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CROSS-LINKING OF HUMAN HEMOGLOBIN WITH DIIMIDO ESTERS

BY

CHEN DIAN-ER

A dissertation submitted to the Graduate Faculty
in Biochemistry in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
The City University of New York

1975

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5/5-75
date

Chairman of Examining Committee

5-15-75
date

Executive Officer

Supervisory Committee

ABSTRACT

Amidination of human hemoglobin, prepared from red cells, with the bifunctional diimidoesters (i.e., diethylmalonimidate (DEM), dimethyladipimidate (DMA), dimethylsuberimidate (DMS), and dimethylsebacimidate (DMSB)) resulted in the cross-linking of lysine ϵ-amino groups.

The reaction of DEM with a 10% human hemoglobin solution produces a mixture of molecules with a wide molecular weight range from 68,000 to 669,000 together with a small amount of very high molecular weight molecules.

The reaction of a 1% hemoglobin solution with DMS and DMSB produces intramolecular cross-linked hemoglobins with a molecular weight of mostly 68,000.

Polyacrylamide gel electrophoresis of these modified hemoglobin molecules in the presence of sodium dodecyl sulfate resolves a set of species with molecular weights equal to integral multiples of monomers. The percentage of monomers and dimers found in each modified hemoglobin is decreased as the chain length of the diimidate reagents is increased.

Preliminary tests of the DMSB-modified intramolecular cross-linked hemoglobin in rabbits showed a longer life span in the circulation than unmodified hemoglobin.

For structural studies, C^{14} dimethylsebacimidate was used

to prepare the intramolecular cross-linked hemoglobin. The C^{14}-tetramer globin was treated with chymotrypsin and the peptides fractionated on a Dowex 50-X2 column followed by two-dimensional paper chromatography and electrophoresis. Several cross-linked peptides were obtained and their structures indicate the presence of α chain lys 99- β chain lys 82, β' chain lys 17- β'' chain lys 17, and β' chain lys 8- β'' chain lys 8 cross-links.

ACKNOWLEDGEMENTS

I would like to acknowledge my deep appreciation to the persons who contributed to the completion of this dissertation.

Professor A. Mazur, my adviser, provided inspiring guidance at all time. His warm and almost parental care for the student deserves my special gratitude. In addition, his grants expedited my study by releasing me from teaching duties which graduate biochemistry students usually assume for financial reasons.

Professors A. Lukton, C. Russell, O. Roels, and H. Schultz all provided useful comments on various aspects of the research.

Behind this undertaking there are also my parents' and husband's encouragement and understanding without which the tiring process would never have led to a successful conclusion.

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INTRODUCTION

Hemoglobin prepared from discarded red cells, have several properties which might be desirable for use as plasma expander. In addition to the large quantity of hemoglobin available from outdated blood, it can transport and exchange oxygen and unlike red cells does not require typing (Hamilton) and cross-matching prior to its use. But the use of some hemoglobin solutions can cause renal damage (Hardaway 1965; Hardaway, Byungkyu 1965) due to the presence of erythrocyte stroma. In 1970 Rabiner prepared a hemoglobin solution which was free of stroma or phospholipid and possessed no coagulant activity, but the property of dissociation of hemoglobin in solution reduced its half life in the blood stream (Lathem). The relatively rapid removal of hemoglobin from the plasma of the recipient animal involves two mechanisms: combination of hemoglobin with serum haptoglobin and removal of the haptoglobin-hemoglobin complex by reticuloendothelial cells of the liver and spleen, and excretion of hemoglobin via the kidneys into the urine. The complexing of hemoglobin by haptoglobin occurs only with the dissociated dimeric form of hemoglobin (Nagel, R;,Gibson 1971) and the excretion of hemoglobin also requires a prior dissociation into dimers.

One purpose of this study is to achieve a chemical modification of human hemoglobin in order to prepare an undissociable hemoglobin to increase its life span in the circulation.

The bifunctional diimido esters have been proven to be highly useful cross-linking agents (Dutton, et al.), since (Hunter, et al.) it produce no significant changes in conformational properties of the protein or biological activities of the protein which were modified. These reagents react specifically with ϵ-amino group of lysyl residues of a protein, and this results in only minor effects on the charge distribution of the molecule which they modified. The protein derivative remains soluble at neutral pH. Various chain lengths of these reagents can be prepared easily from the corresponding dinitriles.

Diethylmalonimidate has been used (Wofsy, Singer) to modify bovine serum albumin and human γ-globulin without altering their antigenic activity or electrophoretic mobilities, and also to cross-link bovine serum albumin and ferritin to human γ-globulin (Dutton, et al.). Dimethyladipimidate (Hartman, Wold) has been used to study the structure of ribonuclease by mapping the distance of lysyl residues within a molecule. Dimethylsuberimidate was also used to study subunit structure of oligomeric protein (Gregg, et al.).

The second purpose of this study was to prepare an intramolecular cross-linked hemoglobin tetramer by using the C^{14} bifunctional sebacimidate reagent and studying the subunit structure of hemoglobin by mapping and identifying the cross-linked residues. Identification of several cross-linked positions in the modified hemoglobin is reported.

EXPERIMENTAL

Materials:

Analytical grade resin AG50W-X2 (200-400 mesh), AG 1-X2 (200-400 mesh), and DEAE-cellulose were purchased from Bio-Rad. Ninhydrin was obtained from Pierce Chemical Company. Radioactive C^{14} sodium cyanide was bought from New England Nuclear. All standard proteins, i.e., thyroglobulin, aldolase, hexokinase, myoglobin, albumin, and glyceraldehyde-6-p dehydrogenase were obtained from Sigma Chemical. Reagent grade pyridine was purchased from Fisher Scientific Company. Human blood was obtained from New York Blood Center. Whatman 3MM paper was obtained from Savant Instrument Inc. All other chemicals (reagent grade) not listed here were purchased from Fisher Scientific Company.

Preparation of Stroma Free Hemoglobin:

Hemoglobin solutions were prepared according to the method of Rabiner, et al. (1967). Erythrocytes were separated from outdated human blood by centrifugated at 2,000 rpm for 10 min. The red cells were washed three times with 1.6% saline and the supernatant removed by suction. These washed cells were lysed with 4 volumes of 5 ideal milliosmolar phosphate buffer, pH 7.4 for each volume of packed red cells. The cells were mixed gently and thoroughly by inverting the flask repeatedly. After standing 30-60 min. at 0°C, the mixture was centrifuged at 16,000 g for 90 min. at 5°C. Supernatant was removed and

centrifuged at 35,000 g for 90 min. in a Sorvall centrifuge. The hemoglobin solution was concentrated in a ultrafiltration cell to achieve a 10% hemoglobin solution which was then dialysed against five changes of a 0.05 M sodium phosphate buffer, pH 7 in 0.10 M NaCl.

Preparation of Diimidate Esters:

These reagents were prepared according to the method of McElvain and Wold. 2 g of malonitrile were added to an ice-cold solution of 20 ml of dry dioxane and 2 ml of dry ethanol both of which had been distilled over CaH_2. The mixture was saturated with dry hydrogen chloride gas which was generated by adding concentrated H_2SO_4 to solid NaCl and passing through concentrated H_2SO_4. The reaction mixture was allowed to stand in the cold overnight, and the diethylmalonimidate dihydrochloride precipitated by adding dry ether and filtered. It was washed with dry ether. The product was dried in a desiccator over NaOH pellets and stored in a freezer. Extreme care is necessary to keep the entire system at all stages free of moisture to avoid hydrolysis of the imidoester. All other diimidate esters, i.e., dimethyladipimidate (DMA), dimethylsuberimidate (DMA), and dimethylsebacimidate (DMSB) were prepared as their dihydrochlorides by using the same method starting with the corresponding dinitriles.

Preparation of Cross-Linked Hemoglobin:

Intermolecular cross-linked hemoglobin was prepared from a 10% solution of stroma-free protein by treatment with DEM

dihydrochloride. 0.08 g of DEM dihydrochloride was added to 10 ml of a 10% hemoglobin solution in a container at 5°C with stirring. The pH was adjusted to 8.5 by adding a 10% aqueous solution of triethylamine. An addition amount of DEM dihydrochloride (0.4 g) was added to the reaction mixture at five 20 min. intervals (0.08 g each time). The pH was maintained at 8.5 by using triethylamine. After 4 hours the pH was stabilized which indicated that the reaction was complete and, an equal volume of saturated $(NH_4)_2SO_4$ solution was added and the precipitated protein centrifuged at 3,000 rpm for 10 min. at 5°C. The supernatant was discarded and minimum amount of water was used to dissolve the precipitate and centrifuged again. The solution and precipitation was repeated three times, the sample was dissolved in a 0.05 M sodium phosphate buffer, pH 7 containing 0.10 M NaCl and dialyzed against the same buffer. Intramolecular cross-linked hemoglobin was prepared from a 1% hemoglobin solution which was reacted with 4 different reagents, i.e., DEM (3 C atoms), DMA (6 C atoms), DMS (8 C atoms), and DMSB (10 C atoms). The same method described above was employed, but the ammonium sulfate precipitation step was omitted.

Molecular Weight Distribution of Modified Hemoglobin on Sepharose 6B:

A 2.5 x 39 cm Sepharose 6B column was packed at 5°C and equilibrated with 0.05 M sodium phosphate buffer, pH 7 in 0.10 M NaCl solution. The void volume was determined by running through 0.5 ml of 0.5% blue dextran (M.W.=2,000,000). Both

kinds of modified hemoglobin were dialysed against the same buffer and sucrose was added to a final concentration of 10% to increase its density; 0.5 ml (12.5 mg) of each sample solution was run through the column. Several proteins of known molecular weights were used to standarized this column, i.e., normal hemoglobin (M.W.= 68,000), Hexokinase (M.W.= 102,000), aldolase (M.W.= 160,000) and thyroglobulin (M.W.= 669,000). The protein concentration used for these experiments was 25 mg per 0.5 ml.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate (SDS):

These experiments were carried out according to the method of Weber and Osborn. The protein solution was incubated at 37°C for 2 hours in 0.01 M sodium phosphate buffer, pH 7, in the presence of 1% SDS and 1% mercaptoethanol. Gels were prepared from the following solutions: 15 ml of gel buffer (7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g $Na_2HPO_4 \cdot 7\ H_2O$, 2 g SDS in one liter), 13.5 ml of 10% acrylamide-bis (22.2 g acrylamide and 10.6 g bis per 100 ml), 0.045 ml TEMED, and 1.5 ml ammonium persulfate (15 mg/ml). Sample solutions were prepared by adding 3 ul bromephenol blue (0.05%), 1 drop glycerin, 5 ul mercaptoethanol and 50 ul buffer solution to 50 ul protein solution. Electrophoresis was carried out at 8 ma per gel at constant current for 5 hours. Gels were stained with Coomassie Blue, and destained with a solution of 75 ml acetic acid, 50 ml ethanol and 895 ml H_2O for two days or longer until the protein bands appeared clearly.

Protein Determination:

Protein was measured by the method of Lowry (1951) using bovine serum albumin as a standard.

Scanning of SDS Gel:

Gels were placed in a special cell of a Zeiss photocolormeter and overlaid with distilled H_2O. Readings were taken at 600 nm, Peak areas were measured in order to estimate the relative proportion of each band on the gel.

Preparation of Radioactive C^{14} Dimethylsebacimidate (DMSB) (Hartman):

A mixture of C^{14} NaCN (by mixing 0.5 g dry NaCN with 1 Mc/ 0.0082 g of C^{14} NaCN) and 5 ml of dimethyl sulfoxide was heated to 90°C, and 0.78 g (4.25 m moles) of dichloroctane was added with vigorous stirring. An exothermic reaction ensued as indicated by a rise in temperature to 130°C. After cooling to 40°C, 20 ml of $CHCl_3$ was added, and this solution was washed with 30 ml of a saturated NaCl solution. The chloroform layer was collected, and the aqueous phase extracted with three 20 ml portions of $CHCl_3$. The combined $CHCl_3$ phase was dried over anhydrous Na_2SO_4 and concentrated to yield 0.49 g of C^{14}sebaconitrile, which was then added to an ice-cold mixture of 10 ml dioxane and 2 ml of methanol both of which had been distilled over CaH_2. This mixture was saturated with dry HCl gas for 30 min. The reaction mixture was allowed to stand in refrigerator overnight, and the C^{14} DMSB dihydrochloride precipitated by addition of

20 ml dry ether. Recrystallization from methanol-ether yielded 0.63 g of C^{14} dimethylsebacimidate dihydrochloride.

