CELL SURFACE CHANGES IN DEVELOPMENT: THE I BLOOD GROUP ANTIGEN IN HUMANS

Thesis by

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-..."Ember:küzdj és bizva bizzál."

Madách, The Tragedy of Man.

-..."but for God's sake also have a good time!"

Zoltán's reply to Madách, 1970.

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ABSTRACT

The I blood group system in humans was investigated in order to study cell surface changes in development.

One advantage in studying the I-antigen in man is the availability of well defined, easily purifiable cold agglutinin molecules with anti-I specificity. Sera from patients with chronic cold agglutinin, post pneumonia cold agglutinin and post viral influenza cold agglutinin disease have been studied. The IgM agglutinins were isolated and their restricted heterogeneity established. These well characterized protein molecules were used in experiments to study the I antigen's development and in experiments designed to reveal information about the molecular basis of I antigen specificity.

The development of I antigen and postnatal changes in hemoglobin were examined in human infants, to see whether the developmental changes in these two attributes are correlated in individual cells in such a way as to suggest that they have common biochemical control mechanisms. The results demonstrated that the expression of I antigen on the erythrocyte surface is independent from the control mechanism of the biosynthesis of the beta chain of hemoglobin. These observations are explained by suggesting two separate modulatory regulations, one for the enzymes involved in the expression of I-specific molecules on the cell surface and another for the regulation of hemoglobin subunit synthesis.

The rates of A-antigen and I antigen site development were compared using I^{125} labeled anti-A IgG molecules in order to test for possible common control or close association. Erythrocytes from postnatal infants were fractionated with respect to their I-agglutinability. Experimental results indicate that cells with well expressed I-specificity have more A-antigen sites; less sites were found on erythrocytes with weak expression of I antigen. These results suggest that enzymes responsible for the expression of these two antigenic specificities are under a common control mechanism or that they are closely associated.

Erythrocyte stromata from human adults and from umbilical cord blood samples were fractionated. Fingerprints of the two membrane preparations indicated that the major protein components are identical. Molecular fractionation of the stomata resulted in fractions with I antigen activity. All fractions with I-activity contained ABO blood activity, but some preparations with ABO activity failed to inhibit agglutinins with I-specificity. Hydrophilic fractions containing glycopeptides with blood group activity were isolated. The I-activity was pronase sensitive. Quantitation of molecular fractions with I blood group activity from cord and adult erythrocytes suggests that I-negative phenotype could be explained by either the two dimensional distribution of molecules on the cell surface or by the covering up of the I-specific molecules in the membrane.

The significance of these findings are discussed with respect to embryonic cell surface specificity and with respect to the recognition of molecular patterns by IgM molecules with multiple binding sites.

TABLE OF CONTENTS

INTRODUCTION

Human blood group antigens

Man's age-old desire to perform blood transfusion as a treatment for various diseases, to expel evil spirits and for rejuvenation led to the foundation of immunohematology. It became a pioneering field to study specific molecules on cell surfaces and their recognition by circulating antibodies.

There are no precise records on the first attempts of blood transfusion, but descriptions from the early seventeenth century already indicated the possibility of transferring blood from one individual to another. In 1615, Andreas Libavius wrote about "the rejuvenation of an old and thin, emaciated man with the blood of a robust young man full of spirited blood (1)". One of the earliest reports on the results of transfusing unmatched blood came from Jean Denys in 1667 (2). Nine ounces of blood was transfused from a lamb into the vein of a young man suffering from "leutic madness". Although the technique was successful, the patient passed urine as black as soot. Incompatability of the red blood cells resulted in complement mediated lysis and release of hemoglobin. Subsequent publications were predominantly empirical observations until KarlLandsteiner's systematic study of red cell agglutination at the turn of this century. He was the first to point out that there are antigenic differences among the

red blood cells of people. The recognition of the ABO blood groups was followed by the development of safe blood transfusion. In 1910 van Dungern and Hirszfeld suggested the Mendelian inheritance of these groups (4), but it was not until the 1920's that the current interpretation of ABO as dependent on a multiple-allelic series was established. Subsequent years led to the discovery of additional blood group antigen systems in man. Today at least eleven well established systems are known with the possibility of discovering more. These systems are the ABO, MNSs, P, Rh, Lutheran, Kell, Lewis, Duffy, Kidd, Diego, Yt and I (5). The antigens of these systems are expressed on the surface of erythrocytes. Some of them also appear on other cells or on secreted molecules of the body fluids.

It seems ironical that after fifty years of experience in matching blood groups prior to transfusion, organ transplantations were performed in the 1950's and early 1960's without sufficient matching of the antigens controlled by the histocompatability locus. These antigens are expressed on lymphocytes and on the surfaces of cells in vital organs. Organ rejection occurs if the donors and recipients are mismatched (6).

The molecular structure of cellular antigens was first studied by inhibition techniques. Monosaccharides and oligosaccharides with known composition were tested for their capacity to inhibit antisera against blood group antigens.

A second approach consisted of chemical and enzymatic modification of the cell surface and examining the effect of such treatment on agglutinability of the cells. Research on the molecular basis of antigenic specificity benefited greatly from the observation that secreted mucopolysaccharides also possess blood group specificity. Most of the biochemical work was carried out on this material, because it is available in large quantities from saliva, gastric mucin, or ovarian cyst fluid. These efforts lead to the establishment of the terminal sugar sequences in polysaccharide chains of glycoproteins which confer A,B and H specificity (7), as summarized on Figure 1.

A-specific chains terminate with an N-acetyl-galactosamine residue and B-specific chains with a galactose residue. The oligosaccharide chains in group O individuals end with α-L-fucosyl-(1-2)-galactose, which also serves as the point of addition for the A and B-specific residues. This chain has the H-specificity. Glycotransferase enzymes, necessary for the biosynthesis of the A and B terminal carbohydrate sequences, are controlled by the ABO locus. α-N-acetyl-D-galactosaminyltransferase, which transfers N-acetyl-D-galactosamine from uridine diphosphate N-acetyl-D-galactosamine to the acceptor oligosaccharide with H specificity, has been shown to occur in submaxillary glands from group A or group AB individuals, but not from group B or group O individuals (8). Similarly the enzyme α-D-galactosyltransferase has been shown to occur in submaxillary glands and in

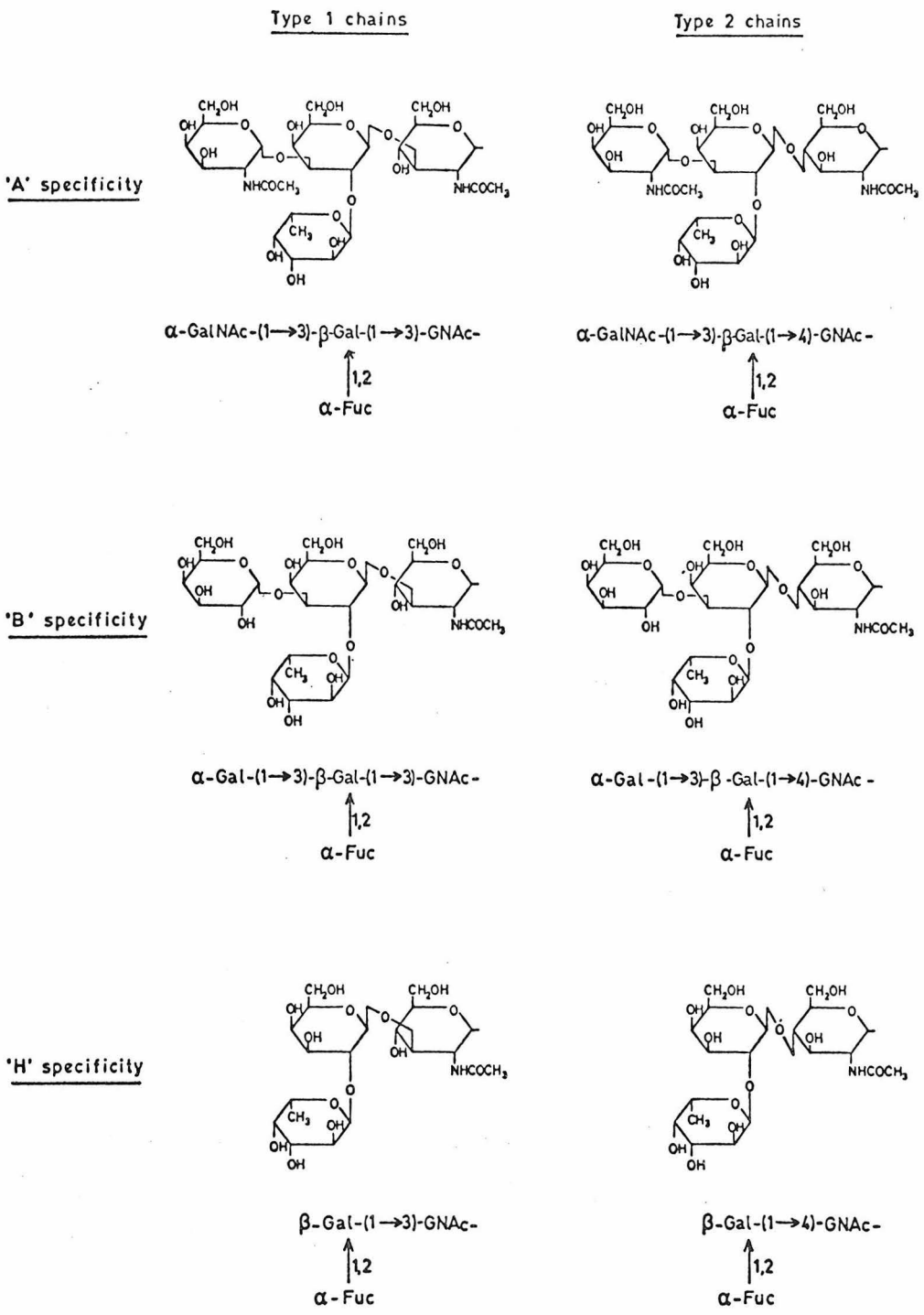


Figure 1. Terminal sugar sequences which confer A,B and H specificity on polysaccharide side chains of glycoproteins. The abbreviations used are: Fuc: L-fucopyranosyl; Gal: D-galactopyranosyl; GNAc: N-acetyl-D-glucosaminopyranosyl; GalNAc; N-acetyl-D-galactosaminopyranosyl. (After Watkins (7)).

gastric mucosal material from group B and AB individuals. This enzyme transfers D-galactose from uridine diphosphate galactose to the terminal nonreducing end of the H-active structure (9). Very little is known about the structure of these enzymes and they have not yet been isolated from erythrocytes. Because the two substrates are almost identical(the only difference is between an OH and an N-acetyl amino group),point mutation or a limited amino acid substitution have been suggested to explain the differences in specificity. The absence of A and B specific molecules in a group O individual might be due to enzyme inactivity as a result of mutation which renders the glycosyl transferase inactive, or due to a complete repression of the enzyme synthesis.

The H gene is responsible for the synthesis of the A and B precurser carbohydrate chain. This locus controls the enzyme which transfers L-fucose to the terminal galactose of the oligosaccharide. Liberation of L-fucose from H reacting substances result in a loss of H specificity. Individuals of the "Bombay" or O_h phenotype do not have any detectable A, B or H activity on the red cells. Homozygosity for an inactive allele h, as suggested by Levine (10), could explain the complete absence of H activity. This locus is independent of the ABO locus. The removal of a galactose residue eliminates the "Bombay" type specificity. This galactose is linked to an N-acetylglucosamine residue either by a β-1,3 or

by a β-1,4 glycosidic bond. The corresponding type 1 and type 2 chains are indicated in Figure 1. The genes controlling the biosynthesis of this precurser oligosaccharide chain have not yet been identified.

The 'Lewis' locus also effects the biosynthesis and hence the antigenic specificity of the water soluble glycoproteins in mucous secretions. Oligosaccharides with Le^a specificity contain an L-fucosyl residue attached in α-1,4 linkage to the penultimate N-acetylglucosaminyl residue in the chain. The carbohydrate chain is the same as that of one of the two types of chains which by addition of an L-fucosyl residue to the terminal galactosyl unit give rise to the H specificity. Le^b specificity is expressed if two L-fucosyl residues are linked to the precurser oligosaccharide; one is attached to the terminal galactose through an α-1,2 and the other through an α-1,4 glycosidic bond in the type 1 precurser molecule (7). One important aspect of the 'Lewis' locus is the lack of participation in the biosynthesis of blood group substances present in the erythrocyte membranes. Lewis specificity is acquired by the cells from the serum unlike the ABO specificity which is under the cells intrinsic control mechanism.

Most of these structural works were done on 'water-soluble' glycoproteins. Structural understanding of molecules carrying blood group specificity in the erythrocyte membrane came from the study of an 'alcohol soluble'

glycolipid fraction (11). In the last two decades large numbers of glycosphingolipids have been isolated and characterized from erythrocytes. These molecules contain various fatty acids, sphingosine, neutral hexose and N-acetylgalactosamine in a molar ratio of 1:1:3:1. Yamakawa, who subsequently named this fraction "globoside", isolated glycolipids which contained neuraminic acid in addition to the usual carbohydrates (12). The currently accepted structure for a typical globoside molecule from human erythrocytes, as suggested by Yamakawa, Nishimura and Kamimura (13), is depicted in Figure 2. Numerous other globosides have now been isolated and characterized. They vary in carbohydrate content; fucose, glucose, galactose,N-acetylgalactosamine, N-acetylglucosamine and sialic acid are present in varying molar amounts. These molecules appear to be specific for blood groups A and B; similar attempts to isolate H-active glycolipids were only recently successful (14). Hakomori and Strycharz were able to purify three different fractions of glucosphingolipids with A blood group activity; they shared a similar basic composition containing glactose, glucose, fucose, N-acetylglucosamine and N-acetylgalactosamine in ratios of 2-4:1:1:1:1. One of these three A-active glycolipids contained a unit or two of sialic acid. Cells from a group A individual with A_2 subgroup specificity contained only two of these glycolipids. The A_2 subgroup is known to have less A antigen expressed on the cell surface

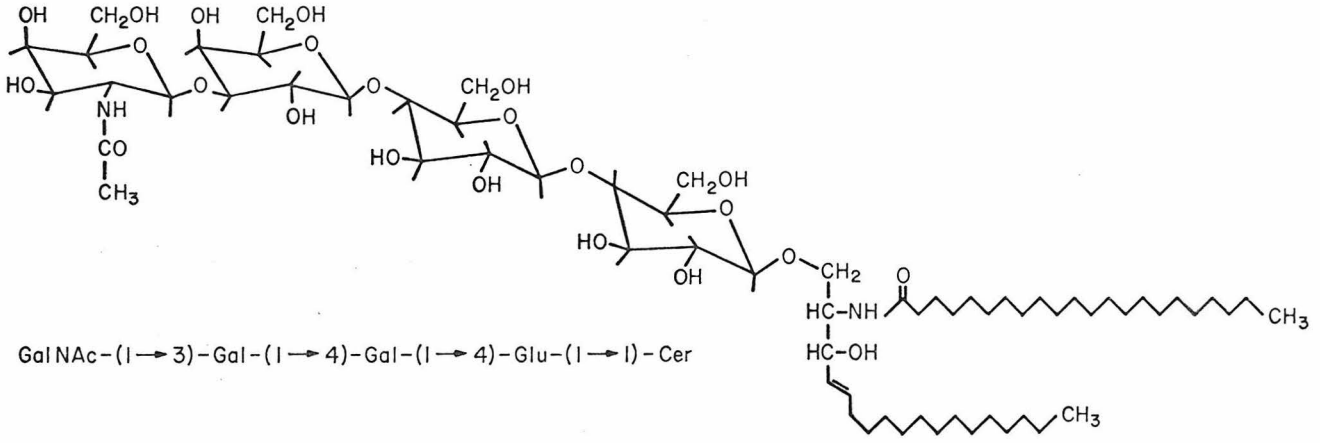


Figure 2. The molecular structure of a typical globoside isolated from human erythrocytes. (After Yamakawa et al. (13)).

than on A_1 subgroup cells (15). This finding of Hakomori suggests that there are qualitative molecular differences in addition to quantitative difference between the two subgroups, as had been earlier indicated by the common existence of A_1-specific antibodies and lectins. 1 mg of glycosphingolipids with A specificity was obtained from five liters of blood. Glycolipids with group B activity were also obtained from erythrocytes in a yield of 1 mg per 30 liters of blood. Only two fractions were found, one of which contained sialic acid. Both fractions were missing N-acetylgalactosamine. One other glycosphingolipid was isolated from group O cells in a yield of 1 mg per 60 liters of blood. This fraction contained glucose, galactose, fucose and N-acetylglucosamine but no sialic acid or N-acetylgalactosamine and was active in the inhibition of both H and Le^b hemagglutination.

The question whether glycolipids are the only fractions carrying A, B and H blood group activity in human erythrocyte membranes has been raised recently. Red blood cell membranes contain approximately 8% carbohydrate by weight; only a fraction of this carbohydrate is present in glycolipids. 35-40% of the total sialic acid of erythrocytes is present as sialoglycopeptides (11). Most of the membrane proteins have carbohydrate attached to them. These glycoproteins have been studied with respect to blood group activity. Zahler (16) demonstrated complete monomerization of stromal proteins

and lipids in 2-chloroethanol. Chromatography of solubilized erythrocyte stromata on LH-20 columns in 95% methycellosolve resulted in a fraction free of lipid, which contained 85% of the membrane protein and most of the stromal sialic acid. This glycoprotein fraction displayed (A and B) blood group activity. Recombination of the glycolipid and glycoprotein fractions resulted in the formation of aggregated which were only slightly less active than the original stromata. MN-active carbohydrate-rich proteins have been also isolated by a 'phenol-extraction method' worked out by Westphal (17). Springer succeeded in the isolation of a homogeneous, highly active MN-fraction (18). Amino acid analyses of these preparations reveal high concentrations of threonine, serine and glutamic acid. The molar proportions of sialic acid:hexosamine:hexose were approximately 1:1:1. Cook and Eylar, in contrast, found a proportion of 1:3:2 in the M-substance and a relation of 2:2:1 in the N-substance (19). Results from enzymatic studies with different neuraminidases allowed the conclusion that sialic acid in a terminal position is largely responsible for the specificity of the M- and N-antigenic site.

Whittemore et al. (20) extracted stromata twice with n-butanol, producing over 80% solubilization of the membrane protein. The initial extract contained 5% of the lipid present in intact stromata. The second extract contained no lipids or glycolipids detectable by thin layer chromatography on silica Gel G using a solvent system of chloroform:methanol: water 56:65:4. The solubilized glycoprotein possessed A, B

and H activity comparable to that of the intact ghosts at the same protein concentration. H activity could not be demonstrated in preparations from group A_1 erythrocytes but was present in glycoproteins prepared from group O and group A_2 erythrocytes; the former exhibited greater activity.

The number of A-specific molecules per erythrocyte surface has been estimated to be 1.2×10^6 A_1 sites per cell (21). Assuming a molecular weight of 1800 for the blood group active glycosphingolipid isolated by Hakomori and Strycharz (14), one would expect to recover 100 or 150 mg of glycolipids from 10 liters of blood if all the antigenic activity were present in this fraction. The reported low yield of 1 mg suggest that other molecules might also be involved and the glycoprotein fraction would qualify best for this role.

Exact information is not yet available on the molecular structure of other blood group antigens. Glycoprotein products of the histocompatibility (H-2) locus have been solubilized from two murine strains (22). Most of the peptides of these glycoproteins from $H\text{-}2^b$ and $H\text{-}2^d$ strains were identical; however, three peptides of the $H\text{-}2^b$ and four peptides of the $H\text{-}2^d$ preparation were unique. The results were consistent with the hypothesis that protein primary structure determine wholly or in part the histocompatibility alloantigenic activities.

We are only at the beginning of membrane protein solubilization and fractionation. The cellular antigen molecules

represent an important class of immunological receptors, and their molecular structure is a key to the understanding of the genetically determined spectrum of individual specificities.

The I blood groups in man

IgM macroglobulins from the sera of patients suffering from chronic cold hemagglutinin disease often agglutinate human adult red blood cells when tested at lower than body temperature. The name "I" was given to the corresponding antigen by Wiener et al. (23). This antigen has a characteristic slow maturation, and an almost universal occurrence. It is a frequent target of 'cold agglutinin type' autoimmune disorders.

Wiener et al. (23) tested erythrocytes from 22,000 random adult individuals with a cold agglutinin serum and found five donors whose red cells were not agglutinated. These adults apparently had very little or no I antigen and their sera contained anti-I isoagglutinins. Erythrocytes which failed to react with anti-I sera were classed as I-negative or i. There are two general types of non reactors: i_1 is usually found among Caucasians while i_2 is usually found among Negroes and only occasionally among Caucasians. In 1960, Marsh and Jenkins discovered a serum later designated as anti-i, with antibodies able to agglutinate I-negative cells preferentially. This would indicate that the absence

of I-specific molecules on the erythrocyte surface is associated with the expression of a different molecular entity with i-specificity.

The relationship between I and i antigens has been studied by Marsh (24), who demonstrated an apparent reciprocal serological relationship on the red cells during the first year of a child's life. He found that infants at birth are of the phenotype i. Erythrocytes of 62 normal infants with ages ranging from two days to four years were tested with anti-I and anti-i sera in parallel. The results demonstrated that shortly after birth the i-reaction becomes steadily weaker, while the I reaction becomes stronger. Normal adult I-i status is established at about 18 months of age. The I-i relationship was also studied by Bingham (25), who made a longitudinal investigation of 127 Negro infants followed from birth through the second year of life. In this study, no evidence was found to suggest a reciprocal relationship between I and i antigens. The gene frequency analysis of the infants, mothers and the mother - child combinations of the proposed i-phenotypes were in agreement with the hypothesis that anti-i identifies an antigen that is inherited as a simple Mendelian autosomal dominant trait. Bingham's results also suggest that the i-antigen of cord red cells is cross reacting but not identical to the i-antigen that appears shortly after birth. These observations are in agreement with the hypothesis that I and i-specificity represent two

blood group systems rather than one, and that the genes responsible for the expression of these antigens are not allelic.

A considerable amount of information exists concerning certain disease states and the I-i antigens (26). The red cells from patients with Thalassemia major react strongly with anti-i sera, just as newborn infants' cells do, but the reaction with anti-I sera is like that of healthy I-positive adults. Similar observations were reported by Giblett and Crookston (27) and by Hillman and Giblett (28) who showed that erythrocytes from patients with hypoplastic anemia, chronic hemolytic anemia and acute leukemia were agglutinated strongly by anti-i sera. They have found no corresponding decrease in I-activity. Their results are indicative of a possible relationship between the intramarrow transition time during erythropoiesis and the amount of i-antigen expressed on red cell surfaces. McGinness et al. (29) observed that in leukemia, the amount of I-antigen was depressed; patients were being misclassified as I-negative. They reported that I expression increased when the disease was in remission. One case of leukemia has been described by Jenkins, et al. (30), in which the I-antigen was depressed and the i-antigen's expression was increased. Observations to the contrary have been published by Ducos (31), who found no apparent difference between normal red cells and those from leukemia patients using and auto-anti-I serum from a normal

I-positive subject. All of these observations were made on the basis of serological techniques, and no molecular studies were conducted to measure the number of antigenic sites per erythrocyte surface. Such a biochemical approach will be necessary because of the uncertainties due to variations in agglutinating capacity among the anti-I sera and the lack of precisely defined anti-i sera.

The molecular structure of I-antigen is not known. Two indirect studies suggest that the specificity is determined by carbohydrates. First, Marcus, et al. reported the action of enzymes from Clostridium terium on the I-antigenic determinant of human erythrocytes (32). Partially purified beta-galactosidase and beta-glucosaminidase were used to modify the stromal surface. This treatment resulted in a decrease in ability to absorb anti-I agglutinins and in the release of galactose and N-acetylglucosamine as monosaccharides. Stromata from erythrocytes of blood group A_1 were altered more rapidly and extensively than preparations from group O cells. Yakulis et al. (33) found that the I-antigen of rabbits and human A_1I erythrocytes was inactivated by α-galactosidase. They tested several galactosides for the inhibition of two different human cold agglutinins with anti-I specificity with no success. These same α-galactosides inhibited experimentally produced rabbit anti-I cold agglutinins. It is difficult to interpret these experiments in terms of the I-antigen's molecular structure; one may only conclude that

the integrity of oligosaccharide chains on erythrocyte surface is necessary for I-specificity.

