ENZYMATIC AND NONENZYMATIC ALTERATIONS OF ERYTHROCYTE SURFACE ANTIGENS

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Introduction 191
Surface of Intact Erythrocytes 194
Morphology and General Chemistry of Erythrocyte Membrane 194
Immunological and Immunochemical Considerations 196
Antigenic Changes Resulting from Enzymatic Action on Erythrocytes 201
Origin and Nature of Enzymes 201
Action of Enzymes 202
Uncovering of "hidden" receptors not reactive with agglutinins prior to treatment 202
Saline agglutination by "coating" antibodies and increase of pre-existing agglutinability 203
Decrease or abolition of agglutinability 204
Action of enzymes on virus receptors of red cells 206
Hemolysis due to enzymes 206
Enzymes in structural chemical analysis of erythrocyte surface antigens 208
Immunological Alteration of Erythrocyte Surface by Agents Other Than Enzymes 210
Changes Caused by Substances Not Antigenic Per Se 211
Silicic acid 211
Periodate 211
Tannic acid 211
Formaldehyde 212
Heavy metals and salts 212
Detergents and saponins 213
Changes Due to Fixation of Antigenic Substances 214
Antibodies and antibodylike substances 214
Bacterial antigens 214
Significance of Enzymatic Research on Erythrocyte Surfaces for Clinical Medicine 216
Hemolytic Anemias 216
Therapeutic and Diagnostic Aspects of Enzyme Treatment of Erythrocyte Surfaces 219
Outlook 219
Literature Cited 220

Introduction

Cell surfaces are of great interest to the physical-chemical and biological sciences. The use of enzymes and of reagents of known specificity and mode of action in the study of cell surfaces presents an indirect approach for obtaining evidence as to the nature of surface structures and specific receptor sites. Under appropriate conditions, antibodies are sensitive indicators of changes on macromolecular surfaces brought about by enzymes.

Much successful investigative effort has recently been spent on bacterial surfaces, walls, and membranes (143), and the classical older work on cell surfaces of higher plants has been fundamental to our concepts of living organisms in general. In contrast, with the exception of the extensive studies of transport across membranes and the recognition of their semipermeable selective nature, little appears to be established regarding the nature of mammalian cell surfaces.

Erythrocytes and their surfaces are relatively amenable to experimental manipulation. Therefore, they may serve as models, and, to a degree, results obtained from observations on them may be extrapolated to other more complete cells of animals. That erythrocytes share antigenic receptors with other cells was shown as early as

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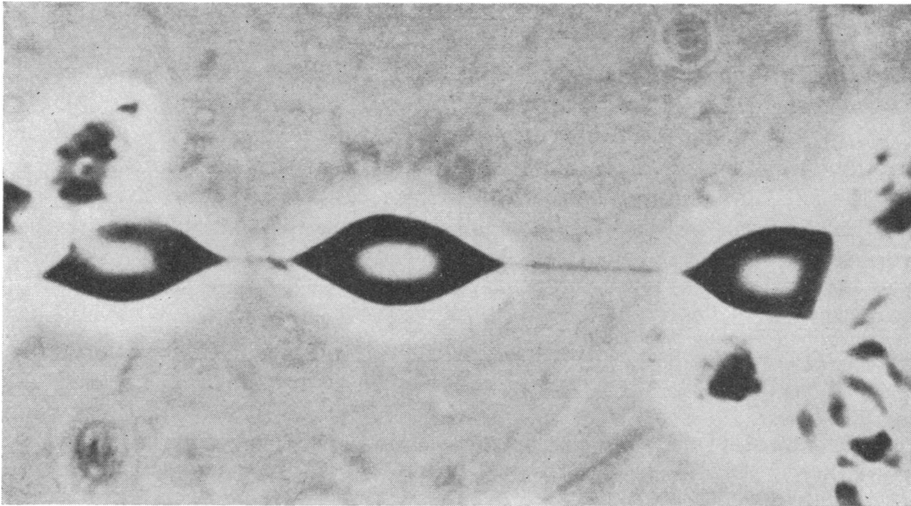

FIG. 1. *Agglutination of erythrocytes by an antiserum. Note spindle and thread formation during separation of cells. Phase contrast, ca. 1,500 ×. After Bessis (149).*

1900 by von Dungern (36), and it is now well-established that certain of the blood group specific receptors are common to erythrocytes and other cells. Similarly, pharmacologically important receptors, such as those for tetanolysin, are common to erythrocytes and to other cells (38). Thus, one may profitably study induced alterations of red-cell surfaces and hope to obtain significant information on the structure and function of animal-cell surface components in general.

Erythrocytes and their membranes will be considered in relation to their antigenic structures. This dimension of their character, which is measurable by antibodies, permits determination of chemical and physical properties, and it should be possible to assess the influence of individual specific receptors on them. Classical antigen-antibody interactions are surface reactions. Antibodies react with specific chemical structures and, therefore, are, to some degree, analytical chemical reagents as well as tools of the physical chemist. Antibodies may or may not grossly deform the erythrocytes with which they react. This effect is markedly influenced by "sphering" and "antisphering" factors in the plasma. Figure 1 demonstrates the physical effect agglutinating antibodies may have on erythrocytes.

In some respects, there is close similarity between antigen-antibody and enzyme-substrate reactions. In both instances, the first step is a stereochemically selective adsorption of the reacting partners, a combination which takes place almost instantaneously (90).

From what is known today, it may be stated in a simplified way that three groups of forces operate in antigen-antibody reactions after the spatial requirements have been met (90, 158): electrostatic interaction between charged groups, hydrogen bonding, and electronic van der Waals attraction.

Enzymatic and other studies on erythrocytes are reported here predominantly from an immunological point of view, although an investigation of antigenic alteration of red-cell surfaces appears to hold considerable promise for fields other than immunology. Information can be obtained by enzymatic action either on the antigen itself or on its immediate environment on the cell surface. Thus, investigation of an enzyme-substrate reaction and of the reaction products will not only help unravel the structure of the erythrocyte membrane but also may simultaneously give a clue as to groupings involved in antigenic specificity.

As often happens in an area of research, some basic knowledge of enzyme action on erythrocytes existed for many years (44) without receiving much attention. The last two decades, however, brought rapid progress in two seemingly unrelated fields: in virology (61) the action of virus enzyme on erythrocytes was clearly demon-

strated, and in blood grouping practical considerations led to renewed and concerted efforts to study and understand the action of enzymes on erythrocytes and the concomitant changes on their surfaces.

Thus, the discovery of the Rhesus factor and the subsequent great interest in "coating" antibodies, i.e., antibodies which merely cover but do not agglutinate erythrocytes suspended in physiological saline, led to attempts to make their detection easier. One of the most successful but least understood procedures for doing this involves the pretreatment of erythrocytes with enzymes. It is usually stated without experimental evidence that the observed effect on the erythrocyte surfaces is due directly to the proteolytic effect of the enzymes employed. It is by no means established that the effect of even crystalline so-called proteolytic enzymes in the majority of cases is due solely to split peptide bonds. For example, the ability of crystallized trypsin and papain to hydrolyze ester linkages in addition to peptide bonds is well-known (105).

The finding that enzyme-treated erythrocytes become agglutinable in ordinary saline medium by coating (blocking) antibodies directed against their surface antigens turned attention from the concept of "incompleteness" of antibody to some structural feature of the surface of the erythrocyte, be it the antigen or the surface area surrounding it, whose alteration leads to agglutinability of the red cell. The two most obvious explanations for increased agglutinability of enzyme-treated erythrocytes by antibodies are the following.

(i) Enzymes render erythrocytes agglutinable by coating antibodies because of the uncovering of additional receptors on or below the erythrocyte surface. The removal of blocking structures from the erythrocyte surface gives the combining sites on the antibody a better chance to unite with complementary antigen structures. Therefore, even the so-called blocking or "incomplete" antibodies would be bi- or multivalent. Coffin and Pickles (24), whose experiments support such an explanation, inactivated erythrocyte Rh_o (D) and S antigens by mild periodate oxidation, and partially restored their specific activity by treatment with crystalline trypsin. The combining groups uncovered by trypsin were visualized as being located at a greater distance below the cell surface than the primary cell receptors. Race, Sanger, and Selwyn (136), who discovered individuals with erythrocytes of the type D--/D-- ($\overline{\overline{Rh}}_o/\overline{\overline{Rh}}_o$), reported "the curious ability of these cells to be agglutinated in saline by incomplete anti-D." This suggested to them "that the incomplete antibody is not monovalent but rather that some hindrance to agglutination in saline by incomplete anti-D is removed when all C and E antigens are absent from the cells." This interpretation has been criticized by some investigators (180).

(ii) The agglutinability of erythrocytes by coating antibodies after enzyme treatment may be due predominantly to a change in the electrostatic bonding forces (of second and third order) on the erythrocyte surface and a subsequent decrease in hydration of the red blood cell. The strong negative charge of ordinary erythrocytes is well-known (53), as is the release of sialic acid from erythrocytes by influenza viruses (80, 156, 67, 153), papain, bromelin, ficin (156), and trypsin (82). The release of this strong organic acid is accompanied by a very large decrease in electrophoretic mobility (53) [except in horse, pig, and ox erythrocytes treated with proteases (150)]. The possibility of a change in electrostatic bonding forces on or in the vicinity of the antigenic site and the ensuing decrease in inter-erythrocyte repulsion and facilitation of agglutination apparently have not been considered sufficiently.

Both explanations, however, fail to account for the fact that a viscous hydrophilic environment *per se*, even if not charged significantly, such as a dextran solution, may have an effect similar to enzymatic treatment in that erythrocytes become agglutinable by coating antibodies in the viscous medium.

It will be difficult in the ensuing discussion to establish in each instance which of the factors mentioned play a role and whether others of importance have not been considered. Furthermore, in many cases, results are influenced, even if other experimental conditions are kept grossly constant, by such parameters as purity of enzyme preparation (112), degree of hemolysis and polyagglutinability caused by the enzyme, source and nature of available antiserum, and, occasionally, source of supposedly closely similar erythrocytes (27). Also, it is known that only a certain proportion of persons of the same blood group, given the same antigenic stimulus, will manu-

facture blood group antibodies which can be inhibited by oligosaccharides (73; Springer and Readler, *unpublished data*).

Not only structural features are of importance in studying enzyme action on the erythrocyte surface, but also the variability in the number of given combining sites for different erythrocyte antigens must be considered. It was calculated, for example, by Boursnell, Coombs, and Rizk (12) that there exist 500,000 heterophile combining sites per bovine erythrocyte, whereas only about 5,500 Rh_o (D) sites are present per A, B, CDe/CDe(R_1R_1) human erythrocyte. By using purified I^{131}-labeled isoantibody, Masouredis (100) found cells homozygous for Rh_o (D) to possess 1.6 times more Rh_o (D)-reacting antigen sites than heterozygous cells. These values were about 30% lower in rh′ (C) than in hr′ (c) cells, indicating an interference of rh′ (C) antigen on the erythrocyte's Rh_o (D) content.

Hoyt and Zwicker (68) showed how carefully one must interpret results in this area of investigation. Human serum contains substances inhibitory to trypsin; according to Hoyt and Zwicker's experiments, it seems quite possible that the clumping of trypsinized cells by a serum may be due to a reaction between its trypsin inhibitor and the trypsin adherent to the cells. Thus, trypsin appears to have a double action on erythrocytes: (i) antigen-modifying, and (ii) sensitizing (coating). This coating effect is likely to be of importance for other enzymes and different substances as well.

Furthermore, polyagglutinability effects ascribed to the action of microbial enzymes might, in some instances, be due to coating of erythrocytes with microbes or their nonenzymatic constituents and subsequent reaction with homologous or cross-reacting antibodies against such products (155, 176).

This introduction indicates the complexity of the subject to be discussed. In view of this complexity, the reviewer intends to do no more than take stock of the facts which have emerged and try to evaluate them with an eye for some points of departure for further experimental attempts which may increase the very fragmentary understanding of the structure of erythrocyte surfaces and the action of enzymes upon them. In doing so, the discussion will be confined to the more basic aspects of enzymatic and nonenzymatic alteration of erythrocyte surfaces. It is not the task of this report to dwell on the importance of enzyme techniques in the routine investigation of erythrocyte-antibody reactions. It should be mentioned, however, that an enormous practical advance in many procedures concerned with the entire field of routine blood grouping in its widest sense (140, 178) has been achieved since the original description by Pickles (129) of the effect of cholera culture filtrate on "incomplete" anti-Rh antibodies.

SURFACE OF INTACT ERYTHROCYTES

Morphology and General Chemistry of Erythrocyte Membrane

The surface configuration of non-nucleated erythrocytes is easily changeable, as erythrocytes are highly flexible; although their normal shape is biconcave (in mammals usually discs but ovals in llama and camel), they may assume spherical or "thorn-apple" forms. Detailed descriptions of the gross appearance of erythrocytes may be found in many textbooks (132). Our understanding of the morphology of erythrocyte membranes (which may be equated with their surfaces) has been much advanced by Wolpers' electron microscopy (185). Apparently the erythrocyte membrane is a balloonlike structure filled with hemoglobin (58, 186, 186*a*), although others like Bessis (149, p. 9–28) think that the cytoplasm (stroma) condenses at the periphery and decreases slowly towards the center. The thickness of the erythrocyte membrane, carefully and gently prepared by successive hemolyses, is approximately 50 A (80), whereas simple osmotic hemolysis leaves a membrane 150 A thick; this disparity is most likely due to attached hemoglobin (186). Wolpers (185) thinks that the structural elements of the erythrocyte membrane are a micellar protein-scaffolding which is filled with lipids.

