A STUDY ON THE AGGLUTINATION OF ERYTHROCYTES

A Thesis

by

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I N D E X

INTRODUCTION

The present investigation arose out of a study on blood preservation which was begun in the Department of Biochemistry at McGill in January, 1940 by Collip and Denstedt. In the course of the investigation it became necessary to evaluate the efficiency of various methods of preservation and the viability of the preserved red cells. The only satisfactory method at that time, and even at the present date, was to transfuse the preserved blood and follow the elimination of the donor cells from the circulation of the recipient. This was done by the method used first by Ashby (1) in 1919 and which has been modified in various ways since that time. Ashby's procedure was to transfuse blood (cells) of a donor of blood group O into a recipient of group A (or B) and periodically thereafter to remove blood samples from the recipient and, after agglutinating the latter's cells with anti-A serum, to count the unagglutinated donor cells in the haemocytometer. It is impossible to agglutinate completely cells of any group with even the most potent sera; there is always a certain number--less than 1% of the cells--which cannot be agglutinated. The proportion of these "free" or "unagglutinable" cells in the recipient's blood, therefore, must be accurately determined prior to the transfusion since they will be

included among the total number of unagglutinated cells in subsequent tests. Experience of various workers had shown that the "free-cell" count of any person remains practically constant provided the same antiserum is used and the technique of agglutination is kept uniform. Thus by deducting the recipient's free-cell count from the total number of unagglutinated cells found in any test, the remainder presumably represents the number of donor cells per mm^3 which still remain in the circulation. Transfusion and cell survival experiments with fresh blood afforded a normal basis for comparison. It has been shown repeatedly that the fresh cells are eliminated from the recipient's circulation usually at a linear rate of about 0.8% per day, and that the last detectable donor cells disappear about the 125th day after transfusion. This figure therefore is taken as the average life span of the human erythrocyte.

With the discovery of the M, N and Rh red-cell agglutinogens it was possible to use these types as well as the A, B, O groups with the Ashby method. Thus a group OM blood (cells) could be administered to a recipient of group ON and agglutination tests be carried out with anti-N serum. Other variations also are applicable by administering ORh- blood (cells) to a recipient of group ORh+ using anti-Rh testing serum. It

is even possible to follow the survival of two donor bloods simultaneously in a recipient. For example donor bloods of group OM and ON have been transfused in close succession into a patient of group BN and the survival of the two followed simultaneously with anti-B and anti-N respectively.

In the course of the studies (2,3) carried out by Denstedt and Osborne from 1943 to 1945 certain anomalies in the results of the differential agglutination method were encountered. In many of the cell survival studies carried out there were periodic increases in the number of unagglutinable cells. This should not occur provided that the recipient's free-cell count remained constant and the transfused cells were being continuously destroyed. These increases were not due to errors in counting (or decrease in potency of the antiserum). That some erythrocytes were being sequestered from the circulation and released again periodically was untenable. A study on the constancy of the free-cell count and the factors which influence it, therefore was carried out by Osborne and Denstedt (3). It was found that the count could be relied upon as remaining constant only if strong test sera were used, and provided the conditions of agglutination, for example, rate of shaking, temperature, etc. were kept

constant. Even at that certain striking and obscure anomalies were encountered from time to time. It was not evident, for example, why markedly avid antisera gave a high free-cell count, especially when there was no lack of antibody. Variability in the free-cell counts was still encountered from day to day, even though they were found to be quite constant on any one day.

In the present investigation an effort has been made to provide an explanation for these anomalies and to study the mechanism of agglutination. The study includes the specificity of the second or aggregation stage of agglutination and the effect of temperature and various other conditions on agglutination. A theory has been developed also to explain the free-cell count and other anomalies of agglutination. In addition, an alternate method of following cell survival using erythrocytes containing sulphaemoglobin has been developed for the purpose of checking the accuracy of the serological method.

I The Properties of Antibodies

1. Relation to gamma-globulin

An antibody, according to the definition given by Topley and Wilson (4), is any substance which makes its appearance in the blood serum or body fluids of an animal in response to an antigen introduced into the tissues, and which reacts specifically with the antigen. Presumably the authors would include also under the definition the various normally occurring antibodies such as the alpha and beta agglutinins of human blood plasma.

That the antibody fraction in the blood is closely associated with the serum globulins has long been known since it is possible to concentrate them in certain globulin fractions by fractional precipitation of the serum proteins. Also, they resemble common proteins in physical and chemical properties, and the susceptibility to denaturation and hydrolysis by proteolytic enzymes.

Knowledge of the nature of antibodies has been extended greatly in the past decade through the use of more refined quantitative analytical methods and with the aid of newer techniques such as Tiselius' electrophoretic and Svedberg's ultracentrifugal methods for studying the size and shape of protein molecules.

The majority of antibodies are included in the gamma-globulin fraction or a closely related fraction of the blood plasma and tissue fluids, since most of the immune bodies studied by electrophoretic analysis in the rabbit, horse, rat, monkey and man, are contained in the least mobile globulin fraction; i.e. the gamma-globulin. For example, Tiselius and Kabat (5) state that "rabbit and monkey antibody (pneumococcal) and rabbit anti-egg albumin antibody were quantitatively contained in the gamma-fraction; that removal of the antibody with antigen produced a decrease in the gamma-globulin and that this decrease corresponded quantitatively to the amount of antibody removed".

It is common knowledge that the concentration of total serum globulin and gamma-globulin in man undergoes a parallel increase in infections. This is seen, for example, in tuberculosis, leprosy, lymphogranuloma, rheumatoid arthritis, and other diseases which are characterized by hyperproteinemia. It is not known to what extent the increase in gamma-globulin is due to the antibody itself. According to Kabat (6) about 1/3 of the total globulin in human serum is gamma-globulin.

Even in normal persons the globulin fractions may contain a large variety of antibodies. Thus

Enders (7) has found at least 16 different antibodies in serum globulin fractions which were more than 90% gamma-globulin.

Cohn (8), Oncley et al (9), in their separation of the protein and lipoprotein components of human plasma, have confirmed their previous finding that the antibodies occur largely in the gamma-globulin fraction.

2. Chemical nature and physical properties

The exact difference between "normal" globulins and immune globulins is still uncertain. It is known at least that both are protein in nature and are very similar in chemical and physical properties. However, the immune globulins possess the property of specificity which does not appear to be possessed by the normal globulins. It is very difficult to make comparisons from the data in the literature since the majority of studies were carried out with impure globulin fractions. Of those workers who used pure globulin some found differences, while others did not. For example, Smith, Brown and Gross (10) and also Calvary (11) found the nitrogen content of purified globulin to be practically identical with that of normal serum pseudoglobulin, purified Type I pneumococcus

antibody and purified diphtheria antiserum. Other workers have obtained evidence of differences in the normal and immune globulins. The antipneumococcus antibody in horse serum, for example, has an isoelectric point of about 4.8, while that of normal horse globulin is about 5.7 (12).

Perhaps the structure rather than the chemical composition is the important determining factor in distinguishing antibodies from normal serum globulins. Pauling (13) suggests that the precursor of a molecule of normal gamma-globulin is a polypeptide chain containing a thousand or more amino acid residues arranged in a sequence determined by the protein-synthesizing enzyme system of the cell. The chain may become either a molecule of normal gamma-globulin, or it may undergo folding and assume a definite pattern under the influence of a given antigen and thus acquire a specificity for the latter. Thus it might be possible to have a close relationship or even identity in the composition of the normal and immune globulins and yet have a wide difference in biological activity due to a difference in folding.

3. Molecular weight and shape

The molecular weight of antibodies in the rabbit (and probably in man also) is of the same order

as that of normal rabbit globulin, viz. about 160,000 (14). The shape of most antibodies is asymmetric. Neurath (15) has estimated that the molecule of human antipneumococcus antibody is a prolate ellipsoid with axial dimensions of 37 Ångström units (A.U.) and 338 A.U. respectively. For the rabbit antibody the axes are 37 and 274 A.U. respectively, and for the horse antipneumococcus, 47 and 950 A.U. The antitoxic pseudoglobulin molecule of the horse also is asymmetric. Petermann and Pappenheimer (16) have calculated the ratio of the major to the minor axis to be 7.0. In shape and size of the molecules, therefore, there is no striking difference between the ordinary and the immune globulin.

4. Rate of production and site of formation

Antibody may be detected within a few days after the injection of antigen and the titre in the serum usually reaches a maximum within a week or two. Thereafter the antibody concentration falls as they are gradually eliminated and their production falls off. The titre in the serum and the rate of formation are generally enhanced by repeated administration of antigen until a level is reached where further antigenic stimulation becomes ineffective, and the response declines and ultimately ceases. Schoenheimer (17) by

administration of glycine containing radioactive carbon (C^{14}) has estimated the survival time of antibodies to be about 30 days. It would seem that the rate of antibody production is at least as great as that at which the body can replace the globulins in the circulation. Madden (18) estimated that a dog weighing 11 kg. produced 13 g. of plasma proteins per week, or about 170 mg. per kg. of body weight per day. However, in certain infections the rate of production of globulins is increased.

Although the site of formation of antibodies is still somewhat in doubt, the evidence on the whole favors the reticulo-endothelial system as the main site of formation (19,20). Madden and Whipple (21), in their review of the subject, consider the liver to be of major importance in the production of all plasma proteins. However, deGara and Angevine (22) claim that antibodies can be manufactured locally in almost any part of the body.

There is considerable evidence that antibodies are formed by the lymphocytes and in the lymphatics. McMaster and Hudack (23) are of the opinion that antibody formation takes place largely in lymph nodes, while other workers (24,25,26) held that the lymphocytes themselves are the main site since they observed that

the antibody concentration in extracts of lymphocytes was higher than that of lymph plasma. White et al (27,28) found that injection of adrenal cortical extract (ACE) into mice, rats, and rabbits, within a few hours caused a rapid disappearance of lymphocytes with a concomitant increase in serum protein, chiefly globulin. They reported also that simultaneous injections of ACE and antigen produced higher antibody titres than with antigen alone. Eisen et al (29), studied the effect of ACE in adrenalectomized rats maintained on sodium chloride and desoxycorticosterone acetate (DCA). The latter substance had been shown to have no effect on antibody levels (25). Their findings show that adrenal cortical activity has only a transitory and perhaps insignificant effect upon the production or release of antibodies and gamma-globulin. However, they did find that shortly after a single injection of ACE into rabbits which previously had been immunized with pneumococcus Type I, there was about a 30% increase in serum antibody nitrogen. Thus the possibility still remains that the adrenal cortical hormone may be part of a protective mechanism that leads to an initial sudden release of antibodies from existing lymphocytes.

The plasma cells, considered by many to be derivatives of lymphocytes, also are claimed to play a

role in antibody production. Kabat (30) recently has reviewed this subject.

5. Mode of formation

Alexander (31) and Mudd (32) were first to propose a satisfactory theory of antibody production. They suggest that as a new molecule of globulin is formed, presumably in the reticulo-endothelial cells, the sequence of the amino acids on part of the surface is influenced by a molecule, or part of a molecule of antigen adjacent to it. The rearranged chemical grouping on the surface of the antibody then could function as a combining group by virtue of its complementary structure. Pauling (13,33) suggests that the antigen influences the folding of the polypeptide chain rather than the sequence of the amino acids in the chain. Burnet (34), on the other hand, thinks it unreasonable that the mere presence of an antigen can give rise to a complementary structure in the forming antibody, but thinks rather that the proteinases undergo modification by the antigens they destroy, and that they synthesize the specific antibodies. These theories, while reasonable, are highly speculative and lack experimental evidence.

Pauling and Campbell (35) claim to have conferred antibody-like properties on normal bovine gamma-globulin by in-vitro reversal of denaturation in

the presence of antigen. They interpret this as confirmation of their theory of antibody formation. Kabat (30) doubts the validity of this conclusion and similar reports of antibody production in-vitro. If it should be successfully demonstrated that a protein, by artificial means, can be made to acquire a specificity for a given antigen, it will be of considerable value to our understanding of protein structure.

Denaturation of normal globulin, followed by renaturation in the absence of antigen, should give rise to a product lacking any serological specificity, since it is unlikely that any portion of the molecule would assume the essential configuration. Neurath and Erickson (36) performed experiments using purified antipneumococcal horse-serum globulin, Type I, and normal horse pseudoglobulin, which had been denatured by, and regenerated from, 8 molar guanidine hydrochloride. The irreversibly denatured antibodies, as well as the regenerated ones, were strongly precipitated with the homologous antigen; while the irreversibly denatured and regenerated normal globulins apparently did not possess any serological activity. This suggests, contrary to Pauling's views, that there is little dependence of antibody activity on a specific structure but that the essential requirement for activity is the nature and sequence of amino acid

residues in the polypeptide chains. Surface-film studies tend to support this but most of the work on protein monolayers and biological activity has been done with antigens (37). Rothen and Landsteiner (38) reported an experiment in which an unfolded film of Types I and III pneumococcal antibodies was observed to react specifically with the homologous polysaccharide. Thus it appears that certain kinds of biological activity require a specific configuration of the native protein, whereas others require a certain amino acid composition of the polypeptide chain, regardless of the mode of folding.

6. The stability and alteration of antibodies

The serological activity of antibodies is destroyed by many agents or treatments which bring about denaturation and modification of proteins in general. Inactivation of antibodies by heat, e.g. has a high temperature coefficient and proceeds rapidly at the coagulation temperature of serum proteins. Many of the effects of alteration in proteins will be discussed in the section on chemically altered antigens. This topic is discussed fully in reviews by Landsteiner (39), Marrack (40), Neurath (41) and Herriott (42).

II The Properties of Antigens

1. Antigenicity

The majority of known antigens are proteins of high molecular weight. Some lipids, however, particularly cholesterol and related compounds, when injected along with an antigenic protein, cause the production of antibodies specific for the lipid. Since cholesterol itself is not antigenic, the protein evidently forms a complex with lipid. In other words, the lipid is what Landsteiner calls the haptenic group. Many polysaccharides are also antigenic. For example, purified polysaccharides in the capsular material of the pneumococcus are antigenic in the mouse and man (43).

According to Boyd (44) the antigenic property of protein tends to be greater the farther the source of the protein is removed from the test animal in the phylogenetic scale. This would imply that fish globulin is more antigenic to rabbits than mouse globulin would be. The absence of antigenicity in certain proteins, for example, gelatine, he attributes to the fact that they are removed rapidly from the circulation. It is significant, however, that gelatine contains little tyrosine and tryptophane. The phenolic hydroxyl of tyrosine is a hydrogen bond forming group and during denaturation of a protein the free phenolic hydroxyls are increased. Perhaps the lack of these groups in

gelatine makes it impossible for gelatine to assume some essential configuration necessary for antigenicity. In addition, gelatine is not denatured in the usual sense, only fragmented.

For a review of the literature on the complex bacterial antigens the reader is referred to Boyd (45) and to Landsteiner (39). The chemical nature of bacterial antigens also has been discussed by Morgan (46). He states that the antigenic complex in the intact bacterial cell contains substances in addition to phospholipid, polysaccharide, and protein components, but it appears that the protein and polysaccharide components are essential for antigenicity.

2. Size and number of reactive groups

Antibodies can be prepared against simple chemical compounds such as p-aminobenzoic acid coupled to protein by injecting these compounds into animals (39). The antibodies will precipitate any protein complex containing the chemical compound (hapten). The reactive group in the antigen is, of course, of known size, since it is the introduced compound. The location and distribution of reactive antigen groups in the natural protein molecule, however, remain unknown.

Attempts to estimate the size of antigenic groups in natural proteins were made by Landsteiner (47).

He hydrolyzed silk with sulphuric acid and obtained peptides with molecular weights ranging from about 600 to 1000. These peptides were capable of inhibiting the precipitin reaction between the antibody and silk in solution. He suggested that these peptides were the antigenic groups of the silk molecule and that in general natural haptens might be of this size. Compounds simpler than the natural hapten however, but chemically related to it can serve as inhibitors.

From analysis of antigen-antibody precipitates Hooker and Boyd (48) estimated the maximum number of antibody molecules that can combine with the antigen molecule. The number of combining groups on the antigen, therefore, cannot be less than this number. These workers have cited values for several proteins, ranging from 5 for ovalbumin with a molecular weight of 40,500, to 231 for Viviparus haemocyanin with a molecular weight of 6,630,000. The number of combining groups appears to vary with the molecular weight of the antigenic material. It is quite possible that because of steric hindrance some of the combining groups on the surface of an antigen molecule cannot react with antibody, so these figures represent minimal values.

3. Chemically altered antigens

Many workers have attempted to determine what parts of the protein structure are essential for the various serological activities. It was assumed that if a specific group were destroyed by a particular reagent, this could be taken as an indication that the group played an important role in mediating the activity. A number of reagents and methods have been used to modify protein. Few workers however, have established that a chemical reaction has been carried through involving specific groups of a protein without denaturing the rest of the protein in the process. Even though it is not possible to define denaturation exactly, if change in the unique structure of protein occurs to any extent, the nature of the change doubtless is complex and no reliable interpretation can be made as to the function of a specific group.

The literature on the subject is vast and since the present investigation has been concerned with only a few reagents, the reader is referred to reviews by Neurath et al (41), Herriott (42) and Olcott (49). In the first the chemistry of protein denaturation is discussed, the second and third review the reactions of native proteins with chemical reagents, especially where denaturation is not to be expected.

The effects of heat on immunological properties

of immune sera, purified antibodies and virus antigens have been investigated by Neurath (41) and Follensby (50). The present author also has been interested in certain aspects of this subject especially relative susceptibility of the red-cell antigens to alteration by various agents. Lubinski (51) found that heating red cells at 56° C for periods ranging from 5 to 20 mins. either diminishes or destroys the capacity of the Rh antigen to react in the normal manner and give rise to agglutination. The A, B, M and N antigens on the contrary are not affected. The Rh antigen also was more labile to formaldehyde. Hubinont (52) reports that he was able to release the Rh antigen from red cells by heating.

The reaction of formaldehyde with proteins is complex since it may involve various functional groups (49). Thus in neutral solution, the immediate reaction is an equilibrium combination with the uncharged amino groups. The formaldehyde tends slowly to become more firmly bound and the amino nitrogen content decreases. The guanidyl group also reacts slowly with formaldehyde at neutrality and room temperature. In alkaline solution, not only the free amino groups, but also the indole nucleus, and the amide and guanidyl groups rapidly combine with formaldehyde. The initial reaction with the amino group is still reversible, while

the later combinations are more stable, thus making it possible to obtain protein derivatives with the amide, indole and guanidyl groups combined with the reagent. The reaction of the -SH group with formaldehyde seems to proceed slowly, although a readily dissociable compound may be formed. The reaction of protein with formaldehyde is complicated by cross-linkages. The cross-linking of guanidyl groups has been demonstrated with protamine (53). The condensation reaction of aminomethylol groups ($RNHCH_2OH$) with amide, imidazole, indole, phenol and perhaps other groups, to give stable methylene linkages, can occur under conditions where neither the amino nor the polar groups by themselves give stable formaldehyde addition products. The reactions of formaldehyde with amino acids and proteins have been reviewed by French and Edsall (54).

Another reaction which can be used is acetylation. Of the two acetylating agents, ketene and acetic anhydride, only the former can be used for tying up the free amino groups on the surface of the red cell. Herriott (42) and Olcott and Fraenkel-Conrat (53) have reviewed the literature on acetylation. From pH 7.0 to pH 5.0 ketene reacts with $-NH_2$, the phenolic hydroxyl of tyrosine and -SH in order of decreasing reactivity. With the amino group, the reaction is as follows:

$$\text{(Protein)} - \underset{\underset{H}{|}}{NH} + O=C=CH_2 \longrightarrow \text{(Protein)} - \underset{\underset{H}{|}}{N} - \underset{\underset{O}{\|}}{C} - CH_3$$

Li (55) achieved acetylation of the primary amino groups in the lactogenic hormone by passing a stream of ketene for 5 minutes into a suspension of the hormone in molar acetate buffer of pH 5.6 at room temperature. Li and Kalman (56) in a later paper, confirm the previous conclusion that free amino groups are essential for the biological activity of lactogenic hormone.

It was also shown by Herriott and Northrop (57) that at pH 5.5 the amino groups of crystalline pepsin were the first to be acetylated and that the acetylation of the phenolic groups became slower as the pH of the medium was reduced, and virtually stopped at pH 4.0. Selective acetylation of the amino group of pepsin was achieved by Herriott (58) who hydrolyzed the acetylated phenolic groups in alkaline medium. It has been suggested (59) that some of the asymmetric carbon atoms of proteins might be racemized by ketene in acid solution after several hours' exposure. In addition it seems that the -SH groups react only very slowly with ketene (60), possibly only as fast as they are unmasked through denaturation at the cell surface.

4. Blood group substances in the stroma of the red cell

Absorption of agglutinins on the red cell apparently leads to some kind of injury to the cell surface. Haemolysis can then be brought about rapidly by the addition of complement or more slowly by mechanical trauma (61). Haemolysis also tends to occur in hypotonic solutions. Surface active agents such as digitonin, saponin, and lysolecithin, may dissolve out fatty material or may denature proteins in the cell surface, leaving holes sufficiently large for the haemoglobin molecules and soluble constituents to diffuse out (62). There remains an insoluble residue, the stroma or "ghost", the membrane of which has a thickness of some 200-300 A.U. Calvin and co-workers (63) have isolated a lipoprotein complex, "elinin", which makes up 40% of the stroma substance. The remaining 60% of the stroma is the insoluble protein constituent, the "stromatin". The Rh antigen was found entirely in the elinin fraction. In addition the content of A and B antigens was 4-5 times higher in this fraction than in the whole stroma. An ether soluble fraction still richer in Rh antigen has been separated by Calvin from elinin.

The other blood group substances such as, A_1, A_2, B and O and the series M, N and S are also associated with the stroma. Morgan (63a) gives the following analysis of the A, B and O substances:

Substance	Total N	Amino N	Hexosamine N	Hexosamine	Reducing Sugar (as glucose)	Ash
A	5.65	2.48	1.81	23.3	45.5	0.76
B	5.33	2.35	1.71	21.7	48.5	0.90
O	5.75	2.91	1.68	21.5	46.5	1.91

Kabat (64) has compared A substances from various human sources such as saliva, stomach and amniotic fluid. They were similar in the content of nitrogen, glucosamine, reducing sugar, and acetyl values. The substances A_1 and A_2 differed in optical rotation. No chemical differences were found between group A and O substances (65). They are similar in viscosity and electrophoretic mobility; both contain l-fucose, d-galactose and d-glucosamine and both also have the same nitrogen, reducing sugar, glucosamine and acetyl content.

III The Mechanism of Agglutination

1. The nature of the antigen-antibody binding

The nature of the chemical bonds which hold atoms together into stable molecules has been summarized in two monographs (66,67). Two atoms, instead of interacting strongly to form chemical bonds, may interact weakly by means of inter-molecular forces. The properties of antigen-antibody systems, especially their reversibility, are such as to indicate that antigen-antibody attraction is due to weaker inter-molecular forces and not to the formation of chemical bonds.

Evidence has been brought forward more recently for the possibility of long range forces between antigen and antibody. The work of Rothen (68) especially seems to point to this possibility. Films of barium stearate or heterologous proteins deposited on top of antigen films do not prevent a specific adsorption subsequently of homologous antibodies. That there may be holes in the screen through which the antibody molecules can project was discounted in view of the closely packed structure of such screens in relation to the size of the antibody molecule. However, most workers do not assume that the whole antibody molecule reacts, but that only a surface "patch" is involved in the binding. The rate of interaction was of the same order whether there was a screen

or not and the nature of the screen did not seem to play an important role. Rothen assumes an effective range between the reactive groups of antibody and antigen of the order of hundreds of A.U., instead of a few A.U., as between individual atoms. Rothen (69) also points out the possibility of such long range forces in enzymatic reactions. It was found that the greater the number of underlying monolayers, the greater was the thickness of barrier necessary for protection against enzymatic action. He suggests that this long range interaction might take place through "resonating extended oscillators", the presence of which according to London (70) is likely to occur in large molecules. Rothen's hypothesis is not clear nor has the idea been further developed. Further evidence is needed to prove that the screens are of uniform thickness and free of holes.

The alternative and generally accepted view presupposes the action of weak intermolecular forces. These forces may be of three kinds, namely (a) attraction of electric dipoles or multipoles, (b) electronic Van der Waals's attraction and (c) hydrogen-bond formation. These polar forces between molecules reach their maximum when the molecules are as close together as possible. The distance of closest approach of two molecules is determined by the "electronic spacial extension" i.e.

field of space of the atoms in the molecules. Polar forces may be considered as arising from the unequal distribution of electrical charges in the molecule; e.g. the hydrogen atoms of water are positively charged and the oxygen atoms negatively charged thus making the molecule as a whole, neutral. Films formed on the surface of water afford a simple example of the orientation of molecules under the influence of polar forces. A polar group such as the carboxyl of a fatty acid is attracted by the water molecules, while the nonpolar hydrocarbon tail is attracted less strongly. If there is insufficient room for the fatty acid molecule to lie on the surface of the water the carboxyl group will be in the water and the hydrocarbon chain will be oriented upwards.