Numerous serological observations indicate a relationship between the ABO and the I system (5). The first clear example was the finding of an anti-I serum which reacted stronger with A_1 than with A_2 or O cells (34). Since then, numerous examples of sera that react stronger with OI than A_1I cells, or with A_1I than with OI, and sera that react preferentially with BI cells have been reported. The increasing number of reports on the possible ABO and I relationship remained a puzzle, because no secreted I-activity has been reported on water soluble glycoproteins with blood group activity. Recently, Marsh et al. (35) reported the inhibition of anti-I sera by human saliva and by hydatid cyst fluid. The concentration of I-inhibitory substance found in the saliva of newborn infants and i adults was comparable to that found in normal I-positive adults. This indicates that the presence of I-substance in the saliva does not reflect the I status of the red cells. A possible relationship between the P and the I blood group systems has been reported by Jenkins et al. (36) and Issitt et al. (40).

The almost universal occurrence of I-antigen on red cell surfaces indicates a possible structural role in the membrane. In this case topographic vicinity could explain some of the interrelations between I and other blood groups. The very frequent 'public antigens' such as I could have their specificities' expressed on molecules which are structural

entities of the red cell membrane, and therefore their biochemical investigation could reveal important information on the molecular assembly of membranes.

At present there is a difference of opinion with regard to the number of I antigenic receptor sites present on red cells. The number of cold agglutinin receptor sites was estimated to be 32,000 by Olesen (37), using formalin treated I-positive cells. Evans et al. (38) estimated the number of I-receptor sites on human erythrocytes as 500,000,using high titered cold auto-anti-I antibodies labeled with radioactive iodine. Rosse et al. (39) determined the maximum number of anti-I antibody molecules which can attach to human red cell. The values represent the minimum number of antigen sites, because the 19S IgM molecules have five or more available binding sites. As many as 500,000 to 700,000 antibody molecules were observed per normal cell. The surface area of red blood cells was measured and found to be 120 μ^2. Assuming an average number of 3 antigen sites combined with each IgM molecule , and a uniform distribution of antigens on the cell surface, one can estimate that the average distance between I-specific molecules may be 100 A°. It is fair to conclude that molecules with such a surface density have a role in the molecular structure of the membrane.

Agglutinins associated with the I blood group system

Two major types of anti-I agglutinins have been

characterized. One occurs in normal individuals who lack an adequate amount of I-antigen and are i-phenotypes; these have been referred to as 'natural' or isoagglutinins. The other type occurs in I-positive individuals usually following certain infections, and has been called auto-agglutinins(5).

Natural anti-I agglutinins have been found regularly in the serum of I-negative individuals of i_1 and i_2 phenotype. Some variations have been found according to the source of the serum. Anti-I antibody molecules from i_2 donors fail to react with i_1 or i_2 cells. Anti-I agglutinins from i_1 phenotype donors do react weakly with i_2 cells. These observations are analogous to the agglutinins of the ABO subtypes, where antibody molecules directed against the A antigen from some A_2 individual are able to agglutinate A_1 cells.These agglutinins are 19S IgM molecules, which are particularly sensitive to the antigen density on the cell surface. Anti-I cold agglutinins have been reported by Adinolfi (41) in a high percent of umbilical cord sera. It is not clear whether these agglutinins are produced in response to maternal I antigen in utero. They behave as 'naturally-occurring' IgM molecules and are of fetal origin. 19S anti-I agglutinins do not cross the placenta; none could be detected in the sera or on the cells of the normal babies of an i_1 or of and i_2 mother (5).

Auto-anti-I agglutinins have been found in the sera of patients suffering from acquired hemolytic anemia of the cold antibody type (26). Cold agglutinins are antibody molecules which agglutinate erythrocytes at lower than body

temperature. These immunoglobulins are synthesized in response to infections by any of a large number of microbial agents. Specific cold agglutinins have been found in connection with Lewis and the P system. Sera which were earlier identified as "nonspecific chronic cold auto-agglutinating" contained anti-I IgM molecules; Jenkins et al. (36) found that all of 50 patients with cold agglutinins had anti-I antibody.

Cold agglutinins with I-specificity are present in the sera of some patients with "primary atypical pneumonia", an upper respiratory tract infection associated with a group of Pleuro-Pneumonia-Like-Organisms (PPLO). The question arises whether these antibodies are produced as a result of biochemical alterations on the host's red cell surface or are directed against an antigen similar to the I-antigen present on the surface of PPLO or viral agents. Schmidt et al. (42) reported that 18 of 25 Mycoplasma (PPLO) strains destroyed the I-receptors of normal red cells after in vitro incubation. This observation could not be reproduced by Altucci et al. (43) who concluded that the interaction of Mycoplasmas and erythrocytes has no effect on the expression of I-antigenicity.

Feizi, et al. (44) found no evidence that the cold agglutinins have antibody activity against Mycoplasma pneumoniae, indicating a lack of I-antigen on the surface as detected by serological techniques. It is possible that the antigen is present on or in the organism in amounts too small

to be detected, but still responsible for the anti-I agglutinin response. The organisms were grown in vitro for these experiments and it is also possible that the I-antigen was lost as a result of culture techniques. Such changes in surface antigens have been reported on chick embryo cells grown in culture. An alternative explanation would be that M. pneumoniae during in vivo infections alters the I-antigen and renders it "autoantigenic". This could be brought about by enzymatic alteration or by hemolytic activity. Evidence in favor of such a mechanism has been reported by Feizi et al. (45), who claim that the reaction product of I-antigen with M. pneumoniae, rather than the microbial organism alone, may be responsible for the production of cold agglutinins in man.

Experimental production of cold agglutinins in rabbits has been reported by Costea, Yakulis and Heller (46). Rabbits with I-positive erythrocytes have been injected with heat-killed Listeria moncytogenes (HKLM), a PPLO-like organism, isolated from a patient with Listeria monocytogenes septicemia and cold agglutinin hemolytic anemia. The cold agglutinins produced by the rabbit were I-specific, with high agglutination titers for autologous and allogeneic rabbit erythrocytes and human adult cells. Newborn rabbits' erythrocytes and human cord cells were only weakly agglutinated by the rabbit cold agglutinins. The authors found that absorption of the immune serum with HKLM at 37°C removed all the

I-specific agglutinating activity - an important finding which suggests that the erythrocytes and the microbial agents share cross-reacting antigens. The antigens from HKLM bind antibody at body temperature while the erythrocyte-associated antigens react only at lower than body temperatures. The same investigators recently reported the results of experiments in which live and formalin or phenol treated suspensions of M. pneumoniae were injected into I-positive rabbits and I-negative CF_1 mice. They observed high titers of I-specific cold agglutinins in both species within one week after immuization. Although these cold agglutinins from humans, rabbits and mice have anti-I specificity, their affinity for oligosaccharide ligands differ as determined by inhibition studies with various model galactosides. The results of these experiments indicate that I-specific cold agglutinins are produced in rabbits and mice in response to M.pneumoniae or Listeria monocytogenes which cross-react with the I-erythrocyte antigen system, and their production does not require steric or structural modifications of the I-antigen of the red cells (47).

Recently, Costea et al. (48) reported the occurrence of cold reacting antibody in NZB/B_1 mice. This inbred strain of mice develops hemolytic anemia of varying severity. Anemia appears at an age of six to nine months among the males and around twelfth month in the females. Affected mice show reticulocytosis, splenomegaly, jaundice, anemia and elevated

antibody titer. This autoimmune condition is inherited but its pattern of inheritance is not simple. The main manifestation of the disease is Coomb's-positive hemolytic anemia with significant similarities to human autoimmune conditions. NZB/B_1 mice throughout life produce cold reacting antibodies with specificity relating to the I-erythrocyte antigen in men. Mouse erythrocytes do not carry the I-antigen, but it is widely distributed in several tissues. Newborn mice from certain strains, such as CF_1, produce cold reacting antibodies when injected with cell-free filtrates from NZB/B_1 mouse spleen. The untreated offsprings of these injected animals also synthesize this type of antibody, suggesting that the immune response is induced by an infectious agent.

High incidence of cold autoagglutinins with anti-I specificity has been reported by Layrisse (49) among the Yanomama Indians of Venezuela. These cold agglutinins react with cells having intermediate I-antigen density. 84% of Yanomama Indians had these autoagglutinins, in contrast with the Warao Indians who had none. These two tribes inhabit similar tropical areas in the Orinoco Delta of Venezuela, where protozoon, bacterial and viral agents have about equal chance to disseminate among the people. These tribes come from one ethnic group, which split up about 4,000 years ago as indicated by blood group frequencies and each represent an inbred population. Genetic implications of the distribution of cold agglutinins have been discussed by Layrisse.

Recently Bloom et al. (50) reported a high frequency of complex inter- and intrachromosomal aberrations in the leukocyte of Yanomama Indians. It is not clear that these observations reflect a natural condition, rather than a technical artifact; there is reason to believe that the chromosome breakage occurred in cell culture, and may reflect an infection. High incidence of cold agglutinins directed against the I-blood group system has been reported in a Melanesian population living in a climate similar to that of the Orinoco Delta in Venezuela (51). Serologically, the I-positive erythrocytes from Melanesians and Caucasians are identical with respect to their I reactions. Neither the Chinese or the Caucasians who live in the same tropical environment have a high frequency of these cold agglutinins. This observation again implies a possible genetic basis for the disorder.

The molecular structure of cold agglutinins with anti-I specificity has been studied extensively. These autoagglutinins belong to the 19S IgM macroglobulin class. The molecules consist of five identical subunits held together by disulfide bonds. Each subunit has two light and two μ type heavy chains (52). The transient cold agglutinins, defined as antibodies produced as a result of microbial infection, are heterogeneous molecules containing both K and L types of light chains (53). Their binding specificity varies, and only a fraction of the antibody population has anti-I specificity.

These cold agglutinins resemble other anti-blood group antigen antibodies. However, cold agglutinins isolated from the sera of patients with chronic cold agglutinin hemolytic anemia contain only type K light chains (53,54). Harboe et al. studied 37 cold agglutinins and found exclusive occurrence of K type light chains. Feizi reported three IgM preparations from patients with chronic hemolytic anemia, which contained light chains of the L type. Only one of these preparations had anti-I activity (55). On the basis of restricted electrophoretic heterogeneity of light chains, and single amino acids observed at the amino-terminus, Cohen and Cooper (56) suggested that the proteins are of monoclonal origin. This would imply that the antibodies are homogeneous, like the myeloma type proteins. However, such conclusions need more careful consideration. Chemical differences among cold agglutinins from individual humans were also reported by Cohen and Cooper. Both heavy and light chains varied as much in amino acid composition as do myeloma proteins of a given type, chosen at random. The light chains had Asp or Glu as N-terminal amino acids. The chemical individuality of cold agglutinin μ-chains was also shown by differnces in hexose, fucose and glucosamine content.

The described restricted heterogeneity of cold agglutinins is similar to the antibodies from antipneumococcal sera as reported by Jaton et al. (100). Antibodies of sufficient

homogeneity for sequence studies were isolated in high concentrations from rabbits immunized with pneumococcal vaccines. A unique sequence for the N-terminal 11 amino acid residues of the light chains indicate a great restriction of heterogeneity. Unequivocal indication of homogeneity, however, will only be obtained through complete amino acid sequence determinations.

Changes of the erythrocyte surface during development.

One of the important aspects of the I blood group system is the slow appearance of the I-antigenic specificity. Fetal erythrocytes have i-phenotype, which gradually changes into an I-positive phenotype during the period of one to two years after birth. The erythrocyte surface without the I-antigen therefore appears as "embryonic-like", and the molecules exposed on this surface could be referred to as "embryonic"-antigens. The antigen molecules do not have to be structurally different; the change in specificity could be a function of a changed topographic arrangement.

The development of the ABH antigens of the red cells has been studied by numerous investigators (5). The A and B antigens are detectable long before birth. The A antigen was found in a 37 day old fetus. A difference in the number of antigenic sites in the adult and cord cell surface, has been reported by Economidou et al. (21). The number of A-antigen sites was estimated as between 2.5 and 3.7×10^5 on A_1 cord red cells, and between 8.1 and 11.7×10^5 on adult red cells. Only a twofold difference was found between A_2 adult and cord cells. A similar increase in the number of B-antigenic sites has been reported (21). A difficulty in typing cells from the umbilical cord is in the determination of subgroup types, because A_1 cord cells frequently type as A_2-like cells. This observation further substantiates the suggestion, discussed earlier in this thesis, that the

difference between the two subtypes may in part by the number of A-specific molecules on the cell surface. Type-A cord cells also have detectable H-antigen on their surfaces, which fits with the suggestion that incomplete oligosaccharide chains are responsible for the lower number of A-antigenic sites. It is not known whether these oligosaccharides are incomplete because of the shortage of enzymes responsible for their biosynthesis or because of a shortened marrow transition time.

Antigens of other blood group systems are well expressed at birth. Very interesting exceptions have been reported by Reed and Milkovich (57), who have tested the accuracy of blood grouping of cord blood specimens with special reference to the ABO, Rh, MNSs, Kell, Duffy, Lutheran and P system. After testing 9,293 individuals at birth and after an age of five years they found in both Negroes and Caucasians a very high frequency of false negatives in the cord blood grouping in the P system and a very significant occurrence of false negatives in Lu^a grouping. These findings indicate that the molecules responsible for these antigenic specificities are not fully expressed on cord erythrocytes. It is interesting to note that these same antigens, together with the I antigen, are almost the exclusive targets of cold autoagglutinins (26). The development of these antigens, or the establishment of the surface molecular distribution characteristic of a mature adult cell, may take place at the time when self-tolerance is established. It is possible that the high

incidence of autoimmune disorders directed against these antigens is related to their immature development or expression at an early age.

Antigenic differences between fetal and adult erythrocytes have been reported in other species as well. For example, in cattle Stormont et al. (58) reported that the J-antigen was missing on the erythrocytes of embryos from J-positive dams. This antigenic specificity is acquired later by absorption of a water soluble substance from the blood plasma.

An antigen on the red cells of young chickens that is not detectable on the red cells of adults was found by Owen and Mallek (59), using a hemolytic test system. Sanders (60), who further investigated this observation, reported that the antigen is present on all red blood cells at hatching. Their disappearence starts about 50 days after hatching and the antigen is undetectable by 120 days of age. Studies aimed at elucidating the mechanism of the developmental antigenic change suggested that the antigen was lost during development rather than coated by a serum component, a phenomenon noted earlier by Sanders with respect to a developmentally changing antigen on the red cells of brown trout (97).

The development of antigens controlled by a histocompatibility locus has been studied by several workers, including Klein (61) and Gold and Freedman (62). The H-2 antigens in mice were detectable only immediately before birth. The

amount of antigens increased rapidly after birth and reached adult strength in a few days. The answer to the question of the presence or absence of H-2 antigens in early embryonic tissues depended on the method used for their detection. The method of hemaglutinin absorption gave a negative answer while indirect techniques provided evidence for the presence of residual amounts of antigens. The antigen density on cell surface increased slowly during early embryonic stages but a very rapid increase was observed immediately before and after delivery. Embryonic cells, cultured in diffusion chambers in an isologous adult host, exhibited a striking increase in the antigen expression which coincided with the period of a hypothetical delivery of the fetus. The results suggest that the embryonic cells retained their own "calendar" of antigenic maturation.

Additional developmental aspects of normal and abnormal cell surface genetics have been recently reviewed by Boyse and Old (63). The particularly interesting TL antigens in mice are controlled by the Tla locus. All mice, except those of thymocyte phenotype Tl.1,2,3 carry repressed Tla structural genes which are derepressed in the event of leukemia. These antigens, detectable on embryonic tissues, are expressed only on normal thymocytes in the adults. Three things are necessary for the expression of TL.1 antigen in normal thymocytes: a structural gene which all mice possess, the allele for the expression of TL.1 and an inducer of TL synthesis. This inducer is presumably present in

the thymus. Only in the event of thymic leukemia are there cells outside of the thymus with TL+ antigens. For example, thymocytes of C57BL/6 strain of mice are then atypically TL-. In the leukemic cell due to a genetic derepression the phenotype becomes identical with the genotype and the cells become TL 1,2,-,4. Repression is re-established in the presence of anti-TL antibody, and the cellular phenotypes can therefore be "modulated" by the experimenter. The molecular mechanism responsible for this antigenic modulation is not yet understood.

The appearance of embryo-specific molecules in patients with primary cancer of the liver has been reported by Tatarinov et al. (64). This phenomenon was first observed in mice and rats with transplantable hepatoma. Animals with the tumor produced an embryonic alpha-globulin, a specific antigenic constituent of fetal serum. Adenocarcinomas have also been reported to produce "carcinoembryonic" antigens, found in the serum of patients with cancer of the colon and rectum (65). Similar constituents were found in normal embryonic and fetal gut, pancreas and liver during the first two trimesters of gestation. It is interesting to note that most of these patients have autoagglutinins with anti-i specificity. The I-negative or i-specificity can be considered as "embryonic specificity"; it would therefore be interesting to find out if the carcinoembryonic antigens in the serum are glycoproteins with I blood group system activity.

The change from I-negative to I-positive red cell specificity takes place about the same time as does the change from fetal to adult hemoglobin inside the cells. A schematic representation of the erythropoietic system's development is illustrated in Figure 3. This simplified diagram is based on the currently accepted understanding of the major events in the differentiation of hematopoietic stem cells, as summarized at the XII Congress of the International Society of Hematology (66).

Five major classes of polypeptide chains make up the hemoglobins of a human individual at various stages of his development. The chains, designated as α, β, γ, δ, and ϵ, account for the different species of hemoglobin. Dimers of the ϵ chain in equilibrium with the tetramer form are referred to as Hb $Gower_1$. This is the first hemoglobin to appear, it is synthesized by nucleated cells differentiated from the yolk sac. During the first month of gestation the cells begin to synthesize α chains. Hb Gower 2 consists of two ϵ and two α chains. Betke (67) claims that cells of the same organ, in a later stage of development, begin to synthesize fetal hemoglobin, which consists of two γ and two α chains. Migrating stem cells appear in the liver and the spleen around the third month of gestation. Fetal hemoglobin is the predominant product in the erythrocytes derived from erythropoietic differentiation in liver and spleen. Around the fifth month of embryonic development, the site of

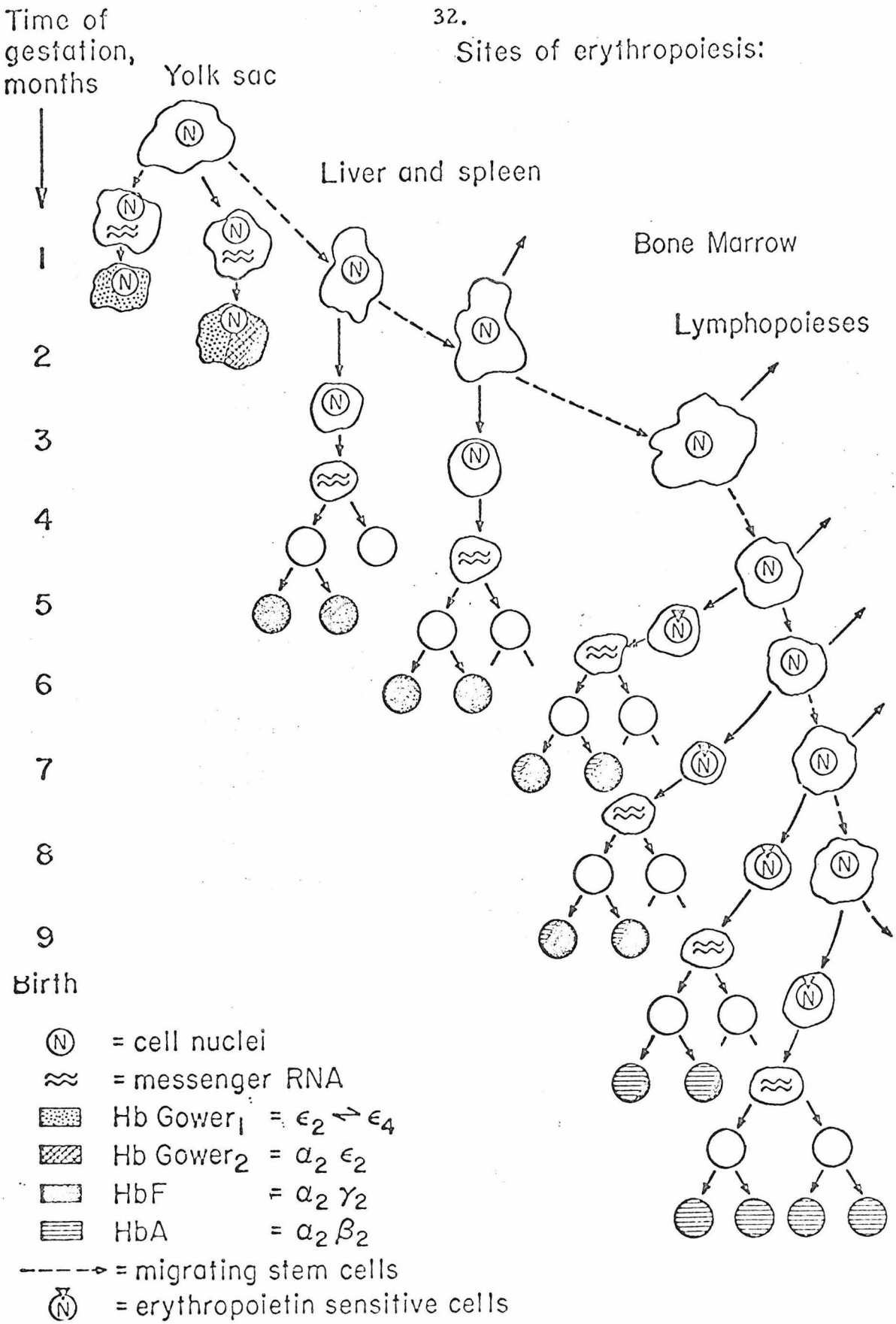


Figure 3. The development of the erythropoietic system.

erythropoiesis changes gradually to the bone marrow. Presumably stem cells of the same organ populate the bone marrow, and by the seventh and eight months of gestation the marrow becomes the major site of erythropoietic activity. About this time the biosynthesis of adult hemoglobin starts, but active synthesis of fetal hemoglobin has been reported in the bone marrow. These results indicate that there is no limitation of the synthesis of a certain type of hemoglobin to a certain hematopoietic site. It is believed that common stem cells give rise to the lymphopoietic proliferation. Figure 3 also indicates the presence of a so-called "early differentiated" cell population in the bone marrow, which is sensitive to specific controllers such as erythropoietin.

In normal adults, the bone marrow is the only site of erythropoiesis. Under pathological conditions in which there is a failure in hematopoiesis in the bone marrow, such as hemolytic anemias, the spleen and liver may again become centers of erythroid cell development during adult life (68).

Erythroid differentiation in fetal mice follows a similar pattern, with the exception of adult hemoglobin synthesis. Nucleated erythrocytes originating in the blood islands of the yolk sac appear during days 8-12 of gestation and synthesize embryonic hemoglobin predominantly (69). A second population of non-nucleated erythrocytes originating in the liver

is present from 12-18 days of gestation and synthesizes predominantly adult hemoglobin.

The sequential derepression and repression of the non-allelic genes responsible for these hemoglobin chains represent an attractive system to study the regulation of protein synthesis during embryological development and tissue differentiation. Several inherited defects have been studied as they effect the biosynthesis of the polypeptide chains of hemoglobin. Among the most extensively studied defects are those collectively known as the thalassemias. They appear to be determined by a series of distinct abnormal genes, in different heterozygous and homozygous combinations. Thalassemias have been classified according to the polypeptide chain primarily concerned in causing hemoglobin deficit. Thus in the so-called beta-thalassemias, the main defect appears to involve beta chain synthesis, whereas in alpha-thalassemias alpha-chain synthesis is primarily affected. It is probable that a number of different mutant genes can cause this type of abnormality and although they each result in the repression of one of the polypeptide-chain syntheses, the degree to which this occurs appears to vary considerably from one gene to another (70). It is interesting to note that in both beta and beta-delta- thalassemia, fetal hemoglobin synthesis increases and is unevenly distributed in the erythrocytes. This observation is in contrast with the observation of even distribution of fetal hemoglobin in

individuals with hereditary persistent fetal hemoglobin disorder. These observations indicate that different non-structural genetic loci are involved with the regulation of hemoglobin subunit biosynthesis. Although the exact molecular mechanisms of these regulatory events are not yet understood, the system provides an excellent approach for the study of regulation of protein synthesis during development.

