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THE PHYSICO-CHEMICAL NATURE OF THE BOVINE ERYTHROCYTE

ISOANTIGENS; SOME ASPECTS OF THE IMMUNOCHEMISTRY

OF THE F AND V ANTIGENS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Charles Louis Hatheway, B. Sc., M. Sc.

* * * * * * *

The Ohio State University

1964

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Studies in Agricultural Biochemistry:
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Studies in Genetics and Biometrics:
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is conceivable that if simple, definable haptenic prosthetic groups were responsible for certain specificities, antisera could be produced against the conjugated hapten specifically, eliminating the need for some of the difficult absorptions which are sometimes necessary. Reagent analysis could be done partially by using the hapten-inhibition technique.

If the antigenic components were of such a nature that they could not be duplicated, but could be isolated and purified from their biological source, these preparations could provide better immunizing materials than the entire erythrocyte, or erythrocyte membrane. They would provide an antigenic stimulus in such a way that the antibodies would be restricted to reactivity against the desired structure. This would permit more widespread use of heteroimmunizations.

The major stimulus for this investigation has been a curiosity to explain the basis for the mysterious reactions observed in the blood typing laboratory which have been so remarkably repeatable and in accordance with the established rules. At the present state of development the cattle blood antigen field provides a vast amount of knowledge of the genetic and serological behavior of entities of whose physico-chemical nature we know almost nothing.
REVIEW OF LITERATURE

Introduction

The scope of this review includes the areas of study which have yielded information on the physico-chemical nature of antigenic determinant groups. After an introduction into the subject a mention will be made of some of the representative and some of the classical works of antigen isolation and characterization of antigenic determinants. Next will be a coverage of studies of bloodgroup reactive materials derived from non-erythrocyte sources. Those studies have provided the bulk of our knowledge on the determinant groups of blood factors up to the present. These sources have easily provided large quantities of materials which were readily purified without loss of activity. Also a look at the biochemistry and physical nature of the erythrocyte seems in order so that the knowledge from the previous areas (antigen isolation and nature of antigenic determinant groups) might be applied to erythrocytes.

It is difficult to draw the line between biochemical studies on the erythrocyte and studies on the erythrocyte antigens, but an attempt is made with the start of the
fifth section of this review, which is limited to the blood group antigens of the human erythrocyte. The last section includes the studies on the antigens of bovine erythrocytes.

The most up-to-date picture of the blood antigen systems of cattle is very well presented by Stormont (1962). This review shows the complexities which have been accumulated by serological studies over the past 24 years and they probably comprise the most involved antigenic systems yet elucidated. Kabat (1956) gives a comprehensive review of the blood group substances and it is restricted almost exclusively to the A, B, H, and Le\textsuperscript{a} soluble substances. Sources include hog gastric mucosa, human saliva, human amniotic fluid, human ovarian cyst fluids, and meconium (fluid from intestine of full term fetus).

Morgan’s Croenian Lecture (Morgan, 1960) integrates the diverse knowledge obtained from the intensive studies on the soluble substances and depicts the processes which take place on the molecular level mediated by the genetic factors. Morgan also attempts to incorporate in the scheme the events taking place in the incorporation of the characters into the red cell membrane.
Uhlenbruck (1961) reviews the work on the studies on the erythrocyte antigens with some emphasis on the M & N factors and the virus receptors, indicating the role of sialic acids in these specificities. The most recent review on the subject of the erythrocyte and its component structures has been provided by Springer (1963). This review is aimed quite a bit at obtaining an overall picture of the erythrocyte membrane. Reference is made to alterations brought about by enzymes, chemical agents, absorption of foreign substances (e.g., bacterial antigens, soluble antigens) and others. The results of attempts to characterize antigens by isolation and degradation techniques are presented. Other reviews which provide insight and inspiring ideas are the Prankerd's monograph entitled The Red Cell (Prankerd, 1961) and a general review on mammalian cell membranes by Weiss (1963).

Studies on antigens - general

Certainly Landsteiner has exerted the greatest influence in this area as is evidenced by the many contributions cited in his monograph (Landsteiner, 1945). Others in the field made much progress by application of the methods which he provided. A classic example is that of Avery and co-workers. Dochez and Avery (1917) noted the
elaboration of a specific polysaccharide antigen into the medium by Pneumococci during their growth. Avery and Goebel (1929) showed first that simple sugars could provide specific determinant groups when conjugated to protein by diazotization (see Landsteiner, 1945, p.156ff). Then applying the technique to the soluble polysaccharide they succeeded in immunizing rabbits, protecting them against Type III pneumococcus and obtaining an antiserum which was specific in its reactivity against the Type III organism and its capsular polysaccharide (Avery and Goebel, 1931). Continued studies on this antigen resulted in elucidation of its structure in various pneumococcal types, and ultimately made the very significant contribution to the field of genetics that the DNA molecule is the carrier of genetic information (Avery, MacLeod and McCarty, 1944). The work with the pneumococcal polysaccharide antigens was simplified by the fact that they are soluble and are extruded into the medium and required no extraction of the cell mass. This is a common situation in bacterial immunochemistry and a more recent example is seen in the work of Lawton et al. (1963) with the V and W antigens of Pasteurella pestis.

The work with the gram negative organisms necessitated use of extraction methods for obtaining the antigens.
The Boivin antigens of Salmonella were first obtained by extraction with trichloracetic acid (Boivin and Mesrobeanu, 1935). These were found to be more complex than the pneumococcal antigens. Other extraction methods were devised for further studies on antigens of gram negative organisms (e.g., see Morgan, 1937). Professor Morgan had much of his earlier experience with the bacterial antigens before becoming involved with the human ABO and Le^a substances. The method of antigen extraction from bacteria which has been carried over into the field of erythrocyte antigens by numerous investigators is that of Westphal et al. (1952). It consists of treatment of the cell mass with a phenol-water (or phenol-saline) emulsion at low temperature (4°C), separating the two phases, and recovering the aqueous phase. An alternate procedure consists of extracting the cell mass with the single phase phenol-saline mixture which exists at higher temperatures (65°C) and then recovering the aqueous phase which results upon cooling to 4°C. Residual phenol is eliminated by dialysis.

**Human blood group substances from non-erythrocyte sources**

The soluble human blood group substances (Kabat, 1956) have provided the material from which the basis for the antigenic specificities of the ABO and Lewis blood
systems were determined. The fluid from human ovarian cysts has provided the best source because of the large quantity (up to several liters volume) which may be obtained from an individual donor (Morgan, 1963). Analysis of the materials possessing the A, B, H, and Le\textsuperscript{a} activities does not show a clear-cut difference in components which would indicate what structures were responsible for the serological specificities. It was found, however, that L-fucose was an effective inhibitor of the anti-H agglutinins of eel serum and plant extracts (Morgan, 1960).

The action of enzymes from \textit{T. fetus} was shown to inactivate all of the blood group substances. It was found that addition of L-fucose to the medium prevented destruction of H while it would not protect A or B from destruction. On the other hand, addition of N-acetyl-D-galactosamine would prevent destruction of A but not B or H, and D-galactose in the medium would protect B but not A or H. (Watkins and Morgan, 1955). This gave a clue to structures involved in the A and B specificities. The N-acetyl-D-galactosamine and the D-galactose by themselves were not sufficient to inhibit the antibody reactions.

Mild hydrolysis of human A-substance produced larger fragments than the monosaccharide units resulting from complete hydrolysis. Cote and Morgan (1956) isolated
five nitrogen containing disaccharides after such treatment. One of them was identified as O-α-N-acetyl-D-galactosaminoyl-(1→3)-galactose. This proved to be a very effective inhibitor of the anti-A isoagglutinin. The others were ineffective. This type of investigation brought about the search for a hydrolysis method which would tend to produce the larger fragments, i.e., oligosaccharides. Painter (1960) has devised a method which has been quite successful in this respect. A soluble polystyrenesulfonic acid which is non-dialyzable catalyzes the hydrolysis of the non-dialyzable substrate inside a dialysis bag. The fragments which result are dialyzable and are recoverable in the outer dialysate.

Rege et al. (1963) reported the isolation of three new tri-saccharides from the partial hydrolysates of A, B, H, and Leα substances. These are in addition to the oligosaccharides reported previously by the Morgan group (Cote and Morgan, 1956; Painter et al., 1962; Painter et al., 1963; Cheese and Morgan, 1961) and by the Kabat group (Schiffman, Kabat and Leskowitz, 1962). Rege et al. summarize all of these contributions by listing the oligosaccharides which are common to the hydrolysates of all four substances (4 disaccharides and 3 trisaccharides), those occurring only in the hydrolysates of A-substance
(1 disaccharide and 2 trisaccharides) and those occurring only in the hydrolysates of B-substance (1 disaccharide and 2 trisaccharides). From these data they are able to propose two alternate possible five-unit chains for each substance A and B and two alternate four-unit chains for the H and Le\(^a\) substances. The chains proposed for the partial determinant segment of the H and Le\(^a\) substances are identical. The difference between the H and Le\(^a\) substances is probably due to the position of fucose residues which are always cleaved off in the hydrolysis procedures in the preparation of the oligosaccharides. In the unhydrolyzed substances there are large amounts of sialic acid and fucose whose glycosidic bonds are very susceptible to acid hydrolysis. These are believed to be attached as branching units to the sugars which make up the main carbohydrate chains. The sialic acid has not been shown to be involved in any of the ABH and Le\(^a\) specificities.

Most recently Schiffman et al. (1964) reported the preparation of an active fragment from A and B substances which is many times more potent than the most active oligosaccharide previously studied. It was prepared by treatment with 0.2M NaOH in 1% NaBH\(_4\) at room temperature. Partial purification was performed by paper chromatography. It is postulated that the active fragment possesses the
entire antigenic determinant. The fragment has not yet been completely purified.

The peptide portion of the blood group substances does not seem to play any part in the serological activity. Pustai and Morgan (1963) have determined the amino acid composition of each of more than twenty blood group specific mucopolysaccharides. They were found to be closely similar irrespective of serological specificity within the ABO and Lewis blood group systems.

Blood group active materials are also derived from a variety of other sources (Springer, 1958). Products of some of the higher plants as well as some of the Salmonella species of bacteria show specific H (O) activity. An active B-substance is produced by E. coli O86 and an A active substance is found in E. freundii. An interesting point was made from the study of one of the H-reactive substances. This material was very effective in inhibiting the anti-H reagent eel serum but was ineffective against the anti-H agglutinins from lotus extract (Springer et al., 1964). Further, it was found that the purified active material lacked L-fucose, which seems to provide the basis for H-specificity. The unit responsible for the H-reactivity (against the eel serum only) was isolated and identified as 3-O-methyl-D-galactose. It was pointed out
that when this compound and L-fucose are viewed in the more realistic models of the "chair and boat" forms which sugars assume, rather than the Haworth structures, it can be seen that an identical surface is provided by a portion of each of the two molecules. In this case, the active site is provided by less than one monosaccharide unit. This also points out that serological reagents which seem to be detecting an identical entity may not in fact be doing so.

Bigley et al. (1963) reported on a glycopeptide from a *Pseudomonas* organism which appears to have some relationship to the RHo(D) antigen. Antisera produced against this substance show specificity for the D factor. A precipitin reaction was demonstrable by a regular anti-D typing reagent against the glycopeptide but another anti-D reagent failed to react similarly.

