACTION NUMBER
4
3
2
1
200
150
100
50
10
20
30
40
50

one molecule of BME have not previously been reported but there seems no reason why they would not be expected to form. A similar fractionation of human hemoglobin treated with BME also gave a peak containing one molecule of BME per tetramer.

The yield of human or horse Hb tetramers containing one molecule of BME was not anticipated and therefore the uptake of reagent was checked by determination of remaining reactive sulfhydryl groups using the methods of Boyer (1954). Figure 50 shows a representative titration of unreacted horse hemoglobin and a sample from tube 29. Table 6 shows amounts of BME bound and amounts of sulfhydryl group remaining for the two fractions of horse Hb treated with BME. It is evident that uptake of BME has been at the expense of sulfhydryl groups. The value of 2.39 for untreated hemoglobin is in agreement with Benesch and Benesch (1962) who obtained 2.4, but is significantly higher than 2.0 reported by Bunn (1967). This increase over a value of 2.0 may represent partial reaction with normally unreactive -SH groups combined with experimental error. The spectrophotometric method (Boyer, 1954) depends upon accurate measurement of small changes in absorbance when both sample and blank already have an absorbance near 0.6, and consequently is lower accuracy than radioactivity counts. Several titrations of human hemoglobin yielded -SH contents

Figure 50: Titration of sulfhydryl groups with p-chloromercuribenzoate (4.01×10^{-4} M). Closed circles, titration of 1.345×10^{-2} micromoles horse hemoglobin. Open circles, titration of 1.520×10^{-2} micromoles horse hemoglobin-BME_1.

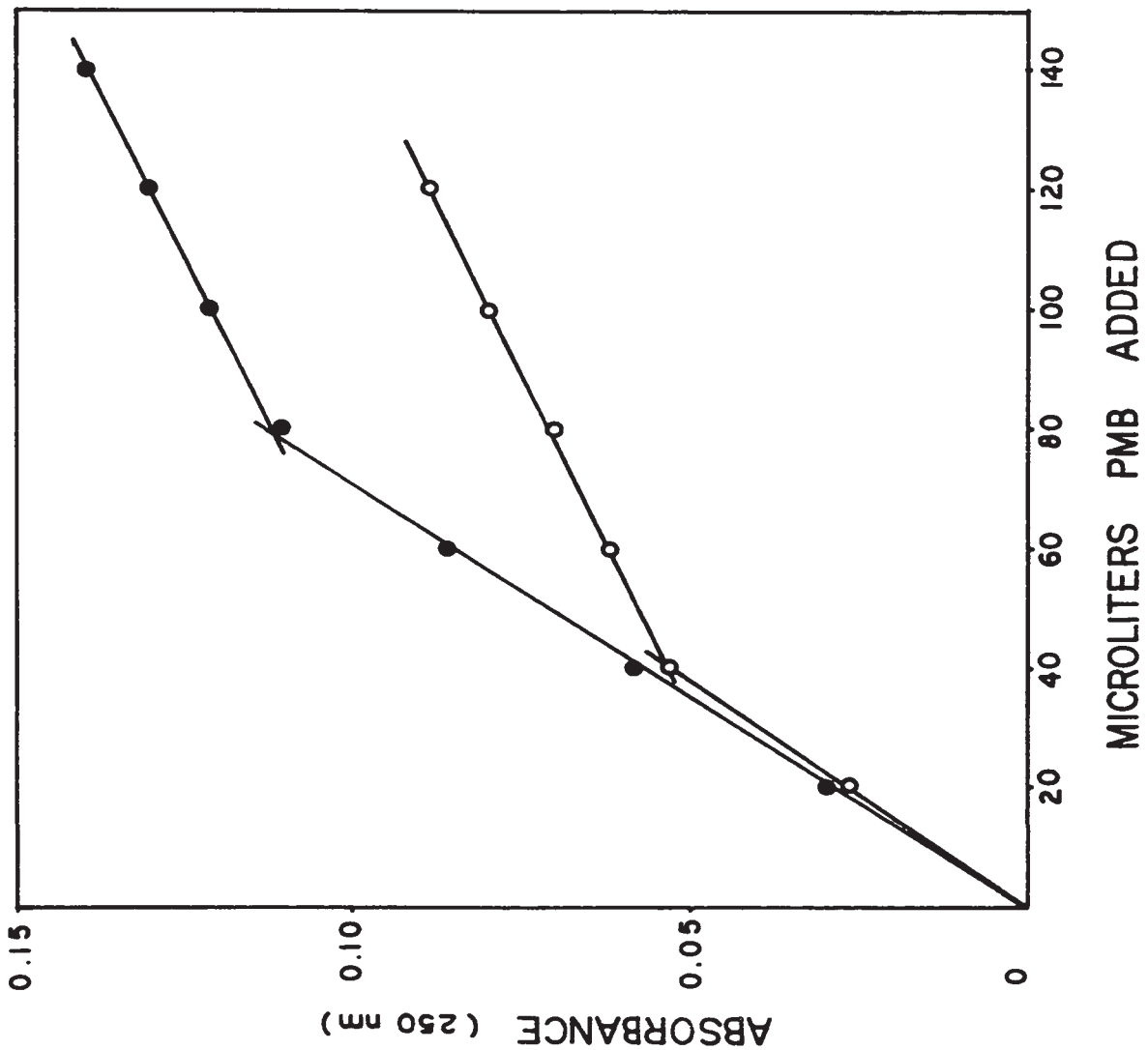

ABSORBANCE (250 nm)
0
0.05
0.10
0.15
20
40
60
80
100
120
140
MICROLITERS PMB ADDED

TABLE 6

Analysis of horse hemoglobin and BME derivatives for BME uptake (as disintegrations per minute per four heme groups), and for free sulfhydryl groups (by titration with p-chloromercuribenzoate)

	Peak 1	Peak 2	Hemoglobin
Moles BME Bound	1.98	0.99	-
Moles -SH remaining	0.16	1.05	2.39

of 1.97 to 2.36 per tetramer. It was concluded from these studies that BME uptake was specific to the reactive sulfhydryl groups at position 93 of β-chains and that horse Hb-BME_1 had one chain so modified while horse Hb-BME_2 had two.

