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I. THE PREPARATION, PROPERTIES AND BIOSYNTHESIS OF RAT TRANSFERRIN

II. THE PREPARATION AND PROPERTIES OF MODIFIED HEMOGLOBIN

By

WINNIE WAI-CHING MOK

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

of

DOCTOR OF PHILOSOPHY

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Abstract

(A) The Preparation, Properties and Biosynthesis of Rat Transferrin

Adviser: Dr. Abraham Mazur By Winnie Mok

The existence of two forms of transferrin in rat serum has been demonstrated using the methods of isoelectric focusing on analytical gels and on sucrose gradient column. The latter method allowed the preparation of each form of rat transferrin in large quantities for further studies.

The two forms of rat transferrin have the same molecular weight, amino acid composition (within limits of the accuracy of the method) and immunological properties. The major ("slow") transferrin is 90% saturated with iron, while the minor ("fast") transferrin is 95% saturated. The isoelectric points of the major and minor forms of transferrin are 5.75 and 5.50, respectively. SDS polyacrylamide gel electrophoresis indicated that neither have a subunit structure.

Maximum accumulation of newly formed transferrin in the rat occurs at about 30 min. in the rough microsomes and at about 40 min. in the smooth microsomes. Newly synthesized transferrin is exported from liver cells into the incubation medium after a minimum period of 30 min. The newly synthesized transferrin does not pick up any iron despite the fact that there is iron within the liver cell in several forms.

Abstract

(B) The Preparation and Properties of Modified Hemoglobins
Adviser: Dr. Abraham Mazur By: Winnie Mok

Diethylmalonimidate dihydrochloride has been used to cross-link the lysyl ϵ-amino groups of hemoglobin to produce high molecular weight hemoglobin polymers (Polyhemoglobin). About one-half to two-thirds of these lysyl residues are cross-linked. These polymers are highly viscous, heterogeneous in sizes and they do not dissociate into dimers and monomers in the presence of sodium dedocyl sulfate.

The polyhemoglobins, when injected into rabbits, have a half-life of 4-5 times (at 0.14 g/Kg) to 10-15 times (at 0.5 g/Kg) longer than an equal quantity of unreacted hemoglobin.

Polyhemoglobin appears to have a delaying action on coagulation. It does not bind as well or at all to haptoglobin as does unreacted hemoglobin. Most of the polyhemoglobins have molecular weights over 300,000. Some 15% have molecular weights between 400,000 to 800,000. The high molecular weight of polyhemoglobins together with their inability to complex with haptoglobin may offer a probable explanation for their longer stay in the circulation.

I. Preparation, Properties and Biosynthesis of Rat Transferrin

INTRODUCTION

Transferrin is a serum protein which has the unique property of binding iron. It functions as a means of transport of iron, at neutral pH, picking up iron absorbed through the small intestine or released from reticuloendothelial cells which contain degraded old red cells and transporting the iron for release to cells which require the element, e.g., the erythroid cells of the bone marrow, and to a lesser extent the hepatic cells where iron is stored in the form of another protein, ferritin.

The existence of such an iron-binding protein in the plasma was first noted by Holmberg and Laurell (1) who named it transferrin (2). Schade and Caroline (3) demonstrated that the bacteriostatic activity of serum was in part due to an iron-binding protein which they named siderophilin (4). This name has been supplanted by transferrin.

Transferrin exists in the plasma in a partially unsaturated state with respect to its potential iron-binding capacity; some two-thirds is unsaturated. All the plasma iron (ca 100µg /100 ml) is bound to transferrin. Transferrin (or serum) binds added inorganic ferrous or ferric iron at neutral pH in the presence of bicarbonate, forming a ferric iron-transferrin complex with an extremely low dissociation constant. The complex has a salmon-pink color, absorbing in the region of 460-470 mu. When saturated, transferrin binds two atoms of iron per gm-molecular weight and one mole of bicarbonate per atom of iron.

Hen's egg transferrin has been reported to have a molecular weight of 80,000 (5), human transferrin of 68,000-78,000 (6,7,8) and rat transferrin of 68,000 (6). Because transferrin has two equivalent iron binding sites (9) and two similar carbohydrate chains (10), the molecule might consist of two subunits. Jeppson (11) subjected reduced, alkylated human transferrin to "approach to equilibrium" centrifugation technique and reported dissociation into two similar subunits of molecular weight 39,000 to 42,000. However, studies in other laboratories (8, 12, 13) of the same material resulted in the finding of a single polypeptide chain. Similarly, Mann (14) reported a single polypeptide chain for human transferrin examined in 6 M guanidine hydrochloride by gel filtration, sedimentation equilibrium and intrinsic viscosity measurements.

Multiple forms of transferrin have been reported. Two forms have been found for hen's egg transferrin (15, 16, 17) and rat transferrin (18), although separation for preparative purposes was not achieved in these studies. Gordon and Louis (19) reported the separation of rat transferrin into two components by electrophoresis in Pevikon c870 followed by DEAE Sephadex chromatography. Wenn and Williams (5) used the technique of isoelectric focusing to demonstrate a major ("slow") and a minor ("fast") component from hen's egg. Two species of transferrin have also been reported for rabbits (20) and frogs (21). Several variants for human (21) turtle and hagfish (8) transferrins have also been reported.

The present studies were undertaken with two objectives in mind:

(a) To explore a relatively rapid and reliable method of separation and preparation of rat transferrins in a pure state for a study of its chemical and physical properties, and for purposes of preparing specific rabbit antibodies to each of the two transferrin components to compare their immunological properties, and,

(b) To study (i) the intracellular site of secretion of rat transferrin in the hepatic cell, (ii) whether iron is added to the transferrin at an intracellular location or after it leaves the hepatic cell.

EXPERIMENTAL

Materials: Pooled rat sera were obtained from Pel-Freeze Biologicals, Inc. ^{59}Fe (as $FeCl_3$) was obtained from Abbott Laboratories. Sephadex was purchased from Pharmacia Fine Chemicals. Ammonium sulfate and sucrose were supplied by Mann Research Laboratories and was of ultrapure quality. Ampholines and equipment for sucrose gradient isoelectric focusing were purchased from LKB Products. Analytical disc gel and isoelectric focusing were performed in a Savant tank. Wistar rats (CFN strain) were purchased from Carworth Farms. All other reagents were of highest available purity.

Fractionation of rat sera: Radioactive ferric chloride was added to rat sera to saturate transferrin completely. (Iron-binding capacity of serum is 3 μg/ml) 50 ml of rat sera were put on a Sephadex G-100 column and eluted with sodium phosphate (0.05 M)-NaCl (0.1 M) buffer, pH 7.4, Fractions were collected via a fraction collector (LKB Producter) and counted in a well-type radiation counter (Nuclear Chicago Company). The fractions with high counts were pooled and made 50% saturated with ammonium sulfate. After centrifugation, the pellet was dissolved in and dialysed extensively against $5x10^{-3}$ M tris-glycine buffer, pH 8.6.

Isoelectric fractionation in a sucrose density gradient: The dialysed proteins were subjected to isoelectric focusing (IEF), using a 110 ml-continuous sucrose gradient column. A sample of 40 mg was applied and a pH gradient of 5 to 7 was chosen. A constant voltage of 600 volts was passed through the column for about 72-84 hours of 4°C.

Preparative isoelectric focusing in polyacrylamide gels: Analytical gels were scaled up 100-fold by using glass tubes 2x50 cm containing a reservoir at the top to serve as a buffer compartment. The gel tubes were suspended in a wooden rack and dipped into a lower buffer compartment. Rubber stoppers were inserted at the bottom of each tube prior to pouring the gel solution and were removed after polymerization. 20% sucrose solution was layered at the bottom and 5% sucrose solution on top to obtain level gel surfaces.

Up to 40 mg protein were incorporated into each gel and electrophoresis was conducted at 500 to 600 volts for 72-96 hours at 4° C. The gels were sliced as close to the pink bands as possible and ground in a mortar with sand as an abrasive for extraction with $5x10^{-2}$ M tris-glycine buffer, pH 8.6.

Analytical isoelectric focusing: Isoelectric focusing was performed in polyacrylamide gels according to Dale and Latner (22) as modified by Wrigley (23). The sample which had been dialysed extensively against $5x10^{-3}$ M tris-glycine buffer, pH 8.6, was mixed with the gel solution prior to polymerization. Samples from 0.1 to 0.4 mg were incorporated into gels which were 8 cm in height and 0.7 cm in diameter. A pH gradient from 5-7 was chosen and electrophoresis was carried out at 50 volts for 84-96 hours at 4° C. Gels were fixed and washed many times with 12.5% trichloroacetic acid to remove the ampholines before staining with Coomassie Brilliant Blue according to the method of Chambrach, et al. (24).

Scanning of analytical size gels: A Joyce-Loebl recording densitometer was used for scanning gels. The relative proportions of each transferrin was estimated by measuring the area under each peak.

Disc gel electrophoresis: Disc gel electrophoresis was performed according to the procedure of Davis (25). The separating and stacking gels were 6 cm and 0.1 cm, respectively. The sample was dialysed against $5x10^{-2}$ M tris-glycine buffer, pH 8.6 prior to electrophoresis. A sample of 0.01 to 0.1 mg was mixed with some sucrose and layered over the stacking gel. Electrophoresis was conducted for 50 minutes at 4 mamp/gel at 4°C. The gels were stained with Amido-Schwartz (Naphthol Blue-Black) and destained with 7% acetic acid electrophoretically.

Molecular weight determination: Molecular weight of the transferrins was measured by the method of sedimentation equilibrium. Each transferrin was run at a concentration of 0.15 mg/ml, using the Beckman Model E Ultracentrifuge at 12,000 rpm at 20^{o} C overnight. A solvent density of 1.005 was used and a partial specific volume of 0.74 was assumed (26).

Preparation of Transferrins for amino acid analysis: Each transferrin was placed on a Sephadex G-75 column and eluted with 5×10^{-2} M tris-glycine buffer, pH 8.6, to remove the ampholines. It was then dialysed extensively against distilled water.

Amino acid analysis: A sample of 1 mg/ml was hydrolysed in 6N hydrochloric acid at 110^{o} for 20-22 hours. The hydrolysis tubes were frozen in acetone and dry ice and evacuated under nitrogen with a water aspirator prior to sealing. Analyses were performed using a Beckman Model 116 Amino Acid Analyzer according to Moore and Stein (27). Tryptophan was determined by the method of Udenfriend and Peterson (28) and checked by the method of Matsubara and Sasaki (29). All buffers, reagents and resins used in these analyses were purchased from Beckman Instrument Company.

Subunit molecular weight: Subunit molecular weights were determined by electrophoresis on dodecyl sulfate-polyacrylamide gel according to the method of Weber and Osborn (30). The acrylamide stock solution contained 22.2 gm of acrylamide and 0.9 gm of bisacrylamide. Standard proteins of known molecular weights were run simultaneously. The mobilities of the standard proteins were plotted against the known molecular weights expressed on a semi-logarithmic scale.

Protein determination: Protein was measured by the method of Lowry, et al. (31) using crystalline bovine serum albumin as a standard.

Iron determination: The iron content of each transferrin was measured by the method of Schade, (32). The samples were dialysed against iron-free water exhaustively before assay.

Immunoelectrophoresis: Immunoelectrophoresis was conducted on cellulose acetate membranes in 0.0375 M barbital buffer, pH 8.6 at 150 volts for 50 minutes, using a Beckman Microzone Electrophoresis Cell.

Immunoelectrophoresis by Osserman modification: The identity of the two rat transferrins was studied by the Osserman Reaction (33). Electrophoresis was conducted at 6 volts/cm for 1-1½ hours.

Preparation of antisera to rat transferrins: Each purified rat transferrin was injected into a rabbit: *First week*: 0.3 mg/ml of transferrin was mixed with 1 ml of Freund's complete adjuvant and injected subcutaneously in the back and footpads of rabbits; *Second week*: 0.5 mg/ml mixed with 1 ml of Freund's incomplete adjuvant; *Third week*: Rest *Fourth week*: 1.0 mg/ml mixed with 1 ml of Freund's incomplete adjuvant; *Fifth week*: Test bleed.