OUTLINE OF THE RESEARCH PROBLEMS INVESTIGATED

The development of I-antigen and postnatal changes in hemoglobin were examined in human infants, to see whether the developmental changes in these two attributes are correlated in individual cells in such a way as to suggest that they have common biochemical control mechanisms. Similarly, the rates of A-antigen and I antigen site development were compared, to test for possible common control or close association.

Experiments were performed to modify the surfaces of erythrocyte stromata with enzymes and chemical reagents in order to obtain information on the possible molecular basis of I antigenic specificity.

Stromata from human adult I-positive erythrocytes were fractionated in order to isolate molecular fractions with ABO and I specificity and to deal quantitatively with the molecular species responsible for blood group activity. Molecular fractions from the erythrocytes of postnatal infants were also compared for ABO and I specificity.

One advantage in studying the I-antigen system in man is the availability of well defined, easily purifiable cold agglutinin molecules with anti-I specificity. These IgM molecules have restricted heterogeneity and their molecular structure can be studied by standard methods of protein chemistry. Sera with anti-I agglutinins were studied from

various patients with chronic cold agglutinin disease, postpneumonia cold agglutinin disease and post viral influenza cold agglutinin disease. IgM cold agglutins were isolated from several of these sera and their restricted heterogeneity established.

These well characterized protein molecules were used in experiments to study the I antigen's development in postnatal infants.

MATERIALS AND METHODS

Characterization of anti-I agglutinins

Anti-I sera were collected for several years from numerous sources and screened routinely for cold agglutinins and specificity. Standard methods of immunohematology were performed as described in the Reference Manual prepared by Hyland Laboratories (71), and as outlined by Campbell et al. (72). All reagent red blood cells and standard anti-blood group sera were obtained from Hyland Laboratories, Los Angeles. Serum concentrations of immunoglobulins were determined using the Immunodiffusion Plates of Hyland Laboratories.

Agglutination was performed with the Microtiter System developed by Cooke Engineering Company, Alexandria, Virginia. Stainless steel loops were used for serial dilution, except in inhibition studies where the surface tension of the solutions tested for inhibition varied considerably. Glass micropipets were used for dilution of stromata suspensions, and the agglutination was carried out in glass test tubes. Each test was carried out in triplicate; in case of marginal agglutination strength 'blind-reading' by an additional individual was obtained.

Cold agglutinins were incubated at $4^{o}C$, unless otherwise specified, and the agglutination strength was determined three times, after one, four and twelve hours of

incubation. The strength of agglutination was scored as follows:

Assigned agglutination stength:	Description:
4+	Agglutinated clumps of cells that do not disaggregate by shaking the sample.
3+	Large clumps of cells with a tendency to dissociate.
2+	Large clumps with loose attachment to each other.
+	All cells are present in clumps which are small but visible. The agglutinated cells sediment rapidly under gravitation.
≠	Weak positive reaction, still visible without the use of a microscope.
-	Negative reaction with easily dissociable single cells.

In all the experiments, only the macroscopically visible agglutination was scored as positive. Agglutinated small cell clumps as observed under the microscope were scored as negative in order to avoid unnecessary questionable interpretations. Evaluation of agglutination strength is not precise, but the use of control cells and serial dilutions of antisera make it applicable for scientific investigation.

Serum from a patient (COH) with postpneumonia cold agglutinin disease was a gift from Dr. J.L. Fahey of the City of Hope. This patient was an 80-year-old Caucasian

woman with a long history of pneumonia; her white cell count was normal but a bone marrow examination was consistent with a diagnosis of hemolytic anemia.

Two sera from patients MAT and HOV, with chronic cold agglutinin disease, became available through the courtsey of Dr. R. Evans of University of Washington, Seattle, and of Dr. E. Shanbrom of Los Angeles. Serum from an I-negative Caucasian boy with thalassemia minor with the gift of Dr. B. Myrie from the Blood Bank of the Los Angeles Red Cross. This patient had numerous operations for congenital glaucoma. His sister's erythrocytes were also I-negative in phenotype. Serum from patient AB3 was also a gift from the Blood Bank of the Los Angeles Red Cross. This patient developed a high titer of cold agglutinin with I-specificity following a viral influenza.

Microdroplet cytotoxicity tests were used to detect antibodies directed against antigens present on lymphocytes (73). Lymphocyte suspensions were incubated with the cold agglutinin sera for one hour at 4°C. Complement was added and the samples were further incubated for an hour at 4°C followed by incubation at 17°C for an additional 30 minutes. Lymphocytes were stained with eosin and the numbers of living and dead lymphocytes were scored under a phase contrast microscope.

Isolation of IgM cold agglutinins was carried out according to the methods described by Miller et al (52), Lamm et al. (74) and Chaplin et al. (75). Combinations of different

purification steps were used in order to obtain the highest yield and the least amount of contamination by other serum proteins. These steps included gel-filtration, ion exchange chromatography, sucrose density gradient centrifugation and starch gel electrophoresis as described by Kunkel (76). Additional purification and protein concentration were achieved by using ultrafiltration on Diaflow membranes (Amicon Corp.). Lyophilization was avoided whenever possible because the macroglobulin preparations were often difficult to redisolve after freeze-drying. The extent of purification was examined in successive steps by means of immunoelectrophoresis and acrylamide gel electrophoresis in 8M urea at pH 2.7. The 19S IgM macroglobulin preparation was reduced and alkylated in 5M Guanidine hydrochloride, using dithiothreitol for reduction and ethylanimine for aminoethylation.

The heavy and light chains of the cold agglutinin molecules were separated on Sephadex G-200 columns in 5M guanidine hydrochloride. The amino terminal sequences of the light chains were determined according to Edman (77) using an automatic Protein Sequenator.

The development of the I antigen

Cord blood samples were obtained through the courtesy of Mrs. E. Prestegard of the Pasadena Clinical Laboratory. Erythrocytes were washed from the clot with buffered saline. The washing procedure was repeated three times at 37°C in order to remove any possible fetal cold agglutinins absorbed

to the cell surfaces. All samples were studied within 24 hours after birth.

Blood samples from postnatal infants were obtained through the courtesy of Dr. J. Wilkins of the U.S.C. Medical Center in Los Angeles. Blood was allowed to clot for four hours. Cells were washed out of the clot at 37°C and the samples were studied within 24 hours of their drawing.

Erythrocytes were fractionated with respect to their I-agglutinability. Cell suspensions (2%) were mixed with equal volumes of different dilutions of previously-characterized anti-I cold agglutinin sera. The samples were incubated at 4°C for two hours and the agglutinated cells were subjected to repeated sedimentation in a clinical centrifuge. Centrifugation for 30 seconds at 1,000 rpm sedimented all the agglutinated cell clumps. The suspended single cells were removed and the agglutinated cells were washed and resedimented several times in cold buffered saline. The degree of fractionation was followed by microscopic observation. Unagglutinated cells were counted in a hemocytometer. The agglutinated cells were designated as "I-positive" in contrast with the single cells, which were called "I-negative". It must be kept in mind that this nomenclature is only for convenience, to describe cells which agglutinate or fail to do so under the conditions applied; the terminology is not intended to imply the absence or the presence of the I antigen on two mutually exclusive categories of cells.

The amount of fetal hemoglobin was determined by the method of Singer et al. (78), using alkali denaturation, and by ion exchange chromatography as developed by Jones and Schroeder (79). The hemoglobin content of single erythrocytes was studied by the microspectroscopic technique of Matioli et al. (80). A Zeiss microscope furnished with an apochromatic condenser, an apochromatic objective (60X N.A. 0.85) and a compensatory ocular (8X) lens was made available by Dr. G. Matioli of U.S.C. Medical School. Fresh blood samples were smeared on a quartz slide (Leitz) and air dried. The samples were fixed first in methanol and later in 80% ethanol. The optical absorption of 100 randomly chosen single cells was determined for every sample. Adult hemoglobin was extracted from the fixed erythrocytes by immersing the slide in a buffer of 0.2 M Na_2HPO_4 + 0.1 M citric acid, pH 3.30, for 20 seconds. The extracted samples were washed with deionized water and air dried. The unextracted hemoglobin was quantitated microspectrophotometrically.

The hemoglobin content of "I-negative" cells was determined in a similar way. An "I-negative" cell population was obtained by mixing on a quartz slide red blood cells with 10 microliters of undiluted anti-I cold agglutinin serum, and agglutinating the samples on the slide at $4^{o}C$ for 15 minutes in a small container saturated with water vapor. After incubation the smear was examined as described. The unagglutinated cells on the slides are referred to as "I

negative". In order to find out if fractionation was achieved, an equal mixture of adult type A_1,I and cold type A_1,I cord blood sample was treated in a similar way. The unagglutinated cells were predominantly of cord type with respect to their hemoglobin content, indicating that fractionation of this mixture was achieved.

The number of A-antigenic sites per erythrocyte was determined by the method of Economidou et al. (21). An IgG fraction of a high-titer anti-A human serum from Hyland Laboratories was prepared by ion exchange chromatography. Fifty milligrams of anti-A containing gamma globulin preparation were reacted with 10 mc of carrier-free NaI^{125} according to the method of Greenwood et al.(81). The labeled globulin was adsorbed onto 5 million type A_1 cells suspended in 2% bovine serum albumin. In some experiments the adsorbed globulin was eluted at 56°C for 20 minutes and the amount of radioactivity in the globulin fraction was determined using a Beckman LS-200B Liquid Scintillation System. In some other experiments the cells with the adsorbed labeled globulin were dissolved in NCS solubilizer (Amersham/Searle) and the radioactivity determined. Group O erythrocytes were used as controls for nonspecific adsorption in every experiment.

The Chemical and Enzymatic Modification of Human Erythrocyte Stromata

Stromata were prepared from fresh blood by washing the

red blood cells three times in 0.15 M NaCl solution followed by a wash in 310 milliosmolar phosphate buffer, pH 7.4. The packed cells were lysed in 15 volumes of 20 milliosmolar phosphate buffer, pH 7.4 for 30 minutes with constant stirring. Stromata were collected by centrifugation in a Servall centrifuge using the GSA rotor head at 18,000 g for 40 minutes. After repeated washings in the low ionic strength phosphate buffer, the intact white stromata were resuspended to an equivalent of 5% cell suspension with an equal mixture of 0.15 M NaCl and 310 milliosmolar phosphate buffer, pH 7.4. Anti-A and anti-B serum specifically agglutinated the stromata prepared from type A or B cells. Anti-I sera of HOV and MAT similarly agglutinated the stromata at 4°C, while normal serum gave no agglutination. This indicated that A, B and I antigens remain unaltered on the membrane surface after the preparation.

Successful agglutination of stromata was achieved with purified macroglobulin molecules from HOV, indicating that the clumping was due to the binding of 19S antibody molecules with the I antigen. The agglutination showed the same temperature response as the intact cells and was completely reversible when the temperature was raised to 37°C. I-activity was retained for several days when kept at 4°C, and for several months if the sample was frozen in 30% glycerin and kept in liquid nitrogen. Stromata were lyophilized and later used in some experiments. The resuspended lyophilized

stromata retained over 50% of their capacity to bind anti-A or anti-I antibody molecules. Fetal stromata were similarly prepared from cord red blood cells. Such preparations failed to agglutinate with anti-I serum.

These experiments proved that the stromata were feasible material for chemical and enzymatic modifications, and could be used to obtain information about the molecular nature of the I antigen.

The specific absorption of antibody molecules by the stromata was measured in a Microtiter System. Anti-I serum was serially diluted into a suspension of stromata or into a solution tested for the presence of I antigen. The dilutions were incubated at 4°C for two to four hours and fresh adult cells were used to detect the remaining uninhibited anti-I cold agglutinins. All tests were carried out in phosphate buffered saline at pH 7.4. In these experiments lyophilized adult stromata, in a concentration of 4 mg per ml, completely inhibited equal volumes of anti-I sera with an agglutination titer of 256. Cord red blood cell stromata at the same concentration inhibited equal volumes of anti-sera with an agglutination titer of 4.

Neuraminidase treatment. (3.2.1.18 Worthington Biochemical Corporation.)

This enzyme, derived from Clostridium perfringens, removes N-acetyl neuraminic acid from the terminal ends of carbohydrate side chains. 1.0 ml of a 2% suspension of

human group O, Rh negative red blood cells was mixed with an equal volume of 0.1 M sodium acetate (ph 5.0), and 0.5 ml of 0.05 mg/ml enzyme in saline was added to the mixture. The mixture was incubated for 30 minutes at 37°C, followed by washing with buffered saline two times. Lyophilized stromata at 4.0 mg/ml was treated in a similar way.

Phospholipase-C treatment (3.14.3. Worthington Biochemical Corporation.)

Phospholipase-C or phosphatidylcholine cholinephosphohydrolase prepared from Cl. perfringens catalyzes hydrolysis of the linkage between glycerol and phosphate in substrates such as lecithin, sphingomylin and phosphatidyethanolamine. 150 microliters of lyophilized stromata suspension was mixed with 200 microliters of 0.1 M Tris-malcate buffer pH 7.3 and 50 microliters of 0.05 M $CaCl_2$. One hundred microliters of 0.1 mg/ml enzyme in 1% saline solution of bovine serum albumin was added and the samples were digested for 30 minutes at 37°C. The digested membrane particles were centrifuged at 50,000g for 15 minutes and both the pellet and the supernatent were tested for the presence of the I antigen.

Phosphlipase-D treatment (Calbiochem.)

This enzyme, prepared from cabbage, digests lecithin and other phosphatides liberating choline, ethanolamine or serine. 0.1 ml of lyophilized stromata suspension was mixed with an equal volume of 0.1 M sodium acetate buffer (pH 5.6).

Ten microliters of 20 mg/ml enzyme suspension in acetate buffer and 1 microliter of 1M $CaCl_2$ solution were added. The sample was digested for 3 hours at room temperature. The digested membrane particles were washed free of enzyme as in the previous treatment.

Proteolytic enzyme treatment. Trypsin, chymotrypsin, subtilisin and pronase. Worthington Biochemical Corporation.

To 0.1 ml of 4 mg/ml lyophilized membrane fractions suspended in 0.1 M Tris buffer pH 8.6, 5 microliters of 5 mg/ml proteolytic enzymes were added. The samples were digested at 37° C for 4 hours and washed with 1% bovine serum albumin in buffered saline three times.

Glycosidic enzyme treatment. A crude preparation of glycosoidic enzymes from Jack Bean Meal by Dr. E. Eylar, Salk Institute, was used in this experiment. The enzyme preparation was dissolved at a concentration of 5 mg/ml in 0.1 M KCL. To 0.2 ml of lyophilized stroma, 0.1 ml of 1M potassium phosphate buffer pH 6.5 was added and the samples were digested with 0.05 ml of enzyme solution for 10 hours at room temperature. The stromata were washed as before.

Chemical alterations of the stromata; modification of the free amino groups.

Free amino groups and sulfhydryl groups can be selectively blocked by KCNO or acetylated with acetic anhydride. 0.2 ml of lyophilized stromata diluted with 0.7 ml of 310 milliosmolar phosphate buffer (pH 7.4) was reacted with 0.1

ml of 0.2 M KCNO for five hours at room temperature. A similar fraction of 0.2 ml of lyophilized stromata was mixed with an equal volume of saturated sodium acetate at $0^{\circ}C$, and 10 microliters of acetic anhydride was added in five equal portions over the course of one hour. After the reaction, the stromata were centrifuged and washed three times with buffered saline.

Periodate destruction of carbohydrates.

A useful way to specifically destroy certain carbohydrates is by periodate oxidation. Under carefully controlled conditions, periodate will oxidize simple glycosides without affecting proteins. Lee and Hager (82) successfully removed over 90% of the hexoses from chloroperoxidase, a glycoenzyme containing approximately 60 residues of hexose and five residues of hexosamine, without affecting the enzyme activity. The method consists of a mild hydrolysis by dialysis at pH 3.5 to break the labilized glycosidic bonds.

Two sets of experiments were performed with periodate. In the first set, the stromata were treated with 0.05 M $NaIO_4$ at pH 4.5 for different lengths of time. 85% of hexosamine was destroyed after 6 hours at $20^{\circ}C$ under these conditions. Oxidation was stopped by adding excess ethylene glycol and the stromata were washed by centrifugation in buffered saline. After three washes, no periodate was detectable with 1 M KI. For control, stromata were mixed

with excess ethylene glycol prior to the addition of $NaIO_4$ and washed immediately. In the second set of experiments, oxidation was carried out in 0.04 M $NaIO_4$ for 140 hours at 4°C, followed by reduction with $NaBH_4$ and hydrolysis at pH 3.5. The stromata were washed free of salts using ultrafiltration and tested for the presence of I-antigen.

Fractionation of erythrocyte and buffy coat cell membranes.

Buffy coat cells were obtained through the courtesy of Dr. Paul Teresaki of U.C.L.A. Forty units of plasmaphoresed blood were used within 12 hours to collect the buffy coat cells and the platelets. Erythrocytes were removed by repeated centrifugation in buffered saline. Membrane preparations were obtained by the "Tris-method" of Warren et al. (83).

Red blood cell membrane fractionation was done according to Tanner et al. (84), Laico et al. (85) and Ruoslahti et al. (86). Membranes were dissociated with 100% formic acid prior to enzymatic digestion. Fingerprints were prepared of the trypsin and subtilisin-digested stromata according to Katz et al. (87). The solubilized membrane components were identified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (88). Protein concentrations were determined by amino acid analysis, using the Beckman Automatic Analyser. Hexosamine content was determined according to Rondle et al. (89), and the amount of hexose was measured with the anthrone method (90).

Molecular fractions obtained after membrane fractionation were tested for the presence of blood group activity. Specific inhibition capacity was estimated as follows: A dilution of an anti-blood group serum with an agglutination strength of 2+ was mixed with an equal volume of the solution being tested for the presence of blood group activity. A decrease in agglutination strength due to inhibition was scored and assigned a numerical value. Every drop in agglutination strength on the scale given on p 34 was noted as 2 inhibition units; for example, a drop from 2+ to + represents two inhibition units; to - represents six units. These values were then calculated back to a unit concentration of 1 mg/ml carbohydrate present in the solution being tested. Therefore, if a preparation had 0.001 mg/ml hexose when it changed an agglutination strength of 2+ to +, the 2 inhibition units would then be multiplied by 1,000 to obtain 2,000 inhibition units per mg of hexose per ml. These values have little absolute significance, but they are useful for comparative purposes.

RESULTS

THE CHARACTERIZATION OF ANTI-I REAGENTS

Over thirty sera were screened for the presence of anti-I cold agglutinins. The final selection was made according to the amount of serum available, the agglutination titer and the capacity to distinguish between human adult and fetal red blood cells. Two chronic cold agglutinin sera and a post-pneumonia serum were chosen on these bases. In addition, serum from a 32 year old Caucasian male who developed cold agglutinins following a viral influenza and serum from a 12 year old Caucasian boy of i blood group type were selected. Normal pooled serum was used as control. The concentrations of immunoglobulins in these sera were determined and the results are summarized in Table 1. All sera will be referred to from here on as indicated in the table. Sera from MAY, HOV and COH had six to tenfold increases in macroglobulin concentration, and three to fourfold decreases in 7S gamma globulin as compared with normal pooled serum. The amounts of gamma-A immunoglobulins were normal in all the sera studied.

All the sera were tested for their agglutination capacity with adult human group A, B and O, Rh positive, and group O Rh negative red blood cells and with pooled group O umbilical-cord cells at four different temperatures. The results for the three cold agglutinin sera are summarized in Figure 4 . The three sera exhibited different agglutination

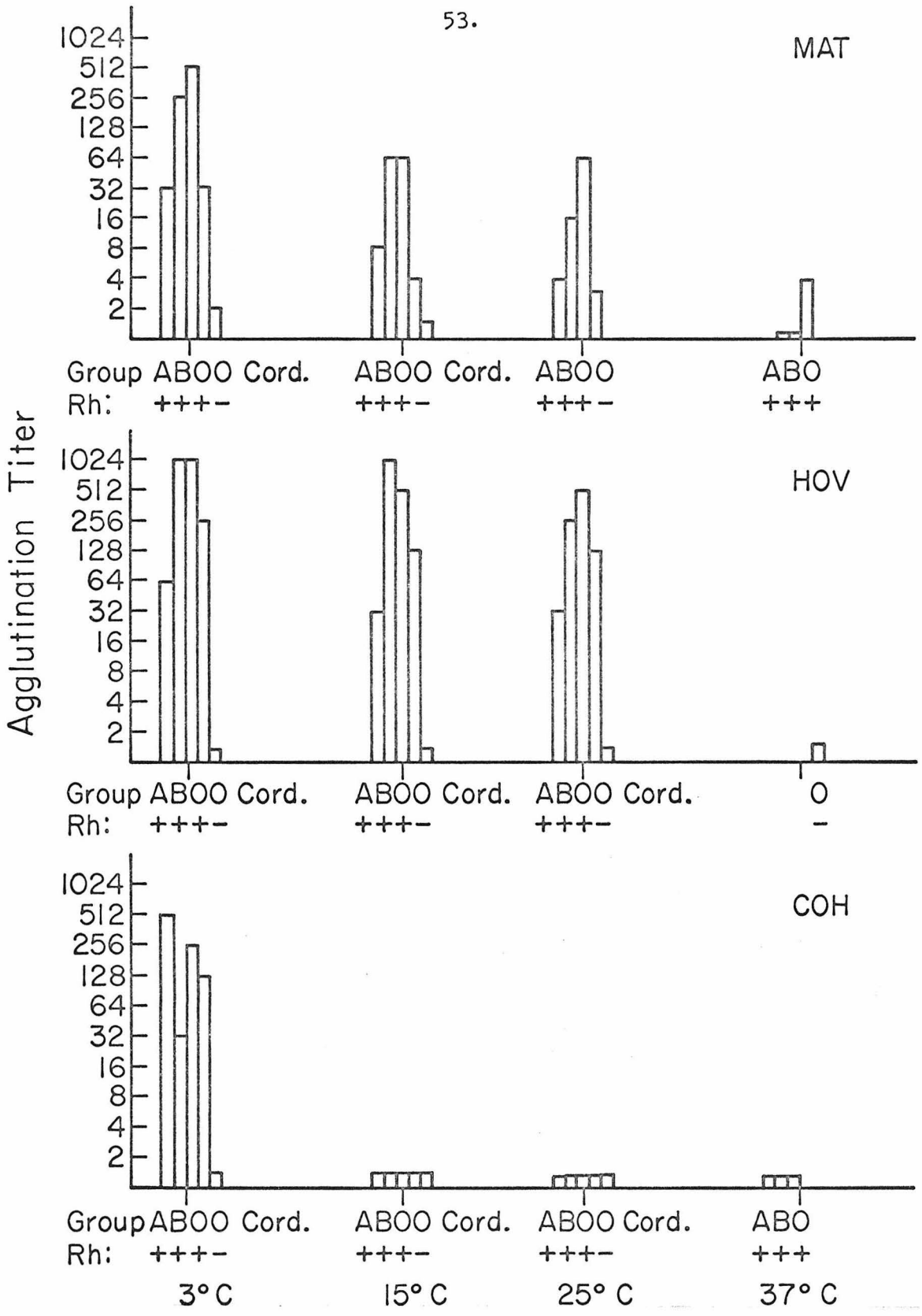


Figure 4. Agglutination characters of the cold agglutinating sera.

Table 1. Immunoglobulin concentrations in the sera studied.