Since BME has the potential for forming intermolecular cross-links, samples of horse Hb-BME_1, horse Hb-BME_2, and human Hb-BME_1 were subjected to gel filtration on Sephadex G-100 but no material eluted before the hemoglobin position and it was concluded that reaction was entirely intramolecular. The possibility of intramolecular cross-linking between chains was investigated by SDS-gel electrophoresis (figure 51) which revealed that both horse Hb-BME_1 and horse Hb-BME_2 contained a cross-linked dimer as well as the major fraction which ran as monomer in SDS. Even heavy loading of the gels failed to reveal any trimer or tetramer. Examination of the 3-dimensional model of horse Hb revealed that -SH β-93 lies on the periphery of the $\alpha_1\beta_2$ contact and the only chain that the second maleimide ring of BME could reach would be the α-chain by bridging the $\alpha_1\beta_2$ contact. It was concluded from gel electrophoresis that both horse Hb-BME_1 and horse Hb-BME_2 were heterogeneous in that both contained a major component reacted entirely within β-chain, and a minor component with a bridge across the $\alpha_1\beta_2$ contact.

Figure 51: SDS-gel electrophoresis of horse hemoglobin BME derivatives.

Left, Hb-BME_1

Center, Hb-BME_2

Right, horse Hb.


DIMER
MONOMER

A preliminary binding study was performed with human Hb-BME_1 (figure 52) and it was evident that binding was reduced by 30-40 per cent, approximately half the reduction observed by Bunn with human Hb-BME_2. No further work was performed with human material and it was concluded that binding was restricted by BME in proportion to the amount of BME incorporated. Moreover, the curvature observed in the binding relation suggested that binding was abnormal. This served to confirm the observation by Bunn (1967) that untreated hemoglobin readily displaced Hb-BME_2 from haptoglobin.

Binding studies with horse Hb-BME_1 and horse Hb-BME_2 are shown in figure 53. It is apparent that essentially the same binding properties existed in horse Hb treated with BME as with human; not only has binding been restricted but it has again been in proportion to BME uptake. This then was the end of the experiment as initially planned; in spite of its ability to dissociate to dimers in 0.25 M $MgCl_2$ (Simon et al., 1967) horse Hb-BME_2 does not bind with haptoglobin to the same extent as unreacted horse Hb.

The apparent presence of a cross-link bridging the $\alpha_1\beta_2$ contact, however, was of interest because of the suggestion by Malchy and Dixon (1969) that this contact might contain the site of haptoglobin binding. It was therefore necessary to determine whether the material containing an interchain bridge could bind with haptoglobin, and this was

Figure 52: Titration of haptoglobin (490 micrograms) with increasing amounts of human hemoglobin-BME_1 (filled circles) and with unreacted hemoglobin (open circles).