Biosynthesis of rat transferrin: In the in vitro experiments, female rats weighing 100-200 gm were used. The livers were removed, washed in cold saline and sliced using a Stadie slicer. The slices were blotted and 1-1.5 gm quantities were weighed and placed in 50 ml Erlenmeyer flasks to which 20 ml of Krebs-Ringer bicarbonate buffer (0.154 M NaCl, 0.154 M KCl, 0.11 M $CaCl_2$, 0.154 M KH_2PO_4, 0.154 M $MgSO_4$, 0.154 M $NaHCO_3$, 20% glucose, 100:4:3:1:1:21:2.5) which had been gassed with 95% O_2- 5% CO_2 for at least half an hour, and to which H^3-leucine (Amersam-Searle) and a mixture of amino acids (except leucine) had been added. The flasks were gassed for one minute with 95% O_2-5% CO_2, stoppered and incubated for five minutes at 37° with shaking. At the end of five minutes, the liver slices from each flasks were quickly filtered by suction and washed extensively with cold saline, then transferred to another flask containing Krebs-Ringer buffer together with a total amino acid mixture, and re-incubated ("chase"). Liver slices from one flask were not "chased", but were filtered, washed with cold saline and homogenized in 10 volumes 0.25 M sucrose. The liver slices from the other flasks were "chased" for 10, 20, 30, 40, 50 and 60 mins., respectively. The slices were filtered, washed with cold saline and homogenized in 10 volumes of 0.25 M sucrose. Aliquots were taken for total counts, protein and RNA determinations. The remaining liver homogenates were centrifuged at 12,500 rpm for ten minutes, using a #40 rotor in the Model L Ultracentrifuge. The pellet obtained contained nuclei and mitochondria. The supernatant was used to prepare rough and smooth endoplasmic reticulum.

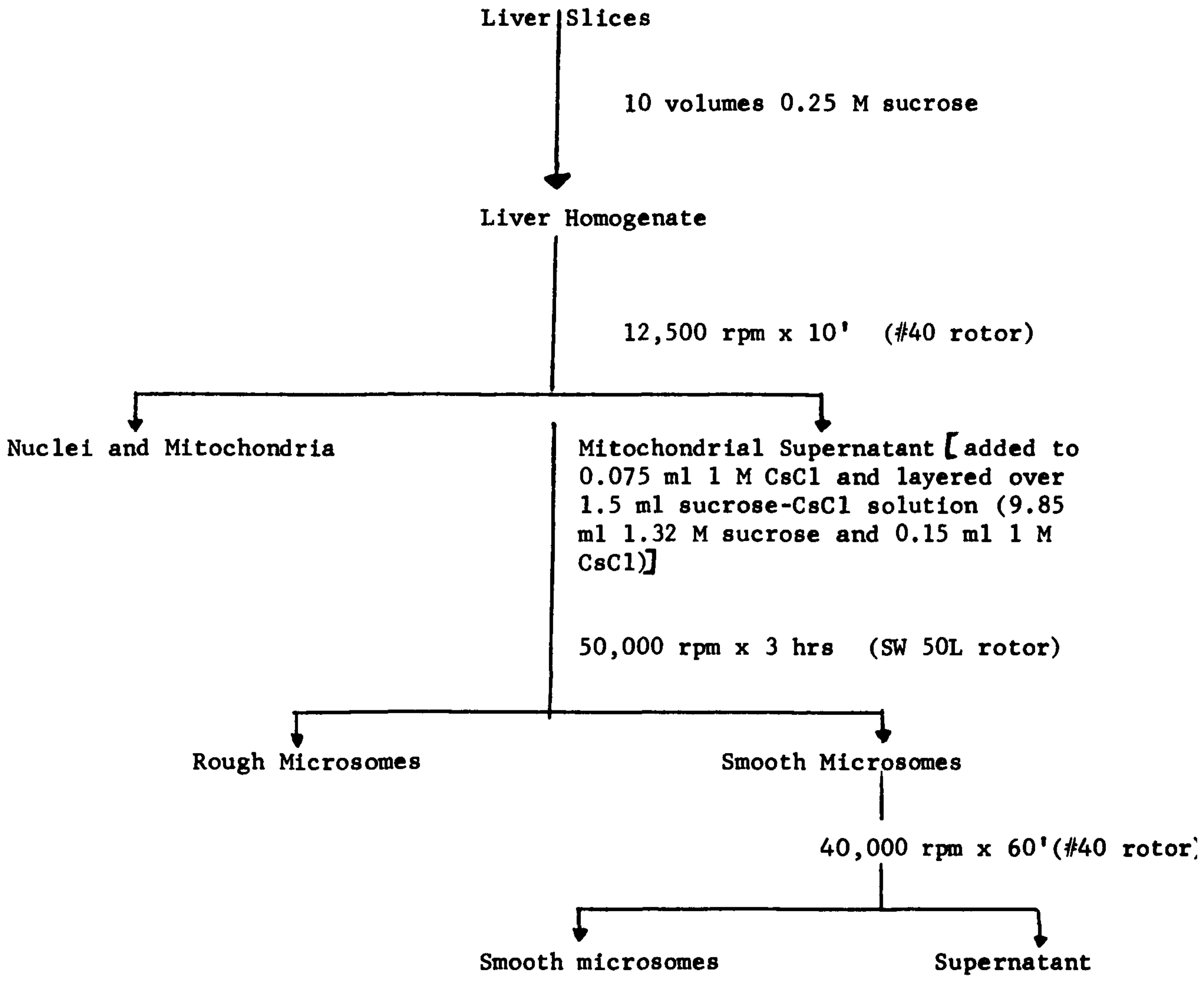
Preparation of Rough and Smooth Microsomes: The rough and smooth microsomes were prepared according to the method of Dallner (35). The supernatant after removal of the mitochondria, was added to 0.075 ml of 1 M CsCl and layered carefully over 1.5 ml of sucrose -CsCl solution (9.85 ml 1.32M sucrose to 0.15 ml 1 M CsCl) and centrifuged at 50,000 rpm for 3 hours, using the SW 50L rotor. The pellet contained the rough microsomes and were resuspended in 10 ml of 0.25 M sucrose. The smooth microsomes formed a ring at the center of the tube and they were pipetted out carefully and suspended to 11 ml with 0.25 M sucrose and centrifuged at 40,000 rpm for 1 hour, using the #40 rotor to remove the contaminating supernatant. The resulting pellet containing the smooth microsomes was resuspended in 10 ml of 0.25 M sucrose. The procedure is summarized in Table II.

Precipitation of rat transferrin with specific antisera: The rough and smooth microsomes were divided into 2 tubes and 0.5 ml of 10% deoxycholate was added to each tube. Chicken serum and antichicken serum (Kallestad) were added to each tube to remove some non-specific radioactivity which would adsorb to specific precipitates. Each tube was incubated for 60 minutes at 37^{o} and left overnight at 0^{o}. The tubes were centrifuged at 2000 rpm for 10 minutes. The pellets were washed 3 times with cold saline, dissolved in 0.5 ml formic acid (97%) and counted in vials with 10 ml Bray phosphate solution, using a Packard Liquid Scintillation Counter. The supernatant from each tube was removed and antisera to rat transferrin and carrier rat transferrin were added to some tubes. As a control, the corresponding tubes were treated a second time with chicken serum and antiserum to chicken serum. The tubes were centrifuged at 2,000 rpm for ten minutes, pellets washed 3 times with cold saline, dissolved in 0.5 ml formic acid (97%) and counted in vials containing 10 ml of Bray phosphate solution.

Incorporation of iron into rat transferrin: These experiments were performed exactly as described for the biosynthesis of rat transferrin except the rat livers were perfused with cold saline (to wash out the blood which contains transferrin) before slices were made. The slices were incubated with serum-found Fe^{59} and total amino acid mixture for 5' and "chased" for 10', 20', 30', 40, 50' and 60 minutes. The microsomes were not cleared with chicken and antichicken serum as was done in those experiments performed with ^{3}H-leucine. The fractions were counted in a well-type radiation counter (Nuclear Chicago Company).

Table I

Preparation of rough and smooth microsomes



RESULTS

(A) Resolution of rat transferrins: When equilibrium was reached using isoelectrofocusing in a sucrose gradient column, two salmon-pink bands were visible (Fig. 1). Each band was syphoned with a pump (Buchler) from the column through Teflon tubing inserted from the top of the column. Small fractions were collected via a LKB fraction collector. Recovery of each component was almost 100%. The isoelectric point of each component was determined by measuring the pH of the solution as soon as it was syphoned from the column.

	Isoelectric Point
"slow"	5.75
"fast"	5.50

Analytical isoelectrofocusing also resolved rat transferrin into two components. The relative amounts of the components were determined from densitometer tracing of gels. The "slow" component is about twice that of the "fast" component (Fig. 2).

Preparative isoelectric focusing also revealed the presence of two components. Due to the work involved in extracting the components from the gels and that the recovery was usually not higher than 70%, isoelectric focusing in a sucrose gradient column was the method of choice to prepare large quantities of rat transferrins for further studies.

The purity of each component was tested by disc gel electrophoresis and analytical isoelectric focusing. In each instance, only one band was observed (Figs. 3 & 4).

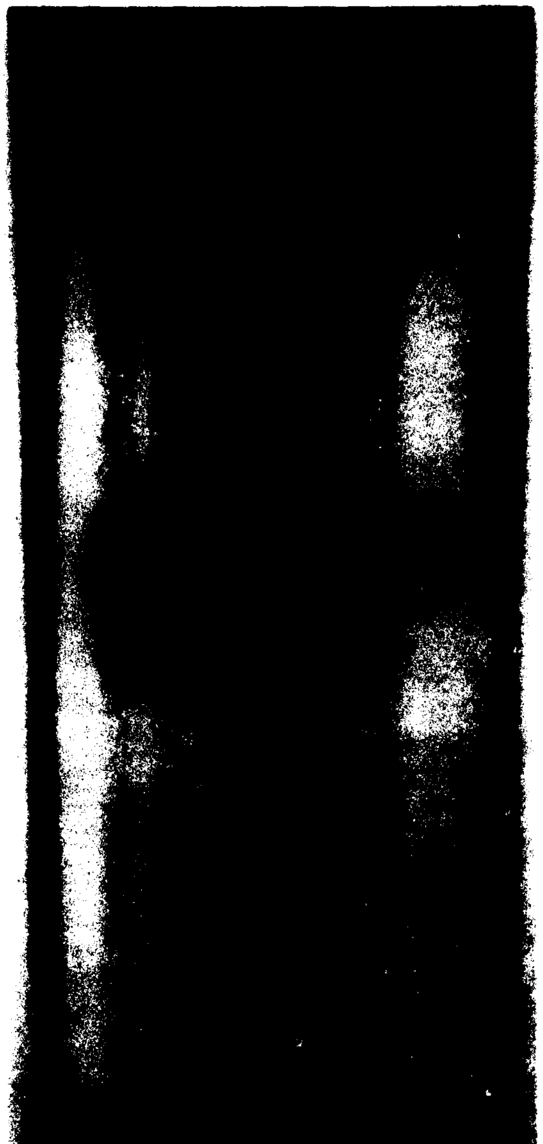


Figure 1. Isoelectric focusing of rat transferrin on a sucrose gradient, pH 5-7 (cathode at top).

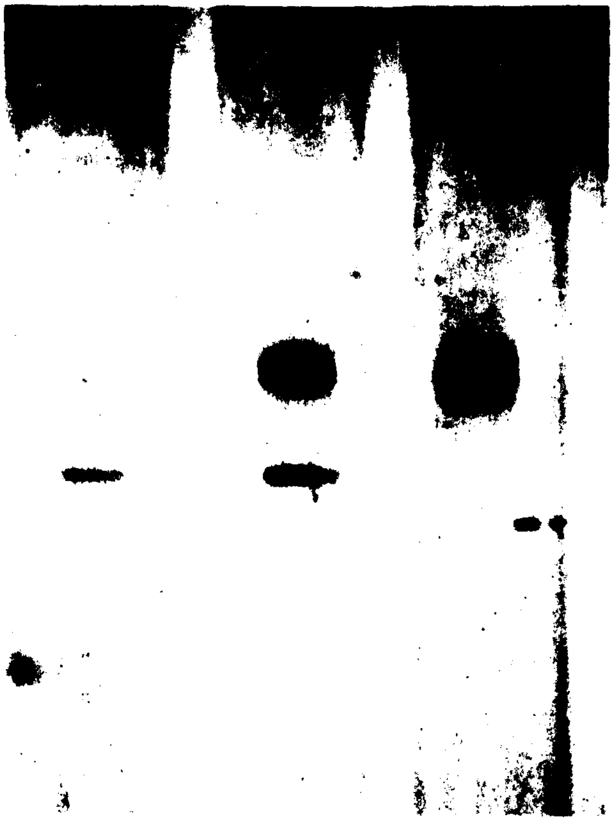


Figure 3

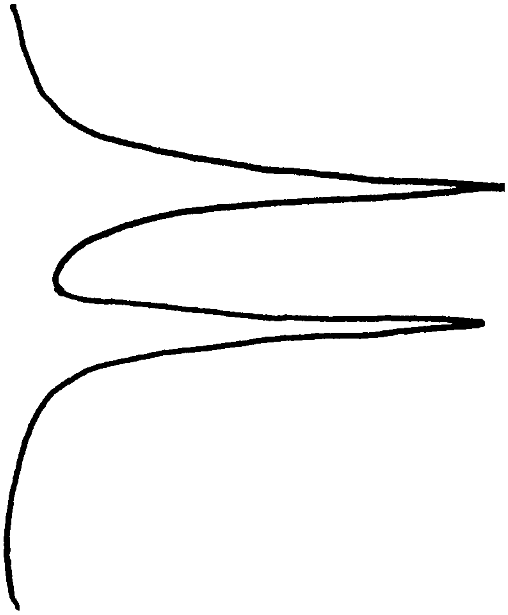


Figure 2

Figure 2. Densitometer tracing of analytical isoelectric focusing gel (centre gel, Figure 3).