**Biochemistry of the erythrocyte**

It is quite a step to go from the blood group substances of non-erythrocyte sources to the antigens as they are on the red cell membrane. Evidence suggests that they are quite different but they possess certain active groups in common. In some cases the possession of this common property by the different biological materials is strictly fortuitous as one must conclude in observing a
similar reactivity of the H substances from plant sources and the H-factor in the human erythrocyte. On the other hand, the similar reactivity of the H-substance in secretions and the H-factor on the erythrocyte of the same individual is no doubt the result of an inherited capacity to perform a specific biosynthetic process. The process in the cell membrane formation might follow (or precede or be concurrent with) a different set of other synthetic processes from the events which take place in the production of the secreted substances. This proposal seems to be indicated because of the difficulties encountered in attempts to isolate some specific substances from erythrocytes. It does not seem that such difficulties would arise if the substances in both sources were more nearly the same. Therefore, it might be helpful to look into the studies on the erythrocyte membrane structure and composition before going very deeply into the study of the membrane antigens.

This appears to be the approach taken by Springer (1963) in his review. First, he presents electron micrograph studies. Later the mechanism of hemolysis is touched on. Also, the ability of the erythrocyte membrane to adsorb components such as bacterial products is discussed. The alteration of serological reactivity of the erythrocytes discussed was not limited to the loss of a serological
reactiveness, e.g., upon exposure to an enzyme, but also to the acquisition of new reactive capacity because of the alteration. The phenomenon of panagglutination is pointed to as an example of this. Numerous studies on antigens of erythrocytes are cited, many of which are included below in this review.

Dourmashkin et al. (1962) reported on electron microscope studies of red blood cells which had been treated with saponin. Hexagonal pits were noted suggesting an erosion of lipid materials from those areas. It would appear from the electron-micrographs that the membrane was more of a mosaic structure than the smooth monomolecular-layer structure which is held by others. Pretreatment with digitonin prevented the formation of the pits, indicating that the saponin was acting on the cholesterol components. Husson and Iuzzati (1963) studied this matter by means of x-ray diffraction and the resulting data appeared to dispute the findings of Dourmashkin et al.

Calvin et al. (1945) and Moscowitz et al. (1950) fractionated erythrocyte stroma by ether extraction and alkaline washings and produced what they called "elinin", a water soluble fraction which appeared to retain all of the blood group activity of the various blood group systems. Dandliker et al. (1950) reported on physical and chemical
studies on elinin. The particles are rod shaped, with a length from 2,500 to 10,000 Å. The average particle weight is estimated to be about 40 million. The elinin particle is a lipid-protein-carbohydrate complex. On the basis of these studies, Moscowitz and Calvin (1952) attempted to postulate the arrangement of component structures in the erythrocyte membrane.

Moscowitz et al. (1950) also recovered a protein from the hemolysate after the stroma were centrifuged out. This protein, which they designated as "S-protein", was recovered by lowering the pH to 6.5. There was no blood group activity associated with this component. Recently, it was reported (Vulpis et al., 1964) that this S-protein component from the hemolysates from certain species caused agglutination of red blood cells. The S-protein from humans did not have this effect. Brusca et al. (1963) describe the action of extracts from bovine stroma on basic proteins. Aqueous extracts of stroma bring about a precipitation in the presence of lysozyme, salmine or thymohistone. It is not clear where the S-protein comes from; it could be an intracellular soluble protein or it might have been a structural component of the membrane before the hemolysis. The aqueous extracts might be similar to the S-protein.
The erythrocyte has been studied by means of electrophoresis. The major component in all hemolysates was hemoglobin. Three others were observed, a, b, and c. Component "a" has a high anodic mobility at pH 8.6, about \( 6 \times 10^{-5} \text{ cm}^2/\text{v/sec} \) (mobility of hemoglobin is \( 3 \times 10^{-5} \)). It was suggested that "a" might be derived from the cell membrane because it has the same mobility as the intact cell-stroma. A second component, "b", has a mobility intermediate between "a" and hemoglobin. Frankerd et al. (1954) investigated the blood group activity of paper electrophoretic fractions of stroma. The A and B activity migrated with the slowest moving fraction.

Other electrophoretic experiments have been performed in attempts to determine the basis for the electrical charge on the membrane surface. It was found that treatment with trypsin reduced the electrophoretic mobility of intact red blood cells (Ponder, 1950). Seaman and Heard (1960) found the same effect as a result of treatment with trypsin and N-bromosuccinimide. They conclude that this loss in negative charge was not due to elimination of phosphate groups but to disappearance of carboxyl groups through bond fission which resulted in either their elimination from the membrane or else a reorientation of surface structures. A loss of carboxyl groups from the erythrocyte surface is
certainly brought about by the action of neuraminidase which cleaves the terminal sialic acids from membrane structures. Experiments of this type were performed by Eylar et al. (1962). Their conclusion was that the sialic acids are responsible for almost the entire net negative change of the membrane surface.

On the more chemical side, several reports of amino acid content of stroma protein are available, one of which (Beach et al., 1939) gives a comparison for five different species. The analysis of the lipid components of the red cell membrane has perhaps attracted more interest than the protein portion. The lipid fraction of the red blood cell membrane resulting from alcohol-ether extraction is listed by Harris (1960) as amounting to 20% of the dry weight. It includes 30% cholesterol, 46% cephalins, 11% lecithin, and 8% sphingomyelin. One thing which must be kept in mind is that there are differences among the species. Frankerd (1961) points out, for instance, that the erythrocytes of cattle possess no lecithin.

Klenk and Lauenstein (1951) investigated the sugar-containing lipids from human erythrocytes and found a component very similar to a ganglioside except that it possessed no sialic acid. Yamakawa and Suzuki (1951) reported the presence of gangliosides in the lipid fraction of horse
erythrocytes. Klenk and Lauenstein (1952) then reinvestigated their findings with human erythrocytes and confirmed the absence of gangliosides in the human cells. The same was the case with the bovine erythrocytes. The same methods, however, did result in the isolation of a ganglioside from horse red cells (Klenk and Wolter, 1952). Yamakawa and Suzuki (1953) extended their studies on the glycolipids of erythrocyte stroma. They found that with some cattle erythrocyte stroma the glycolipid fraction lacked sialic acid as Klenk and Lauenstein (1952) had observed, but several of their bovine stroma preparations yielded glycolipid fractions which did contain sialic acid. In these latter cases they found two types of glycolipids, the type containing hexosamine (globoside) and the type containing sialic acid (hematoside). They found that human, sheep, goat, and swine erythrocyte stroma contained only globoside, dog and horse stroma probably contained only hematoside, cattle stroma contained both, and chicken stroma contained neither. The term hematoside apparently is synonymous with ganglioside. Yamakawa et al. (1960) have further studied the glycolipids of stroma from various species using different solvent mixtures (methanol-ether and chloroform-methanol) and subsequent chromatography on silicic acid columns. Those efforts showed a division of
the glycolipids obtained by each solvent extraction method into two major peaks (by carbohydrate test) in the column eluate. A purification of individual compounds had not been achieved by the time of that report.

Sialic acid was demonstrated in the protein portion of cattle erythrocyte stroma. Klenk and Uhlenbruck (1958) isolated a mucoprotein (mucoid) from cattle stroma by the phenol-water extraction method (Westphal et al., 1952) which contained 13% sialic acid. The sialic acid was found to be in the form of the N-glycolyl derivative. They found that, although the cattle erythrocytes are agglutinated by influenza virus and that the sialic acid (N-glycolyl derivative in this case) is involved in the receptor site, this mucoid was not an effective inhibitor of the virus agglutination. Their analysis indicated that the mucoid had a composition similar to the human blood group substances. A component similar to this mucoid appears to be present in the erythrocyte stroma of all species studied.

Yamakawa (1962) reviewed what is known about mucosubstances of the mammalian erythrocyte stroma. He contrasted two types of substance, erythrocyte mucolipids and erythrocyte mucoids. The former is obtained by extraction with organic solvents and the latter by phenol extraction.
Uhlenbruck (1961) reviewed the studies on the erythrocyte antigens and attempts to piece together a schematic representation of the membrane surface on the basis of the accumulated facts. The earlier attempts to isolate or study antigens from the red cell were concerned with the ABO system antigens, then much interest developed with the Rh factor. More recently the M and N factors came into prominence because of the fact that a chemically defined component, sialic acid, was implicated in these factors (Springer and Ansell, 1958). This development had come about partially as a result of intensive studies on the virus receptor sites.

Alcohol extraction of red blood cells and sometimes the use of other organic solvents has been a popular means of attacking the ABO-antigen problem. Hallauer (1934) extracted erythrocyte stroma with a series of 96, 50, 25, 25, and 10% alcohol. The first (the 96% alcohol extract) was discarded and the subsequent ones were pooled. After recovery and purification of the materials, a product was obtained which showed strong and specific A and B activities, depending upon the type of the erythrocytes. The product from 0-cells was inactive while as little as 1 mg of that
from A or B cells was effective in inhibiting the specific agglutination. Rex-Kiss (1942) studied the properties of the A substance of the erythrocytes in comparison with that obtained from saliva. The cellular A-substances were prepared by (1) hemolyzing red blood cells and then adjusting the sodium chloride content to physiological concentration; this 1:20 blood cell solution was used as the inhibitor; and (2) the method of Kossjakow (Cf. Z. Immunitats. 99:221, 1941) which consisted of a primary extraction of red blood cells with 20 volumes of 16% alcohol and then a complicated procedure for recovery and purification of the extract. The results showed that the materials prepared by the Kossjakow method were only 1/20 to 1/40 as effective as the simple blood cell solution. The comparison of the materials from saliva and erythrocyte sources by inhibition tests did not indicate any qualitative differences.

More recently, Koscielak and Zakrzewski (1960) reported their procedure for the preparation of A substance from erythrocytes by alcohol extraction. In contrast to Hallauer, they made their preparation from the first extract, i.e., with 96% alcohol which was the portion which Hallauer discarded. They did not make subsequent extractions of the residue. After several steps of
purification they produced a fraction which was 25% as active (on a wt. basis) as standard A-substance from ovarian cyst. The analytical results indicate that the blood group substance from erythrocytes is a different chemical entity from that obtained from body fluids. They appear to be glycolipids composed of galactose, glucose, amino sugars, sphingosine and fattyacids. These findings were supported by Hakomori and Jeanloz (1961) who used a different preparation procedure. They purified a glycolipid which they found to possess the A and B blood group activity. This was contrary to a suggestion by Kabat (1956) that the ABO reactivity is due to the adsorption of the soluble antigen substance onto the red cell surface.

Koscielak (1962) elaborated his studies on the erythrocyte A substance. He found that fractionation of an active preparation ("ppt M") produced two subsequent fractions "IA" and "IIA". The activity of IIA in the quantitative precipitin test was increased four-fold over ppt.M, while IA was inactive. However, IIA was not capable of inhibiting the agglutination of A cells by anti A, while ppt.M was capable of inhibiting the hemagglutination. Recombination of IA and IIA by solution in chloroform and evaporation of the solvent restored the hemagglutination.
inhibition capacity to the mixture, comparable to that of ppt. M. Attempts to recombine IIA and IIB by mixing in aqueous medium failed to restore the capacity. Recombination of IIA with IB (the analogous preparation to IA from B-positive cells).resulted in restoration of hemagglutination capacity against anti-A but not against anti-B. These findings seem to indicate something of the complexities involved in antigen effectiveness.