In the co-valent bond two electrons may be shared between two atoms forming a single bond, each atom supplying an electron. The number of electrons in the outer shell of each atom now possesses the stable electron octet. Both electrons may be supplied by one atom to form a co-ordinate bond. In the double bond 4 electrons, and in the triple bond 6 electrons, are shared. The electrons, however, are not stationary and in a co-valent link are not necessarily shared equally between the two atoms. The outer orbit of one atom may be larger and the electrons will spend more time in the orbit, so that the one atom will be relatively negative, and consequently

the other atom will be positively charged.

In a co-ordinate link the atom which supplies the pair of electrons loses them from its system during part of the time, without a corresponding gain as in the co-valent link. This atom is therefore positively charged while the other atom in the link, since it gains two electrons, is negatively charged. The molecules formed on the whole may be neutral, but their constituent atoms may be positively or negatively charged and attract oppositely charged atoms of other molecules. The strength of the fields varies greatly with different groups of atoms.

Another general force of intermolecular attraction, which operates between molecules, is electronic Van der Waals's attraction. A molecule of methane, for example, which has no permanent average electric dipole moment, may have an instantaneous electric dipole, as the centre of charge of the electrons, in their rapid motion in the molecule, swings to one side or the other of the centre of charge of the nucleus. The temporary dipole moment produces a transitory electric field, which tends to polarize any molecule in the neighborhood. The electrons of the latter molecule would move in such a way relative to its nucleus, as to give rise to a force of attraction towards the first molecule. This electronic attraction operates between every atom in a molecule and

every atom in other molecules in the immediate neighborhood and is increased very rapidly as the interatomic distance is decreased. Thus, molecules whose surfaces are largely complementary tend to show strong mutual attraction.

Another type of intermolecular attractive force is that associated with the hydrogen bond. The bond arises from the attraction of a hydrogen atom, when attached to an electronegative atom, for an unshared electron pair of another electronegative atom. The strength of the bond depends on the electronegativity of the two atoms; fluorine, oxygen, and nitrogen, being the most electronegative atoms tend to form the strongest hydrogen bonds.

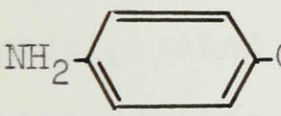
2. Specificity of binding in the first stage of the antigen-antibody reaction

The intermolecular forces in themselves are not specific. The study of Landsteiner and others on various artificial, conjugated antigens provides much qualitative information on the specificity of antibodies. The findings, when considered along with the results of experiments with natural antigens, led to the proposal independently by Breinl and Haurowitz (71), Alexander (72), and by Mudd (73), of the theory of the structural "complementariness" of the antigen and its specific antibody. The idea has

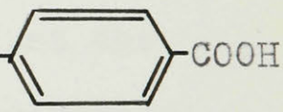
been amplified by Pauling (74). The concept implies that two molecules with configurations such that the surface of one conforms closely to the surface of the other will attract one another provided the oppositely charged groups are brought close together. If in addition the hydrogen-bond forming groups are also so placed as to present the maximum number of hydrogen bonds, then the total energy of interaction will be great, and the two molecules will attract each other strongly. A molecule, therefore, would show strong attraction for another molecule which was completely complementary in surface configuration and in the distribution of charged and hydrogen-bond forming groups over a considerable area. The attraction would be weaker between molecules which approximate a complementary surface, and very weak for all other molecules.

The agglutination of erythrocytes, bacteria and colloidal antigens by specific antisera is commonly regarded as being a two-stage process. In the first stage, antibodies of the serum combine with antigen on the cells or particles, producing a state of "sensitization". In the second or "aggregation" stage, the cells or particles cohere and precipitate out in clumps. The specificity of the first stage was investigated by Landsteiner (39) and later by Pauling who considerably extended the knowledge.

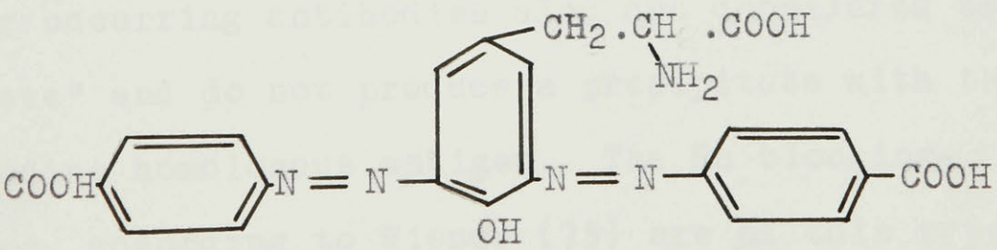
Karl Landsteiner (39) discovered that it is possible to cause an animal to produce antibodies having the power of specific combination with various chemical groups, which he called the haptenic group. He and his collaborators prepared artificially conjugated antigens by coupling relatively simple chemical compounds to proteins. The artificial antigens so formed were usually azoproteins with structure: protein—N=N—R, made by coupling a diazotized amine to the protein molecule. By injecting these substances into animals Landsteiner produced antibodies specific for the protein and antibodies specific for the attached group of known structure. For example: p-aminobenzoic acid



may be diazotized to



and when mixed with a protein such as egg albumin in alkaline solution will combine presumably with tyrosine or histidine in the protein to form an azoprotein. With tyrosine for example the following compound would be produced:



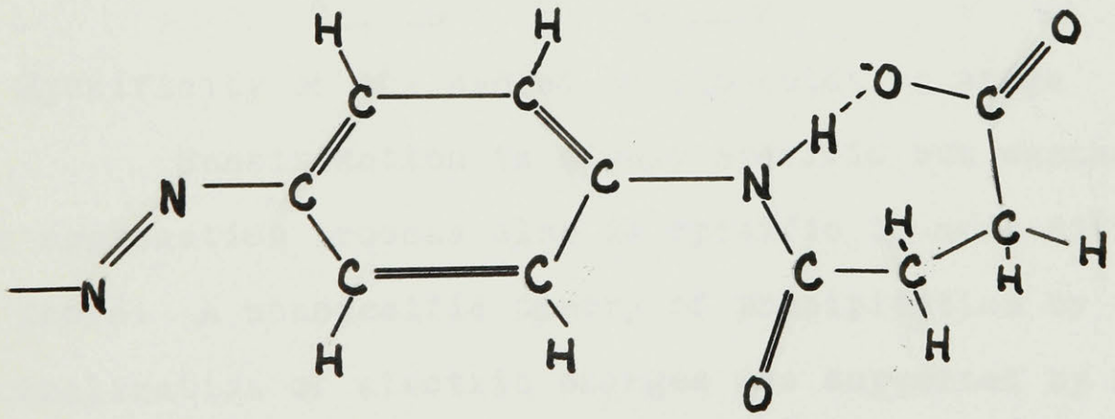
The antiserum made by injecting the azoprotein into a rabbit not only had the power of forming a precipitate with a solution of the azoprotein which was

made from egg albumin and was used as antigen, but could form a precipitate with any other protein coupled with diazotized p-aminobenzoic acid. In other words, an antibody was produced which was specific for the hapten group of the antigen. The antibody however can react weakly with azoproteins carrying closely related haptens such as substituted p-aminobenzoic acid for example, with a group or atom such as methyl or chlorine on the benzene ring.

When benzoic acid itself was added to an anti-p-aminobenzoic acid serum no precipitate was obtained. The serum antibodies apparently were blocked since on addition of the homologous azoprotein it was not precipitated by the antiserum. It is assumed that the antibody has two combining sites and that two molecules of the benzoate ion attach, one to each of the reactive groups. The antibody however is not able to form an aggregate or precipitate with benzoate ion, since the latter possesses only one combining group. Certain naturally occurring antibodies also are considered to be "incomplete" and do not produce a precipitate with the corresponding homologous antigen. The Rh blocking-antibodies, according to Wiener (75) are of this type and thus have only one combining group.

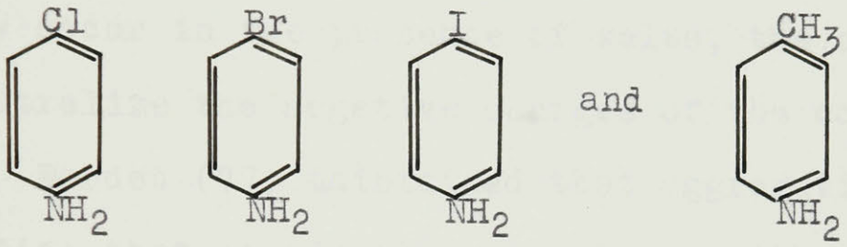
Continuing the work of Landsteiner, Pressman et al (76) have carried out quantitative studies on the

relative inhibiting power of a large number of haptens, and have obtained information about the closeness of fit between the combining group of the antibody and certain haptenic groups of known structure. In general, the replacement of one group by another differing in shape by as much as 1 or 2 A.U. leads to a significant decrease in the capacity to combine with the antibody. Pressman et al hold that the principal forces of attraction between the antibody and the hapten are: (1) the attraction of a positive group for the negative charge of the carboxyl group, (2) attraction for the carboxyl group through formation of a hydrogen bond, and (3) Van der Waals's attraction for the benzene ring. The anti-serum was prepared by immunizing rabbits against an azoprotein made by coupling sheep serum with diazotized p-aminosuccinanilic acid as illustrated below:

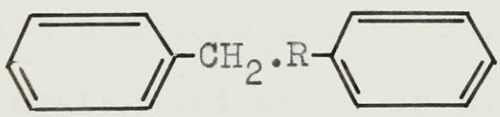


cis p-azosuccinanilate ion group

A further example of the specificity of the first or sensitization stage of the antigen-antibody reaction is shown by the serological equivalence of determinant groups which differ slightly in chemical composition. For example substituted products such as the following are practically equivalent:



The same is true of groups having local similarities of electric field. The oxygen atom and the groups $-CH_2$ and $-NH_2$, for example, are mutually interchangeable in crystals of the substances represented by the structure shown below where R may be O, CH_2 or NH (77):



3. Specificity of the second or flocculation stage

Sensitization is highly specific but whether the aggregation process also is specific is more difficult to prove. A nonspecific theory of precipitation by neutralization of electric charges was supported by many early workers who were attracted by the analogy to the phenomenon of mutual precipitation of oppositely charged colloids. It is now known, however, that under normal hydrogen-ion and salt concentrations, antibodies and most

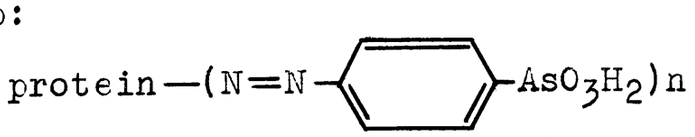
antigens are negatively charged and therefore the theory of neutralization has been abandoned. The failure of antigen-antibody systems to agglutinate in low salt concentration is attributed to the mutual electrostatic repulsion of negatively charged complexes in solution thus preventing the formation of larger aggregates. Agglutination may occur in the presence of salts, the cations of which neutralize the negative charges of the complexes.

Bordet (77) maintained that aggregation is nonspecific; that combination of antigen with antibody so alters the properties of the cells or particles as to render them susceptible to flocculation by the electrolyte in the surrounding medium. Several years later Marrack (78) advanced the theory of specific aggregation, frequently referred to as the "framework" or "lattice" hypothesis, which implies that homologous antibodies constitute the links that hold aggregated cells or particles together. Thus, if cells, differing in antigen composition, be mixed and agglutinated simultaneously with their respective homologous antisera, the aggregates according to Bordet, should consist of a mixture, whereas according to Marrack each type of cell should form separate aggregates.

Heidelberger, Kabat and Kendall (79-83) precipitated polysaccharide, azoprotein, and other protein antigens with immune serum, and showed that aggregation is specific. Topley, Wilson and Duncan (84) used a mixture

of two species of bacteria to demonstrate that simultaneous agglutination with the respective homologous antisera caused the organisms to form separate clumps. Wiener and Herman (85) also obtained discrete clumps with a mixture of Kline antigen and human red corpuscles. However, many of the systems studied are dissimilar in size or properties and probably react at different rates. The classic studies of Pauling and his collaborators (86-91) with coupled azoprotein compounds provide strong evidence in support of Marrack's hypothesis. Boyd (92) criticized Pauling's views on the ground that many of the haptens themselves may exist in solution in associated form consisting of 10 to 12 molecules per aggregate. Pressman et al (76) claim that this is not so.

It seems worthwhile to review briefly some of the work of Pauling's group as it represents a unique approach to problems in serology. Pauling's studies were carried out with antisera homologous to the p-azobenzene arsonate group:



It was found that of 27 protein hapten complexes tested as antigens only those containing two or more p-azobenzene-arsonate-ion groups in the molecule produced precipitates with the antisera. No precipitates were obtained with any of the monohaptenic substances. The formation of these precipitates was regarded as being

evidence for the framework theory.

There is a possibility that in the precipitation of the polyhaptenic molecules only one of the haptenic groups may be engaged by the antibody. Pauling (90) however, showed that both of the haptenic groups of a dihaptenic substance enter into specific combination with antibody in the formation of the precipitate. He prepared the following two antisera:

(1) anti-R serum, made by immunizing rabbits against azoprotein containing R groups (p-azophenylarsonic acid)

(2) anti-X serum, made by immunizing rabbits against azoprotein containing p-azobenzoic acid.

As the precipitating antigen a protein carrier was prepared which had one molecule of R and one of X per molecule. It was found that the antigen complex gave a precipitate only when both antisera were present.

According to the theory of nonspecific agglutination, antibodies have only one combining group per molecule, so that soluble complexes are formed according to the following scheme where ● represents antibody and ⊘ represents antigen:

(a) ●X-X⊘R-R● with $\frac{\text{antibody}}{\text{antigen}} = \frac{2}{1}$

If the antigen molecules have only one combining group, -X or -R, soluble complexes of the form:

(b) ⊘X-X●X-X⊘ or ⊘R-R●R-R⊘ will be formed, with $\frac{\text{antibody}}{\text{antigen}} = \frac{1}{2}$

According to the specific-bonding theory on the other hand, both antibody and antigen must at least be bivalent and capable of forming complexes of the form:

(c) -R⊘X-X●R-R⊘X-X●R-R⊘X- etc.

The objection still might be raised, namely that the antibody might have only one combining group, but when anti-R and anti-X sera together are added to a solution of the antigen complex a precipitate might form, if the complex illustrated in (a) above, has a tendency to associate into colloidal particles.

The antigen-antibody ratio in compound (c) theoretically should be close to 1:1: on analysis the ratio was found to be 1:0.7. Further, the precipitate should be soluble in the presence of an excess of antibody if both antigen and antibody are "bivalent". A decrease in the amount of precipitate actually was found when an excess of both antisera was added to a suspension of the protein complex. Thus, Pauling's studies, in general, support the theory of specific aggregation. The reactions with naturally occurring antigens and antibodies also fit in well with his theory.

As evidence for the theory of nonspecific aggregation, on the other hand, Abramson (93) obtained both homogeneous and heterogeneous clumps with a mixture of sheep red cells and Friedlander bacilli. Likewise, Hooker and Boyd (94) mixed mammalian and avian red corpuscles with the respective antisera and obtained heterogeneous clumps indicative of nonspecific aggregation. Later, however, Abramson and Boyd (95) repeated the experiments with partially haemolyzed red cells and observed a tendency towards specific aggregation. Duncan (96), on agglutinating mixtures of flagellated and nonflagellated bacteria with immune sera obtained evidence that both stages of agglutination were specific. Also, in precipitin tests with mixtures of yeast polysaccharide and purified albumin, there was evidence of both modes of aggregation. It might be suggested that any evidence of specific aggregation would favor the specific aggregation hypothesis, since experimental conditions might lead to some nonspecific aggregation, while nonspecific forces could hardly be expected to become specific.

The kinetics of agglutination based on the Bordet hypothesis has been studied by Teorell (97) and by Morales (98), who have derived equations on the assumption that antibody is univalent and antigen is polyvalent. Analogous studies based on the specific-aggregation hypothesis have been reported by Heidelberger and

colleagues (99). The mathematical treatments have been reviewed by Boyd (100). Further confirmation of specific aggregation in the case of haemagglutination has been obtained by McKerns and Denstedt (101) by using human red cells labelled with sulphaemoglobin.

4. Rate of reaction

That the binding of antibodies to antigen usually proceeds very rapidly can easily be shown by adding red cells to the antiserum and centrifuging immediately. As a rule the cell sediment will be agglutinated. From the Brownian movement it has been calculated (102) that the velocity of the antibody between molecular collisions in the antiserum at room temperature is of the order of half a meter per second. From the observations of many authors (39,44) it seems that most of the antigen in the suspension combines with antibody within a minute or two. It seems logical to suppose that the rate would vary according to the affinity of the antibody for the antigen. The second stage, i.e. the "clumping" of cells, or the formation of a precipitate, is a slower process and varies greatly depending on the potency of the antiserum and on experimental conditions, such as rate of shaking, temperature, pH, salt content, and so forth. For example, de Kruif and Northrop (103) found that the agglutinin for the typhoid bacillus reacts best above pH 6. Below pH 4 the amount of antibody taken up was found

to be much reduced. This suggests that in the reaction between antibody and antigen the presence of ionized carboxyl groups may be essential. Pressman et al (76, 104) also found that optimum precipitation for azoprotein antigens with negatively charged haptenic groups occurred between pH 7 and 8. Kleinschmidt and Boyer (105) studied the effect of pH and ionic strength on the precipitin reaction between egg albumin and rabbit anti-egg-albumin. Complete precipitation occurred between pH 6.25 and 8.45, thus indicating that the charged groups of aspartic, glutamic and lysine play a prominent role in the reaction. They found also that inhibition by electrolytes is increased by NaCl up to 1 molar and by KBr up to 3 molar. This is probably due to the environment of electrolyte ions around the oppositely charged polar ions of the combining groups on the antibody and antigen thus reducing their attraction.

The rate of combination between antigen and antibody may be expected to increase with a rise in temperature up to the point where one or both of the reagents is altered by heat. Osborne and Denstedt (3) have studied the effect of the titre of the antiserum and the rate of shaking on the course of the agglutination reaction and the free-cell count.

IV Agglutinogens of Erythrocytes

In the latter half of the nineteenth century, Landois found that blood from one animal could be transfused into the bloodstream of another animal of the same species without harm. The result was usually fatal, however, if the blood of one species was transfused into an animal of a different species. Landois also demonstrated that if human blood was mixed *in vitro* with the blood of other animals, the human red blood cells suffered haemolysis. It was not apparent at the time however, why the transfusion of the blood of one human into another human frequently led to fatality. In 1900 Landsteiner demonstrated that the serum of certain normal human individuals, when mixed with red cells of certain other individuals, caused the cells to be agglutinated or haemolyzed. This led to Landsteiner's discovery of two main types of naturally occurring agglutinins in human serum and to his classification of humans into three distinct blood groups, according to the antigen present in the red cells. The groups later were designated A, B and AB respectively, according to whether the red cells contained either or both cell factors. The serum of each group lacks the corresponding agglutinins. For example the serum of blood group A contains anti-B (β) agglutinin and no anti-A (α). Also AB blood serum usually contains neither anti-A nor anti-B agglutinins.

In 1902 von Decastello and Sturli, the latter a student of Landsteiner, demonstrated the existence of the fourth group, designated "O". The cells of this group contain neither A nor B factor but the serum contains both alpha and beta agglutinins and thus can agglutinate the cells of the other three groups.

For a classification of the blood groups the reader is referred to standard texts books on the subject (106,107). The Rh agglutinins also have been the subject of numerous recent works (108, 109, 110).

For the purpose of this thesis the following table will suffice to summarize the various agglutinogens and agglutinins in human blood as far as is known at this time.

Table 1

Agglutinogens of Human Red Corpuscles

and Agglutinins in the Plasma

Cell Agglutinogens (Antigens)			Agglutinins in Plasma (Antibodies)	
Groups	Subgroups	Genotypes	Usual	Occasional Additional
O (45%)		OO	α, α_1, β	None
A (41%)	A_1 (80%)	A_1A_1, A_1O A_1A_2	β	α_2 (anti-O), rare, reacts with 95% A_2 and all O cells
	A_2 (20%)	A_2A_2, A_2O	β	α_1 (1-2%) reacts with A_1 and A_1B cells
B (10%)		BB, BO	α and α_1	α_2 (anti-O) very rare
AB (4%)	A_1B (66%)	A_1B	none	α_2 (anti-O) very rare
	A_2B (34%)	A_2B	none	α_1 (25-30%)
Types				
M (30%)		MM	none	anti-N (1 case reported)
N (20%)		NN	none	anti-M (10 cases reported)
MN (50%)		MN	none	none
Rh (85%)	12 in number	78 in number	none	by isoimmunization only
P (76%)		PP	none	rare
Lewis (25%)		LL	none	by isoimmunization only
Kell		KK	none	by isoimmunization only
X (94%)		XX	none	none

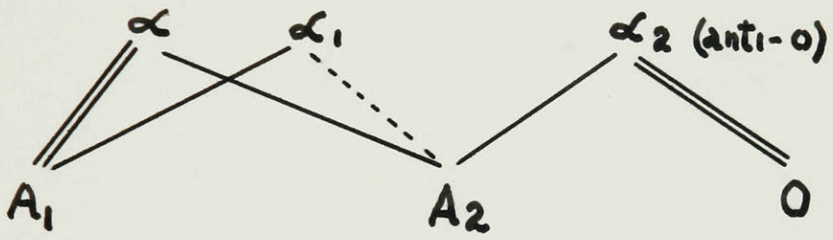
The percentages in the first column of Table 1 represent the frequency of occurrence in the blood of white races--European and American. The percentages in the second column represent the proportions of the subtypes in the groups indicated in column I. From the information given it is obvious that persons of group O can no longer be considered as "universal donors", nor can persons of group AB be regarded as "universal recipients".

Still further subdivisions of the human blood groups have been made necessary by the discovery of additional rarely occurring and weakly reacting agglutinogens. (See Wiener (106)). The A agglutinogen, for example, may have further variants A_3, A_4, A_5 and A_6, which react weakly with even the most potent anti-A serum. Similarly, there is evidence that the agglutinogen B also is a multiple factor. A weakly reacting N agglutinogen, N_2, also has been reported. A recent discovery by Sanger of a further factor related to the M-N type has been reported (111). The new factor has been designated "S".

It is generally accepted that the more commonly occurring alpha agglutinins in group O and group B serum consist of two qualitatively different agglutinins, alpha and $alpha_1$. The subgroups of A also consist of two corresponding agglutinogens, A_1 and A_2. This idea is suggested by the finding that group B serum contains two agglutinins. One of these, alpha, is capable of agglut-

inating both group A_1 and A_2 cells, whereas the other, $alpha_1$, agglutinates only A_1 cells. A third agglutinin, $alpha_2$ sometimes is found in the sera from blood of groups A and B persons.

The following scheme from Wiener (106) indicates the comparative reactivity of the various alpha agglutinins with A_1, A_2 and O cells.



Thus the alpha agglutinin reacts more strongly with A_1 cells than with A_2; $alpha_1$ reacts less strongly than alpha with A_1 cells and only weakly with A_2. It is noteworthy however, that $alpha_2$ agglutinates only 95% of A_2 cells but agglutinates 100% of cells of group O. Thompsen (112) has suggested that $alpha_2$ is really anti-O and reacts with most A_2 cells for the reason they happen to be mostly of the heterozygous genotype A_2O. This is supported further by the observation that $alpha_2$ agglutinates A_2B and A_1A_2 cells very weakly if at all. Theoretically, then, the homozygous A_2 cells (A_2A_2) should not react with $alpha_2$. The latter genotype is present in only about 1.5% of all group A individuals and can be identified with certainty only if both parents are of group A_2B. In tests with the cells of this rare group

they have been found not to be agglutinated with the $alpha_2$ agglutinin (106). It might also be noted that cases of haemolytic reactions have been reported which have been caused by mistaking A_2B cells for group O or group B due to the use of weak antisera.

V The Rh Blood Groups

In 1940 Landsteiner and Wiener (113) tested the red cells of a large number of humans of European descent with an anti-Rhesus serum prepared by immunizing rabbits against red cells of Macaca rhesus monkeys, and found that 85% of their bloods (cells) gave agglutinates while the remaining 15% did not. They called this new factor Rhesus or "Rh" factor.

In 1939 Levine and Stetson (114) had described a case of intragroup agglutination, due to a new immune agglutinin in the serum of a woman who had recently given birth to a dead foetus. The agglutinin reacted with the erythrocytes of 83 out of the 104 group O persons tested. After the discovery of the Rh antigen, it was demonstrated that the atypical agglutinin in this case was anti-Rh.

The dangers of intragroup transfusion reactions soon became apparent from the work of Wiener, Levine and others in the United States, and of Taylor, Mollison, Race, and their colleagues in Great Britain. It soon became realized that the giving of Rh-positive blood to Rh-negative persons who have been sensitized either by pregnancy or a previous blood transfusion may be dangerous and perhaps fatal. In addition, the transfusion of Rh-positive blood to an Rh-negative girl or woman might sensitize her to the Rh antigen, so that any Rh-positive child she may later bear may develop haemolytic disease

of the newborn which often is fatal.