Isolation of Major Human Hemoglobin Component by DEAE-cellulose Chromatography (Huisman, Dozy):

A column measuring 34 x 2.5 cm was used in order to prepare relatively large amounts of hemoglobin. A DEAE-cellulose column was equilibrated with 0.005 M sodium phosphate buffer. pH 8.6 containing 100 mg KCN/liter. 3 g of human hemoglobin solution was dialyzed for 24 hours against the same buffer and chromatographed on the column by use of 0.01 M sodium phosphate buffer pH 8.6, containing 100 mg/1 liter of KCN.

Preparation of C^{14} intramolecular cross-linked hemoglobin:

The purified major hemoglobin component (A_0) was dialysed against deionized water to remove excess KCN. 200 ml of a 1% hemoglobin solution was reacted with 0.62 g of C^{14} DMSB dihydrochloride by the same method as described earlier. After the reaction was completed the sample was dialysed against 0.05 M sodium phosphate pH 7 buffer, and centrifuged at 5,000 rpm to remove any insoluble material.

In order to remove a small amount of high molecular weight hemoglobin, this modified C^{14} hemoglobin was passed through a Sepharose 6B column standardized with normal hemoglobin.

Globin Preparation from C^{14} Labelled Hemoglobin Derivative:

Globin was prepared by the method of Rossi-Fanelli. A 2% (by volume) solution of HCl-acetone was prepared in a tube

inserted into a -60°C dry ice-acetone bath. Hemoglobin (3%) was added drop by drop into the tube and stirred. Before centrifugation, the mixture was maintined at -20°C for at least 2 hours. Occasional stirring was necessary until a white precipitate was formed at the bottom of tube. The mixture was centrifuged at 2,000 rpm for 5 min. (The temperature was not allowed to rise above -15°C). The white pellet was washed with cold acetone (-20°C) 4 times. The last wash was with dry ether. The final globin pellet was dried in a vacuum desiccator.

Radioactivity Determination:

0.1 ml of a sample was added to 10 ml Bray's solution which was prepared from the following: 60 g naphthalene, 4 g PPO (2,5-diphenyloxazole), 200 mg POPOP ((1,4, bis(2-(5-phenyl-oxazolyl)) benzene, 100 ml MeOH, 20 ml ethylene glycol and one liter of dioxane. Radioactivity was measured in a liquid scintillation counter. C^{14} peptides on chromatography paper was measured in the same way using the ninhydrin sensitive portion of the paper.

Denaturation of C^{14} Globin:

(1) The globin was converted to its S-(β-aminoethyl) derivative (Clegg); 500 mg C^{14} globin were dissolved in 1 M Tris solution and the pH adjusted to 9.2 with HCl. 0.02 g of mercaptoethanol was added to make a final concentration of 0.05 M, in order to cleave the disulfide bond. 5.1 ml of ethyleneimine (Matheson, et al.) was added with stirring to

make its concentration 0.5 M. After the reaction was complete (3.5 hours) or until a negative test for free SH-group was obtained with nitroprusside reagent (Katchalski), the pH was brought to 3 by adding conc. HCl. The protein solution was then dialysed against 0.5% formic acid in order to get rid of excess ethyleneimine and salts.

(2) Denaturation in a boiling water bath (Hirs); the sample solution in a test tube was heated in a boiling water bath for 30 seconds, and then lyophilized.

Chymotryptic Digestion (Hill, Konigsberg) of Denatured Globin:

500 mg of C^{14} globin were dissolved in 45 ml of distilled H_2O and 5 ml of 10% (weight per volume) NH_4HCO_3. 7 mg of chymotrypsin in 0.001 N HCl were added to the protein solution. After the pH was adjusted to 8.0 the milky suspension was allowed to digest at room temperature for 6 hours with constant stirring. An additional 7 mg of chymotrypsin dissolved in 0.001 N HCl was added at the end of 6 hours, and the digestion allowed to proceed for 24 hours. 5 ml of glacial acetic acid was added to the reaction mixture to stop the reaction. The digested sample was lyophilized twice in order to remove ammonium acetate, and dissolved in pyridine-acetate buffer pH 3.1, no insoluble materials was found after centrifugation.

Separation of Chymotryptic Peptides by Chromatography on Dowex 50-X2 (Schroeder) with Volatile Developers:

An analytic grade resin, AG50W-X2 (200-400 mesh), was used. Pyridine (reagent grade) was redistilled in order to remove the ninhydrin positive material. A 0.9 x 100 cm AG50W-X2 column was packed and equilibrated with pH 3.1 pyridine-acetate buffer (64.5 ml pyridine and 1114 ml glacial acetic acid diluted to a volume of 4 liters with distilled H_2O). The top buffer of the column was removed and a 250 mg sample of the chymotryptic peptides in 5 ml was carefully layered on the column. When the sample had completely entered the column by air pressure, the pH 3.1 buffer was added to the top of column. The column was then eluted by using a gradient buffer system which consisted of 333 ml of pH 3.1 pyridine-acetate buffer in the mixing chamber and 666 ml of pH 5.0 pyridine-acetate buffer (645 ml pyridine and 573 ml of glacial acetic acid diluted to 4 liters with H_2O) in the reservoir. A ratio of 1:2 of these two buffers were then maintained by using three bottles of the same size, one as the mixer and the other two as the reservoirs. The flow rate was 20 ml/ hour at 38°C, 400 fractions (2ml of each) were collected.

Photometric Determination of Peptides Content:

A modified ninhydrin reagent (Moore, Stein) was prepared by dissolving 20 g of ninhydrin and 3 g of hydrindatin (80 g ninhydrin in two liters of water at 90°C was added to a solution of 80 g ascorbic acid in 400 ml of H_2O at 40°C, hydrindatin is filtered off) in 750 ml of methyl cellosolve.

The stirring should not incorporate air bubbles in the solution. 250 ml of 4N sodium acetate buffer, pH 5.5 was added and transfered immediately into a dark reservoir bottle which was flushed with nitrogen gas. 25 µl of each effluent fraction was added to 1 ml of this modified ninhydrin solution in a test tube. The capped tubed were shaken briefly by hand and heated for 15 min. in a covered boiling water bath. The samples were cooled and read at 570 nm within one hour.

Amino Acid Analysis of Hemoglobin Protein and Peptides:

Approximately 1.5 mg of C^{14}globin and normal hemoglobin (A. component) were hydrolysed in 6 N HCl at 110°C for 72 hours. Before hydrolysis, the sample in ampules were frozen in a dry ice acetone bath, and sealed under vacuum (Moore, Stein). For analysis of the peptide, it was eluted with 6 N HCl from the chromatograph of paper spot and the same method was used to hydrolyze for 23 hours.

Peptide Separation by Two-Dimensional Chromatography and Electrophoresis:

Peptide mapping was performed by the method of Katz et al., with the exception that electrophoresis was conducted prior to chromatography. The salt-free sample, dissolved in distilled H_2O, was applied to a 18 x 22.5 inch sheet of Whatman 3MM chromatography at a point 4.5 inches from the 22.5 inch edge and 4.5 inches from the other edge. The paper was subjected to electrophoresis at 2,000 volts for 2 hours in pyridine-acetate buffer, pH 6.5 (pyridine-acetic acid-H_2O,

10:0.4:90). A Savant high voltage electrophoresis apparatus was employed. The whole system was covered with Varsol and cooled with a water circulator. After air-drying the paper for 2 hours in the hood and folding the edge to allow even solvent flow from chromatogram, it was developed by decending chromatography for 16 hours using the top layer of a solution obtained by mixing 1-butanol-H_2O-glacial acetic acid (4:5:1). The developed peptides were visualized by dipping the dried chromatograms in a 0.05% ethanolic ninhydrin solution, and then heated at 60°C until the components were visible.

Equipment:

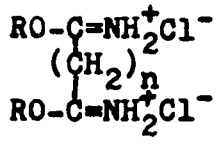
Panglas "Shandon" 500 chromatank was used for paper chromatography. Savant electrophoresis tanks and flat plates with a high voltage power supply, model HV-1000B were used for peptide mapping. Amino acid analyzer model 118 (Beckman Company) was used for amino acid analysis. The liquid scintillation counter was from Nuclear-Chicago. A Beckman Quartz Spectrophotometer and Zeiss M4 Q III 87462 (Germany) were used. A Pharmacia column (2.5 x 39 cm) was used for gel chromatography. An LKB Uvicord I (Sweden) and fraction collector were used for protein and peptide fractionation.

RESULTS

Preparation of Cross-Linked Human Hemoglobin:

Normal hemoglobin has a short half-life in the blood stream of a recipient due to its dissociable properties. An attempt was made to prepare an undissociable hemoglobin of the same molecular weight as normal hemoglobin in order to increase its potential usefulness as a plasma expander. This intramolecular cross-linked hemoglobin was also prepared for the purpose of structural studies. The percentage yield of intermolecular cross-linked hemoglobin made with DEM was 41.7% and the intramolecular cross-linked hemoglobin made with DMS or DMSB was 90%.

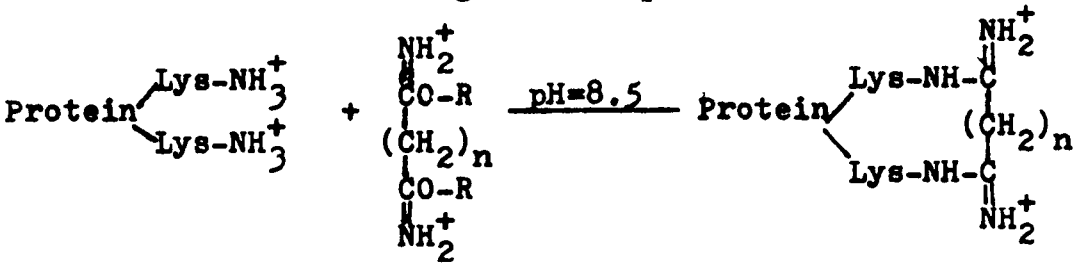
Bifunctional diimidate esters (Hartman and Wold) were used to prepare these two different kinds of cross-linked hemoglobin molecules. Both ends of the reagent can react with lysyl residues to form a bridge across two protein molecules or two different subunits in one protein molecule. These reagents (Dutton, et al.) specifically react with ϵ-amino group of lysine of the protein and does not affect to any great extent of the charge distribution of the protein which is modified. (The synthesis of these reagents from nitriles is summarized in Fig. A on page 32). The molecular structure of the reagent is shown in Fig. 1



R= methyl or ethyl group

These are diethylmalonimidate (DEM) when n=1,
dimethyladipimidate (DMA) when n=4,
dimethylsuberimidate (DMS) " n=6,
dimethylsebacimidate (DMSB) " n=8.

The reaction of this reagent with protein is shown below:



Molecular Weight Distribution of Cross-Linked Hemoglobin on a Sepharose 6B Column:

This experiment indicates that the intermolecular cross-linked hemoglobin has a range of molecular weights from 68,000 to more than 669,000, and a small amount of very high molecular weight protein which eluted from the column with the void volume (Fig. 2). This column was standardized with proteins of known molecular weights (Fig. 3). The intramolecular cross-linked hemoglobin had a molecular weight of 68,000 and a small amount of high molecular weight molecules around 85,000 as compared with normal hemoglobin.