These observations on the association of the ABO activities of the erythrocyte with the lipid fraction are very much in agreement with Yamakawa who has emphasized this point repeatedly (Yamakawa, 1960, 1962; Yamakawa et al., 1960). He showed the ability of globoside preparations to inhibit specifically the hemagglutination reaction by ABO reagents. By specifically is meant that globoside from A erythrocytes would inhibit the agglutination of A erythrocytes by anti-A but not the agglutination of B erythrocytes by anti-B. Further, he precipitated A globoside by anti-A, recovered the precipitate, and after washing and drying the precipitate, it was extracted with ether-chloroform-methanol (1:1:1). The extract recovered in this manner was a slightly better inhibitor of the hemagglutination reaction on a weight basis than the less purified globoside before precipitation. Analysis before and after
precipitation showed that its activity could hardly have been due to an admixture of an active mucoid (Yamakawa, 1960). Yamakawa et al. (1960) showed the presence of ABO blood group activity in the methanol-ether and chloroform-methanol extracts from human erythrocyte stroma but in the silicic acid chromatography the peak of blood group activity did not coincide with the carbohydrate peaks in the eluate. They showed the presence of Forssman antigen activity in analogous extracts from sheep and cat erythrocyte stroma.

Yamakawa (1962) makes an analogy in his discussion of the erythrocyte ABO antigens and the soluble form of the antigens. He states that in the case of certain bacteria, lipocarbohydrates make up the cell wall. This is also the type of compound involved in the erythrocyte membrane. The secreted substance might be something like the capsular polysaccharide of bacteria which is exuded into the medium.

Chemical reagents whose action is quite specific have been used sometimes to obtain insight into the nature of an antigen. Typical of this approach was the use of periodate by Morgan and Watkins (1951) on intact red blood cells. Periodate oxidation takes place with two adjacent carbon atoms each of which are bonded to oxygen (either as a hydroxyl, aldehydic, or keto function). Reactivity is restricted almost entirely to carbohydrate type units.
Inactivation by exposure to periodate indicates that a carbohydrate unit is at least a necessary part of the active group. Morgan and Watkins found that periodate diminished the activity of all factors tested, i.e., the A, B, O, H, Le^A, P, M, N, and Rh_0(D) factors of human erythrocytes.

Kaufman and Masouredis (1963) contrasted the effects of periodate and formaldehyde on the Rh_0(D) antigen. Both were shown to inactivate it. Periodate inactivated the exposed antigen sites as well as the masked sites which could be uncovered by papain treatment. The masked sites, however, were protected from the formalin until they were uncovered by the enzyme action. This experiment made use of I^{131} labeled anti-Rh_0 antibodies for quantitation of the active Rh_0 antigen sites present on the red blood cell surface before and after the various treatments. This sensitivity of the Rh_0 antigen to periodate was also confirmed by Wolff and Springer (1964).

The Rh_0 antigen was shown to reside in the elinin complex along with the virus receptor (Howe, 1951) and the ABO antigens (Moscowitz et al., 1950).

Dodd et al. (1960) reported the relationship of the Rh_0 antigen to sialic acid as a result of finding inhibition of the Rh_0 reagent by N-acetylneuraminic acid and by other
preparations, some of which contained sialic acid and others had some structural relationship to it. Johnson and McOluer (1961) reported that they were unable to duplicate the results of Dodd et al., and further, that treatment with neuraminidase did not alter the reactivity of Rh₀ positive cells. Dodd et al. (1963) repeated their former results and explained that the failure of others to duplicate the results was due to longer incubation of reagent with inhibitor and use of reagent in too high of concentration. In addition, using Rh₀-negative erythrocytes to which the various substances were coupled, absorption of Rh₀ antibodies was accomplished, although the efficiency of absorption was low. The contention by Dodd's group that sialic acid was involved in the Rh₀ specificity received support from the report by Boyd and Reeves (1961) who also found inhibition by sialic acid and by a polymer containing sialic acid, colominic acid. Wolff and Springer (1964) reported their findings which again negated the involvement of sialic acid or complexes containing it in the Rh₀ specificity. In their experiments no inhibition was produced by a number of gangliosides, the glycopeptide from Pseudomonas (Bigley et al., 1963), colominic acid or sialic acids.

While it appears that the status of the Rh₀ antigen is undetermined, the situation with the M and N antigens
seems to be a little more definite. In all of the alcoholic extraction experiments which were performed in the ABO antigen studies the M and N factors failed to show up whenever they were tested for. In noting this, Hohorst (1954) chose to use the phenol-water extraction method, which would not exclude all of the protein bound components from the isolate. He found specific M and N activities in his preparations and concluded that the carbohydrate portion of the material was responsible for the M and N specificities since they were not denatured by the extraction method and were also heat stable. Springer and Ansell (1958) reported that treatment of human erythrocytes with influenza virus or with "Receptor Destroying Enzyme" (RDE) eliminated the M and N factors. Klenk and Uhlenbruck (1960) isolated a sialic acid containing mucoid from human erythrocytes by the Westphal method and analyzed it for its chemical components and demonstrated its activity with respect to the M and N factors as well as the virus receptor site. Springer and Hotta (1963) reported a difference in the M and N factors. Purified M antigen was destroyed by incubation with RDE and by mild acid hydrolysis. The purified N-antigen was not inactivated by such treatments; dialyzable products were released from the macromolecule which were still active and capable of inhibiting the anti-N reagent.
Studies on the nature of the isoantigens of cattle

The erythrocytes provide the only source of the antigens at present with the exception of J (Stone, 1962). The J-substance in serum shows some similarities to the soluble A-substance of humans and has attracted some attention for chemical study because of its ready availability and because of its stability. Bednekoff et al. (1958) purified J-substance from serum by extracting the coagulated serum which resulted from heating at 100° for 30 minutes. The purified extract contained 10% of the original serum protein and all of the J-activity. Electrophoresis indicated that the preparation consisted of 75% alphaglobulin. The cellular J-factor in cattle appears to result from adsorption of the soluble J which is present in the serum (Stormont, 1949). The serum level of soluble J-substance varies considerably between animals with some individuals being apparently absolutely negative as would be suggested by the ability of some to produce a normal anti-J. The quantitative difference makes it difficult to investigate both from a genetic standpoint and also from the aspect of studying it as a cellular antigen from a physico-chemical approach.

Stone and Miller (1955) reported on the effects of
proteolytic enzymes on the antigens of bovine erythrocytes. Such treatment caused a loss of reactivity by many of the B- and C-system factors. Some B-system factors were inactivated by the enzymes when present in certain phenogroups but appeared to be resistant when present in others. Factors in the other systems in general were not affected. Datta and Stone (1963) report on their comparisons of B-system factors in different phenogroups by strictly serological techniques.

The first report on an attempt to isolate the bovine isoantigens from the erythrocytes was given by Royal et al. (1953). They extracted stroma by the method of Hallauer (1934) and showed inhibition of the various reagents by the extracts. Passing the extracts through a column containing adsorption alumina resulted in a partial separation of the inhibiting factors in the eluant fractions.

Patras and Stone (1960) have described a procedure for obtaining active fractions from the red blood cell hemolysate. The fraction which consisted of the insoluble membrane fragments, which were sedimentable without pH adjustment possessed the highest specific activity (units/mg.protein) and the greatest total activity. A second fraction could be obtained by adjusting the pH to 4.7. The precipitate which was recovered had low activity for several factors, notably F and V.
Schmid (1961) gave a report whose title suggested that cattle erythrocyte antigens had been isolated. The report described a method for preparation of stroma which could be stored, and established that the antigenic factors were associated with the stroma materials and remained active throughout the preparation and freeze-drying procedures and after periods of storage.

The title of the dissertation by Beuche (1960) was almost identical to the title of the Schmid article but the contents of the dissertation appeared to merit its caption much better. Beuche used methods such as phenol extraction, enzyme digestion and extraction with acetone and petroleum ether. The phenol treatment was done by the Westphal method and also by a method devised by Mitscherlich and Liess (1958). This latter variation was more successful. It involved use of liquified phenol which had been neutralized to near the neutral point, and several days contact of the stroma with the extraction medium seemed to be necessary.

Many of the preparations by the various methods showed the ability to inhibit selectively, various reagents. Use of the preparations as immunizing reagents often were successful in producing antisera. The specificity of the antibodies produced was almost always directed towards the F-factor, even in cases where the preparation was obtained
from F-negative erythrocytes. Some preparations showed F-inhibition even though they were obtained from F-negative erythrocytes. Beuche concludes that the F-factor is present in the F-negative cells but is masked by the V-factor, and upon treatment it becomes uncovered. Beuche's conclusions on this point were also stated in a report by Tolle (1959).

Uhlenbruck and Schmid (1962) studied the mucoid obtained from cattle erythrocytes by the Westphal phenol-water extraction method (see Klenk and Uhlenbruck, 1958) for its blood group antigen properties. The mucoid was purified by alcohol precipitation which produced a preparation which showed one peak upon sedimentation in the ultracentrifuge. Preparations of this type showed capacities to inhibit various blood typing reagents. Most of the inhibitive capacities were in accord with the type of the cells from which the preparations were made. Several peculiarities were noted. The F-factor was not detected in the preparations even though the starting material (red cells) was F-positive. The preparations were, on the other hand, able to inhibit the V-reagents although the original erythrocytes were V-negative. This appears to be just opposite to the findings of Beuche (1960). They conclude, similarly to Beuche, that factors may be hidden in the interior of the membrane which are not detected by the hemolytic test on the erythrocytes (in this case, the V-factor).
Some of these preparations were shown to possess the virus receptor site (in contrast to Klenk and Uhlenbruck, 1958) while some preparations did not retain it. Studies with neuraminidase showed elimination of the virus receptor while they found no influence on the blood group antigens by this enzyme.

A note added to the above article indicated that the mucoid from cattle erythrocytes reacted with some phytoagglutinins. After exposure to RDE the reactivity was increased. The reactivity of this mucoid with the phytoagglutinins as well as with anti-pneumococcus serum (horse) was studied in detail a short time later by Uhlenbruck and Kruepe (1963).

Uhlenbruck and Schmid (1963) investigated the bovine erythrocyte antigens from another viewpoint; this one involved the use of proteolytic enzymes. Identical results were obtained using trypsin, papain, and pronase. A glycoprotein was released from the surface of intact erythrocytes by each of the named enzymes. Analysis of the carbohydrate portions of each gave almost identical results, roughly, 51% reducing sugar 19% hexosamine and 13.5% sialic acid. While the glycoprotein did not show any activity in the virus hemagglutination system, it was reported that it
could be shown that it possessed the capacity to inhibit certain blood group reagents specific for factors in various blood group systems. Details of this latter finding were not presented.
MATERIALS AND METHODS

The antigens whose nature was investigated from different standpoints were the isoantigens associated with the red blood cells of cattle. No attempt was made to test every factor by every process. Often it was a matter of using one or several representative factors from most of the bloodgroup systems. The choice of factors was directed by the types of available blood materials, the availability of sharp, discrete reagents for the respective factors, the stability of the factor throughout certain processes, the stability of the antibodies in some cases, and the ability of the particular reagents to be specifically inhibited when inhibition tests had to be relied upon for demonstration of serological properties. The J antigen was not studied in these experiments since it does not seem to be intimately associated with the cell membrane, and also because it has received special study by others.

Red blood cells

Cattle red blood cells were used in these experiments for a source of the isoantigens to be studied and also as indicator cells in the hemolytic reactions. All of the red blood cells which were used for source of the antigens were
obtained from cows in the Ohio State University dairy herd. Animals from the Holstein, Jersey, Guernsey, and Brown Swiss breeds were used as donors. Blood from the animals in university herd as well as from Holsteins from some of the state mental and correctional institutions usually provided the source for the indicator cells. Occasionally blood samples on hand in the Cattle Blood Typing Laboratory which had been received for commercial typing purposes were used for indicator cells. Blood collected for these purposes was obtained by bleeding the animals from the jugular vein into vessels containing sufficient isotonic sodium citrate solution to prevent clotting. Before use for the various purposes the erythrocytes were recovered by centrifugation and washed in isotonic saline to remove the plasma.