The complex gene structure of the Rh chromosome is explained by Fisher and Race (115) as being due to adjacent genes along the same chromosome. Wiener (116) has a concept based on a series of multiple alleles at one locus.

The present state of knowledge of the Rh groups shows there are six common Rh antigens in the red cells. These antigens are called by Fisher's nomenclature, C, c, D, d, E and e, and fall into three groups Cc, Dd, Ee. Each of these antigens can stimulate the production of its corresponding antibody by isoimmunization. The same nomenclature is used to classify the corresponding genes.

The relationship between the members of the gene pairs, for example, C and c, is one of genetic allelomorphism, i.e. a chromosome can carry C or c but not both. Since all the nucleated cells in the human body, except the gametes, inherit a double set of chromosomes, they therefore carry two Rh chromosomes. If two chromosomes both carry the gene C, the genotype is then CC (homozygous); one may carry C and the other c, so that the genotype is Cc (heterozygous); or they may both carry c and the genotype is cc (homozygous).

Since the gametes carry only one set of chromosomes, they will carry only one Rh chromosome. Thus the chromosome in the ovum or spermatozoon may carry either

C or c. Which of these it carries will depend on the genotype of the parent. If the inherited genotype is CC, then the sex cells will carry C; if the genotype is Cc, half the gametes will carry C and half c; and if the genotype is cc, all the gametes will carry c.

The gene pairs Dd and Ee are inherited in a similar way.

The three classes of genes C or c, D or d, and E or e, moreover, are carried close together on the same chromosome. If a person inherits, for example, cde from one parent and CDe from the other, his children will inherit either cde or CDe, not Cde or cDe.

Eight combinations of C or c, D or d, and E or e are possible on an Rh chromosome. In England, according to Race (109) the following is the descending order of frequencies of the chromosomes: CDe, cde, cDE, cDe, cdE, Cde, CDE and CdE. Each of these represents a single Rh chromosome, as found in a sex cell. Since 8 things can be paired in $\frac{8}{2}(8+1)$ or 36 different ways, there are 36 possible genotypes. In English people CDe/cde, CDe/CDe, cde/cde, CDe/cDE and cDE/cde account for 90% of the population. The following antisera are now widely available: anti-C, anti-c, anti-D, and anti-E.

Other subdivisions now can be made. Callender and Race (117) discovered a third allelomorph at the C-c locus called C^W. C^W has been recognized in the chromo-

some combinations $C^{W}De$ and $C^{W}de$. The majority of anti-C sera are anti-C + anti-C^{W}, since this mixture is the usual product of immunization by the C antigen.

Fourth and fifth allelomorphs at the C-c locus, C^{u} and C^{v} have been discovered by Race, Sanger and Lawler (118). Also a third alternative to D and d called D^{u} was discovered by Stratton (119). Ignoring the hypothetical chromosomes and those involving C^{u} and C^{v}, there are 12 different Rh chromosomes: CDe, cde, cDE, cDe, $C^{W}De$, cdE, Cde, CDE, $CD^{u}e$, $cD^{u}E$, $cD^{u}e$ and $C^{W}de$, giving rise theoretically to 78 genotype combinations.

The scheme of the eight Rh blood types is shown in the following table, after Cappell (120).

Table 2

The Rh Blood Types (after Cappell)
and Their Reactions with Antisera

Human Iso-Antisera															
Fisher	Wiener	R_o cDe	r cde	R_2 cDE	R" cdE	R_1 CDe	C^wDe	R' Cde	C^wde	R_z CDE	C^wDE	R_y CdE	C^wdE		
Anti-C^W		−	−	−	−	−	+	−	+	−	+	(−)	(+)		
Anti-C + C^W	Anti-rh'	−	−	−	−	+	+	+	+	+	+	(+)	(+)		
Anti-C		−	−	−	−	+	−	+	−	+	−	(+)	(−)		
Anti-C + C^W + D	Anti-Rh_1	+	−	+	−	+	+	+	+	+	+	(+)	(+)		
Anti-C + D		+	−	+	−	+	+	+	−	+	+	(+)	(−)		
Anti-D	Anti-Rh_o	+	−	+	−	+	+	−	−	+	+	(−)	(−)		
Anti-D + E	Anti-Rh_2	+	−	+	+	+	+	−	−	+	+	(+)	(+)		
Anti-E	Anti-rh"	−	−	+	+	−	−	−	−	+	+	(+)	(+)		
Anti-c	Anti-hr'	+	+	+	+	−	−	−	−	−	−	(−)	(−)		
Anti-d	Anti-Hr_o	−	+	−	+	−	−	+	+	−	−	(+)	(+)		
Anti-e	Anti-hr"	+	+	−	−	+	+	+	+	−	−	(−)	(−)		

The latest changes in Wiener's nomenclature (108) bear the following relationship to that of the English workers:

Red Cells Containing the Factor Rh_o (D) (Rh positive)			Red Cells Not Containing the Factor Rh_o (D) (Rh negative)		
Designation of Types			Designation of Types		
Fisher	(Race)	Wiener	Fisher	(Race)	Wiener
cDe	(R_o)	Rh_o	cde	(r)	rh
CDe	(R_1)	Rh_1 (Rh_o')	Cde	(R^1)	rh′
cDE	(R_2)	Rh_2 (Rh_o'')	cdE	(R'')	rh″
CDE	(Rz)	Rh_1Rh_2 ($Rh_o' Rh_o''$)	CdE	(Ry)	rh′ rh″

The short symbols used by Race (109) are included; some of them are based on those used by Wiener.

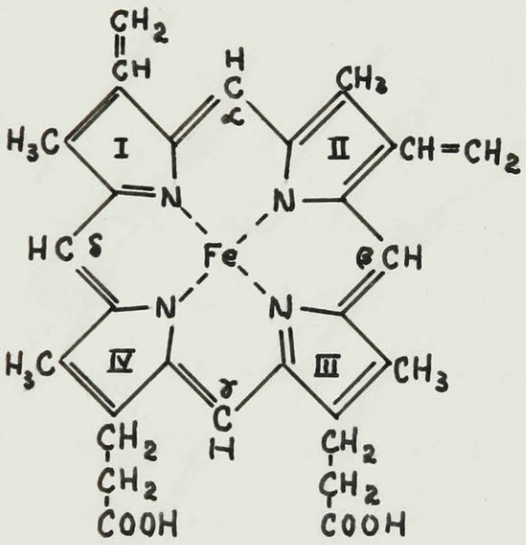
The commonest Rh antibody, anti-Rh_o (anti-D), exists in two forms. One will agglutinate a saline suspension of red cells containing the antigen D. The other form, called "incomplete" by Race (121) or "blocking" by Wiener (122), will not agglutinate D-positive cells suspended in saline. It will, however, block D-positive cells so that they are not subsequently agglutinated by saline-agglutinating anti-D. Diamond and Denton (123) found that blocking anti-D, however, will agglutinate D cells if they are suspended in a protein medium such as a 20% solution of albumin. The agglutinin

which is active in albumin is thought to represent a later product of the immunization process. It is probable that there are incomplete or blocking or albumin-agglutinating antibodies corresponding to all the Rh antibodies which produce agglutination of cells in saline.

Sulphaemoglobin and Related Pigments

Before proceeding with the discussion on sulphaemoglobin (SHb) it will be worthwhile to briefly consider the structure and properties of haemoglobin itself. Haemoglobin constitutes 95% of the weight of the mature red cells. It is a conjugated protein with a molecular weight of 68,000, made up of a large colorless protein portion called globin, with four small haeme molecules as prosthetic group attached on its surface. The colored haeme molecule (iron protoporphyrin-9) is an organo-metal complex consisting of an iron atom in the centre of a porphyrin structure. Protoporphyrin-9 consists of four substituted pyrrole rings attached to one another through methene bridges. This forms an innermost resonating ring with alternating single and double bonds. The individual pyrrole rings also enter into the resonance. All the atoms in this structure lie in the same plane so that the porphyrin molecule is flat. The stabilizing effect of resonance on the porphyrin is enhanced by the presence of the substituent side chains. These are of three kinds namely four methyl, two vinyl and two propionic acid groups. There are fifteen possible arrangements of the side chains around the circumference of the porphyrin and hence there are 15 isomeric porphyrins. The two negatively charged carboxyl groups of protoporphyrin-9 are thought to be attached to two positive groups of the globin,

possibly the guanidine groups of arginine. The iron in the centre of the porphyrin is hexacovalent, i.e. it can bind six atoms. Ferrous protoporphyrin-9 has the following structure (Granick (124)):



The iron atom in ferrous protoporphyrin-9 binds the four pyrrole nitrogens in the plane of the ring and the nitrogen of the imidazole group of histidine in the peptide chain of the globin. The sixth coordination link of iron is capable of combining with O_2 reversibly.

Sulphaemoglobin is formed from oxyhaemoglobin (HbO_2) by the action of hydrogen sulphide according to the following scheme from Michel (125)

$$HbO_2 + H_2S \longrightarrow Hb + S + H_2O_2$$

$$Hb + H_2S + H_2O_2 \longrightarrow SHb + \text{unknown products.}$$

Fairley (126) suggests that SHb is formed from methaemoglobin (HbOH) by the action of H_2S, but Keilin (127) differs with him on this point since he claims to have prepared sulphmethaemoglobin. SHb can also be formed in vivo by the action of drugs in the presence of excessive intestinal putrefaction. Drugs such as acetanilide or phenacetin for example, are supposed to accelerate the formation of SHb.

Sulphaemoglobin can exist in two forms, namely, a ferro or reduced form and a ferri form. The reduced form is quite stable and is nonreactive with O_2, CN, S^-, NH_3, azide $(NH_4)_2S$, $Na_2S_2O_4$, fluorides or alkalies (128). SHb^{++} combines quantitatively with CO (129) to form carbonmonoxide sulphaemoglobin (SHbCO) but does not combine with oxygen. Michel (125) claims that in the formation of SHb one atom of S is introduced into the haemoglobin molecule for each iron atom. Haurowitz (130) found two atoms of S but his product, as Jope (129) points out, was not freed of colloidal sulphur.

Ferri-SHb, containing ferric iron, on the other hand, is an unstable form prepared from ferro-SHb by the action of oxidizing agents, such as ferricyanide. Presumably the ferri form does not combine with CO or O_2.

Judging from the reactions of HbOH, one might expect ferri-SHb to react with CN, F and azide.

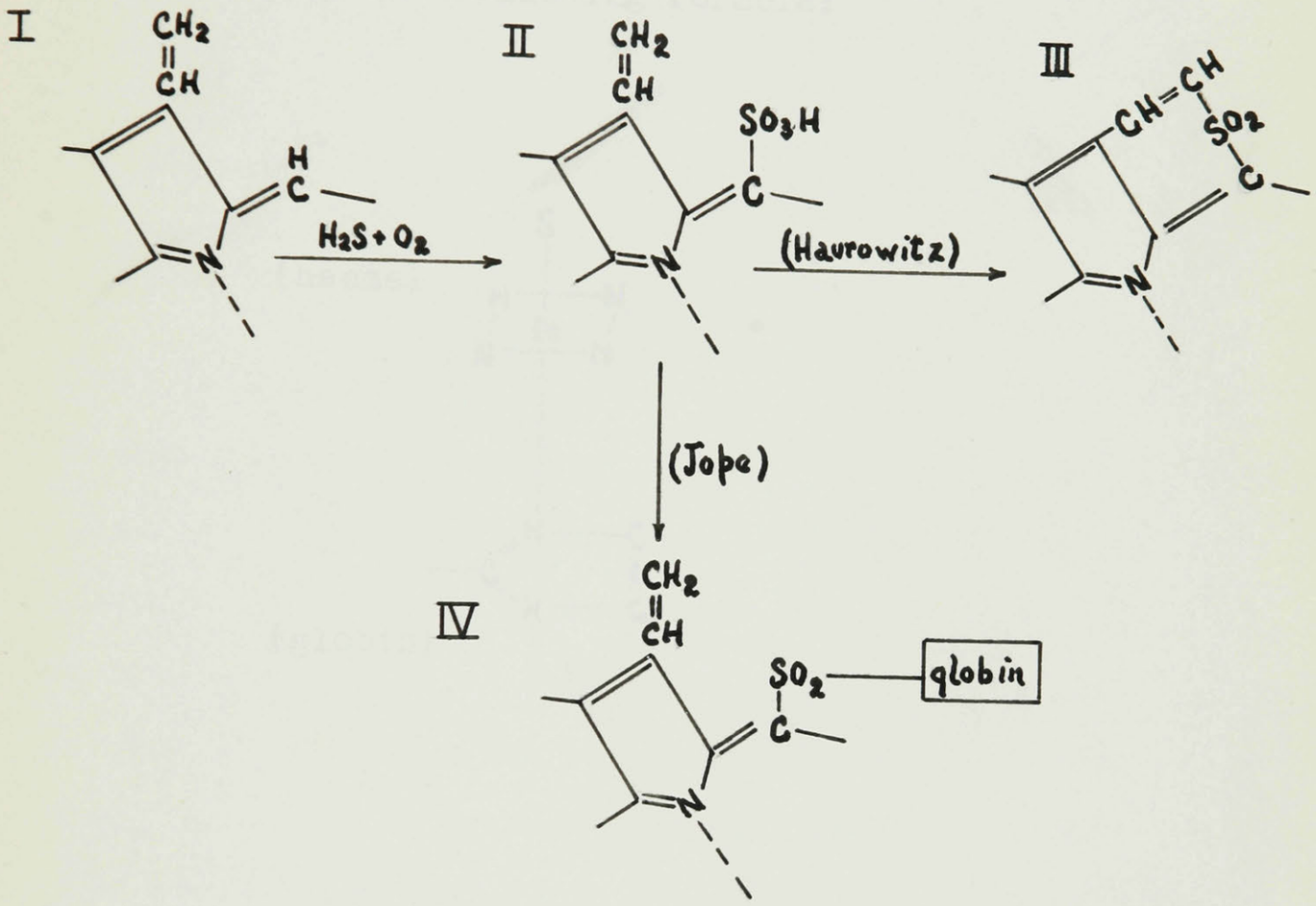
SHb has absorption bands at 6180, 5780 and 5400 A.U. The presence of SHb in a blood sample is readily detectable by the strong band at 618-620 mu, which is unchanged by the addition of 10% KCN. The absorption bands of HbOH at 630 and hematin at 610-630 mu, on the contrary, disappear with the addition of KCN.

As yet, SHb has not been prepared in pure form. Drabkin and Austin (131), who first attempted to define the spectral characteristics of SHb, achieved sulphuration up to about 75% of the total pigment. This is the upper limit of sulphuration achieved by ourselves and other workers. Not all of the abnormal pigment is SHb, however, since the continued action of H_2S and O_2 on haemoglobin tends to rupture the porphyrin ring. This has been shown by Lemberg and co-workers (132). They demonstrated that on prolonged exposure of haemoglobin to H_2S in the presence of O_2, some of the alpha-methene bridges of the porphyrin rings break open without being detached from the protein (globin) carrier. This product they call "choleglobin". Choleglobin gives a visible spectral absorption similar to that of SHb, namely a strong band at 618-620 mu. The spectral absorption of SHbCO at 617 mu is used to measure SHb in the presence of choleglobin. Lemberg reports that in his clinical studies in humans, little or no choleglobin

was found. Jope (129) also failed to find any choleglobin in his studies. Lemberg showed, however, that when HbO_2 is sulphurated with H_2S *in vitro* the more prolonged the treatment, the greater is the proportion of choleglobin to sulphaemoglobin produced. In some cases the choleglobin amounted to 50% of the abnormal products.

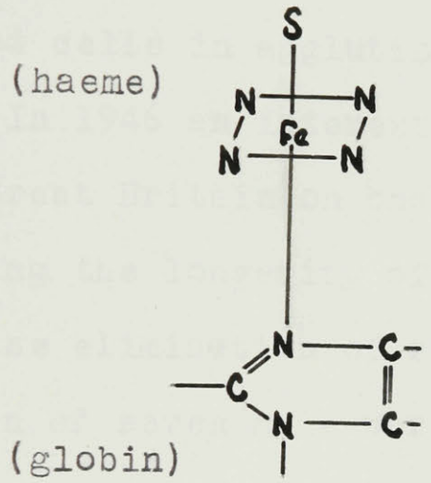
Obviously the chemistry of these reactions is extremely complicated and thus it is not surprising that the constitution of SHb has not yet been established. It appears, however, that the protein carrier, globin, is not altered in the sulphuration process. This has been shown by the electrophoretic studies of Michel (125). Jope (129) unsuccessfully attempted to separate the unchanged Hb and the SHb by chromatography; nor was Michel (125) able by crystallization to separate HbO_2 and SHb in sulphurated rat and dog blood. Lemberg (132) was successful in separating these pigments in the blood of rats which had been fed sulphur and acetanilide. Jope (133), and Lemberg (132), have shown that SHb retains the porphyrin ring structure. Haurowitz (130) found that on acid hydrolysis or peptic digestion of SHb, the haeme group is not split from the protein, as does occur with haemoglobin. Hydrolysis of SHb was found to yield a product consisting of protein still attached to the haeme. Haurowitz concluded that in the formation of SHb, an $-SO_2H$ group combines with each of two methene bridges of the

porphyrin ring and that subsequent condensation takes place with the vinyl groups as shown below in product III:



Jope (129) on the contrary, contends that Haurowitz's interpretation does not explain the strengthened linkage between haeme and the globin in SHb. He suggests rather that the condensation takes place between the $-SO_3H$ and a basic group of the globin, as shown above in product IV.

Stern (134) suggests a third alternative structure for SHb. According to this conception S merely replaces the oxygen of methaemoglobin. This may be represented by the following formula:



This representation might explain why SHb cannot reversibly combine with O_2. The writer, however, fails to see how this compound could account for the observations of other workers, namely the oxidized and reduced forms of SHb, the strengthened linkage between haeme and the globin, and the formation of SHbCO.

EXPERIMENTAL

I Sulphaemoglobin Studies

1. Introduction

For convenience of presentation the work on sulphaemoglobin will be discussed first. By doing this the reader will be able to follow more easily the use of sulphurated cells in agglutination studies.

In 1946 an interesting report appeared by Jope (129) in Great Britain on the use of sulphaemoglobin for ascertaining the longevity of the human red cell. He followed the elimination of sulphaemoglobin from the circulation of seven male workers who had been employed in the manufacture of explosives and had developed sulphaemoglobinemia and mild cyanosis from handling cordite. Using the spectrographic method he found that about 116 days were required for the complete elimination of the sulphaemoglobin-containing red cells. Since this figure agrees well with that by the serological method of Ashby (1) and others, who followed the survival of red cells after transfusion, it would appear that Jope's interpretation is valid, namely, that a small proportion of the haemoglobin of the red cell can be converted to the inert sulphaemoglobin without affecting the normal life-span of the cell. One may infer also that the presence of the abnormal pigment which, in these individ-

uals, rarely exceeds 3% of the total pigment, does not seriously interfere with the oxygen-carrying properties of the remaining haemoglobin in the cells. That red cells containing SHb are capable of functioning or at least of surviving, is, in our opinion, somewhat unexpected. It raises the question whether the SHb is built into the blood cell at the time of its formation and whether artificially sulphurated cells are capable of normal function and survival. SHb is very stable in the ferrous form and unlike carboxyhaemoglobin and methaemoglobin, cannot be transformed back into haemoglobin in the circulating red cells. Presumably, therefore, it remains in the cells until they are destroyed. If artificially sulphurated red cells could be prepared so that the cells retained their normal viability, the technique of labelling cells in this manner could be applied as a useful tool in blood-volume and cell-longevity studies. In brief, the experiments consisted in producing SHb in the red blood cells of rabbits. Two methods were used, namely (a) <u>in vitro</u>, by treating a saline suspension of the cells with H_2S, and (b) <u>in vivo</u>, by forced-feeding of the animals with sulphur and phenacetin. The cell-survival tests also were carried out in two ways, namely (a) by transfusing the sulphurated cells prepared by either of the above methods into a normal animal and determining the time required thereafter for the complete elimination of the cells, and

(b) by determining the time required for the elimination of SHb from the circulation of the experimental animal after discontinuing the feeding of the drugs.

2. Production of sulphaemoglobin

(a) Sulphuration *in vitro*

The fragility of the red cells was found to be greatly impaired if sulphuration was carried out in unbuffered saline medium. The viability of the sulphurated cells as measured by their survival in the blood stream after transfusion is influenced also by the time of exposure to H_2S, the density of the cell suspension and the level of sulphuration. A marked impairment of viability was produced when the SHb content was greater than 15% of the total haemoglobin (Hb). Since secondary alteration of the Hb, possibly through opening of the alpha methene bridge of the porphyrin ring, takes place on prolonged sulphuration, care was taken to limit the duration of exposure of the cells to H_2S to 3-5 minutes. The reaction was stopped at the desired time by the addition of solid $Na_2S_2O_4$ to the mixture, thus converting any remaining oxyhaemoglobin to the reduced form which does not react with H_2S. The procedure finally adopted is as follows: the red cells from a citrated blood sample after removal of the plasma are suspended in a closed vessel in an equal volume of isotonic phosphate

buffer* of pH 7.35. A measured quantity (usually 0.1 of the cell suspension volume) of the H_2S-saturated buffer is added. The vessel is stoppered and slowly and repeatedly inverted for 3-5 minutes until the blood darkens to what is judged to be the desired level of sulphuration (10-15%). A small quantity of solid $Na_2S_2O_4$ sufficient to convert the remaining oxyhaemoglobin (HbO_2) to Hb, is added and mixed. The excess H_2S is then removed by aeration and subsequent washing of the cells with the phosphate buffer. The cells are suspended in the original plasma (which had been saved). To determine whether the sulphaemoglobin content of the cells was uniform a fragility series was set up in a graded series of hypotonic saline solutions in the usual manner. The unhaemolyzed cells in each tube then were separated from the supernatant fluid and analyzed for SHb content (135). It was found that the SHb content of the cells was uniform. It appears therefore that the oldest and youngest cells in the blood sample are sulphurated to the same degree.

(b) Sulphuration <u>in vivo</u>

Rabbits were fed various amounts of phenacetin and sulphur (usually 0.5 g. phenacetin and 2 g. sulphur

*The buffer mixture was prepared by mixing 20 ml. of 4.17% sodium dihydrogen phosphate monohydrate and 16.5 ml. of 0.302N sodium hydroxide (prepared from 18N NaOH).

for a 4 kg. rabbit) three times a week by stomach tube until the desired level of SHb (3-9%) was produced in the circulation. The animals varied considerably in their tolerance of the drugs. It usually took 4 to 6 weeks to reach the desired SHb level. Attempts to speed the process by increasing the drug dosage killed many animals.

3. The osmotic properties of sulphurated red cells

Five ml. of blood was removed from the marginal ear-vein of a rabbit. The cells were washed twice with isotonic phosphate buffer solution of pH 7.35 and the sample made up to original volume with the buffer solution. One ml. was kept as a control sample and the remainder was transferred to a 15 ml. centrifuge tube. H_2S was passed into the air space above the cell suspension and the tube was then stoppered and inverted repeatedly and gently for thirty minutes to ensure completion of the reaction. The cell suspension was then washed several times with the phosphate buffer, and the volume finally adjusted to 4 ml. With experience the final SHb concentration could be regulated by controlling the duration of exposure to H_2S.