Hillier and Hoffman (58) investigated by electron microscopy the orientation of membrane components at the molecular level, attaining resolution down to 20 A. Ghosts of erythrocytes of the human male show a surface covered with cylindrical structures approximately 30 A high with a diameter of 200 A (Fig. 2). These "plaques" appear to be arranged at random. In addition to the plaques, these authors found a fibrous component, the fibers having a diameter of the order of 20 A and a length of about 200 A. These observations were in agreement with the

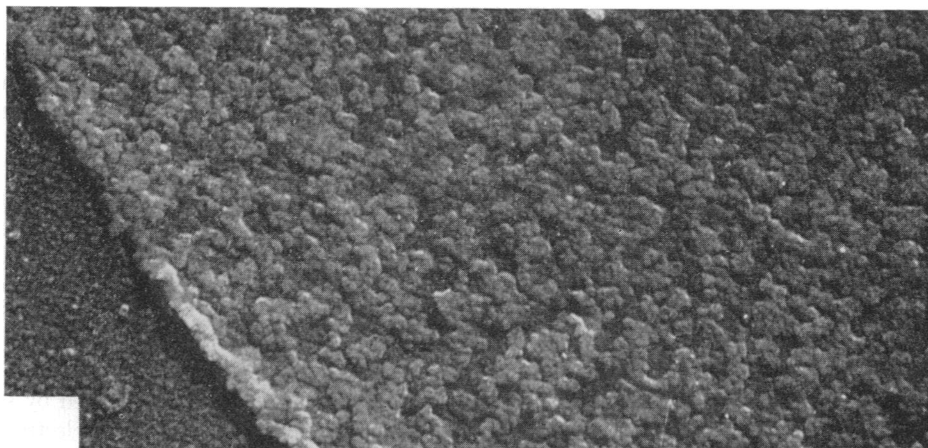

FIG. 2. *Air-dried human erythrocyte ghost. Shadowed, treated with phosphotungstic acid (pH 7.4). 75,000 ×. After Hillier and Hoffman (58).*

earlier qualitative findings of Wolpers. Lipid solvents removed the plaques from the ghost membranes (stroma) without, as judged by electron microscopy, changing their structure. Apparently, plaques are insoluble in hydrophilic as well as lipophilic solvents, but phospholipid material fixes them to the fibrous cell membrane.

Hillier and Hoffman (58) observed positive birefringence of the whole erythrocyte and of the erythrocyte membrane, which is indicative of a high degree of tangential orientation of the surface molecules.

Thus, two levels of organization in the ghosts have been demonstrated: fibers and plaques. The fibers are oriented tangentially to the surface and appear to cover the major part of the exterior surface. The hydrophilic ends of the presumed phospholipid layer would be attached to the fibrous protein layer. This would give rise to a structure, consisting of plaques located on the outside of a fibrous network, which is joined together by ether-extractable lipids. A minor part of the lipid would be radially oriented.

The total number of plaques per human cell was calculated to be about 10^4 to 10^5, and the number of pores separating the plaques was assumed to be equal to or double their number. The pores, which are lined with phospholipids, have been calculated repeatedly to have a minimal size of 4.5 to 35 A. Such a structure was believed to be present also in the intact wet ghost. Hillier and Hoffman made similar findings on red-cell membranes from rabbits, rats, and groundhogs. Interestingly, the mean plaque size varied from one human to the next much more than the range of variations within numerous samples from a single individual. The significance of these variations is obscure; the authors considered a possible association with an individual's blood groups. In general, besides variation in size and shape of the entire erythrocyte, no morphological difference is discernible, even with an electron microscope, between red-cell surfaces of healthy individuals and of those suffering from a blood disease. In thalassemia major, however, a ghost texture different from that of ordinary erythrocytes was found (62).

Obviously, the information obtained on the structure of the dried ghost even with various independent treatments can be applied only with reservation to intact erythrocytes.

Moskowitz, Dandliker, Calvin, and Evans (115) isolated from human stroma a lipoprotein, elinin (most likely identical with the plaques of Hillier and Hoffman), which was characterized by the method of isolation. Blood group A, B, and Rh activity was found to reside in this "elinin" fraction. Electron micrographs showed elinin to be rods of a length between 2,500 and 10,000 A (average, 3,000), with the narrowest rods being approximately 125 A wide (30). The particle weight of elinin was found to be about 40 million, and it was stated to be a lipid-carbohydrate-protein complex. Calvin and his coworkers described an additional fraction, S protein, which was devoid of A, B, and Rh activity (115). These workers proposed a model of the erythrocyte membrane composed of elinin units

parallel to the cell surface and linked together with ether-extractable lipids. S protein and some hemoglobin was thought to be associated with this structure. However, Hillier and Hoffman's observations (58) are only partly compatible with the structural arrangements proposed by Moskowitz and Calvin, the difference being mainly in regard to the orientation of ether-extractable structures.

Chemical information on the nature of the structural elements composing the erythrocyte membrane is of rather recent date. The problem is being approached from two different angles after complete chemical analyses of whole stroma (132, p. 119–127) where free cholesterol was recognized early.

On the one hand, workers have tried with some success to isolate biologically specific erythrocyte surface determinants [whose surprisingly large number was pointed out at an early date (38)], such as blood group agglutinogens and virus receptors in active and pure form, and then to study their chemical composition, especially the groupings responsible for their activity. On the other hand, attempts are being made to extract erythrocyte membrane components and to determine their chemical nature, without, in the beginning at least, any regard to their biological function. Thus, Klenk and his collaborators isolated glycolipids and gangliosides from human erythrocytes (79), in particular a carbohydrate-rich lipid composed of fatty acids (mainly lignoceric acid) 20%, sphingosine 20%, galactose, glucose, and galactosamine. This material was also isolated by Yamakawa and his colleagues who called it globoside and considered it to represent the erythrocytes A and B substances in spite of its low activity (187). Yamakawa, Yokoyama, and Handa consider globoside to be the main mucolipid of human erythrocytes and recently reported its structure as *N*-acetylgalactosaminoyl-β-1 $\rightarrow$ 6 galactosyl 1 $\rightarrow$ 4 galactosyl 1 $\rightarrow$ 4 glucosyl-nervonyl-sphingosine (188*b*). Sialic acid was also demonstrated as a component of erythrocyte stroma (80, 188*a*). Compounds similar to those isolated from human erythrocytes have also been obtained from the stroma of horse and ox red blood cells (141*a*, 187, 188, 170*a*). Cattle, sheep, pig, and horse erythrocytes contain *N*-glycolyl-neuraminic acid (81), whereas human erythrocytes appear to possess acetyl-neuraminic acid only. Red blood cells of man and dog contain considerable amounts of ethanolamine and phosphatidyl serine (6). An average human erythrocyte has 21 to 25% of its phospholipid in a free state. Since the phospholipid is easily removed, it must be situated at the surface of the cell membrane (139).

It was also attempted to correlate chemical structures with the strong electronegativity of erythrocyte surfaces (45). Furchgott and Ponder, in commenting on the very low isoelectric point of human erythrocytes (pH 1.7, μ = 0.172) and the higher ones of stroma (pH 4.7) and of erythrocyte lipid (pH 2.6), concluded that the surface of human erythrocytes is dominated by strongly acidic groups effective at the electrophoretic plane of shear and considered that these may be the phosphoric acid groups of cephalin and related molecules. It is now known, however, that the strong electronegativity of erythrocytes is predominantly due to the terminally located strong organic acid, sialic acid. After removal of much sialic acid by influenza viruses and the complete elution of virus from the erythrocytes (see below), this strong electronegativity decreases, and the isoelectric point moves in the direction of neutrality (53).

Immunological and Immunochemical Considerations

Most or all agglutinogens are developed early in embryonic life. It is long established (76, 164) that human erythrocyte A, B, and H (O) agglutinogens are demonstrable in small fetuses (e.g., 1.8 cm long, 37 days old). The A and B agglutinogens increase quantitatively during fetal life and up to the age of 16 to 20 years. No variation of these antigens was demonstrable in given healthy individuals 20 to 100 years old.

Red-cell surfaces are characterized by multiple genetically determined agglutinogens, at least one of which is species specific. At least ten independent human blood group systems involving over 50 serologically distinct agglutinogens and many more factors have been identified on the surface of human erythrocytes by means of the corresponding antibodies (135). All these factors exhibit a complex serological behavior, in general as a mosaic of substructures characterizing the larger agglutinogen. Studies on blood group chimeras and on transfused erythrocytes showed that blood group agglutinogens are an integral part of the erythrocyte surface. This is true for

all known human blood groups except those of the Lewis system (40); in this system, antigens on the red cells are acquired by adsorption from the plasma. Similar adsorption of the host's own antigens from the plasma onto erythrocytes has been found in cattle (162) and in sheep (120).

Immunological relationships between erythrocyte surface receptors of various animal species have been studied since the beginning of this century (e.g., 39) when Ehrlich and Morgenroth noted that ox, goat, and sheep erythrocytes share certain receptors but not others.

Other receptor sites on erythrocyte surfaces have been revealed by studies of myxovirus hemagglutination (60). Although a considerable range of viruses other than the myxoviruses agglutinate erythrocytes, very little is known about the nature of their receptor on the erythrocyte surface.

Unknown also are the location and true nature of the erythrocyte receptor involved in the "immune adherence phenomenon" (121), in which primate red cells play an important role as opsonizing agents in fixing bacteria sensitized with specific antibodies in the presence of complement. It is also likely that rather specific erythrocyte receptors are present for certain bacterial hemagglutinins, such as the transient one elaborated by *Bordetella pertussis* [in which the agglutinin appears to be related to virulence (77)] and for numerous toxins and poisons, mushroom extracts, and proteins of higher plants (86, 13). Although most of these agglutinins react with receptors whose specificity is unknown, it has nevertheless been established that a number of plant agglutinins, e.g., from potato tuber (98) and notably from leguminous plants, may differentiate between the red cells of various animal species, and some of them even recognize and react with structures responsible for blood group A, B, H (O), M, and N specificity (13, 109). The usefulness (174*a*) and limitations (158*a*) of these plant reagents in structural chemical analysis of blood group active substances have recently been discussed. Specific inhibition of erythrocyte agglutinins has been one of the most informative methods in the elucidation of biologically specific erythrocyte structures. Its usefulness as an aid in the study of red-cell receptors has already been pointed out by Ehrlich (38). One of the early precise studies is that by Fisher (43*a*) who showed it to be likely that the pertussis hemagglutinin has structural requirements for its fixation to the erythrocyte surface closely similar to those of digitonin (see below).

It had become clear by 1951, owing to the studies of Cohn and his group (25), that the component carbohydrates of blood group substances A and B inhibited isoagglutinin action on their specific erythrocyte receptors. Soon afterward, Morgan and Watkins (109) and Kabat and his associates (73) showed which sugars were involved as the terminal determining structures of A, B, H (O), and Le^a specificities. Results obtained with this so-called hapten inhibition technique (73, 152) were in general agreement with those obtained by enzymatic procedures. It has been shown, however, that the hapten inhibition technique, at least with heterologous reagents, may on occasion give only partially correct or even misleading results (158, 158*a*).

The observations on the Lewis blood group in man and the J factor in cattle, namely, that host antigens are readily adsorbed onto red cells (49, 162) and that the Lewis substances may be removed from the erythrocyte surface by washing with physiological solutions, must be considered when the antigenic make-up of erythrocyte surfaces is studied, as must be the loss of blood group antigens (real or apparent) innate to red cells under severe disease conditions (171). The reverse must also be borne in mind; that is, substances may be adsorbed permanently onto the erythrocyte surface although they are not normally a part of it. In this category are body constituents such as antibodies, including those of the incomplete variety, and complement components. They will not be removed by the standard procedures used to wash erythrocytes. Beyond this, it is quite conceivable that substances may be adsorbed onto red blood cells in vivo and that this ability to adsorb substances like toxins may give the erythrocytes an important defensive immunological function (35). Erythrocytes, therefore, may be coated with materials alien to the individual from which the red cells were obtained. These substances may even be blood-group active (138*a*, 155, 163), and they may be sensitized with their individually specific antibodies.

Renton and Hancock (138*a*) found that group O red cells can acquire small amounts of A or B substance when in the circulation of A or B recipients. The uptake became maximal after about 2 weeks. Other foreign substances, such as

acidic polysaccharides of bacterial origin, may coat erythrocytes and reduce substantially their agglutinability. Such an inhibition by an acidic polymer was shown conclusively by Keogh, North, and Warburton for virus hemagglutination (78) and later for passive bacterial hemagglutination by Spaun (151) and for blood group agglutination by Ceppellini and DeGregorio (22*a*). This inhibition by strongly acid polysaccharides such as Vi antigen and colominic acid is explainable by an increase in surface charge and concomitant repulsion of the coated erythrocytes from one another, although Neter considers the "steric hindrance" by the Vi antigen as a possible factor (123).

Attempts to isolate biologically specific receptors from erythrocytes date to the early days of blood group research. Characterization of such receptors would provide information on structure and give indications as to functions of some components of the red-cell envelope.