SDS-Gel Electrophoresis:

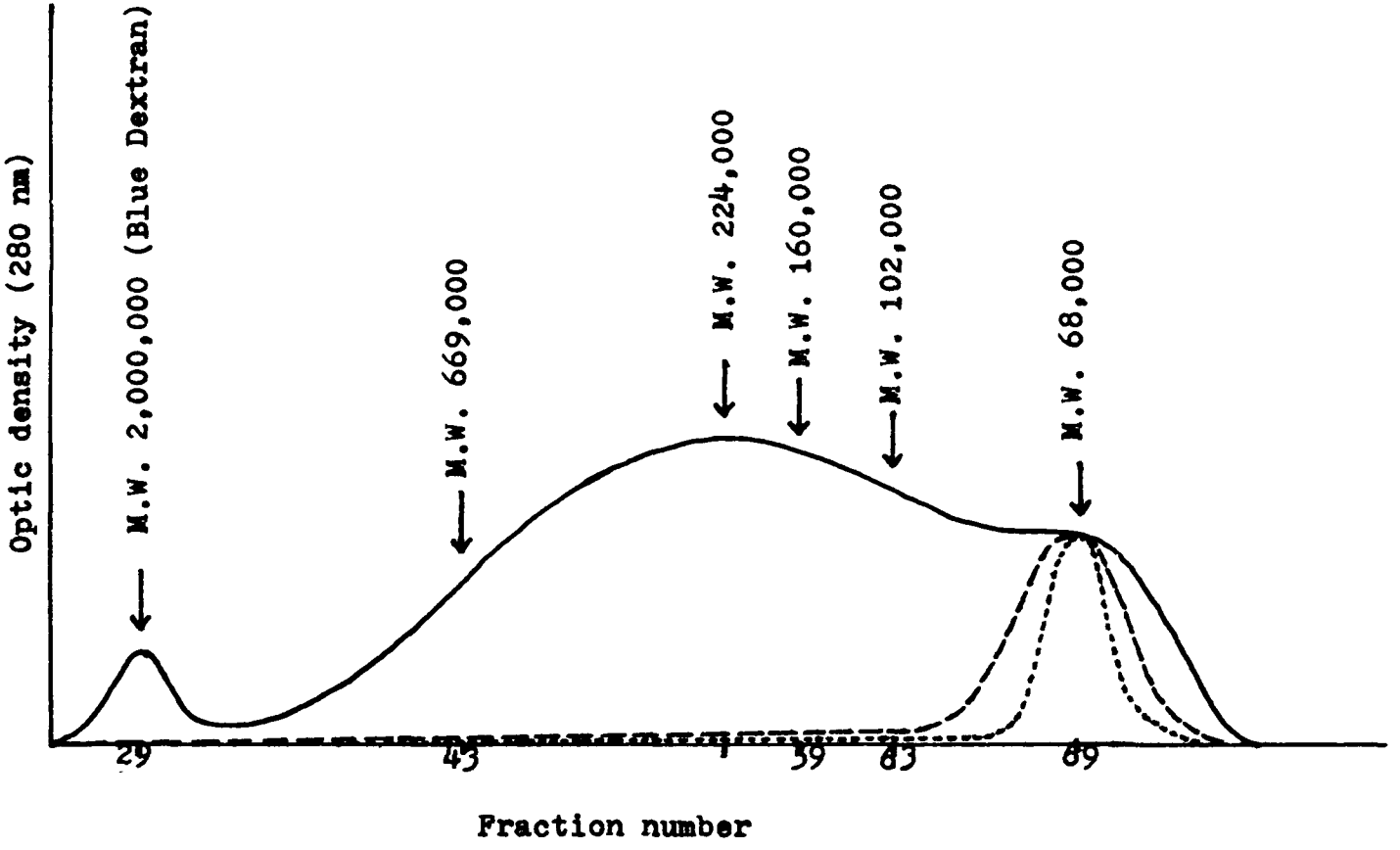
This analysis indicates the relative distribution of dissociable subunits of the two different kinds of cross-linked hemoglobin as compared with normal hemoglobin. Fig. 4 shows the SDS gel pattern of intermolecular cross-linked hemoglobin modified by DEM. Figure 5 shows the SDS gel pattern of cross-

Fig. 2 Molecular Weight Distribution of Modified Hemoglobin on Sepharose 6B

——— DEM-modified (10%) hemoglobin

– – – – Intramolecular cross-linked Hb modified by DMA, DMS, and DMSB

····· Unreacted hemoglobin



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dimethylsuberimidate (DMS) " n=6,
dimethylsebacimidate (DMSB) " n=8.

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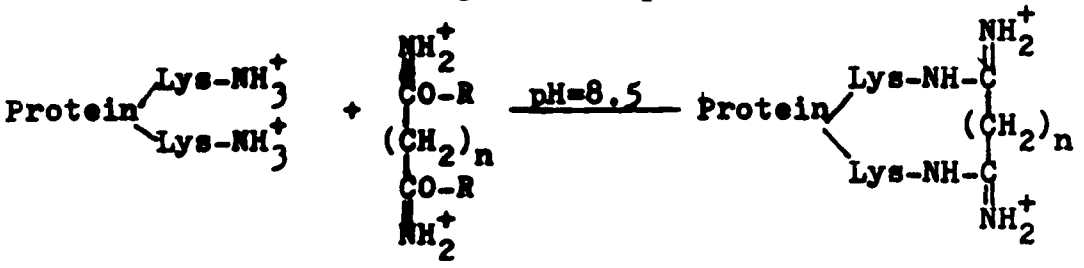

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This analysis indicates the relative distribution of dissociable subunits of the two different kinds of cross-linked hemoglobin as compared with normal hemoglobin. Fig. 4 shows the SDS gel pattern of intermolecular cross-linked hemoglobin modified by DEM. Figure 5 shows the SDS gel pattern of cross-

Fig. 3 Standardization of Sepharose 6B Column with Known M.W. Proteins

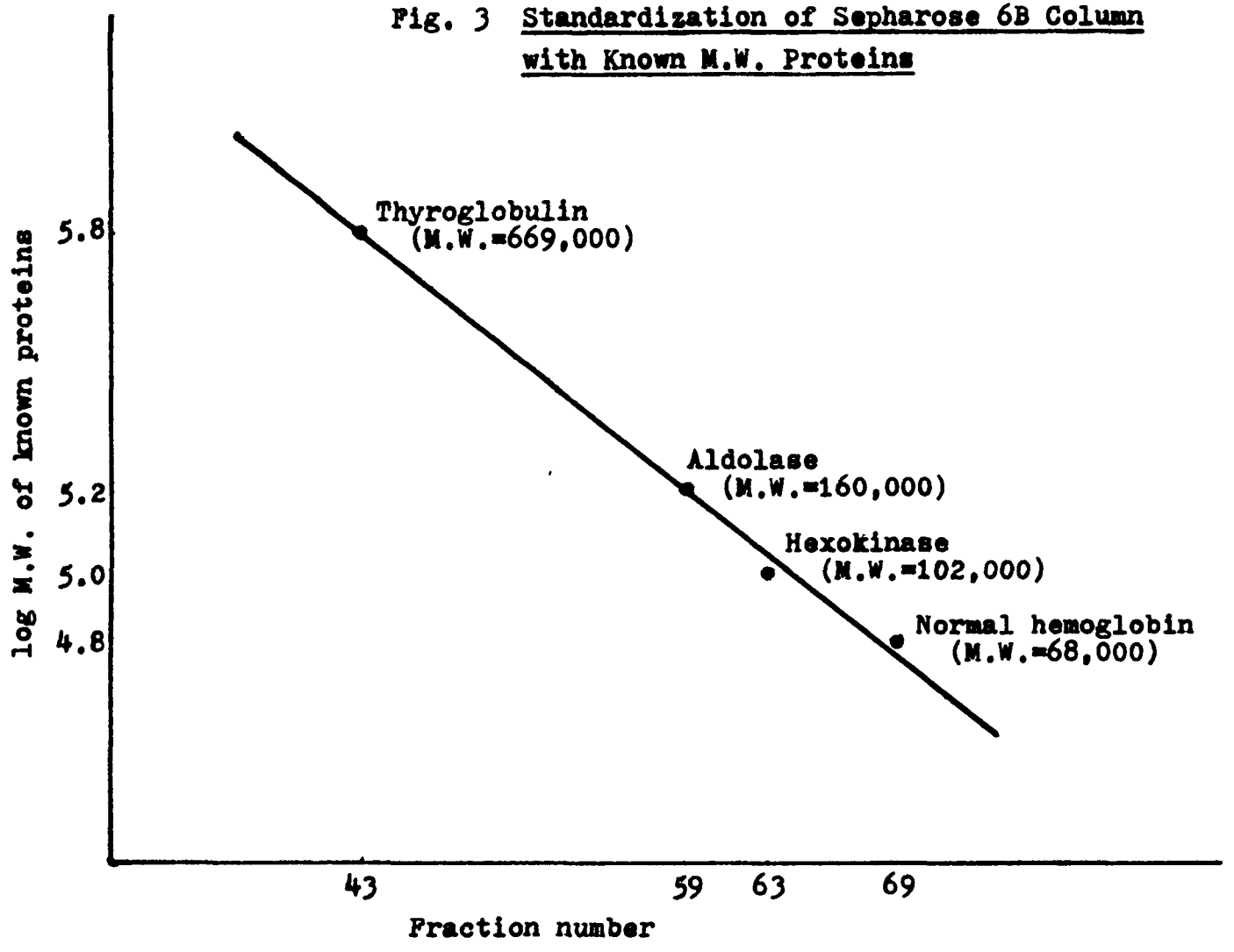
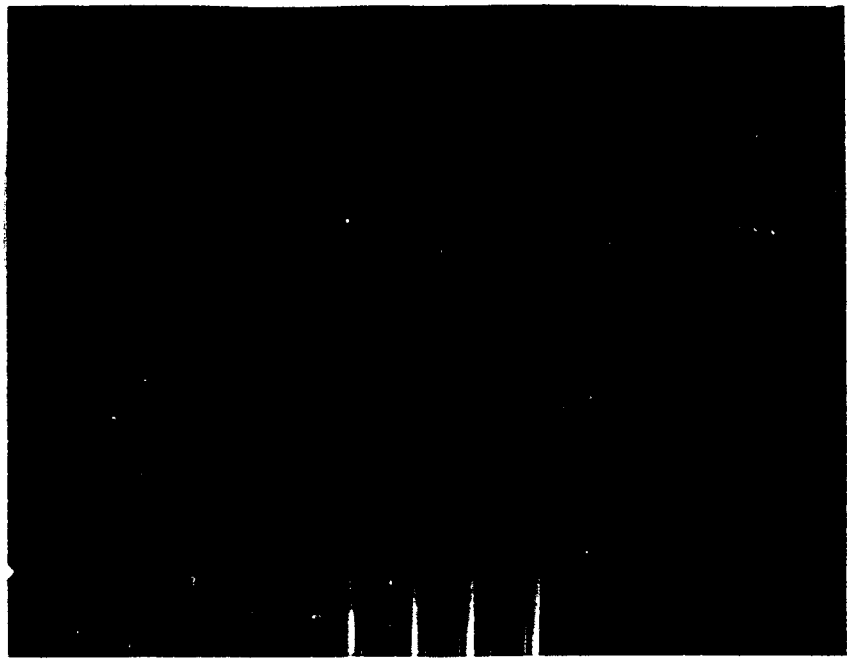


Fig. 4 SDS-Gel Pattern of Intermolecular Cross-Linked Hemoglobin and Control Hemoglobin

1. Myoglobin
2. Control hemoglobin
3. DEM-modified hemoglobin



1 2 3

Fig. 5 SDS-Gel Pattern of Intramolecular Cross-Linked Hemoglobin and Control Hemoglobin

1. Control hemoglobin
2. DEM-modified hemoglobin
3. DMA-modified hemoglobin
4. DMS-modified hemoglobin
5. DMSB-modified hemoglobin

1 2 3 4 5

linked hemoglobins modified by the 4 imidate reagents. A determination of the molecular weight for each band on the gel was done by comparing the mobilities of each band with that of standard proteins of known subunit size, i.e., myoglobin (one subunit with M.W. 17,000), albumin (one subunit with M.W. 64,000), and glyceraldehyde-6-phosphate dehydrogenase (6 subunits with M.W. 37,000 for each).(Fig. 6).

