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ISOLATION, PARTIAL CHARACTERIZATION, AND
ANTIMICROBIAL ACTIVITY OF IgM FROM
BOVINE COLOSTRUM AND MILK.

The Ohio State University, Ph.D., 1971
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ISOLATION, PARTIAL CHARACTERIZATION, AND
ANTIMICROBIAL ACTIVITY OF IgM FROM
BOVINE COLOSTRUM AND MILK

DISSERTATION

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

By
Surinder Kumar, B.Sc., M.S.

* * * * *

The Ohio State University

1971

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I wish to express my sincere appreciation and gratitude to my adviser Dr. E. M. Mikolajcik, for his valuable guidance and encouragement. His affectionate concern for his students will always be remembered with reverence.

At this time, my head bows with respect and gratitude for my parents, Mr. and Mrs. Kanshi Ram, whose sacrifices enabled me to pursue my education.

My special thanks are due to my host country— the United States of America— and its wonderful people whom I had the opportunity to meet.

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To

DR. E. M. MIKOLAJCIK

In recognition of his ingenuous advice and inspiring leadership throughout my Ph.D. program -- this little work is dedicated with admiration and affection.
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INTRODUCTION

Immunoglobulins are a unique group of high molecular weight proteins interrelated chemically and antigenically and, in most instances, possessing antibody activity. They play a significant role in the immune response of the body to alien bacteria, viruses, or other immunogenic materials.

In the human blood serum, three major classes of immunoglobulins have been demonstrated on the basis of chemical structure, antigenic properties, and physical-chemical behavior. These are IgG, IgM, and IgA. Two other immunoglobulin classes, IgD and IgE, are recognized but these are less abundant and poorly characterized. Each class may have several subclasses and numerous genetic variants. A substantial body of information also exists on immunoglobulins in human milk.

For the bovine species, the situation with respect to the immunoglobulins is less clear. Although bovine milk and colostrum are known to contain immunoglobulins, only limited information is available on the nature of the individual classes present and their physical, chemical and antibody properties. In contrast to the human species, it is known that there is little or no placental transfer of
immunoglobulins in the bovine species. The immunoglobulins in bovine milk and colostrum, therefore, assume added importance, and merit a complete investigation.

The role of bovine lacteal immunoglobulins is far from completely understood. However, the immunoglobulins of milk and colostrum are important in providing immunity to the young calf against infectious diseases. Thus, it may follow that the immunoglobulins of bovine milk can influence the growth and metabolic activity of microorganisms used in the preparation of various dairy foods, and thereby affect the final product.

To determine the exact role of the immunoglobulins in milk and colostrum, it is imperative that all the components of the immune system are identified, isolated in pure form, characterized, and their antimicrobial activity examined. As a step in this direction, the IgM class of bovine lacteal immunoglobulins was selected for an in-depth study in order to provide this needed information.
REVIEW OF LITERATURE

Bovine lacteal secretions have been shown to contain inherent materials which influence the growth and metabolic activity of a wide range of microorganisms (95).

The immunoglobulins are one group of agents which have been shown to exhibit antimicrobial activity in bovine milk and colostrum (149). Chemically, the immunoglobulins are heterogeneous (24). Recently, it has been recognized that bovine milk contains in varying amounts at least three major classes of immunoglobulins i.e. IgG, IgA, and IgM. Two subclasses of IgG, IgG1 and IgG2, have also been shown to be present (18).

The present study is concerned with the immunoglobulins, primarily IgM: its chemical and physico-chemical characteristics and its role as an antimicrobial agent in milk and colostrum.

The review of literature is devoted to a general discussion of the chemical nature and the biological activity of the immunoglobulins in milk and colostrum with specific attention to the IgM class.
Immunoglobulins: Nomenclature and General Characteristics

The World Health Organization (190) defines immunoglobulins as "proteins of animal origin endowed with known antibody activity, and certain proteins related to them by chemical structure and hence antigenic specificity. Related proteins for which antibody activity has not been demonstrated are included—for example, myeloma proteins, Bence-Jones proteins and naturally occurring subunits of the immunoglobulins." These proteins occur in blood serum (53), milk (61), saliva (28), tears (190), spinal fluid (28), lymph nodes (85), spleen (190), and urine (190).