It is obviously of great importance to know the number of specific receptor sites per red cell, since success in the chemical elucidation of the nature of these receptors with present-day methods depends on their sufficient quantity. The surface area of the human erythrocyte has been calculated to be about 1.63×10^{-6} cm^2. Perhaps the earliest method to determine the relative numbers of receptors per erythrocyte was devised by Ehrlich in 1901 (38). He determined the ability of erythrocytes to take up a defined number of amboceptors, or a multiple thereof. Wiener and Gordon (179) estimated the relative number of antigenic sites on the human erythrocyte surface to be about ten times as many Rh_o (D) sites as Fy^a agglutinogens, while there were twice as many K as Fy^a agglutinogens. The number of antigenic sites per red cell is under genetic control, and is characteristic for a given person (cf 135). This varying amount of antigen on the erythrocyte surface permits a decision for some blood group systems as to whether an individual is homozygous or heterozygous for a blood group characteristic, as was first noted by Landsteiner and Levine for the M, N system (89). Hughes-Jones, Gardner, and Telford (69*a*) arrived at a value of 65,000 c (hr′) sites per red cell homozygous for this antigen; other calculations of the absolute number per erythrocyte of antigenic sites of the Rh_o (D) and mononucleosis receptors have already been cited (12, 100). Hanig (53), on the basis of his electrokinetic studies, concluded that under optimal conditions only one-eightieth of the human erythrocyte surface is covered by PR8 influenza virus. This corresponds to about 300 virus particles per erythrocyte. Later studies employing different methods are in general agreement with Hanig's observation. Coupling erythrocytes with haptenic groups has shown that as few as about 60 arsonate groups of the agent fixed to the red cells, i.e., about 0.02% of the erythrocyte surface, sufficed for agglutination (133). Similarly, Heidelberger and Mayer found that the optimally sensitized sheep erythrocyte contains about 1,000 antibody molecules (56). These occupy $<0.3\%$ of the cell surface. Sensitization of red cells with antibodies leads to increased destructibility even in the absence of complement.

That serological analysis of antigens on the erythrocyte surface may yield fundamental information on the genetic mechanisms controlling basic structures of the erythrocyte membranes has recently been indicated by Levine et al. (94) for the unaltered basic antigenic Rh substance common to all human and many monkey blood cells. This basic substance appears to have evolved in man by mutations into the complex Rh-Hr situation as it exists today.

In these analyses, somatic variations of a permanent nature arising from admixture of erythropoietic tissue (126) or somatic mutations (e.g., 5), as, for example, the presence of a low frequency of inagglutinable cells in a population composed predominantly of agglutinable cells, may also have to be taken into account. Recent studies have even shown that the presence of a red-cell agglutinogen in certain chickens is the function of the genotype *and* of a hormonal factor extrinsic to the red cells (144).

The first observations pertaining to the physical and chemical nature of the human erythrocyte A and B agglutinogens were those of Schiff and Adelsberger (146), Landsteiner, van der Scheer, and Witt (91), and Witebsky (182), namely, that they could be extracted from the red cells with ethanol. These substances were characterized by their mode of extraction as being lipid in nature. However, there is little to indicate that the serological specificity of the blood group A, B, and O agglutinogens is due to the lipid part of the extracted molecule. Schiff

demonstrated the carbohydrate nature of the blood group specific groupings, when he saponified the lipid part of the extracted A substance without destroying the blood group A activity (145). Hallauer (52) extracted A, B, and O active substances from human erythrocytes and stroma. These materials behaved as water-soluble haptens, and were believed to be of cerebroside-like nature with additional carbohydrates. Kossjakov (85) extracted human erythrocytes with 16% ethanol, and obtained blood group A and B specific preparations corresponding to the blood group specificity of the extracted erythrocytes. He concluded that these substances were pure polysaccharidic haptens. Neither Hallauer nor Kossjakov considered that their method of preparation may have dissociated a more complex structure. More recently, Yamakawa and Iida (187) isolated glycolipid structures of low but specific serological activity from human erythrocytes, and a number of other investigators made similar observations (152). Assuming that no water-soluble contaminants accompanied these glycolipids, their low activity may be explainable in part by the poor solubility of these substances and their correspondingly reduced surface area. Accordingly, Kościelak (84*a*) has recently offered a possible explanation for the failure to obtain highly active blood group A, B, H(O) substances from erythrocytes. He observed that A active substance prepared from human erythrocyte stroma behaved like a two-component complex in the hemagglutination-inhibition assay. Two erythrocyte components were required to complex in order to exert full activity. One of these, which is of lipid nature, was thought to have only carrier function. Kościelak assumed that the carrier material may produce the necessary structural arrangement which permits the chemical groupings of the blood group substances the combination with antibody molecules. However, even the most active complex possessed only 20% of the A activity of a standard ovarian cyst A substance (84*a*). The substances isolated from human A and B erythrocyte stroma showed great similarity in chemical composition and physical structure and, in addition to sphingosine, they contained the monosaccharides thought to be predominantly responsible for A and B specificity.

On the basis of Hirst's (60) concepts, DeBurgh et al. (34) first isolated an acid-labile, influenza virus-inhibiting, ether-soluble substance from human erythrocytes. This material was found in the above-mentioned elinin fraction. It was stated to consist of at least 50% polysaccharides and contained 2.6% N. A soluble virus-inhibitor preparation as active as purified serum-inhibitor material was first obtained from red cells and examined by McCrea (104). This stable "mucoprotein" was not homogeneous physicochemically. It contained amino acids, two hexosamines, galactose, and fucose, and showed blood group H (O) and A activity. No analysis for sialic acid was performed, but more recently a number of similar erythrocyte surface components of high viral inhibitor activity have been extracted from erythrocyte stromata, and they all contained sialic acid (67, 82, 75).

Immunologically specific receptors of the human erythrocyte surface, on whose chemical nature detailed unequivocal information is accumulating, are the M and N agglutinogens, the Lutheran-system antigens of human erythrocytes, and the heterogenetic "infectious mononucleosis receptor" of sheep erythrocytes. Strong evidence has been obtained by enzymatic methods (153, 156) that sialic acids are an intrinsic part of these blood group agglutinogens.

Hohorst (63) first obtained chemical evidence, based on earlier observations by H. Schmidt, for the carbohydrate nature of M and N substances. These observations were confirmed, and the studies were extended to the isolation of potent but physicochemically inhomogeneous M and N active preparations from human and horse erythrocytes. A remarkable feature of these materials is their acidity, explained by their high content of sialic acid (8, 82, 119) and their high potency as inhibitors of influenza virus agglutinins (152). These inhomogeneous M and N receptors were found to contain, in addition to sialic acid, the following monosaccharides: galactose, glucosamine, galactosamine, mannose, and fucose. At least 14 different amino acids were present also, among which serine, threonine, and glutamic acid were predominant. None of these monomers, however, possessed hapten activity, with the exception of unneutralized L-aspartic acid which weakly inhibited Vicia anti-N lectins (119).

The relationship of sialic acid to the determinants of blood group M and N specificity is certainly not a simple one. Circumstantial evidence indicates that it may be more closely re-

lated to M than to N specificity (119). From isolated receptors, 94 to 100% of the sialic acid could be removed by mild acid hydrolysis (158*b*) and subsequent treatment with "receptor-destroying enzyme" without noticeable decrease of their N activity, as measured with the anti-N agglutinin from *Vicia gruminea*. Anti-N antibodies from man and rabbit, however, indicated destruction of the isolated NN antigen by both mild acid hydrolysis and receptor destroying enzyme (158*b*). M activity disappeared during the early stages of hydrolysis. Free sialic acid and numerous sialic acid derivatives did not significantly inhibit any of a number of the anti-M and anti-N agglutinins (152). Also, the M^g erythrocyte antigen, thought to be the product of a gene allelomorphic to the M and N genes, is not inactivated by influenza viruses and receptor-destroying enzyme under conditions which destroy M and N agglutinogens (157).

No unequivocal evidence regarding components of additional human erythrocyte agglutinogens has accrued, with the exception of the apparent sialic acid content of the Lu^a and Lu^b antigens which are also inactivated by influenza viruses (154). The claims advanced for the involvement of sialic acid in Rh_o (D) specificity by two groups of workers have not been substantiated by four other laboratories with inhibitor concentrations up to 10 mg/ml and 4 hemagglutinating doses of serum (152).

There are, however, some recent but as yet fragmentary observations on the chemistry of the infectious mononucleosis receptor of sheep and beef erythrocytes, on the serum sickness receptor of beef erythrocytes, and on the J substance of cattle (152). The isolation of blood group specific receptors from beef erythrocyte stroma was reported by Royal, Ferguson, and Sutton (141*a*) and later by Uhlenbruck and Schmid (170*a*). The extracted materials were stated to contain about 20% hexosamine and approximately 15% sialic acid. They inhibited sera against numerous different blood group factors of cattle. No quantitative comparisons as to the activities against the various antisera were given. Royal, Ferguson, and Sutton reported a rather remarkable chromatographic separation of their material into fractions containing only one to three haptens; they also found their fractions to be nonantigenic in the rabbit. The later authors have not reported any similar studies.

A component of the erythrocytes of some animal species, which is also present in the erythrocyte membrane of humans of blood group A, is the Forssman hapten. It is frequently prepared from sheep erythrocytes. Brunius (19*a*) first noted its hexosamine content which was confirmed by Chase and Landsteiner. More recently Forssman active haptenic glycolipids have been isolated from sheep erythrocytes by Papirmeister and Mallette and by Yamakawa, Irie, and Iwangawa. This glycolipid was stated to contain about 18% hexosamine (predominantly galactosamine) and in addition fatty acids, a lipid base and galactose (152).

The foregoing indicates the major avenues currently employed in the investigation of erythrocyte surfaces. More refined methods and new ideas are badly needed, since the present approaches have obvious limitations. Autolysis and loss of soluble components are an ever-present danger during the preparation of stroma and extraction of stroma once prepared with predominantly organic solvents undoubtedly yields artifacts, as neither the intact erythrocyte nor physiological extractants are being used. Enzymatic methods suffer from the occasional lack of purity of even recrystallized enzymes and from the inhomogeneity of the substrate. Also, the observed effects are frequently interpreted rather uncritically; for example, the removal of an agglutinogen, or of a postulated "layer" of serologically "inactive" material, from the erythrocyte surface by trypsin or papain has been usually taken as proof of the proteinaceous nature of the substance under study. However, one enzyme can have more than one function, as was already pointed out regarding the esterolytic activity of trypsin and papain.

Even if a monospecific enzyme, such as sialidase is believed to be, is used, the proof of the nature and linkage of a substrate attacked by a given enzyme is only circumstantial evidence as to the nature of the specific groups of the agglutinogens. The substrate inactivated by enzymatic action may not be the serologically specific grouping, but may merely link it to the macromolecule or properly orient it on the surface of the macromolecular complex.

Also, the seemingly specific approach of using antibodies has to contend with a number of pitfalls. In spite of the great resolving power and accuracy of antibodies as test reagents when used with proper precautions, it is necessary to be aware of their limitation in certain circumstances,

such as the occasional failure to discern between closely related structures or the general restriction of their reactivity to end groups alone or structures, such as branch points, close to them. Another limitation of antigen-antibody reactions rests in the difficulty of obtaining systems consisting of but a single antigenic specificity and a homogeneous antibody population (74, 152).

Structural peculiarities of erythrocytes, such as "layers" on their surface interfering with a given antibody, may obscure reactions. Thus, certain ox cells were not agglutinated by anti-ox cell sera, even when using the Coombs test, whereas others showed wide variations in degree of agglutination. This reminds one of older observations by Bailey and Raffel, who found ox cells highly resistant to agglutination by infectious mononucleosis antibodies although they did adsorb these antibodies and were fully susceptible to lysis in the presence of guinea pig complement (7). Coombs, Gleeson-White, and Hall divided ox red cells into three groups according to their agglutinability (27). As there was no obvious difference in the amount of antibody adsorbed by any of these three categories, it was concluded that there must be some variable agglutination-preventing character in the structure of the red-cell membrane, rather than an appreciable difference in the number of receptors present in the three classes of bovine erythrocytes. It was suggested that agglutinogens were located at different levels, some of them so deep that even a sensitizing antibody molecule combined with an antiglobulin antibody molecule was not "long" enough to permit agglutination. This hypothesis was supported by building up antiglobulin-globulin lattices from the sensitizing antibody molecules to the "outer effective limit of the cell wall" and subsequent agglutination. A number of alternate treatments were needed for cells which were originally inagglutinable.

These observations as to the nature of the structure of the erythrocyte membrane are not incidental to the subject of this review, but it seems to the writer that a fruitful discussion of antigenic alteration of the erythrocyte surface can only be hoped for if the substrate of enzymatic or other action is defined to the fullest possible extent on a chemical and physical as well as morphological basis, and if not only the possibilities but also the limitations of the combined immunological-enzymatic approach are pointed out.

Antigenic Changes Resulting from Enzymatic Action on Erythrocytes

Origin and Nature of Enzymes

Animals, protozoa, higher plants, bacteria, and viruses have all been shown to possess proteases, esterases, or glycosidases which, acting singly or in combinations, may alter the antigenic structure of red-cell surfaces (73, 152). With the exception of a minority of the animal's (or man's) own enzymes, these are enzymes from sources with which erythrocytes do not come in contact under physiological conditions.

Although much study has been devoted to the energy metabolism of erythrocytes, very little is known of the in vivo location and action of enzymes which synthesize and degrade erythrocyte membranes and their antigenically specific components, even though a number of enzymes have been found to be associated with erythrocyte stroma. Thus, Morrison and Neurath (111) noted the presence of three different protein-splitting enzymes in hemoglobin-free stroma suspensions. The existence, in stroma, of peptidases is well-established (1), and acid phosphatase also appears to be a part of stroma (169). A specific cholinesterase bound to the human erythrocyte membrane and not elutable from it has also been reported (127). No function of these enzymes in relation to the erythrocyte membrane structure has been established other than that the erythrocyte membrane is considered to take part actively in the transport of metabolites and in the regulation of concentration gradients.

There is substantial evidence that damaged circulating erythrocytes are sequestered predominantly in the bone marrow, spleen, and liver (72), where their membranes are destroyed, with carbohydrases and proteases likely to be involved. The flow of blood in the spleen is slow and even stasis is quite common. Stasis is said to lead to the formation, in the red cell membrane, of lysolecithin which hemolyzes (11). There is also some experimental evidence that the cholinesterase of erythrocytes releases acetic acid from acetylcholine, which is abundant in the spleen. This is supposed to lower the pH and result in hemolysis (168).