Patient	Diagnosed disease	Immunoglobulin concentration: mg/100 ml		
		IgM	IgG	IgA
MAT	Chronic cold agglutinin disease	650	300	100
HOV	Chronic cold agglutinin disease	500	450	150
COH	Postpneumonia cold agglutinin disease	800	300	1000
DAZ	i_1 individual, thalassemia minor	80	900	115
AB3	Post viral influenza cold agglutinin disease	140	800	110
NRM	Normal pooled serum*	80	950	110

* Hyland Laboratories have published the mean levels of IgM, IgG and IgA in normal, white adult to be 80 mg/100 ml (S.D. $\pm$ 29 mg/100 ml), 1200 mg/100 ml (S.D. $\pm$ 319 mg/100 ml), 112 mg/100 ml (S.D. $\pm$ 41 mg/100 ml) respectively.

characteristics with respect to temperature. Serum HOV was near its maximum agglutination at temperatures as high as 25°C, the titer for MAT was intermediate at the intermediate temperatures, and COH had no significant titer above 5°C. No warm-reacting antibodies were found in any of the sera when tested at 45°C. The amount of I antigen varies among individuals, thus some of the differences observed with cells of different blood groups may reflect a variation in I antigen rather than a group specific distinction. Although

differences were noted among blood group types, the temperature profiles were the same for a given serum.

Red blood cells from the patient COH were tested with the serum of MAT and with her own serum. The agglutination titers and the temperature responses were identical with those given by normal adult group A cells.

Normal anti-I in an i_1 individual, DAZ, agglutinated I positive cells at 37°C and no additional cold acting antibodies were found in this serum.The titer was very low; a dilution of 1:3 gave 1+ agglutination. The serum from patient AB3 with post viral influenza cold agglutinin disease had a anti-I titer of 128 at 15°C which increased to 256 at 5°C.

The sera were also tested for additional antibodies with other than blood group specificities. A microdroplet cytotoxicity test with human lymphocytes was used to detect anti-HL-A antibodies. Each serum was tested with three different samples of previously typed cells. The results, summarized in Table 2, show that only the post viral influenza serum had detectable anti-leuckocyte antibodies. This patient's lymphocytes were not available for typing, therefore, it is not know if these antibodies had auto-specificities. Serum from DAZ showed positive reaction only at 1:2 and 1:4 dilutions with lymphocytes of type HL-A 2,10,12. Since the undiluted serum failed to show any reaction, these results may represent an artifact. Anti-complementary test were negative for all the sera tested,

Table 2. Microdroplet cytotoxicity test for anti-lymphocyte antibodies in cold agglutinating sera with anti-I specificity.

Serum	Dilution	Lymphocyte types		
		HL-A 2,12	HL-A 2,10,12	HL-A 2,60
HOV	1:10	1	1	1
	1:20	1	1	1
	1:40	1	1	1
COH	undiluted	1	1	1
	1:2	1	1	1
	1:4	1	1	1
DAZ	undiluted	1	1	1
	1:2	1	6	1
	1:4	1	4	1
	1:8	1	1	1
AB3	undiluted	5	6	6
	1:2	6	6	6
	1:4	6	6	6
	1:8	2	6	6
	1:16	1	1	1
AB3	without complement	1	1	1

The code for scoring cytotoxicity: 1= Negative reaction in which the viability is the same as in controls. 2= Doubtful positive reaction with a perceptible increase in barely stained cells over the control level. 4= Doubtful positive reaction with slight but detectable change in viability. 6= Positive reaction, clearly different from controls with 10 to 90% of the cells killed.

indicating that the lack of cytotoxicity was not due to some serum factor interfering with the detection system.

Macroglobulins were isolated from several sera in order to make purified IgM cold agglutinins for later experiments and for further characterization. Different approaches were used for different sera in order to account for the variations in the most frequent contaminants such as α_2-macroglobulin, β-lipoprotein, haptoglobin-2 and aggregates of other immunoglobulins. The yields varied according to procedures used.

Small amounts of I-specific cold agglutinins were isolated by absorbing the sera with human adult I-positive stromata at 5°C for 4 hours. Macroglobulins were eluted at 37°C after the stromata were washed free of serum proteins with cold buffered saline. The eluate was concentrated with pressure filtration and passed through a previously calibrated Biogel P-300 column. The excluded peak containing IgM was sometimes contaminated with membrane derived aggregates. This however did not interfere with the light chain typing of the anti-I macroglobulins. Such preparations from MAT, HOV and COH indicated the presence of kappa type light chains. The yield from AB3 was too low for typing and there was not enough serum from DAZ to perform the stromata absorption.

Miller (52) and Chaplin (75) suggested euglobulin precipitation as a first step for the purification of larger amounts of IgM. Euglobulins precipitate when 1 volume of serum is mixed with 10 volumes of distilled water, or when serum is dialysed against 30 volumes of 2mM- sodium

phosphate buffer, pH 8.0, for 24 hours. The euglobulin fraction contained all of the serum macroglobulins, together with 10-20% contamination of lower molecular weight materials and 20-30% of high molecular weight aggregates. The most serious disadvantage of this initial step was the difficulty of eliminating these nonspecific aggregates. Chaplin's approach (75) of resuspending the euglobulin fraction in 1.0 M NaCl 0.5 M tris buffer pH 8.0, followed by centrifugation in a 1.065 density salt solution, adjusted with NaCl and KBr, was successful in removing the lipoprotein and most of the smaller molecular weight contaminants. The larger aggregates however had to be separated by ion exchange chromatography followed by gel filtration on Sephadex G-200 columns. This procedure gave a low yield from the serum of MAT and the preparation was contaminated with approximately 15% of larger than 19 S components.

The best results were obtained when all protein denaturation procedures such as precipitation and lyophilization were avoided. Sapharose 4B, Sephadex G-200 and Biogel P-300 gels were tried for the first step of purification. Both Sepharose 4B and Biogel P-300 gels separated IgM from IgG but in Sephadex G-200 a considerable overlap was observed. A typical elution curve for the COH serum on a Biogel P-300 column is given in Figure 5a. Portions of the excluded peak were pooled and analysed by immunoelectrophoresis.*

* Rabbit anti-human IgM serum was used to locate the IgM arc.

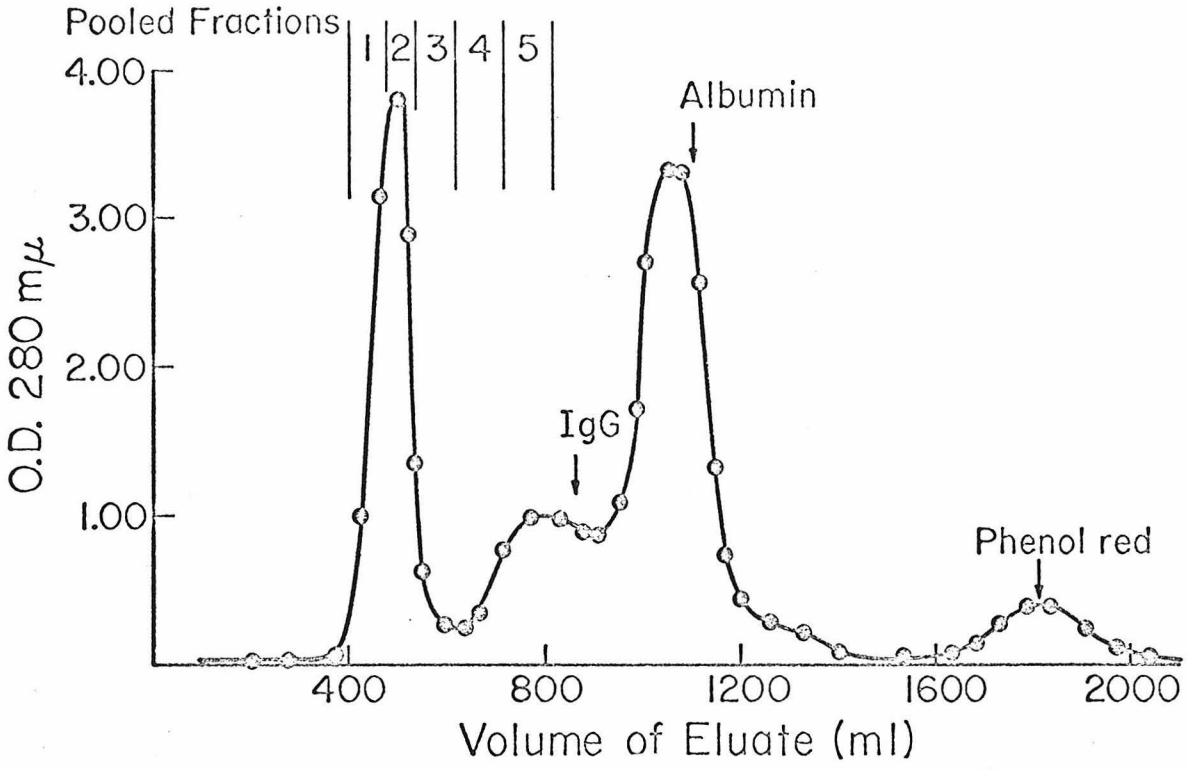


Figure 5a. COH serum fractionation on Biogel P-300 column.

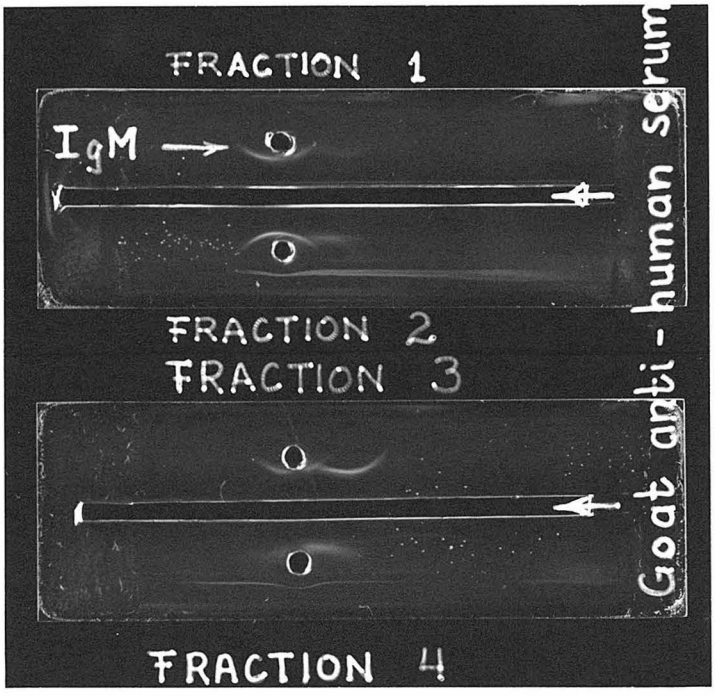


Figure 5b. Immunoelectrophoresis of pooled fractions from P-300 gel filtration.

Figure 5b indicates the extent of IgM purification.

Ion exchange chromatography on DEAE cellulose served as a successful additional step of purification. A typical elution curve, depicted in Figure 6a, shows the separation of IgG, IgA and other contaminants from IgM. The recovery varied from 50 to 75% depending on the macroglobulin solubility in the low ionic strength buffer used for column application. If only limited amounts of partially purified cold agglutinins were available, sucrose gradient sedimentation was a useful procedure for further fractionation. Figure 6b indicates the additional separation of the front peak from Biogel P-300 gel filtration of serum MAT. The major peak had a sedimentation of 19S and showed a single band for IgM by immunoelectrophoresis.

None of the above methods resulted in a pure IgM preparation from normal serum, therefore an additional step of starch-block electrophoresis was introduced. The macroglobulin fraction obtained by gel filtration and DEAE ion exchange chromatography was electrophoresed in 0.2 M borate buffer pH 8.6 using starch as a supporting medium. After the separation 1 cm blocks were cut out and eluted. Figure 7 shows the results and the extent of purification as indicated by immunoelectrophoresis. The final yield was between 8 and 10 mg macroglobulin from 100 ml of normal serum. This represents a 10-12% recovery. The yield from cold agglutinin sera varied with the number of purification steps used. 60-75% of recovery of IgM preparations with better than 80% purity

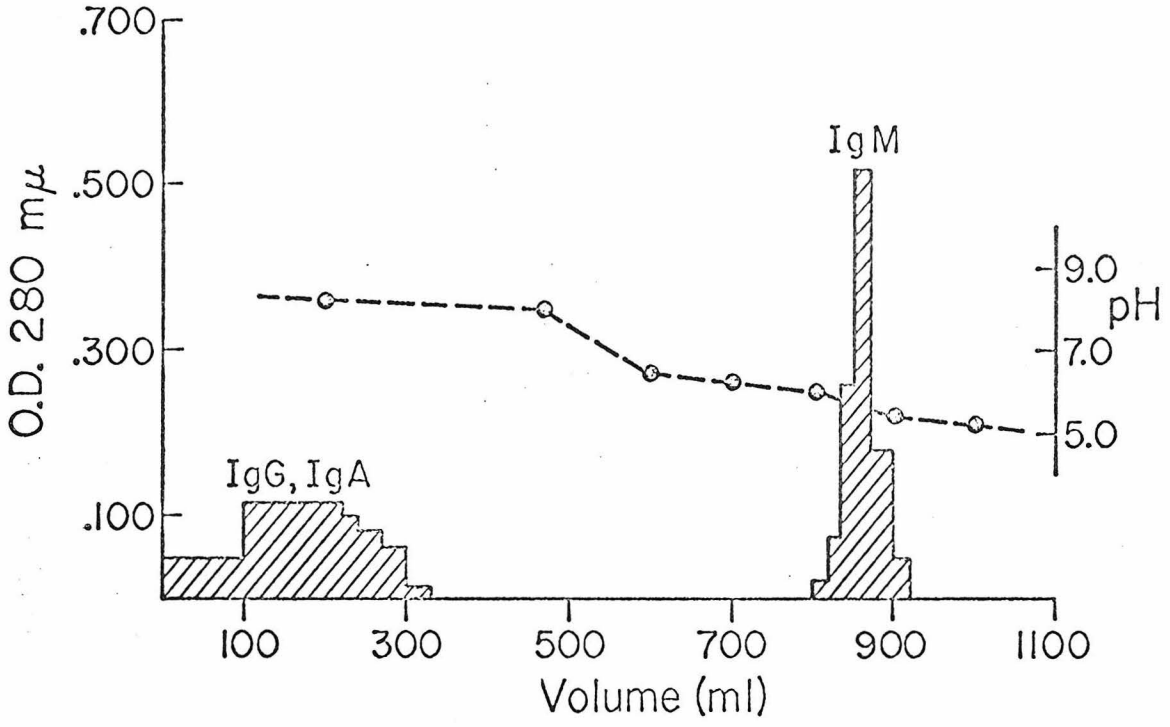


Figure 6a. Further fractionation of partially purified IgM (MAT) cold hemagglutinin on DEAE ion exchange column.

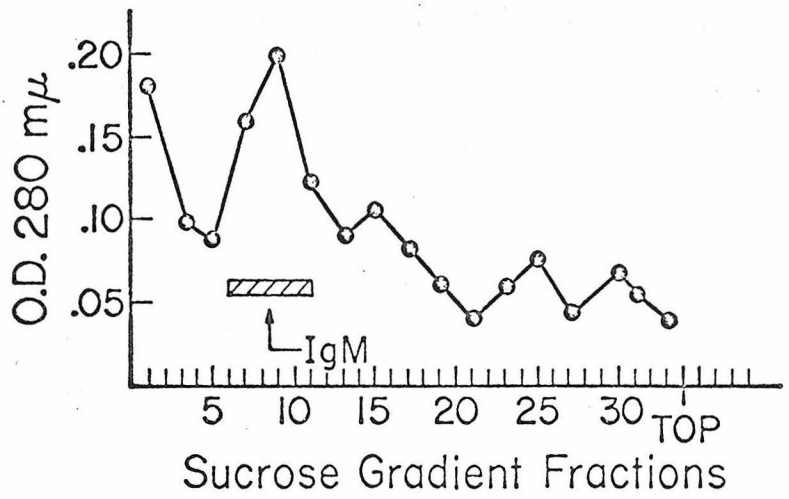


Figure 6b. Further fractionation of Biogel P-300 front peak from the serum of MAT using sucrose gradient sedimentation.

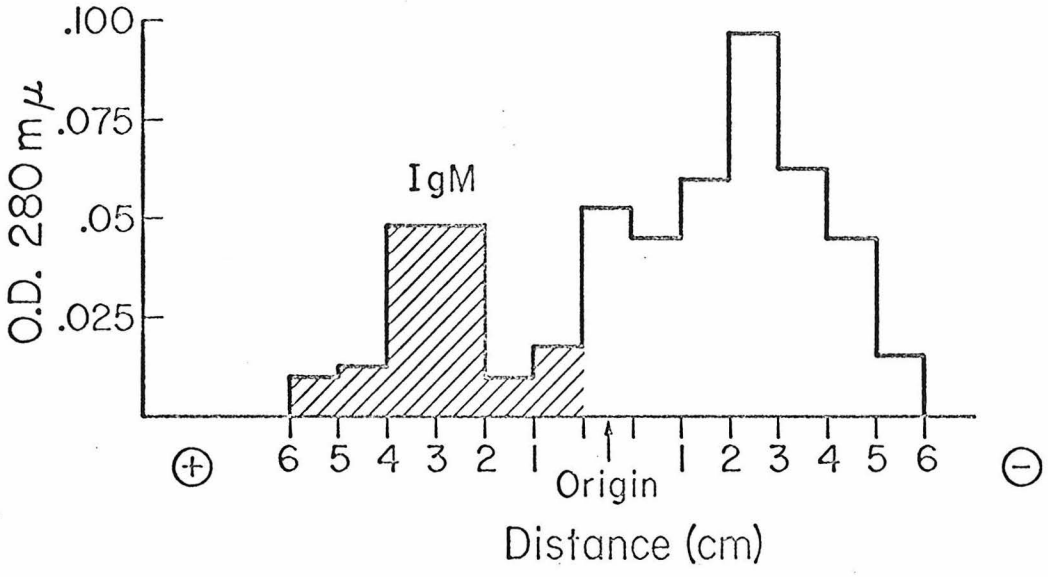


Figure 7a. Starch block electrophoresis of partially purified IgM from normal human serum.

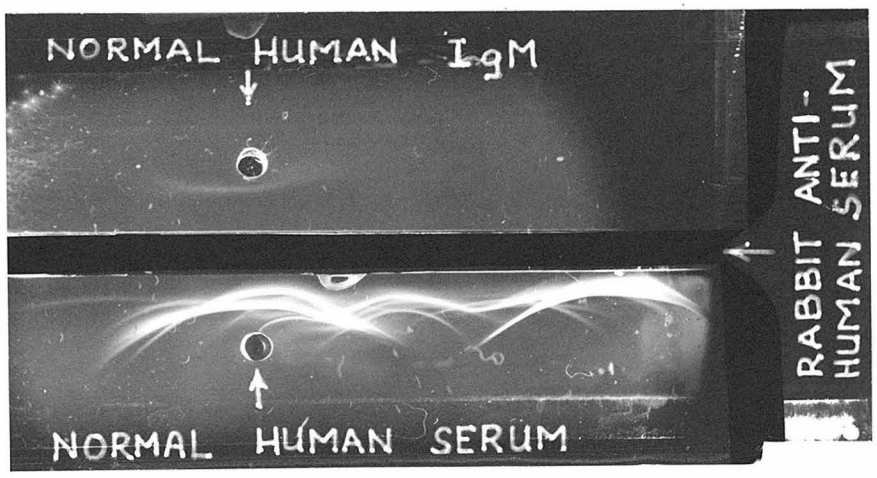


Figure 7b. Immunoelectrophoresis of normal human IgM after starch block purification.

was obtained from the sera of COH, HOV and MAT.

Purified cold-agglutinins behaved like the sera from which they had come in agglutinating human adult red blood cells of the various ABO groups and their stromata. The temperature profiles were also similar. Mild treatment with beta-mercaptoethanol dissociates IgM molecules into five subunits (52). These subunits failed to agglutinate erythrocytes even at ten times the concentrations necessary for agglutination by the intact macromolecule.

IgG obtained from the sera of HOV and COH after DEAE cellulose ion exchange chromatography failed to agglutinate cells at concentrations of 3 mg/ml. Both Coombs and complement-binding tests were negative, indicating that the I-specific agglutination system depends on 19S antibody reactions and does not involve IgG.

IgM from the postpneumonia cold agglutinin serum of COH was purified by disc gel electrophoresis and immunoelectrophoresis as indicated in Figure 9a., the heavy and light chains were separated after reduction of the disulfide bonds with dithiothreitol and alkylation with ethylenimine. The fractionation on a previously calibrated Sephadex G-200 column in 5 M guanidine hydrochloride is depicted in Figures 8,9 . The light chains failed to react with rabbit anti-human lambda serum at a concentration of 3 mg/ml. Their amino terminal sequence and the sequence of normal pooled human light chains are given below. Serine and threonine are together assigned the symbol "Xoh", because they were

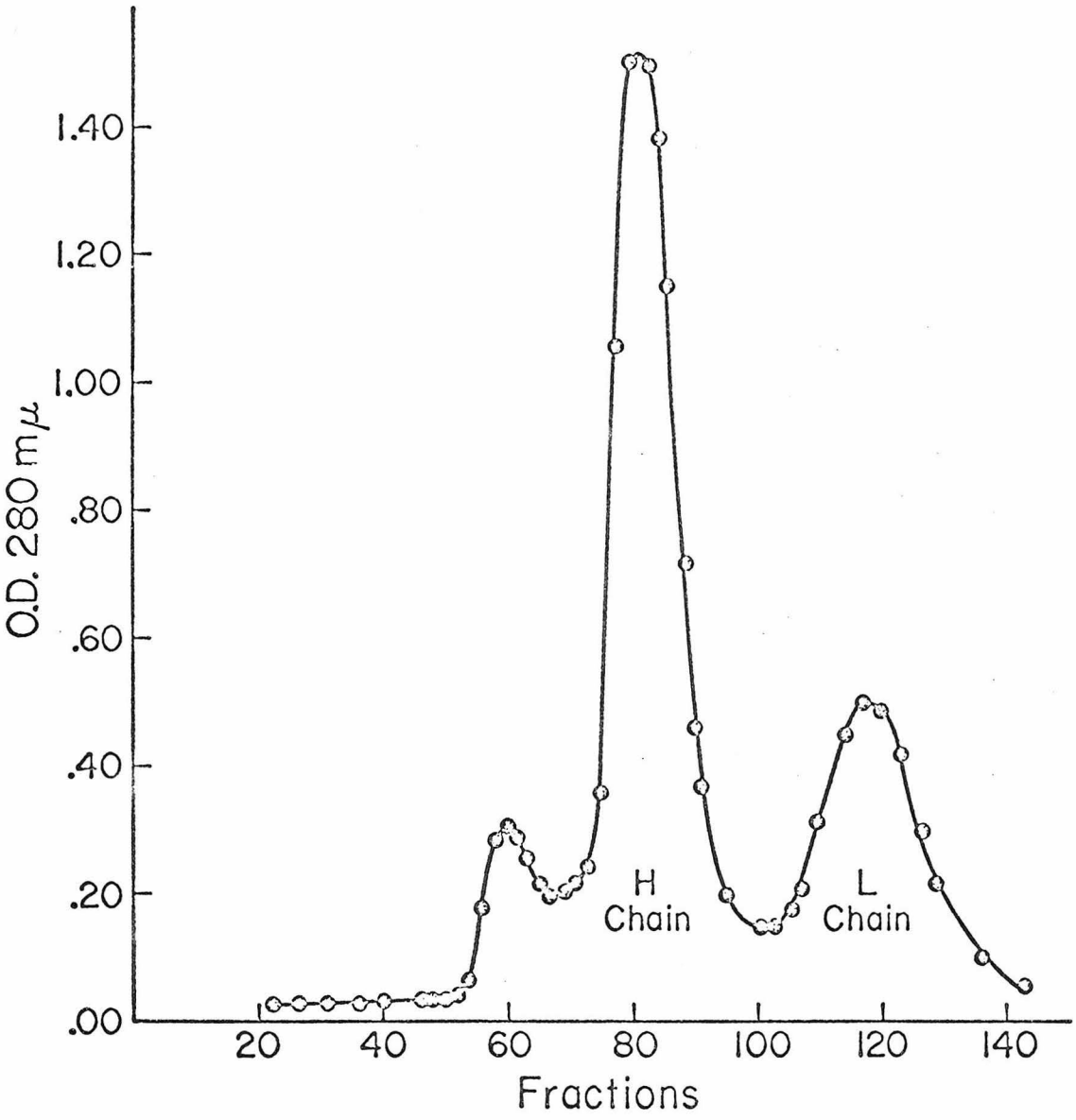
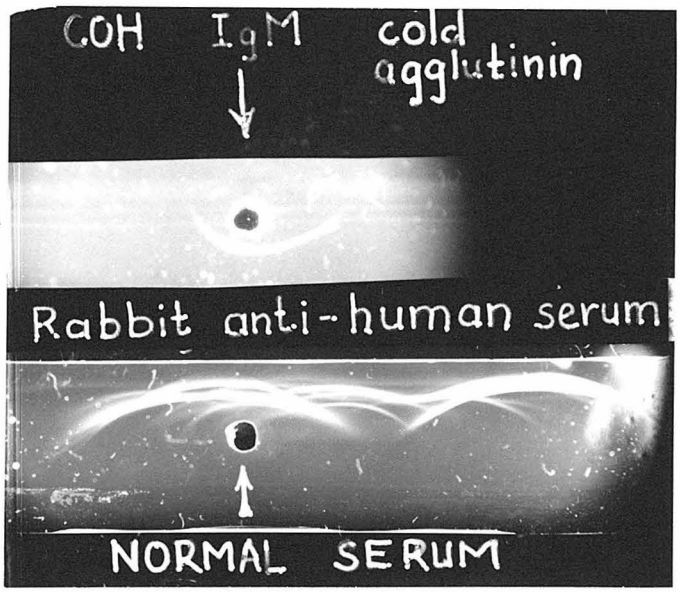


Figure 8. Separation reduced and alkylated heavy and light chains on Sephadex G-200 column in 5M guanidine hydrochloride.



