HEMOPEXIN IN HUMAN SERA: A SEARCH FOR A GENETIC POLYMORPHISM

by

Ray E. Stewart, D.M.D.

A Thesis

Presented to the Department of Pedodontics
and the Graduate Education Committee of the
University of Oregon Dental School
in partial fulfillment
of the requirements for the degree of
Master of Science
June, 1971

APPROVED

[redacted]

Donald R. Porter, D.D.S.
Professor of Dentistry

[redacted]

Everett W. Lovrien, M.D.
Associate Professor of Pediatrics
and Medical Genetics

[redacted]

Gerald Prescott, D.D.S.
Associate Professor of Medical Genetics

[redacted]

Fred F. Cowan, Ph.D.
Chairman, Graduate Education Committee

ACKNOWLEDGEMENTS

The author wishes to express special thanks to Dr. Everett W. Lovrien for the understanding and constructive guidance he provided throughout this project. I am particularly grateful for the many stimulating hours spent in discussing ideas and techniques and his willingness to share his knowledge.

The author also wishes to thank Dr. Donald R. Porter and Dr. Gerald Prescott who gave so generously of their time to provide constructive advice and valuable criticism during the preparation of this thesis.

Last, but not least, I wish to thank my wife and family for their patience and support. To them I am most indebted.

This investigation was carried out during the tenure of a Postdoctoral Fellowship sponsored by the Maternal and Child Health Services Training Grant No. 920.

TABLE OF CONTENTS

LIST OF PLATES

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

This thesis attempts to explore one small facet of a very large and diverse field, that of genetic variability in man. Attention is focused on the phenomena of genetic polymorphism in human serum proteins. More specifically, the purpose will be to determine whether or not there is genetic polymorphism in a particular human serum protein called hemopexin and, if such a variation is present, to determine its frequency and distribution within human populations.

Genetic variation. Genetic variability among humans has been a focus for study since the early part of the twentieth century when it became apparent that Mendel's principles were applicable to man. Variable traits first noted to be genetically determined in man were naturally obvious ones because of variation in phenotype, e.g. hemophilia and achondroplasia. This phenotype variation has been classified in two principle ways depending on whether the trait is continuous (larger vs. smaller, darker vs. lighter) or discontinuous (blue eyes vs. brown eyes, black hair vs. red hair). The distinction between continuous and discontinuous variation is a function of the number of genes affecting the character in question. When a single gene is involved, phenotypic variation will be discontinuous. When several genes are

involved (along with environmental influences), the more continuously variable the trait will appear. Although discontinuous traits, recognizable because of their presence or absence in the individual, have provided the greatest amount of information on genetic transmission, the vast majority of genes affect characters which vary continuously, such as size, body proportions or intellect.

Polymorphism. The term polymorphism is often used to describe a type of discontinuous variation within a population. By the definition of E. B. Ford (8), a genetic trait is said to be polymorphic "when it occurs in a population more frequently than would be expected on the basis of recurrent mutation." Ford has stated that this frequency must be greater than one percent in a given population to be considered polymorphic.

Polymorphism is a relatively widespread phenomena. Every natural population contains significant genetic variability. Most geneticists agree that increased variability enhances the competitive ability of a population in comparison with others. A population which lacks polymorphic diversity is more narrowly adapted, more specialized and therefore more vulnerable to alterations in the environment and hence more susceptible to extinction. (The genetic mechanism which produces and maintains polymorphism in a population is known as natural selection.)

Polymorphism in a population assumes a pool of genetic variability that, in part, provides the material upon which

natural selection acts. An accurate estimate of the extent of this variability, a subject of extensive debate, must await the examination of additional proteins for polymorphism.

Traits which show discontinuous variation and demonstrate impeccable Mendelian transmission in human populations are few in number. The first discontinuous trait to be recognized as a genetic polymorphism in man was discovered by Landsteiner in 1900 (21). This was the ABO blood group system. Since that time, eleven other major blood group systems have been described, the whole of which provide some of the clearest examples of the operation of Mendelian principles in man.

Recent developments in methodology for demonstration of immunological, physiochemical and enzymatic variations in proteins has augmented the search for additional polymorphisms in humans. In particular, the advent of electrophoretic techniques has provided a tool with which to determine (within certain limits) the extent of protein variability in individuals since proteins produced by different alleles of the same locus may differ in their electric charge and be separable by electrophoresis.

Studies on human blood have been particularly fruitful in demonstrating the remarkable diversity which exists between individuals and populations. Approximately thirty genetic polymorphisms have been described in the antigens of red blood cells and serum proteins in humans. The mode

of inheritance for most of the blood group and serum protein variants is determined by co-dominant alleles. Because of their simple pattern of transmission and the variable frequency with which they occur in different populations, these characters are among the most useful tools for studies of genetic linkage and population genetics available in man. The various components of human blood which have been shown to demonstrate sufficient variations to be classified as polymorphic are listed in Table 1.

Heme-binding proteins. Among the serum proteins which have come under close scrutiny are a group classified generally as heme-binding proteins. The clinical significance of this particular group of proteins became apparent when Fairley (6) (7) demonstrated the occurrence of a hematin-albumin complex (methemealbumin) in the plasma of patients with blackwater fever. The presence of this complex in the plasma was suggested as evidence of severe hemolysis of intravascular origin.

Genetic variation in human albumin was first observed by Scheurlen (45) who found in addition to the usual albumin zone, a second band with slower electrophoretic mobility. Since that time, the genetics of this double albumin variant (bisalbumin) has been demonstrated in a number of families of American Indian extraction (28). In all, there are at least six electrophoretic variants which, in heterozygotes, give the appearance of bisalbuminemia.

TABLE 1

Polymorphic Components of Human Blood

SERUM POLYMORPHISMS

- Haptoglobin (Hp)
- Transferrin (Tf)
- Group Specific Component (Gc)
- Pseudocholinesterase
- Alkaline phosphatase
- Albumin
- Ceruloplasmin
- Immunoglobulins
  - Gm
  - Inv
- α_1-antitrypsin

BLOOD CELL POLYMORPHISMS

- Red cell antigens
  - ABO system
  - P
  - MNSs
  - Rh
  - Lutheran (Lu)
  - Kell
  - Lewis (Le)
  - Duffy (Fy)
  - Kidd (Jk)
  - Xg
  - Diego (Di)
- Hemoglobin
- Acid phosphatase
- G6PD
- 6PGD
- Phosphoglucomutase (PGM)
- Adenylate kinase (AK)
- Lactate dehydrogenase (LDH)
- Malate dehydrogenase
- Phosphohexose isomerase
- Carbonic anhydrase
- Red cell esterases
- Peptidases
- Catalase

The presence of a protein in human serum having the capacity to bind hemoglobin was first described by Polonovski and Jayle (41). They characterized the binding substance as a protein with the properties of an β-2 glycoprotein and gave it the name haptoglobin.

In a pioneer study using starch gel electrophoresis, Smithies and Connell (52) demonstrated a polymorphism in haptoglobin of human sera. These patterns, consisting of three distinct banding patterns, were later shown by Smithies and Walker (53) to be genetically controlled by two autosomal alleles.

More recently, another protein has been observed in human serum which also has the ability to bind hematin (1) (2) (9) (38) (39). This protein was identified by Tombs (56) to be a β_1-globulin (Figure 1) and was named hemopexin by Grabar et al. (9).

Heide et al. (15) reported on an attempt to identify serum hemopexin polymorphism in man. They found human serum hemopexin to be heterogeneous using "numerous sera of the haptoglobin 2-2 type." No differences in the electrophoretic mobility of the hemopexin components was observed and no mention was made of the number of samples tested.

Recent studies have shown that a genetically controlled polymorphic protein in the sera of rabbits is able to bind with heme but not whole hemoglobin (10) (11). This serum protein has been identified as hemopexin (11).