Congenital abnormalities of an individual's erythrocyte-metabolism enzyme system may render him susceptible to acute hemolysis when exposed to certain drugs (149). Otherwise, the little information that has accumulated as to the role played by enzymes in the interaction

between the erythrocyte surface and its environment under physiological or pathological conditions will be referred to below.

The studies to be reported first will deal almost exclusively with the strictly controlled in vitro action of enzymes on erythrocyte surface structures.

Action of Enzymes

Uncovering of "hidden" receptors not reactive with agglutinins prior to treatment. The earliest, comparatively neglected, observations of enzymatic action on erythrocytes were probably those dealing with the (Hübener)-Thomsen-Friedenreich phenomenon, in which a pre-existing concealed receptor in the erythrocyte membrane was unmasked. This discovery, which has proved important for blood group research as well as virology, will be discussed as a paradigm of enzyme action on erythrocytes.

In 1927, Thomsen (165) reported that human erythrocytes of any blood group were influenced by a propagative agent and underwent such changes that they were agglutinated by all human sera except those of newborns. The changed corpuscles showed no sign of injury, especially no hemolysis. The agglutinating fraction of the serum, which differed widely in strength from one individual to another, remained remarkably constant for a given individual and behaved exactly like a blood group agglutinin. It could be absorbed only with the altered red cells, which appeared to be provided with a new receptor independent of the A, B, O system.

Occurrence of panagglutination upon prolonged storage of blood had been noticed by a few investigators before. However, an extraneous agent as the cause of this phenomenon was not considered previously. Friedenreich (44) proved the phenomenon observed by Thomsen was associated with the presence of bacteria, which were isolated as pure cultures from dried altered blood and designated as "M" and "J" strains. These caused no direct agglutination, but erythrocyte agglutinability was changed in the characteristic manner by their action on suspended erythrocytes. On examination of many different bacterial species, erythrocyte-transforming power was also noted in vibrios (*Vibrio cholerae* and *V. phosphorescens*), some spirillae, and one phosphorescent coccobacillus. Dead microorganisms had no effect, but with living ones the changes took place even at 2 C, though much more slowly than at ordinary temperatures.

Friedenreich found the transforming agent to be a filterable bacterial product formed during microbial multiplication. He also showed that on "transformation" with "M" bacteria the agglutinability of erythrocytes by serum increased toward a maximum, subsequently decreased, and finally disappeared.

All corpuscles were transformed similarly, including those of newborn infants and human embryos. Red cells removed from the filtrate after 10 min of incubation were transformed as rapidly and thoroughly as those exposed for longer periods, indicating that the active principle was fixed to the erythrocytes at once. It was released into the suspending medium after transformation had become maximal, at which time the erythrocytes could adsorb no more transforming substance. This agent behaved like a catalyst, the fresh and the used filtrates showing the same transforming power. The action of the transforming principle was significant only at pH 5 to 8.

The new condition of the corpuscles after enzyme action was very stable after the action of "J" filtrate. This was in contrast to "M" filtrate-changed corpuscles. "M" filtrate was assumed to contain an additional receptor-destroying factor.

Friedenreich's (44) interpretation of the transformation of red cells as a catalysis implied a pre-existing constituent which changes into a new state after its transitory combination with the catalyst. This view is in accordance with Thomsen's (165) hypothesis that a latent receptor is uncovered by the influence of the catalytic agent. The demonstration of T-receptor antigenicity in a number of animal species (21, 51) also supports Thomsen's hypothesis.

Klenk and Uhlenbruck (82) isolated mucoids from human and beef erythrocytes which, after treatment with receptor-destroying enzymes from *V. cholerae*, inhibited anti-T agglutinins. It is noteworthy that these inhibition experiments were carried out at temperatures of only 12 C, although it had been demonstrated by the discoverers of T agglutinins that they are not related to cold agglutinins.

Burnet, McCrea, and Stone (22) showed that cells treated with influenza viruses become agglutinable by human and other sera which were previously without action on them. These authors, noting the similarity between the Thomsen-Friedenreich phenomenon and the effect observed after influenza virus action, also studied the ac-

tion of *V. cholerae* culture filtrate. This "J" bacillus filtrate induced a red-cell change similar to that of influenza viruses. A preparation of *Clostridium welchii* toxin A also removed the virus receptors and presumably uncovered T antigen, but Friedenreich's "M" bacillus (diphtheroid) was reported not to inactivate the virus receptor.

The question as to whether the uniform effect of these different bacteria was due to identical enzymatic action has been answered indirectly by Friedenreich. He established that the "T" receptors uncovered by those bacteria which he used all absorbed the same "T" agglutinin.

The common denominator of all enzymes which uncover the T antigen appears to be their ability to remove O-glycosidically linked sialic acid; i.e., these enzymes are or contain O-sialidases (neuraminidases; 47, p. 98–102).

Another example of enzymatic action in which removal of terminal structures uncovers previously nonreactive receptors is the transformation of A and B erythrocyte agglutinogens into those having H(O) characteristics (70). This finding is of great importance in the elucidation of the biosynthetic pathway of blood group substances (109).

Saline agglutination by "coating" antibodies and increase of pre-existing agglutinability. The finding by Pickles (129) that washed erythrocytes, previously sensitized with "incomplete" anti-Rh_o (D) antibodies, were agglutinated in saline after incubation with *V. cholerae* filtrate opened an entirely new and, as it was soon found, immensely fruitful supplementary approach to the field of blood grouping. It allowed detection of sensitization by the vast majority of coating antibodies by simply observing agglutination of erythrocytes suspended in physiological saline. Morton and Pickles facilitated the routine use of their new procedure (113, 129) by substituting trypsin for the bacterial enzyme. Trypsin proved to have the same effect as cholera filtrate in regard to the agglutination of the Rh_o (D) receptor by coating antibodies. Both enzymes apparently acted on the red-cell surface without directly affecting the Rh_o (D) hemagglutinogen loci.

Since these original investigations, many enzymes have been used on erythrocytes. Most investigations were directed toward the Rh_o (D) receptor. Wheeler, Luhby, and Scholl (177) pointed out that red cells become agglutinable by incomplete anti-Rh_o (D) antibody not only after the action of the two enzymes mentioned above but also after the action of chymotrypsin, pepsin, papain, bromelin, and Russell viper venom. Morton and Pickles (113), however, found pepsin to be without effect but obtained reproducible enhancement of agglutinability after the action of papain or a preparation from *Streptomyces albus*. Also, these two groups of authors reported enzyme effects on erythrocyte agglutinogens other than Rh_o (D) antibodies for the first time. In their hands, "incomplete" anti-Rh antibodies, namely anti-C (rh′), anti-E (rh″), anti-c (hr′), and anti-e (hr″), showed the same order of increased titration values against trypsin-treated cells as was noted with the incomplete anti-Rh_o (D) antibodies. In the system involving anti-Le^a and anti-Lu^a, no significant differences were noted before and after trypsin treatment.

In the interpretation of these results, it is to be noted that the same enzyme in the hands of different investigators, and different preparations of purportedly the same enzymes used by a single group of researchers, not infrequently showed varying effects. In some instances experimental errors may be implicated, but this seems an unlikely cause in the majority of cases. An observation by Morton and Pickles (113) may serve as an example. They found increased agglutinability of the M, N, and S erythrocyte receptors by their corresponding antisera after treatment with a crystalline trypsin preparation, whereas less purified trypsins completely inactivated the antigens. These varying effects were most likely due to contaminants of the enzyme. Enhancement of agglutinability was noted for the A, B, O, and P factors after exposure of the appropriate erythrocytes to chymotrypsin, trypsin, and papain. In contrast to the observations by Morton and Pickles, enhancement of Le^a and Le^b agglutination was observed by others (140, 177). Red cells treated with trypsin were found to be activated temporarily to an autoagglutinin, similar to T agglutinin.

Ficin (178) was similar to trypsin and papain in its action on human erythrocyte agglutinogens. It also facilitated agglutination of the I antigen by its corresponding antibody (46).

Enzymatic investigations of the surfaces of sheep and beef erythrocytes have also been carried out (e.g., 161*a*, 156, 167), especially in relation to the mononucleosis antigen. The work of Coombs, Gleeson-White, and their colleagues (27) on ox red cells has already been referred to.

It will be recalled that these and other authors reported on a variable character in the structure of ox red cell membranes which in some instances prevented their agglutination by various antibodies, although these antibodies were absorbed equally well by agglutinable and unagglutinable erythrocytes. Trypsin treatment rendered all these cells equally agglutinable. No attention was paid in this and subsequent papers to the possibility that some of the increased agglutinability observed was due to uncovering of T antigenlike determinants. This is especially worthy of consideration, since Klenk and Uhlenbruck (82) reported isolation of some T receptor from beef erythrocytes. Beef and sheep erythrocytes are acted upon similarly by trypsin but not by papain, at least as far as the receptor for antibodies of patients with infectious mononucleosis is concerned (183, 167, 156, 152).

Decrease or abolition of agglutinability. The removal of virus receptors by influenza virus sialidase and receptor-destroying enzymes has already been alluded to. Not only virus receptors but also blood group agglutinogens may be inactivated by enzymes; for example, influenza virus sialidase has been shown to inactivate the heterogenetic mononucleosis receptor of sheep (but not beef) erythrocytes, the M and N agglutinogens, and the antigens of the Lutheran blood group on human erythrocytes (153, 154, 156, 102). Sialic acids are undoubtedly involved in the structures of the receptors inactivated by influenza virus enzyme. In some instances blood group specificity and virus inhibition activity may be part of the same molecule (152).

Enzymatic inactivation of blood group A, B, and H (O) antigens was described long ago, but those reports were largely confined to the specific water-soluble substances (147). Enzymatic inactivation of A erythrocyte receptors (Forssman part) was first observed by Schiff and Akune (147) and by Eisler. Morgan vainly attempted to destroy the group specificity of intact erythrocytes by enzymes (108), although he readily inactivated it on erythrocyte stromata.

Watkins and Morgan (173) have shown that a partially purified enzyme preparation obtained from *Trichomonas foetus* inactivated H(O), M, and N receptors on the red-cell surface but not the A, B, or Rh_0 (D) agglutinogens. This enzyme also uncovered a T-like antigen. The action of the *Trichomonas* enzyme in some aspects paralleled that of trypsin but was different in others, such as in the inactivation of the H(O) erythrocyte agglutinogen. The *Trichomonas* enzyme also destroyed the influenza virus receptor sites on the erythrocytes, whereas the influenza virus did not affect the H(O) agglutinogen under the experimental conditions employed. Partially purified β-galactosidase and β-glucosaminidase isolated from *C. tertium* diminished the amount of I antigen on human erythrocytes (97*a*). It was also reported that more I determinants were removed by these enzymes from A_1 erythrocytes than those of blood group O. The same authors (97*a*) noted the release of galactose and *N*-acetylglucosamine concomitantly with the decrease of demonstrable I receptors.

Other erythrocyte antigens which were found to be inactivated by enzymes were the Duffy (Fy^a) antigen (50), several antigenic factors in the B system, and one in the C system of cattle (161*a*). The infectious mononucleosis receptor of sheep erythrocytes was found to be inactivated or removed from the red-cell surface by ficin (156), papain (183), bromelin, influenza viruses, and receptor-destroying enzymes (156). Interestingly, these enzymes have little or no effect on the infectious mononucleosis receptor of beef erythrocytes (167, 152). It is as yet unknown whether this distinction is due to difference in structure of the serologically active components on sheep erythrocytes or whether some structural feature of the beef erythrocyte membrane shields the receptor from inactivation; the first alternative appears to be more likely, as isolated highly purified receptor from beef erythrocytes was not inactivated by these enzymes (Springer, *unpublished data*).

The action of a large number of enzymes on erythrocyte surface structure has been studied, and some of the most pertinent data are summarized in Table 1. The lack of change and the enhanced agglutinability of an agglutinogen after enzyme treatment are not differentiated in this table, as it has not been established whether enhancement is due to change of the receptor itself or of the area surrounding it. (The latter seems to be more likely.) This is done even though some carbohydrases, such as β-glucuronidase, hyaluronidase, and β-glucosidase, suspected to be involved in the physiological and pathological destruction of erythrocytes, supposedly led to increased agglutinability of red

TABLE 1. *Enzyme effect* on erythrocyte blood group agglutinogens measured by corresponding antibodies*

Enzyme†	Erythrocyte agglutinogens‡																											
	Human																							Sheep			Beef	
	A_1	B	H (O)	Le^a	Le^b	M	M^g	N	S	s	P	Jk^a	K	k	I	Fy^a	Lu^a	Lu^b	C (rh′)	D (Rh_0)	E (rh″)	c (hr′)	e (hr″)	Mononucleosis	Serum sickness	Forssman	Mononucleosis	Serum sickness
Influenza virus type A and B (129, 153, 102, 156, 154, 8, 157)	0	0	0	0	0	+	0	+	0	0	0	0	0	0		0	+	+	0	0	0	0	0	+	0	0	0	0
Vibrio cholerae, receptor-destroying enzyme (155, 154, 157)	0	0	0	0	0	+	0	+	0	0	0	0	0	0		0	0	0		0				+	0	0	0	0
Trichomonas foetus, purified (173)	0	0	+	0?	0?	+		+	0		0									0								
Papain (183, 178, 177, 167, 82, 157)			0			+	+	+												0				+	0	0	0 or ±	0
Bromelin (177, 156)																								+	0	0	0 or ±	
Ficin (178, 50, 156, 46)				0	0	+		+	+	+	0	0	0	0	0	+			0	0	0	0	0	+	0	0	0 or ±	
Trypsin (112, 177, 178, 173, 140, 157, 167)	0	0	0	0	0	+ or 0	+	+ or 0	+ or 0		0		0 or +	0		+	0			0				0	0	0	0	0
Clostridium tertium (66, 70, 97*a*)	+	0	0			+		+					0			0				0								
Bacterium fulminans (70)	0	0	+																									
Clostridium maebashi (70)	0	+	0																									

* Enzymes without effect on any receptor tested, such as β-amylase, lipase, emulsin, hyaluronidases, and lysozyme, are not listed, although the negative information is valuable (177, 156).
† Numbers in parentheses are references.
‡ Agglutinability symbols: + = abolished; 0 = enhanced or not altered; ± = decreased.

cells treated with them (93). Antibodies induced with enzyme-modified human erythrocytes were said to differ in their specificity, depending on the agent employed to change the erythrocyte surface (172).