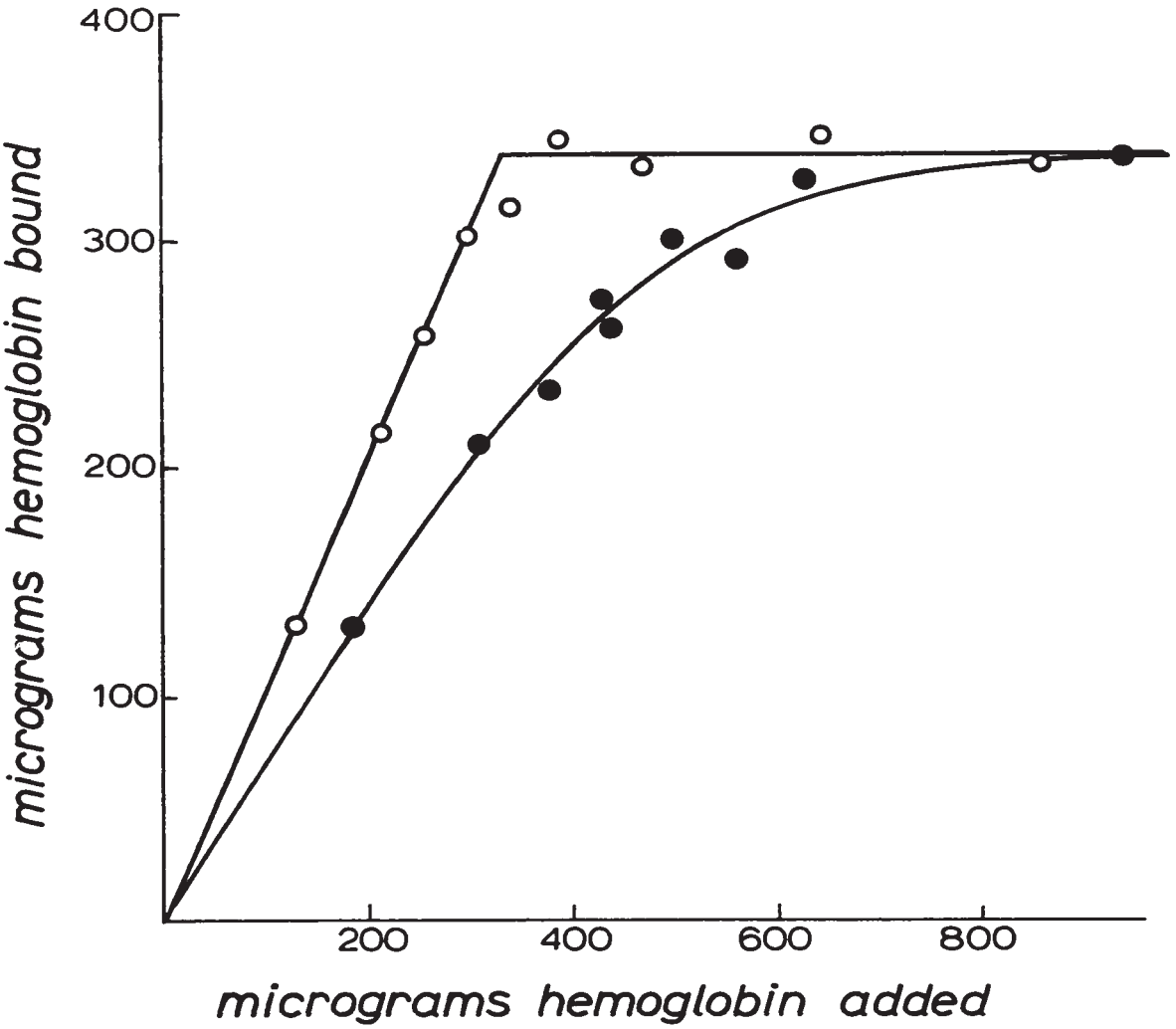
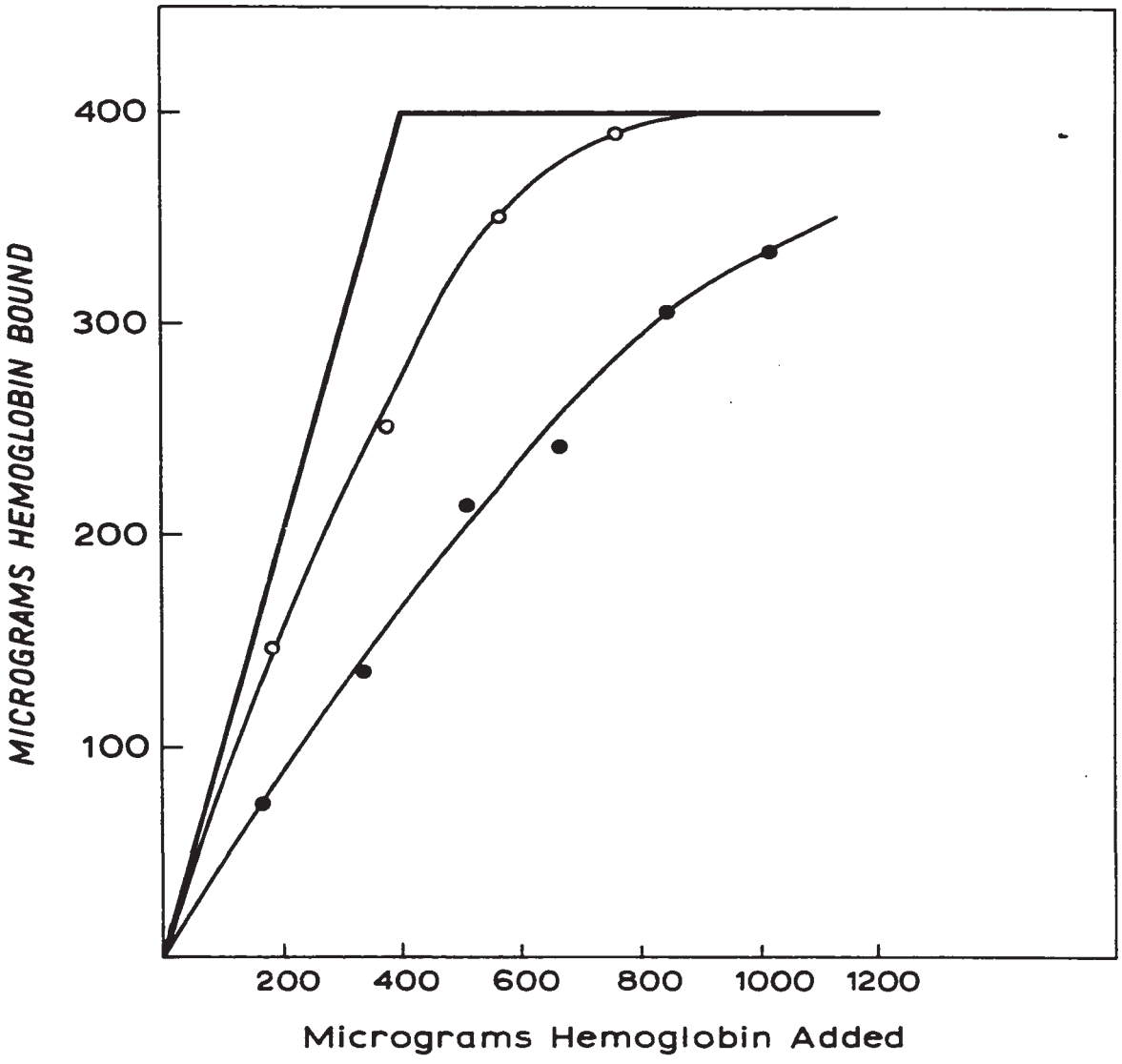

400
300
200
100
micrograms hemoglobin bound
200
400
600
800
micrograms hemoglobin added

Figure 53: Titration of haptoglobin (570 micrograms) with increasing amounts of horse Hb-BME_1 (open circles) and horse Hb-BME_2 (filled circles). Solid line is that expected if binding is not restricted.


MICROGRAMS HEMOGLOBIN BOUND
400
300
200
100
200
400
600
800
1000
1200
Micrograms Hemoglobin Added

done by mixing horse Hb-BME_2 with an excess of Hp and then immediately separating complex from unbound Hb-BME_2 on the Sephadex G-100 column routinely used for binding studies. Figure 54 shows SDS-gels of the complex formed and the unbound Hb-BME_2, and it is evident that the interchain cross-linked material did not bind to any appreciable degree.

Studies were made to determine the amount of horse Hb-BME_2 bound to haptoglobin as a function of time. The results shown in figure 55 were obtained by mixing horse Hb-BME_2 with excess Hp and then assaying aliquots at time intervals. The fraction bound approximately doubled in 25 hours but all had not been bound even at 75 hours. In every case the binding of radioactivity was the same as the binding of heme.

In view of this increase in binding with time, it was necessary to prepare complex after a time lag of 24 hours so that binding species could be identified. For this a 1 x 100 cm column of Sephadex G-150 superfine was chosen as it allowed separation of saturated and half-saturated complex. In the presence of excess Hp, two types of complex are normally formed, that with a full molecule of Hb (thought to be 2 $\alpha\beta$ dimers), and that with only one $\alpha\beta$ dimer (Peacock et al., 1970). For this experiment it was desirable to separate the two types of complex and Sephadex G-100 could therefore not be used. Figures 56 and 57 show the elution

Figure 54: SDS-gel electrophoresis of rapidly separated complex of haptoglobin with Hb-BME_2 (left) and remaining non-binding Hb-BME_2 (center). A gel of untreated haptoglobin is included for comparison (right).