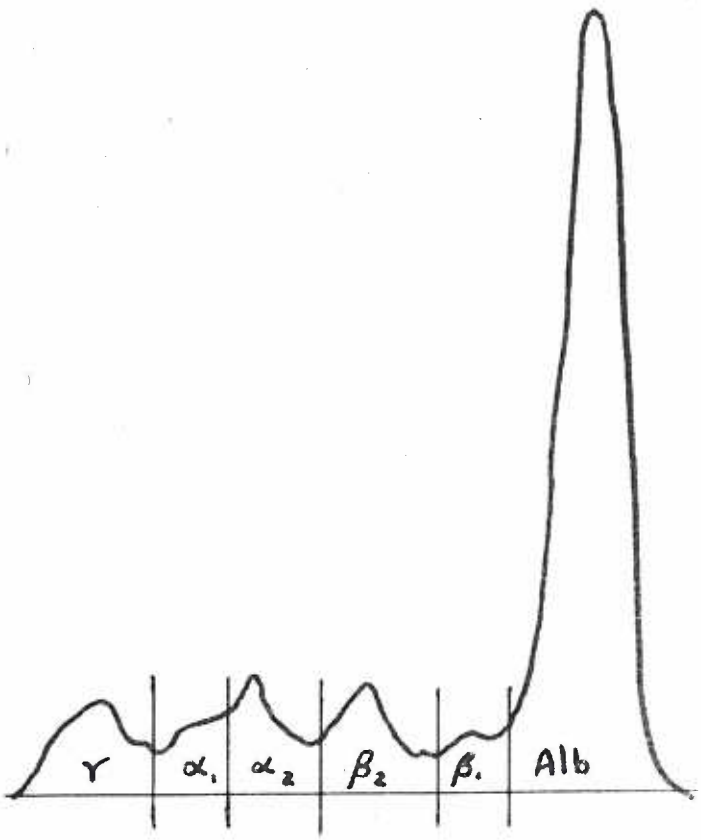


Figure 1

The classification of human serum protein fractions according to migration during starch gel electrophoresis (pH 8.6). The amount of protein in each fraction is calculated from the area under the curve.

Imlah (19) found that similar electrophoretic variants of hemopexin exist in swine, these variants were not demonstrable when the serum was mixed with fresh hemoglobin. The hemopexin bands were observed only when aged hemoglobin which had partially hemolized or alkaline hemoglobin were utilized. Imlah called this protein a "haem-binding globulin". Kristjansen (20) also reported a polymorphism of heme-binding serum protein of swine. He classified the protein as a haptoglobin. Subsequent studies, however, have shown that the protein binds only with heme, thus suggesting it to be hemopexin (12).

Variations in the electrophoretic mobility of hemopexin from the sera of certain canine species have also been demonstrated (unpublished data). The reports of hemopexin polymorphism in the serum of mammals other than man suggested a detailed examination of human serum hemopexin for evidence of a similar genetic polymorphism.

Information on the presence or absence of polymorphism in hemopexin would be helpful in estimating the overall variability in humans.

The identification of additional polymorphic characteristic such as hemopexin could also be useful as a genetic marker in linkage studies. In studies of this type, the more markers one has to use in testing for linkage relationships, the better are the chances for locating two loci on the same chromosome, and thus establishing linkage. Thirty markers allow for $\frac{30 \times 29}{2}$ or 435 possible linkage relationships to

be studied. The addition of one more marker increases this number of possibilities to $\frac{31 \times 30}{2}$ or 465.

The purpose of this study was two-fold:

1. To develop an electrophoretic technique which would allow the optimum separation and resolution of the human serum protein hemopexin.
2. To test the hypothesis that hemopexin is genetically polymorphic in human populations.

REVIEW OF THE LITERATURE

The protein hemopexin, (after the Greek "haima" meaning blood and "pexis" meaning fixation), is a polydispersed non-enzymatic plasma protein. It is synthesized in the liver and is present in the serum of all mammals which have been examined. In man it comprises approximately 1.4 percent of the total serum protein. Hemopexin is one of two serum proteins (the other is albumin) which bind with the heme portion of the dissociated hemoglobin molecule to form a brown-colored complex in vitro.

Hemopexin binds with the heme portion of the hemoglobin molecule to form a complex consisting of one mole of hemopexin and one mole of heme (15) (38). The hemoglobin molecule carries four heme groups per molecule. X-ray crystalography studies by Perutz (40) showed that the hemoglobin molecule consists of four polypeptide chains arranged in a cylindrical fashion with four disc-shaped heme groups fitted into open spaces on the surface of the protein.

One link between heme and globin is a result of a coordinate-covalent bonding of the iron atom with a nitrogen of a histidine side chain in hemoglobin. Other links appear to be the result of hydrogen and hydrophobic bonding. The heme molecules are oriented at an angle which allows the

unbound position on each iron atom to face outward. When the hemoglobin is oxygenated a molecule of oxygen displaces the water molecule previously bonded to each of these four positions:

$$HHb\ (H_2O)_4 + 4\ O_2 \rightleftharpoons HHb\ (O_2)_4 + 4\ H_2O$$

Heme is a colored, iron-containing prosthetic group which, when combined with a basic protein called globin, forms a chromo-protein (hemoglobin) having the ability to transport large volumes of oxygen. It is one of a group of closely related compounds which are ubiquitous in nature. The structure of chlorophyl is very similar to heme. Combinations of heme with various proteins give rise to a variety of oxidizing enzymes such as cytochromes as well as to a large group of vertebrate hemoglobins.

The molecular structure of heme consists of a large iron porphyrin ring composed of four smaller pyrole rings united through methane (= CH-) bridges in such a way that eighteen of the inner atoms make up a ring of conjugated double linkages (Figure 2).

Physical properties. Schultze et al. (46) have characterized human hemopexin as a β-glycoprotein with a carbohydrate content of approximately 22.6 percent consisting of hexose, acetylhexosamine, sialic acid and fucose. Its molecular weight has been estimated as between 70,000 and 80,000 (46) (17).

The amino acid composition of hemopexin was determined by Heimburger et al. (16). Table 2.

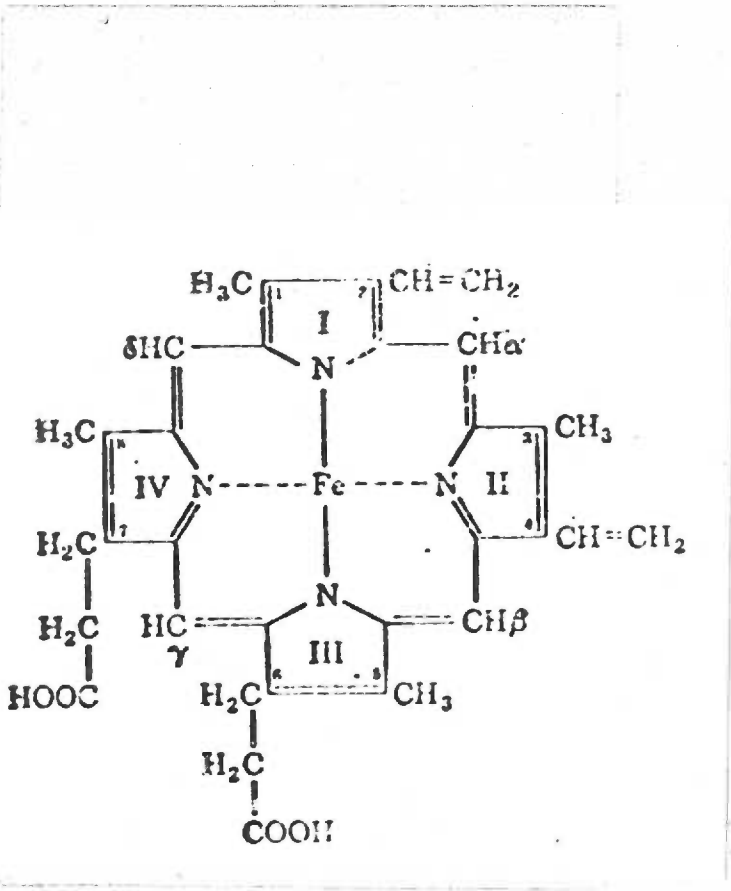


Figure 2

The heme molecule.

TABLE 2

Amino-Acid Composition of Hemopexin
(after Heimburger et al. (16))

	Amino-acid residue (%)	Moles per mole protein
Lysine	4.16	26
Histidine	3.25	19
Amide NH_3	1.24	62
Arginine	4.42	23
Aspartic acid	6.49	45
Threonine	3.46	27
Serine	3.59	33
Glutamic acid	7.74	48
Proline	4.62	38
Glycine	3.18	45
Alanine	2.80	32
1/2 Cystine	1.44	11
Valine	3.45	28
Methionine	1.14	7
Isoleucine	1.59	11
Leucine	5.94	42
Tyrosine	3.74	18
Phenylalanine	3.96	21
Tryptophan	4.30	18
Total peptide	70.51	554

Aber and Rowe (1) confirmed the binding of hematin by both albumin and a "fast β-globulin" in human sera. They compared the relative binding affinities of these two heme-binding proteins and found that hematin was bound by the β-globulin much more strongly than by albumin.

Nyman (37) (38) described three different serum proteins which form complexes with hemoglobin or its heme-containing metabolites. One of these, which Nyman called "heme-binding globulin", was found to migrate with the fast β-globulins on paper electrophoresis. The mobility of the complex was unaffected by the type of heme compound added to the serum, strongly suggesting that no globin was present in the complex.

Wheby et al. (58) also describe a serum protein which is probably hemopexin. This protein reportedly binds the heme portion of the hemoglobin molecule in contrast to haptoglobin which binds the globin fraction. The protein was present in all patients except two who had acute hemolytic anemia.