Action of enzymes on virus receptors of red cells. No detailed account of enzyme action on virus receptors will be attempted. Such action will be considered only if it bears on the antigenic structure of red cells (removal of surface components, or attachment of antigenic substances to the erythrocyte membrane) or if it serves to improve the understanding of changes of antigen structure brought about by enzymes.

Hirst (60) and McClelland and Hare (103) were the first to observe that influenza viruses attach themselves to a receptor substance on the chicken red-cell surface, agglutinate these cells, and subsequently elute. The over-all viral action appeared analogous to one between enzyme and substrate. Hirst found the cellular substrate to be heat resistant and concluded that it was of nonprotein nature. He and others deduced that those receptors on the erythrocyte surface which are changed by viruses also play an essential part in the viral invasion of susceptible cells other than red cells. On the other hand, the suggestion has been made that these microbial enzymes interfere with the defense mechanisms of the host (47). Independent proof of the existence of a specific virus receptor was provided by Burnet, McCrea, and Stone (22), when they demonstrated that treatment of erythrocytes with receptor-destroying enzymes rendered them inagglutinable by influenza viruses; furthermore, erythrocytes pretreated with either influenza virus or receptor-destroying enzyme failed to adsorb both.

Numerous viruses other than the myxoviruses, which include mumps, Newcastle disease, and influenza viruses, cause hemagglutination. It is of interest that lipid extracts from a number of normal tissues agglutinate the same range of red blood cells as vaccinia and ectromelia hemagglutinins (161). All these lipids were found to be phospholipids; they were most effective in combination with cholesterol. The lipid hemagglutinins, however, were stated to be inactivated by dilute normal serum, whereas the virus agglutinins are not. Agglutination is made possible by specific receptors on the red cell in addition to those for the myxoviruses. For example, Fastier (42) has clearly demonstrated that the receptor for the GD VII strain of murine encephalomyelitis virus is quite different from that of the PR8 influenza virus. PR8 virus did not inactivate the GD VII receptor on human red cells, nor did periodate treatment of human red cells prevent elution of GD VII virus, whereas PR8 influenza virus did not elute from red cells treated in the same manner. Numerous other red-cell surface virus receptors which are not impaired by viruses exist, as has been demonstrated for varying groups of the arthropod-borne viruses (2). Some viruses agglutinated red cells of day-old chicks but generally not those from adult fowls, indicating a change of the erythrocyte surface dependent on age.

Those viruses which exert enzymatic action concomitant with an antigenic change of erythrocyte membranes are most relevant to the context of this review, although viruses or viral products which stay adsorbed onto erythrocytes must also be considered by virtue of their change of the erythrocyte surface. Influenza viruses elute virtually completely, but this is not the case for Newcastle disease and mumps viruses; in these a proportion of virus particles apparently unites irreversibly with the erythrocyte surface, thus conveying new properties to it (2).

A number of nonviral enzymes apart from the receptor-destroying enzyme and *C. welchii* and *T. foetus* enzymes referred to are capable of inactivating erythrocyte receptors for influenza viruses; for example, preparations from *C. tertium* (66) may be mentioned. *C. tertium* enzymes also inactivated the human red-cell receptor for one strain of encephalomyocarditis virus (Col. SK; 66), and Chu and others (47, p. 101) found some time ago that enzymes similar in their action to receptor-destroying enzyme are elaborated by many microbes. Recently, inactivation by trypsin but not by receptor-destroying enzyme of the receptor for reo viruses on human erythrocytes has been reported (92*a*).

Hemolysis due to enzymes. Hemolysis may be induced by enzymes, nonenzymatic chemicals, such as numerous metals (48), and by physical agents (see below). Apparently, no firm knowledge of antigenic changes of erythrocyte surfaces as a result of hemolysis of the red globules exists; morphologically, in nearly all cases of hemolysis, the so-called myelin figures occur (see Fig. 3). These are considered to be phospholipids (149, p. 9–28). According to C. Steffen,

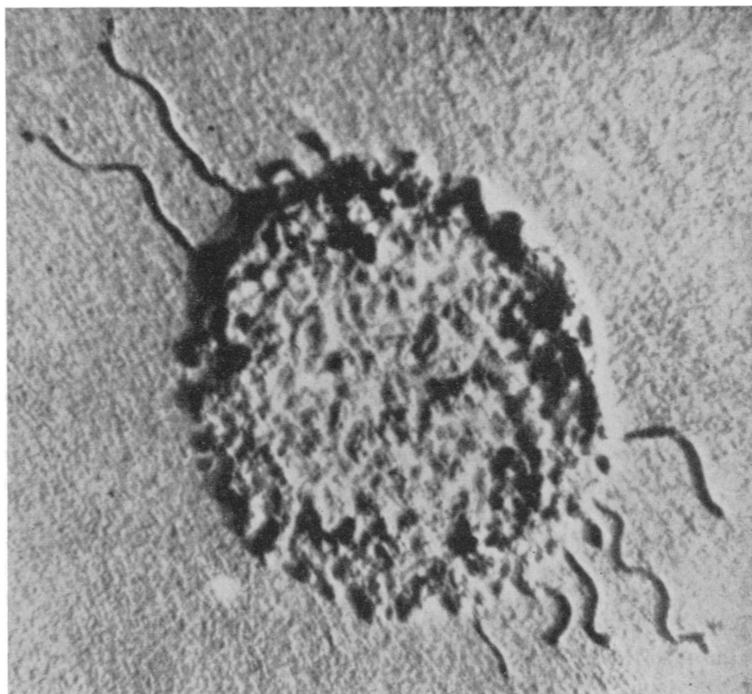

FIG. 3. *Erythrocyte ghost, myeline figure. Electron micrograph, gold-shaded; ca. 10,000 ×. After Bessis (149).*

R. Timpl, and I. Wolff (*personal communication*), coupling of simple azo compounds onto human erythrocytes led to extensive hemolysis by multiple degradation of their membranes; concomitantly the damaged cells became strongly agglutinable by normal human sera. The azohapten alone or coupled to a foreign protein did not inhibit this agglutination, which was thought to be due to uncovering of pre-existing erythrocyte antigens.

Numerous enzymes may induce lysis (direct or indirect) of erythrocytes without grossly altering the erythrocyte surface. Only these will be mentioned here, as a host of enzymes will ultimately "digest" the entire erythrocyte, thus releasing hemoglobin and its breakdown products. Immune hemolysis is considered to be an enzymatic process, as was assumed by P. Ehrlich. From 400 to 600 antibody molecules are required to prepare a red cell for immune lysis (56) and to allow complement to act. Most important is the esterase activity of at least one component of the complement system, C'_1, which is activated by antibody-antigen complexes, although it may not act directly on the red cell membrane but rather activate other complement components (10, 92).

As a result of their experiments, Plescia et al. (130) considered it possible that proteolysis, probably of cell membrane components, plays a part in immune hemolysis, and that peptidase action is in some way connected with C'_2. The detailed mechanism of hemolysis due to complement and antibody is beyond the scope of this review, and the reader is referred to the comprehensive treatise by Mayer (101).

Most extraneous hemolytic agents, such as those of some arthropods and snakes, act by splitting lecithin at the glycerol-fatty acid junction, in many instances leaving lysolecithin which is itself hemolytic (132, p. 329). Accordingly, Turner (170) has differentiated between a directly enzymatic lysis and an indirect one which implicates lysophosphatides formed by the action of venom phospholipases. This author stated that erythrocytes of those animal species which resist venom hydrolysis do not contain lecithin in their membranes, whereas those which are susceptible invariably do. Similar lack of receptor or substrate may be responsible for the

failure of staphylococcus β-toxin to lyse horse erythrocytes, as it hemolysed erythrocytes of other species (134).

The rate of hemolysis of human erythrocytes by carbohydrases suspended in heat-inactivated AB serum was followed (93); β-glucuronidase and β-glucosidase were stated to be potent hemolytic agents, hyaluronidase and lysozyme were much less active, and β-amylase was not lytic. In sera containing antibodies, including some autoantibodies against the erythrocytes, doses of β-glucuronidase and hyaluronidase which were not hemolytic in ordinary AB serum produced significant hemolysis.

Enzymes hemolyzing mammalian red cells are also elaborated by numerous microorganisms, such as bacteria (e.g., 99) and certain viruses. Newcastle disease and mumps viruses hemolyze, besides using the same receptors as influenza viruses. Mumps virus hemolysis has been studied extensively by Moberly et al. (106). It was considered probable that the virus attacked an erythrocyte membrane component involving sphingomyelin. The authors noted that mumps virus hemolysin decreased thickness, rigidity, and strength of red-cell membranes; hemoglobin was released into the solution without visible cell-membrane rupture. The mumps virus receptor was destroyed by this action. The authors believe they have differentiated this enzyme from lecithinase A.

Hemolysis due to lysozyme has also been reported (9). Rather large concentrations of the crystalline enzyme had to be used, however; boiled lysozyme was said to agglutinate erythrocytes, indicating the fixation of this enzyme to red cells.

Enzymes in structural chemical analysis of erythrocyte surface antigens. Information on the chemical nature of erythrocyte-membrane antigens is obtainable through the use of enzymes as analytical tools. This method has proven fairly successful in the analysis of water-soluble blood group substances isolated from epithelial secretions (73, 109). The ultimate aim of chemical analysis of red-cell membrane components released by enzymatic action is their correlation, as well as that of the residual structures, with serological specificity and enzyme-induced serological changes.

If the specificity of an enzyme were exactly known, enzyme action could then be strictly related to the substrate acted upon, i.e., for the present discussion, the changes inflicted upon the erythrocyte surface pattern. This situation may pertain, at least to a degree, to the influenza virus enzyme, sialidase. Usually, however, a mixture of substances is released whose analysis and correlation with structural arrangement on the erythrocyte surface presents a formidable challenge. Due to the small amount of antigen present on the surface of an erythrocyte, stroma is usually prepared, and this may be studied enzymatically. Caution must be exercised, however, in extrapolating findings to ordinary erythrocytes, as Morgan (108) made the unexplained observation that enzymes from *C. welchii* readily inactivated serologically specific receptors on stroma but not the corresponding agglutinogens on the intact red cells. Since most enzyme preparations are mixtures and stromata are crude compounds, it is difficult to decipher the significance of the results obtained for a specific antigen. Consequently, attempts have recently been made to obtain stroma fractions enriched with erythrocyte agglutinogens, and to use these for enzymatic experiments. This approach appears promising, but again conclusions from observations on isolated receptors related to native erythrocytes must be drawn with great caution; the effect of even so well-defined an enzyme as influenza virus sialidase on the serological specificity of a substrate differed for the same agglutinogen depending on whether it was still part of the red-cell surface or whether it had been isolated (119).

Among the earliest statements relating to the chemical nature of erythrocyte antigens were those, based on observation of inactivation of red-cell agglutinogens by "proteolytic enzymes," which equated the inactivated structures with protein. The potential fallacy of this reasoning has already been pointed out in this presentation. In addition, no serious attempt has been made until recently to chemically identify the material released by enzymes or to investigate the serological properties of the released substances. Burnet and Anderson have claimed (21) that receptor-destroying enzyme releases some antigenic (i.e., macromolecular) material from guinea pig and human erythrocytes which is able to induce antibodies against ordinary erythrocyte surfaces; this finding, and more recent

observations by Springer and Hotta (158*b*), who used commercial receptor-destroying enzyme (lot 19 from Behringwerke, Marburg), appears to be in contrast with the general belief that receptor-destroying enzyme splits only sialic acid from macromolecules and oligosaccharides (47); however, the purity of neither of these enzyme preparations has been proved. One of the earliest differentiations of various erythrocyte surface structures by enzymes was reported by Tomcsik and Schwarzweiss (166), who noted that trypsin and pepsin would inactivate the thermolabile, species-specific antigens of sheep erythrocyte membranes but lacked demonstrable influence on the thermostable mononucleosis and Forssman receptors of these erythrocytes. The species-specific receptors were therefore considered to be protein in nature whereas the mononucleosis and Forssman receptors appeared to be something else.

In addition to this, enzymes have yielded chemical information on the nature of the T antigen as already mentioned, whereas the M, N, and Lutheran substances are simultaneously inactivated by influenza virus and receptor-destroying enzyme action (153, 154). Klenk and Uhlenbruck (82) reported that low concentrations of mucoids isolated from human and beef erythrocyte membranes specifically inhibited the T agglutinin after treatment with receptor-destroying enzyme. So far, sialic acids have always been stated to be linked to either D-galactose or *N*-acetyl-D-galactosamine as subterminal structures (47); it is reasonable, therefore, to expect either or both of these sugars to be involved in T-antigen specificity.