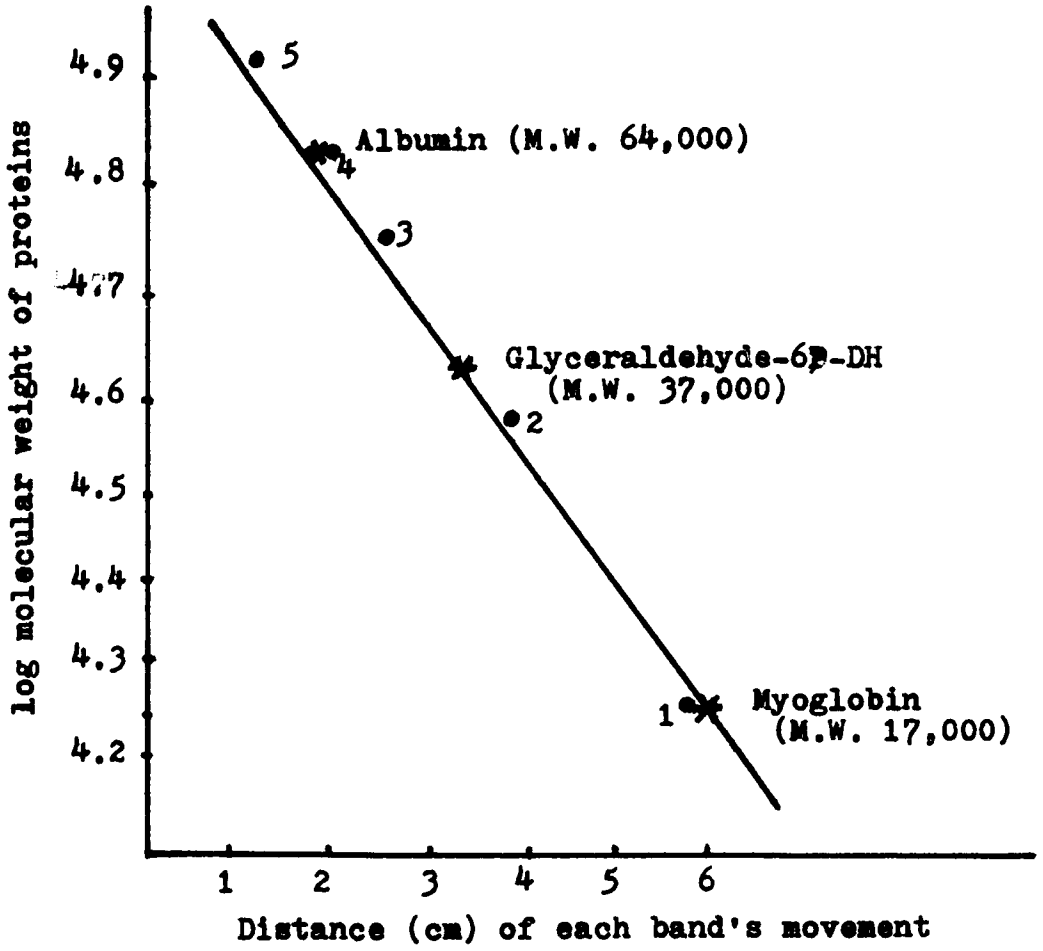
Fig. 7 shows the densitometer tracing of SDS gel of intermolecular cross-linked hemoglobin, and Fig. 8,9,10,11, and 12 show the densitometer tracing of SDS gels of cross-linked hemoglobins prepared with a 1% solution of hemoglobin. Table I shows the relative distribution of the subunits that are dissociable by SDS. The highest percentage of undissociable hemoglobin tetramer was obtained from the sebacimidate-modified hemoglobin which only has a small amount of dimers and monomers dissociated by SDS.

Preliminary tests of the DMSB-modified intramolecular cross-linked hemoglobin in the rabbit show a longer life span in its circulation as compared with untreated hemoglobin.

Preparation of C^{14} Dimethylsebacimidate Dihydrochloride:

In order to study the hemoglobin structure by formation of intramolecular cross-linked hemoglobin molecules, the radioactive C^{14} DMSB reagent was used to span the distance between lysyl residues in the hemoglobin tetramer. The preparation of this C^{14} reagent is shown as follows:

Fig. 6 Standardization of SDS-Gel with Known M.W. Proteins



* Represent the standard proteins
• 1 First band from the bottom of gels
• 2 Second band from the bottom of gels
• 3 Third band from the bottom of gels
• 4 Fourth band from the bottom of gels
• 5 Fifth band from the bottom of gels

Fig. 7 Densitometer Tracing of Intermolecular Cross-Linked Hemoglobin on SDS-Gel (see Fig. 4)

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. Pentamer

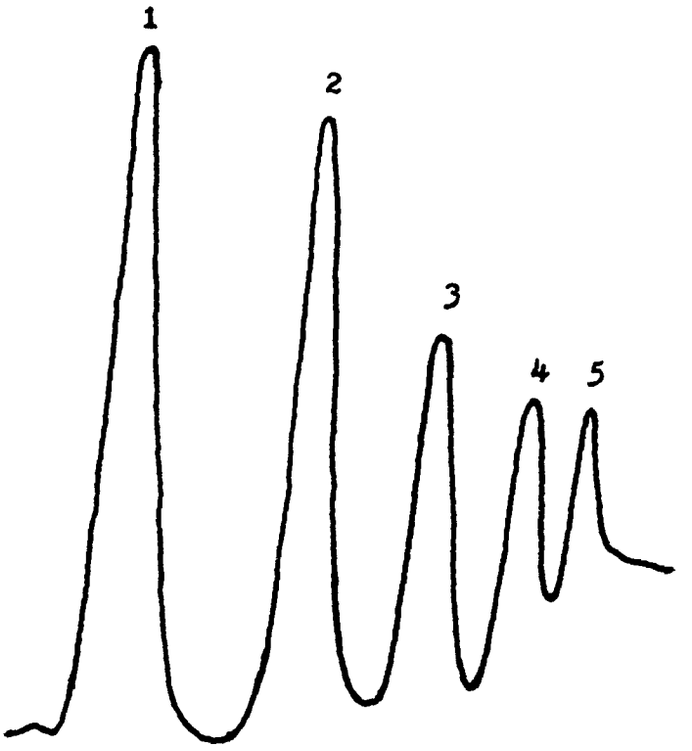


Fig. 8 Densitometer Tracing of Control Hemoglobin on SDS-Gel (see Fig. 5)

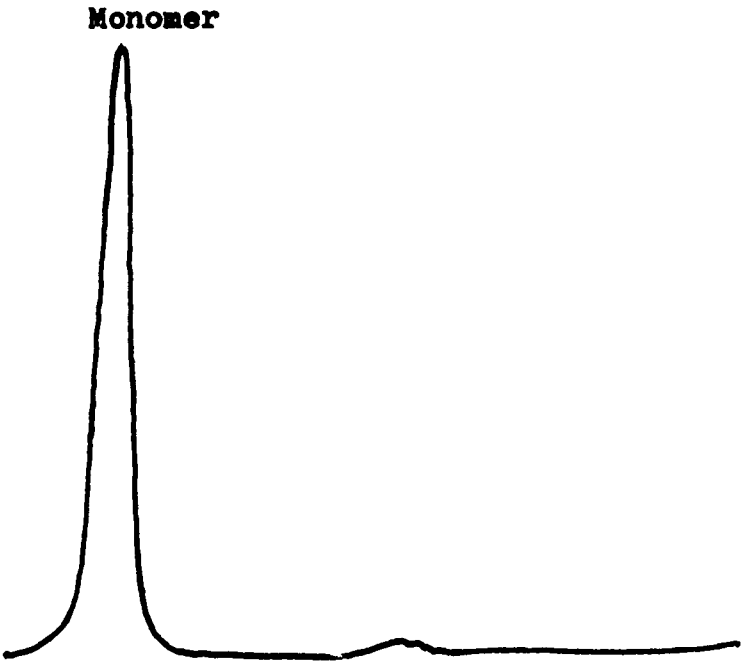


Fig. 9 Densitometer Tracing of DEM-Modified (1%) Hemoglobin on SDS-Gel (see Fig. 5)

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. >Pentamer

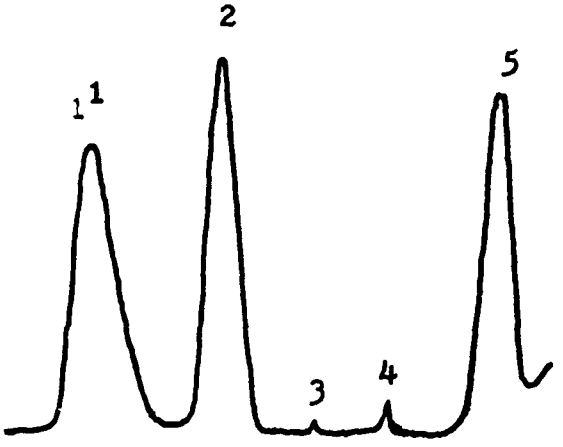


Fig. 10 Densitometer Tracing of DMA-Modified (1%) Hemoglobin on SDS-Gel (see Fig. 5)

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. >Pentamer

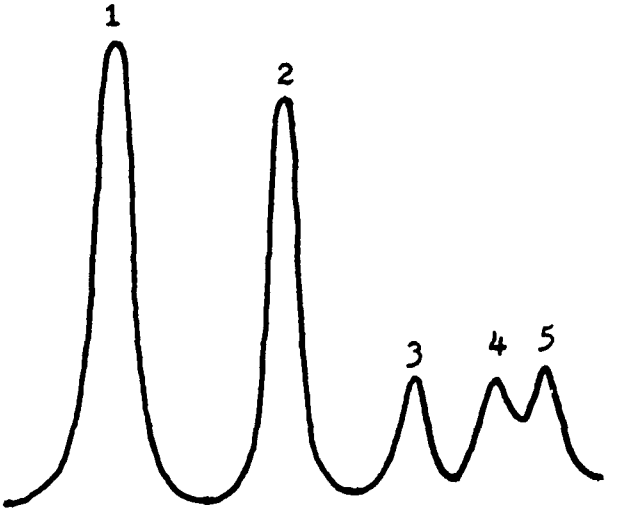


Fig. 11 Densitometer Tracing of DMS-Modified (1%) Hemoglobin on SDS-Gel

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. Pentamer

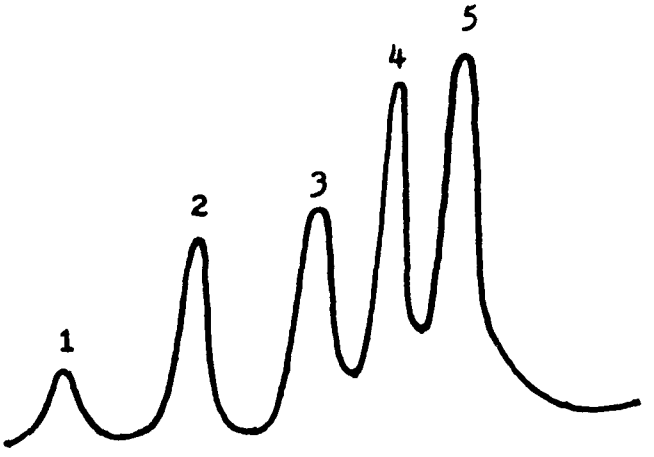


Fig. 12 Densitometer Tracing of DMSB-Modified (1%) Hemoglobin on SDS-Gel (see Fig. 5)

1. Monomer
2. Dimer
3. Trimer
4. Tetramer
5. Pentamer

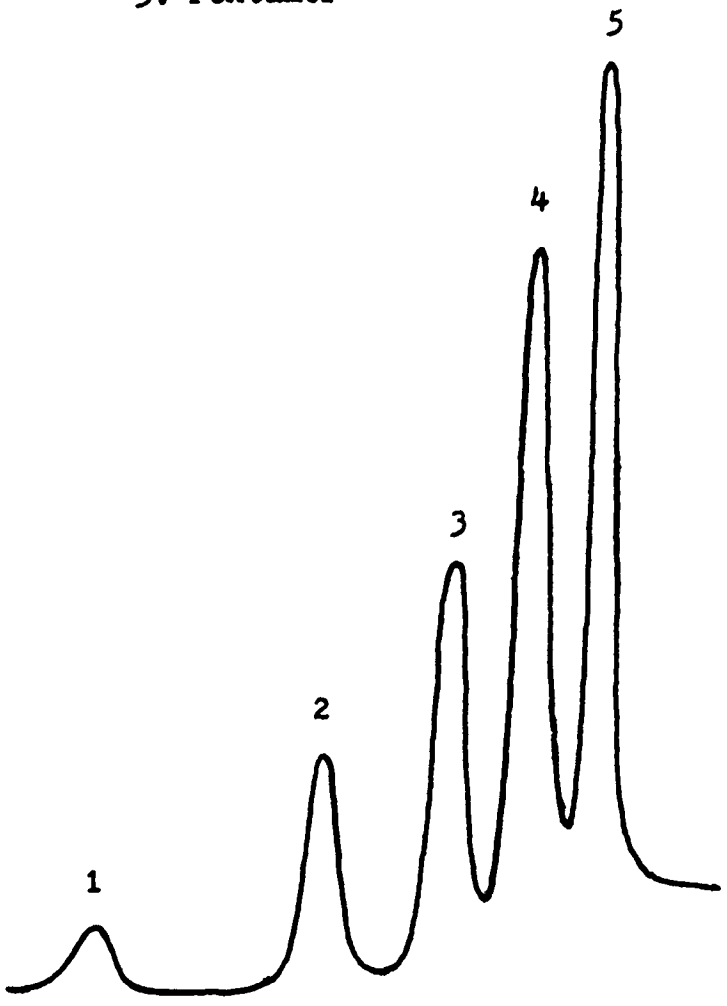


Table I. Relative Distribution of the Dissociable Subunits of Modified Hemoglobin