Heide et al. (15) studied the heme-binding capacity of hemopexin using spectrophotometric and starch gel techniques. They confirmed the findings of Aber and Rowe (1) when they reported that heme has a greater affinity for hemopexin than albumin since no heme was transferred from hemopexin to albumin upon addition of the latter to a heme saturated serum solution. The reverse is true, however, in that heme can be

transferred from albumin to hemopexin in a heme-saturated solution.

Physiologic role of hemopexin. A reduction or absence of hemopexin in the serum of individuals suffering with various hemolytic disorders has been observed by a number of workers (13) (31) (36) (47) (58). These variations in serum levels can probably be ascribed to the formation of a heme-hemopexin complex in vivo. The findings suggest that the function of this protein may be related to the conservation of iron since the heme-hemopexin complex rapidly disappears from the circulation and there is no apparent loss of iron. Variations in serum levels of hemopexin also have been reported as a function of age and ingestion of certain drugs (13) (43).

The physiologic role of hemopexin seems to be similar to that of the other heme-binding serum proteins, haptoglobin and albumin. These heme complexes cannot be considered as actual transport proteins since neither delivers its bound product to a specific receptor site nor do they re-enter the bloodstream once the bound complex is catabolized. It appears that the primary function of both hemopexin and haptoglobin is the conservation of heme iron by preventing the renal excretion of extracorpuscular hemoglobin metabolites.

Several studies (31) (36) (58) have shown that the absence of haptoglobin from the plasma has clinical significance in that it is a sensitive indicator of a variety of

hemolytic anemias in humans.

It is probable that the deficiency of hemopexin in hemolytic disorders can also be ascribed to the formation of a complex of hemopexin with heme or related protoporphyrins which are rapidly eliminated from the circulation.

Deficiency of β_1-heme-binding globulin (hemopexin) in hemolytic disorders, associated with ahaptoglobinemia, supports the conclusion that hemopexin might represent an additional mechanism capable of eliminating hemoglobin metabolites from the circulation.

Sears (50) has reported findings strongly suggesting that heme and hemopexin are eliminated from the circulation as a complex. He concluded that patients who had hemolytic disease or who had received an injection of heme underwent a depletion of hemopexin as well as an elimination of albumin-bound ferri-heme. It is possible that hemoglobin binds to hemopexin after complete saturation of haptoglobin. This possibility may explain the findings of low hemopexin levels in spherocytosis and sickle-cell anemia. However, the binding of heme seems to be the major function of hemopexin (33) (35) (47). The absence of detectable amounts of hemopexin in the presence of haptoglobin as observed in thalassemia major indicates that these two proteins have distinctive functions. Although it cannot be excluded that impaired synthesis of hemopexin may be responsible for the deficiency of this protein in thalassemia, the observation in one case of thalassemia major that the protein was present in decreased

amounts suggests this explanation is unlikely. Moreover, heterozygous individuals revealed no detectable abnormalities of hemopexin (33). It is more probable that the deficiency of hemopexin in hemolytic disorders can be ascribed to the formation of a complex of hemopexin with heme or related protoporphyrins which are rapidly eliminated from the circulation. The deficiency of β_1-heme-binding globulin (hemopexin) in patients with thalassemia major suggests that increased concentrations of a heme compound may be present in this disorder. Future investigations will be necessary to determine whether this compound is a normal or abnormal product of porphyrin metabolism.

The average lifespan of the human red blood cell is 120 days (22). The red cells are withdrawn from circulation and catabolized by special cells in the spleen and liver. The hemoglobin contained within these cells is released as free intravascular hemoglobin.

Heme disposal. The mechanism by which hemoglobin is removed from the bloodstream has been the subject of numerous investigations (35) (48) (50). Studies on the mechanisms of heme disposal are relatively few, however. Recent experimental evidence implicates hemopexin as playing the major role in this process of heme disposal (47) (48) (49) (50) (30) (34).

The current concept of the mechanism for disposal of hemopexin is shown in Figure 3. Soon after the hemoglobin is released from the red blood cell it is bound by the serum

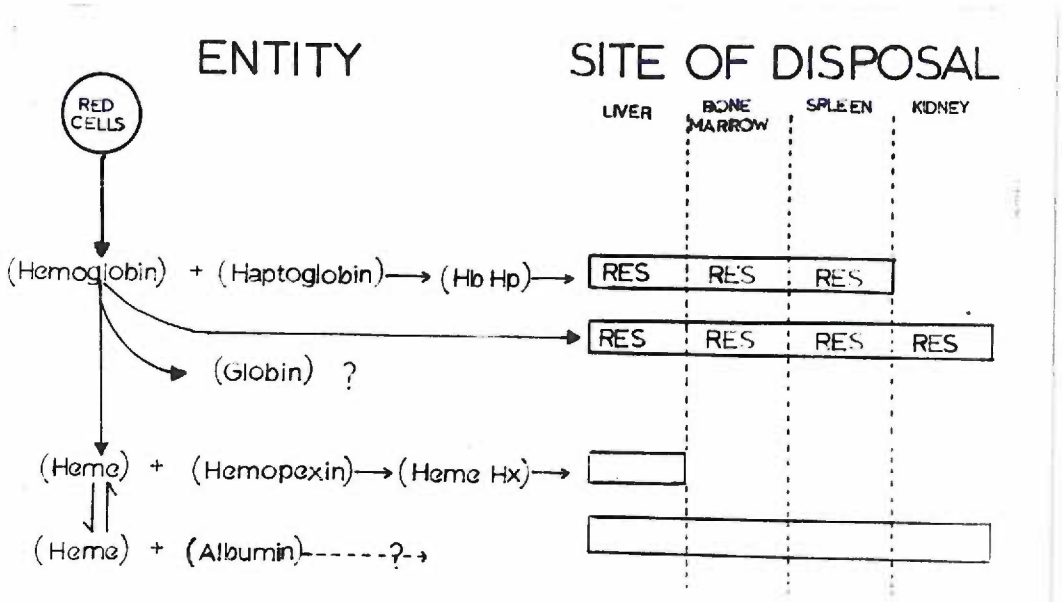


Figure 3

A current concept of the mechanism for the disposal of hemoglobin and its metabolites. RES indicates Reticuloendothelial cells.

protein haptoglobin. This hemoglobin-haptoglobin complex is removed from the circulation by the reticuloendothelial cells of the liver, bone marrow and spleen (30). Unbound, hemoglobin, in excess of the haptoglobin binding system may also be removed by the reticuloendothelial system. A portion of this unbound hemoglobin dissociates into subunits which include two α - β -chain dimers, each of which contain two hemes. These subunits are able to pass glomerular membranes and are reabsorbed in the renal tubules. When the absorptive capacity of the renal tubule is exceeded, hemoglobin subunits appear in the urine giving rise to what is observed clinically as hemosiderinuria (33).

Another part of the unbound hemoglobin dissociates into heme and globin components at which time the iron portion of the heme moiety is converted from a divalent to a trivalent form. The resulting oxidized heme becomes complexed with albumin or hemopexin. Hemopexin, which has a higher affinity for the heme, forms heme-hemopexin complexes which are removed from circulation in the liver and subsequently degraded there by the hepatocytes (30). Heme in the form of the heme-albumin circulates until apo-hemopexin becomes available for its removal or until it is taken up directly by tissue heme proteins and is thus recycled or disposed of at sites of heme catabolism.

MATERIALS AND METHODS

Sample. Sera from 418 unrelated individuals were examined electrophoretically. The specimens were collected from the following populations: University of Oregon Medical School Genetics Clinic, 292; Southern Alaska Eskimo, 64; Papago Indian, 50; and 12 cord blood samples from the Multnomah County Hospital obstetrics service. Samples were collected over a three-year period.

Family studies were initiated in selected cases where it appeared that there was a mating of individuals exhibiting various banding patterns.

Blood samples were collected by venipuncture from the antecubital vein of adults and by jugular or femoral vein puncture in infants. The samples were allowed to clot at room temperature and were centrifuged at 20,000 rpm for 15 minutes.

Preparation of samples for electrophoresis. The samples, when not run on the day of collection, were stored at -20° C. Frozen storage did not visibly affect the electrophoretic characteristics of hemopexin. When a sample was required, it was thawed at room temperature. Three drops (Pasteur pipette) of sera were added to one drop of heme solution and mixed thoroughly. The sample mixtures were

allowed to stand at room temperature for 10 minutes. Filter paper inserts were saturated by immersing in the sample mixture.

Preparation of heme solutions. Crystalline hemin was obtained commercially (Nutritional Biochemicals Corp., Cleveland, Ohio). The hemin was freshly prepared by dissolving 0.01 grams in 10 ml of gel buffer made alkaline with 0.5 ml 0.1 N NaOH.