It was first shown by Springer and Rapaport (156) that papain treatment of intact sheep and beef erythrocytes released predominantly substances which contained nondialyzable, non-heat-coagulable substances which contained sialic acid and other carbohydrates, as well as amino acids. The enzymatic action abolished reactivity of sheep erythrocytes, but not of beef erythrocytes, with anti-infectious mononucleosis sera. Similar results were obtained by these authors with three-times-crystallized ficin, thus making remote the possibility that these substances were released by enzymatically active impurities. The results clearly indicated that "proteases" such as papain, ficin, and bromelin did not merely remove a "protein coat" from the erythrocyte surface, as was widely assumed, but that in addition a substantial part of the liberated material was carbohydrate in nature. Some of the released nondialyzable materials inhibited anti-infectious mononucleosis sera weakly but apparently specifically.

Analogous experiments with quite similar results were performed later on human erythrocytes with trypsin, papain, and bromelin (82, 157), and some weakly M and N active material was reported to be released. Analytical data on a weakly M, N active preparation obtained by Klenk and Uhlenbruck (82) from human blood group O erythrocytes were as follows: sialic acid, 14.7%; reducing sugar as galactose, 47%; hexosamine, 18%; sugars identified by paper chromatography were galactose, glucosamine, galactosamine, and, in smaller amounts, mannose and fucose. These authors also reported inactivation of isolated M, N active erythrocyte material (isolated by the phenol method) by papain. Preparations obtained in this laboratory usually contained >20% sialic acid and isolated NN antigens seemed to contain more readily hydrolyzable fucose than did MM receptors (158*b* & unpublished data).

It was reported (156) that the mononucleosis receptor of sheep erythrocyte membranes was also inactivated by influenza virus sialidase and receptor-destroying enzyme, agents which release exclusively or predominantly the low molecular weight sialic acids. These observations on enzymes with such different actions as papain and influenza virus sialidase point to the necessity of differentiation of the causes of seemingly identical serological results. Thus, sialic acid may be removed from a mucoprotein not only as a terminal unit by neuraminidase, but also by an enzyme like trypsin, which acts on the carboxyl of diamino acids far removed from the end of the molecule.

Inactivation of human erythrocyte agglutinogens M, N, Lu^a, and Lu^b similar to that of the sheep erythrocyte mononucleosis antigen, was obtained with influenza viruses and for the M, N antigens only with receptor-destroying enzyme. Some further relation between the M, N agglutinogens and the sheep mononucleosis receptor is their susceptibility to plant proteases, while only M, N but not the mononucleosis receptor are moved by trypsin from the red cells.

The inactivation of the M, N, Lu^a, and Lu^b erythrocyte agglutinogens by influenza viruses, and the former two by receptor-destroying enzymes as well (153, 102, 154), obviously implicated sialic acids in the structure of these agglutinogens. This implication is supported by the high sialic acid content of M and N active substances isolated from human erythrocyte stroma (cf 152) and by the destruction of the blood group specificity of isolated MM and NN receptors by influenza viruses, "receptor destroying enzyme" and mild acid, as measured with human and rabbit antibodies (152). However, the significance of sialic acid or its derivatives for M and N specificity has, in the opinion of the writer, not been firmly established. Free sialic acid and numerous sialic acid derivatives did not significantly inhibit any of a number of anti-M and anti-N agglutinins (152). Furthermore, release of 80 to 100% of the sialic acid present in isolated M, N receptor by either mild acid or influenza virus treatment (119, 158*b*) led to complete destruction of M activity but to an increase in the pre-existing N activity of these isolated receptors when measured with anti-N agglutinin from *Vicia graminu*. Thus sialic acid susceptible to sialidase action and mild acid may be directly involved in the specificity of human blood group M substance. Also blood group N active, but not M active, dialyzable substances were obtained by mild acid hydrolysis of M substance (119). The release of N active structures from homozygous M erythrocyte antigens concomitant with destruction of their M activity and uncovering of residual N activity may indicate that N contains a basic structural specificity more easily accessible to certain anti-N reagents (158*b*). These structures may be covered in part by M specific groupings. Allen, Corcoran and Ellis (1*a*) on the other hand, suggested that M genes normally produce a small amount of N factor.

Interestingly, the blood group factor M^g, which is thought to be the product of a gene allelomorphous to the M and N genes, was not inactivated by either influenza viruses or receptor-destroying enzyme (157). M^g factor-containing erythrocytes were agglutinated by influenza viruses, as were other human erythrocytes, and subsequently became inagglutinable by the virus after extensive exposure to it, thus indicating no gross difference in regard to influenza virus receptors between M^g erythrocytes and those not possessing this antigen.

The lack of inactivation of the M^g agglutinogen by influenza viruses and receptor-destroying enzyme, in contrast to the M and N antigens, is unexpected in view of the assumed close serological and genetic relation of these blood group receptors. It recalls, however, similar observations for the ABO blood group system in which certain enzyme preparations inactivate all three blood group antigens, whereas others affect only one. Influenza virus enzyme may thus be a more sensitive tool than papain and trypsin in the investigation of fine structural differences in the agglutinogens of the MNS system.

Enzymatic investigations on the human red-cell A, B, H(O) antigens have yielded useful information, and indicate that oligosaccharide structures responsible for the specificity of the much better investigated water-soluble blood group mucoids are also decisive determinants of the red-cell antigens of the same specificity. The clearest indication for this was the observation by Watkins and Morgan (174) that specific blood group-destroying enzymes are inhibited specifically in their action by those haptenic sugars which are responsible, in large part, for a given blood group specificity and which are liberated by these enzymes (73, 109). Some enzymes, while inactivating erythrocyte A and B agglutinogens, concomitantly give the erythrocyte surface H (O) activity; this indicates, on a chemical level, that previously nonreactive α-L-fucopyranosyl structures have become available for reaction with anti-H (O) antibody; these structures were either covered by the A and B specific groupings or, more likely, the A and B determinants "sterically hindered" the action of anti-H(O) agglutinins on the red-cell surface.

IMMUNOLOGICAL ALTERATION OF ERYTHROCYTE SURFACE BY AGENTS OTHER THAN ENZYMES

Changes in the antigenic make-up of an erythrocyte's surface may be caused by agents other than enzymes. The changes may be due either to the intrinsic antigenicity of substances adsorbed onto the erythrocyte surface or to substances not antigenic *per se* which may alter erythrocytes in such a way that they become immunologically distinct in vitro or in vivo from untreated erythrocytes. All these agents, unless they are overwhelmingly destructive, react with specific structures which can frequently be defined immunologically. It is well

known that there exists a quantitative relation between the amount of adsorbed substance and its concentration in solution; frequently the adsorption follows reasonably well the so-called adsorption isotherm of Freundlich [see also the early work of Arrhenius (4)].

Changes Caused by Substances Not Antigenic Per Se

Silicic acid. Landsteiner and Jagič (87) first found certain analogies between antibodies and colloidal silica in their reaction with erythrocytes. They noted that silicic acid (diluted 1:5,000) agglutinated in the presence of salts but not when sugars were substituted instead of electrolytes. Although silicic acid was similar in its reactivity to antibodies, its lack of specificity was in contrast to that of antibodies. Fresh serum (i.e., complement) hemolyzed cells sensitized with silicic acid. From this, the investigators (88) concluded that the effect of complement was due to its "nonenzymatic alteration of the colloidal structure" of the cells. Other inorganic salts and acids had an effect similar to that of silicic acid. The hemolytic activity apparently depends on the particle size of the silica. Particles of a size of 3 mμ or smaller had no hemolytic activity but instead protected cells against agglutination and lysis by larger silica particles, of which those of a mean particle diameter of 30 mμ were especially effective (54). This blocking effect is said to persist after repeated red-cell washing with saline, thus indicating an irreversible adsorption of silicic acid onto the red-cell surface. Such "coated" red cells showed additional changes in that they were more susceptible to immune lysis by rat sera and were not hemolyzed by streptolysin O. The nature of the hemolytic effect of silica has more recently been shown to be caused, probably, by adsorption of lipids and possibly lipoproteins onto the silica from the red cells (19).

Periodate. The periodate ion does not cause extensive damage to the red cells (61), although considerable antigenic alteration does occur. Stewart (159) showed that human red-cell specificity was changed by potassium periodate in such a way that the treated cells induced antibodies in rabbits which were specific for periodate-treated cells and did not react with untreated red cells. This antigenic change was distinct from that brought about by the action of *V. cholerae*, as shown by absorption of immune rabbit sera and absorption tests on normal human sera. Periodate treatment rendered human erythrocytes panagglutinable by normal human sera. Stewart's observations have been confirmed by Moskowitz and Treffers (114). All these experiments indicated the presence in normal sera of a number of panagglutinins rather than a single one. It was also stated that the Rh_o (D) antigen, but not the A and B antigens, was destroyed by 0.001 M potassium periodate at pH 7.0 (114).

Morgan and Watkins (110) systematically investigated the action of periodate ions at different concentrations, times, and pH values on human erythrocyte agglutinogens A, B, H(O), Le^a, Rh_o (D), M, N, and P. They found that treatment resulted in loss of activity (measured by agglutination *and* absorption tests) of all these agglutinogens, but they noted differences in susceptibility of the various receptors to the action of the oxidizing agent. The M and N agglutinogens were the most susceptible. The rate at which an erythrocyte agglutinogen was inactivated by periodate is influenced not only by its chemical nature but also by its quantity and accessibility on the erythrocyte surface. It was also pointed out by Morgan and Watkins that inactivation by periodate ion cannot be taken as proof for the carbohydrate nature of the substrate, since α-amino alcohols in addition to α-glycols are periodate-susceptible; nevertheless, the speed of inactivation of M, N, and Rh_o (D) receptors was taken as an indication that they are most likely carbohydrates. The panagglutinability produced by the periodate ion ("P" agglutinin) is strikingly reminiscent of T agglutination, but can be serologically differentiated from T and from cold agglutinins (159, 114). It is also noteworthy that periodate treatment did not affect the cold agglutinin receptor. The antigenic stimulus for the "P" agglutinin, provided that this serum protein is an antibody, is as unknown as is that for the T agglutinin; like the latter, it is absent in neonates.

Tannic acid. The influence of tannic acid on the immunological properties of red-cell membranes and their antigenic components has been studied by a numbe- of investigators, with contradictory results. This is undoubtedly due to the great inhomogeneity of various tannic acid

preparations and to the concentration-dependent effects of this agent.

Tannic acid agglutinates erythrocytes; it also hemolyzes them in the presence of complement and thus behaves like amboceptor. For this to occur, tannic acid must be present in a final dilution of approximately 1:5,000 to 1:12,000; in contrast, it is said to abolish the agglutinability in the ABO(H)-anti-A, B, H(O) system when diluted 1:20,000 to 1:40,000. The strong agglutination by even high dilutions of tannic acid was thought to be due to a dehydration process, in spite of the large number of hydroxyl groups in the tannic acid molecule. Lecithin lysed both untreated and tanned erythrocytes equally well. The mechanism of lysis here was thought to be one of nonenzymatic solubilization of the lipids of dehydrated erythrocytes (137).

Permanent coating of erythrocytes by proteins and other molecules is made possible by prior tanning with dilutions of 1:20,000 to 1:80,000 (*see* 137) without interference of the above phenomena (e.g., 15, 184). Tannic acid-"coated" erythrocytes may elicit agglutinins specific for the tannic acid "coat" (69).

The influence of tannic acid on pre-existing erythrocyte agglutinogens was first investigated by Spanish workers (3) who noted disappearance of agglutinability of tanned sheep erythrocytes by human heteroagglutinins. Woerner (184) and Brading (17) confirmed these observations and noted abolition of anti-A, B, Rh_o (D), M, and S isoagglutinations, hetero- and iso-anti-P, and a number of other iso-immune agglutinations. Hetero-anti-M, N and blood group specific plant agglutinins showed no reduction in titer when tested with tanned erythrocytes. Hornung and Baer (64), however, did not observe a reduction or abolition of isoagglutination of erythrocytes tanned under conditions similar to those of the earlier investigators. Brading (17) noted that tannic acid-treated human erythrocytes do not absorb their corresponding blood group agglutinins as readily as do untreated erythrocytes. This author also determined that no significant amount of antigenic material was removed from the red cells by tannic acid and that tannic acid was firmly bound to the red corpuscles. The latter was demonstrated by the ability of tannic acid-treated red cells to remove iron from a solution of ferrous ammonium sulfate (iron combines with tannic acid).

Normal human erythrocytes treated with weak solutions of tannic acid are hemolyzed at 37 C by normal human serum. Similar in vitro requirements apparently exist for hemolysis of tannic acid-treated erythrocytes as for untreated erythrocytes from patients suffering from paroxysmal nocturnal hemoglobinuria (59).

Formaldehyde. Moskowitz and Carb (116) stated that formaldehyde acts on red-cell surfaces so as to inhibit red-cell agglutinability by specific antisera. Formaldehyde-treated erythrocytes, however, adsorbed their corresponding agglutinins specifically and to the same extent as untreated erythrocytes; also the adsorbed agglutinins were elutable as from normal red cells. The authors suggested that some of the changes of agglutinability of red cells of patients, reported in the literature (e.g., decrease in A-agglutinability), may be due to an inhibitory effect of some adsorbed substance similar to that observed for formaldehyde.

Májský (97) carefully studied the effect of formaldehyde on human erythrocyte agglutinogens A, B, H, M, and N and on the Rh receptors Rh_o (D), rh′ (C), and rh″ (E). He found that in the first group of antigens agglutinability was decreased by formaldehyde without influencing the ability to absorb the appropriate antibodies. For the Rh receptors, on the other hand, both agglutinability by homologous antibodies and ability to absorb them were decreased or abolished. Repeated washing (up to 20 times) of formaldehyde-treated red corpuscles led to partial regeneration of agglutinability among the first group of agglutinogens but not of the Rh receptors. The formaldehyde effect was directed toward the agglutinogens and not against the antibodies. Májský speculated that this apparently qualitatively different effect on the Rh antigens was due to their lipoprotein nature, in contrast to the mucopolysaccharide character of the other antigens.