Modified Hb / Subunit	Control Hb	DEM-Hb	DMA-Hb	DMS-Hb	DMSB-Hb
	(percentage composition)				
Monomer	100	37.4	31.9	6.8	2.6
Dimer	0	31.2	35.5	19.1	10.2
Trimer	0	0.5	11.5	25.3	18.8
Tetramer	0	0	6.6	21.4	33.6
Pentamer	0	0	0	27.2	34.6
> Pentamer	0	30.0	14.2	0	0

DEM-Hb: DEM-modified hemoglobin
DMA-Hb: DMA-modified hemoglobin
DMS-Hb: DMS-modified hemoglobin
DMSB-Hb: DMSB-modified hemoglobin

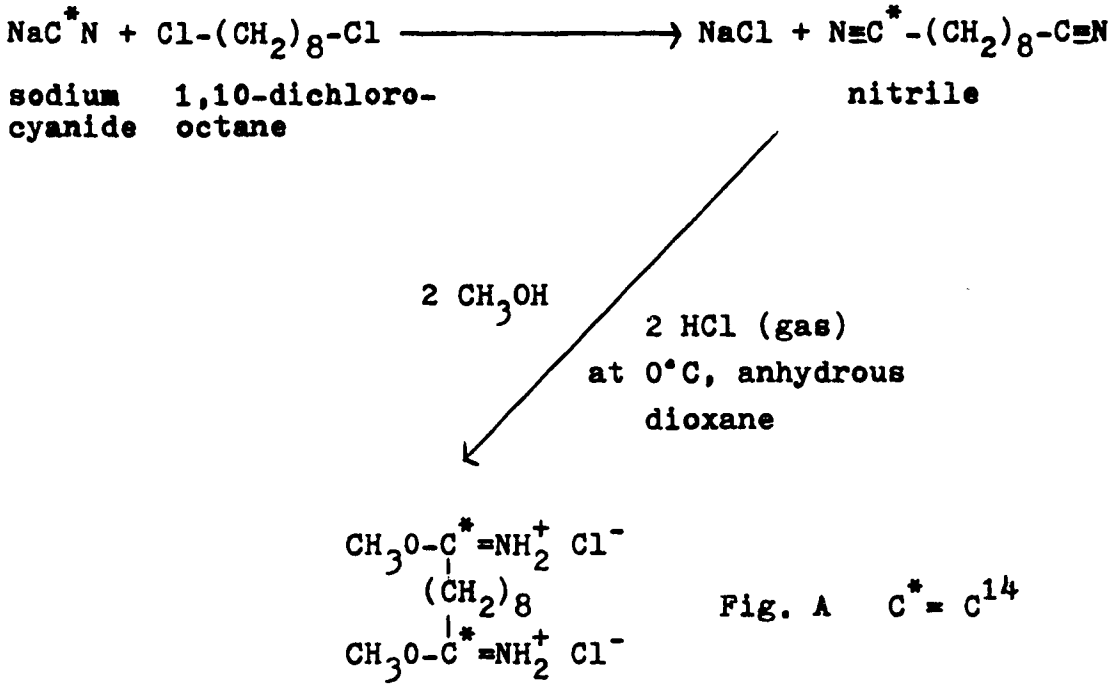


Fig. A $C^* = C^{14}$

C^{14}-sebacimidate dihydrochloride

Isolation of the Major Component of Human Hemoglobin (A_o) on DEAE-Cellulose Column:

In order to study the hemoglobin tetramer structure by using bifunctional cross-linking reagents, the minor components of normal hemoglobin and other possible contaminating proteins in the lysate were removed. Fig. 13 shows the components of a red cell lysate. The A_o (major hemoglobin component), fraction a-b, was pooled to do the structure study. The C^{14} DMSB-modified hemoglobin was further purified by passing through Sepharose 6B column in order to remove the small amount high molecular weight molecules. (Fig. 14).

Amino Acid Analysis of C^{14} Modified Hemoglobin:

This diimidate reagent is a bifunctional reagent which is

Fig. 13 Purification of Human Hemoglobin on DEAE-Cellulose Column

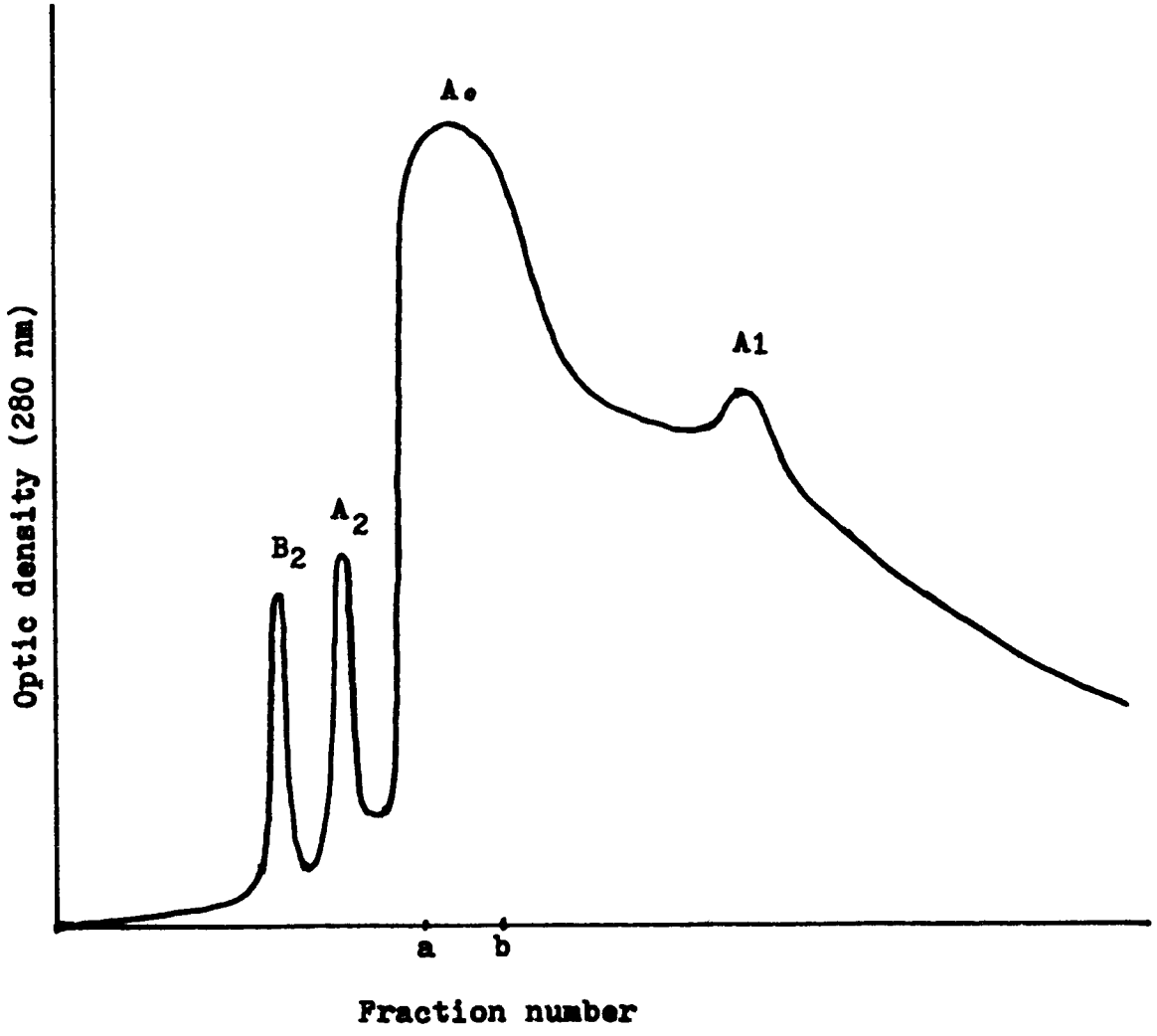
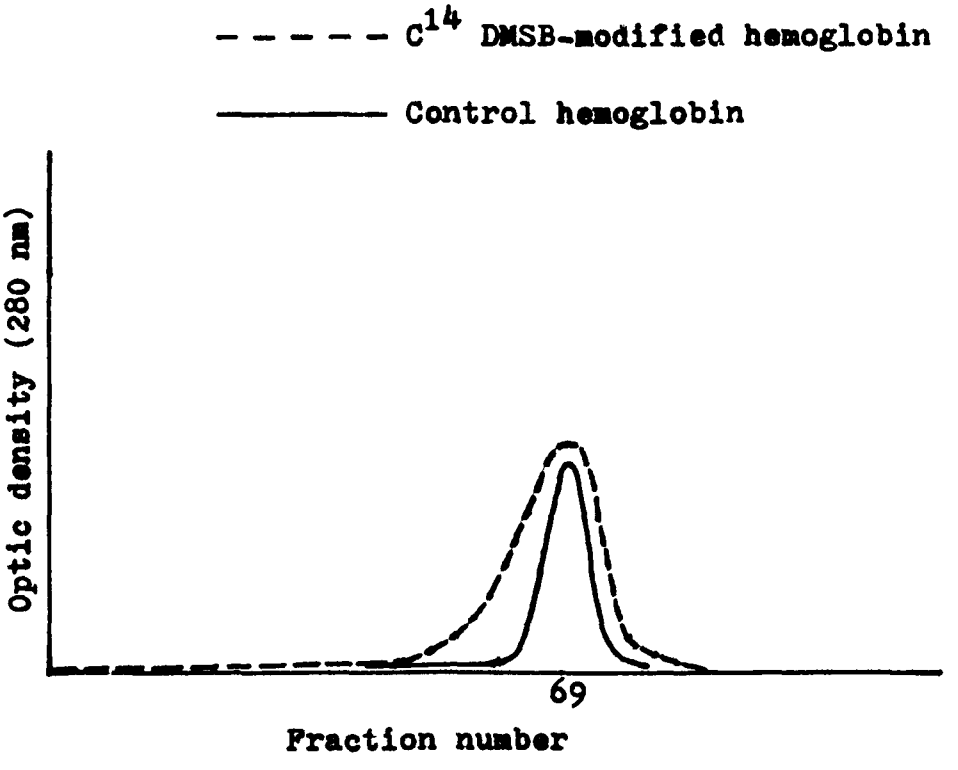


Fig. 14 Isolation of C^{14} Hemoglobin Tetramer from Sepharose Column



known to react specifically with ϵ-NH_2 groups of lysyl residues. Analysis was performed to compare the amino acid composition of modified hemoglobin with normal hemoglobin. In Table II, it can be seen that 31% of the lysyl residues in the modified hemoglobin have been modified, and a new amino acid, dilysyl derivative (Hartman, and Wold) is formed. As for other amino acids, there are no significant differences between the modified and control sample. This dilysyl residue obtained on acid hydrolysis of the modified hemoglobin is shown below:

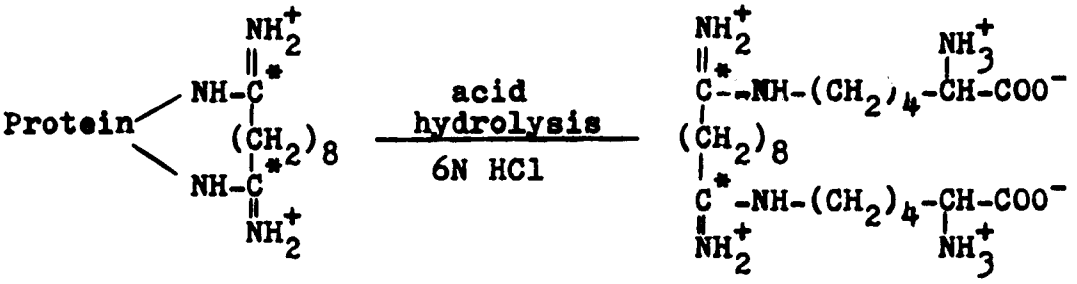


Fig. 15 ($C^* = C^{14}$)

Fig. 16 shows the elution pattern of this dilysyl derivative during amino acid analysis of the hydrolysate. This derivative has a higher pK_a than that of arginine, and is eluted after arginine on the short column of amino acid analyzer.