Electrophoresis. The theory underlying the process of electrophoresis is based on the principle that protein molecules, like any other charged particles, move when exposed to an electrical field. A direct current passed through a solution of serum produces a separation of the variously charged proteins. Components possessing a relatively greater charge at a given pH move faster in the electric field in comparison to components having a lesser charge. Those components having little or no charge will remain relatively stationary while those with a relatively greater charge migrate toward the oppositely charged pole. Figure 1 demonstrates the classification of various proteins according to dispersal in an electric field at pH 8.6.

The net electrical charge on a particular protein is determined, in part, by the type and number of amino acid residues which make it up. These amino acids contain both acidic ($COOH^-$) and basic (NH_3^+) groups. A given protein may be positively charged due to a preponderance of NH_3^+ groups, negatively charged due to a preponderance of $COOH^-$

groups or have a zero net charge by virtue of an equal number of $COOH^-$ and NH_3^+ groups. The pH at which the protein has a zero net charge is known as the isoelectric point of the protein.

The net electrical charge on a protein is also influenced by the pH of the buffer in which it is dissolved. As the pH of the buffer is raised, the amino (NH_3^+) groups are progressively neutralized by the basicity of the buffer so that the carboxyl ($COOH^-$) groups become predominant, thus giving the protein a net negative charge. The opposite is true if the buffer pH is acidic. At pH 8.6 most proteins carry a negative charge and will migrate toward the anode during electrophoresis.

Since the isoelectric point for hemopexin had not been determined, it became necessary to test a variety of buffer systems over a range of pH and ionic strengths to determine the pH which gave maximum separation and resolution.

Recipes for the various buffer systems were derived from reference texts and reports from the literature and are given in Appendix A.

In the study of any protein, selection of a support media for carrying out the electrophoretic separation is very important. Many techniques have been developed which are claimed to be suitable for separation of different proteins.

Starch gel electrophoresis was introduced by Smithies in 1955 (51). Due to the excellent resolving power of the starch gel technique, it has all but replaced the moving

boundary technique of Tiselius and is rivaled only by the more recently developed acrylamide gel methods.

In choosing an electrophoresis system, consideration was given to (1) the complexity of the apparatus, (2) the amount of "know-how" required, and (3) the scale on which the particular separation was to be carried out. The system sought was to be one in which the apparatus was inexpensive, readily available and easy to use without extensive preparative procedures. Although starch gel does not fit all of these criteria, it was selected as the media of choice for several reasons. A single gel can be used for the separation and analysis of multiple serum protein samples. The process of electrophoresis takes only a few hours; with starch gel, extremely small amounts of proteins (10-50 μg) can be separated and clearly visualized.

Starch gel electrophoresis. Optimal separation was obtained when serum samples were examined by horizontal starch gel electrophoresis. A variety of buffer systems were tested at pH's ranging from pH 3.3 to pH 9.2. A modification of the discontinuous buffer system described by Kristjansson (20) gave the best resolution. The reservoir buffer (pH 8.7) consisted of 0.30 M boric acid and 0.10 M NaOH. The gel buffer, pH 7.5 consisted of 0.014 M Tris (Trizma base, Sigma Chemical Company, St. Louis, Missouri) and 0.004 M monohydrate citric acid. Each gel contained 29 grams hydrolized starch (Connaught Medical Research Laboratories, Toronto, Canada) per 250 ml gel buffer.

Samples were prepared for electrophoresis by diluting three parts serum with one part hematin solution. The samples were absorbed onto filter paper inserts (Whatman 3 mm) and placed 6 cm from the cathodal end of the gel at right angles to the direction of current. Electrophoresis was carried out at 4° C. by applying 4 volts/cm for 10 minutes at which time the sample inserts were removed. Electrophoresis was resumed at 6 volts/cm for 4 hours at which time the gel was removed from the gel mold and prepared for slicing.

Slicing. Slicing of the gel was accomplished with a six-inch microtome blade. The gel is placed on a slicing tray with 3 mm walls, covered with a weighted plastic sheet to prevent distortion and the blade drawn smoothly from one end of the gel to the other. The slices were 3 mm thick and were handled most easily when allowed to adhere to a thin sheet of polythene.

Staining. A specific method of testing for the presence of heme-containing compounds (i.e., hemopexin, haptoglobin, hemoglobin) following electrophoresis is with a benzidine stain.

This reaction depends on the oxidation of benzidine by peroxidase to a blue or brown-colored product. The reaction is illustrated in Figure 4. Two molecules of benzidine are linked together following dehydrogenation forming a blue component. In the second step of this reaction, the blue compound is reduced to a brown, high molecular weight polymer of a quinoid structure.

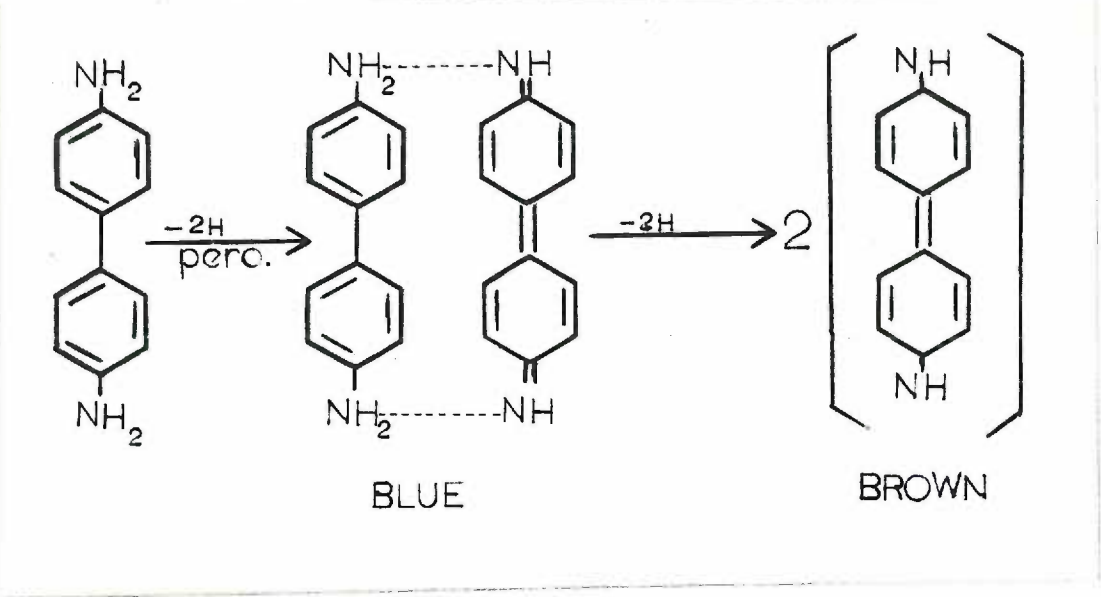


Figure 4. The benzidine staining reaction.

The staining solution was prepared by mixing: 200 ml H_2O; 0.4 grams benzidine; 0.4 ml 30 percent H_2O_2; 1 ml glacial acetic acid. The H_2O_2 was added just prior to use.

The presence of heme-containing compounds in the starch gel was revealed clearly by immersing the thin slice of the gel in the staining solution and allowing it to develop for 15 minutes. The gel was then rinsed with tap water and photographed using Polaroid High Contrast, Black and White film. It was necessary to photograph the gels immediately after staining since the bands faded rapidly. The hemopexin bands were visualized on the gels as blue areas (Plate 5).

Immunoelectrophoresis. Immunoelectrophoresis technique combines the principles of zone electrophoresis with those of immunochemical analysis utilizing a supporting media of agar gel. The procedure allows simultaneous characterization of the serum proteins by electrophoretic mobility and immunologic specificity. As in starch or acrylamide gel

systems, the use of specific stains add yet another dimension to the analysis. Since the resolution obtained with these techniques is a function of both electrophoretic mobility and immunological specificity, it is possible to distinguish between proteins which have the same electrophoretic mobility but are immunologically different.

Immunoelectrophoresis was carried out on one percent agar in barbitone buffer (pH 8.5) on microscope slides using the technique of Scheidegger (44). A voltage gradient of 88 volts at 40 milliamperes was applied for two hours. Rabbit anti-serum to human hemopexin (Behringwerke Diagnostic Reagents), full strength, was used to develop the precipitation lines. The slides were stained for 10 minutes with Amido Schwartz, destained overnight with acetic acid solution and dried. Plate 3 shows a typical slide with the hemopexin precipitin band.

Radial immunodiffusion. In order to investigate possible quantitative variations in the amount of hemopexin present in individual samples, single radial immunodiffusion techniques as described by Mancini (26) were employed. Mancini showed that the area covered by a quantity of precipitated antigen, allowed to diffuse radially from a well in a uniformly thin layer of antibody-containing agar, is directly proportional to the amount of antigen employed, and inversely proportional to the concentration of antibody when sufficient time is allowed for all the antigen to combine. The temperature at which the samples are incubated during the

reaction has no perceptible influence on the results. In the experimental system employed, the method was able to detect antigen in amounts as small as 0.0025 µg in a concentration of 1.25 µg per ml (26).