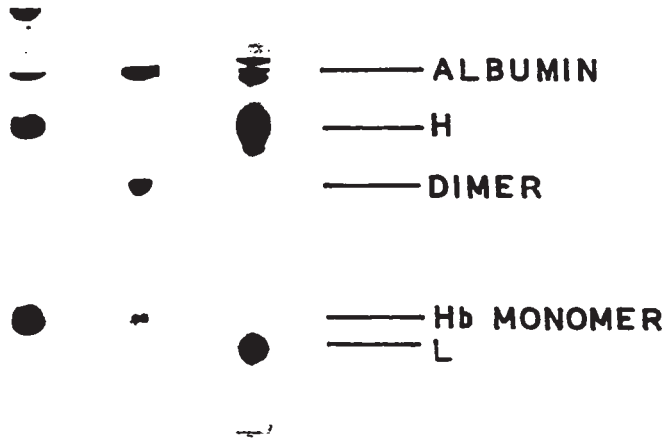
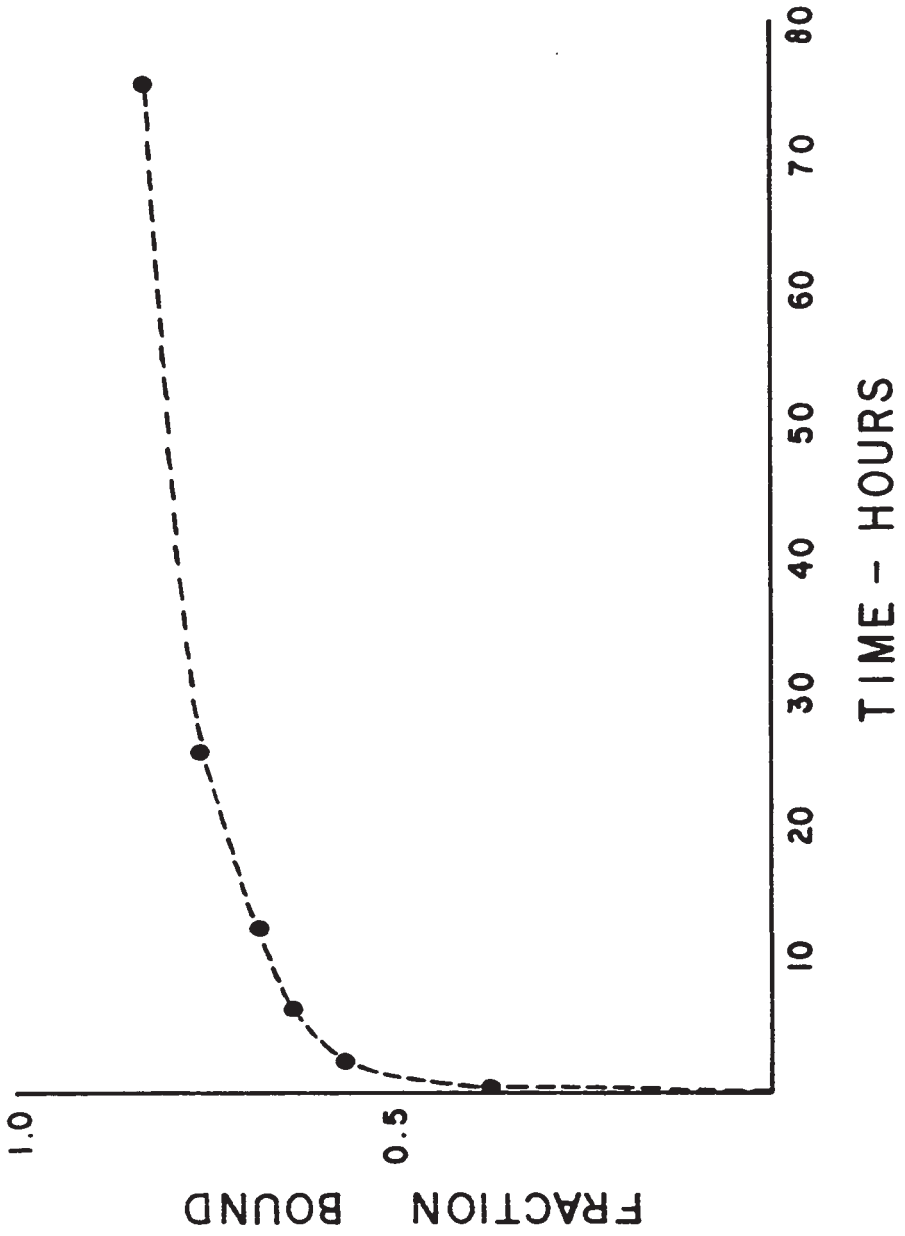

ALBUMIN
H
DIMER
Hb MONOMER
L

Figure 55: Binding of horse hemoglobin-BME_2 as a function of time.

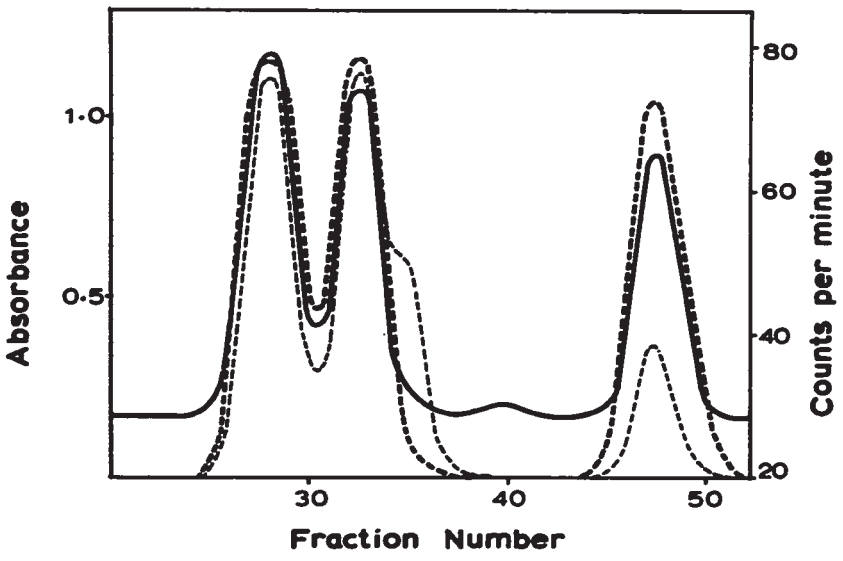

FRACTION BOUND
1.0
0.5
10
20
30
40
50
60
70
80
TIME - HOURS

patterns obtained when horse Hb-BME_1 and horse Hb-BME_2 respectively were run with excess haptoglobin. In the case of Hb-BME_2, a third component was partially resolved as a shoulder preceding saturated complex. No such material was observed in the case of Hb-BME_1 although it may not have been resolved because somewhat larger fractions were collected in order that greater amounts could be taken for radioactivity counts. In both cases the excess haptoglobin can be seen trailing half-saturated complex. Radioactivity counts revealed no differences in terms of counts per heme throughout either separation.