Figure 3. Analytical isoelectric focusing gel of rat transferrin, pH 5-7(cathode at top): "Fast", "Fast" and "Slow", "Slow" left to right.

Fig. 4. Disc gel electrophoresis of rat transferrin, pH 8.6. (Cathode at Top) "Slow", "Slow" and "Fast"; "Fast" components. (Left to Right)

Molecular weight determination: The molecular weight of each component was measured by sedimentation equilibrium to determine whether the observed heterogeneity of rat transferrin might be due to aggregation. It has been shown that frog and turtle hemoglobins produced multiple bands on electrophoresis as a result of aggregation (4). Both rat transferrin components were homogeneous during ultracentrifugation and each has an apparent molecular weight of 72,000 daltons.

Since rat transferrin has a high molecular weight and since it has two equivalent iron-binding sites and also two identical carbohydrate chains, it might be expected to have subunit structure. The existence of subunit structure was explored by electrophoresis in polyacrylamide containing sodium dodecyl sulfate, which is known to dissociate polymeric proteins into their monomers. Each rat transferrin component showed a single band by this procedure with an Rf value corresponding to 76,000 daltons (Fig. 5).

Amino acid analyses: The possibility that the observed heterogeneity may be due to differences in the amino acid compositions of the two transferrins was considered. The amino acid composition of each component was determined. The data reported in Table II are the averages of three determinations. It is obvious that the amino acid compositions of the two transferrins are almost identical, within limits of the accuracy of the method.

Specificity of rabbit antisera against rat transferrin: the specificity of rabbit antisera against rat transferrin was tested by immunoelectrophoresis on cellulose acetate membrane. Results indicated that the antisera were specific for rat transferrin (Fig. 6). Only one precipitin band was observed when rabbit antisera against rat transferrin were reacted with whole rat serum (a), or with purified (e) or crude (d) transferrin preparations. More than one precipitin band was observed when rabbit antisera against whole rat serum were reacted with whole rat serum (b) or with crude preparation of rat transferrin (c).

Fig. 5 SDS polyacrylamide gel electrophoresis of "Fast", "Slow" rat transferrin components, bovine serum albumin (M.W.=68,000) ovalbumin (M.W.=43,000) and aldolase (M.W.=40,000) left to right, cathode at top.

Table II

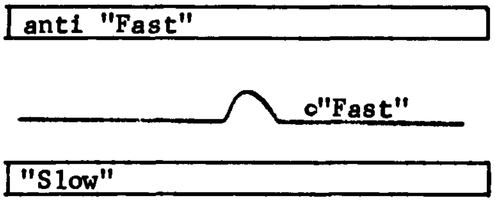
Amino Acid Composition of the Rat Transferrins

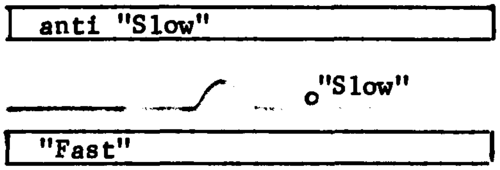
	"Fast"	"Slow"
Lysine	55.4 (0.4)	55.6 (0.1)
Histidine	17.4 (0.3)	17.2 (0.3)
Arginine	25.7 (0.5)	25.8 (0.5)
Aspartic Acid	69.6 (0.2)	70.6 (0.3)
Threonine	36.7 (0.9)	36.3 (0.9)
Serine	41.9 (0.5)	42.9 (0.3)
Glutamic Acid	60.5 (0.9)	60.7 (0.8)
Proline	35.3 (0.1)	35.8 (0.4)
Glycine	58.4 (0.5)	58.4 (0.4)
Alanine	53.4 (0.8)	55.5 (1.2)
Cysteine	16.5 (0.4)	17.1 (0.7)
Valine	38.7 (0.3)	39.7 (0.3)
Methionine	4.5 (0.1)	4.7 (0.1)
Isoleucine	20.6 (1.0)	21.0 (0.3)
Leucine	60.5 (0.2)	61.4 (0.7)
Tyrosine	20.4 (0.8)	21.0 (0.8)
Phenylalanine	34.5 (1.0)	34.7 (1.3)
Tryptophan	8.8 (0.1)	8.9 (0.0)

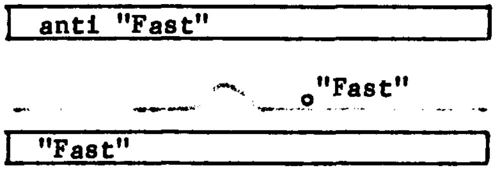
Values are expressed as residues per molecular weight of 72,000 daltons. Average deviations from 4 determinations are given in parenthesis.

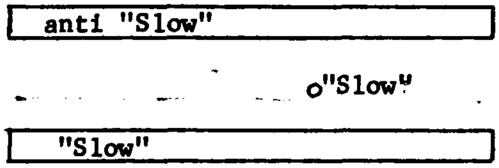
Fig. 6 Immunoelectrophoresis on cellulose acetate membrane. (a) rabbit antisera to rat transferrin against whole rat serum; (b) rabbit antisera to whole rat serum against whole rat serum; (c) rabbit antisera to whole rat serum against crude preparation of rat transferrin; (d) rabbit antisera to rat transferrin against crude rat transferrin; (e) rabbit antisera to rat transferrin against purified rat transferrin.

<u>Immunochemical properties of rat transferrin:</u> Immunochemical properties of the rat transferrin components were compared by immunoelectrophoresis on cellulose acetate membrane. Results indicate that the two rat transferrins have identical or very similar antigenic determinants (Figure 6). The experiment was set up as follows: (Results are traced in red)









In all cases there was identity shown between the two rat transferrin components since the lines fused.

Figure 6. Immunoelectrophoresis of rat transferrins by the Osserman modification. Only experimental results of a and b are shown.

RESULTS

(B) Biosynthesis of rat transferrin: The site of biosynthesis of rat transferrin has been studied and the results are reported in Fig. 7. The results indicate that the maximum radioactivity (biosynthesis of transferrin) occurred at about 30 mins. in the rough microsomes and at about 40 mins. in the smooth microsomes. Therefore, transferrin moves from the rough to the smooth microsomes during the course of its synthesis. Very little H^3-transferrin was found in the nuclear and mitochondrial fraction, and also the supernatant fraction. This may be due to some contamination of these fractions with microsomes.

The time it takes for the transferrin to be secreted into the medium was studied by filtering the incubated liver slices and collecting the filtrate and adding specific antisera. The results are shown in Fig. 8. Transferrin was found to be released into the incubating medium after a minimum period of 30 min. The amount of transferrin released increased linearly with time up to 60 min.

The incorporation of iron into transferrin: The incorporation of iron into transferrin was studied by incubating liver slices with ^{59}Fe for 5 mins. and "chased" in "cold" iron for different time periods. Each fraction was precipitated with specific antisera. The results in Table III showed that there was no difference between the control, which consisted of chicken serum precipitated with antichicken sera, ahd the sample, which was precipitated with specific sera to rat transferrin, at any time point during the experiments.

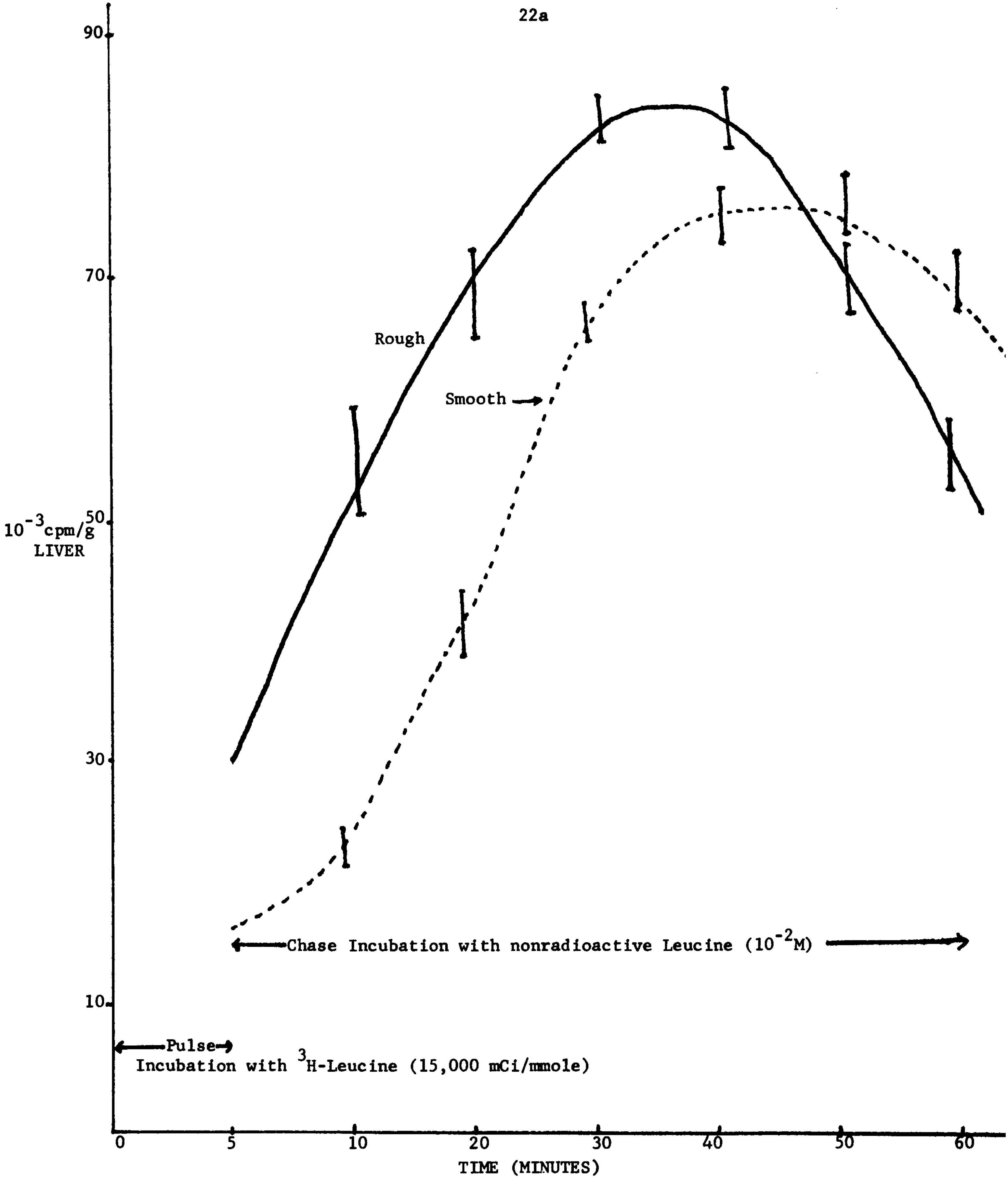


Fig. 7 Time course of appearance of ^{3}H-leucine in transferrin in Rough and Smooth Microsomes