The agar-supporting media was prepared using 0.8 percent agarose in phosphate buffer. The required amount (10 ml) of agarose was melted in a boiling water bath and allowed to cool at 45° C. Sufficient anti-hemopexin was added to make a 5 percent antibody solution and mixed gently. The melted antibody-containing agar was poured into a partigen plate and allowed to cool in 100 percent humidity. Twenty-four wells were cut in the agar and each one filled with three microliters of serum. The sera, containing hemopexin as an antigen, were allowed to diffuse radially for a sufficient length of time to allow all antigen to be reacted. The ring-shaped precipitates which form around the antigen wells grew in size for a few days, after which no increase in dimensions was observed. Measurements of ring diameters were made when the precipitates had reached their final size. The final area reached by the precipitin ring was interpreted as being directly proportional to the amount of antigen employed and inversely proportional to the amount of antibody (26). A typical plate with antigen wells is shown in Plate 4.

RESULTS

No variation in electrophoretic mobility of hemopexin was observed in any of the 418 samples tested. Some variation in the staining intensity and band number was observed, however (Plate 5). The most common electrophoretic pattern consisted of three distinct bands (samples 1, 2, 3 and 6, Plate 6). The first band was moderately intense; the other two faster bands were sharper and of greater intensity. Seven of the three-banded samples showed a variation consisting of an unusually dark middle band (samples 1 and 3, Plate 6). Seventeen samples appeared with an additional distinct fourth band which migrated faster than the third (samples 4 and 7, Plate 6). Other samples had a fourth band which was less distinct, smudged and faster (samples 5 and 8, Plate 6). One sample from the Oregon group had five bands (sample 9, Plate 6) and one Eskimo sample lacked the slowest band showing only two distinct bands (sample 10, Plate 6). In two instances it was possible to collect additional specimens from individuals who initially demonstrated a four-band electrophoretic pattern. Repeat fresh sera from these people showed only three distinct bands. The observed variation of band number in these individuals did not correlate with onset or recovery from illness, drug ingestion, or other known changes in physiological conditions. The segregation

among progeny of various types of matings based on band number is illustrated in Table 3.

Of the 292 samples from the Oregon population, 24 were from patients with a variety of chromosomal anomalies including Trisomy 21, Turner's Syndrome, Cat Cry Syndrome, and No. 18 Deletion Syndrome. None of these individuals' sera showed variation in mobility or staining intensity of hemopexin on electrophoresic treatment of their sera.

Cord blood specimens showed a wide range of variability. Two samples showed no peroxidase activity in the hemopexin region on starch gel. The remaining ten samples showed the characteristic three-band pattern seen in adults; however, these bands were considerably reduced in staining intensity (samples 11 and 12).

Samples showing variability in band number or staining intensity on starch gel were examined by agar gel immunoelectrophoresis. No variation was detectable by this method. A single precipitin band was observed in all samples.

Radial immunodiffusion, by the method of Mancini (26) was used to investigate possible quantitative differences among these samples. The results showed distinct variation in the size of the precipitin rings ranging from 0.5 cm to 1.5 cm diameter. Cord sera, which had shown no peroxidase activity on starch gel, showed no precipitin ring in one instance and a faintly perceptible ring at the well margin in a second instance. Relatively large rings (1.5 cm diameter) precipitated around the wells containing the samples

TABLE 3

No.	Type	3 B	3 + B	4 B
3	3 B x 3 B	1	3	3
3	3 B x 3 + B	6	2	0
1	3 + B x 3 + B	0	2	1
2	3 B x 4 B	4	3	1
1	3 + B x 4 B	1	0	3

Segregation of offspring resulting from various matings based on the number of hemopexin bands seen on electrophoresis.

The number of matings observed and the type of parental band patterns are given in the first two columns.

The combined number of offspring for each mating type (3 bands, 3 bands plus a smudge, and 4 bands) is given in the last three columns.

which had four bands on starch gel electrophoresis. Samples with three bands on electrophoresis had precipitin rings intermediate in size (1 cm diameter).

DISCUSSION

Muller-Eberhard and English (32) isolated and purified hemopexin from human plasma and found it to consist of two components as judged by mobility during immunoelectrophoresis and starch-gel electrophoresis. The first component corresponded to hemopexin as it occurs in whole serum and exhibited a single band on starch-gel electrophoresis at both pH 3.5 and pH 8.6. The second component consisted of four to six bands which was considered to be a product of aggregation of the monomeric form of native hemopexin to form polymers. By using a discontinuous buffer system to improve resolution, in this investigation two to five bands were demonstrable on starch gel electrophoresis of human serum. This finding suggests the existence of hemopexin polymers, in vivo.

The observation that the samples with greater number of bands and greater staining intensity gave larger precipitin rings on radial immunodiffusion suggests that there may be quantitative variation in serum levels of hemopexin which is reflected in the number of bands present and the relative staining intensity of these bands. None of this variation appears to be under genetic control.

Ross and Muller-Eberhard (43) reported that hemopexin levels were increased in the serum of rabbits two to eight

days after the injection of three different agents. These compounds, 3-methylcholanthrene, allylisopropylacetamide, and benzopyrene appeared to induce the synthesis of hemopexin. The investigators noted that hemopexin, by binding heme, may alter the functional capacity of the heme-containing enzyme, cytochrome P-450. They proposed that increased synthesis of hemopexin may result in the regulation of intracellular heme and relate to induction of microsomal, heme-containing enzymes which participate in detoxification mechanisms. Data from this study further suggests that quantitative variation in serum hemopexin may occur in the absence of drug ingestion or a hemolytic episode. Hemopexin serum levels may be influenced by a variety of physiological phenomena such as infection, pregnancy or subclinical intravascular hemolysis; however, these relationships were not investigated.

The observation of reduced levels of hemopexin in the cord serum is contrary to the findings of Fine et al. (7), who reported that hemopexin is present in normal amounts at birth. The findings, however, are in accord with Hanstein and Muller-Eberhard (13) and Weippl et al. (57) who observed that hemopexin levels in the newborn period ranged from 8 to 33 percent of the mean adult values. The reduced quantities of hemopexin observed in cord samples could be attributed to either the incomplete prenatal development of this protein or the presence of perinatal intravascular hemolysis resulting in the formation of a heme-hemopexin complex.

The reduced hemopexin levels may be pharmacologically important to the newborn if the heme-containing enzymes involved in drug detoxification have not fully developed. This fact may be involved in increased drug sensitivity in the newborn human.

The lack of variation in electrophoretic mobility of human serum hemopexin in 418 unrelated individuals indicates that this protein is not polymorphic in humans. The absence of electrophoretic variants in hemopexin indicates that the frequency of polymorphism is less than 0.003 in an Oregon population and less than 0.02 in the Eskimo and Papago Indian populations.

Absence of hemopexin polymorphism on starch gel electrophoresis is not surprising when one considers the results of investigations of other protein systems for the presence of polymorphism. Lewontin and Hubby (23), using electrophoretic separation to detect protein variants in populations of Drosophila pseudoobscura, showed that approximately 30 percent of all loci demonstrate segregation of alternate alleles. They further noted that only about 12 percent of the loci are heterozygous in a typical individual. Their data are in agreement with Harris (14) who, in a preliminary survey of ten randomly selected enzymes in human blood, found only three of them to be demonstrably polymorphic. Crow (4), on the other hand, states that it is highly probable that no more than a minute fraction (1 percent) of the loci in a given population are occupied by dissimilar alleles. An

accurate estimation of the proportion of loci which are polymorphic in man can only come after surveying a large number of enzymes and other proteins for phenotypic variants.

The apparent lack of polymorphism in human serum hemopexin places it among the 60-70 percent proteins which have been found not to be polymorphic in man. This number is quite possibly an under-estimate, however, due to the reluctance of some investigators to report negative results. Publication of negative results in search for human polymorphisms should be encouraged in order to expand our information about the nature of gene products.

SUMMARY AND CONCLUSIONS

Hemopexin was investigated in the sera of 418 individuals for evidence of genetic polymorphism. No variation in electrophoretic mobility on starch gel was observed although differences in staining intensity and the number of bands were noted. There appears to be no genetic basis for the variation in band number or staining intensity. The results indicate that although hemopexin polymers exist in human serum, the protein is not polymorphic.

BIBLIOGRAPHY

1. Aber, G. M. and Rowe, D. S. The binding of haematin by serum proteins. Brit. J. Hematology. 6:160, 1960.

2. Allison, A. C. and Rees, W. The binding of hemoglobin by plasma proteins. Brit. med. J. 2:1137, 1957.

3. Bremner, K. C. Studies on haptoglobin and haemopexin in the plasma of cattle. Aust. J. Exp. Biol. Med. Sci. 42:643, 1964.