**Blood typing reagents**

The blood typing reagents used were ones used regularly in blood typing cattle for commercial and experimental purposes by the Cattle Blood Typing Laboratory. They were produced by personnel of the laboratory mostly by isoimmunizations using animals of the university and state institution herds as donors and recipient. At least one reagent was produced by heteroimmunization with rabbits serving as the recipients.
Stroma

The term stroma usually refers to the red blood cell membrane. Stroma preparations were prepared by lysing washed red blood cells by adding them to ten times their packed volume of distilled water and then collecting the fraction which sediments when centrifuged at 30,000 xg. Usually the stroma were collected by passing the hemolysate through the continuous flow apparatus of the Servall RC-2 supercentrifuge. Most of the time the stroma material collected in this manner was freeze dried in 500 ml centrifuge bottles with stoppers adapted for attachment to a Virtis vacuum manifold which contains a dry ice vapor trap. Freeze-dried stroma preparations were stored on the shelf at room temperature. No loss of serological activity was noted after periods of storage of over three months. The freeze-dried stroma preparations contained a considerable amount of hemoglobin which could be easily removed from reconstituted aqueous suspensions by washing repeatedly with saline or distilled water.

S-protein

S-protein is a component derived from the hemolysate of cattle erythrocytes after removal of the stromal components
by high speed centrifugation. The S-protein is removed by adjusting the pH of the supernatant to just less than 6.4. The precipitate which forms is collected and can be redissolved by addition of alkali to a suspension of the precipitate, adjusting the pH to about 7.5. This S-protein is analogous to a component obtained from a hemolysate of human erythrocytes and described by Moscowitz et al. (1950).

Elinin

The term elinin refers to a product derived from erythrocyte stroma by extraction of the freeze-dried stroma with anhydrous ether and then after removal of the residual ether, the extracted residue is subjected to suspension and solution in alkaline (pH 9.0) aqueous medium and removal by ultracentrifugation (100,000xg) after adjusting the sodium chloride concentration to 0.15M. The sedimented material is redispersed in alkalinized distilled water and resedimented several times. This method was devised by Moscowitz et al. (1950) as a modification of an earlier procedure (Galvin et al. 1946) from which the term elinin was coined. In the experiments in this study, the ultracentrifugation was performed sometimes only three times, and sometimes only two times because of difficulties in
obtaining the use of a Spinco model L preparative ultracentrifuge. Moscowitz et al. describe their procedure as employing five ultracentrifugations.

**Hemolytic test**

The hemolytic test used was the standard blood typing test (Ferguson, 1941) in which 0.05 ml of a 3% suspension of washed cattle erythrocytes in saline was added to a tube which contained 0.1 ml of a reagent. After 15 minutes incubation 0.05 ml of normal rabbit serum was added as the source of complement. The tubes were observed at 30 minutes, 2 hours, and 4 hours after the addition of complement, and the degree of hemolysis recorded for each tube each time. The tubes were shaken after each addition of a component, and after each of the first two readings. In these studies, the hemolytic test was not used simply as such but as a part of the inhibition test or as a means of titration of an antibody medium such as an eluate (see antibody elution, below) to quantitate the relative antibody content.

**Antibody elution**

Elution of antibodies from stroma preparations was used as a means of demonstrating the capacity of the
preparation to combine with antibodies of known specificity. The method of recovery of absorbed antibodies was the acid elution of Landsteiner and van der Scheer (1936). A quantity of stroma (treated or untreated in some process) was suspended in a reagent of high titer which was specific for the factor being studied, and then was sedimented from the medium after about 15 minutes of incubation at room temperature. The specific sites which were capable of combining with the antibodies were considered to be saturated. The stroma material which was thus "sensitized" was then washed repeatedly (4 to 5 times) with saline to remove the occluded serum and unabsorbed antibodies. The washed, "sensitized" stroma material was then suspended in a measured amount of saline and the pH was adjusted to 2.9 - 3.0 by adding 0.1 N HCl. The acidified medium was centrifuged to sediment the stroma and the supernatant was recovered and neutralized with 0.1 N NaOH or a suitable buffer. The neutralized supernatant contained the antibodies which were released from the antigen sites of the stroma under the low pH conditions. This medium containing the eluted antibodies is termed the eluate.
Titration of eluates

An indication of the quantity of antibodies in an eluate can be obtained by titration. Doubling-dilutions of the eluate in saline were made. Each dilution of the eluate was then used as a reagent in the hemolytic test, i.e., 0.1 ml of each was employed against 0.05 ml of a 3% suspension of indicator cells and subsequently the complement was added. In these cases, only one indicator cell suspension would be necessary in the test against the eluate because the antibodies used for sensitization were specific for one factor. A battery of indicator cells is used when a complex of antibodies is being titrated. Here the indicator cells had to possess only the factor in question; however, several positive and at least one negative indicator cell suspension were employed. The extent to which the eluate can be diluted and still retain its ability to cause hemolysis of a suspension of positive cells is an indication of the quantity of antibodies it possesses. In turn, the amount of antibodies able to be carried over into an eluate is an indication of the effectiveness of the antigen sites on the stroma.

Hemolytic scores of eluates were calculated as follows: the degree of hemolysis at the termination of the test was considered 0, 1, 2, 3, 4 or 5 (instead of the more
conventional 0, tr, 1, 2, 3, 4) in the case of each dilution; if the first three dilutions of the eluate each produced a score of 5 (complete hemolysis) the fourth dilution produced a 2 (1/4 to 1/3 hemolysis) and all subsequent dilutions failed to cause hemolysis of the positive indicator cells, the hemolytic score would be $5 + 5 + 5 + 2 = 17$.

**Inhibition test**

The inhibition test is a simple modification of the hemolytic test. After 0.1 ml of the reagent had been placed in a serological tube, 0.05 ml of an isotonic solution or suspension of the inhibitor or dilution thereof was added and the materials were mixed by shaking. After incubating the mixture for about 20 minutes, 0.5 ml of the indicator red blood cell suspension was added and mixed and 15 minutes later the complement was added. From this point on the procedure was handled as described under hemolytic test except that the second reading was taken at one hour and a half after addition of the complement instead of two hours and no four-hour reading was made.

Most of the reagents used in the inhibition test were employed at the same strength at which they are used in the standard blood typing test which is a minimum of "two hemolytic doses". Some have been routinely used at much
higher strengths. An example is reagent 82 which is the standard V reagent. This is generally diluted to 1/8 for use. It was not so easily inhibited as most of the other reagents by what should have been effective V inhibitor. It was found that when diluted to 1/64 it was still capable of producing complete hemolysis of V-positive cells in one half hour in the uninhibited reaction. It was employed at a dilution of 1/32 in the inhibition tests and seemed to give very reliable results.

Certain reagents do not make good inhibition test reagents because their antibodies do not seem to "bind" well. Some reagents have been observed which contain antibodies which cannot be removed by repeated absorption attempts with a particular positive red blood cell mass, but subsequent testing of the "absorbed" antisera against the red blood cells from the same source results in rapid hemolysis of the test cells. This type of reagent cannot be used in inhibition tests. Others appear to lose some of their reactive ability just by the presence of something foreign in the medium.

When testing the inhibitory capacity of a substance, doubling dilutions of the inhibitor were made in saline. In some cases the concentration of inhibitor was known for the 1/1 dilution, so in each subsequent dilution it was
halved. The amount of inhibitor employed in any of the tubes in the series was calculated by multiplying the ug of inhibitor per ml in that dilution by 0.05 since 0.05 ml of each dilution was used. In some cases the initial concentration per ml was not determined, so for comparison only the dilution values are used. In one case the term inhibition test score is used. This is analogous to the term previously defined, titration score. If the uninhibited reaction, i.e., the system which received 0.05 ml of saline instead of the inhibitor produced a 5 value hemolysis, where the presence of inhibitor reduced the value to zero, an inhibition value of 5 was given; if a tube which received inhibitor had a reduction from 5 to 2, an inhibition value of 3 was added. The inhibition score is the sum of the individual inhibition values observed for the entire dilution series of the inhibitor.

Electrophoresis

The elinin and S-protein preparations as well as hemolysates were subjected to analysis by "disc electrophoresis" according to the procedure of Ornstein and Davis (1962). This method which is well adapted for the study of serum proteins involves the electrophoresing of a protein preparation in a small cylinder of polyacrylamide gel.
The apparatus was assembled from easily acquired materials by Drs. Rausch and Weseli of the Dept. of Dairy Science. Detection of the protein bands was accomplished by suspending the gels in a solution of amido black in acetic acid, and then destaining by suspending the gels in 7% acetic acid solutions with changes of acid as needed until only the protein bands in the gels retained the stain.

**Papain digestion**

Degradation of elinin and stroma substrates was performed using a very crude papain preparation obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. It contained a very large amount of carbohydrate reactive material as indicated by the phenol-sulfuric acid reaction, and the Polin test indicated that it contained only about 13% protein. It seemed to accomplish the purpose well enough and it was used without prior purification. The contaminants in the papain preparation most probably were separated from the active fractions obtained in the Sephadex column procedure. The results of a trial column passage indicated so.

The papain reaction mixture consisted of 100 ml of 0.1 M citrate buffer, pH 5.5, which was 0.005M in cysteine-hydrochloride and 0.001M in EDTA. Initially 20 mg of the
crude papain were added and 0.8 to 1.0 g of substrate (elinin or stroma) was mixed in. At intervals, additional 20 mg aliquots of the enzyme were added. Incubation was usually continued for about 18 hours at 37°C. The choice of cofactors and buffer used in the reaction medium was made on a basis of the report of papain studies by Kimmel and Smith (1954). The extent of the proteolysis was done by the periodic assay of a small aliquot (0.05 to 0.2 ml) of the medium for ninhydrin reactivity by the method of Rosén (1957). When there was no further increase in ninhydrin reactivity when active enzyme had been added to the medium the enzyme action was considered exhaustive. In one of the first experiments, it was found that after this point had been reached, washing of the insoluble residue from the reaction mixture and resuspending it in a fresh reaction mixture did not give rise to renewed production of ninhydrin reactivity in the medium.

**Column fractionation**

The soluble portion from the papain reaction mixture was fractionated on a Sephadex G-25 column (32 x 3.8 cm). The solution had been previously freeze-dried and it was dissolved in about 7 ml of distilled water for placing it on the column. It was passed through the column with
distilled water and the effluent was collected in uniform fractions which were varied from one experiment to another from as small as 3 ml to as large as 8 ml. The effluent fractions were assayed for carbohydrate (phenol-sulfuric acid reaction on 0.1 ml), protein (Folin test on 0.1 ml), and amino acids (ninhydrin reaction on 0.04 ml). In one experiment a longer column (64 cm) was used. It gave a greater separation of peaks (carbohydrate), although the same number of peaks (two) was observed.

**Chloroform - methanol extraction**

Extraction of stroma and an F-active subfraction (papain fraction 2B) was performed with chloroform - methanol 2:1 (v/v) according to the Folch method which is described by Bogoch (1958). Either dry material or an aqueous suspension thereof was extracted with the solvent mixture, sometimes using a mortar and pestle and other times using a hand homogenizer. The insoluble residue was removed by filtering the material through glass wool and a sintered glass filter. In attempts to obtain a glycolipid from the solvent extract the chloroform - methanol medium was either extracted with 0.2 volumes of 0.1% salt solution or else the medium was overlayed with the salt solution and the two phase system was allowed to come to equilibrium.
The aqueous phase was removed and evaporated and reconstituted to 1/9 the volume of the salt solution used in the phase partition (resulting in about 0.9% NaCl in the reconstituted solution). The organic phase was evaporated and the residue was suspended in physiological saline. Both products were employed in inhibition tests.