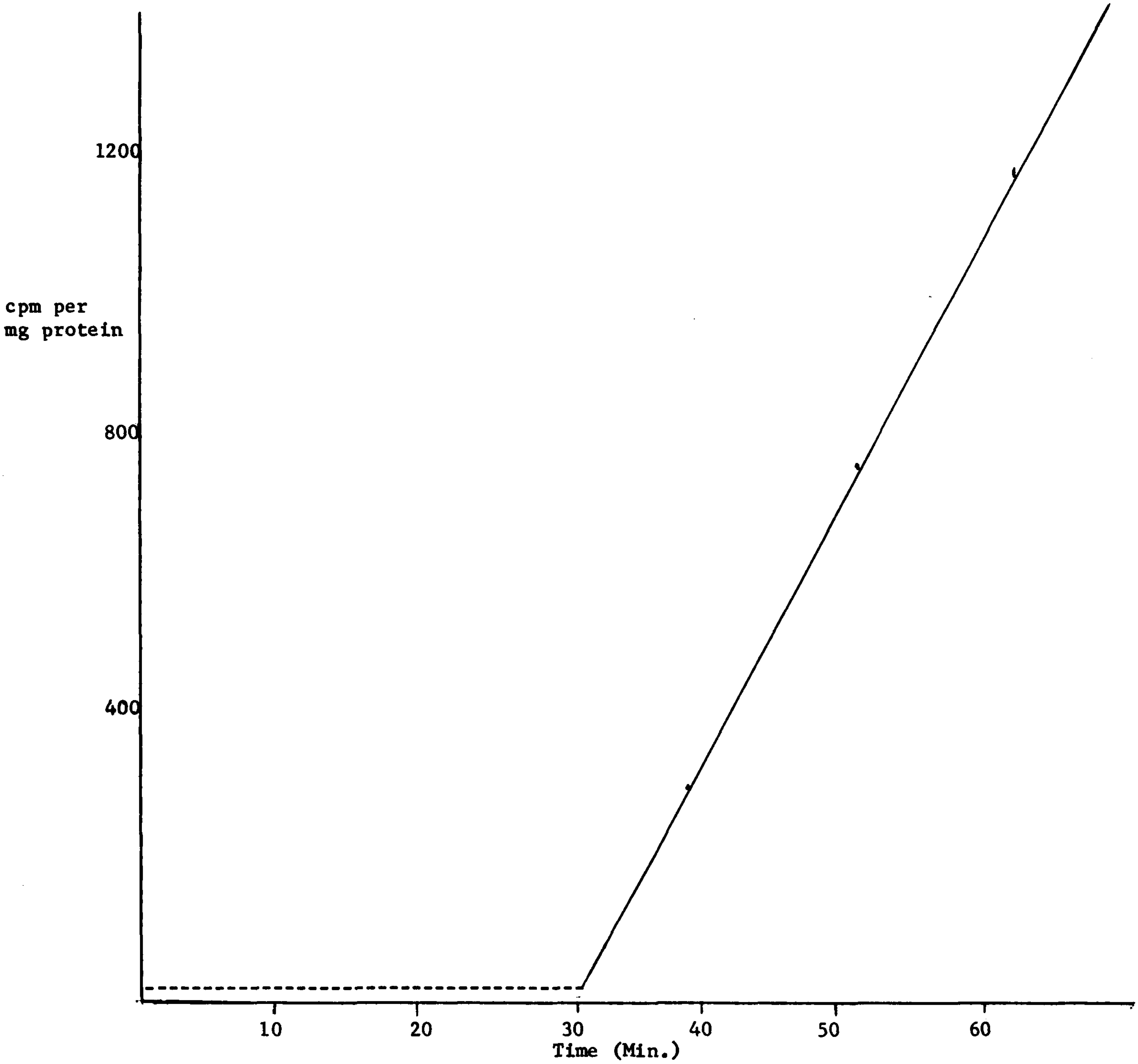


Fig. 8 Time course of appearance of ^{3}H-leucine in transferrin in incubating medium

Table III

Incorporation of ^{59}Fe Cl_3 into microsomal Transferrin

Time (Mins.)	Control (cpm/mg) Protein	Sample (cpm/mg) Protein
10	90	94
20	101	98
30	88	95
40	112	120
50	140	152
60	104	111

DISCUSSION

(A) Preparation and Properties of Rat Transferrins

The method of isoelectric focusing employed in this study yields two forms of transferrin from rat serum in good yield and with a minimum of effort. Two methods have been employed. At first, rat serum, saturated with ^{59}Fe, was subjected to a preliminary separation by means of a very large column of Sephadex G-100 equilibrated with 5×10^{-2}M sodium phosphate, 1×10^{-1}M NaCl, pH 7.4. Fractions containing the radioactive iron were pooled, made 50% saturated with ammonium sulfate and centrifuged. The pellet was dissolved and dialyzed against 5×10^{-3}M tris-glycine buffer, pH 8.6. The sample was then subjected to isoelectric focusing along a sucrose gradient and a pH gradient from 5.0 to 7.0. Each of the salmon-pink transferrin components could easily be removed in a pure state. In later studies, instead of subjecting the sample to column chromatography, it was pre-fractionated on the isoelectric focusing column, each transferrin separated and each fraction subjected again to the same procedure. In both instances, the two proteins were found to be pure.

The major ("slow") transferrin has an isoelectric point of 5.75 whereas the minor ("fast") transferrin, which represented one-third of the total, had an isoelectric point of 5.50, illustrating the remarkable sensitivity of this method. As has been found in other instances of multiple forms of proteins (36, 37), the amino acid analyses of the two forms of transferrin revealed no apparent differences in amino acid content. This finding was further emphasized by the fact that both transferrins react to a rabbit antibody prepared against one of them in an identical fashion, suggesting similar or identical antigenic determinants. Similar results were reported for hen's egg ovotransferrins by Wenn and Williams. (5).

Determination of the iron content of the two forms of rat transferrin revealed that the major component contained 1.39-1.41 μg Fe/mg, equivalent to 1.8 atoms Fe per 72,000 gm of protein (90% saturation). The minor component contained 1.47-1.50 μg Fe/mg protein, equivalent to 1.90 atoms of Fe per mole of protein (95% saturation). Direct determination of the extent of iron-binding of the transferrins revealed 5% unsaturation for both. Gordon and Louis (19) reported that the "slow" rat component of rat transferrin contained 1.4 μg Fe/mg protein, which represents 83% saturation of iron per 68,000 gm of protein. Wenn and Williams (5) reported 1.80 atom Fe/80,000 gm of the major ovotransferrin, which could be raised to 1.96 after fully saturating the protein with iron. None of these workers determined the iron content of the minor ("Fast") component.

After subjecting the rat transferrin components to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, each transferrin migrated as a single component with a Rf value corresponding to 76,000 daltons. Thus it can be concluded that rat transferrins have no subunit structure. Determination of the molecular weights of each of the rat transferrin components yielded a value of 76,000 compared with 68,000 for rat transferrin (6) and 68,000-78,000 for human transferrin prepared by other workers (6,7,8).

Recent studies by Palmour and Sutton (8) reported a molecular weight of 44,000 for hagfish transferrin and a binding capacity of one atom of iron per mole. These workers reported a molecular weight of 76,000 and a binding capacity of two iron atoms per mole for transferrins from human, rabbit and frog. Turtle transferrin was found to have a molecular weight of 92,000 and a binding capacity of two atoms of iron per mole. Sedimentation of reduced and carboxymethylated transferrin in 8 M urea indicated a possible subunit structure for turtle transferrin. No subunits were detected in any of the other transferrins studied. These data have led to the suggestion that a partial gene duplication with subsequent fusion has occured, resulting in an elongated protein with some repeating sequences. Ohno (38) reported that lamprey erythrocytes contain twice as many chromosomes as hagfish erythrocytes, suggesting that the whole genome may have duplicated early in vertebrate evolution.

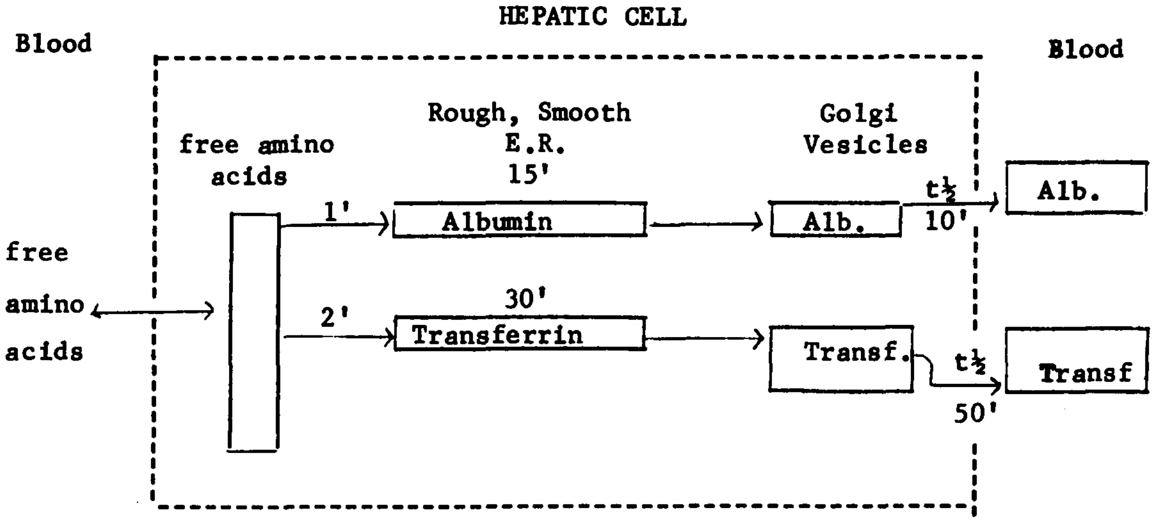
Another possible explanation for two forms of transferrin involves the conversion of one form to another within the cell by the action of some enzyme, a phenotypic expression of the cell. One amino acid side chain might be altered, e.g. conversion of - Co NH_2 to - COOH by a deamidase, or the addition or subtraction of a sugar residue carrying a charged group, e.g. sialic acid.

A detailed study of the amino acid sequences of multiple transferrins will be required in order to determine minor differences between the two transferrin components.

(B) Biosynthesis of Transferrin

Many of the serum proteins are synthesized in the liver cell. Using serum albumin as an example of a protein synthesized for export from the cell, and ferritin as an example of a protein synthesized for use within the cell, Redman (39) arrived at the generalization that proteins synthesized for export are put together on ribosomes attached to the endoplasmic reticulum (bound polysomes), whereas proteins made for use within the cell are synthesized on "free" ribosomes (unbound polysomes). After synthesis of the protein on the membrane-bound ribosomes, the protein moves inside the endoplasmic reticulum (40, 41, 42, 43, 44, 45, 46). The secretory pathway for a particular serum protein---transferrin, has been studied in the present studies. The results indicated that the maximum radioactivity of transferrin occurred at about 30 min. in the rough microsomes and at about 40 min. in the smooth microsomes. Transferrin was first found in the medium after a minimum period of 30 min.

Morgan and Peters (47) reported that the secretion of newly synthesized rat transferrin requires a minimum time of about 30 min. and an average of about 80 min. They compared the rate of biosynthesis of rat albumin and rat transferrin, both of which are serum proteins. They found that the time of the maximum rate of appearance of C^{14} in protein lags behind the peak in C^{14}-leucine by 1 min. for albumin and 2 min. for transferrin. Thus they suggested that it takes about 2 min. to form a molecule of transferrin in contrast to 1 min. for a molecule of albumin. They also found that the maximum C^{14} activities were reached at about 30 min. for microsomal transferrin and 16 min. for microsomal albumin. Their results are summarized in the following diagram:



These workers did not fractionate microsomes into rough and smooth as was done in the present studies.

Transferrin is a glycoprotein whereas albumin is not. Various workers have reported that the first sugar, N-acetylglucosamine, is incorporated while the nascent protein is still attached to the polysomes. The second and the third sugars, mannose and galactose, are incorporated after the newly formed protein has left the polysomes and is on the intracellular pathway which leads to secretion (48, 49, 50, 51, 52, 53, 54). Recently, Redman and Cherian (55) reported that N-acetylglucosamine and mannose are incorporated in the rough endoplasmic reticulum, and that mannose is incorporated immediately after the nascent protein is released from the polysomes but while the glycoprotein remains attached to the membrane of the endoplasmic reticulum. They also found that albumin passes directly from the attached polysomes to the cisternae of the rough endoplasmic reticulum without remaining attached to the membrane of the RER as in the case of serum proteins. These findings may explain why Morgan and Peters, (47) found that it takes longer for transferrin to appear in the blood than albumin, yet both serum proteins follow the same pathway for secretion.