The shoulder preceding saturated complex in figure 57 showed a distinct increase in the ratio of absorbance at 412 nm than at 280 nm. By using unbound Hb-BME_2 to establish the ratio of these absorbances due to Hb alone, and by assuming that saturated complex contained 4 Hb chains, it was possible to construct a curve showing ratio of absorbances as a function of the number of Hb chains bound. This procedure revealed that the shoulder preceding saturated complex contained between 6 and 7 Hb chains per Hp molecule. In addition, SDS-gel analysis of peaks (figure 58) revealed that the fraction containing an interchain bridge was bound by haptoglobin but it appeared only in saturated complex, or in the shoulder preceding saturated complex. It was never observed in the half-saturated complex.

Figure 56: Elution of horse Hb-BME_1 plus excess haptoglobin from 1 x 100 cm column of Sephadex G-150 superfine.
Solid line, counts per minute.
Heavy broken line, absorbance 412 nm.
Light broken line, absorbance 280 nm.

Figure 57: Elution of horse Hb-BME_2 plus excess haptoglobin from 1 x 100 column of Sephadex G-150 superfine.
Solid line, counts per minute.
Heavy broken line, absorbance 412 nm.
Light broken line, absorbance 280 nm.


Absorbance
1·0
0·5
80
60
40
20
Counts per minute
30
40
50
Fraction Number

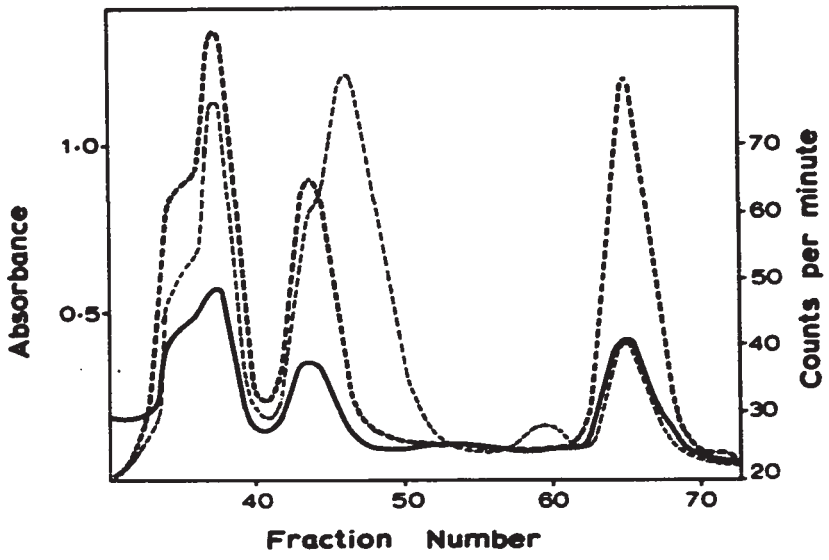

Absorbance
1·0
0·5
70
60
50
40
30
20
Counts per minute
40
50
60
70
Fraction Number

Figure 58: SDS-gel electrophoresis of Hp-binding and non-binding components of horse Hb-BME_2.

a horse Hb-BME_2

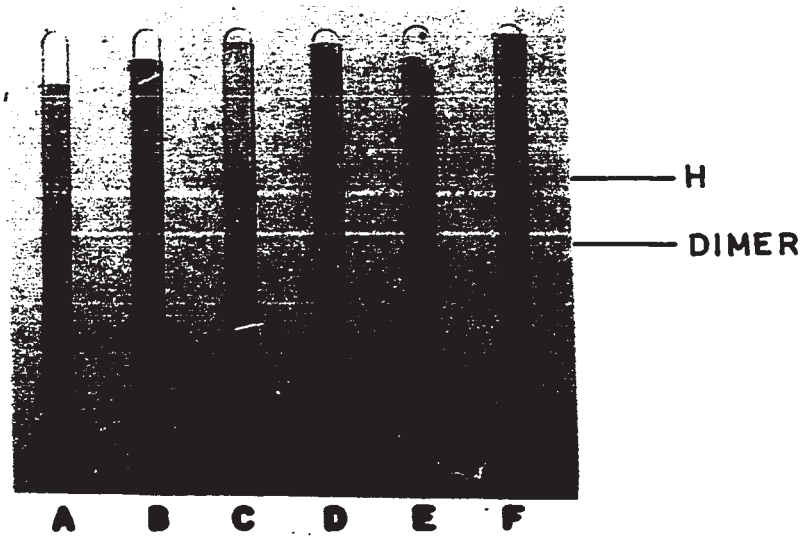
b rapidly eluting band preceding saturated complex

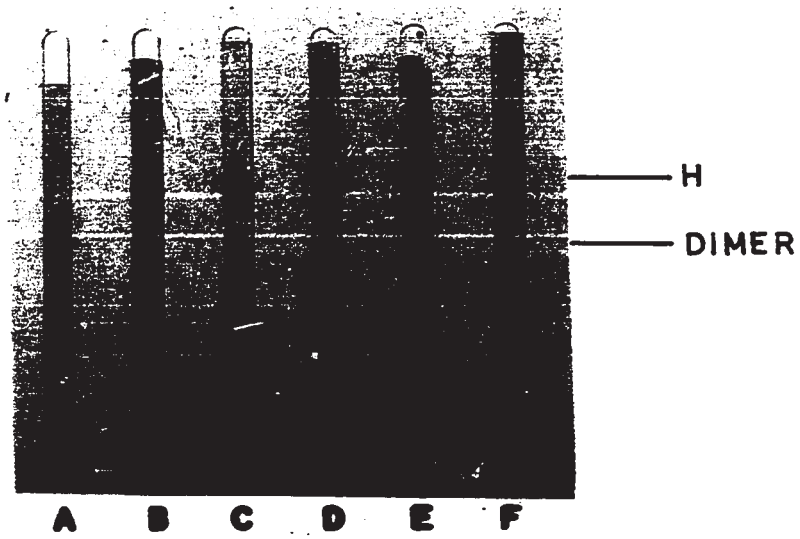
c saturated complex

d half-saturated complex

e non-binding Hb-BME

f haptoglobin


H
DIMER
A
B
C
D
E
F


H
DIMER
A
B
C
D
E
F

These diverse observations may be summarized before proposing an explanation for them.