In other experiments extraction was performed with the chloroform - methanol solvent which was evaporated directly with no water partition and the residue was used in the inhibition test. The extracted stromal residue was also used in the inhibition test.

Evaporation was done in a rotary vacuum evaporator at about 40°C.

Neuraminidase treatment of red blood cells and stroma

The neuraminidase used was what is referred to as "receptor destroying enzyme" (RDE) from Vibrio cholerae. The RDE preparations were either from General Biochemicals, Inc., Chagrin Falls, Ohio, or from Sigma Chemical Co., St. Louis. The latter carries the name Cholera Filtrate and is a more potent preparation although it appears to be more crude. The Sigma product contained some component which contributed somewhat to the thiobarbituric acid
reaction assay so an adjustment had to be made in the assay of the sialic acid liberated from stroma by that enzyme preparation.

The reaction mixture for this enzyme was that prescribed by Eylar et al. (1962). For treatment of intact red blood cells isotonic conditions had to be maintained. In general, the reaction medium consisted of 0.15 M cacodylic acid [(CH₃)₂ASO₂OH] buffer, pH 6.4 which was 0.002M in calcium acetate. The substrate (erythrocytes or stroma) was suspended in this medium and an aliquot of the enzyme solution was added. For the treatment of red blood cells, 0.1 ml of washed packed cells was incubated with 1.8 ml of the buffer medium to which 0.2 ml of the enzyme (the G.B.I. product) was added. Incubation was carried out for 15 minutes at 37°C after which the erythrocytes were washed with saline three times and employed in the hemolytic test against various reagents.

When stroma were treated, several variations were used. The first was done by putting all of the components of the reaction mixture (buffer, a known amount of stroma, and an aliquot of the enzyme) inside a dialysis bag in a total volume of 10 ml, and suspending the dialysis bag in 50 ml of the buffer. The action of the enzyme was followed by assaying aliquots (0.2 ml) of the outer dialysate with
the thiobarbituric acid reaction. Incubation was done at 37°. The free sialic acid content of the medium was followed up to 36 hours. The outer dialysates were analyzed by paper chromatographic methods for qualitative differences. The media were also used as inhibitors after adjusting the pH to about 7 but could not be used concentrated because of the problem of concentrating the salts in the media.

In one experiment suspensions of the various types of stroma (FF, FV, VV) were incubated in 0.05M buffer in a total volume of 3.0 ml. After about 20 hours incubation the residues were sedimented at 20,000xg and the supernatants were recovered and assayed. The supernatants from some reaction mixtures were freeze dried and reconstituted to 1 ml and used in the inhibition tests against F and V reagents. These supernatants would not exclude a non-dialyzable soluble component from being used in the inhibition test, if in some manner one were released by the action of the enzyme preparation and its loss were responsible for the disappearance of the F-activity.

In less fastidious experiments in which the objective of the trial was merely to note if the enzyme would alter the specific inhibitive properties of the stroma, an arbitrary unit of stroma was suspended in about 5 ml of buffer and about 0.5 ml of the enzyme solution was
added. Incubation was carried out at 37° for 2 hours in the first experiments and in later ones for about 14 hours as it was noted that 2 hours was insufficient, at least when using the G.B.I. preparation.

In all of these experiments a control was provided in which an aliquot of substrate was incubated in a medium which lacked only the enzyme.

Special materials for inhibition tests

The following materials were kindly donated by Dr. R. H. McCluer, Dept. of Physiological Chemistry, Ohio State University for use in inhibition tests:

(1) N-acetylneuraminic acid (crude, prepared from bovine submaxillary mucoprotein, containing about 85% NANA and 15% NGNA) — employed against 26 different reagents.

(2) N-glycolylneuraminic acid (preparation of Sigma Chemical Co., St. Louis). — employed against 10 different reagents.

(3) Human ganglioside 1-G, purified. — employed against F. V. and C2 reagents. Maximum amount employed was 260 ug.

(4) Human ganglioside 3-G, purified. — employed
against F. V. and O₂ reagents. Maximum amount employed was 540 ug.

(5) Mixed bovine brain ganglioside preparation—employed against F, V, and O₂ reagents. Maximum amount employed was 500 ug.

The maximum amount of the simple sialic acids used (items 1 and 2) is not stated. Due to an error they were dissolved in unbuffered saline and they affected the pH of the systems in which they were employed so that in the higher concentrations they inhibited the hemolytic reaction by the pH effect. It was noted then that item 1 had been prepared by crystallization from acetic acid. In systems to which the lower concentrations were added there was no inhibition of the antibody reaction by as much as 100 ug of the respective compounds. It was concluded that if the pH effect masked any ability of the compounds to inhibit a particular reaction the inhibiting capacity would not have been very significant. The ganglioside preparations, on the other hand, were dissolved in saline buffered with veronal buffer. The final pH of those inhibitor solutions was about 7.5.
Mild acid hydrolysis of stroma for release of sialic acid

The method described by Whitehouse and Zilliken (1960) for hydrolysis of sialic acids from biological products was used. Sulfuric acid was added to a concentration of 0.1N and the mixture was heated for 1 hour at 80°. The temperature was never noted to go above 82° during the heating. The mixture was then centrifuged and the supernatant was recovered for thiobarbituric acid assay and paper chromatography.

Considering the possibility of O-acetyl derivatives being present, milder conditions of acidity and higher temperatures were tried. Blix (1962) describes methods which can be used which will release such complexes without hydrolyzing the O-acetyl groups from the compounds. Adjustment of the pH to 3.3 and heating the mixture at 100° for one hour is recommended for recovery of O-acetyl derivatives from bovine submaxillary mucin.

Paper chromatography

Identification of sialic acid derivatives released by enzyme and acid hydrolysis was attempted by paper chromatography. A solvent system listed by Whitehouse and Zilliken (1960) seemed ideal for this purpose. The solvent
is 1-butanol-acetic acid -H₂O, 4:1:5. The Rf values for NANA, NGNA, and an O-acetyl derivative are listed as 0.09, 0.19, and 0.45 respectively. Fairly good agreement was achieved with the NANA standard in this solvent system. Rf values of 0.07 and 0.097 were obtained on two different occasions. A second component was noted in the chromatogram of the crude NANA preparation obtained from Dr. McCluer. The second component had an Rf value of 0.18 with the major component exhibiting the Rf value of 0.097. These would correspond to NGNA and NANA respectively, both by the Rf values and the relative concentration indicated by the intensity of the color of the spots.

Spraying of the chromatograms was done by the Resorcinol and Ehrlich methods of Svennerholm and Svennerholm (1958) and by the thiobarbiturate method of Warren (1960). A fourth method was used in an attempt to detect an ester type of configuration which would be indicative of O-acetyl compounds or the lactone form of the sialic acids which according to Gibbons (1963) is induced by acid conditions at high temperatures. This spray procedure is the hydroxylamine method suggested by Block et al. (1955) for the detection of sugar lactones.
Biochemical assays

Quantitation of free sialic acids was always done using the thiobarbituric acid (TBA) method of Warren (1959). Optical densities were determined either using a Beckman DU or a Beckman DB spectrophotometer. The optical density was related to a standard curve based on synthetic N-acetyl-neuraminic acid obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

Protein estimation was done by the Folin method (Lowry et al., 1954) obtaining the optical density measurements on a Klett-Summerson photometer. The quantitation was done by relating the photometer reading to a standard curve obtained with crystallized bovine serum albumin (Armour Pharmaceutical Co., Kankakee, Illinois).

Carbohydrate estimations on effluent column fractions were made by means of the phenol-sulfuric acid reaction (Dubois et al., 1951) on aliquots of the fractions. Optical densities of the reaction mixtures were obtained either on a Klett Summerson photometer with a 42 filter or else on a Bausch-Lomb Spectronic-20 photometer at 490 mu. If quantitation were desired the optical density of the medium was related to a standard curve derived from a standard galactose solution.
RESULTS AND DISCUSSION

Early experiments

Results from some early experiments involving antibody elution from stroma are presented here first because they give some insight into the stability and lability of some of the factors. The elution experiments were an extension of previous work (Hatheway, 1961; Hatheway et al., 1963). The acid elution method of Landsteiner and van der Scheer (1936) was limited in these experiments because certain antibodies were inactivated by exposure to low pH (2.5 pH 3.0). Antibodies against A, B, I, K', R, and Z were stable throughout the pH adjustment while antibodies against C, F, V, L, S and H' were at least partially inactivated by the treatment. Antibodies against A, B, I, K', R, and Z could be eluted by pH adjustment from stroma which had been sensitized with the respective reagents and could be demonstrated in the resulting eluates. It was demonstrated that if the pH of a stroma suspension sensitized with B-system reagents (B, I, K') were lowered to 3.0 and then immediately neutralized to 7.0 the supernatant which resulted after centrifugation and removal of the stroma contained the
antibodies. On the other hand, stroma which were sensitized with anti-A, anti-R, or anti-Z appeared to re-absorb the antibodies released by the low pH conditions if the medium were neutralized before centrifugation. The supernatants resulting after centrifugation did not show any antibodies. If those suspensions were centrifuged while the pH was 3.0 and then the supernatants were neutralized after centrifugation, the eluates possessed strong antibody activity. This suggested that the B-system factors were destroyed by short exposure to pH 3.0 while the A, R and Z factors were not.

A comparison was made on the pH stability of the Z and the I factors. A preparation of stroma which possessed both factors was subjected to different levels of acidity and then neutralized and washed. The material from each pH treatment was divided into two equal parts and one part was sensitized with anti-I and the other with anti-Z. The eluate from each was prepared as described above and each eluate was titrated. The results of the titrations are listed in Table 1. The pH inactivation curves for the I and Z factors are depicted in Figures 1 and 2.

It is seen that the B-system factor (I) is rapidly destroyed at pH levels less than 5 and completely inactivated by pH 3.0. The degree of inactivation appears to be quite
**TABLE 1**

TITRATION SCORES OF ELUATES FROM STROMA SUBJECTED TO DIFFERENT pH LEVELS AND SUBSEQUENTLY SENSITIZED WITH ANTI-1 OR ANTI-Z

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<th>Per cent of maximum</th>
<th>Anti-Z</th>
<th>Per cent of maximum</th>
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<td></td>
<td>Titration score</td>
<td></td>
<td>Titration score</td>
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<td>--</td>
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<td>1.2</td>
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<td>10.9</td>
<td>23</td>
<td>96</td>
<td>24</td>
<td>109</td>
</tr>
</tbody>
</table>

24 = 100%  22 = 100%
FIGURE 1. RELATIVE ACTIVITY OF I FACTOR AFTER EXPOSURE TO VARIOUS pH LEVELS (Data from Table 1.)

FIGURE 2. RELATIVE ACTIVITY OF Z FACTOR AFTER EXPOSURE TO VARIOUS pH LEVELS (Data from Table 1.)
linear. The situation appears to be similar with the Z-factor except that it is more tolerant of acid conditions and that appreciable inactivation does not occur until the pH is below 3.0 at which point the B-system factor is completely inactivated. At a pH of about 1.0 the Z-factor is completely inactivated. Both factors give indication of being rather tolerant of the higher pH levels.

Using the same general procedure, the stability of the Z-factor was studied in relation to a variety of treatments, among them exposure to oxidizing agents, formaldehyde, trypsin, extraction of the stroma pulp with ether, and heating at 100° for 3 minutes. The results of these experiments are indicated in Table 2.