Since the function of transferrin is to act as a transport agent for plasma iron, it seemed likely that the newly formed intracellular transferrin in the liver cell would not pick up any iron from the cell. This indeed was found to be true in the present studies despite the fact that there is iron within the liver cell in several forms, e.g., heme-iron bound to heme proteins, ferritin and an unidentified low molecular weight iron complex which may be Fe-ATP(Mazur, unpublished). These results can be explained in two ways. First, the newly synthesized transferrin is always bound within vesicles during its secretory pathway and the tyrosyl residues which are believed to bind the iron are blocked. Second, the completed glycoprotein (transferrin) may be the only form which can bind iron and this completed protein occurs only inside the golgi membranes and is therefore unavailable for iron binding until it moves into the plasma.

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II. The Preparation and Properties of Modified Hemoglobins

INTRODUCTION

Much effort has been employed and many synthetic compounds have been tested in an attempt to find an adequate substitute for whole blood in the treatment of acute clinical conditions resulting from massive loss of blood, e.g., traumatic injury and hemorrhagic shock, burns, and for an external circuit as is required in open-heart surgery. The design of these substitutes is aimed at maintaining osmotic activity for proper water and electrolyte balance, and to maintain adequate kidney function. These "plasma expanders" have proven to be of very limited usefulness. Instead, plasma or the albumin fraction prepared from pooled plasma has proven to be much more useful. Such materials are available from donor blood which has "outlived" its shelf-life of 21 days because of a deterioration of the red cells. Because plasma obtained from out-dated blood can be utilized efficiently, this leaves the red cells as the only fraction which needs to be discarded in a well-run blood bank, although rare type red cells can be preserved for future use.

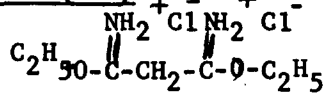
The use of hemoglobin, prepared from red cells, as a plasma expander has been investigated in a number of laboratories (1,2,3,4). Despite poor preparations which revealed coagulation-promoting properties due to the presence of red cell stroma (5,6,7,8,9), Rabiner (10,11) has succeeded in preparing hemoglobin solutions which are non-toxic, and which carry oxygen reversibly. However, it has one property which reduces its potential usefulness; it has a half-life (in the dog) of only 4-5 hours in the blood stream. The hemoglobin binds to the serum protein haptoglobin and this complex is removed by the reticulo-endothelial cells of the liver and spleen and the remainder, after binding to all of the serum haptoglobin, is excreted via the kidneys. The removal of hemoglobin by the kidneys is probably due to the fact that the tetrameric molecule dissociates into dimers (M.W.=34,000) and monomers (M.W.=17,000), and these can be removed by the kidneys.

The object of this study was to synthesize a polymeric form of hemoglobin by use of a bifunctional coupling agent forming molecules of high molecular weight which are incapable of dissociation and excretion by the kidney at a rapid rate.

EXPERIMENTAL

Materials: Malononitrile was purchased from Eastman Kodak company. Sephadex was purchased from Pharmacia Fine Chemicals. All other reagents were purchased from Fisher Scientific Company. Millipore filters and apparatus for sterile filtration were obtained from Millipore Company.

Preparation of diethylmalonimidate dihydrochloride (DEM):

Diethylmalonimidate dihydrochloride (DEM)  $C_2H_5O-\overset{NH_2^{+}Cl^{-}}{\overset{\|}{C}}-CH_2-\overset{NH_2^{+}Cl^{-}}{\overset{\|}{C}}-O-C_2H_5$ was prepared according to McElvain and Schroeder (12). 13.2g of malononitrile and 9.2 g of dry ethanol were dissolved in 240 ml of dry dioxane. The solution was cooled in ice and then treated with dry hydrogen chloride gas which was generated by adding concentrated H_2SO_4 to solid NaCl, and passing through concentrated H_2SO_4. Extreme care was taken to protect the entire system at all stages from moisture to avoid hydrolysis of the imidoester. The resulting DEM was allowed to precipitate overnight at 0°C since imidoesters decompose at higher temperatures. DEM was filtered,washed with dry ether and then dried in a dessicator over NaOH. The dessicator was stored at -20°C.

Preparation of hemoglobin solution: Hemoglobin was prepared according to Rabiner, et al.(10,11) . Fresh or out-dated human or rabbit blood was centrifuged at 2,000 rpm for 10 min. to obtain red blood cells (RBC). These were washed three times with 1.6% NaCl. The washed cells were lysed with 4 volumes of 5 ideal milliosmolar phosphate buffer, pH 7.4, for each volume of packed RBC. The mixture was mixed gently and thoroughly by inverting the flask repeatedly. After standing for 3-60 min. at 0°C, the mixture was centrifuged at 16,000 g for 90 min. at 5°C. Supernatant was removed and recentrifuged at 35,000 g for 90 min. in a Sorvall centrifuge. The hemoglobin solution was concentrated in dialysis tubing, by packing with Sephadex G-100, to produce a solution containing 10-12% hemoglobin. A portion of the hemoglobin solution was adjusted to pH 7.4 and dialysed with many changes against 310 mOsm sodium phosphate buffer, pH 7.4, for use as a control in *in vivo* experiments. The remainder of the hemoglobin was used to prepare cross-linked hemoglobin.

Preparation of cross-linked hemoglobin: 2.4 g of DEM (diethylmalonimidate dihydrochloride) were added to 5 ml of 0.05 M sodium phosphate buffer, pH 8.5 in a beaker at 5°C containing a magnetic stirrer. 5N NaOH was added dropwise until the solution became milky. 50 ml of hemoglobin solution (10-12%) were added and the pH was adjusted to 8.5 with 5N NaOH. When the pH was very close to 8.5, 1N NaOH was used instead. The reaction was allowed to proceed for 1-1½ hrs.and the pH maintained at 8.5 with 1N NaOH. After the reaction was over, the volume of the solution was measured and an equal volume of saturated ammonium sulfate was added to give a solution 50% saturated with ammonium sulfate. The mixture was centrifuged and the pellet dissolved in distilled H_2O. An equal volume of saturated ammonium sulfate was again added. The pellet obtained was dissolved in 310 mOsm sodium phosphate, pH 7.4, and dialysed with many changes against the same buffer. Any insoluble material present was removed by centrifugation at 35,000 g.

Infusion of hemoglobin solutions: Untreated hemoglobin solutions as well as the cross-linked hemoglobin solutions were filtered through a 0.2µ millipore filter under sterile conditions to remove any stroma. 10 ml of blood were removed from the recipient rabbit ear vein. Hemoglobin solution was infused via an infusion pump (Harvard Apparatus Company) at a rate of about 0.8 ml/min. Blood samples were collected in 1 ml of heparin — saline 5 min. after infusion was completed. Blood samples were collected in the same manner thereafter at one hour intervals.

Determination of the half-life of hemoglobin in the plasma: Blood samples were centrifuged, plasma removed, treated with CO, and read at 418 mμ in the spectrophotometer. Hemoglobin values were expressed as percentage of the 5 min. sample after absorption of the control sample (obtained before infusion) was subtracted.

Viscosity measurements: The viscosity of the normal and the cross-linked hemoglobin solutions was determined with a viscometer suspended vertically in a constant temperature water bath (Lapine Scientific Company) maintained at ± 0.05°C. An accurate volume (10 ml) of the hemoglobin solution was introduced with a pipet. After temperature equilibrium was reached, the solution was sucked up to a point above the upper mark and then allowed to flow. The time elapsed between the passage of the meniscus from the upper to the lower mark of the viscometer was measured with a stopwatch. At least four determinations were made which agreed within 0.4 second.

Measurement of the binding of hemoglobin to haptoglobin: The binding of normal and cross-linked hemoglobins to haptoglobin was compared by the method of Grunbaum, (13) and Valeri, (14) using a Microzone Cell (Beckman Instrument Company). Known amounts of rabbit hemoglobin were added to a fixed amount of rabbit serum and incubated at 37°C for 30 min. Electrophoresis was conducted at a constant voltage of 150 volts for 40 min. on cellulose acetate membranes, using 0.05 M sodium phosphate buffer, pH 7.0. Each membrane was then stained for 5 min. in fresh stain reaction mixture containing 20 mg o-dianisidine (Eastman Kodak Company) dissolved in 50 ml ethanol. 30 ml of pH 4.7 acetate buffer and 0.2 ml of 30% hydrogen peroxide were added and the solution was allowed to equilibrate for 5 min. The membrane was washed in running tap water for 5 min. and then in 5% acetic acid for 20 min.

Densitometer tracing of gels and cellulose acetate membrane: A Joyce-Loebl recording densitometer was used for scanning gels as well as for cellulose acetate membrane used for electrophoresis.

Amino acid analysis of hemoglobins: Hemoglobin samples of 1 mg/ml were hydrolysed in 6N HCl at 110° for 20-24 hrs. The hydrolysis tubes were frozen in acetone and dry ice, and evacuated under nitrogen with a water aspirator prior to sealing. Analyses were performed using a Beckman Model 116 Amino Acid Analyzer according to Moore and Stein (15). All buffers, reagents and resins used in these analyses were purchased from Beckman Instrument Company.

Molecular weight determination: A specimen of cross-linked hemoglobin was subjected to centrifugation in the Model E Analytical Ultracentrifuge by the method of sedimentation equilibrium. The sample was run at a concentration of 0.15 mg/ml at 8,000 rpm at 20° overnight. A solvent density of 1.000 was used and a partial specific volume of 0.75 was assumed (16).

Attempts to form anti-hemoglobin antibodies: Two kinds of immunization procedures were tried using normal rabbit hemoglobin or modified rabbit hemoglobin (polyhemoglobin). In the first method the hemoglobin was mixed with Freund's complete adjuvant and the mixture injected into the back and footpads during the first week at a level of 0.3 mg/ml of protein and mixed with Freund's incomplete adjuvant and injected at levels of 0.5 mg/ml and 1.0 mg/ml during the second and fourth week. Blood specimens were taken on the fifth week for antibody testing.

The second method involved the intravenous injection of 40 mg hemoglobin per kg of body weight on alternate days for one week and blood removed for testing on the ninth day.

Determination of Sedimentation Constant (S): Unreacted rabbit hemoglobin and cross-linked rabbit hemoglobin were subjected to centrifugation in the Model E Analytical Ultracentrifuge by the method of sedimentation velocity to determine the Sedimentation Constant (S). The samples were run at a concentration of 0.5 mg/ml at 32,000 rpm at 20° for 4 hours. A solvent density of 1.000 was used and a partial specific volume of 0.75 was assumed (16).

Coagulation Studies: To study the effect of hemoglobin solutions on the clotting of intact plasma, either 0.1 ml of hemoglobin solution or saline (control) was added to 0.1 ml of normal intact plasma in plastic tubes. The test tube was gently mixed, placed in a 36° C water bath and 0.1 ml of 0.1M $CaCl_2$ was added. At 1-min. intervals the test tube was picked up and tilted very gently to note clot formation. Results give the recalcification time.

Experiments on the thrombin time were performed similarly to that described for recalcification time, except that 0.1 ml of 2μ/ml thrombin instead of $CaCl_2$ was added to 0.1 ml of hemoglobin solution or saline (control).

Spectral Studies: The spectra of normal hemoglobin and phoyhemoglobin were taken using a Cary 15 Spectrophotometer.