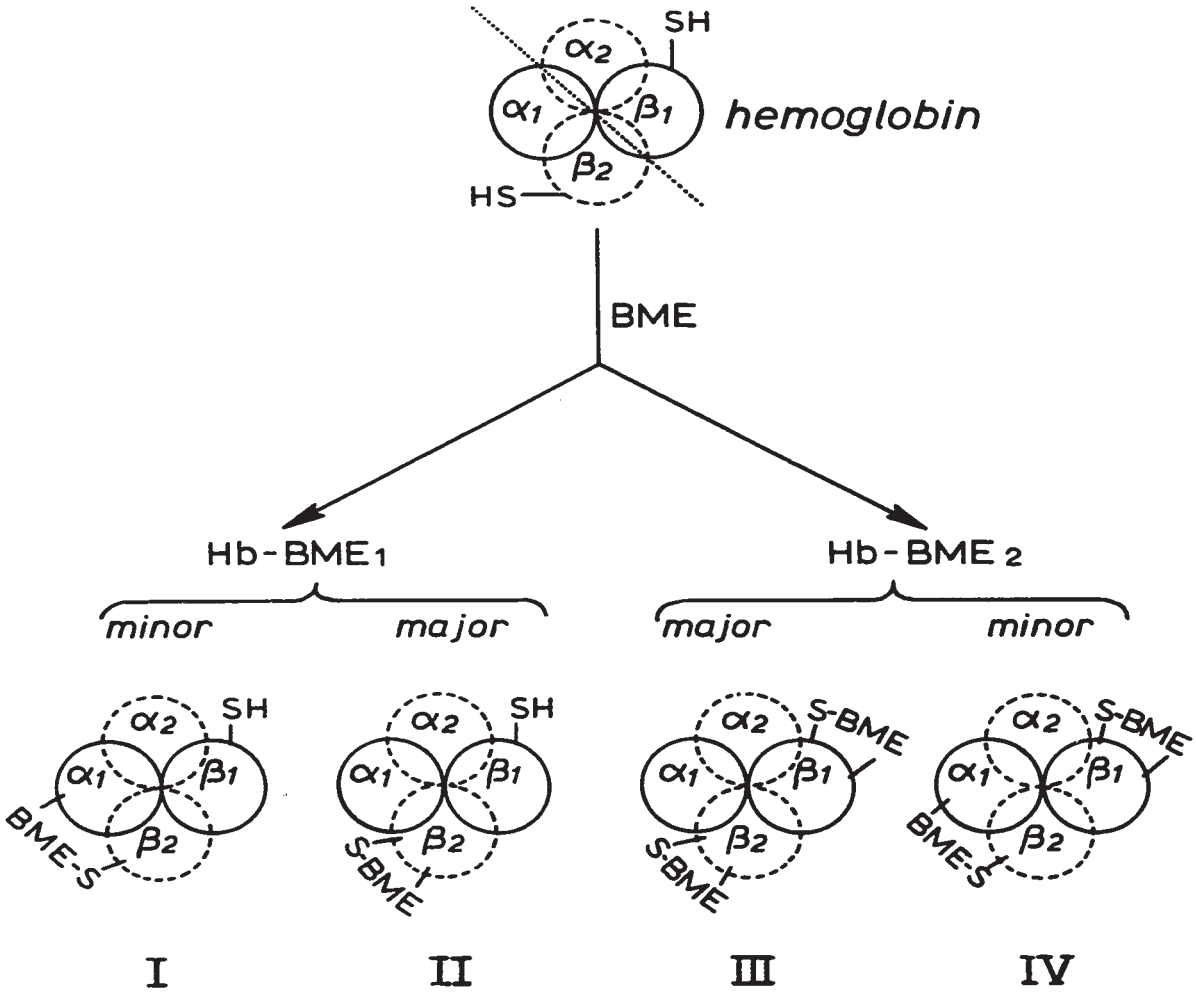
1. Horse hemoglobin reacted with BME yielded two products, $Hb\text{-}BME_1$, and $Hb\text{-}BME_2$.
2. Loss of reactive sulfhydryl groups paralleled uptake of BME. This may be taken as evidence that BME has reacted with one ($Hb\text{-}BME_1$) and two ($Hb\text{-}BME_2$) reactive -SH groups per Hb tetramer.
3. $Hb\text{-}BME_1$ and $Hb\text{-}BME_2$ were heterogeneous in that both contained a fraction with an interchain bridge giving rise to dimers in SDS gel electrophoresis.
4. $Hb\text{-}BME_1$ and $Hb\text{-}BME_2$ both suffered loss of Hp-binding ability, the loss by $Hb\text{-}BME_2$ being approximately twice that of $Hb\text{-}BME_1$.
5. The amount of $Hb\text{-}BME_2$ bound to Hp increased as time of exposure to Hp was increased, but it was not all bound even at 75 hours.
6. The fraction of $Hb\text{-}BME_2$ which was bound rapidly consisted only of those molecules having no interchain cross-link.
7. When exposed to excess Hp for sufficient time, even those components (of $Hb\text{-}BME_1$ and $Hb\text{-}BME_2$) with interchain bridges were capable of binding to Hp but they were excluded from the half-saturated complex.

8. In the case of binding by Hb-BME_2, a third component of complex was resolved, larger in size than saturated complex and containing 6 or 7 Hb chains per molecule of Hp.
9. In all cases of binding by either Hb-BME_1 or Hb-BME_2 to Hp, radioactivity was bound to the same extent as heme.