4. Crow, J. F. Population genetics. Am. J. hum. Genet. 13:137, 1961.

5. Fairley, N. H. and Bromgield, R. J. Laboratory studies in malaria and blackwater fever. Part III. A new blood pigment in blackwater fever and other biochemical observations. Trans. Roy. Soc. Trop. Med. Hyg. 28:307, 1934.

6. Fairley, N. H. Methaemalbumin (pseudo-methaemoglobin). Nature. 142:1156, 1938.

7. Fine, J. M., Imperato, C., Battistini, A., and Moretti, J. Etude immunologique des haptoglobines. Nouvells Rev. Franc. Hematol. 1:72, 1961.

8. Ford, E. B. Genetic Polymorphism. London, Faber, 1965.

9. Grabar, P., DeVaux St. Cyr, C., Cleve, H. Presence de β -globulin dans les extraits perchloriques de serums humains normaux. Bull. Soc. Chim. Biol. 42:853, 1960.

10. Grunder, A. A. Inheritance of a heme-binding protein in rabbits. Genetics. 54:1085, 1966.

11. Grunder, A. A. Hemopexin of rabbits. Vox Sang. 14: 218, 1968.

12. Grunder, A. A. Distinction between heme and hemoglobin-binding proteins of swine and rabbits by electrophoresis. Vox Sang. 14:236, 1968.

13. Hanstein, H. and Muller-Eberhard, U. Concentration of serum hemopexin in healthy children and adults and those with a variety of hematological disorders. J. Lab. Clin. Med. 71:232, 1968.

14. Harris, H. Enzyme polymorphisms in man. Proc. R. Soc. Biol. 164:294, 1966.

15. Heide, K., Haupt, H., Storiko, K., and Schultze, H. On the heme-binding capacity of hemopexin. Clin. Chim. Acta. 10:460, 1964.

16. Heimburger, N., Heide, K., Haupt, H. and Schultze, H. E. Bausteinanalysen von humanserumproteinen. Clin. Chim. Acta. 10:293, 1964.

17. Hrkal, Z. and Muller-Eberhard, U. Physiochemical characteristics of human and rabbit hemopexin. Proceedings of the Pacific Slope Biochemical Conference, Seattle, Washington. June 25-27, 1969.

18. Hochwald, G., Thorbecke, G., and Asofsky, R. Sites of formation of immune globulins and of a component of C'_3. J. Exptl. Med. 114:459, 1969.

19. Imlah, P. A study of blood groups in pigs. Proceedings of the ninth European blood group conference. The Hague, The Netherlands, W. Junk, 1964.

20. Kristjansson, F. K. Genetic control of three haptoglobins in pigs. Genetics. 46:907, 1961.

21. Landsteiner, K. Zur kenntnis der antifermentativen lytischen und agglutinierenden wirkunger des blutserums und der lymphe. Zbl. Bakt. 27:357, 1900.

22. Lathem, W. and Worley, W. E. The distribution of extracorpuscular hemoglobin in circulating plasma. J. Clin. Invest. 38:474, 1959.

23. Lewontin, J. L. and Hubby, R. C. A molecular approach to the study of genetic heterozygosity in natural populations. Genetics. 54:595, 1966.

24. Laurell, C. B. Purification and properties of different haptoglobins. Clin. Chim. Acta. 4:79, 1959.

25. Lundh, B., Oski, F. A. and Gardner, F. H. Plasma hemopexin and haptoglobin in hemolytic diseases of the newborn. Acta Paediat. Scand. 59:121, 1970.

26. Mancini, G., Carbonara, A. and Hermans, J. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry. 2:235, 1965.

27. Meiers, H. G. and Ippen, U. Ahaemopexinamie bei porphyia cutanea tarda. Klin. Wschr. 46:560, 1968.

28. Melartin, L. and Blumberg, B. S. Albumin Naskapi: a new variant of serum albumin. Science. 153:1664, 1966.

29. Muller-Eberhard, U. and Bashore, R. Assessment of Rh disease by ratios of bilirubin to albumin and hemopexin to albumin in amniotic fluid. New Eng. J. Med. 282: 1163, 1970.

30. Muller-Eberhard, U., Bosman, C. and Liem, H. H. Tissue localization of the heme-hemopexin complex in the rabbit and rat as studied by light microscopy with use of radioisotopes. J. Lab. Clin. Med. 76:426, 1970.

31. Muller-Eberhard, U. and Cleve, H. Immunoelectrophoretic studies of the β_1-heme-binding globulin (hemopexin) in hereditary hemolytic disorders. Nature. 197:602, 1963.

32. Muller-Eberhard, U. and English, E. Purification and partial characterization of human hemopexin. J. Lab. Clin. Med. 70:169, 1967.

33. Muller-Eberhard, U., Javid, J. and Liem, H. H. Plasma concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases. Blood. 32: 811, 1968.

34. Muller-Eberhard, U. and Liem, H. H. Exchange of heme between human hemopexin, human albumin, rabbit hemopexin and rabbit albumin. Fed. Proc. 27:723, 1968.

35. Muller-Eberhard, U., Liem, H. H. and Hanstein, A. Studies of the disposal of intravascular heme in the rabbit. J. Lab. Clin. Med. 73:210, 1969.

36. Neale, F. C., Aber, G. and Northam, B. The demonstration of intravascular hemolysis by means of serum electrophoresis and a modification of Schumm's reaction. J. Clin. Path. 11:206, 1958.

37. Nyman, M. Serum haptoglobin: methodological and clinical studies. Scand. J. Clin. Lab. Invest. 39:1, 1959.

38. Nyman, M. On plasma proteins with heme and hemoglobin binding capacity. Scand. J. Clin. Lab. Invest. 12: 121, 1960.

39. O'Hagan, J. E. The attachment of hematin to human albumin and globulin. Third Congr. Int. Biochem., Brussels, 1955.

40. Perutz, M. F. X-ray analysis of hemoglobin. Science. 140:863, 1963.

41. Polonovski, M. and Jayle, M. F. Existence dans le plasma sanguin d'une substance activant l'action peroxydasique de l'hemoglobine. C. r. Soc. Biol. 129: 457, 1938.

42. Reich, M. The electrophoretic mobility of hemoglobin and its derivitives and the effect of their addition to the serum in various animal species. Aust. J. Exp. Biol. 34:343, 1956.

43. Ross, D. and Muller-Eberhard, U. Pharmological induction of serum hemopexin. Clin. Res. 18:133, 1970.

44. Scheidegger, J. J. Une micromethode de l'immunoelectrophorese. Intern. Arch. Allergy Appl. Immunol. 7:103, 1955.

45. Scheurlen, P. G. Uber serumweissenveranderungen beim diabetes mellitus. Klin. Wschr. 33:198, 1955.

46. Schultze, H. E., Heide, K. and Haupt, H. Charakterisierung von hochgereninig tem hemopexin. Naturwissenschaften. 48:696, 1961.

47. Sears, D. A. Plasma heme-binding in patients with hemolytic disorders. J. Lab. Clin. Med. 71:484, 1968.

48. Sears, D. A. Depletion of plasma hemopexin in man by hematin injections. Proc. Soc. Exper. Biol. Med. 131: 371, 1969.

49. Sears, D. A. Disposal of plasma heme in normal man and patients with intravascular hemolysis. J. Clin. Invest. 49:5, 1970.

50. Sears, D. A. and Huser, H. J. Plasma hematin-binding and clearance in the rhesus monkey. Proc. Soc. Exper. Biol. Med. 121:111, 1966.

51. Smithies, O. Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. Biochem. J. 61:629, 1955.

52. Smithies, O. and Connell, G. E. Biochemical aspects of the inherited variations in human serum haptoglobins and transferrins. Biochemistry in Human Genetics. Boston, Little, Brown, p. 178, 1959.

53. Smithies, O. and Walker, N. F. Notation of serum protein groups and the genes controlling their inheritance. Nature. 178:694, 1956.

54. Sutton, H. E. Human Genetics in Annual Review of Genetics. Annual Reviews Inc. Palo Alto, Vol. 1, 1967.

55. Tavill, A. S., Grayzel, A. I. and London, I. M. The role of heme in the synthesis and assembly of hemoglobin. J. Biol. Chem. 243:4987, 1968.

56. Tombs, M. P., Cooke, K. B., Burston, D. and MacIagen, N. F. The chromatography of normal serum proteins. Biochem. J. 80:284, 1961.

57. Weippl, G., Pantlitschko, M. and Hammelmann, C. Hemopexin in cord blood. Clin. Chim. Acta. 29:338, 1970.

58. Wheby, M., Barrett, I. and Crosby, W. Serum protein binding of myoglobin, hemoglobin and hematin. Blood. 15:1579, 1960.

59. Witz, I. and Gross, J. Purification and partial characterization of mouse hemopexin. Proc. Soc. Exp. Biol. Med. 118:1003, 1965.