It is seen that the Z-factor is eliminated by periodate oxidation, the action of trypsin and by high temperature. It is not significantly affected by dichromate, or formaldehyde as well as the low pH.

The inactivation of the Z-factor by the periodate reagent was interesting because this indicated that a carbohydrate residue must be involved in the structure of the Z-factor. The use of the dichromate reagent with the resulting negative effects served to confirm that specific Malapradian oxidation had taken place with the periodate reagent. In this case the site of reaction is at two
# TABLE 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Titration Score</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>17</td>
</tr>
<tr>
<td>Acid (pH 2.5)</td>
<td>17</td>
</tr>
<tr>
<td>0.05 M Potassium Dichromate (pH 6.5, 25°C, 20 min.)</td>
<td>12</td>
</tr>
<tr>
<td>0.05 M Sodium Periodate (pH 6.5, 25°C, 20 min.)</td>
<td>0</td>
</tr>
<tr>
<td>1% Formaldehyde, 25°C, 20 min.</td>
<td>15</td>
</tr>
<tr>
<td>1% Trypsin, 25°C, 20 min.</td>
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</tr>
<tr>
<td>100°C, 3 min.</td>
<td>0</td>
</tr>
<tr>
<td>Aqueous Stroma Suspension Extracted with Ether</td>
<td>0</td>
</tr>
</tbody>
</table>
adjacent carbon atoms each of which is bound to oxygen (either as a hydroxyl, keto, or aldelyde function), or else one to oxygen and the adjacent one to an amino group.

In a later experiment the effect of periodate on a variety of factors was tested, this time treating stroma with periodate (0.05M) and then testing for the presence of the factors in the inhibition test. Factors from the A, B, C, F, and Z systems were tested by this method and all appeared to be readily inactivated by the periodate reagent. The data are presented in Table 3. The susceptibility of the various factors to periodate suggest that a carbohydrate moiety is a necessary component of each specific structure.

At this point the pursuit of the antigen study was directed toward breaking down the red blood cell membrane into subunits which retain their serological properties. The first step was the study of the elinin and S-protein fractions of the erythrocyte.

**Elinin and S-protein**

The elinin and S-protein fractions were prepared according to the procedures outlined in the methods section. There was a question of whether or not the ether extraction procedure might destroy the reactivity because extraction of stroma in aqueous suspension with ether in a two phase
### TABLE 3

INHIBITION TEST SCORES OF AN UNTREATED STROMA PREPARATION AND OF ALIQUOTS EXPOSED TO DICHROMATE AND PERIODATE REAGENTS

<table>
<thead>
<tr>
<th>Factor</th>
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<th>0.05M K-dichromate</th>
<th>0.05M Na-periodate</th>
</tr>
</thead>
<tbody>
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<td>A</td>
<td>30</td>
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<td>7</td>
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<tr>
<td>C</td>
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<td>T*</td>
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<td>0</td>
</tr>
<tr>
<td>C</td>
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<td>F</td>
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<td>5</td>
</tr>
<tr>
<td>Z</td>
<td>17</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

*The red blood cells from which the stroma preparation was made were negative for this factor.*
system eliminated the Z-factor as is seen in Table 2. Immunizations with freeze-dried stroma which had been ether extracted in the wet state were unsuccessful. The extraction of the freeze-dried stroma with ether did not seem to have this effect, although some elinin preparations did not prove to be effective inhibitors of the Z-reagent while the stroma from which they were prepared inhibited the Z-reagent quite well. Some other elinin preparations were good Z-inhibitors. In general, the elinin preparations seemed to retain all of the serological properties.

The testing of the S-protein preparations for serological properties posed some problems. If all of the stroma fragments had not been sedimented by the initial centrifugation, these would be collected when the S-protein was precipitated out by pH adjustment. Any indication of inhibition by the S-protein preparation could possibly be due to contamination by some membrane fragments.

S-protein which had been precipitated at pH 6.0 was redissolved in distilled water by adjusting the pH to about 7.5. This solution was then centrifuged at 30,000 xg for one hour and the supernatant from this procedure was tested in the inhibition test. It was found that before such a treatment an S-protein preparation showed inhibition against
B and C system factors as well as A, F, S, and Z system factors, but after the centrifugation the B and C system properties were lost. Most consistently the F reagent could be inhibited by the S-protein. No S-protein preparations were made from F negative blood so the selectivity of the inhibition is not demonstrated by the available data. The report of Patras and Stone (1960) stated that the supernatants from their hemolysates retained low levels of some factors which could be demonstrated by the inhibition test, and that F and V were the factors most notable in this observation. The observations in this study appear to confirm this and suggest that the serological activity is associated with something analogous to the S-protein fraction of Moscovitz et al. (1950).

Sedimentation of one S-protein preparation in a Model E. Spinco analytical ultracentrifuge showed a single sharp peak. An estimation of the sedimentation rate was about 3.7 S under the conditions present (0.5% solution, pH 7.5, 20°C).

Electrophoresis was performed on both the S-protein and elinin preparations by the method of Ornstein and Davis (1962). Both elinin and S-protein showed two fast migrating components on the polyacrylamide gel. It is difficult to say if the components which showed up are the same or
different. The elinin particle has a particle weight of about 40 million. The size of such a particle would not permit it to enter into the polyacrylamide gel pores as such. Either the conditions in the gel such as pH or ionic strength or else the electric field must cause a dissociation of some of the components of the huge complex. Some of the components of the elinin complex may be identical to the S-protein. The origin of the S-protein in the hemolysate does not seem to be determined. It might have been a portion of the membrane structure which was released by the hemolysis procedure, or it might have been an intracellular soluble protein.

Electrophoresis was performed on hemolysates prepared in several ways. Washed erythrocytes were hemolyzed (1) by addition of distilled water and (2) by addition of digitonin to a saline suspension. On aliquot of the distilled water hemolysate was extracted with chloroform to remove stromal components. The digitonin hemolysate would have had the stromal components precipitated out by the formation of the digitonin complex. All three hemolysates showed the same components and are indicated by the one sketch in Figure 3 designated as hemolysate. The two fastest migrating components of the hemolysate appear to be identical with the major constituents of the S-protein.
FIGURE 3. DISC ELECTROPHORETIC COMPARISON OF S-PROTEIN WITH HEMOLYSATE AND PLASMA PREPARATIONS
preparation. The components with the Rf values of .97 might be artifacts caused by the tracking dye complexing with some protein, but it was always absent from the gels on which plasma proteins were run. The gels on which the elinin preparations were electrophoresed showed bands very similar to the S-protein bands. The only possible difference might be that the two bands showing up were not spaced quite so far apart from one another. No gels were available from which accurate Rf values could be calculated for the elinin.

**Papain degradation**

Noting that the elinin complex possessed a considerable amount of carbohydrate, and further, that from the periodate oxidation experiments the serological specificities seemed in general to be associated with carbohydrates, it seemed desirable to obtain a carbohydrate rich subfraction of the elinin and to test it for serological activity. Montgomery and Wu (1963) and Marks et al. (1962) used methods of enzymic degradation of glycoproteins. They employed the proteolytic enzymes papain and pronase (the latter from Streptomyces griseus) for obtaining the carbohydrates of egg glycoproteins relatively free from peptide components. This method seemed worthwhile trying.
An elinin preparation was made up as described previously and freeze-dried. This material (988 mg) was suspended in 100 ml of the papain reaction mixture which is described in the methods section. The extent of proteolysis was checked periodically by assaying an aliquot by the ninhydrin reaction. After 17 hours there was no further increase in ninhydrin activity of the medium. The reaction mixture was centrifuged at 30,000xg for 30 minutes. The supernatant was a clear, bright yellow solution. A considerable amount of insoluble material was sedimented. The supernatant (designated as papain fraction 1) was carefully removed and freeze-dried. The insoluble portion was washed with citrate buffer and resuspended in a papain reaction medium. This time there was no increase in ninhydrin reactivity of the medium over a period of several hours. Thus, the first treatment apparently had been exhaustive. This papain-resistant material was designated as papain fraction 2. It was recovered by centrifugation and washed two times in distilled water. It separated into two distinct layers which could be easily separated from each other because the lower layer packed tightly on the bottom while the upper layer sedimented only with difficulty, especially after several washings with distilled water. Addition of salt to a concentration of 0.15 M rendered it much more
easily sedimentable. The two layers were separated from each other and washed separately several more times. The rapidly sedimenting dense material was designated as papain fraction 2A and the light, fluffy material which appeared to consist to a large extent of lipid material was designated as papain fraction 2B.

The freeze-dried soluble portion (fraction 1) was dissolved in about seven milliliters of water and placed on a Sephadex G-25 column and eluted with distilled water. The eluant fractions were assayed for carbohydrate (phenol-sulfuric acid test), protein (Folin reaction) and ninhydrin reactivity. The elution pattern with respect to carbohydrate reactivity is shown in Figure 4. The fractions involved in the first carbohydrate peak were pooled, freeze-dried and designated as 1A while those involved in the second peak were handled similarly and designated as 1B.

The papain fractionation is summarized in Figure 5. The four fractions designated as 1A, 1B, 2A, and 2B were dissolved or suspended in saline and employed as inhibitors in the inhibition test against the following reagents: A, G, Y2, C2, X2, F, V, H1, Z, B2, and O2. The red blood cells from which the substrate was prepared were positive for all those factors except B2 and O2. Fraction 1B inhibited all reactions completely out to the fifth dilution tube. This
FIGURE 4. COLUMN FRACTIONATION OF SOLUBLE PORTION OF PAPAIN DIGEST OF ELININ PREPARATION. Sephadex G-25 column (64 x 3.8 cm). Typical elution pattern.

Optical density is result of assay of 0.1 ml. of fractions by Phenol-Sulfuric Acid test for carbohydrates.
ELININ

papain digestion

SOLUBLE PORTION (Papain Fraction 1)
- Sephadex G-25 column fractionation

1st CARBOHYDRATE PEAK (1A)
- V-factor only
- m.i.d. = 5-10 ug

2nd CARBOHYDRATE PEAK (1B)
- Non-specific

INSOLUBLE PORTION (Papain Fraction 2)
- washing and centrifugation (repeatedly)

LOWER DENSE LAYER (2A)
- Non-specific in higher concn.; specific for F in lower concn.
- m.i.d. = 25-40 ug

UPPER LIPOID LAYER (2B)
- Specific for F
- m.i.d. = 3.5 ug
- C inhibited by about 100 ug

**FIGURE 5. PAPAIN FRACTIONATION OF ELININ PREPARATION.** All fractions were tested against A, G, Y, X, C, X, F, V, H', Z, E, and O, reagents in inhibition tests. The elinin was prepared from blood of the type: A/D GY2E/CX2 C1EX, F/V H' Z'. The data and details in this figure were presented previously (Hatheway et al., 1964).

*m.i.d. = minimum inhibiting dose*
was non-specific and was probably partly due to the presence of citrate in high concentration which would prevent the action of complement by tying up essential ions. The large amount of amino acids present would no doubt exert an anti-complementary effect in other ways. Fraction 2A also seemed to show an anti-complementary effect but to a lesser degree. It inhibited all reactions out to the second dilution. Beyond this point the only reagent which was inhibited significantly was the F-reagent. Complete inhibition of the F-reagent was accomplished by a minimum of 25 to 40 ug of the 2A material.