Purification of Chymotryptic Peptides on the Dowex 50-X2 Column:

Fig. 17 shows the C^{14} peptides which are poorly separated from the normal peptides. Fraction A has higher radioactivity which is almost all free C^{14} unreacted and hydro-

Table II. Comparision of Amino Acid Compositions of C^{14}DMSB-Modified Hemoglobin with Control Hb

Amino acid	Control hemoglobin	C^{14}DMSB-modified hemoglobin
Lysine	44.0	30.6
Histidine	41.8	39.7
Arginine	12.5	12.8
Aspartic Acid	48.6	48.9
Threonine	26.3	27.4
Serine	21.3	25.0
Glutamic Acid	30.9	27.4
Proline	24.8	26.1
Glycine	38.1	35.0
Alanine	70.0	74.0
Cysteine	6.0	5.9
Valine	58.0	55.0
Methionine	5.1	5.0
Isoleucine	0	0
Leucine	72.0	72.0
Tyrosine	10.9	10.6
Phenylalanine	29.0	31.1
Dilysyl-derivative	0	6.0

Values expressed are the number of residues per 576 amino acid residues of a hemoglobin tetramer

Fig. 16 Basic Amino Acid Elution Profile of the Acid Hydrolysate of DMSB-Modified Hemoglobin

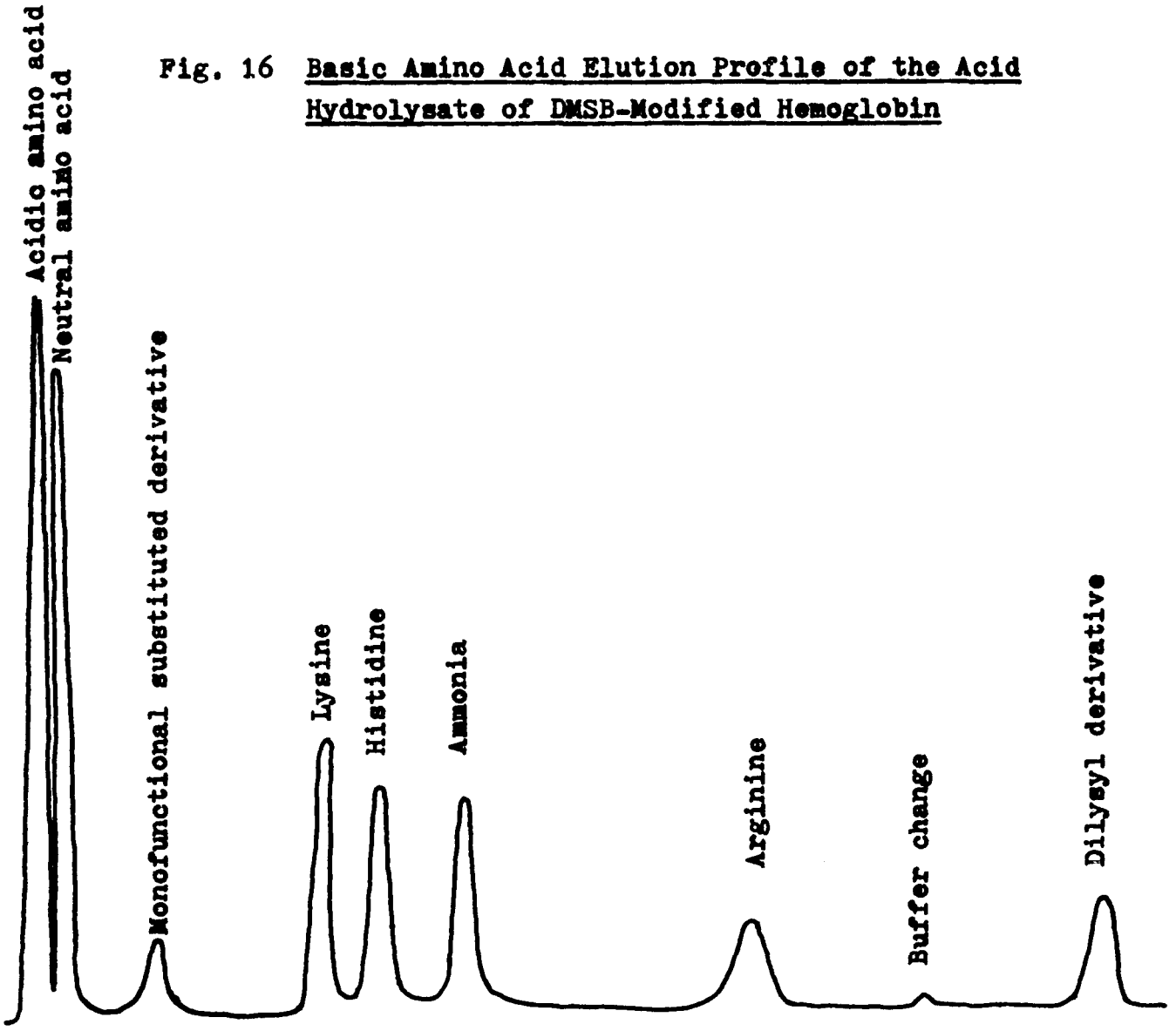
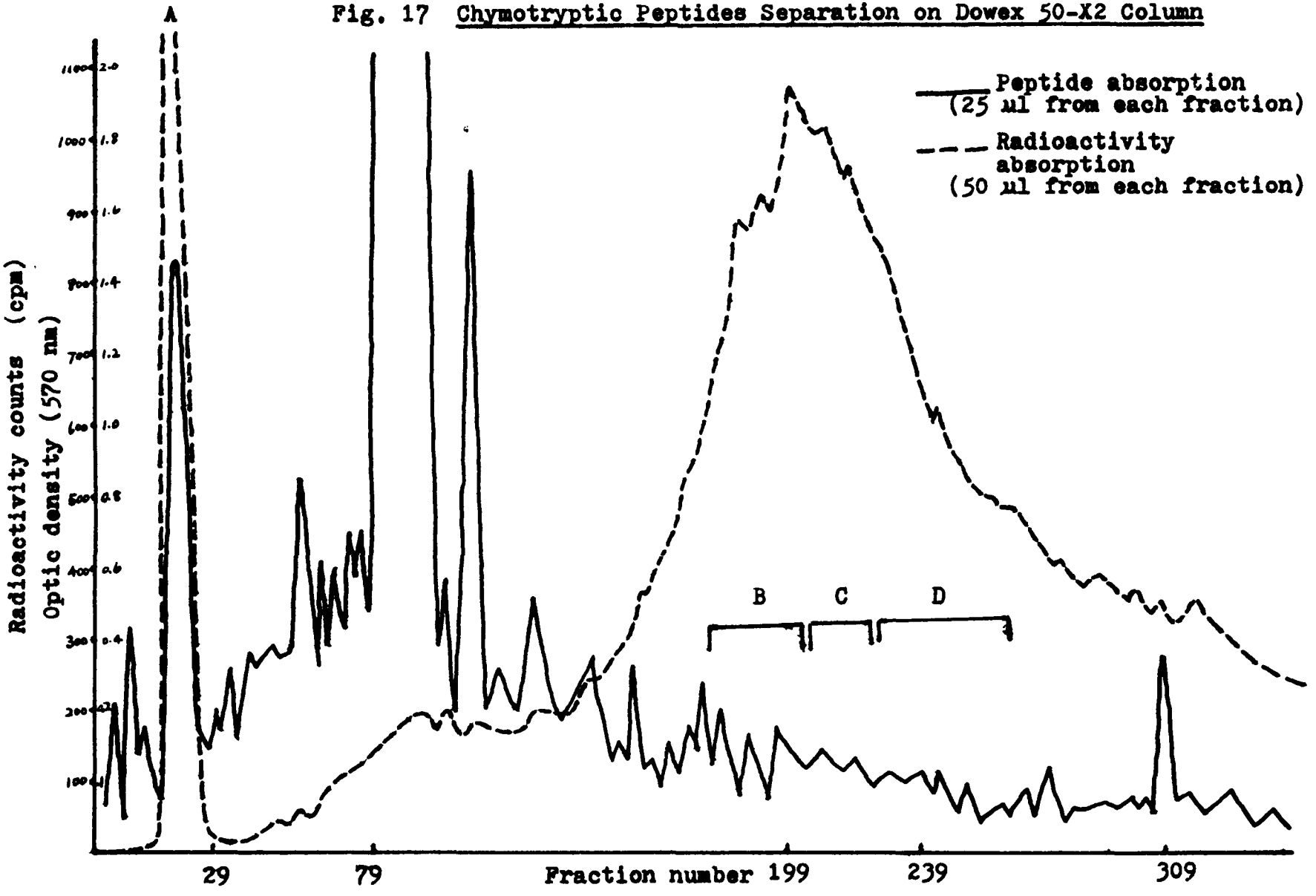


Fig. 17 Chymotryptic Peptides Separation on Dowex 50-X2 Column



lyzed reagent. Further analysis of this fraction A on the AG 1-X2 (200-400 mesh) column showed no detectable peptide absorption under the radioactive peak. Fraction B,C, and D were pooled to do further purification by peptide mapping.

Peptide Separation by Two-Dimensional Paper Chromatography and Electrophoresis:

Peptide separation by electrophoresis is based on the charge difference and chromatography is based on the polarity difference of each peptide. With this method it is possible to detect the difference as slight as replacement of a single amino acid among the peptides. Peptide separation maps of fraction B,C, and D obtained from the Dowex 50-X2 column are shown in Fig. 18, 19, and 20. The dark spots on the map represent the radioactive peptides. The light spots are ninhydrin-positive non-radioactive peptides.

Anino Acid Analysis of C^{14} Peptides:

C^{14} peptides were eluted with 6 N HCl and hydrolyzed for 23 hours. There are two possibilities, one is a bifunctional substitution which indicates a cross-linked peptide, yielding a dilysyl derivative in the hydrolysate. the other one is a monofunctional substitution with only one end of the reagent reacting with a single lysyl residue while the other end is hydrolyzed.

The following C^{14}-peptides were identified:

(1) Amino acid composition of C^{14} peptide C-2 (Table III).