RESULTS

Preparation of cross-linked hemoglobin: Since hemoglobin solutions have been found by Rabiner (10 11), to be an ideal plasma expander, attempts were made in the present study to prolong the half-life of hemoglobin in the circulation to augment its value as a plasma expander. A bifunctional reagent, diethylmalonimidate dihydrochloride (DEM) was used in this study to produce cross-links between different tetrameric hemoglobin molecules so as to yield a high-molecular weight polymer. The synthesis of DEM and its reaction with the protein are summarized in Fig. 1:

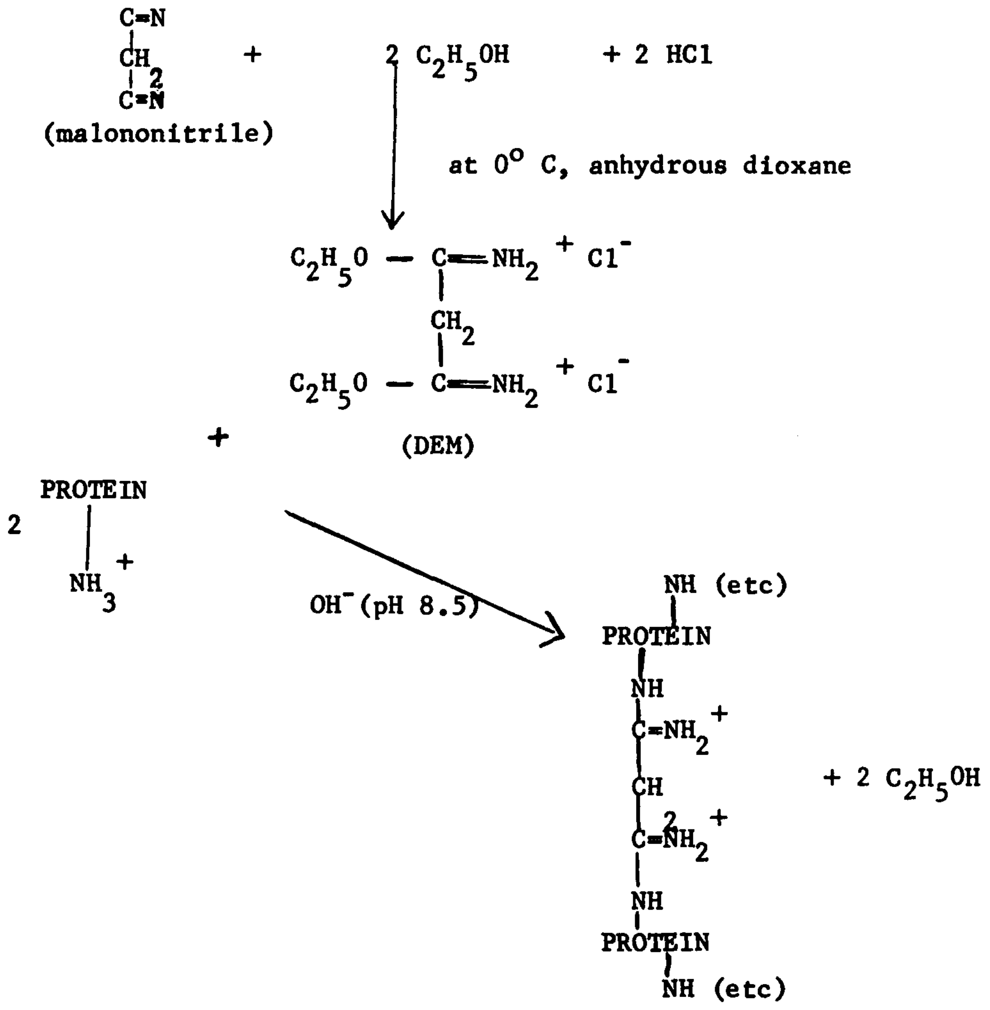


Fig. 1

Disc gel electrophoresis: Disc gel electrophoresis was performed to study the nature of the reaction products resulting when a 10-12% hemoglobin solution was reacted with DEM. Fig. 2 indicates that the products were very heterogeneous. The cross-linked hemoglobin showed up as bands near the top of the gel and moved very slowly in comparison to the normal hemoglobin. There was some unreacted hemoglobin which moved as did the control normal hemoglobin.

Amino acid analysis of hemoglobins:DEM is a bifunctional reagent that is known to react specifically with the lysyl residues of the protein. Amino acid analyses of the normal as well as the cross-linked hemoglobins were performed. The extent of the reaction of hemoglobin with DEM was determined by measuring the number of free lysyl residues present in comparison to that found in the unreacted hemoglobin. Data reported in Table I indicates that about half of the lysyl residues were free, the other half of the lysyl residues appeared as a derivative in a new peak near the neutral amino acids (Fig. 3). There were no significant differences for the other amino acids between the normal and the cross-linked hemoglobins (Table I).

SDS-polyacrylamide gel electrophoresis: Free hemoglobin has a half-life of 4-5 hrs. since it is permeable to the glomerulus and is excreted in the urine. It is not known why albumin, which has the same molecular weight as free hemoglobin, is not permeable to the glomerulus. Hemoglobin-haptoglobin complexes which have a molecular weight of 300,000, are not permeable to the glomerulus and appear to be sequestered by reticuloendothelial cells of the liver and spleen. The objective of the present study, therefore,was to prepare a hemoglobin derivative that had a high molecular weight and was relatively impermeable to the glomerulus. It has been suggested that free hemoglobin is permeable to the glomerulus only after it is dissociated into subunits (Lathem, 1959).

SDS polyacrylamide gel electrophoresis was performed to study whether hemoglobin cross-linked with DEM could be dissociated into subunits with SDS. Results shown in Figs. 4 & 5 indicate that the cross-linked hemoglobins moved as bands near the top of the gel and they were not dissociated in the presence of SDS. The bands corresponding to dimers and monomers were due to the dissociation of some small amount of unreacted hemoglobin in the presence of SDS, similar to the control using normal hemoglobin. Figs. 6 & 7 are the densitometer tracings of normal and cross-linked human hemoglobin, respectively.

<u>Determination of the half-life of the hemoglobins</u>: Since SDS-polyacrylamide gel electrophoresis indicated that cross-linked hemoglobin was not dissociated into subunits while normal hemoglobin was, it was of interest to determine the half-life of the high molecular weight hemoglobin in comparison to the normal hemoglobin. Both the normal and cross-linked hemoglobins were infused into different normal rabbits and the half-life was determined. Table II shows that the half-life of the cross-linked hemoglobin was longer than that of the normal hemoglobin in all the experiments performed. Depending on the dosage of the hemoglobin administered, the half-life of the cross-linked hemoglobin was 4-5 times or 10-15 times longer than the normal hemoglobin.

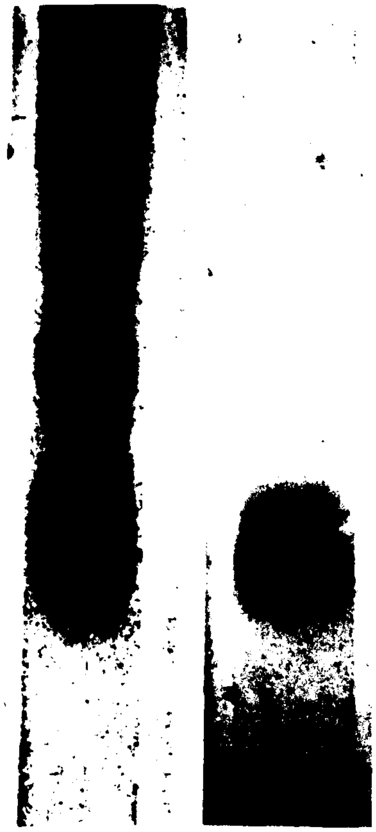


Figure 2a Disc gel electrophoresis of cross-linked and normal rabbit hemoglobins (left to right, cathode at top).

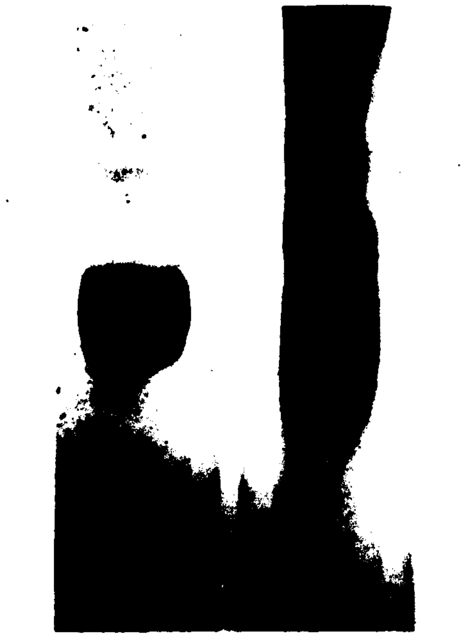


Figure 2b Disc gel electrophoresis of normal and cross-linked human hemoglobins (left to right, cathode at top).

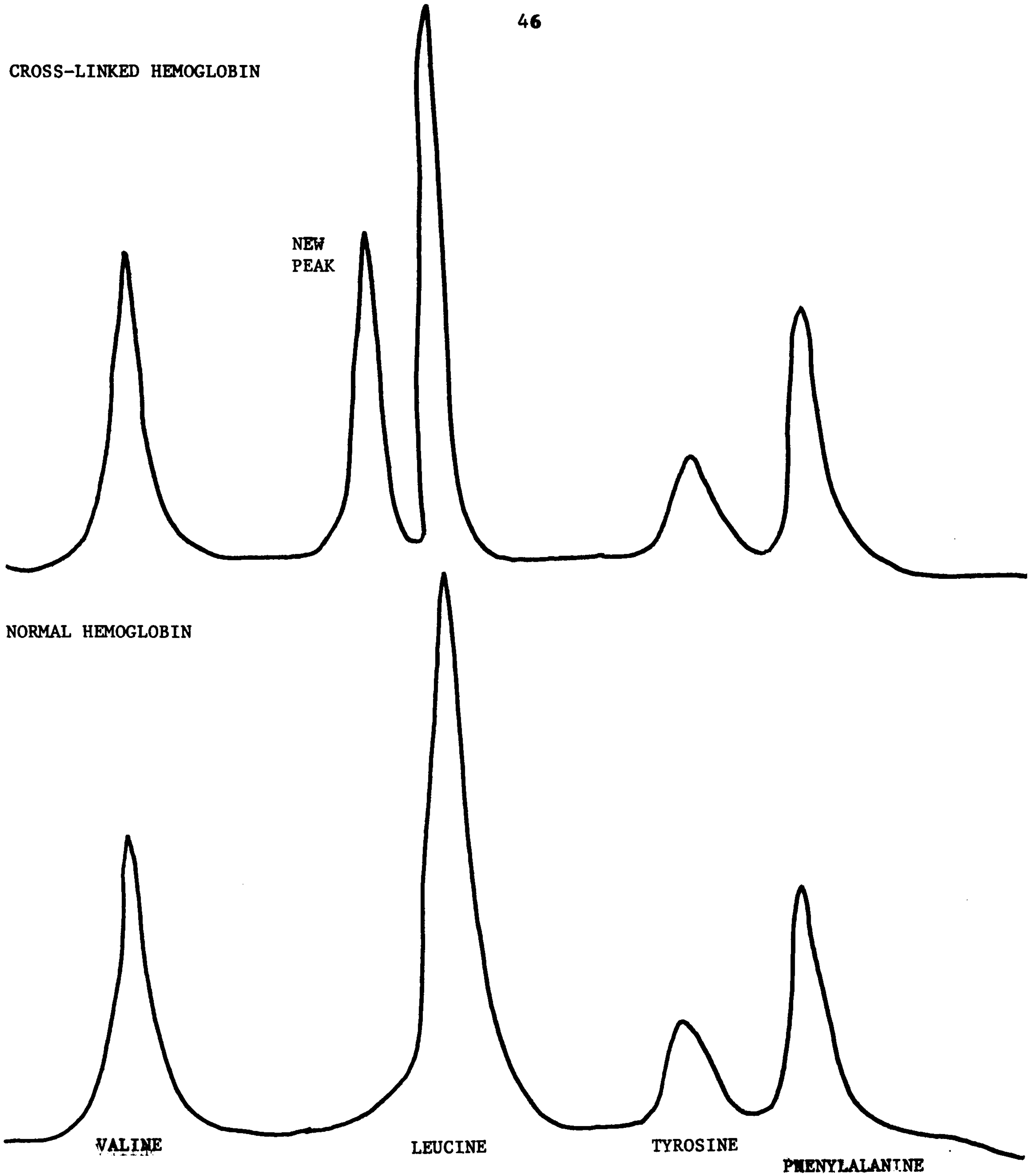


Figure 3. Ion-exchange chromatography of normal and cross-linked human hemoglobins on the Beckman Amino Acid Analyzer long column. (Not drawn exactly to scale)

Table 1 Amino Acid Composition of Normal and Cross-Linked Human Hemoglobins

	Normal	Cross-Linked
Lysine	46.4 (0.1)	19.1 (0.8)
Histidine	39.8 (0.6)	37.2 (0.5)
Arginine	13.6 (0.6)	12.9 (0.3)
Aspartic Acid	33.8 (0.8)	38.0 (1.8)
Threonine	33.2 (0.2)	33.3 (0.7)
Serine	34.0 (0.5)	31.2 (0.5)
Glutamic Acid	29.0 (0.4)	26.6 (2.1)
Proline	30.4 (0.3)	31.9 (0.1)
Glycine	43.7 (0.6)	44.3 (0.4)
Alanine	79.3 (0.6)	78.5 (0.7)
Cysteine	6.6 (0.3)	6.7 (0.1)
Valine	59.0 (0.9)	59.5 (0.7)
Methionine	6.6 (0.3)	6.7 (0.1)
Isoleucine	0.3 (0.3)	0.0 (0.0)
Leucine	78.9 (0.3)	80.7 (1.5)
Tyrosine	11.8 (0.4)	12.1 (0.1)
Phenylalanine	32.8 (0.2)	32.7 (0.1)
New Peak	0.0 (.0)	24.9 (0.1)

Number of lysine lost in cross-linked hemoglobin is approximately equal to number of new derivatives in the new peak.