Very specific results were obtained with the 1A and 2B fractions. The 1A fraction inhibited one reagent only, and that was the V reagent. As little as 5 to 10 ug of the 1A material was able to inhibit this reagent completely. The 2B fraction was a very effective inhibitor of the F-reagent while it was completely ineffective against the V reagent and all others with the exception of the C reagent. The smallest amount of this material used in the inhibition test was 3.5 ug and it completely inhibited the F reagent. A minimum of approximately 100 mg was successful in inhibiting the C reagent. The results of the inhibition test with the 1A and 2B fractions are given in Table 4.
### TABLE 4

**INHIBITION TEST WITH 1A AND 2B FRACTIONS FROM PAPAIN FRACTIONATION OF ELININ**

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<th>Dilution of Inhibitor</th>
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<th>O₂</th>
<th>X₂</th>
<th>F</th>
<th>V</th>
<th>H'</th>
<th>Z</th>
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### 1A

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### 2B

Plus reactions indicate that hemolysis took place; the zero indicates no hemolysis.
It appears that all of the factors other than F, V, and C are destroyed by the papain treatment. It is interesting that the F and V factors can be separated from each other because since they substitute for each other in the genetic scheme they would seem to be physicochemical analogues of each other. Locating the F and V factors in such different fractions, and each quite uncontaminated with the other suggests that they are quite dissimilar. It was quite fortunate that the substrate in this experiment was derived from F/V erythrocytes because it permitted the results to be so spectacular.

When a V-negative (F/F) substrate was used in a similar experiment a similar elution pattern was obtained from the Sephadex G-25 column. The material from the first carbohydrate peak (1A) was ineffective in the inhibition test against the V reagent as well as the eight other reagents employed. The papain-resistant, insoluble material (papain fraction 2) was effective only against the F reagent. The substrate in this case was C-negative.

An experiment was performed with F negative (V/V) stroma in the papain digestion procedure. A 1A fraction was obtained, similarly as when elinin was used as the substrate, although the yield was not so great. The 1A fraction was very effective against the V reagent only. The stroma material which had been incubated in the papain
reaction mixture was used as an inhibitor against the F and V reagents. It had lost almost all of its capacity to inhibit the V reagent and had not acquired any new capacity to inhibit the F reagent. An aliquot of the V/V stroma was incubated in a reaction mixture which contained all of the co-factors and buffer but no enzyme, for the duration of the incubation of the stroma in the complete reaction medium. The material incubated in the absence of the papain retained all of its capacity to inhibit the V reagent. It is seen that it is specifically the action of the papain which releases the V factor from the complex.

It is also seen that the stroma material does not take on the F property upon loss of the V property. Beuche's findings suggested that, perhaps all cattle red blood cells were F positive, but in the case of the V/V erythrocytes, all of the F sites were masked, possibly by the V entities. This does not seem to be indicated by the data of these experiments.

The glycoprotein released from the surface of cattle erythrocytes by proteases which was studied by Uhlenbruck and Schmid (1963) apparently is the same thing as the 1A fraction in these experiments. They used trypsin and pronase as well as papain, obtaining identical results with all three. They obtained the glycoprotein from the enzyme reaction supernatant by extracting the supernatant with 90%
phenol. They reported the demonstration of "clear inhibitory properties in various bloodgroup systems of cattle" with this preparation. They did not present any details on the serological properties. In the experiments here, the only demonstrable bloodgroup factor associated with this component was the V factor when it was present in the native red blood cells. There was an exception in one experiment in which the V activity was contaminated with a low level of F activity. When V-negative cells were used, no bloodgroup factors could be demonstrated.

Uhlenbruck and Schmid (1963) presented analytical data pertaining to the carbohydrate portion of the glycoprotein only. No accurate analytical data were obtained in these experiments but the 1A material always showed the presence of carbohydrate and protein in the ratio range of 1:1.33 to 1:2 on a milligram basis. One preparation was subjected to sedimentation in the analytical ultracentrifuge and was shown to be polydisperse.

In a later experiment it was noted that the use of a longer column, 64 cm instead of the usual 32 cm, resulted in a better separation of the 1A and 1B peaks. It appears that if subsequent work is to be done on this problem the greater length of column would be desirable.
Noting the association of the F factor with a lipid rich material (23) an attempt was made to isolate it in a chloroform-methanol extract. An extraction with this solvent was attempted similarly to the method described by Bogoch (1958 - Folch extraction method) which has been employed for isolation and purification of glycolipids and gangliosides. Extractions were made on a quantity of the papain fraction 2B as well as on quantities of F-positive stroma. In general, exposure of F containing materials to the chloroform-methanol solvent seems to inactivate the F factor. On two occasions preparations were obtained from chloroform-methanol extracts which were weakly inhibitory against the F reagent but this was not very repeatable. Generally, both the extract and the extracted residue (of stroma) were rather ineffective in the inhibition test against the F reagent.

The role of sialic acids in serological specificity of the bovine bloodgroup antigens

Much information from the literature suggested that the question of involvement of sialic acids in the specificity of the bovine antigens should be studied. The role of sialic acids in the virus receptor sites has long been established. These sites occur in the red blood cells of perhaps all species. The discovery by Springer and Ansell
(1958) of the role of sialic acids in M and N specificities of human erythrocytes promises much in view of the analogy between the FV system of cattle and the MN system of humans. The isolation of a mucoid containing sialic acid from human red blood cells which possessed the M and N properties, and also, isolation of a similar mucoid from cattle erythrocytes (Klenk and Uhlenbruck, 1958) seemed to make this question ideal for investigation. It was only a matter of time until Uhlenbruck and Schmid (1962) attacked the problem directly. Unaware of the latter work at the time (autumn 1963) an answer to the sialic acid question was sought in this project. Further impetus was given by the reports of Dodd et al. (1960, 1963) and of Boyd and Reeves (1961) on the ability of sialic acid and related compounds to inhibit Ph(D) antibodies.

A simple inhibition test was set up using two sialic acid derivatives, N-acetylenuraminic acid and N-glycolylneuraminic acid, as inhibitors against 26 different typing reagents for factors in seven different systems. No indication of inhibition was detected in any case.

Red blood cells containing various factors were exposed to a reaction mixture of neuraminidase and incubated at 37°C for 20 minutes. Control suspensions were provided which were incubated at 37°C in media which did not contain
the enzyme. The treated red blood cells, after washing thoroughly with saline, were tested against 25 different typing reagents. This was unsatisfactory because after exposure to the enzyme the red blood cells became hemolyzable by the complement serum alone. Apparently the rabbit serum which is used as the complement contains a normal antibody which is effective against enzyme treated cattle red blood cells but not against the native red blood cells.

The next approach was to treat a suspension of stroma with neuraminidase and then use it as an inhibitor in the various systems. A similar quantity of stroma was incubated in a control medium which contained no enzyme. Incubation was done at $37^\circ$ for two hours. After washing the stroma preparations and suspending them in saline, the treated and control suspensions were employed as inhibitors against the $A, B_2, O_2, T, O_2, W, F, S, H^1$ and $Z$ reagents. The native red blood cells were positive for all the respective factors except the $S$. There resulted no significant difference between the inhibitory capacities of the treated and untreated stroma preparations so at that time it was concluded that there was probably no involvement of sialic acids in six major blood group systems ($A, B, C, F, S$ and $Z$).
At a later date, after the progress had been made with the papain fractionation with the resulting isolation of the F and V factors, the possibility of sialic acid being involved in some manner was again given consideration. It was noted that the neuraminidase had not been used against a V-positive stroma. It was also thought possible that a sialic acid could be involved in the case of F but might not be susceptible to the enzyme. If a different derivative might be involved in the V or perhaps attached by a different linkage, it might be cleaved by the enzyme. Gibbons (1963) found that the O-acetyl derivatives were quite resistant to the enzyme in bovine submaxillary mucopolysaccharides.

A quantity of V-positive stroma was treated with neuraminidase as described previously with a control provided, and after two hours incubation, the suspensions were washed and used as inhibitors against the A, G, Y₂, C₂, X₂, F, V, and Z reagents. The native red blood cells from which this stroma preparation was obtained were positive for all of these factors. This time there was a significant difference in the ability to inhibit one reagent only, the F reagent. This was surprising in view of the previous failure. The treated stroma still partially inhibited the F reagent but permitted weak reactions to occur with the
indicator cells from the 1:4 dilution of the stroma suspension while the control suspension completely inhibited the F reagent up to the 1:128 dilution. The action of the enzyme was apparently not complete. The treated and control stroma suspensions were placed in fresh media and incubated for an additional 14 hours at 37°. The following inhibition test showed almost complete lack of ability to inhibit the F reagent, while the control suspension maintained strong inhibitory capacity. The ability to inhibit the V reagent was not diminished in the least. In fact, the treated stroma appeared to be a slightly more effective inhibitor of the V-reagent than the untreated stroma. This effect was consistent in duplicate experiments.

Table 5 illustrates the effect of neuraminidase on the different types of stroma. In the experiment performed it is seen that the enzyme had greatly diminished the F property from the homozygous F stroma but had not eliminated it completely over the incubation period allotted. It had, however, eliminated it almost completely from the heterozygous type stroma. This probably indicates why the first attempt failed and the second attempt was partially successful. The earlier trial was done with a homozygous F type stroma.

Table 5 also shows that the V-positive stroma increased slightly their capacity to inhibit the V reagent by
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^a Plus reactions indicate that hemolysis took place; the zero indicates no hemolysis.

^b 0.05 ml of the indicated dilution of the stroma preparation was added to the F and V reagents 20 minutes prior to addition of the indicator rbc's.
exposure to the neuraminidase. It should be noted, however, that the V-negative stroma does not acquire inhibitory capacity by exposure to the enzyme.

Uhlenbruck and Schmid (1962) found that their preparations which were derived from V-negative erythrocytes developed the capacity to inhibit the V reagent. It suggested that the red blood cells which did not show the V factor actually possessed the factor but it was masked. This seems to be the direct opposite situation to that described by Beuch (1960) where the F property appeared in preparations from F-negative sources. Neither of these phenomena have been observed in this study.

It is interesting to note that in the preparation of the mucoids by Uhlenbruck and Schmid (1962) the F factor was always lost in the process. The preparations showed serological properties other than the F, some of which were not detectable by hemolytic test on the erythrocytes. They treated their preparations with neuraminidase but found no loss of any of the demonstrable bloodgroup factors. This is in agreement with the findings presented here. The only bloodgroup factor affected by neuraminidase is the F factor. Since it had been previously lost, no effect of the enzyme on it could be observed. They were forced to conclude that the bloodgroup antigens were not influenced by neuraminidase.
Having established that sialic acid is involved in F specificity, the question is presented as to what is the structure of the active F unit. Supernatants from the neuraminidase reaction mixtures which assayed high in sialic acid by the thiobarbituric acid method of Warren (1959) were concentrated and employed in the inhibition test against F and V reagents. They were completely ineffective in inhibiting either reaction, even though their contents resulted concurrently with the disappearance of the F factor from the surface of stroma in some cases.

The use of gangliosides in the inhibition test provided a way of using sialic acids which were bound to other units. Three ganglioside preparations (obtained from H. McCluer, Department of Physiological Strym) were employed. Two were from human sources and purified and designated as 1-G and 3-C respectively. The other was a mixed ganglioside preparation from bovine brain. These were employed against the F, V, and C2 reagents. Negative results were obtained in all cases.

The presence of a complex containing sialic acid associated with a lipid-rich fraction would suggest possibly a ganglioside type of compound. This idea occurred, but then was discarded when it was noted that Klenk and Lauenstein (1952) had demonstrated that bovine erythrocyte stroma
possess no gangliosides. It was concluded that the sialic acid involved in the F specificity must be connected to a glycopeptide unit which is associated with the lipid fraction. A crude estimate on the F-active 2B fraction indicated about 15% protein by the Folin test.