The amino acid content of this peptide indicates that it is

Fig. 18 Purification of C^{14} Fraction B on Peptide Map

Dark spots (B-1, B-2, B-3, B-4, and B-5) represent radioactive peptides

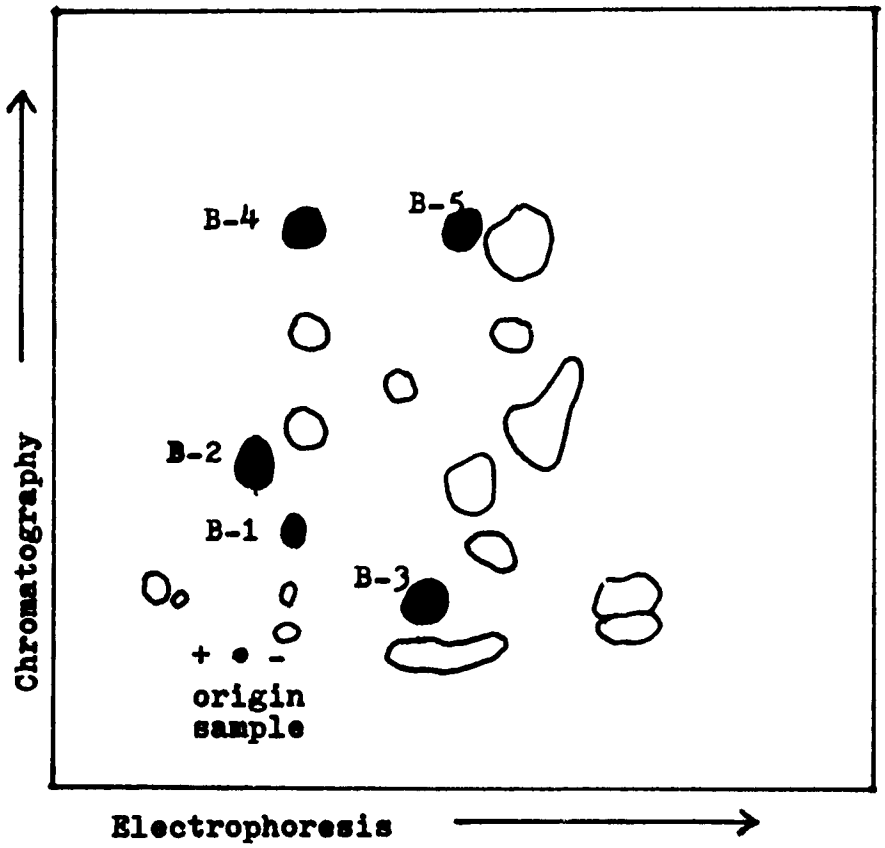


Fig. 19 Purification of C^{14} Fraction C on the Peptide Map

Dark spots (C-1 and C-2) represent radioactive peptides

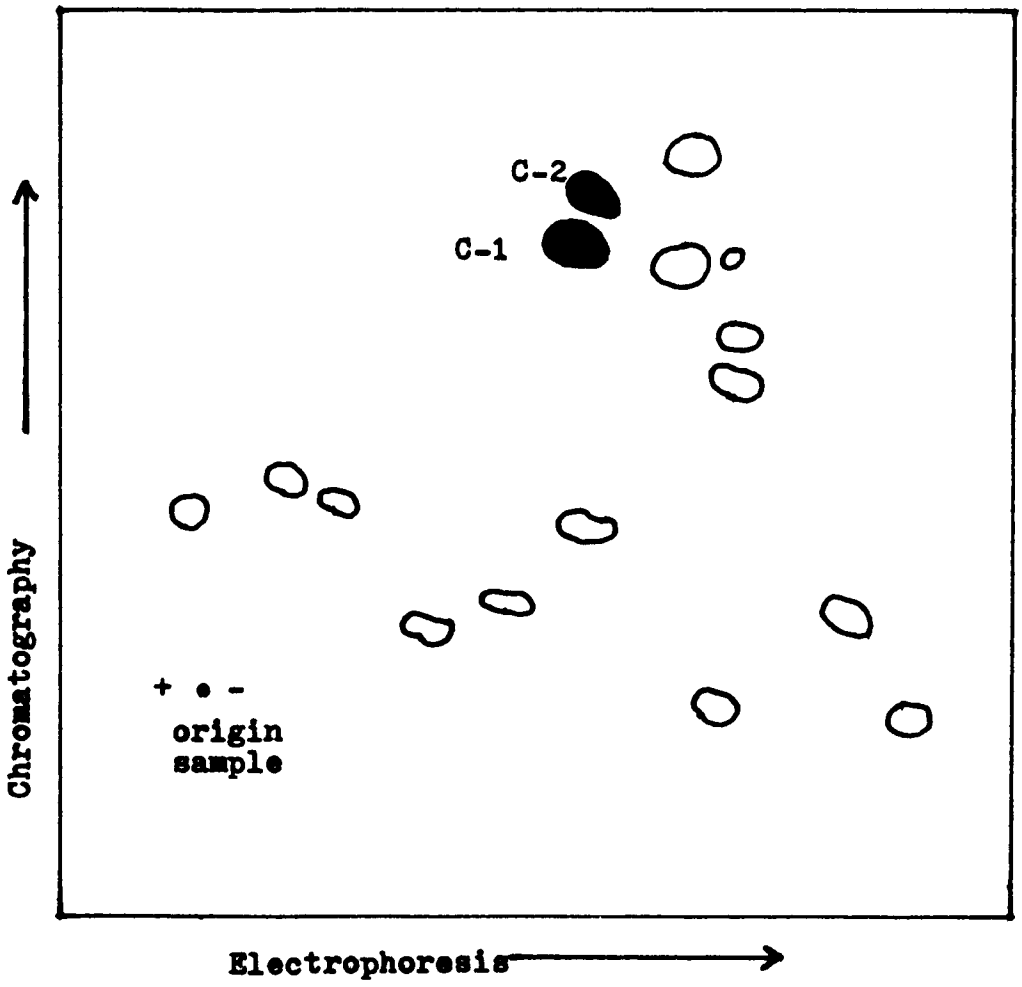


Fig. 20 Purification of C^{14} Fraction D on Peptide Map

Dark spots (D-1, D-2, D-3, and D-4) represent radioactive peptide.

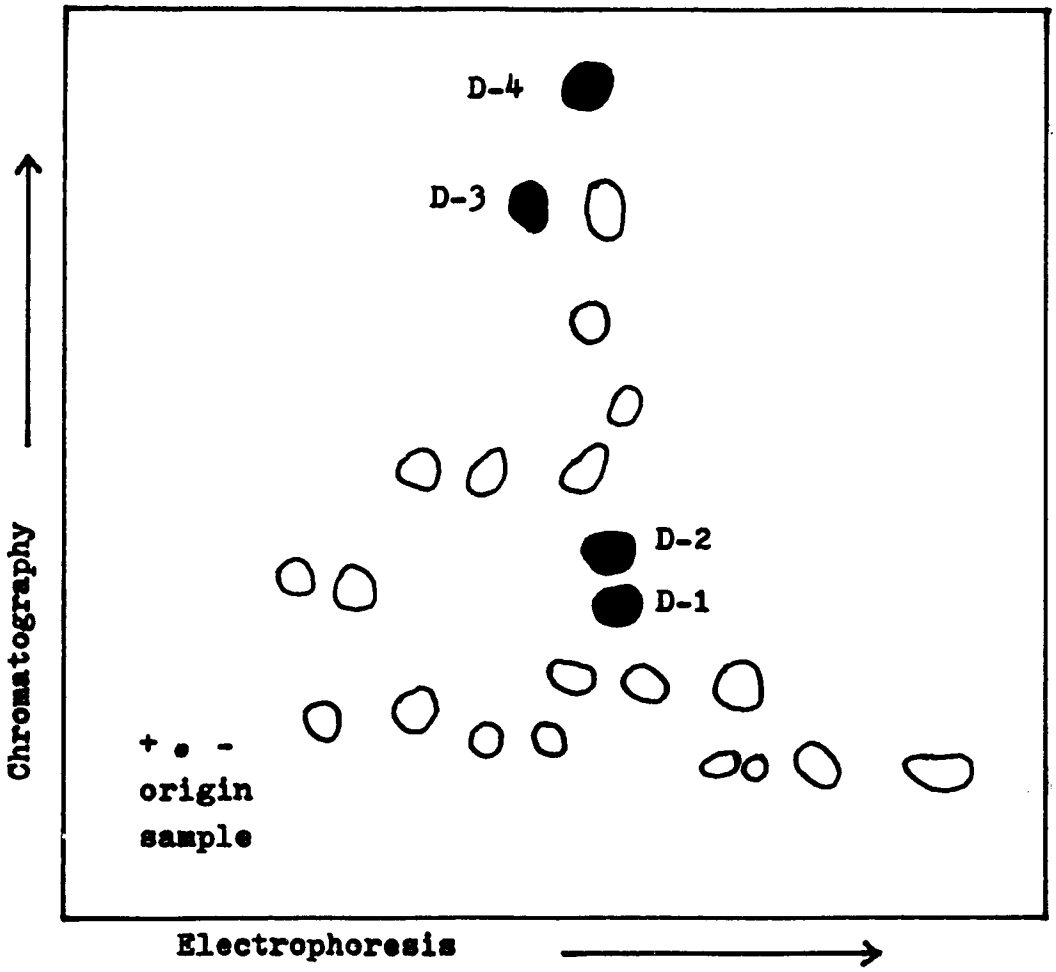


Table III. Amino Acid Composition of C^{14} Peptide C-2

	Number of residues	μmole/ml
Lysine	0	0
Dilysyl derivative	1	0.0051
Threonine	1	0.0063
Glycine	1	0.0061
Leucine	2	0.0130
Serine*	1	0.0050

* Serine is partially destroyed during acid hydrolysis

a cross linked peptide and contains residues 99-101 in an α chain and residues 82-84 in a β chain. No other fragments exist which would yield the amino acid composition which was obtained:

$$\begin{array}{lll} & & 99 \\ \alpha & \text{chain} & \text{lys-leu-leu-ser} \\ & & \quad | \\ \beta & \text{chain} & \text{lys-gly-thr} \\ & & 82 \end{array}$$

A lys 82 (β chain)— lys 99 (α chain) cross-linking would account for the presence of a peptide containing the above amino acids.

(2) Amino acid composition of C^{14}peptide B-2 (Table IV)

This peptide is consistent with a peptide comprising two identical peptides with residues 16-28 in the β chains. Because it contains 1 dilysyl residue, this peptide suggests a lys 17-lys 17 cross-link between two β chains:

$$\begin{array}{ll} 16 & 28 \\ \text{gly-lys-val-asn-val-asp-glu-val-gly-gly-glu-ala-leu} & (\beta' \text{ chain}) \\ \quad | & \\ \text{gly-lys-val-asn-val-asp-glu-val-gly-gly-glu-ala-leu} & (\beta'' \text{ chain}) \end{array}$$

(3) Amino acid composition of C^{14} peptide B-3 (Table V)

Because of the absence of a dilysyl residue in its hydrolysate, it is a monosubstited derivative. It shows a extra new peak near neutral amino acid region in the basic amino acid elution pattern (Fig. 16). This peptide corresponds to residues 15-24 in an α chain.

$$\begin{array}{ll} 15 & 24 \\ \text{gly-lys-val-gly-ala-his-ala-gly-gly-tyr} & (\alpha \text{ chain}) \end{array}$$

Table IV. Amino Acid Composition of C^{14} Peptide B-2

	Number of residues (a)	µmole/ml
Lysine	0	0
Dilysyl derivative	1	0.013
Aspartic Acid	4	0.047
Glutamic Acid	4	0.05
Glycine	6	0.068
Alanine	2	0.026
Valine	6	0.068
Leucine	2	0.027

a. amino acids with number of residues less than one tenth were ignored

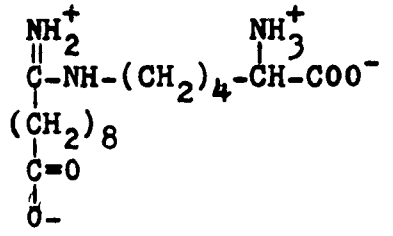
Table V. Amino Acid Composition of C^{14} Peptide B-3

	Number of residues	umole/ml
Lysine	1	0.019
Dilysyl derivative	0	0
Histidine	1	0.018
Glutamic Acid	1	0.022
Glycine	3	0.055
Alanine	2	0.035
Valine	1	0.023
Tyrosine*	1	0.010
Mono-lysyl[x] derivative	0.3	0.006

* Tyrosine is partially destroyed during acid hydrolysis.

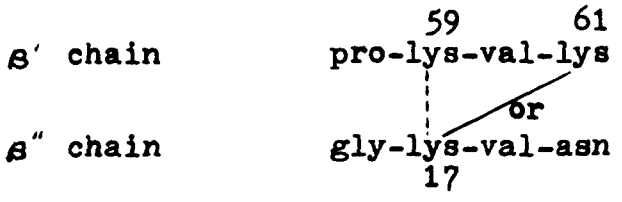
x Mono-lysyl derivative was partially hydrolyzed to lys.

The structure of a monosubstituted derivative is shown below:



(4) Amino acid composition of C^{14} peptide D-1 (Table VI)

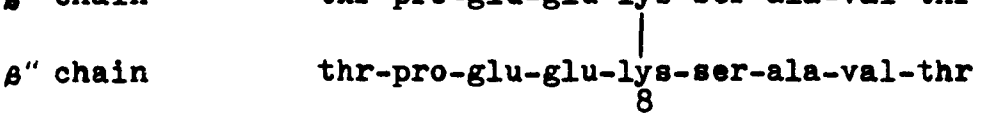
The amino acid content indicates that two peptides are cross-linked together:



There are two possibilities, either lys 59-lys 17 or lys 61-lys 17 is cross-linked and the bridge can be either within a single β chain or between two different β chains. The cross-link between lys 61-lys 17 within a β chain is more likely while comparing with the three dimensional structure as determinated by X-ray.