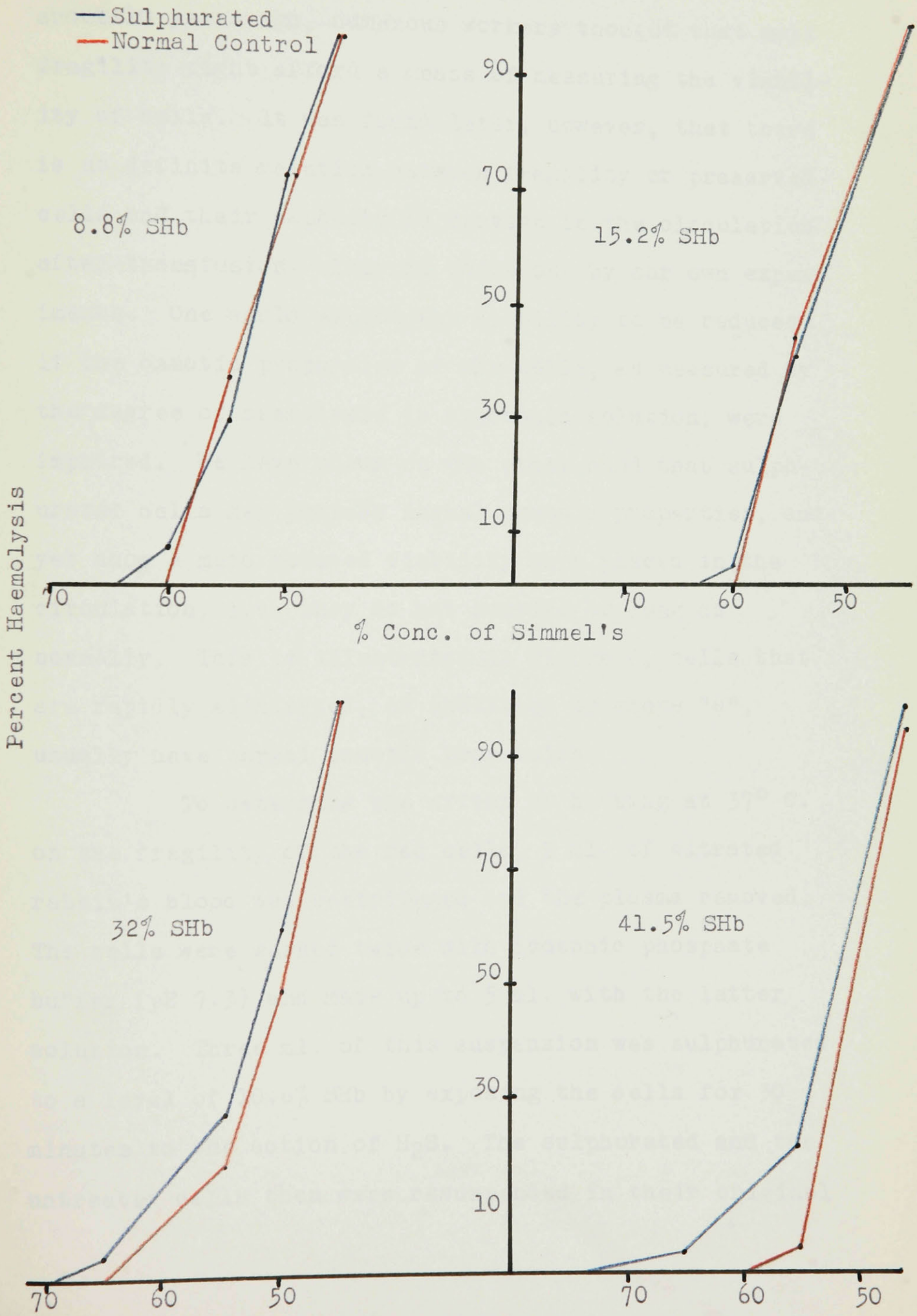
From an isotonic stock solution of a buffered Simmel's solution at pH 7.35 various dilutions were made with distilled water to give a series of hypotonic solutions representing 70, 65, 60, 55, 50, 45, 40 per cent respectively of the isotonic concentration.

The fragility of the control blood sample and of the corresponding sulphurated samples was determined by adding 0.02 ml. of red cell suspension to 10 ml. of each solution in the above series in 15 ml. centrifuge tubes. After standing for 5 minutes the tubes were centrifuged at about 2000 r.p,m. for 5 minutes and the supernatant then discarded. The unhaemolyzed cells were lysed in 10 ml. of M/60 phosphate buffer of pH 6.6, transferred to colorimeter tubes and the total haeme pigment determined by Evelyn's cyanmethaemoglobin method (135). A complete fragility series was carried out on each sulphurated sample and compared with the corresponding control. The values were calculated as percentage of the total haemoglobin (in an isotonic sample). The percentage SHb was determined in a separate sample.

The results represented in the graphs in Figure 1 show that cells containing more than 15% of their pigment in the form of SHb are less stable than normal cells in hypotonic solution. At SHb concentrations less than 15% there is little difference between sulphurated and normal cells. The sulphurated cells apparently are slightly more resistant to 55% and higher concentrations of Simmel's solution.

Figure 1

Haemolysis of Rabbit Red Cells in Simmel's Solution



In the earlier studies on blood preservation about 10 years ago, numerous workers thought that cell fragility might afford a means of measuring the viability of cells. It was found later, however, that there is no definite relation between fragility of preserved cells and their capacity to survive in the circulation after transfusion. This is borne out by our own experiments. One would expect the viability to be reduced if the osmotic properties of the cells, as measured by the degree of haemolysis in hypotonic solution, were impaired. We have shown on the other hand that sulphurated cells may possess normal osmotic properties, and yet show a much reduced viability when placed in the circulation, i.e. they do not survive as long as normally. This is illustrated in Figure 2, cells that are rapidly eliminated, as indicated by slope "a", usually have normal osmotic properties.

To determine the effect of heating at 37° C. on the fragility of the red cells, 5 ml. of citrated rabbit's blood was centrifuged and the plasma removed. The cells were washed twice with isotonic phosphate buffer (pH 7.3) and made up to 5 ml. with the latter solution. Three ml. of this suspension was sulphurated to a level of 10.6% SHb by exposing the cells for 30 minutes to the action of H_2S. The sulphurated and the untreated cells then were resuspended in their original

plasma and heated at 37° C. in a water bath for 20.5 hours. Samples of 0.2 ml. were removed at intervals and the fragility to 100% Simmel's solution was determined by the method already described.

Table 3

The Stability of Sulphurated Cells at 37° C. *in vitro*

	Control Cells		Sulphurated Cells	
Time at 37° C. (hrs.)	Total pigment as cyanmethaemoglobin gm %	% haemolysis	Total pigment as cyanmethaemoglobin gm %	% haemolysis
0	12.10	0	12.10	0
3	12.10	0	12.10	0
20.5	8.80	27.1	0	100

These results were in good agreement with the *in-vivo* survival of transfused red cells which had been sulphurated in a similar manner, namely, given 30 minutes exposure to H_2S without the addition of $Na_2S_2O_4$. On the other hand, red cells sulphurated in the same way and stored at 5° C. for 30 days, showed less haemolysis than with the normal whole blood similarly stored. An increase in temperature appears to increase greatly the osmotic instability of sulphurated cells. The finding that the sulphurated cells retain normal agglutinability suggests that the cells do not suffer any damage to the membrane

until the concentration of SHb exceeds 50%. This aspect is discussed in a later section (II). It is likely, however, that some choleglobin is formed in the cells on sulphuration. Lemberg (132) has shown that the concentration of choleglobin varies with the exposure to H_2S. It seems reasonable to suppose that the opening of the porphyrin ring of Hb would affect the viability of the sulphurated cells. With this idea in mind our main concern has been to sulphurate red cells to a SHb level which can be estimated easily after transfusion (10-15%) and at the same time keep the formation of choleglobin or other breakdown products of Hb at a minimum.

4. Influence of sulphaemoglobin on oxygen-carrying properties of the red cell

Our observation that cyanosis soon appears in rabbits on feeding phenacetin and sulphur, and that, in many instances, the interference with the oxygen transport of the cells is greatly out of proportion to the amount of SHb present, led us to investigate the oxygen-carrying capacity of red cells containing SHb. The oxygen-carrying capacity was determined both by the volumetric and by the manometric methods of Van Slyke (136). The average mean error in comparing the total haemoglobin by the Evelyn cyanmethaemoglobin method and the Van Slyke oxygen-carrying method was 1.1%, using the volumetric apparatus and 0.53% using the manometric technique.

(a) Red cells sulphurated *in vitro*

The relationship between the amount of SHb and the impairment of oxygen-carrying capacity is indicated in Table 4.

Table 4

Loss of Oxygen-Carrying Capacity of Rabbit Red Cells Sulphurated *in vitro*

	Colorimetric Method				Manometric Method	
	(a)	(b)	% pigment	(a-b)		
	HbO_2 + SHb g.%	SHb g.%	as SHb	HbO_2 g.%	HbO_2 g.%	% loss O_2 capacity
1.	13.70	1.20	8.8	12.5	12.5	8.8
2.	13.25	2.00	15.2	11.3	11.4	14.0
3.	11.50	2.20	19.2	9.3	9.2	20.0
4.	9.60	2.20	23.0	7.4	7.2	25.0
5.	9.95	3.18	32.0	6.8	6.1	38.5
6.	13.53	5.60	41.5	7.9	6.9	49.0

By comparing the % SHb with the % loss in oxygen-carrying capacity it is seen that in artificially sulphurated cells the impairment in oxygen-carrying capacity is proportional to the percentage of SHb. A slight additional loss of oxygen-carrying capacity occurs when the SHb concentration exceeds 20%. In other words the presence of SHb apparently does not interfere seriously with the

capacity of the remaining haemoglobin to take up and give off oxygen.

(b) Red cells sulphurated *in vivo*

The blood from the rabbits (average weight 4 kg.) which had been fed phenacetin and sulphur also showed a proportionality between the level of SHb and decrease in oxygen-carrying capacity, except when relatively large doses of the drugs had been given over a period of four days.

Table 5

Loss of Oxygen-Carrying Capacity of Red Cells in Rabbits Fed Small Doses of Phenacetin and Sulphur

	Colorimetric Method				Manometric Method	
	(a)	(b)	% pigment	(a-b)		
Rabbit	HbO_2 SHb g.%	SHb g.%	as SHb	HbO_2 g.%	HbO_2 g.%	% loss O_2 capacity
A	18.23	0.36	2.0	17.9	18.1	0.72
B	9.15	0.24	2.6	8.9	9.0	1.70
	10.16	0.28	2.7	9.9	10.0	1.58
C	13.63	1.03	7.5	12.6	12.7	6.83

After drug feeding the animals usually show a drop in their total haemoglobin, but occasionally there

is a very rapid overnight rise in haemoglobin concentration which can be due only to haemoconcentration. Thus, the haemoglobin of rabbit A was increased overnight from 13.1 g. per 100 ml. to 18.3 g. after a single large dose of the drug (1 g. phenacetin and 3 g. sulphur). The animal died within a few days from haemorrhage in the lung. In the case of rabbit B by the third day after two doses of drugs had been given, the blood was dark, viscous, and was "sludged". The cells also were observed to sediment out very rapidly.

In two animals, which had received three large doses each of 1 g. phenacetin and 3 g. sulphur over a period of four days, the loss of oxygen-combining capacity was considerably greater than the loss of Hb from conversion to SHb would account for. This is shown in Table 6.

Table 6

Loss of Oxygen-Carrying Capacity of Red Cells on Feeding Rabbits Large Doses of Phenacetin and Sulphur

		Colorimetric Determination				Volumetric Determination	
		(a)	(b)	% pigment	(a-b)		
Rabbit	Day	HbO_2 + SHb g.%	SHb g.%	as SHb	HbO_2 g.%	HbO_2 g.%	% loss O_2 capacity
D	2	12.62	1.62	12.8	11.0	11.3	10.6
	3	13.21	2.77	21.0	10.4	10.1	24.0
	4	18.05	3.53	19.6	14.5	10.9	39.8
E	4	14.93	4.07	27.2	10.8	7.9	47.4

Table 7 shows the daily change in the blood of a rabbit after two large doses of drugs given two days apart.

Table 7

Loss of Oxygen-Carrying Capacity of Red Cells after Giving a Rabbit Two Large Doses of Phenacetin and Sulphur

		Colorimetric Method				Manometric Method	
		(a)	(b)	% pigment	(a-b)		
Rabbit	Day	HbO_2 + SHb g.%	SHb g.%	as SHb	HbO_2 g.%	HbO_2 g.%	% loss O_2 capacity
F	1	15.20	0.04	0.24	15.2	15.1	0.6
	3	12.95	0.07	0.56	12.9	12.8	1.2
	4	12.53	0.14	1.94	12.4	12.3	1.8
	5	12.20	0.28	2.28	11.9	11.9	2.5

Table 7 (Cont'd.)

		Colorimetric Method				Manometric Method	
		(a)	(b)	% pigment	(a-b)		
Rabbit	Day	HbO_2 + SHb g.%	SHb g.%	as SHb	HbO_2 g.%	HbO_2 g.%	% loss O_2 capacity
F	6	12.75	0.37	2.92	12.4	12.1	5.1
	7	9.02	0.37	4.12	8.7	5.7	36.8

On the seventh day after the first drug feeding was started and even though the SHb level was only 4.1% of the total pigment the loss of oxygen-combining capacity was 36.8%. The blood in animals D, E and F was very dark and viscous, giving rise to a pronounced cyanosis. In animal D the haemoglobin had increased from 12.6 g. per 100 ml. to 18.0 in three days. The dark color of the blood (F7) was found to be due to reduced haemoglobin. The specimen would not take up oxygen when aerated in a thin film. However, after the cells were freed from the plasma by washing three times with isotonic phosphate buffer solution (pH 7.35), they assumed the normal bright red color on exposure to air. After a further washing with the isotonic buffer, the cells recovered the normal oxygen-carrying capacity with respect to the residual Hb content. The amount of the SHb present was 4.1% and the corresponding loss in oxygen-carrying capacity compared with the original capacity of the cells was 4.1%.

It is evident from the above findings that the interference with oxygen-carrying properties of the red cells in the heavily drugged animals was caused not by the presence of SHb in the cells but by some metabolic derivatives of sulphur or phenacetin in the cells or in the plasma. When these substances are removed by washing the cells the normal oxygen-combining capacity of the cells is rapidly restored. Jope (129) also observed certain patients with cyanosis and with no abnormal pigments in the red cells and no disorder of the circulation. The cyanosis in these patients was attributed to excessive amounts of reduced haemoglobin in the peripheral blood. Jope suggests that in these cases a toxic agent or an abnormal metabolite may be present which alters the oxygen dissociation curve of HbO_2, or that a loose compound may be formed between the metabolite of the drugs and the haemoglobin. We have been unable by feeding phenacetin alone to alter the oxygen uptake of the red cells. For example, a rabbit weighing 2.5 kg. was fed by stomach tube 0.75 g. of phenacetin daily for seven consecutive days. No abnormal pigment was formed, nor was there any loss of oxygen-combining capacity.

5. Use of sulphurated red cells in cell survival studies

(a) Transfusion of sulphurated cells into normal animals

The general procedure in these experiments was

to remove 40-50 ml. of blood from a rabbit by cardiac puncture and then transfuse into the marginal vein of the ear the same quantity of sulphurated red cells. Small samples were removed daily thereafter and analyzed by the method of Evelyn (135) for total pigment, methaemoglobin, sulphaemoglobin and haemoglobin. No methaemoglobin was present in the sulphurated specimens used for transfusion.

(i) Survival of artificially sulphurated red cells

Red cells sulphurated in unbuffered isotonic saline solutions were shown by fragility tests to be much less stable than normal red cells. Since H_2S is a weak acid, the low pH of the suspending medium during sulphuration favors membrane damage and breakdown of the haemoglobin. This tends to disturb the osmotic properties. Possibly the cell metabolism is also affected. Cells sulphurated in such a manner invariably suffered loss of viability as indicated by the rapid disappearance from the circulation after transfusion. As a rule the cells were no longer detectable in the recipient's circulation in 5 to 13 hours. The use of an isotonic phosphate buffer solution of pH 7.4 as suspending medium reduced the damage to the cells during the treatment and extended the survival time to 24-30 hours. It occurred to the writer that perhaps the presence of a protein colloid in the simple buffer medium might provide further protein to the cells during sulphuration and washing. Accordingly,

purified gelatine was added to the buffer. This innovation, however, proved ineffective in increasing the survival time of the transfused cells. The best survival was achieved by suspending the cells in an equal volume of isotonic phosphate buffer and quickly adding and mixing 1/10 volume of the buffer saturated with H_2S, followed by gentle rotation of the closed vessel for 3 minutes. Sufficient solid $Na_2S_2O_4$ was then added to reduce the remaining HbO_2. The excess H_2S was removed by drawing a current of air over the contents and finally washing with phosphate buffer. The cells were reconstituted in their original plasma. The cells sulphurated in this manner could be detected in the recipient's circulation up to seven days. In the graphs in Figure 2 it can be seen, in curves c. and d., that 50 to 60% of the cells had an apparently normal survival time, i.e. the final slope of the elimination curves resembled the slopes of curves A and B.

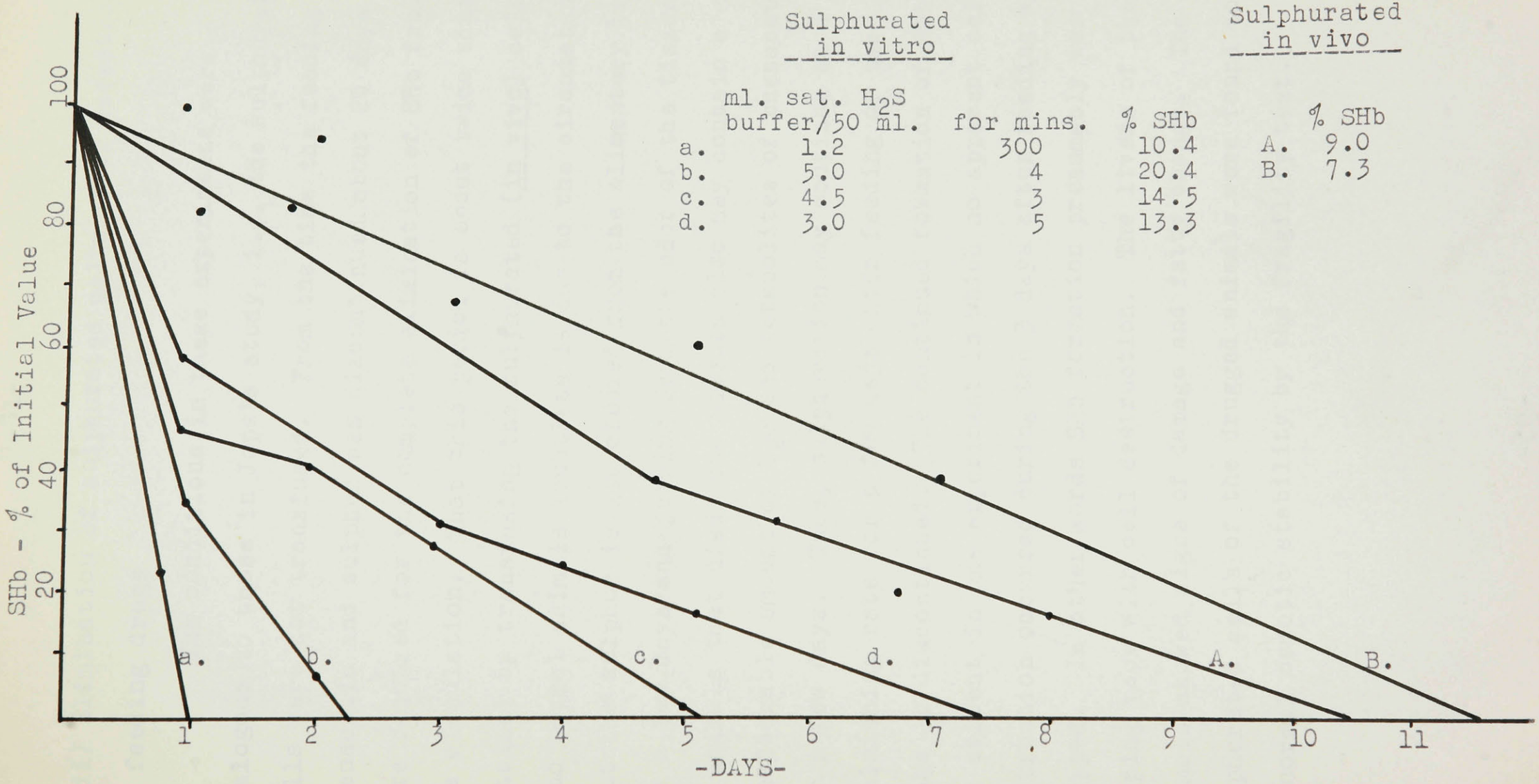
(ii) Survival of sulphurated (in vivo) cells in the normal animal

These tests were carried out with sulphurated red cells produced in the rabbit by forced-feeding of drugs (phenacetin and sulphur). The cells, transfused into normal recipients (rabbits), were eliminated from the circulation within 11-12 days. Whether these cells can be regarded as behaving in a normal manner, i.e.

whether 11-12 days represents the normal life-span of the red cell in the rabbit, obviously cannot be established by this type of experiment alone. That the cell elimination curve is linear indicates that all destruction proceeds slowly and at a steady rate. As a rule 35 ml. of blood containing 7 to 10 per cent of the haemoglobin in the form of sulphaemoglobin was used in these experiments. The cell survival times in two such experiments (A and B) are shown in Figure 2. The slope of the elimination curve in A is steeper in the first 5 days than in the subsequent period, indicating a more rapid initial elimination.

Figure 2

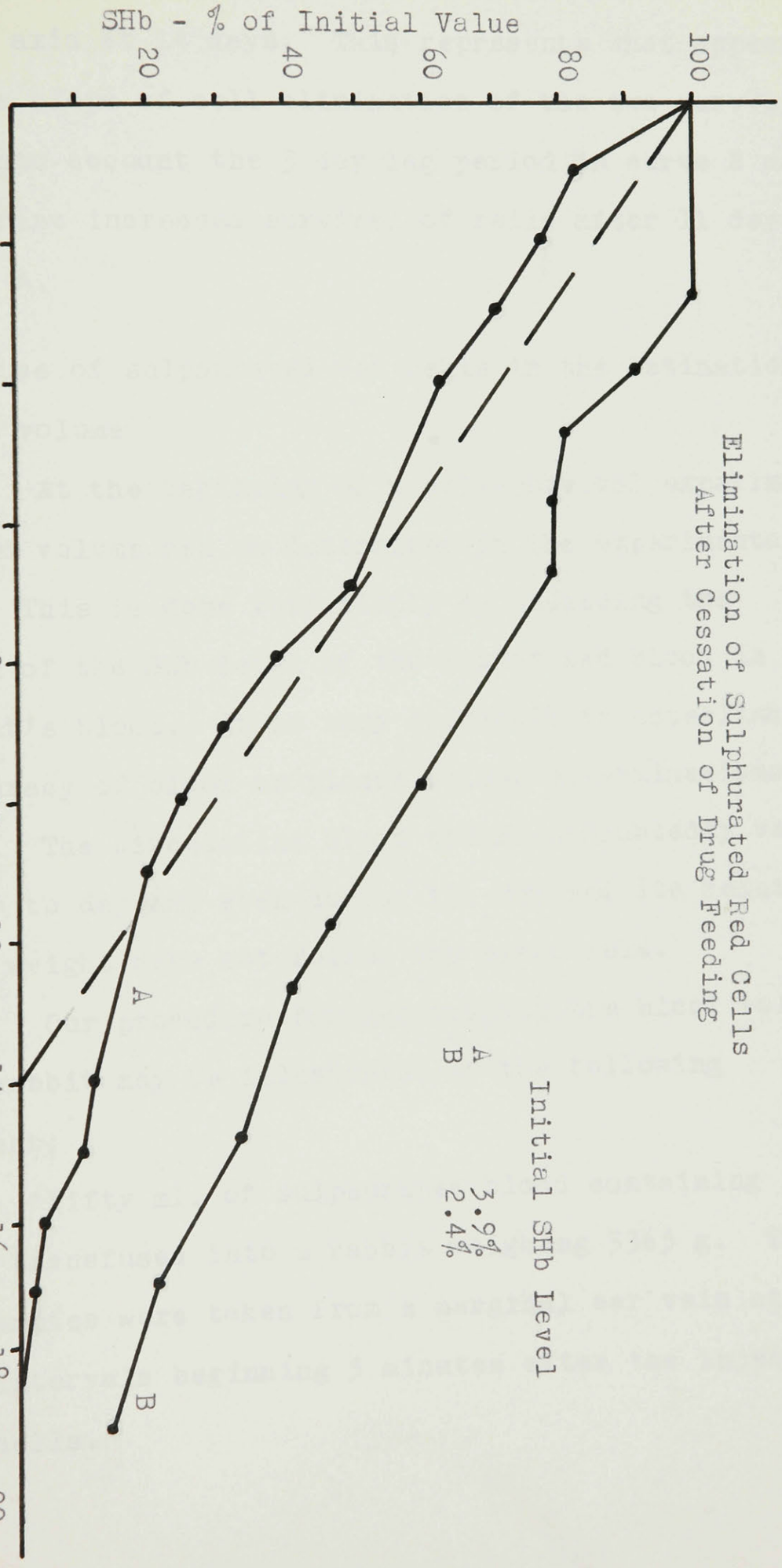
Survival of Sulphurated Cells after Transfusion



(iii) Elimination of sulphurated cells after cessation of feeding drugs

The conditions in these experiments were analogous to those in Jope's study, i.e. the sulphurated cells were not transfused. From the time the feeding of phenacetin and sulphur was discontinued about 20 days were required for the complete elimination of SHb from the circulation. That this period is about twice that obtained by transfusing the sulphurated (in vivo) cells into normal animals doubtless is due to the circumstance that the sulphur is not cleared from the alimentary tract for a few days and judging from the odor of the tissues in animals that died, the tissues also may contain a considerable amount of H_2S and metabolites of phenacetin for a few days. Thus, sulphuration may continue at a diminishing rate for a few days after feeding of the drugs is discontinued. The continued formation of SHb was evident in one experiment in which no apparent cell destruction occurred during the 3 days after feeding was stopped. In other words SHb formation presumably was keeping pace with cell destruction. The livers of these animals showed signs of damage and fatty change. The sulphurated cells of the drugged animals were found to be of normal osmotic stability by the fragility test.