60. Work, T. S. and Work, E. Laboratory techniques in biochemistry and molecular biology. Vol. 1. Amsterdam, North-Holland Publishing Co. 1969.

APPENDIX A

Buffer Recipes

pH	Composition		
2.0	50 ml-M HCl	+	51.8 ml-M glycine
2.2	50 ml-M HCl	+	67 ml-M glycine
2.4	50 ml-M HCl	+	86.6 ml-M glycine
2.6	2 gram NaOH	+	684 ml-M formic acid
2.8	2 gram NaOH	+	442 ml-M formic acid
3.0	2 gram NaOH	+	294 ml-M formic acid
3.2	2 gram NaOH	+	203 ml-M formic acid
3.4	2 gram NaOH	+	146 ml-M formic acid
3.6	2 gram NaOH	+	650 ml-M acetic acid
3.8	2 gram NaOH	+	428 ml-M acetic acid
4.0	2 gram NaOH	+	288 ml-M acetic acid
4.2	2 gram NaOH	+	200 ml-M acetic acid
4.4	2 gram NaOH	+	145 ml-M acetic acid
4.6	2 gram NaOH	+	110 ml-M acetic acid
4.8	2 gram NaOH	+	87.7 ml-M acetic acid
5.0	2 gram NaOH	+	73.8 ml-M acetic acid
5.2	2 gram NaOH	+	65.0 ml-M acetic acid
5.4	2 gram NaOH	+	59.5 ml-M acetic acid
5.6	2 gram NaOH	+	56.0 ml-M acetic acid
5.8	2 gram NaOH	+	53.8 ml-M acetic acid
6.0	371 ml-M KH_2PO_4	+	42.9 ml-M Na_2HPO_4
6.2	323 ml-M KH_2PO_4	+	59 ml-M Na_2HPO_4
6.4	267 ml-M KH_2PO_4	+	77.5 ml-M Na_2HPO_4
6.6	210 ml-M KH_2PO_4	+	96.5 ml-M Na_2HPO_4
6.8	157 ml-M KH_2PO_4	+	114 ml-M Na_2HPO_4
7	50 ml-M HCl	+	53.6 ml-M TRIS
7.2	50 ml-M HCl	+	55.7 ml-M TRIS
7.4	50 ml-M HCl	+	59.1 ml-M TRIS
7.6	50 ml-M HCl	+	64.4 ml-M TRIS
7.8	50 ml-M HCl	+	72.9 ml-M TRIS
8	50 ml-M HCl	+	86.1 ml-M TRIS
8.2	50 ml-M HCl	+	107 ml-M TRIS
8.4	50 ml-M HCl	+	141 ml-M TRIS
8.6	50 ml-M HCl	+	194 ml-M TRIS
8.8	50 ml-M HCl	+	279 ml-M TRIS
9	50 ml-M HCl	+	414 ml-M TRIS
9.2	50 ml-M HCl	+	627 ml-M TRIS

(continued)

APPENDIX A continued
Buffer Recipes

pH	Composition		
9.4	2 gram NaOH	+	154.8 ml-M glycine
9.6	2 gram NaOH	+	116.2 ml-M glycine
9.8	2 gram NaOH	+	91.6 ml-M glycine
10	2 gram NaOH	+	76.2 ml-M glycine
10.2	2 gram NaOH	+	66.4 ml-M glycine
10.4	2 gram NaOH	+	60.2 ml-M glycine
10.6	2 gram NaOH	+	56.0 ml-M glycine
10.8	2 gram NaOH	+	53.4 ml-M glycine

All solutions to 1 liter with distilled water.

APPENDIX B

Preparation of Starch Gel

1. 29 grams of powdered hydrolized starch is placed in a dry vacuum flask. To this 250 ml. of buffer is added and mixed thoroughly.

2. This mixture is heated over a low flame of a bunsen burner with constant shaking until it begins to bubble gently (about 10 minutes). The mixture passes from a cloudy watery mixture to a thick viscuous mass which becomes less cloudy.

3. The flask is removed from the heat and degassed with suction for 60 seconds.

4. Immediately following degassing the gel is poured into a gel mold, covered with a siliconized glass plate and allowed to cool.

ILLUSTRATIONS

PLATE 1

Electrophoresis apparatus

PLATE 2

Stained slide following immunoelectrophoresis of two serum samples showing numerous anti-human serum precipitin lines.

PLATE 3

Stained slide following immunoelectrophoresis of two serum samples showing the single anti-human hemopexin precipitin line

PLATE 2

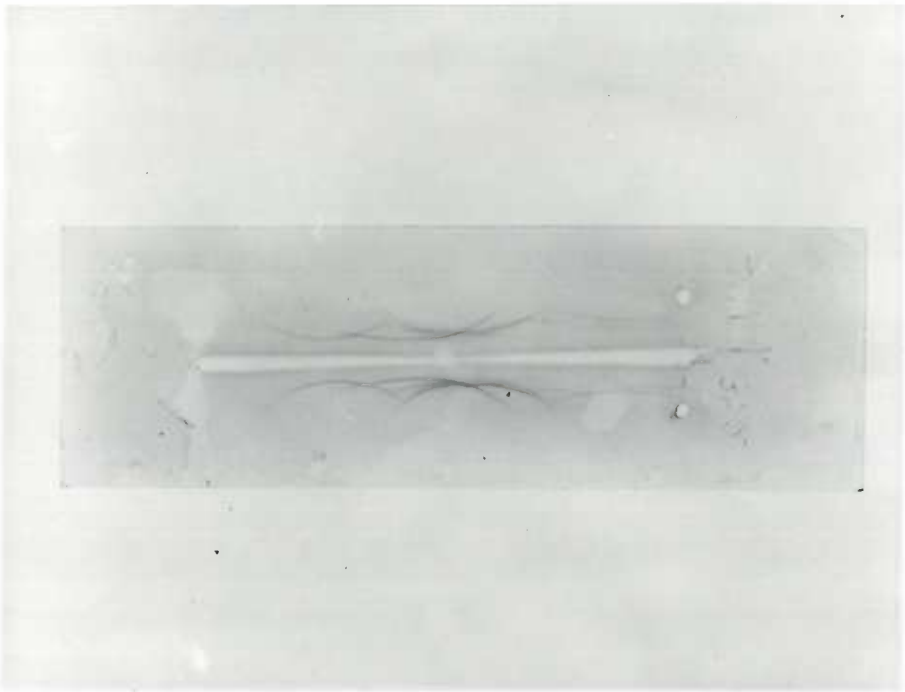


PLATE 3

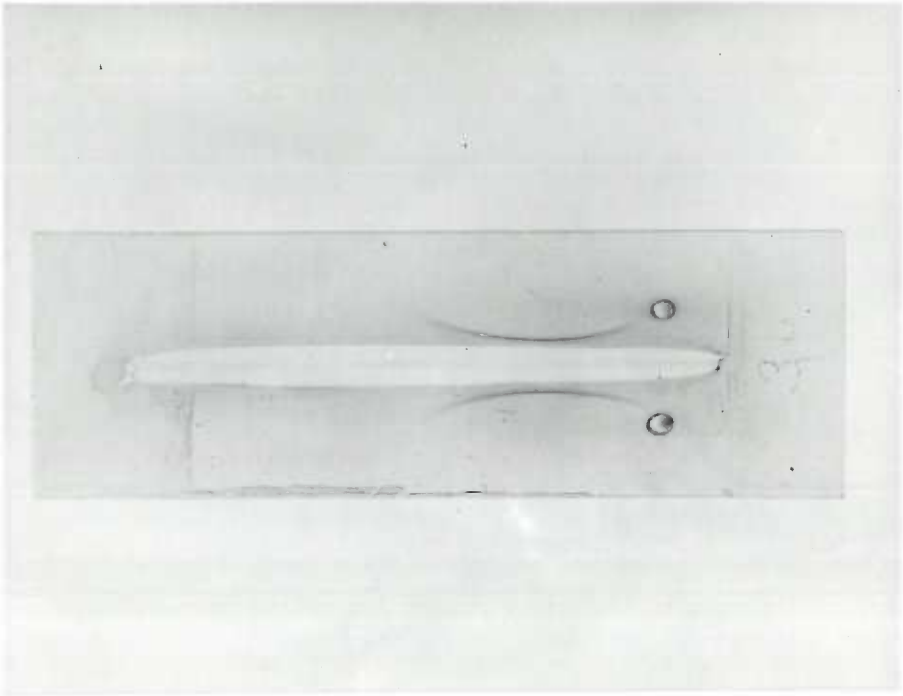


PLATE 4

Radial immunodiffusion gel after staining and drying showing distinct variation in the size of precipitin rings.

PLATE 5

Photographs of starch gels following application of benzidine stain demonstrating the variability in band number and intensity in the hemopexin region.