Heavy metals and salts. Recent investigations confirmed and extended observations made on the action of such substances upon red-cell membranes since the beginning of this century (87, 124). Numerous metals and their salts hemolyze (e.g., 48); thus, copper acetate and copper sulfate hemolyzed and agglutinated erythrocytes, the former effect being given by lower concentration of the salts. Serum prevented the effect. Similarly, Jandl and Simmons

(71) found the majority of multivalent cations capable of agglutinating washed red cells. These authors confirmed in essence the findings of Neufeld and Etinger-Tulczynska (124), who first noted that certain inorganic colloids and neutral salts, notably "alum," had a strongly agglutinating effect. In contrast to tannic acid, however, there was little or no hemolysis of "alum"-treated cells in the presence of complement.

Addition of subagglutinating amounts of cations to red cells suspended in various proteins led to sensitization of the red cells with these proteins, as determined by the Coombs test (71). Sensitization of red cells with proteins via cations, such as Cr^{3+}, was specific for the protein fraction employed. Cations, under appropriate conditions, are thus a suitable agent to couple proteins to the red-cell surface. During sensitization with metalloprotein complexes red cells did not bind guinea pig complement and were not thereafter hemolyzed by complement. The experiments of Jandl and Simmons (71) indicate that the cations are bound by the erythrocyte surface carboxyl groups. The attachment of about 8×10^7 molecules of C^{3+} to each red cell was sufficient to induce agglutination, providing no protein was present. The agglutination may thus be due in part to a reduction in the surface potential of the red cells. Similar agglutinating and also lysing effects have been found for positively charged substances of higher molecular weight, such as polylysine (125), which was thought to act mainly by nonspecific electrostatic attractive forces. Adsorption may take place not only on the exterior of the cell membrane but inside the structure as well. This is made likely by the ability of polylysine to cause hemolysis.

Tomcsik and Scherrer-Gervai (167*a*) reported that high concentrations of neutral salts fragmented trypsin-treated, but not untreated, beef erythrocyte membranes. The process began with formation of globules and ended in the production of small granules. No explanation of this phenomenon was given. In in vivo hemolytic conditions, however, erythrocyte fragmentation is not rare, and fragments, "schistocytes" (consisting of membrane and hemoglobin), and their genesis were described by Ehrlich as early as 1892 (37).

The interesting observations on the action of hydrazine and phenylhydrazine on erythrocytes by Muirhead, Groves, and Bryan also must be mentioned here (117). The authors noted positive Coombs tests in dogs injected with phenylhydrazine, and observed hemolysis, agglutination, or a positive Coombs test in vitro with hydrazine sulfate. The mode of action of these agents was considered to be either an uncovering of proteins in the erythrocyte surface capable of reacting with the antiglobulin reagent, or an alteration of the erythrocyte surface enabling it to adsorb normal plasma proteins.

Detergents and saponins. Detergents and saponins are powerful hemolytic agents. The detergents may be either ionic or nonionic, and the saponins are either triterpenoid glycosides or steroid saponins (e.g., digitonin).

Valuable chemical information has been obtained by studying the action of surface-active compounds on erythrocytes. Thus, digitonin reacts on an equimolecular basis with all the cholesterol of the erythrocytes. Digitonin hemolysis is strictly specific in that it does not react with the lecithin of the erythrocytes (148). The interaction of digitonin with cholesterol derivatives appears to depend on the appropriate configuration of the substituents at C3; of ancillary importance is the position of substituents at C5 (131). Digitonin hemolysis removes all hemoglobin from the erythrocyte membrane and leads to a complete dissolution of the erythrocyte membrane structure. This is in contrast to hemolysis with water where the membrane remains coherent. It was also shown, by surface precipitation with an anionic dye, that hypotonic lysis causes one single defect in the red-cell membrane, whereas lysis due to surface-active agents such as saponins is associated with multiple regions of membrane breakdown (83).

Rideal and Taylor (139; *see also* Pethica and Shulman, 128) found that synthetic anionic detergents destroyed red cells by two mechanisms, one rapid and the other slow. Fresh red cells, placed in saline, gradually lost a component of their membrane whose presence was apparently necessary in the cell membrane for rapid lysis to occur. The substance removed rapidly by the detergent was most likely a phospholipid, since β-lecithin but not cholesterol or bovine serum sensitized washed cells to rapid lysis. The slow hemolytic process, on the other hand, consists of diffusion of the adsorbed detergent

into the lipoprotein-cholesterol complex in the cell membrane and the progressive breakdown of the membrane complex. The erythrocyte can replace phospholipid which it has lost by diffusion from the cell membrane; it appears that phospholipid bound as lipoprotein (the site of attack for slow lysis) is released when necessary to form free lipid on the cell membrane.

A related observation recently reported by Dingle and Lucy (32*a*) must be mentioned here: the hemolytic effect of the polyene, vitamin A. The changes were similar to those observed after exposing erythrocytes to saponin. The authors concluded that the site of action of vitamin A is at the lipoprotein membrane of the cells. Hemolysis, which is likely to be due to an oxidative quantum process (11*a*), was described as early as 1905 by Sacharoff and Sachs (142*a*) who observed the photodynamic effect of fluorescent dyes on erythrocytes.

Changes Due to Fixation of Antigenic Substances

Antigenic substances adsorbed onto erythrocyte surfaces may be from quite various sources, including the host's own tissues. Adsorption frequently occurs in vitro and may play an important pathological role in vivo. Adsorption may be reversible or irreversible under physiological conditions, as has been shown repeatedly for bacterial products (122).

In vitro changes of the erythrocyte surface due to fixation of antigenic substances will be considered first. The effects can be twofold, namely bestowal of new antigenicity to red cells and interference with the analysis in vitro of indigenous erythrocyte antigens. Thus, Hornung and Baer (64) made the interesting statement that pre-existing A or B but not Rh_0 (D) agglutinogens reacted less with antiserum when the red cells were coated with B or A substances, respectively, subsequent to tanning. This would indicate blocking of the pre-existing receptors by the adsorbed blood group substances.

Antibodies and antibodylike substances. The most obvious change of the erythrocyte surface is that due to fixation of proteins onto specific receptors. These proteins usually are antibodies and the fixation is immunologically specific. However, fairly firm nonspecific absorption of serum globulin also appears to occur (e.g., 12, 129*a*). Antibodies, being proteins, are of course antigenic. Antibodies of different function and originating from the same species are antigenically so similar (as long as compared within the two main fractions of globulin types) that distinctions need not to be made for the purpose of this review. The immunochemical similarity between the specific antibodies of one species forms the basis for the widespread use of antiglobulin antibodies to detect "coating" of erythrocytes. Antibodies in turn may fix other proteins such as complement components onto red cells. As already mentioned, a large number of plant proteins may react in vitro similarly to antibodies. These reagents are useful in the elucidation of erythrocyte receptors provided appropriate precautions in interpretations of results are taken (158*a*). An additional observation of interest is the finding that antibodies (Springer et al., *to be published*) and plant agglutinins (94*a*) may have a considerably tighter fit with closely corresponding erythrocyte antigens than with related ones and can only be eluted readily by classical procedures if they are adsorbed on the latter structures.

Bacterial antigens. The two major classes of antigens, proteins and polysaccharides, generally behave differently as far as their ability to adsorb onto erythrocytes is concerned. Purified, not denatured, protein preparations are not usually adsorbed by ordinary erythrocytes in vitro, except those reacting with strictly specific receptors (mentioned above). Boyden (15) found that treatment of erythrocytes with those preparations of inulin which, in high dilutions, agglutinated erythrocytes rendered them capable of adsorbing proteins. Tannic acid which also agglutinates proved to be most effective in enabling red cells to adsorb proteins. Protein antigens may also be adsorbed onto formaldehyde-treated erythrocytes. Tuberculin purified-protein derivative has been coupled onto such cells (26). It has also been stated that protein becomes fairly strongly adsorbed to erythrocytes sensitized with polysaccharidic fractions of tubercle bacilli (189).

Most, if not all, polysaccharide complexes from bacteria are fixed onto untreated red cells under the appropriate conditions in vitro (122). Thus, Boyden (14) found that erythrocytes of various species adsorbed serologically specific substances from a steam-killed culture of *Pfeifferella mallei* in synthetic broth. Human, horse,

sheep, guinea pig, and fowl red cells were rendered agglutinable by antimallein antibodies. Ox and pig red cells, the antigen from mallein solutions, took up but were not agglutinated by antimallein sera, although they adsorbed the antibodies. Sheep and fowl red cells required about eight times more mallein for sensitization than did human and horse erythrocytes. There was no quantitative relationship in the various preparations between the amount of sensitizing antigen and the amount responsible for complement fixation.

As yet, little is known about the nature of the erythrocyte receptors to which bacterial antigens attach, which part of the bacterial molecule fixes itself to the red-cell membrane, or by what mode. In general, the fixation of polysaccharide complexes to human erythrocytes is said not to interfere with the erythrocytes agglutinability by either anti-A, anti-B, or anti-Rh_o (D) reagents (122). It must be assumed, therefore, that they attach to receptors which are different in location and specificity from these antigens.

Interestingly, blood group substances of human or animal origin, with the possible exception of some from meconium (152), will not coat ordinary erythrocytes, whereas blood group active bacterial substances which are normal O-somatic antigens will readily do so (155). The water-soluble mammalian blood group substances consist of about 75% polysaccharide, with the remainder being peptide.

The first reported efforts to characterize the red-cell receptor responsible for adsorption of bacterial antigens appear to be those of Boyden (14), who noted that an alcohol-ether (3:1) extract of horse red cells contained some substance(s) capable of inhibiting competitively the sensitization of horse red cells by mallein to agglutination by antimallein sera. An imaginative investigation, which has yielded at least a limited amount of information on the mode of attachment of bacterial antigens to erythrocytes, is that of Davies et al. (32), who based their studies on the well-known observations that both heating and alkali treatment of bacterial polysaccharides, isolated by any of the commonly employed procedures, enhances sensitizing activity (122). The absence of a direct relation between coating ability and the lipid component of bacterial polysaccharide complexes was noted; the specific polysaccharide of *Aerobacter aerogenes*, which contains no lipid, requires alkali treatment for activity. The effect of alkali was shown in this instance to be the removal of O-acetyl. When alkali treatment was not required for maximal coating activity, no loss of O-acetyl was observed upon alkali treatment, as for instance with a *Pasteurella pestis* product which contained about 50% phospholipid but was as active with as without alkali treatment. Reacetylation of alkali-activated polysaccharides led to inhibition of their ability to be adsorbed onto red cells. The serologically specific activity, on the other hand, was frequently decreased by alkali treatment of the polysaccharide complexes. Based on these results, Davies and his colleagues considered it unlikely that bacterial lipid plays a significant part in prevention of adsorption onto erythrocytes, as had been claimed by a number of other investigators. Some fractionation of antigens by adsorption of lipopolysaccharides onto red cells was also reported; approximately 25% of serologically active material remained unadsorbable (32, 96) under varying experimental conditions and in the presence of an erythrocyte excess. It was observed recently (136*a*) that the type-specific pneumococcal polysaccharides S II, S VI, and S XVIII, after periodate oxidation, fixed to erythrocytes of most mammals tested, as measured with antipneumococcal horse sera. The fixation of the oxidized polysaccharides was believed to be due to combination of the oxidation-produced aldehyde groups with amino groups on the red-cell surface. The periodate oxidation did not destroy the serological specificity. Reduction of the aldehyde groups of the oxidized polysaccharide to alcohols or oxidation to carboxylic acids eliminated demonstrable red-cell fixation, which was also absent prior to oxidation.

These studies have shed some light on which groupings on bacteria interfere with adsorption onto red cells, but in general they do not allow a conclusion as to which structures on the microbial polysaccharides and on the red cells interact to result in the phenomenon of adsorption. Also, it is not easy to picture the mechanism of the adsorption, as it has been stated that at least 11 lipopolysaccharides of different serological specificity might be fixed onto the same cells in maximal concentration without interfering with one another, so that a specific erythrocyte receptor, possibly of lipid nature, for each of these 11

substances must be considered. However, some investigators maintain that several different lipopolysaccharides compete for the same receptors (e.g., 94*b*).

Interference between agglutinability of two adsorbed microbial antigens from the same bacterium, though one was not a lipopolysaccharide, has been observed; the Vi antigen of *Salmonella typhosa* strongly interferes with the agglutinability of the O antigen of this bacterium and with other lipopolysaccharides (123). This effect, like that of some other acidic bacterial polysaccharides which in addition adsorb faster than O antigen, is most likely due to the increase in negative surface charge and subsequent repulsion of the red cells to which they are adsorbed, as has been shown clearly by MacPherson, Wilkinson, and Swain (96) This increased negative surface charge interferes also with the agglutinability of red cells by antibodies directed against preexisting receptors (152).

On the other hand, neither adsorption of antibodies nor hemolysis in the presence of complement are interfered with by coating with such acidic polymers. Neither of these two latter processes requires linkage between two or more erythrocytes. These findings are reminiscent of those reported above on the inagglutinability of native ox erythrocytes. It is significant that Vi antigen coated onto any of the red cells, including the poorly agglutinable ones of ox and alligator, leads to red-cell agglutinability by anti-Vi antibody (123) which apparently is directed *a priori* against groupings of a highly charged molecule.

That erythrocytes from different animal species may behave differently after coating with the same bacterial polysaccharides, with regard to agglutination and hemolysis, has been pointed out repeatedly by Neter and his colleagues (122). This group of investigators also noted that treatment of coated erythrocytes with proteolytic enzymes usually does not enhance significantly the agglutinability of already well-agglutinable red cells but much improves that of poorly agglutinable alligator and ox red cells.