Fig. 3


SHb - % of Initial Value
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
-Days-
A
B
Elimination of Sulphurated Red Cells
After Cessation of Drug Feeding
Initial SHb Level
A 3.9%
B 2.4%

In Figure 3 a broken line is drawn cutting the time axis at 14 days. This represents what appears to be the slope of cell elimination of the two curves, taking into account the 3 day lag period in curve B and the apparent increased survival of cells after 11 days in curve A.

6. The use of sulphurated red cells in the estimation of blood volume

At the beginning of a cell-survival experiment the blood volume can be determined in the experimental animal. This is done very simply by measuring the dilution of the SHb level of the transfused blood in the recipient's blood. It is very difficult to establish the accuracy of blood or plasma volume determinations however. The circulating blood volume undoubtedly varies from day to day and even during the day and its relation to body weight does not follow any exact rule.

Our procedure for determining the blood volume in the rabbit may be illustrated by the following experiment:

Fifty ml. of sulphurated blood containing 18.5% SHb was transfused into a rabbit weighing 3365 g. Three blood samples were taken from a marginal ear vein at 10 minute intervals beginning 5 minutes after the injection of the cells.

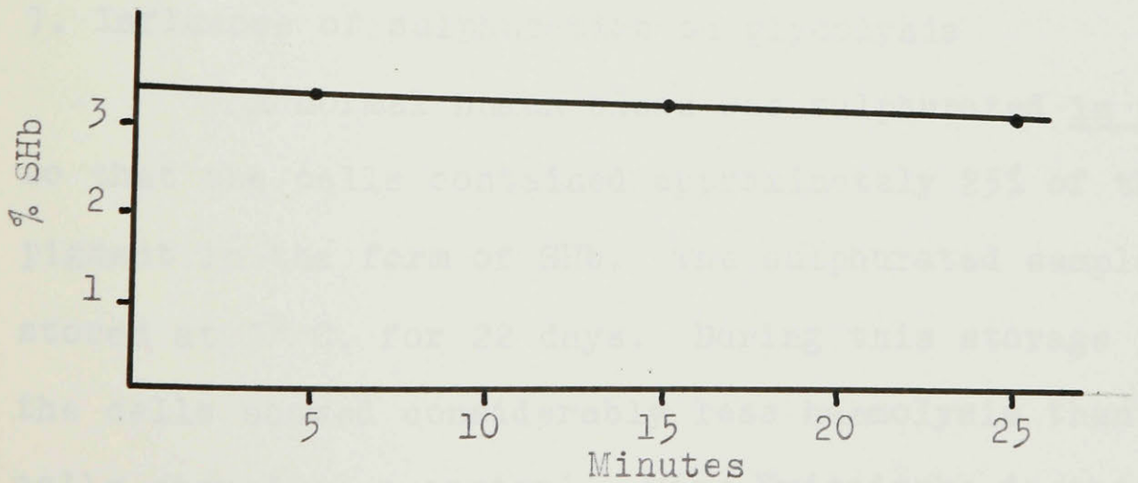
The following values for SHb and total haemoglobin (THb) were obtained:

		SHb g.%	THb g.% (Total pigment in circulation after transfusion)*	
Sample I	(5 mins.)	0.45	13.68	or 3.30% SHb
Sample II	(15 mins.)	0.44	13.68	3.22% SHb
Sample III	(25 mins.)	0.43	13.88	3.12% SHb

*SHb and Hb as cyanmethaemoglobin

Figure 4

Slope of the Initial Elimination of Red Cells Sulphurated <u>in vitro</u>



As can be seen in Figure 4 the drop in SHb level of the blood after 25 minutes was so slight that the first value (5 minute level) was taken as being equal to the theoretical 0 level (which is actually 3.38% when determined graphically). The blood volume is then:

$$3.3x = 50 \times 18.5 = 310 \text{ ml.}$$

If only a blood volume estimation is required, a small volume of highly sulphurated cells may be used. These cells will be nonviable but the number of cells that are eliminated within the time required for the estimation of blood volume is insignificant especially if the calculation is based on a single blood sample taken 5 minutes after injection of the cells. One ml. of blood containing 40% of the pigment as SHb, can be easily detected in 100 ml. of the circulating blood.

7. Influence of sulphuration on glycolysis

A normal human blood was sulphurated <u>in vitro</u> so that the cells contained approximately 25% of their pigment in the form of SHb. The sulphurated sample was stored at 5° C. for 22 days. During this storage period the cells showed considerably less haemolysis than normal cells stored as a control. Miss Kwiecinska in this laboratory showed that glycolysis was apparently unaffected by the presence of the high SHb content of the cells.

The normal glycolytic cycle was functional in spite of the slight excess of H_2S that was present in the suspending medium. Alivasatos, in this laboratory showed on the other hand, that glyoxalase is inhibited in the sulphurated cells. The activity can be restored by washing the cells, however, so the inhibition must have been due initially to incomplete removal of excess H_2S. These findings are interesting in view of the fact that the cells were found to have a markedly lowered viability when transfused.

8. Use of sulphuration in testing viability of oxalated cells

Miss Kwiecinska observed that glycolysis can be arrested in red cells during storage by use of oxalate in place of citrate as the anticoagulant. It was found that the glycolytic activity can be restored by resuspending the cells in fresh serum, i.e. presumably by restoring the magnesium ions. The question arose whether the aging of the cell is arrested when important chemical processes like glycolysis are inhibited. To test this a collaborative experiment was undertaken in which a rabbit's cells containing SHb (by feeding the animal sulphur and phenacetin) were stored in oxalate for two days at 5° C. and then reactivated and transfused. Cell survival was followed by estimating SHb, in the manner previously outlined. Cells were eliminated in a 10 day period

instead of the usual 12. It was evident that the aging of the cells had not been arrested during the period of storage in oxalate.

Summary

The life span of rabbit red cells has been estimated at 12 days. This was done by following the elimination of red cells, sulphurated *in vivo*, from the circulation after transfusion. Red cells sulphurated *in vitro* have a lowered survival time. The relatively longer apparent cell-survival time in the experiments, in which the elimination time was determined after discontinuing the feeding of the drugs, doubtless was due to the circumstance that the tissues of the animals were saturated with the drugs and that sulphuration of newly formed erythrocytes continued for some time after the feeding was stopped.

It seems likely that secondary oxidant changes, leading to the opening of the porphyrin ring structure of haemoglobin, are mainly responsible for the lowered survival time of the *in vitro* sulphurated cells.

The fragility and the oxygen-combining capacity of sulphurated cells have been measured. It is interesting to note that the presence of a fairly large proportion (up to 20%) of SHb, whether produced by the *in vitro* or *in vivo* method, does not interfere with the oxygen-carrying capacity of the remaining haemoglobin. The

presence in the circulation of metabolites from phenacetin and sulphur feeding may, however, interfere seriously with the oxygen-combining properties of the remaining haemoglobin in the red cells.

Rapoport (137) has shown that the physical structure of the cell is changed during storage. As the time of storage is prolonged there is a greater degree of haemolysis in hypotonic solutions, increased thickness and crenation of the cell. We have shown by the rapid elimination of cells, sulphurated to a high level in vitro, and possessing normal stability in hypotonic solutions, that such changes are secondary to other unknown factors.

The fact that glycolysis is unaffected and glyoxylase activity can easily be restored, raises the question of the role which the energy released in glycolysis plays in the maintenance of the red cell. Hb is slowly converted into methaemoglobin in the intact erythrocytes during storage at 37° C. It has been suggested (124) that methaemoglobin is reduced to the functional ferrous form by reduced diphosphopyridine nucleotide arising during glycolysis and that the adenosine triphosphate produced, acts in regenerating the pyridine and flavine enzymes which slowly undergo hydrolysis.

II Agglutination of Erythrocytes Containing Sulphaemoglobin--Further Evidence of Specific Aggregation

If it were possible to label the red cells in such a manner that they could easily be distinguished visually from normal red cells it would be simple to observe the clumps under the microscope and to see directly whether the second stage of agglutination is specific. The evidence we obtained by using partially haemolyzed cells (ghosts) or cells stained with dyes was not conclusive. However, further confirmation of Marrack's hypothesis of specific aggregation has been obtained in our laboratory by use of a mixture of normal and sulphurated erythrocytes. As shown below, red cells can be sulphurated in vitro to a high content of sulphaemoglobin without altering their agglutinability. The sulphurated cells are pale green and are slightly larger in diameter than normal cells. On agglutination they undergo less crenation and shrinkage in diameter than normal cells, and thus are readily distinguishable from the latter in an agglutinated mixture. The experiments here reported concern the influence of sulphuration on agglutinability of human red corpuscles and use of sulphurated cells in confirming the specific aggregation hypothesis.

1. Preparation of sulphurated red cells

A suspension (about 5%) of human red cells from a few drops of blood was made with isotonic phosphate

buffer of pH 7.35 in a small serological test tube (10 x 75 mm.). The air in the tube was displaced with hydrogen sulphide, and the tube was stoppered and slowly and repeatedly inverted. Addition of hydrogen sulphide and mixing were repeated two or three times depending on the degree of sulphuration desired. After standing for 20 min. at room temperature with occasional mixing, the red cells assumed a deep greenish color. To remove residual hydrogen sulphide the tube was centrifuged lightly, the supernatant fluid removed by aspiration, and the cells washed with the phosphate buffer solution. Washing was repeated until the supernatant gave a negative test with lead acetate. The proportion of sulphaemoglobin to haemoglobin in the cells was determined by the method of Evelyn and Malloy (135). No attempt was made to distinguish between sulphaemoglobin and choleglobin (132), which, if present, would be measured as sulphaemoglobin. To ensure ready differentiation of the sulphurated and normal cells in agglutination experiments, the sulphaemoglobin content of the cells should be between 50 and 75% of the total pigment.

2. Stability and agglutinability of sulphurated red cells

It is important in agglutination studies that the stability and agglutinability of the cells should not be greatly altered by sulphuration. The following tests were performed to ascertain the effect of the treatment

on these properties.

(a) Stability

One ml. of lightly packed washed cells was suspended in 2 ml. of isotonic phosphate buffer (pH 7.35); 0.5 ml. portions of the suspension were dispensed into serological tubes. Hydrogen sulphide was introduced into the space above the liquid and the tubes were stoppered and agitated gently for 20 min. The excess hydrogen sulphide was then removed with the aid of an aspirating tube.

Osmotic stability was tested by the fragility method of Waugh and Asherman (138), who recommend the use of a buffered saline (Simmel's) in place of simple sodium chloride solutions.

The sulphurated cell suspension was pipetted in 0.02 ml. portions into 15 ml. centrifuge tubes containing isotonic Simmel's solution and a range of hypotonic concentrations. After five minutes the tubes were centrifuged for five minutes. The supernatant solution was discarded and the cell sediment suspended in 10 ml. of M/60 phosphate buffer of pH 6.6. One drop of each of the following solutions: concentrated ammonium hydroxide, 20% potassium ferricyanide, and 10% potassium cyanide was added in that order. The concentration of cyanmethaemoglobin (cyanhaemochromogen) was determined by Evelyn's method (135), with a 540 mμ filter.

Table 8

Cell Haemolysis after Five Minutes

in Simmel's Solution

Pigment as SHb, %	Dilution, % of isotonic				
	100	70	60	50	40
	Haemolysis, %				
0	0	0	0	0	81
35	0	0	0	0	84
45	0	0	0	11	85
75	0	0	10	54	95

It will be observed from Table 8 that the osmotic fragility of the cells to hypotonic saline solutions is not greatly increased by sulphuration *in vitro*. For the present purpose the slightly increased fragility is of no consequence, since cells containing from 50 to 60% of sulphaemoglobin are stable for several hours in saline even down to 60% of the isotonic concentration. As all the agglutination tests were carried out in isotonic medium no breakdown of the sulphurated cells occurred during the 15 min. period of the test.

(b) Agglutinability

Two criteria were adopted to validate the use of sulphaemoglobin for labelling the cells: (1) comparison of sulphurated and normal cells in titre tests with a given potent antiserum and (2) comparison of the

number of free cells after agglutination of the sulphurated and normal cells.

(i) Titre tests

When the potency of various strong antisera, including anti-A, anti-B, anti-M, anti-N, anti-Rh_o (anti-D), and anti-rh" (anti-E), was tested with appropriate cells, both normal and sulphurated cells from a given donor were found to give the same titre values except when anti-Rh sera were used. The Rh agglutinogens were found to be labile to numerous types of treatment whereas the A, B, M, and N antigens are very stable. Thus a lower titre was obtained with the Rh antisera especially when the sulphaemoglobin level in the cells was more than 50%. Obviously there is no change in the potency of the serum but the impaired reactivity of the Rh antigen results in a lower titre value.

(ii) Free-cell counts

Fresh blood was drawn up to the 1.0 mark in a white-cell pipette, which then was filled to the 11 mark with isotonic phosphate buffer of pH 7.4. After the pipette was shaken, the contents were transferred to a serological tube and an equal amount of buffer was added. The contents were sulphurated and gently agitated mechanically for 20 min. Finally, excess hydrogen sulphide was removed. Colloidal sulphur frequently is formed during sulphuration but is of no consequence.

Duplicate cell suspensions were prepared from blood from various Group A individuals according to the procedure outlined. One sample from each pair was sulphurated and then all samples were agglutinated with potent anti-A serum and the unagglutinated cells were counted according to the following procedure.

Blood is drawn up to the 1.0 mark in a white-cell pipette, which then is filled to the 11 mark with agglutinating serum. The pipette is shaken and the contents transferred to a serological tube. The tubes are gently agitated four or five times during a two minute period and then centrifuged for exactly two minutes at 1500 r.p.m. After flicking the tube to dislodge the agglutinated cell sediment the sample is agitated mechanically for 40 min. at the rate of 230 vibrations per minute using a specially designed shaker.*

For counting the free cells, the larger clumps are permitted to settle for about three seconds and a sample of the supernatant fluid is transferred to the haemocytometer by means of a white-cell pipette filled to the 1.0 mark.

*The shaker is provided with a Graham variable speed motor which gives reproducible rates of shaking over a wide range. A constant temperature bath is part of the apparatus.

The free cells in the entire field (144 large squares) are counted and the number of cells per cu. mm. calculated as follows:

$$\text{Free cells per cu. mm.} = \frac{\text{total count} \times 11 \times 22 \times 10}{9}$$

$$= \text{total cell count by } 269.$$

Results:

(a) Reproducibility of free-cell count

The following figures were obtained from four successive agglutination tests on Group A red cells containing 71% sulphaemoglobin: 19,900; 18,300; 20,200; 20,100. Mean 19,625.

(b) Comparison of free-cell counts on normal and sulphurated cells

The cell counts given in Table 9 represent the means of duplicate counts. The cells were taken from different donors, all of group ARh_o. In all instances anti-A serum of 512 titre was used.

Table 9

Free-Cell Count Before and After Sulphuration

Subject	Cell specimens	SHb, %	Duration of agitation at 230 vibrations per min.	Free-cells per cu. mm.
I	Normal	0	55	14,200
	Sulphurated	60	55	15,000
II	Normal	0	40	21,000
	Sulphurated	75	40	22,600
III	Normal	0	30	39,900
	Sulphurated	67	30	38,000
IV	Normal	0	45	53,000
	Sulphurated	70	45	51,400

It is evident that the reproducibility of the counts is satisfactory and that the agglutinability of the cells is not altered by the sulphuration treatment.

3. Use of sulphurated red cells for testing Marrack's hypothesis

Sulphurated red cells, which are thus shown to be comparable in stability and agglutinability with normal cells, can be used for testing the specificity of aggregation. The general procedure was as follows: light saline suspensions of normal and sulphurated human erythrocytes were adjusted by dilution so that the

concentration of cells in all tubes was the same. To facilitate differentiation of the normal and sulphurated cells in the aggregates, the serum was diluted so as to favor the production of small rather than large clumps. One drop of each of the two cell suspensions was introduced into a serological tube with two drops of the appropriate antiserum or sera. The tube was centrifuged at low speed (about 1500 r.p.m.) for two minutes. The cell sediment was then broken into small clumps by flicking the tube. The microphotograph in Figure 5 illustrates the difference in appearance of the normal and sulphurated erythrocytes. If a daylight lamp is used, the sulphurated cells appear larger, paler, and slightly greenish in color. Table 10 gives the results of a typical experiment.

Figure 5

Appearance of Normal and Sulphurated
Red Corpuscles in an Agglutinated Mixture

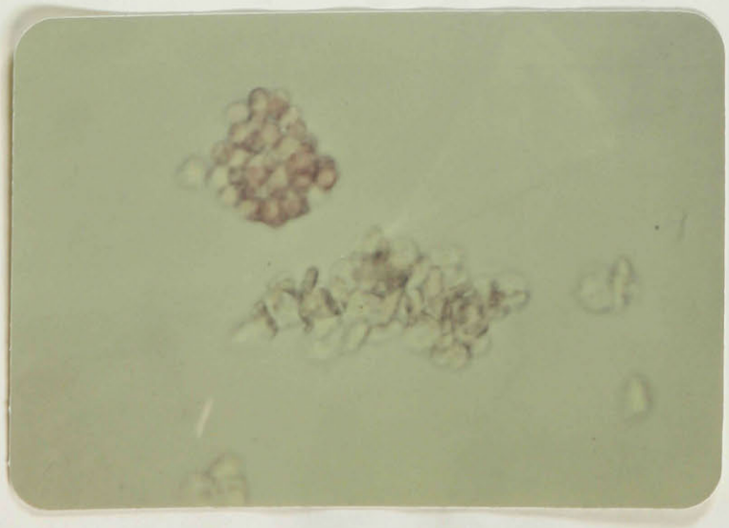


Table 10

Specific Agglutination with A and B Agglutinogens

Blood group of red cells in mixture	Agglutinating serum	Composition of resulting clumps
$\underline{A}$ + A	Anti-A (titre 512)	Mixed $\underline{A}$A
$\underline{A}$ + B	Anti-B (titre 512)	B only
$\underline{A}$ + B	Anti-A	$\underline{A}$ only
$\underline{A}$ + B	Anti-A + Anti-B	Separate $\underline{A}$ and B

Underlined letters designate sulphurated cells.

Analogous results were obtained with cell mixtures $\underline{B}$ + B, $\underline{B}$ + A, $\underline{M}$ + M, $\underline{M}$ + N, M + $\underline{N}$, $\underline{Rh_o}$ + Rh_o, and $\underline{Rh_o}$ + rh". with the appropriate sera.

For the M- and N-tests, red cells of group $BMRh_o$ (50% SHb) and group $ANRh_o$ (46% SHb) were used with anti-M and anti-N sera (titre 32). The anti-M serum was absorbed with a light suspension of "N" cells, and the anti-N, with "M" cells, prior to using.

In the case of the Rh antigens the Rh_o cells were sulphurated to a level of 45% SHb. Anti-Rh_o (anti-D) and anti-rh" (anti-E) sera of titre 64 were used.

Discussion and Conclusions

From the foregoing results it is clear that sulphuration *in vitro* with hydrogen sulphide does not seriously alter the osmotic stability of human red cells, nor does it affect the agglutinability except with anti-Rh serum. The Rh antigen, being much more labile to various treatments than the A, B, M, or N antigens, is altered by sulphuration, especially when the sulphaemoglobin is about 50% of the total pigment. The A, B, M, and N antigens, on the other hand, are stable up to 75% sulphuration, which is the maximum level attainable by our method.

Examination of the clumps obtained by agglutinating mixtures of normal and sulphurated red cells of various types with appropriate antisera showed consistently that cells of different types formed discrete aggregates. In other words, both sensitizing and aggregating phases of the agglutination reaction appear to be specific, as postulated by Marrack and others.

III An Hypothesis to Explain Certain Anomalies of Agglutination

When erythrocytes are exposed to a specific antiserum a few cells invariably escape agglutination even with highly potent sera. These cells, usually referred to as "free" or "unagglutinable" cells, represent less than one percent of the total number of cells in the sample and their number remains practically constant in repeated tests provided the conditions of agglutination are kept constant. Under variable conditions of temperature, rate of agitation and size or shape of container, and especially with antisera of low titre, the free-cell count is subject to wide fluctuation. The failure of these cells to be agglutinated is of no consequence in blood typing and other qualitative tests but, in certain quantitative procedures, it is necessary to know accurately the free-cell count and to keep the conditions of agglutination constant. For example, in the Ashby method of differential agglutination for following the rate of elimination of the donor cells from the recipient's circulation after transfusion, the unagglutinable-cell count of the recipient's blood must be accurately determined prior to the transfusion.

In the course of our studies on the survival of preserved red cells after transfusion we had occasion to investigate the course of the unagglutinable-cell phenomenon and the factors which influence the count.

Among the alternative explanations that appear plausible are the following:

(1) Failure of some of the cells to come in contact with others. This is highly improbable except in very dilute cell suspensions.

(2) Insufficient antibody to produce complete agglutination of the cells.

(3) Lack of agglutinogen on the cells or the presence of defective or weakly reactive agglutinogen.

The first of these explanations is ruled out by the persistence of free cells even after centrifuging and resuspension of the cell precipitate. The second also is ruled out by two observations, namely, (a) that the free-cell count cannot be reduced by the addition of more antiserum, and (b), that after the initial agglutination with a potent antiserum by the method used here, there usually is sufficient antibody remaining to produce agglutination after four or more further additions of cells. Nor is the third hypothesis satisfactory since, as is shown later, the free cells are "unagglutinable" only among themselves, but are capable of reacting with fresh cells. The latter observation suggested a further hypothesis which since has been confirmed by numerous pieces of evidence.

For convenience the theory to be discussed may be referred to as the "saturation hypothesis". In

accordance with present-day concepts the agglutinins are assumed to be at least divalent and to be capable of reacting with specific receptor groups (agglutinogens) on the red cells, and to constitute the connecting links holding the cells together in aggregates which tend to precipitate out as visible clumps. Thus in the process of the reaction of agglutinogen (antigen) with agglutinin (antibody), sometimes referred to as the "sensitization" of the cells, the majority of the cells become agglutinated but a few become more or less saturated with antibody. The completely saturated ones, which are the so-called unagglutinable cells, having all their receptor groups occupied, are incapable of agglutination among themselves. However, according to the hypothesis, the free reactive groups of the attached agglutinins are capable of uniting with the receptor sites on fresh cells, or on partially saturated ones, to yield aggregates. The capacity of the incompletely saturated cells to be agglutinated, therefore, should vary inversely with the degree of saturation. Thus the presence of partially saturated cells explains why the free-cell count falls logarithmically on prolonged agitation or on repeated centrifuging.

The capacity of the so-called unagglutinable cells to react with fresh cells has been demonstrated in our laboratory by using sulphaemoglobin-labelled red

cells. The latter, by virtue of their size and pale green color, are readily distinguishable from normal cells in a mixture under the microscope. As shown in a previous study (101) red cells can be sulphurated up to a level of over 50% (i.e. more than 50% of the Hb can be converted to sulphaemoglobin) without altering their agglutinability with anti-A, -B, -M or -N sera. Thus when sulphurated cells of one of these blood groups are added to the supernatant fluid of an agglutinated sample of normal cells (of the same group) it can be shown that the free cells in the latter combine with the sulphurated cells to form mixed aggregates. It is evident, therefore, that the free cells are not lacking in agglutinability. That the free cells have taken up antibody is shown, furthermore, by the fact that the washed cells can be agglutinated by the addition of the Coombs antiglobulin serum.

Agglutination procedure

A red-cell suspension is prepared by drawing blood up to the 0.5 mark in a white-cell pipette. The latter then is filled to the 11 mark with isotonic sodium chloride (0.9% solution) and the contents, after mixing by agitation, are transferred to a small serological test tube. Into a dry white-cell pipette, the suspension is drawn up to the 1.0 mark and the pipette

then filled to the 11 mark with the agglutinating serum. The contents are mixed and transferred to a serological tube which is then placed in a shaker and agitated at 230 vibrations per minute for 15 minutes in a water bath maintained at a constant temperature (20° C.). The tubes are centrifuged at 1500 r.p.m. in a table model machine for 2 minutes. After breaking up and suspending the cell sediment by flicking the tubes, the contents are again centrifuged. With potent and avid antiserum the procedure usually gives fairly low and reproducible free-cell counts which are not diminished by the addition of more serum or by further shaking. The tests are performed in duplicate and the free cells are counted in a haemocytometer. The count over the entire field, when multiplied by 269, gives the number of cells per mm^3. The counting is done under a medium power objective, and care is taken to avoid counting white cells, especially the monocytes which are similar in size.

Characterization of antiserum

The following three criteria have been used in our study for the quantitative characterization of antisera: (1) titre, (2) free-cell count and (3) the rate of fall in the free-cell count during agglutination. The first of these reflects the concentration of antibody in the serum; the second, the number of saturated cells, which, in turn, reflects the rate of sensitization, i.e.,

the rate of combination of antigen and antibody; the third also reflects the rate and degree of sensitization. The more complete is the saturation of the cells with antibody during sensitization the more slowly the free-cell count will fall with continued agitation, and the higher will be the final free-cell count. It would appear that the ill-defined property "avidity", which is commonly used to imply rapidity of agglutination by a serum, actually reflects rapidity of the sensitization phase. It is well known that certain antisera may have a high titre and yet be relatively slow in agglutinating action, i.e. they are of low avidity. We have observed also that certain sera may retain the titre during storage but suffer a decrease in avidity. As yet the nature of this type of alteration in the properties of the antibody is not clear.