Values are expressed as number of residues per total of 576 amino acid residues
Average deviations are given in parenthesis

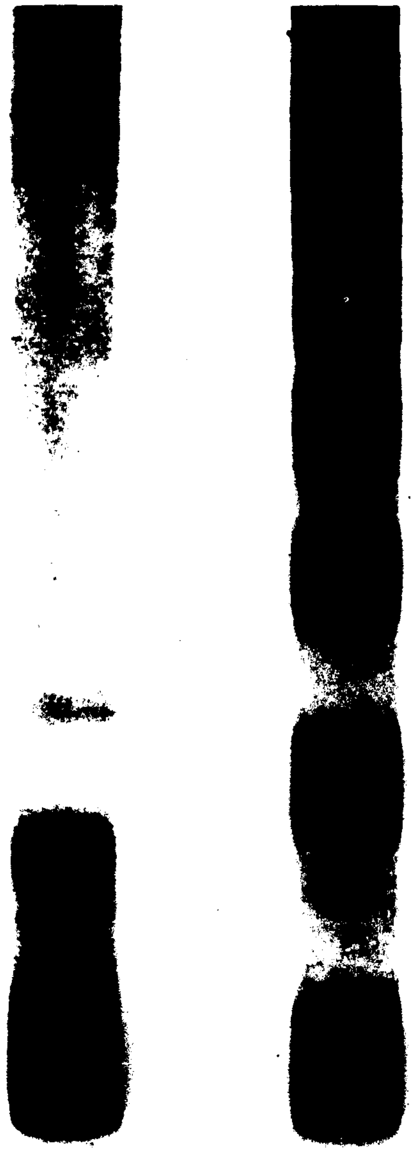


Figure 4. SDS polyacrylamide gel electrophoresis of normal and cross-linked human hemoglobins (left to right, cathode at top).

Figure 5. SDS polyacrylamide gel electrophoresis of normal and cross-linked rabbit hemoglobins (left to right, cathode at top).

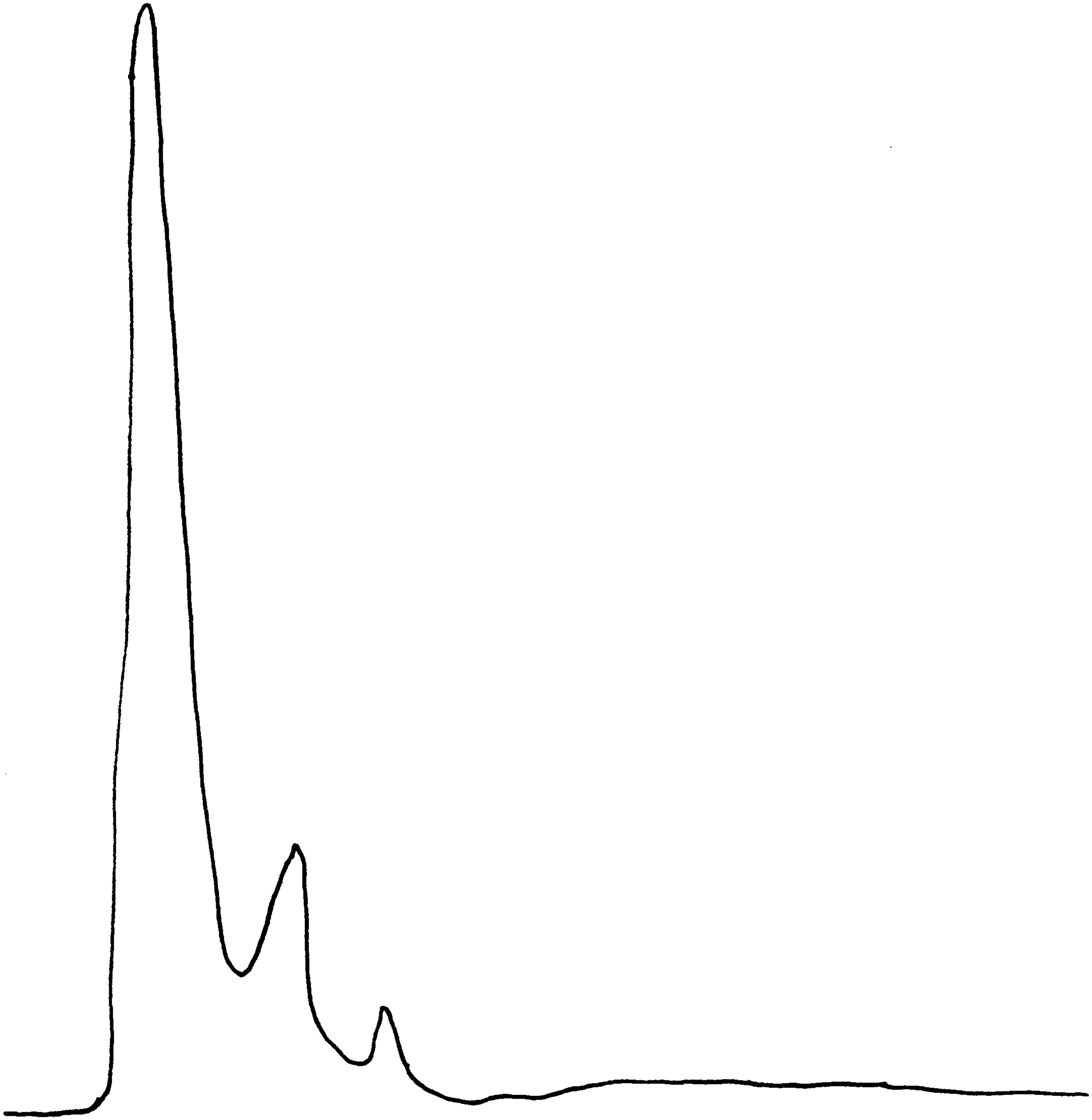


Figure 6. Densitometer tracing of normal human hemoglobin. (See Fig. 4)

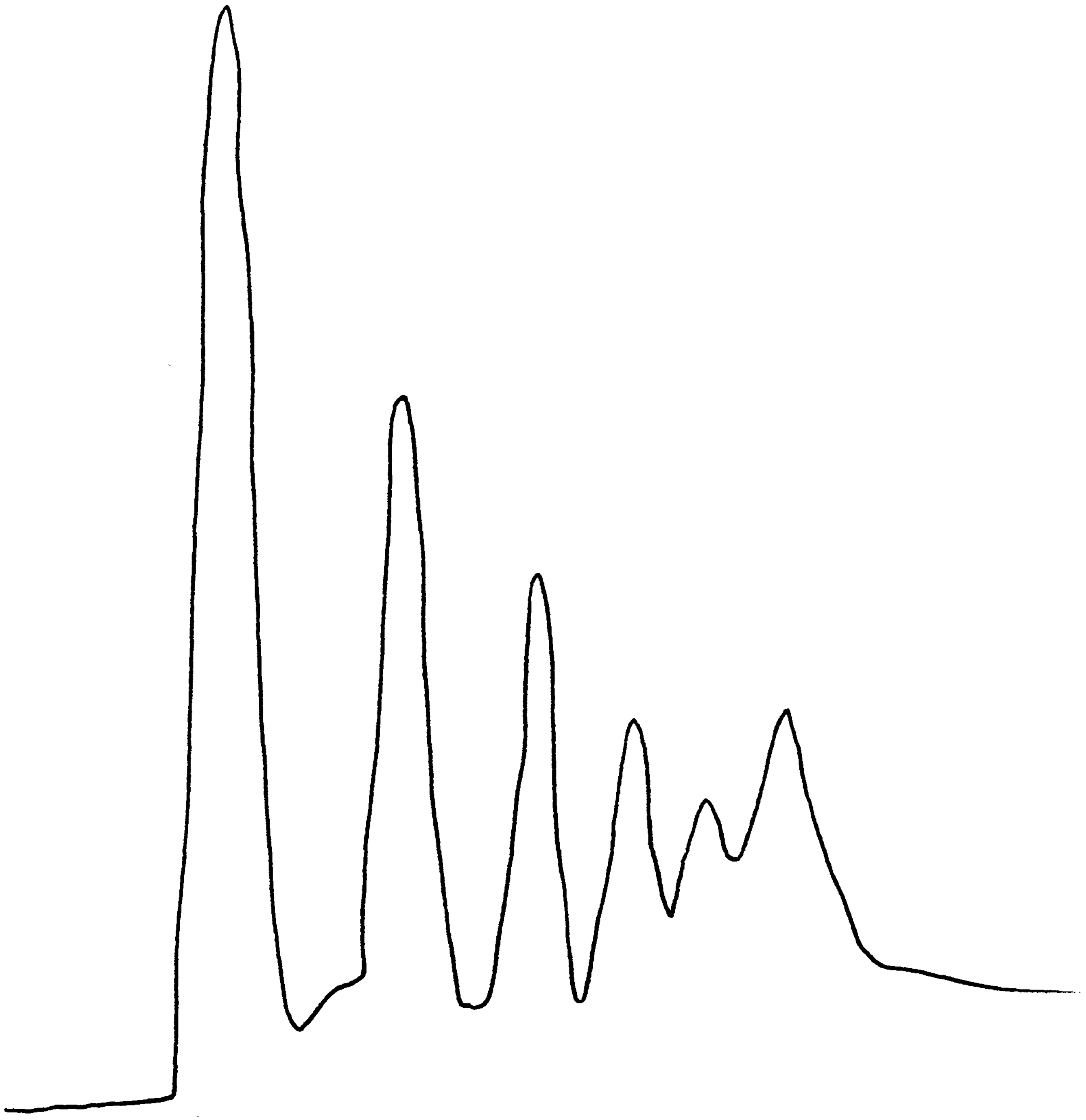


Figure 7. Densitometer tracing of cross-linked human hemoglobin (See Fig. 4)

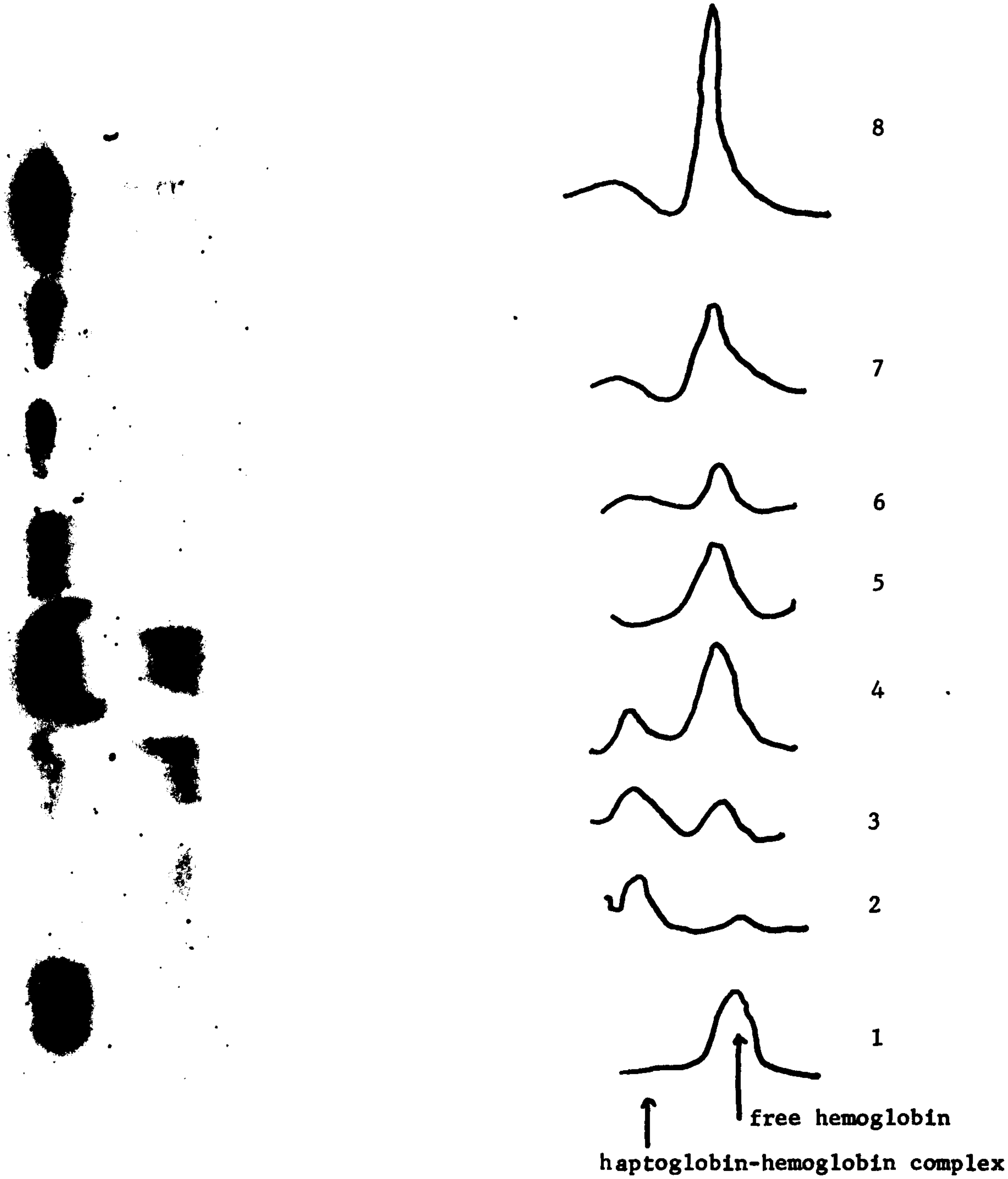


Figure 8. Cellulose acetate electrophoresis of plasma and hemoglobin: Samples 1 and 5 are normal rabbit normal and cross-linked hemoglobins respectively. Samples 2, 3 & 4 are normal hemoglobin in different concentrations with a fixed amount of plasma. Samples 6,7 & 8 are cross-linked hemoglobin in corresponding concentrations to samples 2,3 & 4 with the same fixed amount of plasma