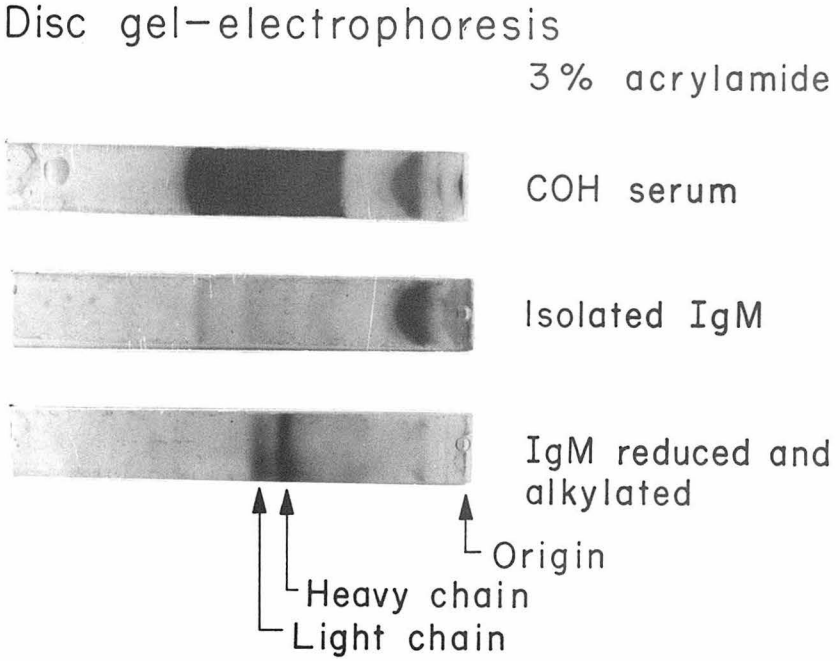


Figure 9. Disc gel electrophoresis and immunoelectrophoresis of purified IgM from the serum of COH.

not distinguishable using thin plate chromatography.

Normal human light chains:

Asx-Ile-Val-Met-Xoh-Glx-Xoh-Pro-Xoh-Xoh-Leu-Xoh--
Glx Glx-Leu

Light chains from COH IgM:

Asx-Ile-Val-Met-Xoh-Glx-Xoh-Xoh-Leu-Xoh-Leu-Xoh--

The light chains from COH IgM belong to the $kappa_{II}$ subclass reported by Gray et al (99). Absence of lambda chains in the IgM preparation and single amino acid at each of the three positions in the sequence at which there are two alternatives in a normal pooled sample suggest that the cold agglutinin is a homogeneous monoclonal product or that it has a very restricted heterogeneity. The results also indicate that over 80% of the IgM globulin, the limit of detection in this system, is I-specific cold agglutinin in the serum of COH.

THE DEVELOPMENT OF THE I ANTIGEN

Two significant changes in postnatal erythrocyte development were studied; one was the development of the I antigen and the other was the rate of adult hemoglobin appearance. The arrangement for obtaining samples made it impossible to follow the same infant from birth on; therefore, the study was carried out on separate individuals. Umbilical cord blood samples and postnatal infants' samples were ABO typed and the percent of fetal hemoglobin was determined using alkali denaturation. The percent of "I negative" cells was determined for all the blood samples studied and the results are summarized in Table 3. "I negative" cells were defined as the portion of erythrocyte population which fails to agglutinate with anti-I sera diluted to 32 times the minimal concentration necessary to produce complete agglutination of adult I-positive cell suspension.

Agglutination titers with anti-A and anti-I (from HOV) sera were determined for the "I negative", "I positive" subpopulations and for the unfractionated cells. Two characteristic examples of infants' cells are summarized in Table 4. Normal adult I cells all agglutinate under these conditions; the table therefore gives only the results for "I positive" adult cells. Quantitative differences were observed in I-agglutinability of the two subpopulations of cells which indicates that fractionation has been achieved. However, the term "I negative" is used only for convenience

Table 3 . Blood samples of postnatal infants characterized with respect to ABO type, fetal hemoglobin content, and percent of "I negative" cells.

Age in days after birth	Blood group	Percent of HbF in red blood cells	Percent of "I negative" cells
H cord cells	O	50	58
H cord cells	O	64	84
43	A_2B	49	47
45	A_1	62	61
51	O	38	71
57	A_1	30	78
70	O	24	21
75	O	40	23
80	B	21	24
87	A_1	18	18
88	A_1	17	18
89	A_1	26	9
105	A_1	40	81
107	A_1	14	34
114	A_1	17	58
117	O	5	8
124	O	6	5
127	B^+	3	54
159	O	5	11
174	A_1B	4	20
185	A_1B	10	49
210	A_1B	11	22
352	O	2	5
Adult	A_1	2	0
Adult	A_1	2	0

Table 4. Characteristic behavior of cells before and after fractionation with respect to their agglutinability with anti-A and anti-I sera.

Sample	Cell population	Antisera	Dilutions of antisera 2	4	8	16	32	64	128	256	512
A.C. male age: 2 mos. A_1, I, Rh+	unfractionated	anti-A	2+	2+	2+	+	+	±	±	-	-
		anti-I	3+	3+	2+	+	+	+	±	±	-
	"I positive"	anti-A	3+	2+	2+	+	+	+	±	-	-
		anti-I	3+	3+	2+	2+	2+	+	±	-	-
	"I negative"	anti-A	2+	2+	2+	+	+	+	±	-	-
		anti-I	2+	+	+	+	+	±	-	-	-
	unfractionated	anti-A	2+	2+	2+	+	+	±	±	-	-
		anti-I	3+	3+	2+	+	+	+	±	±	-
	"I positive"	anti-A	3+	2+	2+	+	+	+	±	-	-
		anti-I	3+	3+	2+	2+	2+	+	±	-	-
	"I negative"	anti-A	2+	2+	2+	+	+	+	±	-	-
		anti-I	2+	+	+	+	+	±	-	-	-
J.G. male Age: 4 mos. A_1, I Rh+	unfractionated	anti-A	2+	2+	2+	+	+	+	±	±	-
		anti-I	2+	+	+	±	+	±	±	-	-
	"I positive"	anti-A	2+	2+	2+	+	+	+	±	-	-
		anti-I	2+	2+	2+	2+	+	±	-	-	-
	"I negative"	anti-A	2+	2+	2+	+	+	+	±	±	-
		anti-I	2+	+	+	±	-	-	-	-	-
Z.T. male normal adult A_1, I Rh+	unfractionated	anti-A	3+	3+	3+	2+	2+	2+	+	±	-
		anti-I	3+	3+	3+	3+	3+	2+	+	+	±
	"I positive"	anti-A	3+	3+	3+	2+	2+	+	+	±	-
		anti-I	3+	3+	3+	3+	2+	2+	+	+	±

Legend for Figure 10, page 71.

The absolute values obtained for the HbF content appear to be lower than the values reported in the literature. This may be due to the difference in the methods used. As a result of it, the curve indicating the decrease in % HbF in the whole blood may have shifted, but would not differ significantly in its character.

Similarly the absolute values obtained for the number of Hb molecules per single erythrocyte cell are higher by a factor of three. This difference is probably due to artifacts caused by the cell fixation to the quartz slides, but will not affect the conclusions drawn with respect to the rate of decrease in HbF content in postnatal infants.

in describing the cells that fail to agglutinate under the specified conditions. Anti-I sera diluted to 32-64 times the minimal concentration necessary to produce complete agglutination of adult I cell suspension seemed to be the most promising for cell fractionation. Less diluted sera would give a lower percent of "I negative" cells, which in many instances would result in a shortage of cells to work with. Many of these cells actually have some I antigenic sites on their surfaces and have a limited capacity to absorb anti-I antibodies.

The fractionation with anti-I cold agglutinins did not interfere with the A-agglutination. Both fractions had the same agglutination titer with anti-A serum as did the unfractionated cell population. No difference was observed between the two subpopulations with respect to the A antigen. Does this mean that the A antigen is evenly distributed on the cells of these two subpopulations? This question was further studied with I^{125}-labeled anti-A antibodies and will be discussed later.

The rate of adult hemoglobin appearance and the rate of disappearance of "I negative" cells as a function of age are summarized in Figure 10 . The results show a much greater variation in the appearance of "I positive" cells than in the disappearance of HbF. At an age of 120 days a variation as high as 45% was observed, whereas the greatest variation in HbF was on the order of 20%. The percent of HbF as a function of the percent of "I negative" population

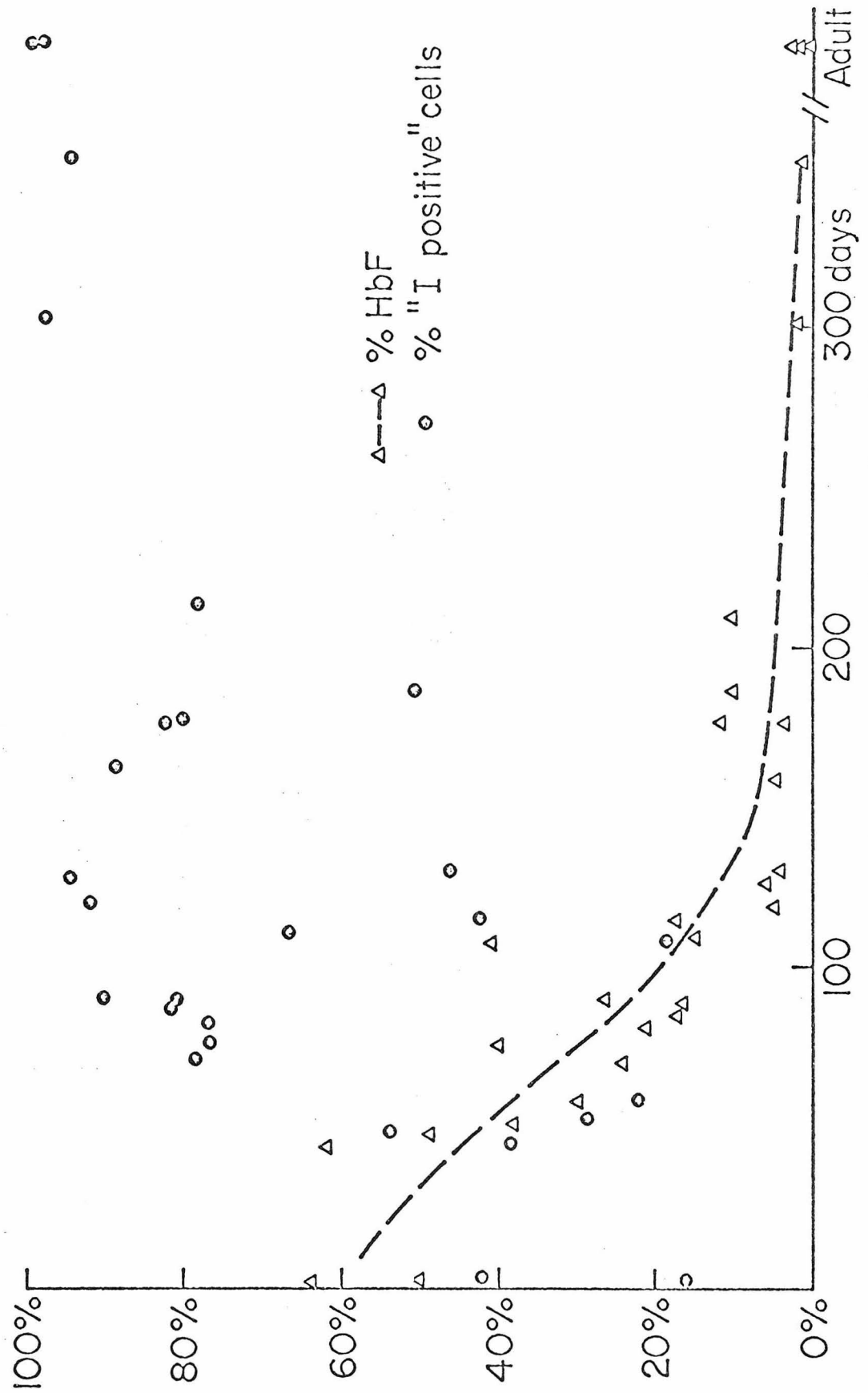


Figure 10. The rates for the appearence of "I positive" cells and for the disappearance of HbF is a function of age.

is indicated in Figure 11 . Two hypothetical relationships are also indicated in this graph. In one case if the two events were closely associated in a one to one proportion with 20% deviation allowed, the values should have fallen between the two dotted lines. For example, blood with 10% of "I negative" cells would have 10 ± 2 % HbF and 30% of "I negative" cells would have 30 ± 6% HbF. The mean values would lie on a straight line with a slope of 1. In the second case a two to one proportion is assumed; blood with 10% of "I negative" cells would have 5 ± 1% HbF, and so on. The values should fall between the two solid lines in this case, and the straight line of the mean values would have a slope of 0.5. Slopes of less than 1 would indicate that the appearance of HbA takes place prior to the expression of I antigen and a slope of greater than 1 would mean that the I antigen expression precedes the biosynthesis of HbA. As the figure indicates, the observed values do not fall into either hypothetical category with 20% deviation, thus there is no evidence of close association between the two processes.

To further test the above conclusion fetal and adult hemoglobin contents were determined in the postulated two subpopulations of cells. The results are summarized in Table 5 . Similar values were obtained using ion exchange chromatography on IRC-50 resins. Three of ten samples, at scattered postnatal ages and with no sign of a trend in time, showed significantly more fetal hemoblobin in the

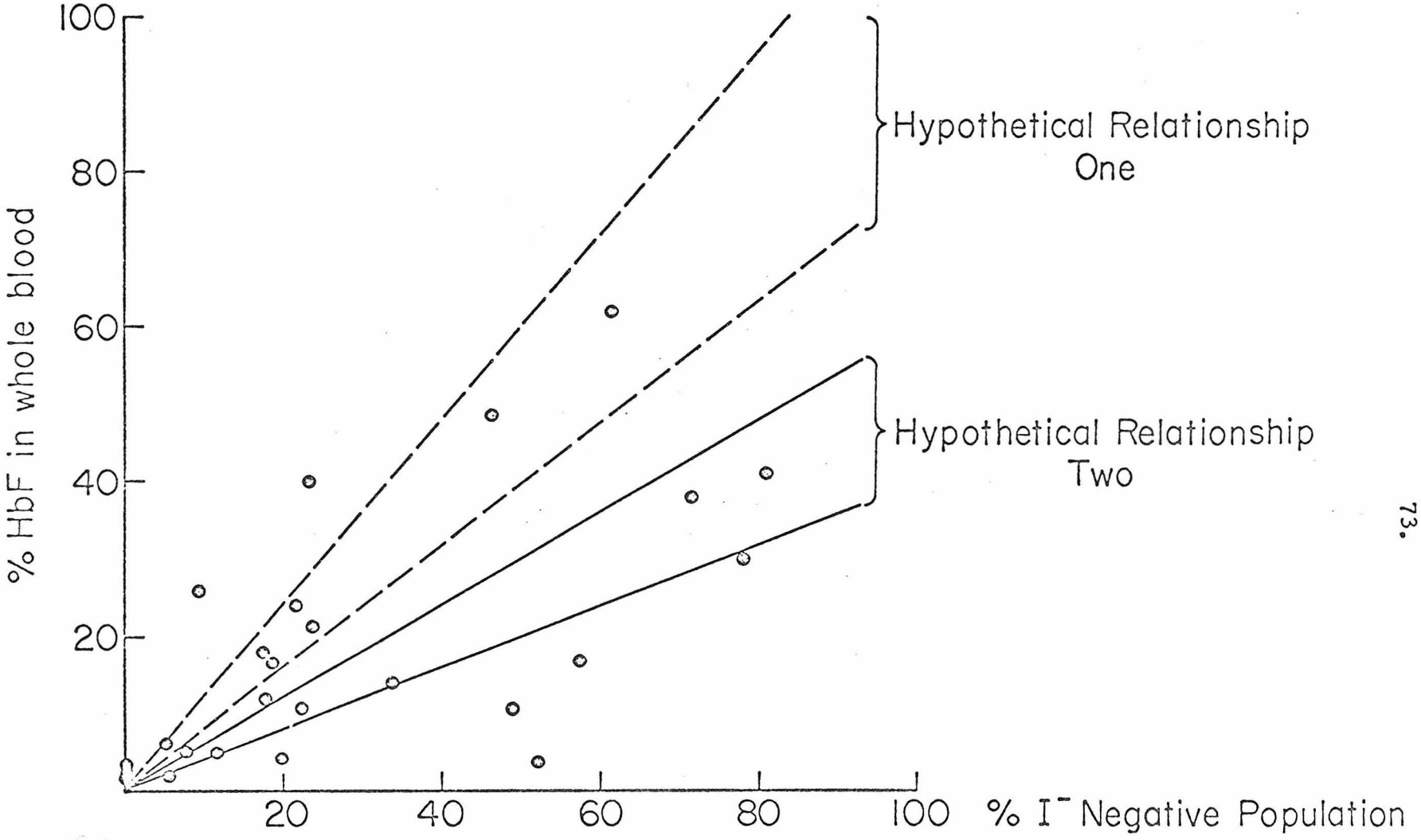


Figure 11. Percent of HbF in postnatal infants' whole blood samples as a function of the percent of "I negative" cell population.

cell fraction defined as "I negative" than in the fraction defined as "I positive". The HbF content can vary from 8% to 90% in "I negative" cells and can be as high as 83% in "I positive" cells. These results are consistent with the previous indication that the two developmental processes are not closely associated.

Table 5 . The percent of HbF in the cells fractionated according to their agglutinability with anti-I cold agglutinins.*

Age of postnatal infant in days	Percent of Fetal hemoglobin† in "I positive" cells	"I negative" cells
1	83 ± 8	90 ± 8
45	42 ± 4	60 ± 5
51	42 ± 4	40 ± 4
57	29 ± 4	30 ± 4
75	30 ± 5	70 ± 6
89	21 ± 5	56 ± 9
105	52 ± 4	54 ± 6
114	15 ± 5	20 ± 4
127	5 ± 2	8 ± 2
185	14 ± 3	22 ± 4
Adult	3 ± 1	- -

* The amount of HbF was determined by the alkali denaturation method.

† Results are the mean values with standard deviations calculated from three separate determinations.

All of the preceding experiments were performed on pooled cells. The amounts of hemoglobins present in single

cells were determined as a further extension of this study. At the outset an attempt was made to identify "I positive" cells with the help of fluorescein tagged anti-I IgM molecules, followed by a microspectroscopic determination of the kinds and amounts of hemoglobins present inside the cell. Successful staining was achieved with a "sandwich technique" using labeled human anti-I IgM and tagged rabbit anti-human IgM molecules. However, the hemoglobin contents of these cells could not be determined because of extensive lysis and cell degradation in the fixing procedure. The only method found successful in distinguishing cells with respect to their I antigen expression was the agglutination of cells with undiluted anti-I sera on a quartz slide. In this manner only the unagglutinated "I negative" single cells could be used for hemoglobin characterizations. Any dilution of the anti-I sera resulted in an uneven drying of the cells with frequent cell rupture.

The absorption spectra of single erythrocytes were similar to the spectrum of oxyhemoglobin in solution. The maximum absorption peak was at 415 mμ, the wavelength at which all the measurements were performed. Extraction with low pH buffer did not change the spectrum, indicating that hemoglobin was qualitatively unchanged by this procedure. Using the absorption coefficient of 124 per millimole of heme at 415 mu wavelength, the number of hemoglobin molecules per cell was calculated to be 3×10^8. Measurements on a single cell had a standard deviation of $\pm$ 5.1%.

Figure 12 shows the optical absorption for 100 adult and 100 umbilical cord cells. After extraction with low pH buffer the absorption decreased as indicated. Cord cells contained 65% HbF and adult cells less than 3% as determined by alkali denaturation. The mean of 0.297 for the adult cells decreased to 0.096 as a result of extraction for 20 seconds. This amounts to the removal of 68% of the HbA. Over 80% of the HbA was removed during a 40 second treatment; however, it became increasingly difficult to locate and to focus on cells as less hemoglobin remained on the slide.

Cord cells had a mean absorption of .287, which decreased to .199 after extraction for 20 seconds. This corresponds to a 31% loss in hemoglobin content. The difference between the adult and cord cells is due to the insolubility of HbF in the low pH buffer. Any possible interference from cell membranes can be ruled out on the basis of the work by Betke and Kleihauer (98). They extracted known mixtures of hemoglobin solutions from filter papers and demonstrated that the amount eluted was directly proportional to the HbA concentration.

99% of the adult cells had an optical absorption of less than .160 after the extraction. Approximately 10-15% of the cord cells fall into this category, indicating that only in this proportion of cells is the hemoglobin essentially all HbA. Blood samples containing only HbF were not available, therefore it is difficult to identify cells which contain

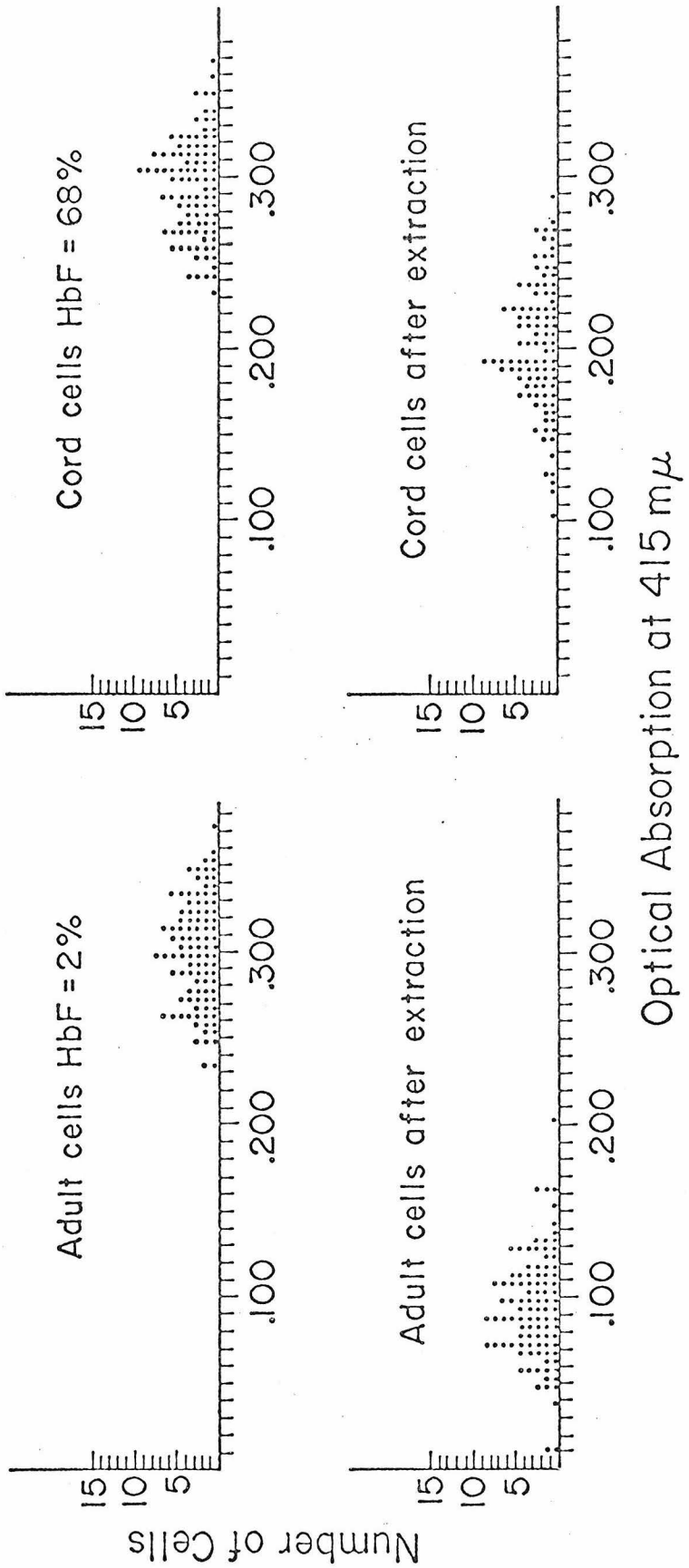


Figure 12. Extraction of HbA from adult and cord erythrocytes as studied by microspectroscopy on single cells.