Evidence for the saturation hypothesis

The following observations appear to support the saturation hypothesis:

1. The high free-cell count with potent (avid) antisera.

It seems paradoxical that the number of free cells should be high with certain very potent antisera. Anti-B serum for example, and especially, heterologous immune sera such as anti-N, are notable for their avidity. Even when the titre is considerably reduced by dilution these sera frequently produce very rapid agglut-

ination. Rapidly acting antisera such as these may be expected to give rise to a high incidence of cell saturation and thus to a high free-cell count provided the concentration of antibody is not too low.

It is reasonable also that the prozone phenomenon, which is shown by certain highly potent antisera, may be explainable on the basis of cell saturation. While this phenomenon is not often seen with haemagglutinating sera, it is well known that practically all highly potent antisera produced by immunization do not produce maximum agglutination of red cells until they have been diluted. In other words the tendency towards saturation of the cells with antibody presumably should be reduced by dilution of the serum.

2. The constancy of the free-cell count during repeated agglutinations.

The following experiment illustrates the tendency of the free-cell count to remain practically unchanged after additional agglutinations are performed with a given amount of potent serum, i.e. in the presence of a large excess of antibody.

A suspension of red cells (ANRh) was prepared by drawing whole blood, from a finger-prick sample, up to the 0.5 mark in a white-cell pipette and then filling the latter to the 11 mark with isotonic saline. After

shaking, the contents were transferred to a small serological test tube. Using the same pipette (which was washed and dried after each use) the cell suspension was drawn up to the 1.0 mark and transferred to each of three serological tubes, which may be designated A, B and C respectively. A fourth aliquot of the suspension was pipetted (to the 1.0 mark) and the pipette then filled to 11 mark with the agglutinating serum (in this case, anti-N, titre 64, diluted 5 times with physiological saline at 25° C.). The contents, after being mixed, were transferred to a serological tube, D (at 25° C.) and then placed in the shaker for agglutination as previously described. In the meantime the cells in tubes A, B and C were centrifuged for 2 minutes at about 1500 r.p.m. and then the supernatant fluid was removed by careful aspiration with a capillary tube. These tubes, containing the cell sediments, were placed in a water bath at 25° C. for temperature equilibration. After the prescribed agglutination period the agglutinated cells in tube D were permitted to settle for two or three seconds and the supernatant fluid, containing the free cells, was transferred to tube A. The contents, after being mixed, were placed on the shaker and permitted to agglutinate in the prescribed manner. The free cells from A, in turn, were transferred to tube B and likewise from B to C. In this way the volume and other conditions were kept uniform in

the four runs. The free-cell counts in the successive runs are given in the table below in Experiment 1. This experiment was repeated using different antisera. In Experiments 2 and 3 however, the free-cell count was first determined in the usual manner and then the supernatant fluid containing the free cell was added to two volumes of sedimented cells and the mixture agitated as previously described.

Table 11

Constancy of the Free-cell Count

During Repeated Agglutinations

Experiment	Antiserum Type	Titre	Free-cell Counts (per mm^3) in the Series A 1 vol. cells	B 2 vols.	C 3 vols.	D 4 vols.
1	Anti-N	12	12,900	12,700	14,000	13,300
2	Anti-A	512	6,200		6,650	
3	Anti-B	128	10,200		10,100	

Thus with a large excess of antibody the number of free cells tends to remain remarkably constant after each addition of fresh cells. This finding indicates also that the free cells in one run must have undergone agglutination with the fresh cells in the subsequent runs. In other words the free cells, while being incapable of

agglutination among themselves are capable, by virtue of the attached antibody, of reacting with fresh cells. This was established further by labelling the free cells from one tube with SHb and demonstrating the presence of the labelled cells in the clumps formed in the succeeding runs. The saturation hypothesis is consistent with these observations.

3. Increase in the free-cell count with increase in Temperature

On the assumption that the combination of antigen and antibody is fundamentally a reaction between chemical groups, it might be anticipated that the rate of reaction should be more rapid with increase in temperature and thus the number of saturated cells should be greater with rise in temperature. We have observed that the free-cell count with group-A cells and anti-A serum can be reduced to a minimum at temperatures below 20° C. As the temperature is increased, on the other hand, the count rises. Above 40° C. the free-cell count is further increased presumably because of alteration in the cellular antigen.

The effect of temperature is illustrated in the following experiment in which the supernatant fluid containing free cells from an agglutination experiment at 30° C. were mixed with fresh cells and the mixture

agglutinated further at 20° C. The new free-cell count was compared with that in a sample agglutinated at 20° C. Thus, two lots of cells (ANRh) were mixed in the usual manner with antiserum and were agitated at 230 vibrations per minute for 50 minutes, one sample (a) at 20° C. and the other (b) at 30° C. (Since blood cells from different individuals react differently at 30° C., any other temperature that gives a marked increase in free-cell count, may be used in this type of experiment.) The specimen then was centrifuged for 2 minutes at 1500 r.p.m., the cells resuspended, and the sample again centrifuged. After determining the number of unagglutinated cells, the supernatant (containing the free cells) from specimen (a) was added to the sedimented fresh cells (saline removed) in a sample (c) and the suspension agglutinated at 20° C. The free-cell counts in the three samples were found to be as follows:

Table 12

Saturated Cells Obtained at 30° C.

Agglutinated with Fresh Cells at 20° C.

Sample	Temperature	Free Cells per mm^3	
		Experiment (1)	Experiment (2)
(a)	30° C.	23,600	19,400
(b)	20° C.	10,500	8,600
(c)	20° C.	10,500	9,400

The figures in Experiment (1) represent the data obtained with cells of group ANRh using anti-N serum (titre 64, diluted 1:5) and those in Experiment (2) the results using anti-A (α)*, (titre 64, diluted 2 times). It is interesting that the results under the conditions represented in (c) agree so closely with those in (b).

The effect of temperature on the free-cell count is shown in Figures 6 and 7. The free cells were counted after 20 minutes shaking at 230 vibrations per minute followed by two centrifugations. The free-cell counts at 35° C. and 40° C. were high and the free cells consisted mostly of saturated cells. The counts were reduced only slightly on continuing the shaking for 10 minutes at 20° C. The slowly falling free-cell count is considered to be due to the slow aggregation of partially saturated cells. It is well known that Rh antibodies can be released from their attachment to antigen by heating Rh agglutinated cells at 56° C. for 3 to 5 minutes. The Rh antibody

*Prepared by absorbing anti-A ($\alpha + \alpha_1$) with A_2 cells until there is no further reaction. The absorbed serum = α_1. The clumps were washed 5 times with saline and suspended in a small volume of saline at 56° C. for 3 minutes then centrifuged rapidly for 2 minutes at 56° C. and the cells discarded. The saline had an α titre of 1:64 in this experiment.

appears unaffected by this treatment and its release is presumably due to denaturation of the labile Rh antigen. Hence, the possibility was considered that heating at 40° C. might alter the reactivity of the antigens A, B, M and N or their corresponding antibodies. However the free-cell counts obtained at 20° C. were the same as the counts obtained after a preliminary heating of the cells and antiserum in separate tubes, at 40° C. for 30 minutes. The free-cell counts obtained with anti-A, -B, -M and -N sera at 20° C. or lower, generally are smaller and subject to less fluctuation than above this temperature.

Fig. 6

Differences in the Free-Cell Count from 5 to 40° C.

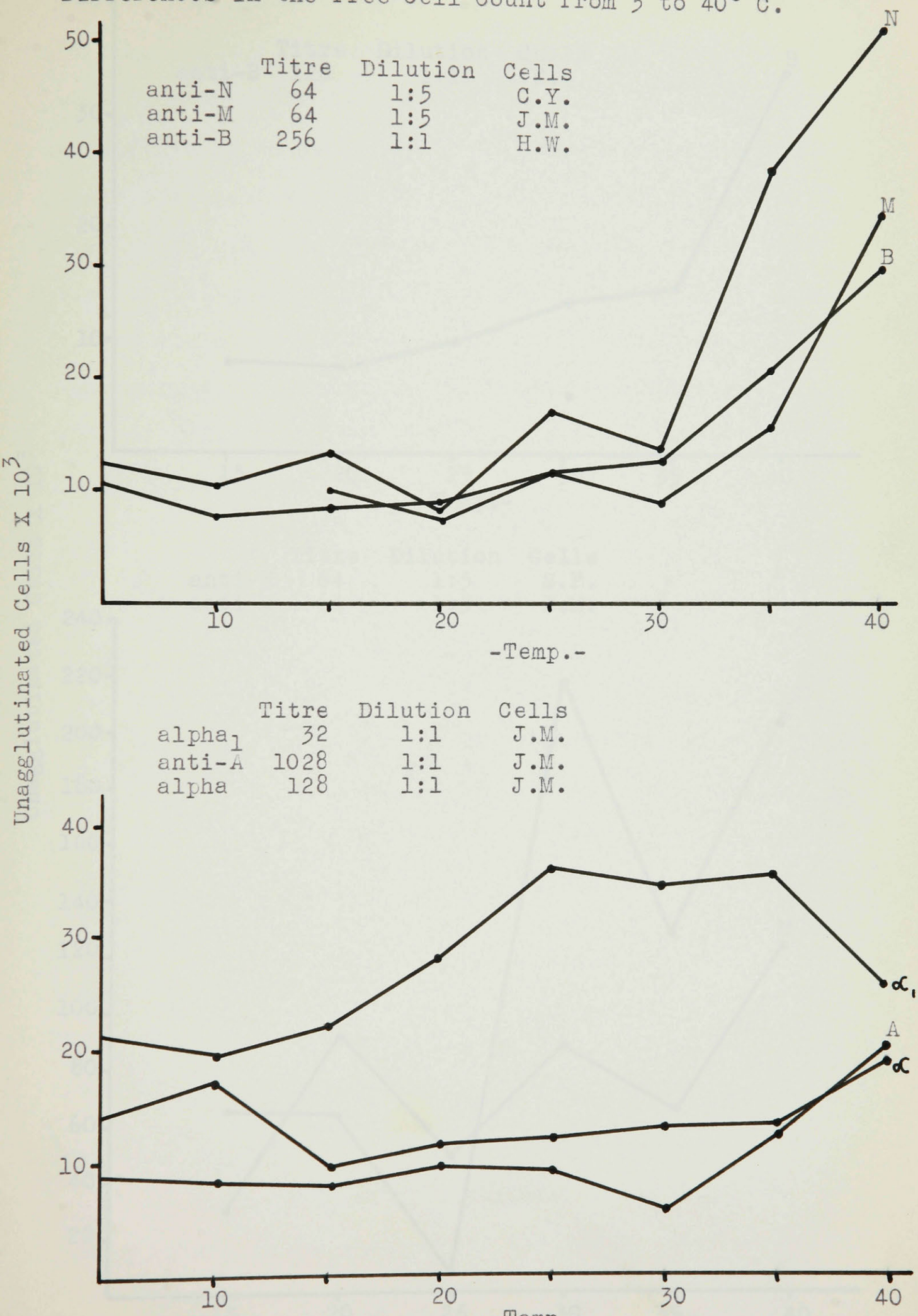
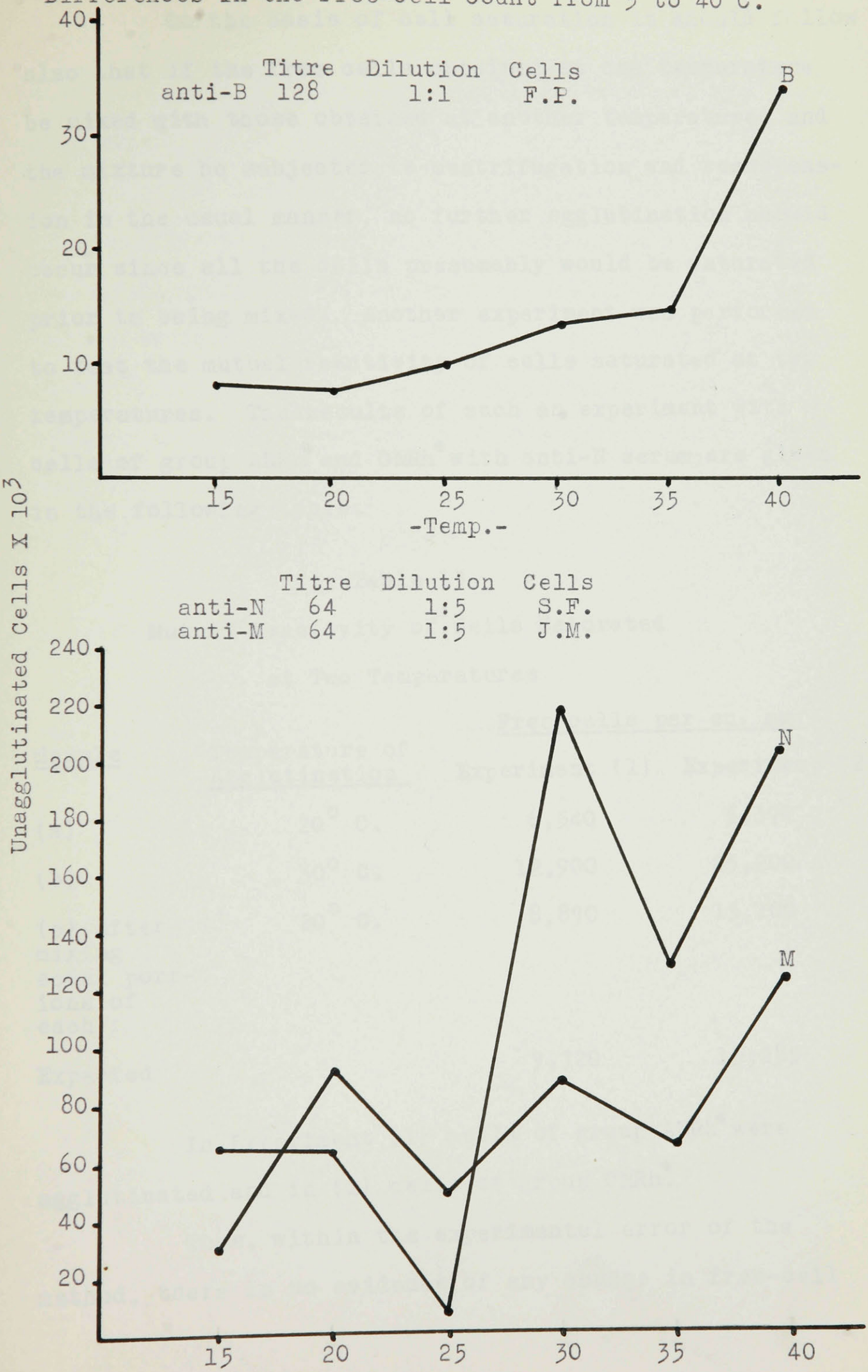


Fig.7

Differences in the Free-Cell Count from 5 to 40°C.



On the basis of cell saturation it should follow also that if the free cells obtained at one temperature be mixed with those obtained at another temperature, and the mixture be subjected to centrifugation and resuspension in the usual manner, no further agglutination should occur since all the cells presumably would be saturated prior to being mixed. Another experiment was performed to test the mutual reactivity of cells saturated at two temperatures. The results of such an experiment with cells of group ANRh$^{\bullet}$ and ONRh$^{\bullet}$ with anti-N serum are given in the following table.

Table 13

Mutual Reactivity of Cells Saturated at Two Temperatures

Sample	Temperature of Agglutination	Free cells per cu. mm. Experiment (1)	Experiment (2)
(a)	20° C.	6,540	5,370
(b)	30° C.	12,900	25,200
(c) After mixing equal portions of each	20° C.	8,870	15,100
Expected		9,720	15,285

In Experiment (1) cells of group ANRh$^{\bullet}$ were agglutinated and in (2) cells of group ONRh$^{\bullet}$.

Thus, within the experimental error of the method, there is no evidence of any change in free-cell

count in these experiments.

4. The relationship between avidity, titre and the free-cell count

The term avidity is generally used to express the rapidity with which visible clumping of red cells occurs in agglutination on a slide or test plate after antiserum is added. In our work, as shown later, we have obtained evidence that the property called "avidity" applies mainly to the first or sensitization stage of the reaction, i.e. it reflects the rapidity with which antibody combines with agglutinogen on the cell surface.

Highly avid sera of high titre may be expected to give rise to a high degree of cell saturation and thus to a high free-cell count. Sera of low avidity and low titre of course may give rise to a high free-cell count due to incomplete reaction. Sera of low avidity, on the other hand, with a high antibody concentration (high titre), may give low free-cell counts. In such circumstances the aggregates usually tend to be small, which suggests that the agglutinogen on the cells is saturated with antibody.

An attempt has been made to develop a method of measuring the avidity of each serum used by obtaining a standard or reference free-cell count. This was done in the following manner: red cells containing the appropriate agglutinogen were agglutinated with the antibody

under study by shaking the sample for 15 minutes at 20° C. at a rapid and constant rate (230 vibrations per min.) The tubes then were centrifuged twice (at 1500 r.p.m.) each time for two minutes duration. The clumps were broken up after each centrifugation by sharply flicking the tube. Counting was done in the usual manner. This procedure was repeated with various antibody concentrations obtained by diluting the antiserum with isotonic saline. The concentration of antiserum used for the reference free-cell count was that which gave the lowest (free-cell) count by the procedure described. The 15 minute period of agitation was used for convenience, since with our special shaking apparatus, the reaction is essentially complete, even with sera of low avidity, within that time.

Using the same antiserum and the same concentration an agglutination was carried out by shaking the cells (and antiserum) at 230 vibrations per minute for one minute at 20° C., followed by centrifugation for one minute. The higher count in the second case is due mainly to incomplete reaction and partly to saturated cells. A comparison of the two free-cell counts gives a measure of the speed with which the antibody combines with the corresponding antigen receptor.

Examples of this method for measuring avidity are given in Table 14. It can be seen that the α_1 and

the anti-B antibodies appear to be the most avid and the anti-M the least, i.e. the greater the avidity of the antiserum the closer the agreement tends to be in the free-cell counts.

Table 14

Comparison of the Free-Cell Counts at 20° C. and at Two Intervals of Shaking

a The free-cell count with 15 mins. shaking, followed by 2 centrifugations of 2 mins. each

b The free-cell count with 1 min. shaking, with 1 centrifugation of 1 min.

c "b" with 2 additional 2 min. centrifugations

Antibody	Titre	Dilution	(a)	(b)	(c)
Anti-A ($\alpha+\alpha_1$)	2048	1:1	6,100	26,600	12,600
Anti-B	256	1:1	10,200	14,250	10,500
Anti-M	64	1:5	6,700	54,000	11,200
Anti-N	64	1:5	6,200	15,900	6,300
Anti-A_1 (α)	128	1:5	10,700	22,600	14,300
Anti-A_2 (α_1)	64	1:4	29,600	22,600	38,000

The counts obtained by using α_1 serum rose sharply when the clump was broken up and recentrifuged. Thus although the avidity of the serum was high the bonding apparently was weak. The counts in column (b) were lowered by the same treatment. While this particular

anti-A serum showed a low avidity, many other anti-A sera were found to have a much higher avidity. A longer shaking time will compensate for a lower avidity, and is necessary when the titre also is low. Even a 1:40 dilution of the above anti-A serum gave a low free-cell count when the shaking time was increased.

The following free-cell counts from various high-titred anti-A sera serve to show the wide variations in free-cell count due to different concentrations of antibody.

Table 15

Anti-A Sera of Various Dilutions Agglutinated with A_1 Cells by 15 Mins. Shaking at 20° C. Followed by 2 Centrifugations

Serum	Titre	Free-Cell Counts at Various Serum Dilutions						
		1	1:1	1:2	1:5	1:10	1:20	1:40
1	2048	98,400				80,500	16,100	
2	2048				62,100	59,600	50,500	
3	512			22,200			7,800	
4	2048			70,200			22,400	10,750
5	1024		23,400				13,700	

It is evident in Table 15 that these potent anti-sera gave rise to a high incidence of cell saturation up to the 1:40 dilution in the series.

5. Variations in the avidity of anti-A sera

Many sera especially when improperly stored or when contaminated with molds, will show reduced avidity on storage. The following anti-A serum was quite avid when fresh but a short time later showed a marked drop in avidity. Concurrently there was a decrease in the α_1 titre. Also α_1 sera prepared by absorbing sera from group-B persons with A_2 cells, showed a rapid drop in avidity along with an ability to strongly agglutinate A_2 cells.

Table 16

Cells ANRh, Agglutinated With Anti-A Serum (titre 2048) at 20° C., 15 Mins. Shaking Followed by 2 Centrifugations

(a) free-cell count on the fresh serum

(b) free-cell count on the same serum 25 days later

Serum Dilution	Free Cell Count (a)	(b)
1:2	72,300	8,600
1:20	22,400	10,200
1:40	10,750	12,600

Whereas in the first series (a) the free cells represented "saturated" ones, many of the so-called free cells in the 1:20 and 1:40 dilutions in the second series (b) were not all saturated and they were agglutinated

after further agitation, reducing the count to approximately that obtained at the 1:2 dilution.

6. Variations in the avidity of anti-B sera

Table 17 shows the variations in the free-cell counts obtained with an anti-B serum and cells from one individual. In three experiments performed on different days the following free-cell counts were obtained with 1 minute shaking and with 15 minutes shaking.

Table 17

Free Cell Counts Obtained with an Anti-B Serum and Cells from One Individual on Different Days

Date:	(1) 19 May /49	(2) 10 June /49	(3) 17 June /49
Free cell count with 1 min. shaking (230 vibrations per min.) and 1 centrifugation of 1 minute	12,400	221,000	65,400
Free cell count with 15 mins. shaking (230 vibrations per min.) and 2 centrifugations of 2 mins.	8,900	16,700	6,200

It will be observed that the figures in column (1) indicate a highly avid serum but at the time indicated in column (3), the avidity had dropped considerably.

7. Effect of avidity on the free-cell count

Figure 8 shows the widely differing free-cell counts obtained with the same blood cells but using two different anti-M agglutinating sera which differed in avidity. The more avid serum (B) had a higher incidence of saturated cells.

The avidities of the two sera are indicated in Table 18.

Table 18

Comparison of the Avidities of

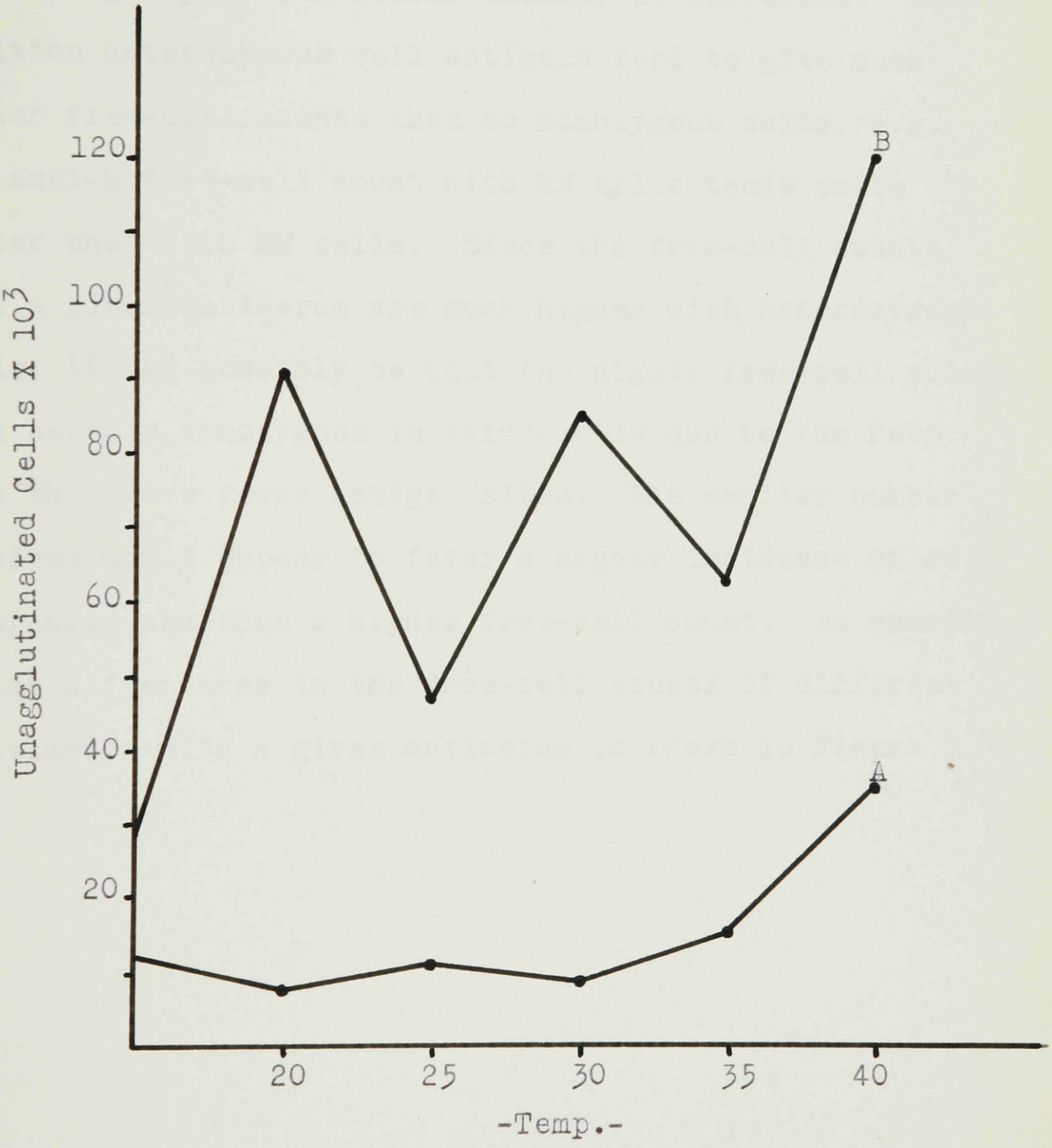
Two Different Anti-M Sera

	Serum A	Serum B
Free-cell count with 1 min. shaking and 1 centrifugation.	54,000	13,000
Free-cell count with 15 mins. shaking and 2 centrifugations.	6,700	4,050

Fig.8

Effect of Avidity on the Free-Cell Count

Anti-M Serum	Titre	Dilution Used	Cells Used
A.	64	1:5	J.M.
B.	64	1:5	J.M.