Table II Half-Life of normal and cross-linked hemoglobins

	g/Kg	T½(min.)	g/Kg	T½(min.)	g/Kg	T½(min.
RABBIT HEMOGLOBIN INTO RABBITS						
NORMAL	0.137	80	0.134	55	0.5	85
Cross-linked	0.137	300	0.134	245	0.5	900
HUMAN HEMOGLOBIN INTO RABBITS						
NORMAL	0.27	80	0.42	110		
Cross-linked	0.27	900	0.42	1605		

Measurement of the binding of hemoglobin to haptoglobin: Hemoglobin is normally bound in the plasma by haptoglobin, which is a glycoprotein. The binding capacity of haptoglobin is on the average some 128 mg per cent. When this capacity is exceeded, free, unbound hemoglobin appears in the plasma and is excreted in the urine. The haptoglobin-hemoglobin complex which has a M.W. greater than 300,000, is not excreted (17). Electrophoresis on cellulose acetate membrane was performed to determine whether cross-linked hemoglobin has the ability to bind to haptoglobin. Fig. 8 indicates that the cross-linked hemoglobin binds haptoglobin to a much smaller extent than normal hemoglobin. When equal quantities of normal and cross-linked hemoglobin were added to a fixed amount of plasma, more free hemoglobin and less hemoglobin-haptoglobin complex was observed in the case of the cross-linked hemoglobin, while the contrary was observed for the normal hemoglobin.

Viscosity measurement: DEM cross-links different tetrameric hemoglobin molecules to produce a high molecular weight polymer. It is obvious that the physical properties of such a polymer are quite different from those of the normal hemoglobin molecule. The viscosity of the normal and the cross-linked hemoglobins was studied using a viscometer suspended in a constant temperature water bath. The densities of the hemoglobins and the solvent were measured by weighing these solutions in a 10 ml pycnometer. The viscosities were calculated using the following equation:

$$n_{relative} = \frac{t_{solution}}{t_{solvent}} \times \frac{d_{solvent}}{d_{solution}}$$

$n_{relative}$ =relative viscosity

t= time elapsed between passage of the meniscus from the upper to the lower mark of the viscometer

d= density

$n_{specific}$ =specific viscosity = $n_{relative} - 1$

It is clear from the results reported in Table III that the cross-linked hemoglobin has a higher viscosity than the normal hemoglobin.

Coagulation properties of hemoglobin solutions: The coagulation activities of the normal and cross-linked hemoglobin solutions were compared. The results are reported in Tables IV and V. It is clear from the results that the cross-linked hemoglobin solution gave a much longer recalcification time than the normal hemoglobin solution. The thrombin time for the cross-linked hemoglobin solution was also significantly higher than the normal.

Molecular weight determination: Most of the polyhemoglobin had a molecular weight above 300,000 with a variation between 350,000 to 400,000. Some 15% of the material had molecular weights between 400,000 and 800,000.

Absence of antibody production by polyhemoglobin: By use of either two methods, neither normal hemoglobin nor DEM-reacted polyhemoglobin of rabbits produced circulating antibodies in the rabbit.

Determination of Sedimentation Constant (S): The sedimentation constant (S) of unreacted rabbit hemoglobin was 4.31 s while that of the cross-linked rabbit hemoglobin was 34.6s.

Spectral Studies: The spectra of normal hemoglobin and polyhemoglobin are shown in Fig. 9. Both hemoglobins showed identical peaks at 412, 540 and 576 $m\mu$.

TABLE III

VISCOSITY OF NORMAL AND CROSS-LINKED RABBIT HEMOGLOBINS

	SPECIFIC VISCOSITY (^{n}specific)				
	65 mg/ml	32.5 mg/ml	16.25 mg/ml	8.125 mg/ml	0.216 mg/ml
NORMAL	0.262	0.131	0.071	0.051	0.005
CROSS-LINKED	0.894	0.330	0.161	0.095	0.029

TABLE IV Recalcification Time

		Time
CONTROL	0.1 ml Control human normal citrated plasma 0.1 ml saline 0.1 ml $CaCl_2$ (0.1 M)	3' 17"
NORMAL RABBIT Hemoglogin	0.1 ml Control human normal citrated plasma 0.1 ml normal rabbit hemoglobin 0.1 ml $CaCl_2$ (0.1 M)	4'6"
CROSS-LINKED Rabbit Hemoglobin	0.1 ml Control human normal citrated plasma 0.1 ml cross-linked rabbit hemoglobin 0.1 ml $CaCl_2$ (0.1 M)	30 min.

TABLE V Thrombin Time

CONTROL	0.1 ml Control human citrated plasma 0.1 ml saline 0.1 ml thrombin (2μ/ml)	33"
NORMAL Hemoglobin	0.1 ml Control human citrated plasma 0.1 ml normal rabbit hemoglobin 0.1 ml thrombin (2μ/ml)	31"
CROSS-LINKED Rabbit Hemoglobin	0.1 ml Control human citrated plasma 0.1 ml cross-linked rabbit hemoglobin 0.1 ml thrombin (2μ/ml)	44"

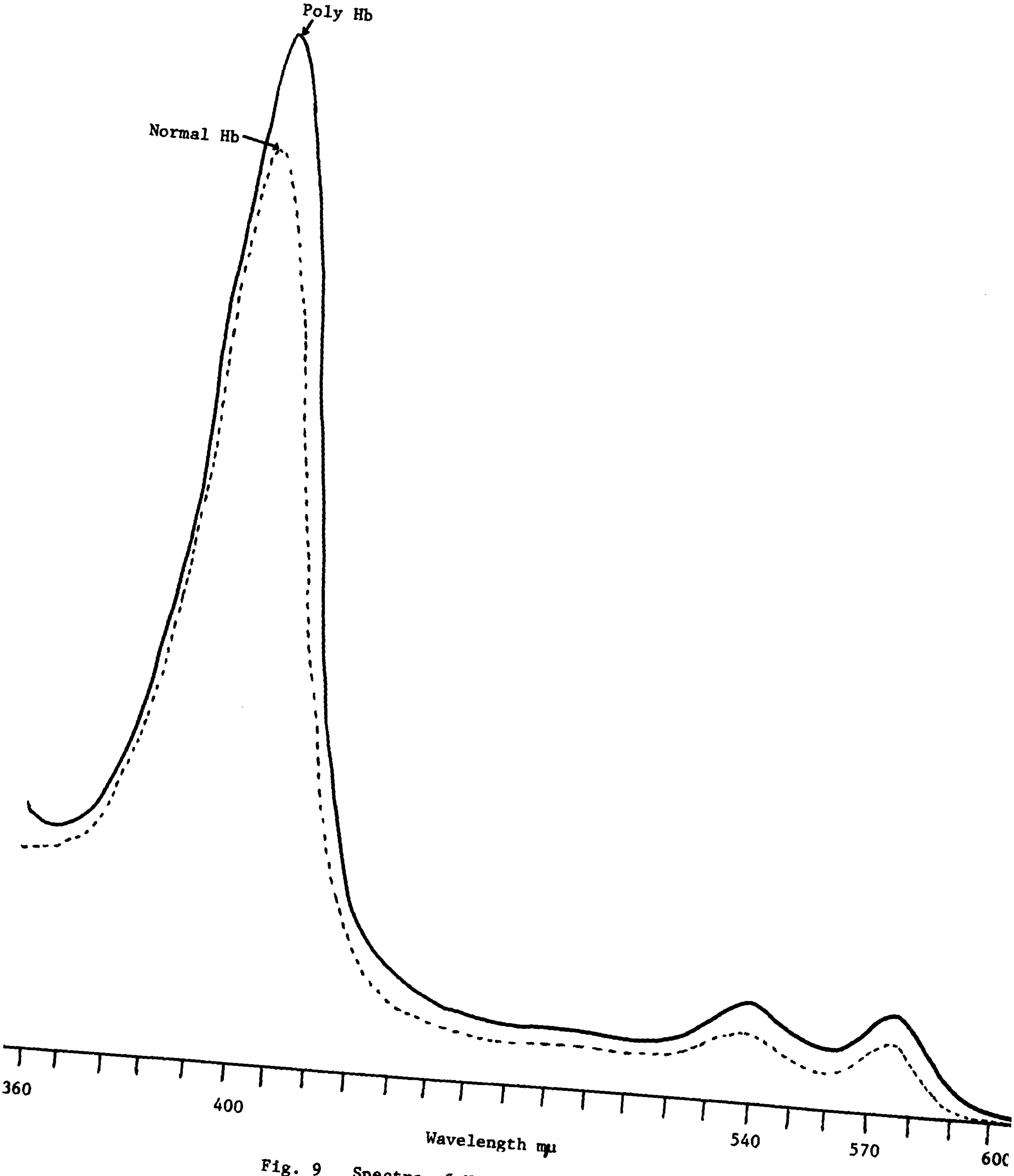


Fig. 9 Spectra of Normal and Poly Hemoglobins

DISCUSSION

Several workers (18, 19) were able to demonstrate that diethylmalonimidate could couple with the ϵ-amino groups of exposed lysyl residues to form protein-protein complexes. This reagent was used in the present study to produce a family of polyhemoglobins of high molecular weight.

This study has demonstrated that the reagent binds lysyl ϵ-amino groups to the extent of about one-half to two-thirds of the total present in hemoglobin. Following hydrolysis a new amino acid "peak" is obtained, which is believed to be the di-lysyl derivative of the reagent, although it has not been separated in pure form nor in the quantity required for a proof of its structure.

The polyhemoglobin, injected into rabbits, has a half-life many times longer than that of an equal quantity of unreacted hemoglobin. At a concentration of 0.5 g/Kg of body weight of rabbit, polyhemoglobin had a $T\frac{1}{2}$ more than 10 times that of unreacted hemoglobin when injected into a normal rabbit. Since, at a dose level of 0.14 gm/Kg, the increase in $T\frac{1}{2}$ was only 4-5 times, it is likely that higher dose levels will result in a much greater half life for the polyhemoglobin.

The polyhemoglobin does not bind as well or at all to haptoglobin as does unreacted hemoglobin. This result supports the finding of Nagel and Gibson (20) who reported that binding to haptoglobin by hemoglobin required that the hemoglobin dissociate into dimers.

The molecular weight distribution of the polyhemoglobin, as measured by the analytical ultracentrifuge, together with the inability of haptoglobin to complex with it offer a probable explanation for the longer stay of this compound in the circulating plasma.

Although the polyhemoglobin does not promote coagulation, it does have a delaying action on coagulation, and thus may be either a good or bad feature of the material, depending on the clinical circumstances.

Further studies in animals will be necessary before the material can be given a clinical trial for absence of toxicity at higher dose levels and for its ability to release oxygen to the tissues.

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