HbF exclusively. An optical absorption of .250 or higher after extraction for 20 second can be classified as belonging to cells containing mainly fetal hemoglobin. This would amount to about 10% of the population in cord blood; the remainder of the cells would contain varying amounts of HbA and HbF. The results confirm previous observations on the absence of two distinct populations of cells containing exclusively HbA or HbF. If there were two clearly distinct populations, one would expect that 32% of the cells would have given optical absorption of less than .160, and the remainder would be unaffected by the extraction. Such results were not found in any samples from umbilical cord or from postnatal infants.

In order to study the hemoglobin content of "I negative" cells, certain controls were necessary to find out if fractionation was achieved. Equal mixtures of adult and cord cells with and without anti-I serum were fixed on quartz slides. Figure 13 shows the results. Adult and cord cells were indistinguishable prior to extraction. A bimodal distribution with some overlap was obtained following extraction. If successful fractionation is achieved with anti-I serum, the single unagglutinated cells designated as "I negative" should resemble the cord cells after low pH treatment. The results shown on Figure 13 confirm that "I negative" cord cells are separated from "I positive" adult cells in a mixture by the fractionation procedure. Approximately 15-20% of the "I negative" cells

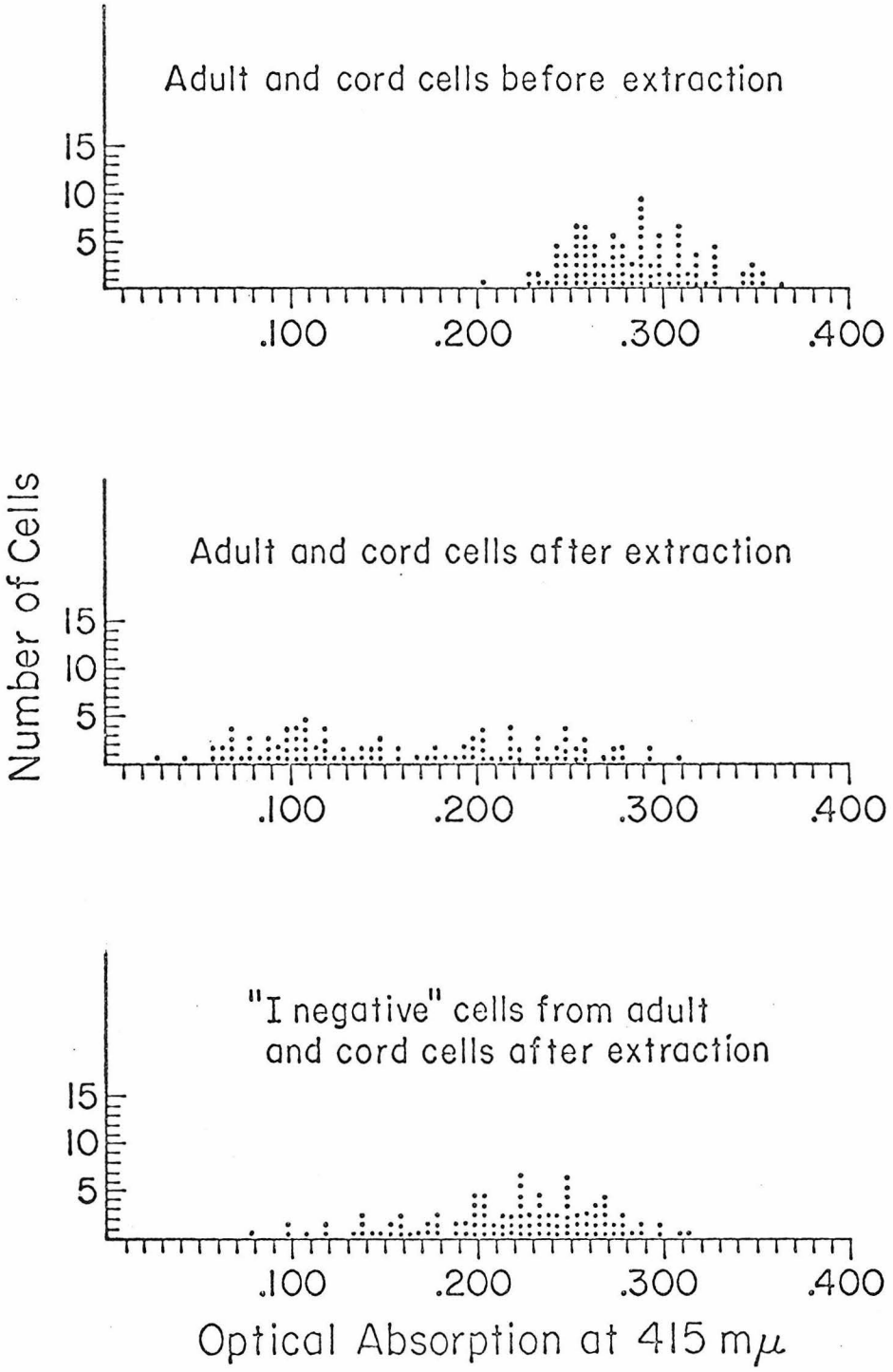


Figure 13. Fractionation of a mixture of adult and cord red blood cells with anti-I serum (HOV) and the determination of their hemoglobin content.

possessed only HbA. Since normal cord cells contain 10-15% such cells, the results indicate that only about 5% adult "I positive" cells contaminate the portion of the cells designated and fractionated as "I negative".

Results for two representative blood samples from infants studied in this manner are summarized in Figures 14 and 15 . Blood from Acuna and Xavier contained 14% and 8.7% HbF respectively. None of the samples had any cells containing HbF exclusively, as determined from the optical absorptions of the unfractionated cells following extraction. HbF was always present with different proportions of HbA in the same cells. The "I negative" cells contained insignificantly higher amounts of HbF. This subpopulation also included cells containing HbA exclusively. These results prove that the expression of the I antigen is independent of the hemoglobin content of the cell during postnatal development.

The relationship between the development of I and ABO antigens was further pursued. The rate of A and I antigen expression was studied with I^{125}-labeled anti-A immunoglobulins from immunized human sera. This method has been used successfuly to study the distribution of ABO and Rh antigens on the red blood cell surface. It is assumed that the amount of antibody absorbed is a direct indication of the number of antigenic sites available. The success of the experiment lies in the binding specificity and strength of the antibody used. The labeling procedure can alter the

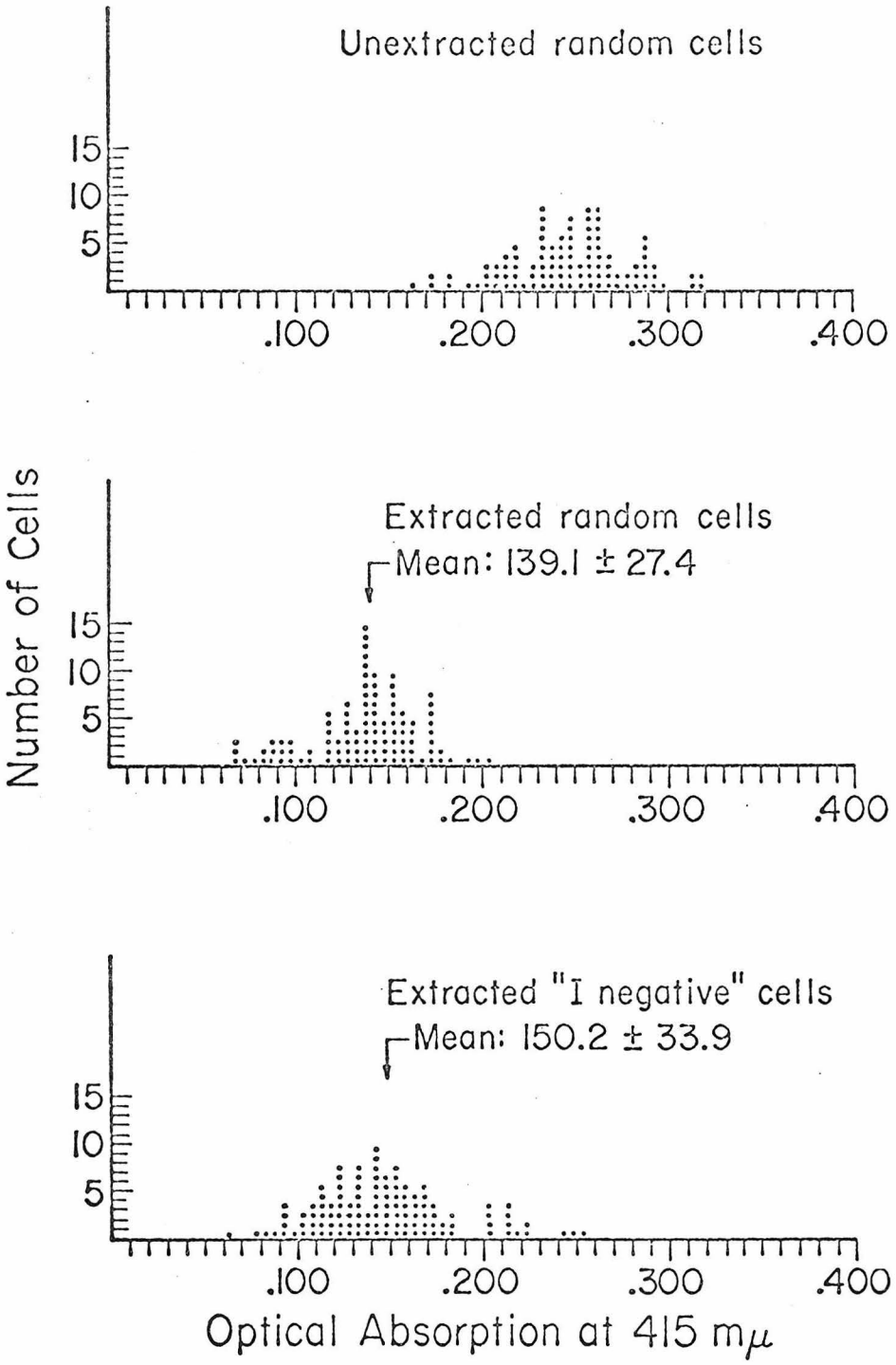


Figure 14. Hemoglobin content of single red blood cells. Patient: Acuna, female, Caucasian, age: 4.5 months. Fetal hemoglobin content: 14%. "I negative" cells: 33%. Blood Group: O,I, Rh+.

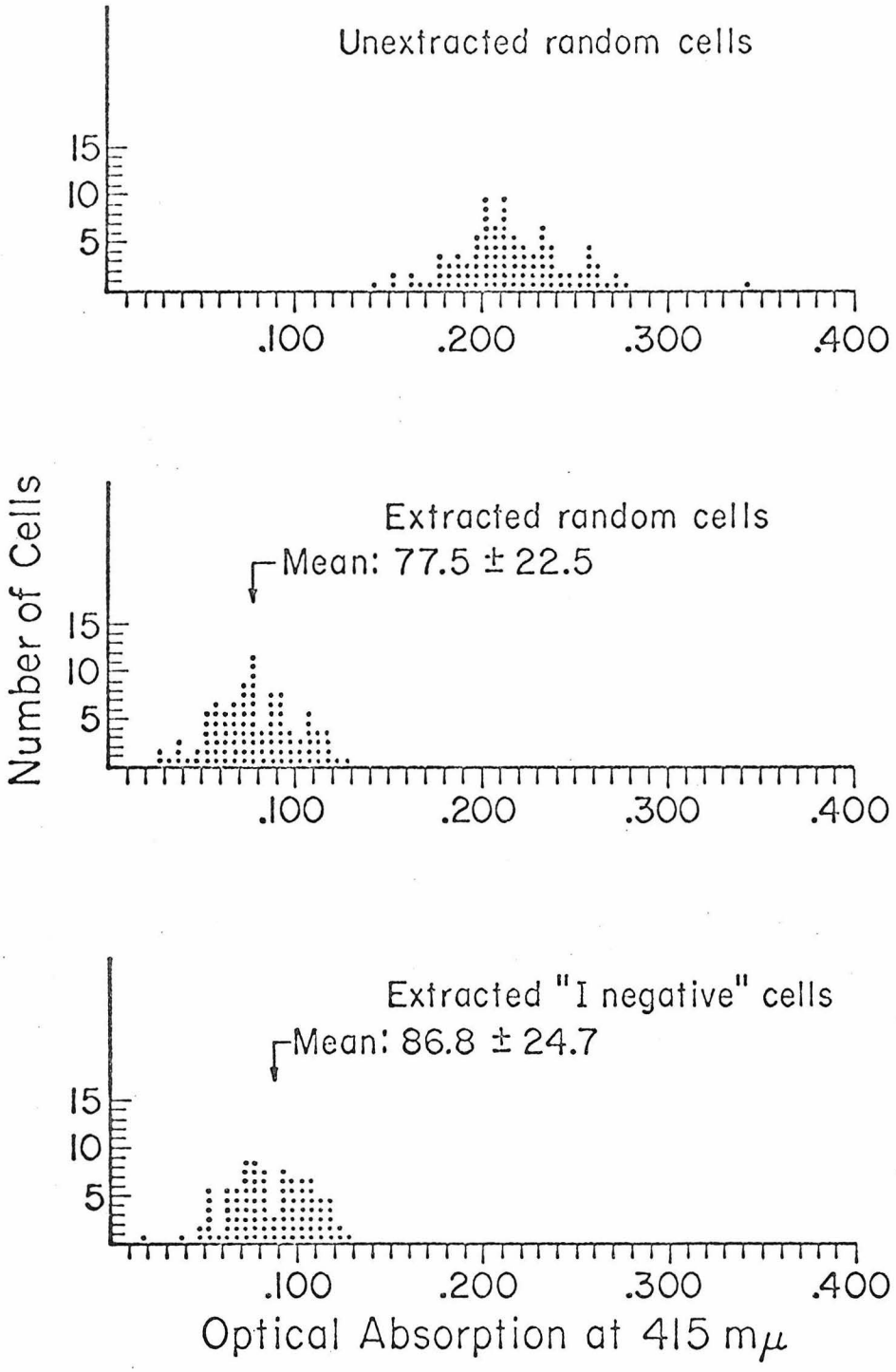


Figure 15. Hemoglobin content of single red blood cells. Patient: Xavier, female, Caucasian, age: 9 months. Fetal hemoglobin content: 8.7%. Blood group: A_1, I, Rh+.

binding capacity unless it is carried out under very mild conditions. The "hypochlorite method" developed by Greenwood (81) proved to be the most useful. Each preparation of labeled antibodies was absorbed using type A or O cells to eliminate most of the nonspecific binding. Specific absorption was greatly improved after the cells were formaldehyde treated, which also helped to minimize lysis caused by washing procedures.

Two different approaches were used to determine the number of antibodies absorbed to the cells. The more conventional approach is to elute antibodies for scintillation counting at slightly elevated temperatures of 45-50^{o}C. This elution is never complete, indicating that significant amounts of antibody remain firmly bound to the cell surface. Data for experiments I, II and III summarized in Table 6 were obtained by this method. In the other approach, used in experiment IV, cells with absorbed antibodies were solubilized together and the amount of radioactivity determined. Unreasonably high values were obtained this way, but the difference between the two subpopulations of cells remained comparable to the results of the previous experiments. The number of I^{125}-labeled anti-A IgG molecules per group A cell was calculated after subtracting nonspecific absorption to group O cells.

Umbilical cord cells were studied in experiment I. The number of A-antigenic sites was approximately 30,000 per cell. This is about the lowest limit of determination

Table 6 . The rate of A antigen development in postnatal infants and its relationship to the expression of the I antigen.*

Experiment	Age	Blood group type	Number of I^{125}-labeled anti-A IgG molecules absorbed per cell x 10^{-5}		
			"I negative" cells	"I positive" cells	Cells not treated with anti-I serum
	Cord cells	A_2	0.32 ± 0.05	0.24 ± 0.05	---
Exp.I	Cord cells	A_1	0.33 ± 0.03	0.28 ± 0.06	---
	Adult cells	A_1	---	3.14 ± 0.36	---
	80 days	A_1	1.05 ± 0.09	1.70 ± 0.10	1.22 ± 0.09
Exp.II	184 days	A_1B	3.35 ± 0.60	3.75 ± 0.60	---
	Adult cells	A_1	---	5.46 ± 0.80	6.19 ± 0.30
	105 days	A_1	4.60 ± 1.01	13.10 ± 3.00	---
Exp.III	185 days	A_1B	5.44 ± 1.81	6.61 ± 1.96	---
	Adult cells	A_1	---	27.60 ± 4.00	31.10 ± 5.00
Exp.IV	185 days	A_1B	60.10 ± 8.00	76.20 ± 3.00	---

*The mean values and standard deviations were calculated from three separate determinations. --- = not determined.

with this method. Standard deviations as high as 21% made it impossible to detect any significant difference between the "I negative and positive" cell populations. Adult cells exhibited a tenfold increase in the number of antigenic sites.

Erythrocytes from postnatal infants were studied in experiment II and III. Although all samples indicated higher numbers of A antigenic sites on "I positive" cells, only two out of four were statistically significant. Separate batches of labeled antibodies were used in these experiments and they accounted for the differences observed in the values for the adult cells. Cells which had not been treated with anti-I cold agglutinins were included in both experiments. They served as controls for any interference from cell fractionation, such as steric hindrance due to unremoved anti-I IgM molecules. A 10% decrease in both experiments following cold agglutination indicated minimal interference.

The values obtained in experiment IV were tenfold higher than in the previous experiments due to the different method used to measure absorbed radioactivity. The percent difference between the two cell populations was however, of the same order as in the previous determinations. This indicates that there was no artifact due to the elution technique used to evaluate the amounts of labeled antibody molecules absorbed to the two subpopulations of cells.

CHEMICAL AND ENZYMATIC ALTERATIONS OF HUMAN ADULT STROMATA AND THEIR EFFECTS ON THE I ANTIGEN.

Modifications of I antigen can be demonstrated by the elimination of inhibition capacity of I positive stromata in the agglutination system of anti-I cold agglutinins and human adult erythrocytes. In all the following experiments, serum from HOV was diluted into samples containing altered and washed stromata suspensions. Untreated human adult and umbilical cord cell stromata were used as controls. The results of several experiments are summarized in Table 7 .

Two reagents, KCNO and acetic anhydride, capable of reacting with free amino groups, failed to alter the I antigen. Lyophilized stromata treated with these reagents absorbed anti-I IgM molecules to the same extent as the untreated samples. The partial destruction of neuraminic acid by either 0.2 N H_2SO_4 or by neuraminidase treatment also failed to eliminate the specific absorbing capacity of the stromata. No detectable effect was seen as a result of phospholipase treatment. Pronase treatment lysed the test cells, so no conclusions could be drawn. Partial digestion with trypsin and chymotrypsin failed to eliminate the inhibition capacity of the stromata. Prolonged treatment with proteolytic enzymes disrupted the stromata. The undigested membrane pieces, sedimented by centrifugation, had no anti-I absorption capacity. The supernatant was tested for released inhibiting activity, but again considerable lysis occured. This was probably due to lecithin, known as a lytic agent,

Table 7 . Inhibition of anti-I serum from HOV with human I positive stromata before and after chemical and enzymatic modifications.

Additions to the serum, treatment of stromata	Dilutions of Anti-I serum											
	2	4	8	16	32	64	128	256	512	1024	2048	4096
No addition	+	+	+	+	+	+	+	+	+	+	≠	-
Lyophilized stromata	+	+	+	≠	-	-	-	-	-	-	-	-
KCNO	+	+	+	+	≠	≠	-	-	-	-	-	-
AC.ANH.	+	+	+	≠	-	-	-	-	-	-	-	-
0.2N H_2SO_4	+	+	+	+	≠	≠	-	-	-	-	-	-
$NaIO_4$ (17 hrs)	+	+	+	+	+	+	+	+	≠	≠	-	-
Lyophilized stromata	+	+	+	≠	≠	-	-	-	-	-	-	-
Glycosidic Enz. from J.B.M.*	+	+	+	+	+	+	+	≠	-	-	-	-
Neuraminidase	+	+	+	≠	≠	-	-	-	-	-	-	-
Phospholipase-C	+	+	+	+	≠	≠	-	-	-	-	-	-
Phospholipase-D	+	+	+	+	≠	-	-	-	-	-	-	-
Pronase	+	≠	L	L	L	L	L	L	-	-	-	-
Lysozyme	+	+	+	+	≠	-	-	-	-	-	-	-
Cord RBC stromata	+	+	+	+	+	+	+	+	+	+	≠	-
No addition	+	+	+	+	+	+	+	+	+	+	≠	≠

Agglutination was scored as follows: + = well defined agglutination; ≠ = weak, partial agglutination; - = no observable cell clumps; L = lysis.

* Jack bean meal

liberated from the membrane.

Crude preparation of glycosidic enzymes from jack bean meal gave pronounced destruction of I antigen, quantitatively similar to the destruction of I antigen by periodate oxidation to the polyaldehyde form. The alteration of A and I antigens by periodate oxidation was further studied in experiments in which the polyaldehyde products were reduced with $NaBH_4$ to their corresponding polyalcohol forms and the labile glycosidic bonds were cleaved by mild acid hydrolysis. 42% of the membranes' carbohydrate was oxidized in this procedure, as measured by the Anthrone method. The concentrations of the treated and the untreated stromal samples were adjusted to the same protein concentrations. The results for the antigen degradation are summarized in Table 8 . In these experiments, the antisera were diluted to give an agglutination strength of 2+ and then mixed with samples of serially diluted stromata. If the stromata had no capacity to absorb anti-I igM the agglutination would remain 2+ in all the dilutions. The untreated stromata could be diluted 640 times to inhibit the anti-I serum, whereas after the periodate treatment a forty-fold dilution was needed to cause comparable inhibition. Anti-B serum was used as a control to test for nonspecific binding to A positive stromata.

Table 8 . Tests for alteration of A and I antigens on human adult stromata by periodate oxidation.*

Antisera dilutions	Test cells	Agglutination without stroma	Dilutions of A_2I stroma						
			1	2	4	8	16	32	64
Untreated stromata									
Anti-A serum 1:200	A_1I	2+	-	≠	≠	+	2+	2+	2+
Anti-B serum 1:120	B I	2+	2+	2+	2+	2+	2+	2+	2+
Periodate treated stromata									
Anti-A serum 1:200	A_1I	2+	-	+	+	+	2+	2+	2+
Anti-B serum 1:120	BI	2+	1.5+	2+	2+	2+	2+	2+	2+
			20	40	80	160	320	640	1280
Untreated stromata									
Anti-I serum 1:125	A_1I	2+	-	-	-	-	-	≠	2+
Periodate treated stromata									
Anti-I serum 1:125	A_1I	2+	-	≠	+	+	+	2+	2+

*Stromata were prepared from human adult A_2,I erythrocytes. 50 microliters of diluted antiserum was mixed with 50 microliters of serially diluted stromata suspension in buffered saline, incubated for two hours at 4°C and the unabsorbed cold agglutinins were tested for with 50 microliters of fresh human adult type A or B cells. Agglutination was scored as previously described, after an additional hour of incubation at 4°C.