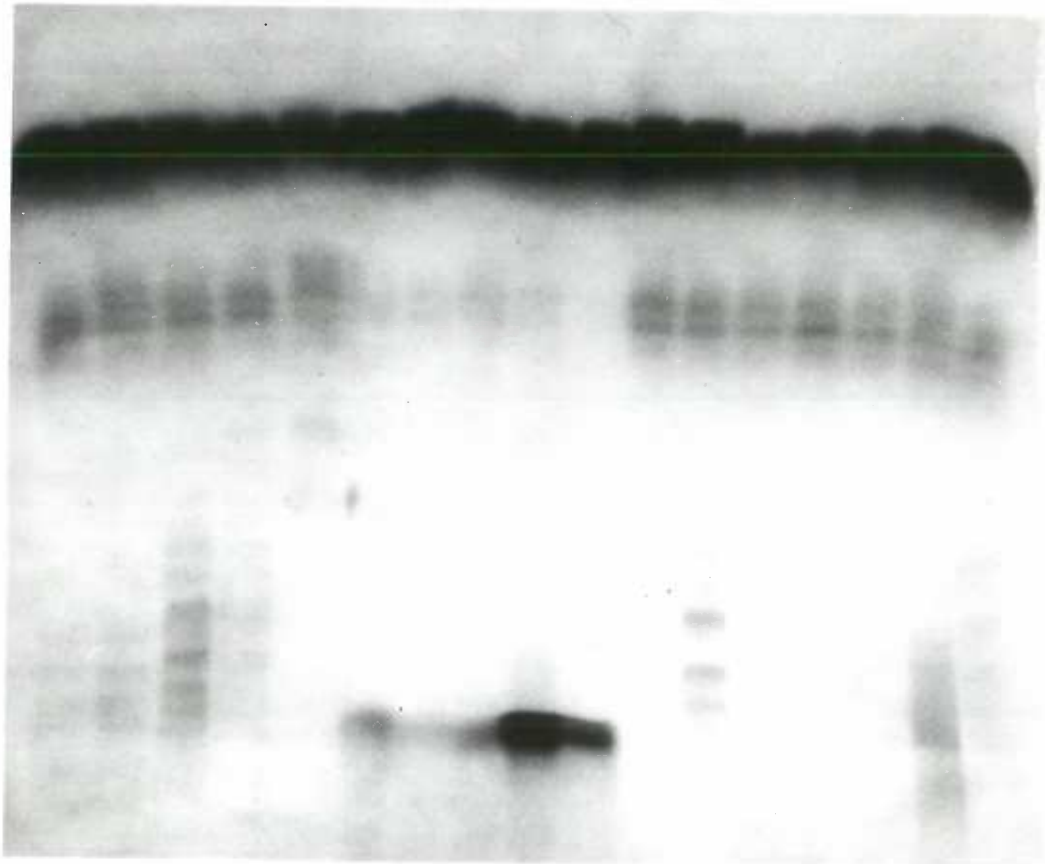
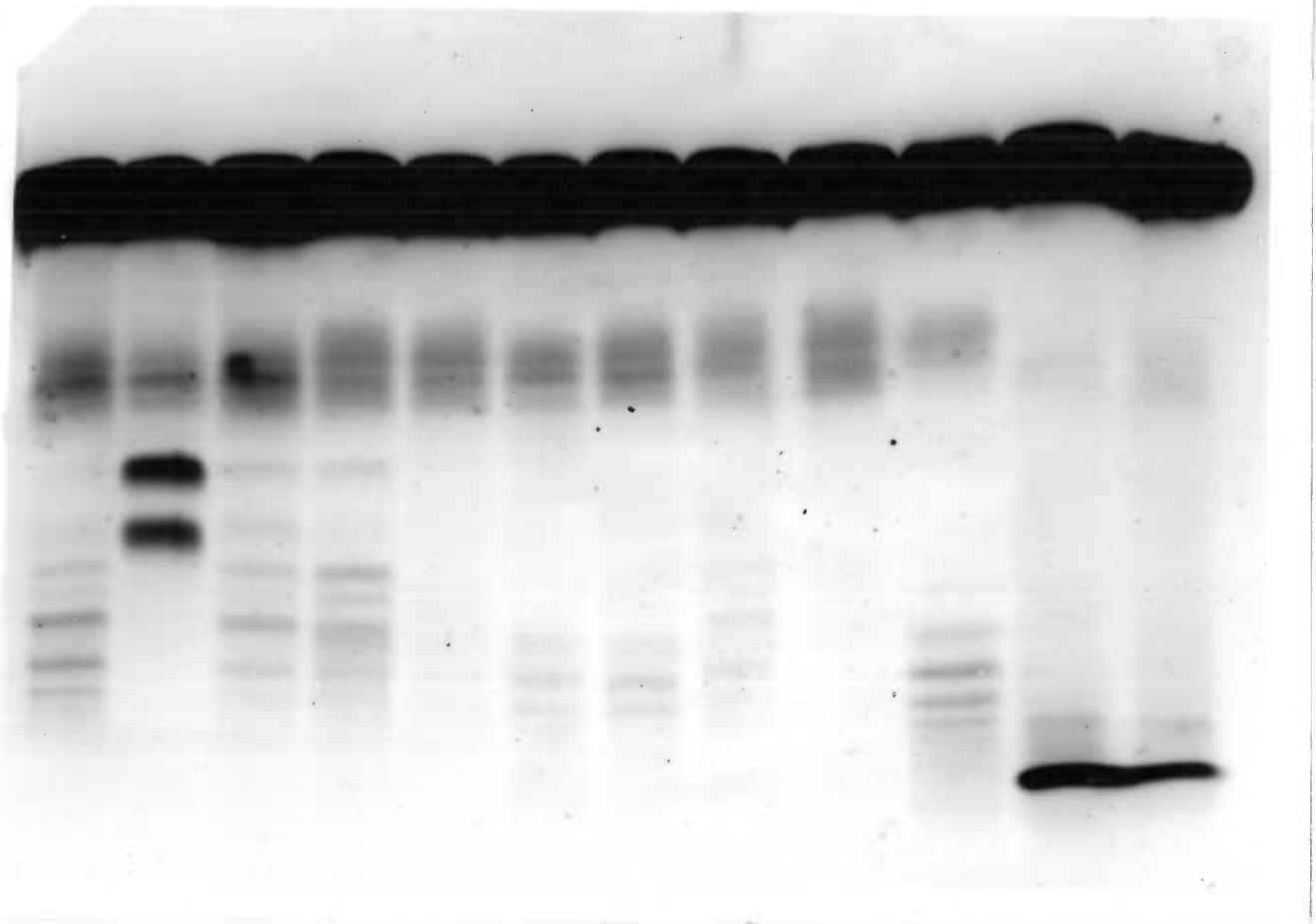
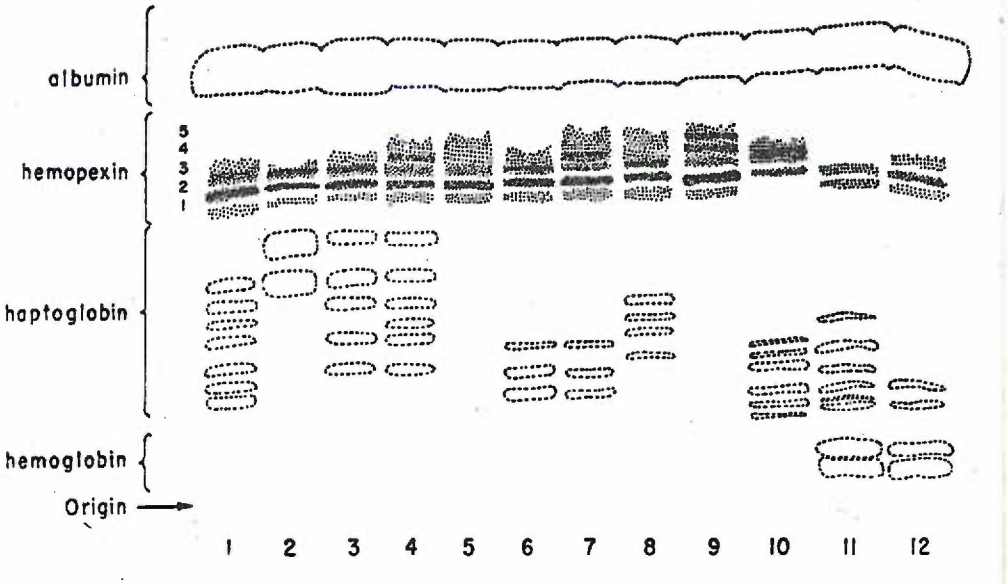


PLATE 6



Stained starch gel following electrophoresis at pH 8.6.
See schematic diagram of this gel in Plate 7.

PLATE 7



Schematic diagram of PLATE 6.