At the time of this writing it had been pointed out that Yamakawa and Suzuki (1953) had found lipid complexes which contain sialic acid (hematosides -- their term which seems to be synonymous with the term ganglioside) in some samples of bovine stroma. This varies, apparently, among the individuals of the species. However, in view of the failure of the chloroform-methanol extraction to isolate an active F unit, a glycolipid component by itself apparently is not a complete enough unit to possess the F property. It might possibly be an integral part, but requires an additional component which is separated from it by the extraction method. The fact that the extracted residue does not possess F activity suggests that a necessary component has been removed from it by the organic solvent. Perhaps this is a situation similar to that observed by Koscielak (1962) where two components of the erythrocyte A antigen could be separated from each other. Neither component by itself was active in the serological system.
The fact that sialic acid is involved in F specificity would lead to hypothesizing that the F-positive erythrocytes possess hematoside type of glycolipid while F-negative erythrocytes lack it and possess only the globo-side type. One objection to this hypothesis is that F-negative cattle are generally rare, although in some segments of the cattle population there might be many F-negative individuals. Klenk and Lauenstein (1952) might have chanced on such individuals as subject (donors) for their studies. Yamakawa and Suzuki (1953) also obtained blood stroma which lacked hematoside, and one might surmise from reading the literature that this type is the more common.

Noting this discrete difference in red blood cell membrane among members of the species, and keeping in mind the repeated implication of glycolipids in serological specificity, it seems certain that this glycolipid difference in bovine stroma will be reflected in an isoantigenic difference. The factor involved might be one, other than F, which was not able to be tested for its susceptibility to the neuraminidase in these experiments. On the other hand, although sialic acid is in a complex, its glycosidic linkage might not be hydrolyzed by the enzyme. The workers who found the hematoside in bovine stroma did not report on this aspect of it. In this case the enzyme would not
inactivate the factor. The factor involved might be reasonably common. It ought to be quite discrete and not be involved in a system in which there is a high degree of cross-reactivity.

This problem could be attacked in a simple manner at present by surveying the glycolipid make-up of stroma from various cattle by the techniques described in the literature (Yamakawa and Suzuki, 1953). If a variation in the glycolipid picture is able to be found among the bloods surveyed, a comparison of their blood types might show a correlation between an antigenic factor and the glycolipid type.

Attempts were made to find a difference in the sialic acid picture of F-positive and F-negative stroma. A quantitative difference was found. The best data were obtained in an experiment in which the sialic acid, which was released from stroma by neuraminidase, was measured. A quantity (15-20mg) of stroma from each of the three F system types was incubated in neuraminidase reaction mixture and the extent of the reaction was followed by assaying the medium with the thiobarbituric acid reaction. After incubation for about 20 hours the enzyme action appeared to be exhaustive. The total amount of sialic acid calculated for the reaction medium was divided by the
amount of stroma (dry weight determined after 48 hours at 100°C) which had been incubated in the medium. The results are presented in Table 6.

An experiment with mild acid hydrolysis (0.1N H₂SO₄, 80°, 1 hour) of FF and VV stroma gave similar findings, although lower values for each. The FF stroma yielded 13.5 µg of sialic acid per mg while the VV stroma yielded 6.5 µg of sialic acid per mg. The lower values might have been due to several reasons, one of which could be less accurate dry weight determinations.

Attempts were made to find qualitative differences in the sialic acids present in the different F system types of stroma. Enzymic and acid hydrolysates were analyzed by paper chromatography. Identical components were detected on the chromatograms whether the hydrolysates were obtained from F-positive or F-negative stroma. Some of the components showing up with the various chromatogram sprays in the case of the acid hydrolysates could not be identified. One component from the 80° hydrolysate by 0.1N-sulfuric acid which showed up very strongly with the resorcinol-TCA spray and less strongly by the Ehrlich spray was not detected by the thiobarbituric acid spray method. This could be a modification of the sialic acid induced by the hydrolysis conditions which is described by Karkas and Chargaff (1964).
TABLE 6

SIALIC ACID RELEASED FROM STROMA OF DIFFERENT BLOOD TYPES (F-SYSTEM) BY NEURAMINIDASE FROM VIBRIO CHOLERAE

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<th>Type of Stroma</th>
<th>Sialic Acid Released/mg Stroma</th>
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<td>F/F</td>
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<td>V/V</td>
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*aAverage of 2 determinations; enzyme hydrolysis of duplicate aliquots of the same stroma preparation; 15.5 and 17.7 ug respectively.

*bSingle determination.

*cAverage of 2 determinations; enzyme hydrolysis of aliquots of 2 different stroma preparations from the same animal; 7.45 and 8.65 ug respectively.
This modified compound does not react in the thiobarbituric acid assay. Perhaps this is the lactone which is formed under hydrolysis conditions which Gibbons (1963) describes. It was noted here that other hydrolysis conditions such as pH adjustment to 2.5 or 3.3 and heating at 100°C for one hour did not produce such a component. However, the total amount of TBA-reactive material produced by the less acidic conditions was considerably less. The lower value obtained for the sialic acid content by the acid hydrolysis (when compared to the enzyme hydrolysis) might be due to these complications. It seems that analysis of sialic acids by a combination of acid hydrolysis and thiobarbituric acid assay might not be as desirable as other possible methods.

A review and discussion of the results

From the early experiments it is indicated that the isoantigens are quite labile. The B system factors are probably more susceptible to destruction than some of the others. A comprehensive study of the pH stability of the blood factors was not done but results obtained indicate that this might be a worthwhile study. A clear difference was noted between B system factors and the Z factor with the former being the more sensitive. There is indication that the R and A factors resemble the Z more than the B
system factors in their pH stability. A knowledge of the stability of various factors under various conditions is necessary when contemplating methods for isolation and study. A systematic study on heat stability of the factors should be followed. The high degree of lability of B system factors under some of the conditions observed makes one question the validity of inhibition of B system reagents by preparations which have been obtained by a process such as the Westphal extraction method.

Stability to extraction procedures using organic solvents must be determined. It appears that the antigenic factors are not destroyed when dried stroma is extracted with dry ether. However, extraction of an aqueous suspension of stroma with ether eliminates the serological activity. When a serological property is lost from a preparation it must be checked whether the treatment destroyed the property, or merely released it from the bulk and it was lost in subsequent handling.

Periodate treatment destroyed the activity of every factor which was checked. This indicates that a carbohydrate moiety is an essential component of the various factors. More refined techniques should be employed in a further study of this kind.
The experiments with the elinin subfraction of the erythrocyte membrane show that a situation similar to that which exists with human erythrocytes is present with the bovine erythrocytes, i.e., a large complex makes up areas of the membrane which possess the serological properties. Electrophoretic experiments indicate that the elinin complex can be broken down into smaller units by electrophoresis. A method of preparative electrophoresis should be followed and the resulting subfractions should be recovered and tested for activity. Preparative methods might be carried out on starch block, starch gel, polyacrylamide gel or by a free boundary method. Preparative electrophoresis might also provide the ultimate method for obtaining a satisfactorily pure S-protein preparation.

In the papain degradation experiments the only factors which were observed to remain active throughout the treatment were the F, V, and C factors. Factors in the A, B, S, and Z systems were shown to disappear because of the treatment. The factor X, which is in the C system also was eliminated. There is the possibility, however, that some of the factors might not have been destroyed, but were in active form in the 1B fractions which contained the small molecules and fragments. The 1B fractions were not able to be employed effectively in the inhibition test because the
non-specific inhibitive properties would mask any specific inhibitive capacity.

The report by Stone and Miller (1955) that proteolytic enzymes were capable of eliminating certain factors, notably B system factors, was with respect to the antigens on the intact erythrocyte. It was stated that "only some of the factors in two or three of the eleven genetic systems were affected by the enzymes." In the experiments here, factors in all systems were affected (with the possible exception of the L, M, and R' systems which were not checked). In the F system, while neither the F nor the V was destroyed by papain, the V factor was eliminated from the membrane surface. This difference in results could be due to a number of reasons: (1) incubation in these experiments was probably extended over a much longer period of time; (2) the substrate in this case was a subunit of the cell membrane and the smaller complex probably had many more sites exposed which were susceptible to the enzyme; (3) exposure to the conditions of the incubation other than the specific action of the enzyme over the extended period of time might have been responsible for the loss of some activities.

The separation of the F and V factors from each other when they occurred together in the erythrocyte membrane of
the heterozygous animal is amazing. The V factor appears to be associated with a large fragment which is released from the elinin particle by the action of papain. This was recovered by fractionation of the soluble portion of the papain digest on a Sephadex G-25 column. The V-active component emerged from the column shortly after the void volume of eluant had passed through. This indicates that it was of a size large enough that it was excluded from the interior of the gel. Since it had passed through the column unimpeded, this indicates that the particle which carried the V property had a minimum molecular weight of 5000. It appears to be a glycoprotein or glycopeptide and is no doubt the same as the product obtained by Uhlenbruck and Schmid (1963). The only active blood group factor found here has been the V, except where there was a slight admixture of F. Uhlenbruck and Schmid imply that they found more than one active factor associated with their glycoprotein, which was obtained from the supernatant of an enzyme reaction mixture by phenol extraction, but they do not indicate which ones. When V-negative substrate was fractionated in the papain procedure a similar component appeared in the column eluate which did not show any blood group properties. It seems that this glycoprotein structure is not the V antigen, or a component only of V-positive cell membranes, but it carries the V factor whenever the membrane possesses it.
The F factor remains with what appears to be mainly a lipid fraction of the papain-resistant insoluble residue. No good analysis of either the F or the V fraction was obtained in this study, but this will be the first item which must be accomplished in a continuation of F and V antigen study. The involvement of sialic acid in F specificity seems to be clearly demonstrated. The experiments were suitably controlled so that the specific action of the enzyme neuraminidase was implicated in the elimination of F activity from stroma preparations. The association of the F factor with a lipid-rich fraction and the involvement of sialic acid strongly suggests that a "hematoside" complex is responsible for F activity. An attack on this problem seems justified by these findings and could be carried out as suggested in the previous section.

The limited amount of data presented show a quantitative difference in sialic acid associated with the various F system type of erythrocyte stroma. The data presented were obtained on sialic acid which was released from the complexes by neuraminidase. The FF stroma yielded twice the amount obtained from VV stroma and the FV stroma yielded an intermediate amount. In an experiment with acid hydrolysis the thiobarbituric acid assay showed, again, about twice the amount of sialic acid in the hydrolysate from the FF stroma as in the hydrolysate from the VV stroma. The assay
values for the acid hydrolysates gave lower amounts of sialic acid per unit of stroma for both types of stroma than the enzyme hydrolysates. This might be due to conversion of some hydrolyzed sialic acid to a lactone form which is non-reactive in the thiobarbituric acid assay. Paper chromatograms of acid hydrolysates showed spots which were reactive with the resorcinol and the Ehrlich reagents but not with the TBA reagent. That component was not identified; it gave a very slight reaction with the hydroxylamine reagent which should react with a lactone. Paper chromatograms of enzyme hydrolysates were not satisfactory because there was streaking from the origin and the areas of the spots were not definite. It seems that it will be necessary to purify the hydrolysates, perhaps by ion exchange chromatography before attempting to identify sialic acid derivatives on paper chromatograms. After purification, the products can be concentrated to a satisfactory degree in order to detect a minor component if one is present. The paper chromatograms of the various hydrolysates showed identical patterns regardless of the FV type of substrate.

The quantitative difference observed in sialic acid among the FV types is consistent with the involvement of
sialic acid in the F antigen. The biggest question which arises is: Why do the F and V conditions substitute for each other when the F and V entities do not seem to substitute for each other structurally in the erythrocyte membrane?


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