FRACTIONATION OF ADULT AND UMBILICAL CORD RED BLOOD CELL MEMBRANES WITH RESPECT TO THE LOCALIZATION OF THE I ANTIGEN.

Adult and cord erythrocyte membranes were digested with trypsin and subtilisin for fingerprinting. This method was used as an initial test to determine if there was any detectable difference between the two preparations. The quality of fingerprints improved if the membrane preparations were denatured and lipids were removed by chloroform-methanol extraction prior to digestion. Lyophilization after concentrated formic acid treatment was used for disrupting the stromata. The tryptic fingerprints so obtained are reproduced on Figure 15 . The major peptides visible are from the "miniprotein" component of the stromata demonstrated by Laico et al (85). No differences are observed on the fingerprints obtained after trypsin or subilisin digestion. This indicates that proteins of cord and adult erythrocyte membrane which are present in 10% or higher molar amounts are identical.

Previous procedures to isolate molecular fractions with blood group activity have started with extraction with various mixtures of organic solvents. The insoluble protein aggregates formed as a result of the extraction are discarded. These aggregates do not show ABO or I activity, but in our hands digestion with trypsin revealed blood group activity. Since digestion did not destroy the ABO and I activity of the stromata, experiments were performed to locate on the fingerprints molecular fractions responsible

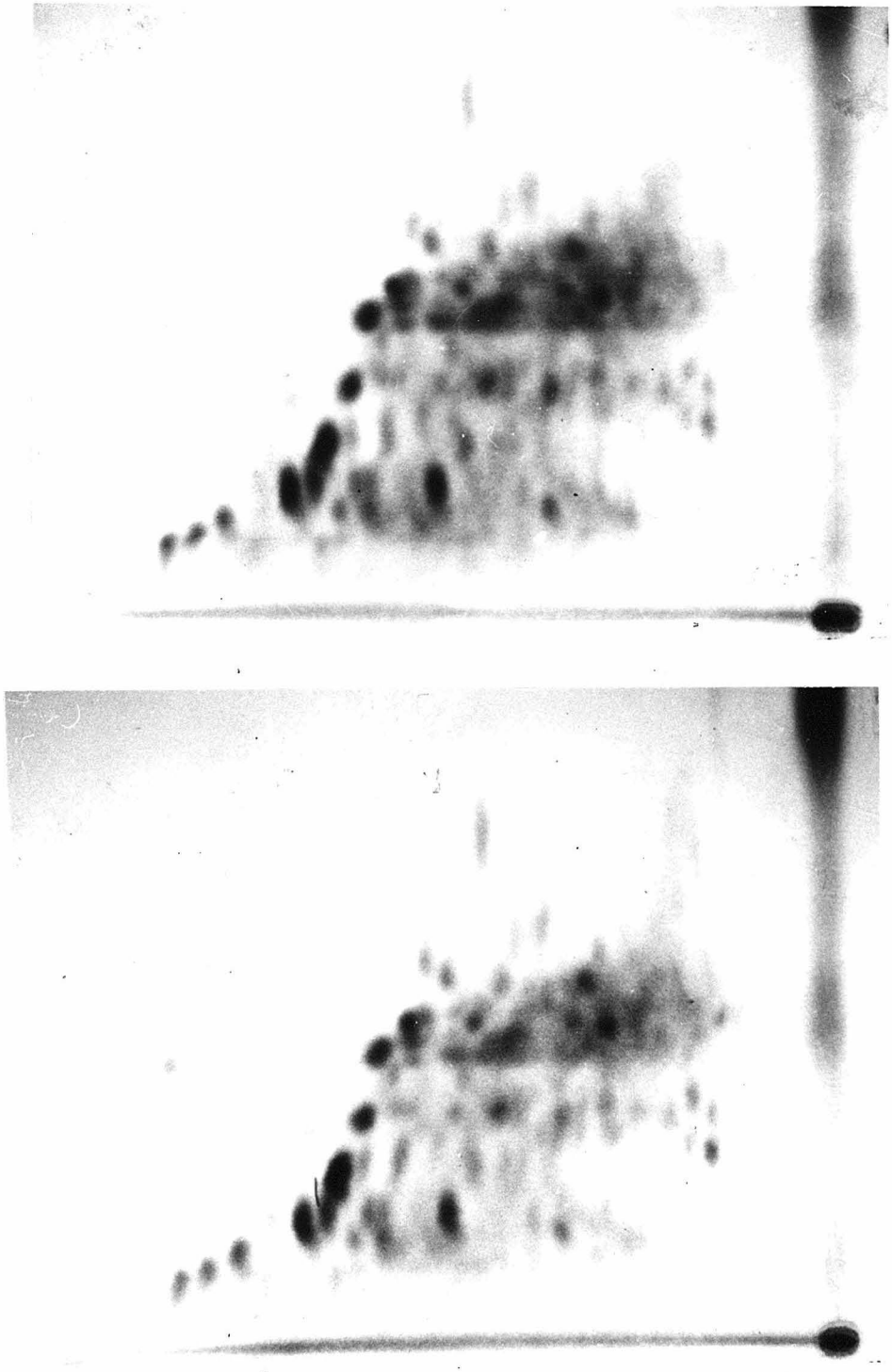


Figure 15. Tryptic fingerprints of adult and cord red blood cell stromata.

for them. The advantage of this approach is in having all the membrane materials present with no fractions discarded.

Denatured stromata were trypsin digested and applied to Whatmann 3MM filter paper in a streak using approximately 2 mg stromata per centimeter. The sample was chromatographed with the organic phase of the mixture of n-butanol: acetic acid : water ; 4:1:5 until the solvent front moved to the end of the paper. The dried paper was divided into five equal strips and the peptides eluted. The fractions were designated according to their characteristic Rf values, defined as the relative rate of migration with respect to the solvent front. The distribution of blood group activity is summarized in Table 9 . The most hydrophilic fraction , R_f 0.0-0.2, and the most hydrophobic fraction, R_f 0.8-1.0 were the only ones with blood group activity.

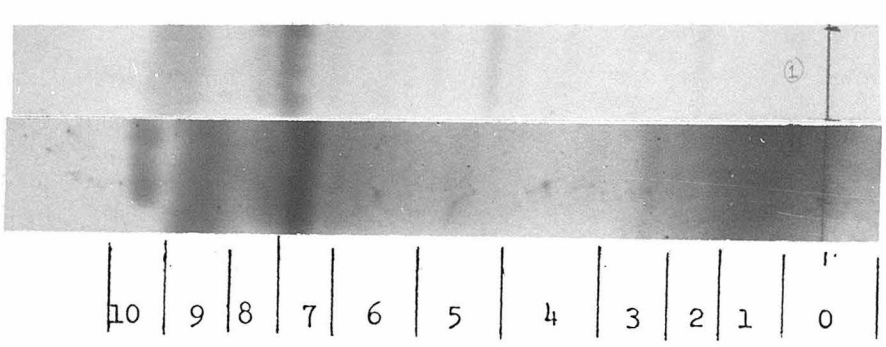
The hydrophobic fraction contained all the neutral lipids and a small number of peptides with high amounts of leucine, isoleucine and valine. In addition, 1-1.6% carbohydrate by weight was present as determined with the anthrone method. The presence of A or B blood group activity in this fraction was always marginal or questionable because of the very high nonspecific inhibition presumably by lipids which coat the test erythrocytes. No I antigen activity was ever found even in concentrations of 10 mg/ml.

The eluate from the hydrophilic region, R_f 0.0-0.2, was paper electrophoresed at pH 1.7. The results of the fractionation and the distribution of blood group activity

Table 9 . The distribution of blood group activity following chromatography of typsin-digested type B,I stromata.*

Samples	Anti-sera	No addition	Dilution of samples, 2 mg/ml				
			0	2	4	8	16
R_f 0.0-0.2	Anti-B	2+	-	-	-	≠	1+
	Anti-A	3+	-	2+	2+	3+	3+
	Anti-I	2+	-	-	-	≠	+
R_f 0.2-0.4	Anti-B	2+	1+	2+	2+	2+	2+
	Anti-A	3+	2+	3+	3+	3+	3+
	Anti-A	2+	1+	2+	2+	2+	2+
R_f 0.4-0.6	Anti-B	2+	2+	2+	2+	2+	2+
	Anti-A	3+	2+	3+	3+	3+	3+
	Anti-I	2+	2+	2+	2+	2+	2+
R_f 0.6-0.8	Anti-B	2+	2+	2+	2+	2+	2+
	Anti-A	3+	2+	3+	3+	2+	3+
	Anti-I	2+	2+	2+	2+	1+	2+
R_f 0.8-1.0	Anti-B	2+	-	≠	+	1+	2+
	Anti-A	2+	≠	≠	≠	1+	2+
	Anti-I	3+	2+	3+	3+	3+	3+
Control	Anti-B	2+	2+	2+	2+	2+	2+
	Anti-A	1+	1+	1+	1+	1+	1+
	Anti-I	3+	3+	3+	3+	3+	3+

*The control was the eluate of an unused filter paper strip equal in size to the samples. Agglutination was scored as described in Methods section. An attempt was made to dilute all the antisera to the same agglutination strength, but some variation was unavoidable.



Inhibition of anti-A, anti-B and anti-I (HOV) sera by the eluted fractions.

	anti-A serum					anti-B serum					anti-I serum				
	dilution of the fractions														
Fractions	1	2	4	8	16	1	2	4	8	16	1	2	4	8	16
0	≠	≠	+	1+	2+	-	-	≠	+	2+	-	-	-	≠	1+
1	≠	+	1+	2+	2+	-	≠	+	1+	2+	-	-	-	≠	1+
2	1+	2+	2+	2+	2+	+	1+	2+	2+	2+	+	2+	2+	2+	2+
3	2+					2+					2+				
4	2+					2+					2+				
5	2+					2+					2+				
6	2+					2+					2+				
7	2+					2+					2+				
8	2+					2+					2+				
9	2+					2+					2+				
10	2+					2+					2+				
Control	2+					2+					2+				

Figure 16. Electrophoresis of the hydrophilic fraction from subtilisin digested human adult B,I stromata.

following elution are summarized in Figure 16. The two samples with A,B and I activity contained approximately equal amounts of carbohydrate and amino acid by weight. Both fractions were rich in serine and threonine and contained 30-40 mole-percent hexosamine. Ser, asp, glu and ala were the amino acids labeled with densyl chloride after amino terminal analysis, indicating considerable peptide heterogeneity.

In order to compare the amount of I blood group activity present in different membrane preparation, the chromatography step alone was satisfactory. Two such comparisons were made. Adult red blood cell stromata were compared with cord cell stromata and with buffy coat membrane preparations.

Stromata from pooled cord blood samples and lyophilized adult, I positive, stroma were dried to constant weight. Both samples contained 2.7 $\pm$ 0.2% carbohydrate and 48 $\pm$ 2% protein. Adult stromata absorbed 64 times more anti-I specific cold agglutinins than the stromata from cord cells.

After chromatography of subtilisin-digested samples, the hydrophilic fraction represented 4-5% and the lipophilic material 35-45% of the stromatal dry weight. The relative amounts of anti-I inhibiting capacity determined from the hydrophilic fractions are summarized below.

Adult: 1500 inhibition units/mg of hexose in the stromata
94 inhibition units/mg of protein in the stromata
40 inhibition units/mg of stromata

Cord: 850 inhibition units/mg of hexose in the stromata
49 inhibition units/mg of protein in the stromata
25 inhibition units/mg of stromata

Inhibition units were calculated from the decrease in Sanger agglutination units and end-point dilutions as described in Matierals and Methods section.

Similar comparisons were made with membranes of buffy coat cells. The results are summarized in Table 10 . Hydrophilic fractions from both trypsin and subtilisin digestions of adult stroma were able to inhibit anti-I sera diluted to an agglutination titer of 2+, at a concentration of 0.16 mg/ml. The same fraction from buffy coat cells failed to inhibit, or showed only marginal inhibition at 5 mg/ml concentration. If the I antigen-carrying molecules are the same in every membrane, these results indicate that there are 30 times less I-specific molecules in the membranes of buffy coat cells. This is in agreement with the observation that leukocytes from pooled blood samples failed to agglutinate with undiluted anti-I sera of HOV and COH and is also consistent with the negative cytotoxicity tests with chronic cold agglutinin sera.

Erythrocyte membrane fractionation was further pursued in order to isolate molecules with I antigen specificity and to further study the relationship between ABO and I blood group specificity. These experiments reflect the efforts in Dr. Dreyer's laboratory to develop techniques for membrane fractionation. The work was done in

Table 10 . The inhibition of anti-I cold agglutinins (HOV) by hydrophilic fractions prepared by chromatography of trypsin and subtilisin digested membrane preparations.

Sample Dilutions	Hydrophilic fractions prepared from: RBC stromata tryptic digest	Buffy coat cell tryptic digest	Buffy coat cell subtilisin digest	RBC stromata subtilisin digest
5.0 mg/ml	-	2+	1+	-
diluted 1:2	-	2+	2+	-
diluted 1:4	-	2+	2+	-
diluted 1:8	-	2+	2+	-
diluted 1:16	-	2+	2+	-
diluted 1:32	≠	2+	2+	≠
diluted 1:64	1+	2+	2+	1+
No additions	2+	2+	2+	2+

50 microliters of anti-I serum, diluted to an agglutination strength of 2+, were mixed with 50 microliters of sample; incubated for 4 hours at 4°C. Anti-I agglutinins not inhibited by the sample were tested for with 50 microliters of 2% adult,I red blood cell suspension.

collaboration with Dr. M. Tanner and Dr. E. Ruoslahti. Preliminary fractionations were done according to Tanner, Gray and Dreyer (84). Treatment of stromata with 0.1 M EDTA (Na_2) failed to solubilize the I-antigen. Lipid fractions obtained by 90% 2-chloroethanol or with chloroform-

methanol extraction did not have any capacity to inhibit anti-I cold agglutinins. Stromata from which lipids had been removed were partially solubilized in 5M guanidine hydrochloride, reduced and aminoethylated. The samples were centrifuged to remove insoluble aggregates and to separate the floating lipoprotein layer. Both of these fractions displayed marginal ABO and I blood group activity. The dissolved membrane components were further separated on a Sephadex G-200 column in 5M guanidine hydrochloride, but none of the fractions obtained had any capacity to inhibit anti-I, anti-A or anti-B sera.

Successful stromal fractionation was achieved using a procedure developed by Ruoslahti (86). Human adult B,I stromata were stirred for an hour in 100% formic acid at $4^{o}C$. Recrystallized urea was added to make the concentration 10 molar. The final solution in 25% formic acid and 10 M urea was chromatographed on a SE-Sephadex column using salt and acid gradients for developing. A typical elution curve and the distribution of blood group activities are shown in Figure 17 . Bovine and human type A stromata had similar elution patterns.

Pooled fractions 1,2 and 3 of the front peak from SE-Sephadex column separation contained only proteins with lower than 6,000 molecular weight as determined by SDS gel electrophoresis. Proteins with larger molecular weights, especially hemoglobin, were found in fractions 5 and 6. The peak eluted with 90% formic acid, fractions 8 and 9,

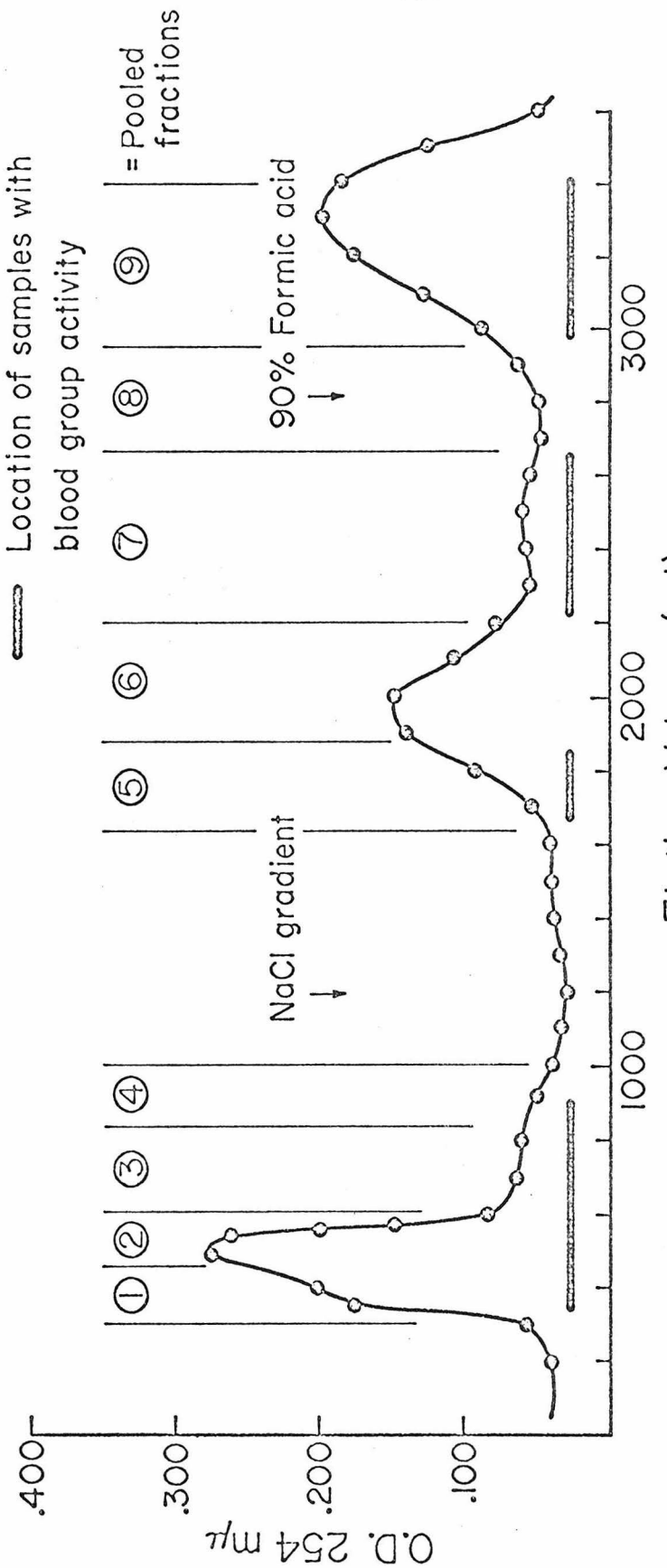


Figure 17. Fractionation of human adult B, I stromata on SE-Sephadex column.

contained predominantly larger molecular weight proteins in addition to residual amounts of smaller proteins. Amounts of blood group activity found in the fractions did not follow the optical absorption curve. Table 11 summarizes the carbohydrate content and the capacity to inhibit anti-B and anti-I antisera in the pooled fractions. A similar distribution of A blood group activity was obtained from type A,I stroma. Fractions 1,2,3 contained most of the I activity. The A or B activity was four to eight times lower. The relative amount of I activity was lower in fractions 4,5 and 7. This was particularly noticeable in fraction 7 which lacked the capacity to inhibit anti-I cold agglutinins. Fraction 9 contained an unusually high amount of carbohydrate in this illustrated separation. This was due to a slower than usual flowrate which resulted in a breakdown of SE-Sephadex resin in 90% formic acid. This explains the low specific blood group activity. With the exception of 6, all pooled fractions contained varying amounts of lipid.

Fraction 2 was further studied because it contained the highest specific I-inhibition capacity. Figure 18 shows the results of SDS gel electrophoresis and the fingerprint after digestion with subtilisin. Thirty percent of the digested sample was recovered in the lipid fraction with R_f 0.8-1.0 following chromatography. Both the hydrophobic and the hydrophilic fractions with R_f 0.0-0.2 were tested for the anti-A, anti-B and anti-I inhibition capacity.

Table 11 . Analysis of pooled fractions from SE-Sephadex chromatography of human adult B, I stroma.

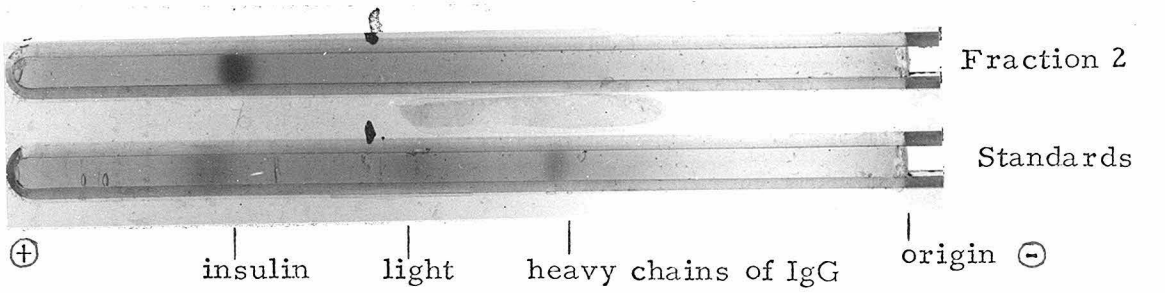
Pooled fractions	Dry weight	Percent carbohydrate	Specific inhibition capacity, inhibition units/mg carbohydrate	
			anti-B	anti-I
1.	70 mg	5.9%	1,700	6,800
2.	100 mg	4.9%	2,400	19,400
3.	95 mg	4.9%	2,000	16,400
4.	22 mg	5.1%	1,500	1,000
5.	7 mg	3.3%	1,500	3,300
6.	40 mg	2.8%	--	--
7.	22 mg	3.7%	2,600	--
8.	15 mg	4.5%	--	--
9.	450 mg	38.4%	100.	100.

---

100 ml of human adult stromata was fractionated on SE-Sephadex. The elution profile is given in Figure 17 . Carbohydrate was determined using the anthrone method. Specific inhibition capacity was calculated as outlined in Materials and Methods section. Nonspecific inhibition of anti-A serum was subtracted.

The specific inhibition per mg of protein was not determined in this fractionation. In separate column fractions the blood group activity failed to correlate with the optical absorption curve, indicating that some of the blood group activity is not related to the protein concentration of the fraction.

SDS gel electrophoresis of pooled fraction 2 from SE-Sephadex column.