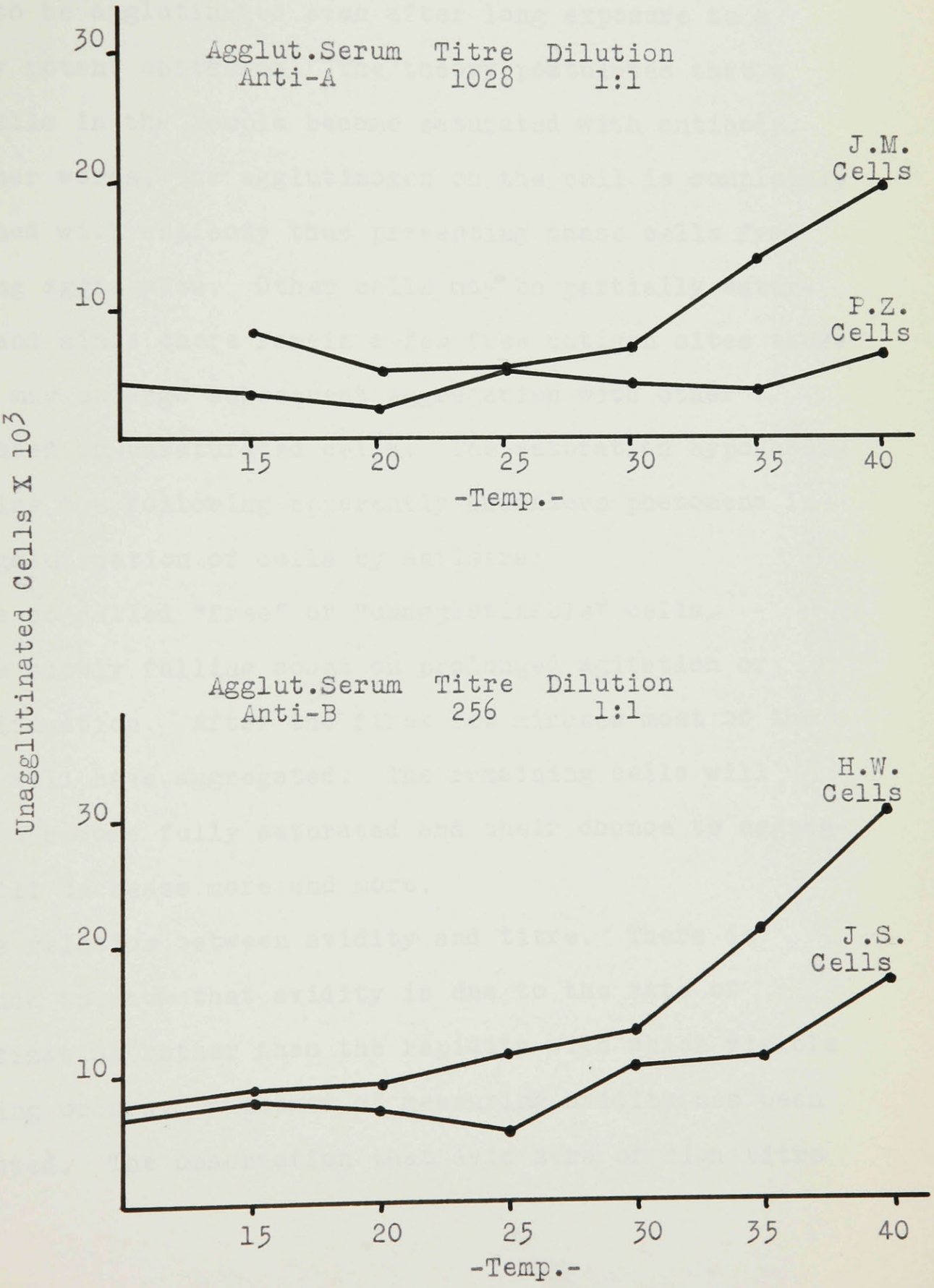


8. Differences in the free-cell count of two individuals

It is well known that blood cells from various individuals may differ considerably in reactivity of the antigen to a given antiserum. For example, with a given anti-A serum the free-cell count obtained using cells from two group-A individuals usually is different. In addition heterozygous cell antigens tend to give much higher free-cell counts than do homozygous cells, e.g. the anti-M free-cell count with MN cells tends to be higher than with MM cells. Since the free-cell counts with a given antiserum are much higher with heterozygous cells, it may possibly be that the higher free-cell counts with certain homozygous individuals is due to the fact that they have fewer antigen sites. The smaller number of sites would appear to favor a higher incidence of cell saturation and thus a higher free-cell count. An example of the differences in the free-cell counts of different individuals with a given antiserum is shown in Figure 9.

Fig.9

Differences in the Free-Cell Count of Two Individuals with a Given Antiserum



Summary

A theory referred to as the "saturation hypothesis" has been proposed to explain why some cells fail to be agglutinated even after long exposure to a highly potent antiserum. The theory postulates that a few cells in the sample become saturated with antibody. In other words, the agglutinogen on the cell is completely combined with antibody thus preventing these cells from forming aggregates. Other cells may be partially saturated and since there remain a few free antigen sites these cells may undergo subsequent aggregation with other saturated or unsaturated cells. The saturation hypothesis explains the following apparently anomalous phenomena in the agglutination of cells by antisera:

1) The so-called "free" or "unagglutinable" cells.

2) The slowly falling count on prolonged agitation or centrifugation. After the first few minutes most of the cells will have aggregated. The remaining cells will tend to become fully saturated and their chance to aggregate will decrease more and more.

3) The relation between avidity and titre. There is evidence to show that avidity is due to the rate of sensitization rather than the rapidity with which visible clumping occurs. A method of measuring avidity has been presented. The observation that avid sera of high titre

give high free-cell counts is explained by this theory. In other words, if the avidity is high the antibody concentration (titre) must be reduced to prevent excessive saturation.

4) Increase in the free-cell count with increase in temperature. It is suggested that the rate of reaction between antigen and antibody is increased as the temperature is raised. Thus, the number of saturated cells will be greater at higher temperatures.

5) The variable counts with one individual and a given antiserum on different days. The avidity of any given antiserum may vary considerably during storage. The avidity generally falls during prolonged storage even at 5° C. Any change in avidity will of course change the incidence of cell saturation.

6) The variable count with two individuals and a given antiserum on the same day. It is suggested that one individual's cells may give a higher unagglutinable-cell count if there are fewer antigen sites on the cells. Thus with fewer sites the chance is increased that a cell may become saturated before it aggregates.

The Antigen-Antibody Binding

1. The stability of agglutinogens to heat

As might be expected when red cells are placed in a boiling water bath for a few seconds, the proteins undergo rapid denaturation and spontaneous coagulation. Saline suspensions of washed red cells, when heated at 56° C. for various intervals of time, however, undergo interesting changes with regard to the agglutinability by various antisera. The effect of the heat treatment in our experiments was tested by comparing the degree of clumping of the cells when exposed to heat with the clumping of a normal control. All agglutination experiments were carried out with potent antisera by shaking for 5 minutes followed by centrifuging and resuspending the cells. The degree of clumping was graded as follows: the formation of a single "button" which could be released from the bottom by flicking the tube was designated as 4 agglutination, aggregation in the form of a few large clumps was taken as 3, numerous small clumps as 2, and a large number of very small clumps, as 1. The results of a typical experiment are shown in Table 19. No nonspecific clumping due to the heat treatment was encountered even after 26 minutes exposure.

Table 19

Influence of Heat Treatment on

Red Cell Agglutinability

Time of Exposure at 56° C. (Minutes)	Degree of Aggregation with Undiluted Antiserum				
	Anti-A (titre 1024)	Anti-B (titre 128)	Anti-M (titre 64)	Anti-N (titre 64)	Anti-Rh_o (Anti-D) (titre 32)
0	4	4	4	4	3
1	4	4	4	4	1
2	4	4	4	3	0
4	3	3	4	3	
5	3	3	3	2	
10	3	3	3	2	
15	3	2	2	1	
18	2	2	2	0	
20	2	2	2		
22	2	2	2		
26	2	2	2		

Thus, of the agglutinogens tested, the A, B and M are remarkably stable to heat treatment. The N agglutinogen is somewhat less stable while the Rh_o antigen is inactivated by heating at 56° C. for less than 2 minutes.

2. The effect of reducing agents on agglutinogens

Red cell suspensions in isotonic phosphate buffer were treated with the following reducing agents: $Na_2S_2O_4$, NaCN, and ascorbic acid. No effect on the agglutinability of the A, B, M or N agglutinogens was detectable even when sufficient solid reagent was added to cause partial haemolysis of the sample and complete reduction of the Hb. The Rh agglutinability, however, was seriously diminished by treatment with NaCN, and often was readily abolished after a short exposure.

Hydrazine was the most effective reducing agent, since a 1.0 molar solution impaired the agglutinability of the A agglutinogen and abolished the agglutinability by anti-Rh. A 0.5 molar solution of the agent decreased the Rh agglutination while the A antigen was unaffected. A buffer mixture of molar concentration with respect to the hydrazine was prepared as follows: 10.0036 g. of $(NH_2)_2.H_2O$ was dissolved in 100 ml. of 4.17% NaH_2PO_4 solution and adjusted to pH 7.3 with concentrated HCl. This solution was made up to 200 ml. with isotonic phosphate buffer of pH 7.3. The solution was molar with respect to hydrazine though slightly hypertonic in salt concentration.

The results obtained by suspending human red cells (ARh_o) for 35 minutes in the solution are given in Table 20.

Table 20

Effect of Exposure of the A and Rh Agglutinogens to Molar Hydrazine

	Agglutinating Serum Anti-A (titre 512)		Agglutinating Serum Anti-Rh_o (titre 64)	
Serum Dilution	Control Clumping	Treated Clumping	Control Clumping	Treated Clumping
1:2	4	3	3	1
1:4	4	2	3	0
1:8	4	2	3	0
1:16	3	1	2	0
1:32	3	0	2	0
1:64	3	0		

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3. Influence of formaldehyde on cell agglutinogens

Saline-washed red cells were exposed for 15 minutes at 25° C. to solutions of formaldehyde ranging in concentration from 0.15 to 1.4 % in 0.9% sodium chloride solution. The Rh agglutinogen again proved to be the least stable and was altered even in a 0.15% solution of the reagent. The A, B, M and N agglutinogens, on the other hand, were unaffected even by 1.4% formaldehyde. The results of a typical experiment are given in Table 21.

Table 21

Influence of Formaldehyde on the A and Rh Agglutinogens (1.4% Formaldehyde in 0.9% NaCl)

	Agglutinating Serum Anti-A (titre 64)		Agglutinating Serum Anti-Rh_o (titre 32)	
Serum Dilution	Control Clump	Treated Clump	Control Clump	Treated Clump
1:2	4	4	4	0
1:4	3	3	3	0
1:8	3	3	3	0
1:16	2	2	2	0
1:32	2	2	1	0
1:64	1	1		

The formaldehyde treatment was found to cause haemolysis to some of the cells. The remaining intact cells were washed 3 times with saline before testing. The treated cells also were more labile and were haemolyzed rapidly when placed in serum, especially in the 1:2 and 1:4 (serum) dilutions. They were more stable in saline.

4. Influence of sulphuration on cellular antigens

When red cells are sulphurated in isotonic phosphate buffer solution of pH 7.35, up to 75% of the haemoglobin can be converted to SHb or related pigments. The cells show normal agglutinability with anti-A, -B, -M, and -N sera. The Rh agglutinability on the other hand

again is altered, especially when the level of SHb exceeds 50% of the total pigment of the cell. As with the other treatments mentioned, acid (below pH 4.0) or alkaline (above pH 10.0) solutions increase the susceptibility of the Rh agglutinogen to damage by these treatments. For example, in unbuffered saline solutions, as the sulphuration proceeds and the SHb content of the cells is increased, the pH falls and the Rh agglutinability is rapidly diminished. When red cells are sulphurated in unbuffered saline to a level of 50% SHb or greater, the osmotic state of the cells is greatly changed (i.e. fragility to hypotonic solutions is increased).

5. Influence of vital dyes on cell agglutinogens

To test whether various vital dyes might combine with the agglutinogens so as to interfere with the agglutinating action of agglutinins, the following dyes were used in 0.1% concentration in physiological saline at pH 7.0: trypan blue, methylene blue, brilliant cresyl blue, naphthol yellow S, light green S.F., pyronine methyl green, Nile blue, and vital red. The series included also a saturated solution of the fat stain, Sudan IV. A 2% suspension of washed red cells was made in the dye solution and the cells were permitted to remain for various periods up to 60 minutes on a Borner Rotator. The tubes then were centrifuged and the cells washed twice with

normal saline and then tested for agglutinability.

Of the dyes tested, Nile blue, vital red, brilliant cresyl blue, and Sudan IV were the only ones that altered the agglutinability of the cells. The effect of Sudan IV which is sparingly soluble in water, was relatively slight. Vital red also showed a feeble interference. A 15 minute exposure of the cells to brilliant cresyl blue at 27° C. reduced the titre of the anti-Rh serum from 32 to 8 without altering the agglutinability by anti-A. A 60 minute exposure reduced the anti-Rh activity to a titre of 2 (i.e. equivalent to the activity of the antiserum diluted with an equal volume of saline), and reduced the anti-A titre from 128 to 16. Nile blue was slightly less effective than brilliant cresyl blue. Heating (at 37° C.) intensified the action of the dyes while cooling (to 5° C.) decreased the effect. The inactive dyes were found to remain inactive at the above temperature limits.

The effect of temperature on the combination of Nile blue with the antigen is illustrated in Table 22.

Table 22

The Effect of Nile Blue at Two Temperatures on the A- and Rh-Agglutinogens

10 Mins. Exposure to 0.1% Nile Blue

Anti-A Serum Titre 256
Anti-Rh_o Serum Titre 64

Dilution of Antiserum	Agglutination at 5° C.		Control		Agglutination at 37° C.	
	A	Rh_o	A	Rh_o	A	Rh_o
1:2	4	3	4	3	2	0
1:4	4	3	4	3	2	0
1:8	4	2	4	3	2	0
1:16	3	2	4	3	1	0
1:32	3	1	3	2	0	0
1:64	3	0	3	1	0	0

Nile blue and brilliant cresyl blue are basic dyes of the following formulae:

Nile Blue $Cl(C_2H_5)_2N = C_6H_3(ON)C_{10}H_5.NH_2$

Brilliant Cresyl Blue $(C_2H_5)_2N(CH_3)\ C_6H_2(ON)C_6H_3.NH_2.Cl$

These dyes probably interfere with agglutination by reacting with acidic groups such as carboxyl and phenolic hydroxyl groups on the antigen. The interference effect was most marked at pH 7-8, where the carboxyl groups are almost entirely dissociated. The effect of a wide range

of H-ion concentration has not been studied. Acid or alkaline solutions below pH 4 and above pH 10 damage the agglutinogens.

6. Influence of acetylation on the reactivity of the agglutinogens of the cell

The effect of acetylation of the cell membrane with ketene was investigated in our study.

Red cells were suspended in molar acetate buffer of pH 5.6. Ketene gas was bubbled through the suspension. As the reaction proceeds the cells turn pinkish, then brick red. Ketene first abolishes the property of the cells to be agglutinated by specific antiserum, then produces weak nonspecific clumping, and finally, the cells undergo haemolysis. The results are consistent with our previous finding, namely, that the Rh agglutinability is destroyed much more rapidly than that of the A, B, M and N agglutinogens.

A typical experiment was carried out as follows: A rapid stream of ketene gas was passed for 1 minute through a red cell suspension in the acetate buffer. The buffering capacity of the medium was such that the pH was lowered only slightly. The cells were then washed with the acetate buffer and suspended for 30 minutes in isotonic phosphate buffer of pH 7.4 before adding the agglutinating serum.

Presumably the amino groups are preferentially acetylated and -COOH, -SH and phenolic-OH groups are little affected under these conditions. (See p. 21) The effect of acetylation on the agglutinating capacity of the cells is shown in Table 23.

Table 23

Effect of Acetylation

on the Agglutinability of the Cells

Agglutinating Serum	Degree of Clumping with Full Strength Antiserum	
	Control	Treated
Anti-A (titre 512)	4	3
Anti-B (titre 512)	4	2
Anti-N (titre 64)	4	2
Anti-M (titre 64)	4	2
Anti-Rh_o (titre 32)	4	0

The agglutinability with anti-Rh_o serum was abolished by acetylation. The clumps produced by the anti-A, -B, -M and -N sera were weakly held together and more easily dispersed than the clumps obtained with normal cells.

Summary

In this investigation an effort has been made to find out the nature of the antigen-antibody binding. The method of approach has been to try to mask the reactive chemical groups on the cell surface and study the effect on the agglutinability of the cell by specific antisera. It would appear from our findings that the attraction of antibody for antigen is due to electrostatic forces. Any agent which masks the charged groups in the antigen sites reduces the agglutinability of the cells with homologous agglutinins. It would appear probable that the electrostatic linkages involving amino and carboxyl groups play a major role in Rh agglutination whereas in the case of the other antigens the binding is more complex and consequently less labile.

The Arrangement of Agglutinogens on the Cell Membrane

Practically nothing is known about the distribution of the agglutinogens in the red cell. Landsteiner pictured them as being disposed in the form of a systematic pattern or mosaic. In an endeavour to obtain information concerning the disposition of cellular agglutinogen, Osborne (139), formerly of this laboratory, performed a large series of experiments in which red cells of type AMNRh were exposed to a concentration of one antibody that was just beyond the titre limit, i.e. that would not produce agglutination, e.g. anti-Rh_o. The cells subsequently were tested for agglutinability with another antibody, e.g. anti-A, anti-B, anti-M or anti-N. Although the interpretation of the data was difficult, the evidence from Osborne's experiments seemed to warrant the following conclusions:

(a) That partial sensitization of AMRh cells with very dilute anti-Rh does not interfere with subsequent agglutination by anti-A serum. In fact in some instances the presence of the Rh antibody on the cell surface facilitated the agglutination by anti-A. Partial sensitization with anti-M does interfere with subsequent agglutination with anti-A, i.e. the presence of anti-M on the cell surface tended to reduce the titre of the anti-A serum.

(b) That partial sensitization of group ABRh cells with dilute Rh also tends to reduce the agglutinability of the

cells by anti-A.

Some of these experiments have been repeated by the writer in the present study but without confirmation of Osborne's findings. In other words no evidence was obtained of any change in agglutinability as a result of initial mild sensitization of the cells. The results of a typical experiment are given in Tables 24 and 25. This work is more quantitative and closely controlled. It is likely that although Osborne used very dilute antiserum for sensitization, the sensitized cells aggregated in the second phase of the experiment.

Table 24

Influence of Anti-A Sensitization

	Test Serum	Titre Found
3% suspension of washed cells group ANRh	Anti-Rh_o (titre 64)	64
The same cells after sensitization with anti-A serum (titre 1024, diluted 1:1024 in normal saline) by exposure for 5 mins. at room temperature (27°C.)	Anti-Rh_o (titre 64)	64

Thus the anti-Rh agglutinability of the cells apparently was unaffected by previous sensitization with dilute anti-A.

Table 25

Influence of Anti-Rh_o Sensitization

	Test Serum	Titre Found
3% suspension of cells ANRh	Anti-A (titre 2048)	2048
The same cells after sensitization with anti-Rh_o serum (titre 64, diluted 1:64 with normal saline) by exposure for 45 mins. at 37° C.	Anti-A (titre 2048)	2048

The anti-A agglutination of the cells also appears to be unaffected by sensitization with anti-Rh_o.

It is surmised, however, that any change in agglutinability following exposure to a very dilute antiserum would be too small to be measured accurately by change in titre since measurements of titre may be subject to a 100% error.

It seems likely that either the agglutinogens occur in the cell as "strips", one antigen being adjacent to another, or that they are more or less evenly distributed throughout the cell stroma. If the first arrangement were true it might be expected that the attachment of an antibody to one antigen would interfere with the subsequent attachment of a second antibody, either by steric hindrance or by overlapping of the antibody molecule onto the adjacent antigen site. If this were so one would expect

that cells of group AB or MN would be especially liable to interference; in other words, the attachment of anti-A agglutinins to A sites on a cell of group AB, might interfere with the subsequent attachment of anti-B agglutinins. On the other hand if the second arrangement were true no interference of one antibody with another should take place.

Accordingly, several experiments were designed to endeavour to bring out these effects in a more clear-cut manner. Counting the free cells was considered to be a reliable method of detecting and measuring any interference with agglutination. The free-cell count of the red cells of one individual (e.g. group AM) was determined separately with anti-A and anti-M agglutinating sera. The counts were then compared with the count obtained in a similar experiment using a mixture of both antisera of the same concentration as previously used. It is reasonable to suppose that the results could be interpreted in the following manner: If one antibody should interfere with another in its attachment to antigen, the result would be equivalent to no change in the number of antigen sites and merely a doubling of the antibody concentration. If there was no interference the number of antigen sites would be in effect doubled. Thus there would be a greater chance for the cells to aggregate and consequently the free-cell count would be lower.

When red cells of group AN, AM, BM, BN, and MN, respectively were tested as described, in all cases there was a lower count when the cells were agglutinated with a mixture of the two antisera.

The results of agglutination of group AN cells with anti-A and anti-N, and the results of agglutinating MN cells with anti-M and anti-N are illustrated in Tables 26 and 27 respectively.

Table 26

A Comparison of the Free-Cell Counts Obtained With AN Cells and Anti-A (Titre 1024) and Anti-N (Titre 64) Sera, Separately and as a Mixture

(20 mins. shaking at 20° C. followed by centrifugation for 1 minute)

Agglutinating Serum	Dilution	Free-cell Count Obtained
Anti-A	1:16	13,450
Anti-N	1:4	24,210
Anti-A N	1:16, 1:4*	7,260

*Equal quantities of Anti-A serum (1:8) and anti-N serum (1:2) were mixed to give a mixed agglutinating serum.

It can be seen in Table 26 that there is no interference when a mixture of the two antisera is used

with AN cells. Thus the free-cell count with the mixed serum is lower than either of the other counts.

Table 27

A Comparison of the Free-Cell Counts Obtained With MN Cells, and Anti-M (Titre 64) and Anti-N (Titre 64) Sera Separately and as a Mixture

(20 mins. shaking at 20° C. followed by centrifugation for 1 minute)

Agglutinating Serum	Dilution	Free-Cell Count Observed
Anti-M	1:2	30,935
Anti-N	1:2	26,900
Anti-M N	1:2, 1:2	10,532

Again no interference was observed. It would appear therefore, that the M agglutinogen is not closely associated with the N agglutinogen.

Owing to the scarcity of group AB cells we have not yet tested the association of the A and B agglutinogens. Nor has the association of the Rh subgroups been tested by this method. We do not think that the relation of the Rh types with the groups A, B, M and N can be accurately estimated by this method, since the reactivity of the Rh agglutinins is much weaker.