In order to explain these observations the model shown in figure 59 is proposed. In this model Hb would dissociate in the plane of the page to yield an $\alpha_1\beta_1$ dimer moving out from the page and an $\alpha_2\beta_2$ dimer moving into the page. The other possible plane of dissociation is represented by the diagonal dotted line through Hb to give rise to dimers $\alpha_1\beta_2$ and $\alpha_2\beta_1$, but this dissociation does not occur (Perutz, 1969). Reaction with BME would be heterogeneous because one maleimide ring of BME would react with cysteine β-93 and the other either within the β-chain or with some residue in the nearby α-chain. This would give rise to a minimum of 4 products:

I Hb-BME_1 containing an $\alpha_1\beta_2$ cross-link,
II Hb-BME_1 containing a cross-link within β-chain,
III Hb-BME_2 containing 2 cross-links each within its own β-chain,
IV Hb-BME_2 containing 1 $\alpha_1\beta_2$ cross-link and 1 cross-link within the second β-chain.

Figure 59: Model proposed for reaction of BME with horse hemoglobin.


SH
α2
α1
β1
β2
HS
hemoglobin
BME
Hb-BME1
Hb-BME2
minor
major
major
minor
BME-S
S-BME
I
II
III
IV

An additional product may have formed in Hb-BME_2, namely that having two interchain cross-links.

This model would account for appearance of products corresponding to Hb-BME_1 and Hb-BME_2 and it would predict that the former should show a free -SH group while the latter would not, in accordance with the results of table 6. Both Hb-BME_1 and Hb-BME_2 would be expected to show a dimer band in SDS-gel electrophoresis because of products I and IV containing interchain bridges (figure 51). Hp-binding by products I and IV would be expected to proceed slowly if at all because dissociation in the plane of the page would be possible at $\alpha_2\beta_1$ contacts only. This would explain failure to observe dimers in SDS-gels of complex in figure 54. Incorporation of 1 molecule of BME per tetramer (product II) might reduce the rate of binding by the altered $\alpha_2\beta_2$ dimer by some amount, and similarly both altered dimers of product III would show approximately twice the reduction (figure 53). However, radioactivity was bound at the same rate as heme, indicating that incorporation of even one molecule of BME as in product II reduces the rate not of binding by the modified dimer but of dissociation by hemoglobin tetramers. Hence product III might be expected to dissociate to dimers only half as fast as product II and cause the binding curves of figure 53.

Given sufficient time even products I and IV were capable of binding, but both were excluded from

half-saturated complex (figures 56-58). This would argue that these products bound as intact tetramers without breakage of $\alpha_1\beta_1$ or $\alpha_2\beta_2$ contacts since binding by tetramer would increase the molecular weight to that of saturated complex even if only one site on Hp were occupied. Binding by tetramer to one site of Hp might be expected to allow binding by a dimer (or even a second tetramer) to the second Hp site to yield a complex larger than normal and such a species was observed in figure 57 with 6-7 Hb chains per Hp molecule. A similar large complex would be expected in figure 56 but none was observed. Somewhat larger fractions had been collected in that case to allow greater volumes for scintillation counting.

In view of its success in explaining a series of diverse observations presented here, the model for BME reaction given in figure 59 seems suitable, however, it does not agree with the results of Arndt et al. (1971) who determined sites of BME reaction in horse hemoglobin using the 'slow' electrophoretic component of Kilmartin and Clegg (1967). Arndt et al. (1971) were able to isolate peptides containing 70 per cent of radioactivity and consisting of amino acid sequences around cysteine β-93. These peptides were deficient in one residue of histidine and they concluded that histidine β-97 had alkylated the second maleimide ring of BME and that no other cross-links were formed. This same

cross-link was observed by Perutz (1970a) by x-ray analysis of reacted hemoglobin. However crystal growth might well be selective for one component of a mixture. Also 30 per cent of the radioactivity remained to be accounted for in the results of Arndt et al. (1971). The observation by Simon et al. (1967) that horse Hb-BME_2 dissociated in 0.25 M $MgCl_2$ to the same extent as unreacted horse Hb would not be expected for preparations used here, because of the inter-chain bridge. The minor products containing such bridges might not be detected by osmotic pressure, or they could be the result of using horse hemoglobin not previously fractionated into 'fast' or 'slow' electrophoretic components. However, the difference between electrophoretic components involves only the substitution of glutamine for lysine at position α-60 (Kilmartin and Clegg, 1967) and would not be expected to alter reaction with BME since α-60 is removed from the contacts between chains.

These experiments have shown that horse hemoglobin treated with BME suffers reduced haptoglobin-binding ability in spite of reports that it can dissociate to dimers as readily as unreacted horse hemoglobin (Simon et al., 1967). It also contains a heretofore unreported interchain bridge which gives rise to a dimer band on SDS-gel electrophoresis. Since reaction was with reactive sulfhydryl groups, the interchain bridge probably spans the $\alpha_1\beta_2$ contact because

that is the only one near the reactive sulfhydryls. Consideration of a structure containing such a cross-link (products I and IV of figure 59) reveals that the only internal surface which could be exposed is the second $\alpha_1\beta_2$ contact, by allowing the tetramer to open using the cross-link as a hinge. Since these products could still bind it is suggested that the $\alpha_1\beta_2$ contact contains the binding site for haptoglobin, in agreement with Malchy and Dixon (1969). Restriction in binding by the major components (products II and III of figure 59) would be expected due to conformation restraint imposed by BME (Perutz, 1970a) combined with a slow rate of dissociation into dimers, which might be tested by analytical gel filtration. It may be concluded that dissociation into dimers or at least exposure of internal dimer surfaces is necessary for binding, and that conformational flexibility is also necessary.

SUMMARY AND CONCLUSIONS

It has been possible to react hemoglobin with maleic anhydride and achieve virtually quantitative modification of lysyl residues, however, undesirable side effects in physical properties accompanied reaction. Maleyl hemoglobin gave no indication of ability to bind with haptoglobin but it was not possible to discern whether this loss was caused by modification at lysyl sites or by accompanying structural alterations and it has been concluded that maleic anhydride, and hence bifunctional anhydrides are not suitable reagents for the purposes of this study.