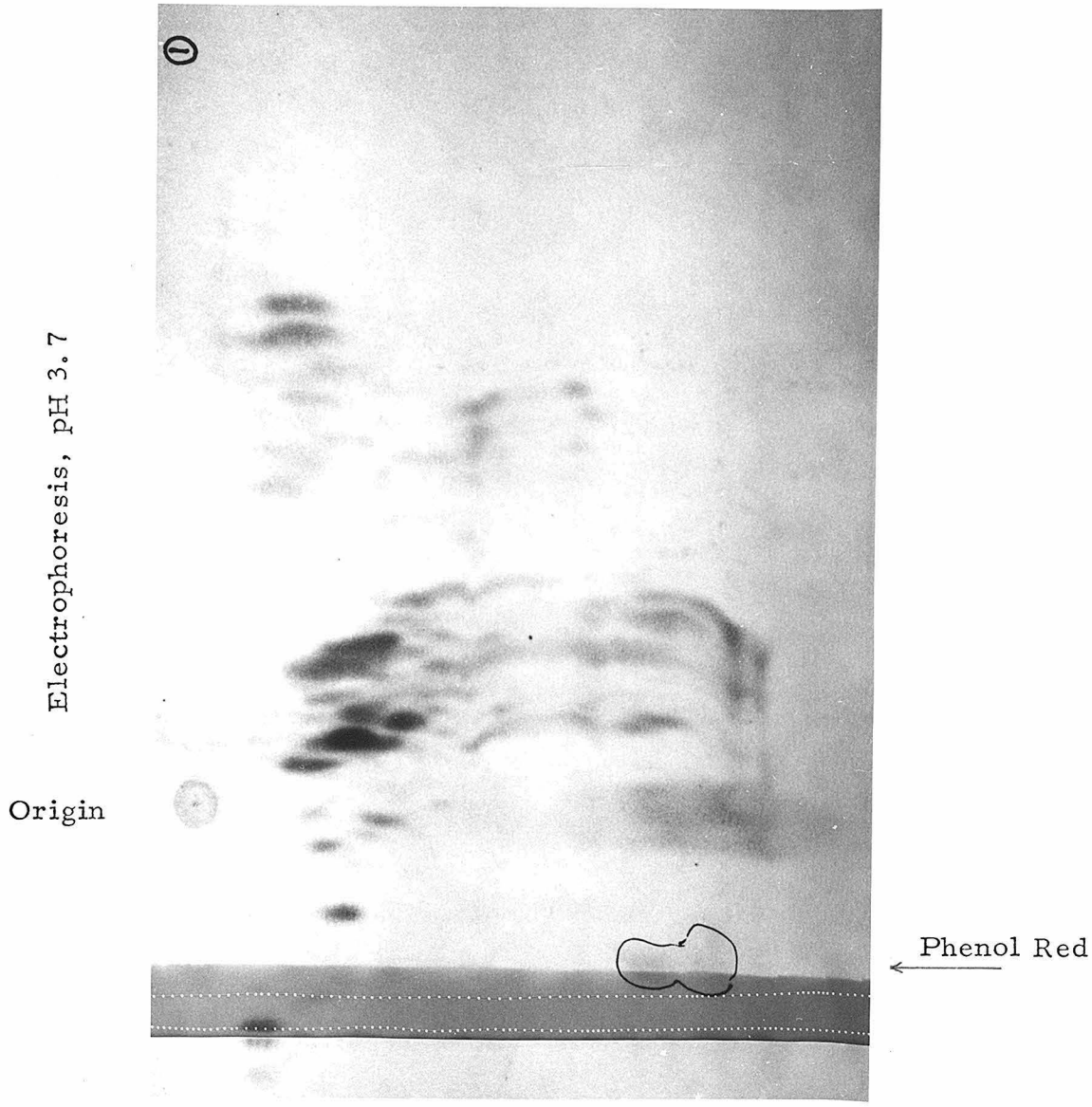


Figure 18. SDS gel electrophoresis of pooled fraction 2 from SE-Sephadex column separation of human adult stromata, and its fingerprint after subtilisin digestion.

Only the hydrophilic fraction had A or B and I blood group activity. Nonspecific inhibition in the lipid fraction was too high to establish the presence of antigens. The hydrophilic fraction was free of positively charged peptides, as determined by paper electrophoresis at pH 1.7. The only region with blood group activity was ninhydrin negative and peptide bond stain positive.

After water elution the sample was further separated on Biogel P-6 column. The results are shown in Figure19 . Only the excluded peak with larger than 6,000 molecular weight components had both A or B and I blood group activity. A second peak with molecular weight between 3-5,000 had only A or B but no I activity. Most of the carbohydrate was found in the excluded peak, but subsequent fractions contained decreasing amounts. The specific anti-B inhibition in fraction 13 was 7-9,000 and in fraction 19 4-6,000 inhibition units per mg of hexose. These values are approximately three times higher than the specific inhibition values of SE-Sephadex fractions. Anti-I inhibition was somewhat lower -- 9,000 inhibition units per mg of hexose instead of 19,000. Amino terminal analysis of the excluded fraction with dansyl chloride revealed Ser as the main N-terminal amino acid, with minor amounts of Asp and Glu as possible contaminants. The amino acid analysis of the pooled fractions and the composition of a typical "mini-protein" preparation from human red blood cell membranes are summarized in Table 12 . Fraction 11 was high in Ser and

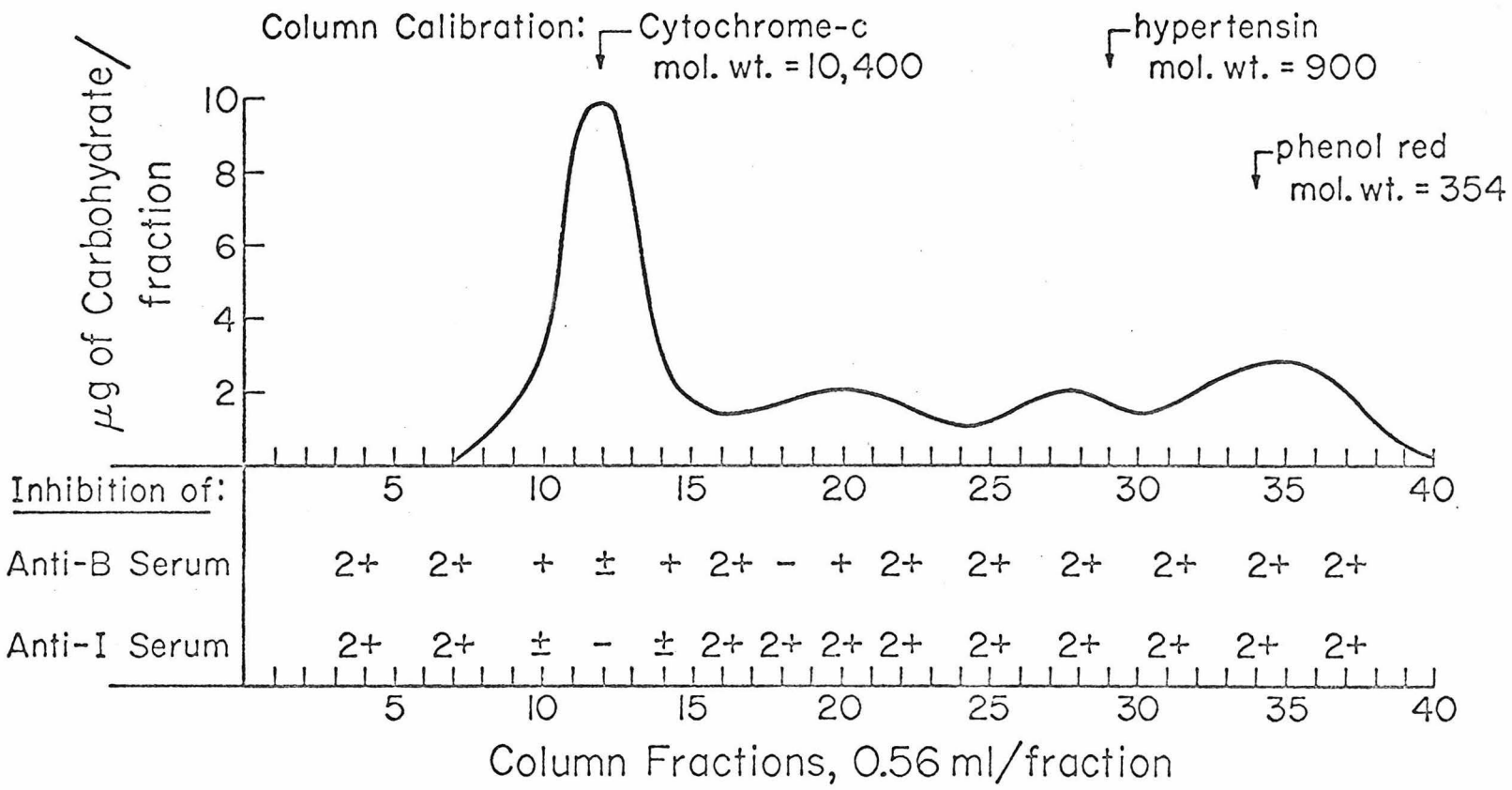


Figure 19. Biogel P-6 column fractionation of the hydrophilic sample from subtilisin digested SE-Sephadex fraction 2.

Figure 19 .:

Human B,I adult stromata were fractionated on SE-Sephadex. Fraction 2 from the front peak was subtilisin-digested and chromatographed. Material with R_f 0.0-0.2 was eluted with water and 1.0 mg was fractionated on P-6 biogel column (1.2 x 27 cm) in 0.05 N NH_4HCO_3. Samples were analysed by the anthrone method for carbohydrates. Blood group activity was located by agglutination inhibition assay, using 25 microliters of antisera with 2+ agglutination strength, and 1/3 of the lyophilized fractions which had been redissolved in 25 microliters of buffered saline.

Table 12 . Amino acid composition of Biogel P-6 column fractions.

Amino acids	nmoles per samples:			
	fraction 11	fraction 17-18	fraction 23-29	miniproteins from human rbc stromata *
CSA	1.20	--	1.34	4.26
ASP	6.68	3.58	3.87	5.67
THR	12.1	1.86	2.31	5.42
SER	12.7	2.48	3.22	8.40
GLU	9.85	3.78	5.36	7.11
PRO	4.10	--	1.52	--
GLY	4.16	2.03	4.24	5.44
ALA	5.53	1.94	3.18	4.17
CYS	0.82	--	1.21	--
VAL	5.47	1.80	2.07	6.22
MET	0.48	--	0.38	1.02
ILE	1.89	0.56	1.31	3.60
LEU	3.35	2.05	4.56	6.10
TYR	2.19	0.53	0.89	2.16
PHE	1.19	0.30	1.40	1.86
TRP	--	--	--	--
LYS	2.35	1.60	1.87	3.32
ARG	6.09	0.05	1.86	2.53
HEX-NH_2	19.34	2.07	2.85	n.d.
HEXOSE#	39.0	3.0	4.2	n.d.

* = unpublished data from Dr.T.Laico, Dr. E.Ruoslahti, Dr. W. Dreyer. # = determined by anthrone method. n.d. = not determined. -- = none detected.

Thr, as was the sample prepared from the hydrophilic fraction of the subtilisin digested whole stroma. The hexosamine content was higher in this fraction than in others, with a ratio of total hexose to hexosamine of 2. Subsequent fractions showed marked differences in amino acid composition; the amounts of Ser and Thr were always lower then Asp and Glu.

Fractions were treated with pronase in order to find out if complete degradation of peptides had any effect on the inhibition capacity. The results, summarized in Table 13, show that the I activity was completely destroyed by pronase, without any significant change in A or B blood group activity.

Table 13. The effect of pronase treatment on the blood group activity in factions from Biogel P-6 column separation.

Fraction 13	Dilution of the samples					
	1/2	1/4	1/8	1/16	1/32	1/64
Anti-B serum						
Untreated sample	-	-	≠	+	+	2+
Pronase treated	-	-	-	+	+	2+
Anti-I (HOV) serum						
untreated sample	-	-	≠	+	2+	2+
Pronase treated	+	2+	2+	2+	2+	2+
Fraction 19						
Anti-B serum						
Untreated sample	-	≠	2+	2+	2+	2+
Pronase treated	-	≠	2+	2+	2+	2+
Anti-I (HOV) serum						
Untreated sample	2+	2+	2+	2+	2+	2+
Pronase treated	2+	2+	2+	2+	2+	2+

Samples were digested with pronase(3% by protein weight) for 30 minutes at 37°C; mixed with an equal volume of anti-I sera diluted with 20% bovine serum albumin to an agglutination strength of 2+. The agglutination inhibition assay was carried out at 4°C; adult,I red blood cells in 20% bovine serum albumin were used to test for the anti-I cold agglutinins.

DISCUSSION

One advantage in studying the I-antigen system in man is the availability of well defined, easily purifiable cold agglutinin with anti-I-specificity. These IgM molecules have restricted heterogeneity, and their molecular structures can be studied by standard methods of protein chemistry.

The cold agglutinins studied have exhibited a wide range of thermal amplitudes as indicated in Figure 4. The normal anti-I isoagglutinin present in the serum of DAZ was active at 37°C, and no increase in agglutination titer was found at lower temperatures. This is in accord with previous observations that only agglutinins synthesized in response to microbial infections are cold acting. The agglutination characters of the sera from COH and AB3 well demonstrated the results of viral influenza or infections by other agents responsible for pneumonia.

These well-characterized protein molecules are now available to study the development of the I-antigen in post-natal infants and the status of this antigen in patients with hemolytic anemias. The IgM molecules offer an additional advantage in their multiple binding sites. In order to bind to a cell surface sufficiently strongly to produce agglutination, these agglutinins must attach to more than one antigenic site on the same surface. This requirement for having in the vicinity more than one antigenic site with blood

group specificity automatically makes the anti-I reagents also sensitive to the site distribution on the cell surface.

The importance of the two-dimensional distribution of molecules on the membrane surface has been recognized by numerous investigators. Recent experiments, for example, by Linscott (91) indicate that the density of antigenic determinants on a cell has a profound effect on the hemolytic efficiency of the complement system. This work suggests that the affinity with which IgM molecules bind the C_1 component of the complement system is proportional to the number of antibody combining sites occupied by the antigen. Low antigen density on the surface results in reduced C_1 fixation.

Hoyer and Trabold (92) have developed a model passive hemagglutination system, using red cells coupled with sulfanilic acid groups to study the importance of antigen site density. Relative site numbers were estimated from the covalently linked S^{35}-sulfanilic acid and the "effective" antigen site numbers were determined with I^{125}-labeled rabbit IgG anti-sulfanilic acid. Cells which had fewer than 20,000 antigen sites per cell were not agglutinated. The agglutination titers of purified IgM antibody were 10-20 times greater than those of IgG antibody when preparations with the same protein concentrations were compared. These findings emphasize the need to consider differences in antigen site density when comparing blood group systems. The type of antibody molecule must also be taken into consideration, because of the differences between IgG and IgM.

In the I blood group system cells with less then 20,000 antigen sites would be typed as I-negative. This does not mean that molecules with I-specificity are not expressed on the surface, but only implies a low-density distribution. The changes from the phenotype i cord to the I-adult have to be viewed in this molecular perspective.

The presence of anti-I isoagglutinins in the fetus with I-positive genotype presumably represents no danger to the individual as long as the cell surface is low in antigen sites. The rapid erythropoietic proliferation during late embryological development might be responsible for the low distribution. The presence of incomplete oligosaccharide chains on the cell surface explains the expression of H-specificity on cord cells of a group A individual. IgM with anti-A specificity in an A_2 type individual represents an analogous situation. The low density of antigen distribution could however, be the result of some additional differences in the molecular structure of some membrane structural component. An example would be having two different carrier molecules for the oligosaccharide side chains. The A_2 individual would have the capacity to attach sugars to only one of the carrier molecules, while an A_1 individual could complete both molecular structures. The findings of Hakomori et al. (14) are in agreement with such an hypothesis.

Are the embryonic antigens related to unfinished molecules exposed to the cell surface? It is possible that some

serologically determined embryonic specificities could be explained by such a molecular interpretation.

The enzymes responsible for the biosynthesis of molecules exposed on the surface appear to be regulated by a different molecular mechanism than the one responsible for the regulation of the hemoglobin subunit synthesis. This conclusion is drawn from the study of the correlation between the appearance of adult hemoglobin and the expression of the I-agglutinability. The rate of synthesis of molecules responsible for the I-specificity was not closely associated with the rate of beta polypeptide chain synthesis in postnatal infants. Although the two events take place at approximately the same time in development, such wide variation is found in the percentage of HbF in the"I-negative" cell population that one has to rule out a regulatory mechanism which would exercise full and direct control over both developmental changes. The single-cell studies provided additional evidence. Cells which appear as "I negative" on their surface can have predominantly adult hemoglobin content. The pooled cell studies also demonstrated that cells with sufficient I-specific molecules on their surface to appear as the 'adult-like' phenotype can contain considerable amounts of fetal hemoglobin. These findings are in agreement with the previous observations on erythrocytes from patients with persistent fetal hemoglobin disease. In these individuals the genetic locus for the structure of beta polypeptide chain

remains depressed and the synthesis of a gamma chain persists throughout life. So far there has been no evidence reported that such a condition has any effect on the biosynthesis of cell-surface-related molecules. These individuals' erythrocytes type serologically as normal "I-positive". However, because exact molecular fractionation of membrane-specific molecules and their precise quantitation has not yet been achieved, one has to accept this information with some caution.

The rate of A antigen expression on the erythrocytes from postnatal infants seems to be related to the rate of I antigen expression. This was demonstrated by the quantitation of A antigenic sites on "I-negative" and "I-positive" cells, using labeled anti-A antibody molecules.

The site of erythropoiesis may have a significant role in regulating the type of hemoglobin synthesis. Such a relationship could be best studied in mice, where there are indications that the type of hemoglobin synthesized is dependent on the erthropoietic site (93). Is the I-antigen exclusive to the bone marrow-derived erythrocytes in humans? It is difficult to answer this question without molecular fractionation of erythrocyte membranes from an embryo of 5-6 months of age. Serological tests would only tell if the I-antigen carrying molecules are available to the antibody population used for the tests.

The results from the experiment in buffy coat cell

membrane fractionation suggest that the I-specific molecules are not present on the surface of cells derived from the lymphopoietic cell proliferation, or if they are present their amount is less than 5% of the red blood cell's. Antigens expressed on the lymphocytes are not necessarily present on erythrocyte surfaces. Transplantation antigens are for example very difficult to detect on human red blood cells, and lymphocytes are not agglutinated by anti-A or anti-B sera. The presence of ABO antigens of lymphocytes has, however, been demonstrated by immunoabsorption methods (94). The molecules carrying the ABO specificity on the lymphocyte surface have not yet been characterized or isolated.

There are indications that the lymphopoietic and erythropoietic proliferation originates from a common stem cell (66). An example of the kind of experiments used to prove this differentiation scheme has been described by Till et al. (95). Bone marrow cells with identifiable chromosomal markers are injected into irradiated mice. Stem cells present in the bone marrow are capable of repopulating the tissues of the immune and of the hemopoietic system. Questions arise with respect to the differentiation of this stem cell. When and by what mechanism do certain specific events show an effect on the differentiating cell's surface? Is I-antigen agglutinability an exclusive property of the mature erythrocytes or does this surface specificity develop gradually during erythropoietic differentiation? Currently

there are no answers to these questions, but indirect observations suggest that the marrow transition time may play an important role in the expression of surface specificity. The I-antigen system may serve as an excellent tool to answer the question. Hillman and Giblett (28) have already reported that when there is a shortage of red blood cells, premature red cells are delivered into the circulation. This shortening of normal maturation is associated with an increase of i-activity. This observation may explain why patients with anemia due to a variety of disorders also have an elevated level of i-activity. Exact molecular quantitation of antigenic sites as a function of maturation time would be of utmost interest.

The chemical and enzymatic alterations of human adult stromata revealed very little information on the possible molecular bases of I-antigenic specificity. The experiments that the specificity requires the integrity of carbohydrate molecules on the surface, and ruled out sialic acid as an integral part of the I specificity. Acetylation of free amino groups had no detectable effect on the antigen.

Molecular fractionation of cell membranes and the localization of fractions with blood group activity proved more promising. The success of any isolation approach depends on the detection methods available. In all these determinations one has to keep in mind that a lack of inhibition of a blood group specific agglutination system does not prove that the

antigen-carrying molecular species are not present. Conversely, the inhibition of the agglutination does not necessarily prove the presence. The finding that insoluble aggregates could show blood group activity only following trypsin digestion illustrates the first point; the very high nonspecific inhibition by the hydrophobic fraction illustrates the second. In all experiments, careful controls were included to assure reliable conclusions.

The first important observation in comparing erythrocyte membranes from adult and cord blood samples was the striking similarity between their tryptic fingerprints. Their similarity was also noted in the observations on subtilisin digested membrane samples. This suggests that the major protein components of the cells do not change much from birth to adult maturation. The second important observation was related to the quantitation of I-inhibition capacity by the adult and cord erythrocyte stromata. Both samples have approximately equal amounts of total carbohydrate and protein. However, the stromata prepared from adult I-positive erythrocytes absorbed 64-128 times more anti-I cold agglutinins than did the stromata prepared from cord red blood cells. After disruption of the membrane and separation of the hydrophilic components by paper chromatography, the difference was only twofold. There are several possible explanations for this observation. The recovery of blood group activity could vary for the two samples, or the

distribution of the activity could be such that more adult I-specific molecules are present in the hydrophobic fraction, in which their detection would be difficult because of the high nonspecific inhibition by this fraction. Another explanation could come from the hypothesis that the I-antigen is present in larger quantities in the cord rbc membrane but it is not exposed to the surface, or it is covered up by other surface molecules. Another explanation would be on the basis that the display of the molecules responsible for the specificity is the critical factor in anti-I agglutinin absorption. This would mean that the two dimensional distribution of the molecules is responsible for the difference of IgM binding capacity on the cell surface. Once this molecular pattern is disrupted the difference becomes less noticeable and the specific inhibition of the two preparations may reflect only the number of molecules present in the membrane. The average distance between I-antigen sites on a cell surface, assuming uniform distribution, was estimated to be 80-100 ^{o}A. This distance is the same as the distance between the binding sites of the IgM macroglobulin molecules, as measured on electron micrographs. Parkhouse et al.(96) reported the overall diameter of IgM molecules to be 300-375 ^{o}A. Each molecule consists of five Y-shaped subunits; the two arms of the subunits correspond to the F_{ab} pieces with one binding site for each piece. The nearest binding sites are 70-80 ^{o}A apart. Cooperative binding would be

observed if such an IgM molecule were to approach a surface with an antigen distribution of 80-100 ^{o}A. A twofold decrease in antigen sites would have greater effect on IgM binding if the antigens are distributed on a surface in a regular molecular pattern rather than being randomly distributed in a solution. Accordingly the binding of 7S IgG molecules with only two binding sites would be less affected by such a molecular arrangement. No 7S anti-I IgG molecules have been detected so far; therefore it would be difficult to test the validity of this hypothesis. Antibodies directed against the A antigen system do however, confirm the relationship outlined with the I system. Anti-A 7S IgG molecules do not distinguish between the A_2 and A_1 subtypes as well as do the 19S IgM molecules (15). The IgM molecules agglutinate only the A_1 cells with the higher antigen density distribution.

The fractionation of erythrocyte stromata produced several membrane fractions with blood group activity. It is important to note that all the fractions containing I-activity also had ABO activity, but not all the ABO activity-containing fractions had I-activity. Part of the difficulty in evaluating this observation is rooted in the problem of antigen detection. I-activity was detected by inhibition of an anti-I serum in which all of the specific antibodies belong to the 19S IgM molecules with multiple binding sites, whereas the anti-A or B sera contain both IgG and IgM

antibody molecules. It may be important to observe that the major I-containing sample after separation on SE-Sephadex column was the so-called "miniprotein containing" fraction. This fraction contains lipid in addition. All of the protein molecules in this fraction, as determined with SDS gel-electrophoresis, were low molecular weight "miniproteins". The hydrophilic preparation from this fraction yielded a glycopeptide-containing sample which was further fractionated on a Bigel P-6 column. This separation revealed the presence of two different fractions, with different molecular weights. Only the larger molecular fraction contained I-activity, but both had A or B activity. Pronase treatment destroyed I-inhibiting capacity of the larger molecular weight fraction but did not have any effect on the A or B activity in either fraction. Although these observations are in agreement with the possibility that the I specificity is located on a glycopeptide, and the integrity of the peptide portion is essential for the blood group specificity, interpretation must wait until possible contamination by glycolipids is precisely investigated. The destruction of inhibition capacity by pronase treatment indicates that the role of polypeptides may be as carrier molecules, but does not prove it. The difference in pronase sensitivity could be explained by the variation in the detecting antibody population. 19S IgM molecules are better inhibited by larger aggregates and therefore the pronase

activity would be more noticeable in relation to the inhibition capacity of the I antigen than to the ABO specificity. Further purifications of these glycopeptides are in progress and will eventually reveal the molecular basis of the specificity. The amino acid composition of the larger hydrophilic molecular fraction is similar to the composition reported for the M, N blood group activity-carrying glycopeptides.

In addition to the previous observations it is important to point out the nature of protein fractionation on SE-Sephadex column. In all separations performed, various amounts of so-called "miniproteins" were recovered in fractions 1ø3 and in fraction 9. The variations in specific blood group activity indicate that fractionation was achieved which behaved independently from the protein distribution. Although low specific activity per mg of hexose was found in fraction 9, the total amount of blood group activity was significant. Approximately 10% of the total I activity was recovered in this fraction and about 40% of the B activity. The amount of specific activity per mg of protein or per mg of lipid was not determined.

The light chains purified from the cold agglutinin IgM molecules had only one amino acid residue in the first eleven N-terminal positions. This indicates a restricted heterogeneity and a possible monoclonal origin for the antibody molecules. Such findings have been previously reported by Cohen et al. (56). The production of restricted antibody heterogeneity might be similar to the production of structurally homogeneous antibodies against capsular polysaccharides of pneumococci (100).

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