In the present study Rh blocking sera were used to show this relationship. Since anti-D blocking serum

does not agglutinate D-positive cells in saline medium, it is possible to saturate the cells with the blocking antibody by exposing them for 45 minutes at 37° C. to potent undiluted serum. The effect of this saturation with blocking antibody on the agglutination by anti-A, -B, -M and -N then was tested. It is difficult, however, to obtain blocking sera which meet the desired requirements for this type of experiment. Preferably one should use a serum from an AB person (i.e. lacking α and β agglutinins) with a high titre of blocking antibodies, and free of saline-agglutinating Rh antibodies. Obviously, such sera are very rare. To use a more readily obtainable serum such as that of a group B individual and to attempt to neutralize exactly the alpha antibodies with Witebsky's A substance without overtitrating would be difficult. The presence of an excess of the latter substance would interfere with the final agglutination tests. However, by using blocking serum from a group-A person one can sensitize $ANRh_o$ or $AMRh_o$ cells. Likewise, blocking serum from a group-B individual can be used to sensitize cells of group $BMRh_o$ or $BNRh_o$. The effect of saturating the Rh agglutinogen with blocking antibody on the agglutinability of A, B, M and N cells may then be measured, either by counting the free cells or by comparing the degree of clumping obtained. It is thought, in this case, that after exposure of the cells to full-strength blocking

serum any interference with subsequent agglutination by anti-N, -M, -A or -B sera should be sufficient to affect the size of the clumps obtained. The experiments were done as follows: Packed washed red cells were drawn up to the 1.0 mark in a white-cell pipette and the latter then filled to the 11 mark with isotonic saline. The contents were transferred to a serological tube. A second tube as a control, was prepared in the same manner. To the first tube was added an equal quantity of blocking (anti-D) serum of the appropriate type. To the second tube was added an equal quantity of isotonic saline. Both samples were placed in the water bath at 37° C. for 45 minutes and occasionally shaken to keep the cells suspended. A few drops from both suspensions were then subsequently agglutinated in the usual way with anti-A and anti-M serum respectively. The results of an experiment with cells of group $AMRh_o$ which were sensitized with a blocking serum (titre 16) from a group-A individual and subsequently agglutinated with anti-A or anti-M serum are given in Table 28.

Table 28

The Effect of Blocking Anti-D Serum on the Subsequent Agglutination of $AMRh_o$ Cells

Agglutinating Serum	Dilution	Degree of Clumping Obtained: Cells Sensitized with Blocking Antibodies	Degree of Clumping Obtained: Control
Anti-A (titre 2048)	1:1	3	4
Anti-A (titre 2048)	1:16	1	3
Anti-M (titre 64)	1:5	2	3

It will be seen that the presence of Rh antibodies on the cells appears to interfere with the subsequent agglutination especially with anti-A serum. Washing the sensitized cells five times with saline restores their normal agglutinability to anti-M or -A as measured by the degree of aggregation obtained. This would indicate that either the attachment of the blocking antibody is weak or the interference is due to a nonspecific coating of the cells with serum protein.

Summary

Two methods have been devised for indicating the arrangement of agglutinogen in the red cell. One method consists of comparing the free-cell counts obtained using two different agglutinating sera, separately and together

as a mixture. It appears that the A, B, M and N agglutinogens are not closely associated.

The second method consists of exposing red cells to blocking anti-D serum and subsequently testing the agglutinability of the cells with anti-A, -B, -M or -N sera. Some interference was observed, especially with anti-A serum. This, however, needs further confirmation.

GENERAL DISCUSSION

In our previous discussion the term "viability" has frequently been mentioned. As used by various authors it is more of a convenience than an accurate term. It frequently is used to imply the capacity of red blood cells to remain in the circulation after transfusion. Strictly speaking, it implies the capacity of cells to live and perform their normal function. To what extent a red cell, particularly a non-nucleated cell as occurs in the majority of mammals, is a living organism, is a controversial question. To imply that certain red cells are nonviable simply because they are removed from the circulation is still more liable to error. It is generally assumed that the body has some way of differentiating between viable and nonviable cells and it is usually assumed that when the functioning of the red cell is impaired the body promptly destroys it. It has been shown, however, from work in our laboratory that the so-called nonviable cells can transfer oxygen and carbon dioxide in an apparently normal manner. By what standards the body can tell when cells are unacceptable is one of the major enigmas. In the case of preserved red cells it is well known that the osmotic changes occur as a result of glycolytic and other metabolic processes and give rise to the entrance of water with subsequent swelling of the cell. It has been shown that some of the

cells, after several days will break down even when mixed with normal plasma in vitro because of the sudden influx of water. There is evidence also that the chemical processes in these cells are not destroyed, yet the cells undergo haemolysis when introduced into the blood stream. This is an example of destruction of a cell that is still living, in other words, cells which retain a large measure of their function can be destroyed. These cells by the present test, i.e. survival after transfusion, are regarded as being nonviable. It is clear, therefore, that the term viability is used rather loosely. Much more work will have to be done on the chemistry of the red cell before this ambiguity can be cleared up.

It is not known whether the changes in the cell membrane itself are responsible for destruction of the cell after transfusion. When a fresh cell is exposed to hypotonic solution, it becomes spherical, the membrane breaks open in one or more places and the haemoglobin flows out through the breaks, leaving a stroma residue or "ghost". When the cell is returned to isotonic solution, the cell shrinks somewhat depending on the amount of residual haemoglobin, if any, remaining. A large measure of the normal agglutinability is still retained. On prolonged storage in vitro, the membranes of some of the cells at least appear to become consolidated and when haemolyzed the cell retains its shape. This is evident in 10-25% of the cells even after storage for 3-4 years.

In agglutination tests on preserved cells we have found that the normal agglutinability to anti-A, -B, -M and -N is retained long after many of the cells have become nonviable, i.e. they would be removed promptly if transfused. The Rh agglutinability, on the other hand, does suffer alteration with storage. We are inclined to think the changes in cell membrane during storage are not a very important factor in the impairment of viability.

Whether there is any difference between the sulphurated pigments formed in vivo with drugs and that in vitro with hydrogen sulphide, has not been definitely settled. The present writer inclines to the view that the sulphaemoglobin formed by the two processes is the same. If there were a difference, the absorption bands of the pigments certainly would give evidence of it. Assuming that the product is the same the difference in the cell survivals obtained by the two processes would appear to be due to conditions of sulphuration. This conclusion is supported by the improved cell survival obtained by decreasing the intensity and length of exposure in the in vitro method. It is hoped that further control of these conditions will give results comparable with sulphuration in vivo.

The greatly impaired oxygen-carrying capacity of the blood under heavy drug dosage and the increase in

the capacity after washing the cells with saline, suggests that some of the clinical conditions of cyanosis after administration of drugs, such as sulfonamides, may be caused by interference in oxygenation by metabolites in the circulation, rather than by the formation of inert pigments such as methaemoglobin. This could be tested easily by washing cells of these patients and testing whether the oxygen-carrying capacity is restored.

The results of the study give sufficient evidence that the approach and the method of study are productive and worthy of further research. The mechanism of agglutination is amenable to being clarified.

SUMMARY

The investigation discussed in this thesis arose out of studies carried on in this laboratory during the war, on the survival of preserved red blood cells after transfusion. The main object of the present study was to elucidate the mechanism of agglutination and the manner in which the agglutinogens are distributed on the cell. An effort was made also to find out why a considerable number of red cells fail to be agglutinated even with potent antiserum. These so-called "unagglutinable cells" constitute the "free-cell" count in the Ashby serological method for the estimation of transfused cells in the recipient's circulation. The accuracy of the Ashby method depends on the constancy of the free-cell count and the accuracy of this determination.

In the present study Jope's method using sulphaemoglobin-labelled cells for estimation of red cell survival was studied as an alternative to the Ashby method. It was discovered that sulphuration can be performed *in vivo* by the feeding of drugs, or *in vitro* by exposing cell suspensions to hydrogen sulphide, without altering the cell fragility or the agglutinating properties. The oxygen-carrying capacity of the cells is reduced by the amount of sulphaemoglobin formed and the remaining haemoglobin is capable of functioning in the normal manner.

The formation of sulphaemoglobin *in vivo*, under the influence of drugs, up to about 10% of the total cell pigment does not injure the viability of the cells. The life span of the rabbit erythrocyte by following the survival of these cells after transfusion is about 12 days. It was found that about 20 days are required for the complete elimination of the sulphaemoglobin from the cells after cessation of feeding drugs. The longer apparent survival of the sulphurated cells under these conditions is explained by the fact that sulphuration doubtless continues for a few days until the drugs are eliminated from the alimentary tract.

Sulphuration *in vitro* with hydrogen sulphide, on the other hand, does somewhat injure the viability of the cells. By improving the method of sulphuration it was possible to obtain a cell survival with fresh cells of about 8 days. The difference in the two modes of sulphuration is not yet clear.

Red cells sulphurated to a high level *in vitro* with hydrogen sulphide are readily distinguishable from normal cells under the microscope because of their larger size and pale greenish color. By labelling the cells in this manner it was possible to confirm Marrack's hypothesis, namely, that the second or aggregation stage of the agglutination reaction, like the first stage, i.e. the sensitization of the cells, is specific.

Sulphurated cells also were used with complete success for the determination of blood volume in the rabbit. A known quantity of the labelled cells was injected and allowing 10 minutes for distribution in the circulating blood, the sulphaemoglobin concentration was estimated spectographically.

An hypothesis designated the "saturation hypothesis" is advanced to explain the unagglutinable cell and other phenomena of the agglutination reaction. It is postulated that a few cells, when exposed to potent antiserum become completely saturated with antibody and thus are incapable of aggregating among themselves. They can, however, react and be agglutinated with cells which are not completely saturated. The unagglutinated cells, i.e. the completely saturated ones, constitute the so-called free cells. This has been confirmed by using sulphaemoglobin-labelled cells and by other techniques. The saturation hypothesis satisfactorily explains the following observations:

1. The free cell phenomenum.
2. The prozone phenomenum.
3. The variation of the free-cell count with the temperature, the count being increased at the higher temperatures.
4. The relation of avidity and titre.
5. The higher free-cell count with heterozygous cells.
6. The higher free-cell count with certain homozygous cells.

Experimental evidence has been provided to support the above anomalies.

The agglutinability of red cells has been studied after masking chemical groups of the agglutinogen. The Rh agglutinogen is the most labile to various treatments. From our findings it would appear that the attractive forces are electrostatic, with charged amino and carboxyl groups forming the major Rh bonding.

With reference to the agglutinogen distribution in the red cell the evidence obtained indicates that the A, B, M and N agglutinogens are far enough apart so that the attachment of one agglutinin does not interfere with the subsequent attachment of a different agglutinin.

CLAIMS TO ORIGINALITY

1. An hypothesis designated the "saturation hypothesis" has been developed to explain the unagglutinable-cell count and why it is impossible to obtain complete agglutination of cells with potent antisera.
2. The hypothesis also has been applied experimentally to explain five other hitherto obscure phenomena connected with agglutination.
3. It is shown that sulphuration of the haemoglobin of the red cell can be achieved without altering the agglutinability.
4. The use of sulphaemoglobin for visibly labelling red cells.
5. The application of sulphaemoglobin to demonstrate the specificity of the aggregation stage of the agglutination reaction.
6. The improvement of the technique for sulphurating red cells *in vitro*.
7. The demonstration of peculiar lability of the Rh agglutinogen.
8. The application of sulphaemoglobin for estimating blood volume.
9. The use of *in vitro* sulphurated cells in cell survival studies.
10. The development of techniques for studying the distribution of red cell agglutinogens.

CLAIMS TO ORIGINALITY (CONT'D)

11. Further elucidation of the nature of chemical groups in cellular antigens.

REFERENCES

1. Ashby, W. J. Exptl. Med. 28: 267, 1919.
2. Denstedt, O.F. Osborne, D.E. Stansfield, H. and Rochlin, I. Can. Med. Assoc. J. 48:477, 1943.
3. Osborne, D.E. and Denstedt, O.F. J. Clin. Invest. 26: 655, 1947.
4. Topley, W.W.C. and Wilson, G.S. The Principles of Bacteriology and Immunity, Edward Arnold, London, p. 137, 1936.
5. Tiselius, A. and Kabat, E.A. J. Exptl. Med. 69: 119, 1939.
6. Kabat, E.A. J. Immunol. 47: 513, 1943.
7. Enders, J.F. J. Clin. Investigation, 23: 510, 1944.
8. Cohn, E.J. Strong, L.E. Hughes, W.L. et al. J. Am. Chem. Soc. 68: 459, 1946.
9. Oncley, J.L. Melin, M. Richert, D.A. Cameron, J.W. and Gross, P.M. J. Am. Chem. Soc. 71: 541, 1949.
10. Smith, M.L. Brown, A.M. and Gross, C. Biochem. J. 26: 1473, 1932.
11. Calvery, H.O. J. Biol. Chem. 112: 167, 1935-36.
12. Heidelberger, M. Pedersen, K.O. and Tiselius, A. Nature, London, 138: 165, 1936.
13. Pauling, L. Endeavour, 7: 43, 1948.
14. Kabat, E.A. and Beyer, A.E. J. Exptl. Med. 82; 207, 1945.
15. Neurath, H. J. Am. Chem. Soc. 61: 1841, 1939.
16. Petermann, M.L. and Pappenheimer, A.M. J. Phy. Chem. 45: 1, 1941.
17. Schoenheimer, R. J. Biol. Chem. 144:545, 1942.
18. Madden, S.C. et al. J. Exptl. Med. 71: 283, 1940.
19. Sabin, F.R. J. Exptl. Med. 70: 67, 1939.
20. Doan, C.A. J. Lab. Clin. Med. 26: 89, 1940.
21. Madden, S.C. and Whipple, G.H. Physiol. Rev. 20: 194, 1940.

22. de Gara, P.F. and Angevine, D.M. J. Exptl. Med. 78: 27, 1943.

23. McMaster, P.D. and Hudack, S.S. J. Exptl. Med. 61: 783, 1935.

24. Harris, T.N. Grimm, E. Mertens, E. and Ehrich, W.E. J. Exptl. Med. 81: 73, 1945.

25. Dougherty, T.F. Chase, J.H. and White, A. Proc. Soc. Exptl. Biol. Med. 58: 135, 1945.

26. Kass, E.H. Science, 101: 337, 1945.

27. White, A. and Dougherty, T.F. Proc. Soc. Exptl. Biol. Med. 46: 26, 1944.

28. White, A. and Dougherty, T.F. Endocrinology, 36: 207, 1945.

29. Eisen, H.N. Mayer, M.M. Moore, D.H. Tarr, R.R. and Stoerk, H.C. Proc. Soc. Exptl. Biol. Med. 65: 301, 1947.

30. Kabat, E.A. Ann. Rev. Biochem. 15: 505, 1946.

31. Alexander, J. Protoplasma, 14: 296, 1931.

32. Mudd, S. J. Immunol. 23: 423, 1932.

33. Pauling, L. J. Am. Chem. Soc. 62: 2643, 1940.

34. Burnet, F.M. The Production of Antibodies, MacMillan, Melbourne, 1941.

35. Pauling, L. and Campbell, D.H. J. Exptl. Med. 76: 211, 1942.

36. Neurath, H. and Erickson, J.O. Science, 98: 284, 1943.

37. Rothen, A. Adv. in Prot. Chem. 2: 123, 1941.

38. Rothen, A. and Landsteiner, K. J. Exptl. Med. 76: 437, 1942.

39. Landsteiner, K. The Specificity of Serological Reactions, Harvard Univ. Press, Cambridge, 1945.

40. Marrack, J.R. The Chemistry of Antigens and Antibodies, Med. Research Council, London, Special Report Series, No. 230, 1938.

41. Neurath, H. Greenstein, J.P. Putnam, F.W. Erickson, J.O. Chem. Revs. 34: 157, 1944.

42. Herriott, R.M. Adv. in Prot. Chem. 3: 169, 1947.

43. Dubos, R.J. The Bacterial Cell, Harvard Univ. Press, Cambridge, 1945.

44. Boyd, W.C. Fundamentals of Immunology, Interscience Publishers, New York, 1947.

45. Boyd, W.C. J. of Allergy, 18: 125, 1947.

46. Morgan, W.T.J. Brit. Med. Bull. 2: 281, 1944.

47. Landsteiner, K. J. Exptl. Med. 75: 269, 1942.

48. Hooker, S.B. and Boyd, W.C. J. Immunol. 45, 127, 1942.

49. Olcott, H.S. and Fraenkel-Conrat, H. Chem. Rev. 41: 151, 1947.

50. Follensby, E.M. and Hooker, S.B. J. Immunol. 55: 205, 1947.

51. Lubinski, H.H. and Portnuff, J.C. J. Lab. Clin. Med. 32: 178, 1947.

52. Hubinont, P.O. Nature, London, 61: 642, 1948.

53. Fraenkel-Conrat, H. and Olcott, H.S. J. Am. Chem. Soc. 68: 34, 1946.

54. French, D. and Edsall, J.T. Adv. in Prot. Chem. 2: 277, 1945.

55. Li, C.H. Simpson, M.E. and Evans, H.M. Science, 90: 140, 1939.

56. Li, C.H. and Kalman, A. J. Am. Chem. Soc. 68: 285, 1946.

57. Herriott, R.M. and Northrop, J.H. J. Gen. Physiol. 18: 35, 1934.

58. Herriott, R.M. J. Gen. Physiol. 19: 283, 1935.

59. Jackson, R.W. and Cahill, W.M. J. Biol. Chem. 126: 37, 1938.

60. Desnuelle, P. and Rovery, M. Compt. rend. acad. sci. 224: 235, 1947.

61. Dameshek, W. N.Y. Acad. Sc. Annals, 48: 685, 1947.

62. Ponder, E. Hemolysis and Related Phenomena, New York, Greene and Stratton, 1948.

63. Calvin, M. Evans, R. Behrendt, V. and Calvin, G. Proc. Soc. Exper. Biol. Med. 61: 416, 1946.

3a. Morgan, W.T.J. Brit. Med. Bull. vol. 2, Nos. 8 & 9, 1944.

64. Kabat, E. Bendich, A. Beyer, A. and Beiser, S. J. Exptl. Med. 85: 685, 1947.

65. Bendich, A. Kabat, E.A. and Beyer, A. J. Am. Chem. Soc. 69: 2163, 1947.

66. Pauling, L. The Nature of the Chemical Bond and the Structure of Molecules and Crystals, Cornell Univ. Press, Ithaca, N.Y. 2nd ed. 1940.

67. Rice, O.R. Electronic Structure and Chemical Binding, McGraw-Hill Book Co. New York, 1940.

68. Rothen, A. Science, 102: 446, 1945.

69. Rothen, A. J. Biol. Chem. 163: 345, 1946.

70. London, F. Surface Chemistry, The American Association for the Advancement of Science 21: 141, 1943.

71. Breinl, F. and Haurowitz, F. Z. Physiol. Chem. 192: 45, 1930.

72. Alexander, J. Protoplasma 14: 296, 1931.

73. Mudd, S. J. Immunol. 23: 423, 1932.

74. Pauling, L. J. Am. Chem. Soc. 62: 2643, 1940.

75. Wiener, A.S. Am. J. Clin. Path. 15: 106, 1945.

76. Pressman, D. Bryden, J.H. and Pauling, L. J. Am. Chem. Soc. 70: 1352, 1948.

77. Bordet, J. Traite d l'immunite. Masson et Cie, Paris, 1920; 2ieme ed. 1939.

78. Marrack, J.R. The Chemistry of Antigens and Antibodies. Med. Research Council, London. Special Report Series, No. 230, 1938, Chap. 4.

79. Heidelberger, M. and Kabat, E.A. J. Exptl. Med. 67: 181, 1938.

80. Heidelberger, M. and Kendall, F.E. J. Exptl. Med. 61: 563, 1935.

81. Heidelberger, M. and Kendall, F.E. J. Exptl. Med. 62: 467, 1935.

82. Heidelberger, M. and Kendall, F.E. J. Exptl. Med. 62: 697, 1935.

83. Heidelberger, M. and Kendall, F.E. J. Exptl. Med. 64: 161, 1936.

84. Topley, W. Wilson, J. and Duncan, J.T. Brit. J. Exp. Path. 16: 116, 1935.

85. Wiener, A.S. and Herman, M. J. Immunol. 36: 255, 1939.

86. Pauling, L. Pressman, D. Campbell, D. Ikeda, C. and Ikawa, M. J. Am. Chem. Soc. 64: 2994, 1942.

87. Pauling, L. Pressman, D. Campbell, D. and Ikeda, C. J. Am. Chem. Soc. 64: 3003, 1942.

88. Pauling, L. Pressman, D. and Ikeda, C. J. Am. Chem. Soc. 64: 3010, 1942.

89. Pressman, D. Brown, D.H. and Pauling, L. J. Am. Chem. Soc. 64: 3015, 1942.

90. Pauling, L. Pressman, D. and Campbell, D. J. Am. Chem. Soc. 66: 330, 1944.

91. Pressman, D. Grossberg, A.L. Pence, L.H. and Pauling, L. J. Am. Chem. Soc. 68: 250, 1946.

92. Boyd, W.C. and Behnke, J. Science, 100: 13, 1944.

93. Abramson, H.A. Nature, 135: 995, 1935.

94. Hooker, S.B. and Boyd, W.C. J. Immunol. 33: 337, 1937.

95. Abramson, H.A. and Boyd, W.C. Journal of Bact. 50: 15, 1945.

96. Duncan, J.T. Brit. J. Exp. Path. 19: 328, 1938.

97. Teorell, T. J. Hyg. 44: 227, 1946.

98. Morales, M.F. Botts, J. and Hill, T.L. J. Amer. Chem. Soc. 70: 2339, 1948.

99. Heidelberger, M. Bact. Rev. 3: 49, 1939.

00. Boyd, W.C. Fundamentals of Immunology, pp. 268-274, Interscience Publishers, New York, 1947.

01. McKerns, K.W. and Denstedt, O.F. Can. J. Res. Sect. E, 27: 164, 1949.

02. Boyd, W.C. and Hooker, S.B. Proc. Soc. Exptl. Biol. Med. 39: 491, 1938.

03. de Kruif, P.H. and Northrop, J.H. J. Gen. Physiol. 5: 127, 1922.

04. Pressman, D. Swingle, S.M. Grossberg, A.L. and Pauling, L. J. Am. Chem. Soc. 66: 1731, 1944.

05. Kleinschmidt, W.J. and Boyer, P.D. Fed. Proc. 8: 213, 1949.

.06. Wiener, A.S. Blood Groups and Transfusion, Charles C. Thomas, Springfield, Illinois, 1948.

.07. Stitt, E.R. et al Practical Bacteriology, Hematology and Parasitology, Phila. Blakiston, 1948.

.08. Wiener, A.S. Laboratory Digest, May, 1949.

.09. Mollison, P.L. Mourant, A.E. and Race, R.R. Med. Res. Coun. Mem. No. 19, 1948. His Majesty's Stationery Office, London.

.10. Hill, J.M. and Dameshek, W. Rh Factor in the Clinic and the Laboratory, N.Y. Grune, 1948.

.11. Race, R.R. International Society of Hematology, Buffalo, 1948.

.12. Thomsen, O. Acta. Soc. Medic. Fenn. Ser. A, No. 9. 15: 1, 1932.

.13. Landsteiner, K. and Wiener, A.S. Proc. Soc. Exptl. Biol. Med. 43: 223, 1940.

.14. Levine, P. and Stetson, R. J.A.M.A. 113: 126, 1939.

.15. Fisher, R.A. and Race, R.R. Nature, 157: 48, 1946.

.16. Wiener, A.S. Am. J. Clin. Path. 16: 477, 1946.

.17. Callender, S. and Race, R.R. Ann. Eugen. Camb. 13: 102, 1946.

.18. Race, R.R. Sanger, R. and Lawler, S.D. Nature, London, 161: 316, 1948.

.19. Stratton, F. Nature, London, 158: 25, 1946.

.20. Cappell, D.F. Brit. Med. Jour. 2: 601, 1946.

.21. Race, R.R. Nature, London, 153: 771, 1944.

.22. Wiener, A.S. Proc. Soc. Exptl. Biol. Med. 56: 173, 1944.

.23. Diamond, L.K. and Denton, R.L. J. Lab. Clin. Med. 30: 821, 1945.

.24. Granick, S. Blood, 4: 404, 1949.

.25. Michel, H.O. J. Biol. Chem. 126: 323, 1938.

.26. Fairley, N.H. Quart. J. Med. 10: 95, 1941.

.27. Keilin, D. Proc. Roy. Soc. Lond. B, 113: 393, 1933.

128. Haurowitz, F. Z. Physiol. Chem. 151: 130, 1926.

129. Jope, E.M. Brit. J. Indus. Med. 3: 136, 1946.

130. Haurowitz, F. J. Biol. Chem. 137: 771, 1940.

131. Drabkin, D.L. and Austin, J.H. J. Biol. Chem. 112: 51, 1935.

132. Lemberg, R. Holden, H.F. Legge, J.W. and Lockwood, W.H. Austral. J. Exptl. Biol. Med. Sci. 20: 161, 1942.

133. Jope, E.M. BSc. Thesis, Oxford, 1941.

134. Stern, K., Seminar, Dept. of Phy. Chem. Harvard Univ. 1948.

135. Evelyn, K.A. and Malloy, H.T. J. Biol. Chem. 126: 655, 1938.

136. Peters and Van Slyke, Quan. Clin. Chem. Vol. 2

137. Rapoport, S. J. Clin. Invest. 26: 591, 1947.

138. Waugh, T.R. and Asherman, E.G. J. Lab. Clin. Med. 23: 746, 1938.

139. Osborne, D.E. and Denstedt, O.F. Unpublished data.