Immune adherence has already been mentioned. Antigenic substances may become attached to the surface of primate erythrocytes, provided that homologous antibodies and complement are present (121). This phenomenon is not only of importance when antigenic changes of erythrocyte surfaces are to be considered, but it may point to a possible defense function of erythrocytes and their receptors in vivo (121), in that erythrocytes may aid in opsonizing, transportation, and detoxification.

Antigens may also be fixed, at least in vitro, onto erythrocytes after they have been coupled to nonagglutinating antibodies which in turn are adsorbed onto their homologous erythrocyte surface antigens (28). The attachment of (antigenic) substances onto erythrocytes by purely chemical means has also been accomplished (133; subsequent to the work by Landsteiner and van der Scheer on erythrocyte stroma), and may be a valuable aid in elucidation of number and nature of those erythrocyte structures with which the chemicals react.

SIGNIFICANCE OF ENZYMATIC RESEARCH ON ERYTHROCYTE SURFACES FOR CLINICAL MEDICINE

Although this review deals with fundamental aspects of erythrocyte membranes and their antigenic structures without regard to the practical usefulness of such investigations, it can confidently be predicted that ultimately clinical medicine will benefit by comprehension of pathophysiological processes underlying disease. This usefulness may not be restricted to the disease processes of red cells but may extend to other cells as well, even to those outside the circulation. Thus, further comprehension of some properties of membranes of malignant cells and of the mechanisms of metastatic processes, such as those determining the local invasiveness of cancer cells, may be achieved (e.g., 142). In addition, the mode of fixation of a pharmacologically active agent to its specific receptors [e.g., tetanus toxin to both erythrocytes and receptors in the brain (38)] may be elucidated.

The applicability of knowledge gained during the study of erythrocyte surfaces and their alteration by enzymes already involves three major aspects: (i) the pathogenesis of some hemolytic anemias, (ii) the related involvement of erythrocytes in the organism's defense mechanism, referred to earlier, and (iii) the usefulness of enzymes in diagnostic and therapeutic aspects of erythrocytes.

Hemolytic Anemias

Hemolytic anemias are due either to destruction of normal red-cell membranes by some

pathological process (including accelerated physiological mechanisms) or by toxic compounds within the organism, or to some congenital defect in the internal red-cell enzyme metabolism, which makes the membranes more susceptible to ordinary enzymatic hemolysis. The latter appears to be the case in paroxysmal nocturnal hemoglobinuria (59).

In general terms, it may be said that the integrity of red-cell structure depends on the metabolic state of the cell. Lysis of erythrocytes results because the lytic agents interfere with metabolic-enzymatic reactions which are necessary to maintain structural integrity, e.g., drugs, such as the 8-amino-quinolines, in susceptible individuals, or enzymes like the complement complex. Lysis may also be due to direct impairment of the structure, such as that caused by detergents and saponins.

The common denominator for *all* specifically erythrocyte-damaging substances is the presence of appropriate receptors; the noxious effect (direct or indirect) is always preceded by the fixation of the agent. Hence, lack of specific receptors and natural resistance are closely interconnected (38).

Not much is known about the enzymatic mechanism involved in the decreased membrane resistance of the congenital anemias; therefore, they will not be discussed here. Instead, the reader is referred to the treatise by Dacie (29) and a recent symposium (149).

Hemolytic disorders may be caused by innumerable toxic agents (38) such as those described in the in vitro experiments above, but they may also be due to disease involving *de novo* production of antibodies, in most instances due to virus or bacterial infection. These antibodies may cross-react with pre-existing erythrocyte structures and damage them. The damaged erythrocytes may then be subject to subsequent attack by enzymes present in the organism under physiological conditions. Although a similar erythrocyte-eliminating mechanism is probably involved in the notorious hemolytic anemias of erythroblastosis fetalis and transfusion reactions, one must be cautioned against overemphasis of the importance of autoimmunization as primary pathogenetic factor.

The importance of pre-existing lytic substances acting on abnormal erythrocytes was first recognized clearly by Ehrlich and Morgenroth (39), who assumed that all blood sera contained various enzymelike agents, some of which were endowed with complement-like activities, and that these substances probably were of fundamental importance in the pathogenesis of certain severe anemias. They concluded (39) that autolysins would not normally be able to exist; "only when the internal regulatory mechanisms are no longer intact can serious dangers arise" (reviewer's translation). Ehrlich clearly considered the possibility of autoimmune hemolysis and actually observed experimentally induced autolysins, although only in a single goat (38). This historically important fact contrasts with the accepted opinion that Ehrlich considered autoantibodies to be an impossibility.

The production of antibodies against the host's own cells is looked upon as an autoimmune process. Although autoagglutination in vivo is a rare phenomenon, it was observed as early as 1890 in man (138) and somewhat later, by Landsteiner, in animals. A careful analysis of autohemagglutination has been published by Rosenthal and Corten (141). That autoimmune processes may cause disease in man was first shown by Donath and Landsteiner (33) for "paroxysmal cold hemoglobinuria."

Lüdke, in 1918 (95), was probably the first to state that lysins in severe streptococcal or malarial infections were responsible for hemolytic conditions. He also stated that he found "autohemolysins" in the serum of some dogs after he reinjected them with their own blood. Spleen particles from dogs with autolysins hemolyzed the animal's own erythrocytes in vitro and induced anemia when injected into healthy animals. Neither effect was noted with spleen extracts from healthy dogs.

Hemolytic anemias with high-titer cold hemagglutinins have been found associated with many viral and bacterial infections (43, 65). These cold agglutinins may become fixed to the erythrocyte by complement components. In a second variety of autoimmune hemolytic anemia, the autoantibodies preferentially react at body temperature. They are commonly found during or after virus infection, and there are indications that such warm autoantibodies are frequently directed against the "nucleus" of the Rh-Hr substance (181). Hemolytic anemia complicating infectious disease may result from several mechanisms. In some diseases, hemolytic anemia is accompanied by autoagglutinins; a secondary hemolytic anemia may also be due to delayed

passage of erythrocytes in the spleen (see above) and resulting enzyme or lysolecithin action; in addition, there may be hereditary inferiority of the red cells. Further reasons are given below.

Some of the hemolytic anemias are apparently caused by antibodies induced by extraneous agents which possess antigens closely similar to those inherent to the host's red cells. Thus, cross-reacting antibodies may be responsible for so-called acquired immune hemolytic anemias, and study of the affinity of these antibodies to the red-cell surface frequently permits an accurate assessment of the severity of the patient's hemolytic disease (72). The extraneous antigens responsible for induction of such antibodies may even become attached to the erythrocytes. It may thus not be necessary that the antigenic substrate against which the hemolytic system is directed be an intrinsic part of the erythrocyte surface. A striking example is the case of numerous gram-negative bacteria which possess blood group specificity (155, 158*c*). Constituent parts of these fix to erythrocytes under appropriate conditions in vitro, cross-react with human isoagglutinins, and induce ordinary blood group agglutinins in man and in animals (158*c*, 158*d*). Several British workers have reported the acquisition of blood group B-like antigens by human erythrocytes in vivo (163). This acquisition was thought to be possibly due to bacterial products, especially since it occurred only in patients with severe intestinal disorders or gangrene (increase of permability of body surfaces); it was not reported whether there was any in vivo hemolysis. There are indeed indications that, under severely pathological conditions, blood group active bacterial products are able to coat erythrocytes in vivo; however, studies on man and animals indicate that such coating effects are exceptions rather than the rule (155). The in vivo fixation of products from helminths (84), bacteria (16), and viruses (107) onto erythrocytes has been claimed to follow infection of man or injection of these organisms into experimental animals.

These observations need confirmation, but they point to the possible role of antigens adsorbed to the erythrocyte surface in the pathogenesis of hemolytic disorders, and have actually been observed in chick embryos for the virus of louping ill (20). Numerous antigenic substances may be adsorbed to the erythrocyte surface, inducing antibodies against themselves. Such antibodies may then destroy the red cells to which the extraneous antigens are adsorbed (122). Haptenic substances may act similarly, imparting a new antigenic specificity to the erythrocytes to which they are adsorbed. Many drugs have been implicated in such a mechanism. As was first shown by Harris (55), based on Ackroyd's finding with platelets, in vitro demonstration of the immune mechanism in many instances is contingent, in addition to the usual three factors (antibody, complement, and antigen), on the addition of some of the haptenic drug against which the organism is sensitized.

After considering antibodies directed against intrinsic and adsorbed antigens of erythrocytes, a third possibility must be mentioned, namely, that enzymes act on erythrocytes in vivo and may thus render them autoantigenic. Abnormally agglutinable erythrocytes may occur in individuals with hemolytic syndromes suffering from severe infections of bacterial or viral origin (31, 41). The erythrocytes are poly- or panagglutinable at 37 C, and it is assumed that this condition may be due to enzymatic action of the infecting organism on erythrocyte-membrane antigens (31), in analogy to the Thomsen-Friedenreich phenomenon. It is not surprising that these T-like receptors were not completely identical in all cases (e.g., 57), since it is well known that "T enzymes" (sialidases) from various sources show slight variations in their action. The enzymatic origin of some instances of abnormal agglutinability is made likely by experiments of Ejby-Poulsen (40). These experiments were said to demonstrate that subcutaneous and intraperitoneal injection into guinea pigs of pathogenic pneumococcus XIX led within a few days to T transformation of the erythrocytes of these animals. Further, it was claimed that their sera induced T transformation of erythrocytes in vitro. Intravenous injection of this "T enzyme" led to hemolytic anemia, but experimental details were not given and the hemolytic disease was not continuing (18, 40).

Also, various authors showed that animal erythrocytes, after in vitro treatment with influenza virus (160) and receptor-destroying enzyme, trypsin, or papain (18), were quickly removed from the circulation upon reinjection. As was to be expected, the panagglutinins disappeared from the circulation during elimination of erythrocytes. Antibodies to influenza virus were not involved. The occurrence of the

Thomsen-Friedenreich phenomenon has also been observed (23) in vivo in a septic patient, from whose circulation a T-transforming *Corynebacterium* was stated to have been isolated.

The involvement of carbohydrases has been considered in physiological as well as pathological red-cell destruction, especially since such an in vitro effect has been claimed for β-glucuronidase, β-glucosidase, lysozyme, and hyaluronidase (93).

The potential clinical-therapeutical importance of those enzymes which transform blood group A and B antigens into H(O) specific substances must be mentioned, as it appears desirable to increase the amount of H(O) specific blood available.

Therapeutic and Diagnostic Aspects of Enzyme Treatment of Erythrocyte Surfaces

The general usefulness of enzymes in routine blood banking to facilitate recognition of antibodies has already been mentioned. An enzymatic test in the diagnosis of infectious mononucleosis was originated by Wöllner (183), and a modification of it (156) has proved most sensitive and reliable in the author's laboratory for routine diagnosis of infectious mononucleosis; 338 patients with infectious mononucleosis and over 100 control cases have been studied. No false positive and, as far as can be determined by the usual hematological and clinical criteria, no false negative reactions were obtained. A lower incidence of positive reactions or a lower titer in patients of blood group A, as has been claimed by some authors, was never found. Similar observations on the reliability and sensitivity of enzymatic procedures in the diagnosis of infectious mononucleosis were made subsequently by other workers (118, 167). The principle of this procedure is the selective destruction of the mononucleosis receptor of sheep erythrocytes by plant proteases, such as papain, bromelin, or ficin; if the human serum in question is absorbed with sheep red cells from which the infectious mononucleosis receptor has been selectively removed, then all anti-sheep cell antibodies will be taken up by these erythrocytes except those against the infectious mononucleosis receptor. If such an absorbed serum is now titrated against ordinary sheep erythrocytes, any antibody (titer) found in properly controlled experiments is directed against the heterogenetic mononucleosis agglutinogen.

That enzymes elaborated by microorganisms may alter erythrocyte surfaces so as to render them unsuitable for blood grouping and typing has been noted in numerous laboratories, and has led to stringent conditions for their storage and preservation.

OUTLOOK

The studies reported here indicate the central position the erythrocyte occupies in cell research. Investigation of the erythrocyte surface and its antigenic structure has involved a number of disciplines from physical chemistry to clinical medicine, and has pointed to the relation between genetically determined receptors on the red-cell membrane and on innumerable different cells, including those in the mammalian brain and on bacteria. Also, it points to the genetic determination of many of its structural and functional properties and their dependence from and interaction with their environment. While the breadth of the problem has been delineated, it has become evident during the course of this review that there is as yet little depth to our insight into structure, function, and metabolism, as well as into the antigenic determinants, of red-cell surfaces. The apparent simplicity of any reaction, whether it is caused by periodate, enzyme, or antibody, brings, in reality, a multitude of changes to the erythrocytes, some facets of which are quite unexplored. But the abundance of advanced methods which may now be applied to investigation of the erythrocyte membrane, and the firmness of the knowledge already acquired, which has brought the essential problems into clearer focus, make rapid progress likely from here onward. It does not seem too much to expect that from the study of enzyme action on red-cell membranes a deeper insight into the basis and meaning of antigenic specificity of cell surfaces and of synthesis, function, and degradation of erythrocytes and cell receptors in general may result. When that understanding is approached, much of what is "fashionable" today, and, therefore, subject to redundancy, will be viewed in its proper relation to the whole. The red cell can serve as a model of basic processes in host-virus relationships, and in those between a toxin or a drug and the receptor they need before they can begin to exert their influence.

Also, the action of some vitamins and hormones under physiological conditions may well be brought closer to comprehension by studying their in vitro effect on erythrocyte-membrane receptors. Understanding of the function of a receptor and its integration into the entirety of a cell and its host may then be advanced above that of today, which is still essentially that of Paul Ehrlich and Karl Landsteiner.

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