Quantitative modification of lysyl residues of hemoglobin has also been possible with ethyl acetimidate HCl, without concomitant alterations in physical properties. Ability to bind haptoglobin was quantitatively retained by fully acetamidinated hemoglobin and it has been concluded that lysyl residues play no significant role in binding to haptoglobin. Consideration of a three-dimensional model of hemoglobin constructed in this laboratory revealed that lysyl residues were well distributed over the external surface of hemoglobin tetramer and it was concluded that

haptoglobin-binding sites must be located on the interior of the hemoglobin tetramer. Retention of binding activity by amidinated hemoglobin dictated the choice of an imidoester as a cross-linking reagent sufficiently mild to cross-link amino groups without accompanying structural changes.

Dimethyl adipimidate HCl was chosen since detailed methods for its synthesis and analysis were known. Suitable conditions were developed for its reaction with hemoglobin and these were extended to haptoglobin and hemoglobin-haptoglobin complex.

Cross-links were formed between hemoglobin tetramers at protein concentrations greater than 0.5 per cent and polymers so produced retained ability to form complex with haptoglobin. This confirmed the conclusion reached earlier from monofunctional amidination because tetramers cross-linked by various points on their external surfaces must have those surfaces largely blocked sterically. Cross-links between chains within tetramers were formed but their detailed analysis could not be achieved by conventional methods. The only technique found to give satisfactory resolution of cross-linked products was polyacrylamide gel electrophoresis in sodium dodecyl sulfate. By this means it was possible to demonstrate four major components in cross-linked hemoglobin, corresponding in mobility to monomer, dimer, trimer, and tetramer. The dimer region could be resolved into two different components which have

been assumed to be different dimers. The more rapidly migrating one has been tentatively identified as $\alpha_1\beta_1$ because a product of identical mobility resulted when hemoglobin was cross-linked at high dilution and is known to exist predominately as $\alpha_1\beta_1$ dimers. Although electrophoretic bands were discrete each may have been heterogeneous due to multiple cross-linking possibilities.

Studies on the binding properties of cross-linked hemoglobin have revealed that all fractions could bind to haptoglobin but that none bound quantitatively. A linear relation was found when a fixed amount of haptoglobin was titrated with increasing amounts of cross-linked hemoglobin and this has been taken as evidence that binding was altered only in quantity. Since amidination itself did not alter binding, loss of binding by cross-linked hemoglobin has been attributed to the additional cross-linking function of this reagent. The most probable effect of cross-linking would be to reduce possible conformations which hemoglobin or its subunits could adopt. Failure to bind quantitatively by even that fraction capable of dissociation to monomer has been taken as evidence that conformation restraint reduces binding. A corollary to this is that hemoglobin subunits must undergo conformation changes in order to bind firmly to haptoglobin.

Haptoglobin was also cross-linked with dimethyl adipimidate and the resulting cross-linked products were separable on gels of reduced N,N'-methylenebisacrylamide content. Mobilities of fractions obtained proved to exactly coincide with molecular weights of expected cross-linked species. Difficulty in increasing the scale of gel electrophoresis with such fragile gels precluded preparation of components in amounts sufficient for conventional analysis. Based on mobility, fractions obtained were L_2H_2, H_2, L_2H, LH, H, and L. No band corresponding to L_2 was observed.

Separation of hemoglobin-haptoglobin complex cross-linked with this same reagent also required gels of reduced methylenebisacrylamide but in addition the acrylamide content was halved. Three readily recognized bands were obtained corresponding to free monomers (Hb monomers and possibly L chains of Hp), a dimer of hemoglobin, and heavy chain of haptoglobin. A series of additional products moving more slowly than heavy chains was observed but identification of these was not attempted due to the large number of combinations possible with its inherent likelihood of errors. Recovery of cross-linked hemoglobin dimer led to the conclusion that at least 2 hemoglobin chains were bound within 8.6 Å of each other. Since this band corresponded closely to the more rapid of hemoglobin dimers, it probably represents a cross-linked $\alpha_1\beta_1$ species. This is the first

evidence that, on haptoglobin, hemoglobin chains are bound in close proximity to each other.

An investigation on the structure of haptoglobin has been made in order to discover whether this protein from pigs can dissociate to partial molecules as has been reported for human haptoglobin. Analysis of shapes of leading and trailing edges of elution profiles from Bio-Gel P-150 revealed no dissociation. Elution volume and sedimentation rate were essentially independent of protein concentration and consistent with a molecular weight near 100,000. It was concluded that porcine haptoglobin did not dissociate upon dilution. This was confirmed by SDS-gel electrophoresis which revealed intact haptoglobin to be highly sensitive to reduction. A series of intermediates were formed by incomplete reduction and these had mobilities characteristic of L_2H, LH, and L_2. With increasing 2-mercaptoethanol these and intact L_2H_2 completely disappeared leaving only bands of L and H. This dictated a change in the original model of haptoglobin to show a disulfide bridge between light chains but none between heavy chains since no band of H_2 was found. These studies have also demonstrated the sensitivity of cross-link analysis by SDS-gel electrophoresis. In the course of these experiments haptoglobin of exceptionally high purity was prepared by gel electrophoresis and analysis for N-terminal amino acids by the 'dansyl' method revealed

dansyl-valine and dansyl-isoleucine. Titration with p-chloromercuribenzoate revealed no sulfhydryl groups, both in agreement with human haptoglobin.

Cross-linking experiments on horse hemoglobin were carried out with a second bifunctional reagent, bis(N-maleimidomethyl)ether which was known to cross-link cysteine β-93 to histidine β-97. Hemoglobin-BME was fractionated to yield two derivatives, one containing 2 molecules of BME and no sulfhydryl groups, and a second containing 1 molecule of BME and 1 sulfhydryl group per tetrames. SDS-gel electrophoresis of these revealed further heterogeneity in that each contained a significant proportion of cross-linked dimer. It was concluded that this must represent a bridge at the periphery of the $\alpha_1\beta_2$ contact because cysteine β-93 is situated in that region. The binding properties of these derivatives were investigated and it was shown that the product with an interchain bridge bound with haptoglobin more slowly than the product that could be fully dissociated by SDS. A structure, $\alpha_2\beta_2$- $\alpha_1\beta_1$, was proposed for this cross-linked product and its binding properties were shown to be those expected of such a structure. Those internal surfaces which such a structure could expose would be little more than the $\alpha_2\beta_1$ contact and it was therefore proposed that in native hemoglobin, the sites for binding haptoglobin are in the regions of $\alpha_1\beta_2$ and $\alpha_2\beta_1$ contacts.

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