(5) Amino acid composition of C^{14} peptide B-1 (Table VII)

The amino acid content of this peptide indicates that there is a bridge between two different β chains, lys 8-lys 8.



All above cross-linked peptides were treated with 0.8 ml of concentrated ammonium hydroxide-glacial acetic acid (15:2)

Table VI. Amino Acid Composition of C^{14} Peptide D-1

	Number of residues (a)	µmole/ml
Lysine	1	0.024
Dilysyl derivative	1	0.023
Aspartic Acid	1	0.024
Proline	1	0.022
Glycine	1	0.024
Valine	2	0.032

a. amino acids with number of residues less than 0.3 were ignored

Table VII. Amino Acid Composition of C^{14} Peptide B-1

	Number of residue	µmole/ml
Lysine	0	0
Dilysyl derivative	1	0.015
Threonine*	4	0.048
Serine	2	0.028
Glutamic Acid	4	0.058
Proline	2	0.027
Alanine	2	0.034
Valine	2	0.031

*Threonine is N-terminal residue which is partially destroyed by ninhydrin

at room temperature for 8 hours (Ludwig and Byrne) to remove the cross-linking reagents, and the ammonium acetate, was removed by repeated lyophilization. The residue was then subjected to peptide mapping. These cross-linked peptides showed two non-radioactive peptide spots after this treatment, although peptides B-2 and B-1 showed only one spot. An attempt to analyze the amino acid composition of these separated spots was not successful, because of the small quantities present and ease of contamination.

DISCUSSION

The formation of intermolecular cross-linked hemoglobin polymers using DEM and a 10% hemoglobin solution is due to the relatively higher concentration of hemoglobin solution in the reaction as well as to the fact that this reagent is too short to span the distance between lysyl residues of different subunits in a single hemoglobin tetramer. This polymer, however, shows a great degree of potential dissociation to dimers and monomers.

The intramolecular cross-linked hemoglobin modified by DMSB is much less dissociable and retains a molecular weight of about 68,000 and shows a longer life span in the rabbit circulation than that of unmodified hemoglobin. It may serve as a satisfactory plasma protein extender. Further studies of oxygen dissociation and the physiological effect of this modified hemoglobin in animals are necessary.

The presence of the pentamers after SDS treatment of those modified hemoglobin is probably due to an undissociable tetramer cross-linked to a single subunit belonging to another dissociable molecule.

Comparing these four different lengths of diimido ester-modified hemoglobin, we conclude that the longer length of the reagent, the better chance of cross-linking within a hemoglobin tetramer. There are two diimidate reagents, i.e.,

dimethyldodecanimidate (12 carbon chain) and dimethyltetradecanimidate (14 carbon chain) which were also prepared and tested as intramolecular cross-linking reagents for hemoglobin, but the low solubility in H_2O at pH 8.5, limits their usefulness.

Two classes of reaction products (Dutton, et al.) can form in the reaction of bifunctional diimido esters with lysyl-residues of protein. In one class, both imido ester groups react with two lysyl residues to form a diamidine cross-linked product:

$$2\ \text{Protein-}NH_3^+ + CH_3O\text{-}\overset{\overset{NH_2^+\ Cl^-}{\|}}{C}\text{-}(CH_2)_n\text{-}\overset{\overset{NH_2^+\ Cl^-}{\|}}{C}\text{-}OCH_3 \longrightarrow$$

$$\text{Protein-NH-}\overset{\overset{NH_2^+}{\|}}{C}\text{-}(CH_2)_n\text{-}\overset{\overset{NH_2^+}{\|}}{C}\text{-NH-Protein}$$

In the other class, (Roger, Neilson) only one imidoester group of this reagent reacts with a single lysyl residue whereas the second is hydrolyzed.

$$\text{Protein-}NH_3^+ + CH_3O\text{-}\overset{\overset{NH_2^+\ Cl}{\|}}{C}\text{-}(CH_2)_n\text{-}\overset{\overset{NH_2^+Cl}{\|}}{C}\text{-}OCH_3 + H_2O \longrightarrow$$

$$\text{Protein-NH-}\overset{\overset{NH_2^+}{\|}}{C}\text{-}(CH_2)_n\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH + NH_4^+ + H^+ + CH_3OH$$

The reagents of this family are so unstable that no unreacted, unhydrolyzed imidoester group could remain. Since the di-lysyl derivative has a higher pK_a than that of arginine, the short column of the amino acid analyzer, after emergence

of arginine in the normal pH 5.26 sodium citrate buffer, was eluted with a pH 9.7 borate buffer to reveal the dilysyl derivative.

Amino acid analysis of C^{14} peptide B-3 shows another new peak in neutral amino acid region (see Fig. 16), but no dilysyl derivative peak appeared after changing to pH 9.7 borate buffer. This peptide represents a mono-functional substitution derivative and this extra peak is believed to be the monolysyl derivative of DMSB. It has not been separated in pure form yet.

The number of moles of incorporated C^{14} reagents (from radioactivity measurement) and the number of moles of modified lysyl residues (from amino acid analysis of the lysyl content before and after the reaction) were calculated. The ratio of lysyl residues modified to the moles of incorporated reagents is 1.6 which indicates that 60% of the incorporated C^{14} DMSB reagent is present as DMSB-dilysyl derivatives and that there are 5.3 cross-linked bridges per tetramer on the average. The number of cross-links introduced as calculated from direct measurement of dilysyl derivative by automatic amino acid analysis indicates that the average number of cross-link bridges is 6 per hemoglobin tetramer. It was also found that an increase in the amount of the reagent added during the reaction with hemoglobin will increase the radioactive incorporation and also the degree of cross-linking.

The reason why chymotrypsin, instead of trypsin, was used is due to the fact that the modified lysyl residues are resistant to trypsin catalysis (Hartman). Chymotrypsin (Hirs) rapidly catalyzes the hydrolysis of peptide bonds in which the -COOH groups are due to the aromatic amino acids, i.e., tyrosine, phenylalanine, and tryptophan. Slow hydrolysis has been found to occur at the -COOH groups of numerous amino acids such as asparagine, glutamine, histidine, leucine, lysine, methionine, serine, and threonine. Cleavage at these sites is usually significant only if a higher concentration of chymotrypsin was employed or when the time of digestion was prolonged. During the digestion of the C^{14} cross-linked hemoglobin, three portions of the enzyme was used and the time of digestion was extended to 30 hours. Under these conditions all of the possible cleavage sites can be hydrolyzed. Some of the C^{14} peptide are small and the cleavage sites are all consistent with the possibilities described above and the chymotryptic digest of human α chains and partial digestion of β chains. (Robert, Konigberg).

There are two possibilities to explain the nature of the di-lysyl residues cross-linked in the dilysyl peptides identified in the C^{14}-DMSB modified hemoglobin. The first would be due to cross-linking within a single tetrameric molecule by bridging the distance with the reagent. Such a product would indeed be an undissociable tetrameric hemoglobin. The other possibility would involve a dissociation of the hemoglobin tetramer due to the low concentration of hemo-

globin in a 1% solution and the effect of pH (8.5). Hemoglobin dissociates reversibly into half-molecules, each of which contains an α - and a β -chain, to give the equilibrium

$$\underset{\text{Hemoglobin}}{\alpha\alpha\,\beta\beta} \rightleftharpoons \underset{\text{Half-molecules}}{\alpha\beta \;+\; \alpha\beta}$$

This equilibrium was driven toward right hand side due to the fact that these cross-links werarformed between dimers. Such dissociated dimers would now react, involving all kinds of surface lysyl residues, and in some instances with a single subunit in addition. The latter would account for pentamers which appeared during SDS-gel electrophoresis.

In order to determine which of the above two possibilities were actually involved, Dr Cyrus Levinthal of Columbia University kindly allowed us to view a three-dimensional projection on a computer screen. Each of the α and β lysyl residues in the peptides which were identified by us was identified on the screen. The projection model of the tetramer was rotated to show clearly the lysyl residue positions cross-linked in our peptides. Only lys 61-lys 17 cross-linked within one β chain is possible, in other instances the lysyl residues in the cross-linked peptides were too far apart to have been found with the 10 carbon span in the sebacimidate molecule. Indeed in two instances, as for example in the β' lys 17-β''lys 17 and β'lys 8-β''lys 8, the lysyl residues are on the opposite sides of the tetramer which are 65 Å far apart.

The conclusion to be drawn from the results of the present study and the three-dimensional structure of Perutz obtained from X-ray diffraction studies, is that a dilute solution of hemoglobin at pH 8.5 dissociates sufficiently, and most probably as dimers, to allow for intra-dimer cross-linking but yielding a fairly highly proportion of tetramers with a molecular weight approximately the same as that of unmodified hemoglobin. We also concluded that the tertiary structure of human hemoglobin in solution is similar to that of hemoglobin in crystalline state as determined by X-ray studies but the quaternary structure is different.

However the modified tetramers are much less dissociable than normal hemoglobin and may serve as adequate plasma protein expanders.

BIBLIOGRAPHY

Barratt, J.U.W. and Yorke, W., British Medical Journal, 1, 235, (1914).

Carpenter, F.H. and Harrington, K.T. Journal of Biological Chemistry, Vol. 247, No. 17, pp. 5580-86. (1972).

Clegg, J.B. and Naughton, M.A. Journal of Molecular Biology, 19, pp. 91-108 (1966).

Davies, G.E. and Stark, G.R. Proceedings of the National Academy of Sciences, Vol. 66, No. 3. pp. 651-56 (1970).

Dutton, A., Adams, M., and Singer, S.J. Biochemical and Biophysical Research Communications, Vol. 23. No. 5 (1966).

Hamilton, P.B., Farr, L.E., Hiller, A. and Van Slyke, D.D., Journal of Experimental Medicine, 84, p. 445 (1947).

Hardway, R.M., Byungkyn, C. and Rutherford, R.B. Acta. Chir. Scand. 130+157 (1965).

Hardway, R.M. American Journal of Surgery, 110, 298, (1965).

Hartman, F.C., Biochemistry, Vol. 6 No. 8 (1967)

Hill, R.J., and Konigberg, W. Journal of Biological Chemistry Vol. 237, No. 10 (1962)

Hirs, C.H.W. Method in Enzymology,XXI (1967)

Huisman, H.J., and Dozy, A.M., Journal of Chromatography, 7, pp.-180-203 (1962).

Hunter, M.J. and Ludwig, M.L., Journal of American Chemical Society, 71, p. 40 (1949).

Hunt, J.A., and Ingram, V.M., Biochimia Biophysica Acta, Vol. 28, (1958).

Katchalski, E., Benjamin, G.S. and Gross, V. Journal of American Chemical Society, 79, pp. 94-96. (1957).

Katz, A.M., Journal of Biological Chemistry, Vol. 234, No. 11 (1959).

Konigsberg, W. et al. Journal of Biological Chemistry, Vol. 238 (1963).

Lathem, W. Journal of Clinical Investigation, 38, 652 (1959).

Lowry, O.H., Rosenbrough, N.V. Farr, A.L., and Randall, R.J., Journal of Biological Chemistry, 193, 720 (1951).

Ludwig, M.L., and Byrne, R. Journal of American Chemical Society, 84, p. 4160 (1962).

Moore, S., and Stein, S.H., Method in Enzymology, VI, 819 (1963).

Moore, S., and Stein, S.H., Journal of Biological Chemistry (1965).

McElvain, S.M., and Schroeder, J.P., Journal of American Chemical Society, 71 (1949).

Nagel, R., and Gibson, P.H., Journal of Biological Chemistry 246:69 (1971).

Rabiner, S.F., and Helbert, J.F., Lopas, H. and Friedman, L., Journal of Experimental Medicine, 126, p. 1127 (1967).

Roger, R., and Neilson, D.G., Chemical Review, 61, p. 179 (1961).

Rabiner, S.F., O'Brienk., and Peskin, G.W., Annals of Surgery, 171, 615 (1970).

Rossi-Finelli., Advances in Protein Chemistry, 19 New York Press, (1964).

Shapiro, A.L., Vinela, E. and Maizel, J.V., Biochem Biophy Res Comm, 28, p. 815, (1967).

Weber, K., and Osborn, M. Journal of Biological Chemistry Vol. 244, p. 4406 (1969).

Wold, F. Method in Enzymology, XI, 617 (1967).

Wofsy, L. and Singer, Biochemistry, Vol. 2 p. 104 (1963).

Journal of Biological Chemistry, Vol. 250, p. 87 (1975).