Tiselius and Kabat (180) showed by free boundary electrophoresis that antibodies were associated with the serum γ-globulin fraction. The γ-globulin fraction has now been shown to consist of a number of related but heterogeneous proteins called immunoglobulins. Each immunoglobulin molecule is made up of one or more monomer units of approximately 150,000 to 160,000 molecular weight (34, 52). The monomer unit is composed of two heavy and two light polypeptide chains bonded through disulfide linkages (94). Light chains are of two antigenic types, termed kappa (κ) and lambda (λ) (94). All classes of immunoglobulins have identical light chains, approximately two-thirds of any single immunoglobulin class having the κ type and one-third
having the \(\lambda\) type (33). For each class, however, heavy chains are different structurally and antigenically. Structural dissimilarities are reflected in functional differences among the classes. Thus, immunoglobulins are classified primarily on the basis of antigenic uniqueness of the heavy chains (34). For each of the five types of heavy chains \(\gamma\), \(\alpha\), \(\mu\), \(\delta\), and \(\varepsilon\), the immunoglobulin class is IgG, IgA, IgM, IgD, and IgE, respectively. This classification system for human immunoglobulins has been adopted by the World Health Organization (WHO) (191).

A summary of the main characteristics of each class of human immunoglobulins is given in Table 1. The nomenclature committee of the American Dairy Science Association, in its most recent report (157) has recommended that the WHO classification system be followed for the bovine immunoglobulins.

The four-chain structure first proposed by Porter (143) for rabbit IgG has been found to apply to immunoglobulins of all vertebrates (34). The structure is normally studied by reduction and alkylation followed by fragmentation with proteolytic enzymes or cyanogen bromide (18). Porter (142) showed that digestion of rabbit IgG with papain, in the presence of cysteine, decreased the \(S_{20}\) value from 7S to 3.5S. By ion-exchange chromatography, he obtained three fragments, two of which are now known as Fab (antigen-binding fragments), and the third is called Fc (crystalizable fragment). The Fab fragment contains the N-terminal half of the heavy
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aData were compiled from the following sources (11, 18, 24, 25, 33, 34, 51, 52, 90, 171, 190, 191)
chain and one of the light chains (18), whereas the Fc fragment contains the C-terminal half of the two heavy chains (18). When a Fab fragment is reduced with sulfhydryl reagents, the light chain and the heavy chain portions of Fab are separated (18). The latter is called the Fd fragment. The schematic diagram in Figure 1 points out the structural complexity of immunoglobulins.

Specific Classes of Immunoglobulins

Because of their related structures, immunoglobulins share several physico-chemical characteristics (34). Hence immunoglobulins of different classes may even possess similar antigenic properties (52, 64). However, each class of immunoglobulin has specific physico-chemical and antigenic features distinguishing it from other classes. A review of information of the individual immunoglobulins, therefore, merits attention.

IgG. IgG is, probably, the most extensively studied immunoglobulin. It is the predominant immunoglobulin of human blood sera (18, 28, 32), and human internal secretions, i.e. aqueous humor of the eye (171), cerebrospinal fluid (171) synovial fluid (171), amniotic and peritoneal fluids (171, 191). In human blood sera, IgG constitutes approximately 75% of the total immunoglobulin content.

IgG consists of a single monomer unit, with either
Figure 1. Proposed four-chain general structure of immunoglobulins (18).
or \( \lambda \) light chains and \( \gamma \) heavy chain (142). Based upon minor differences in the antigenic and physico-chemical properties of the \( \gamma \) chain, human IgG has been subdivided into four subclasses, i.e. IgG1, IgG2, IgG3, and IgG4 (33). The principal difference among these subclasses is the number and position of inter and intra-chain disulfide bonds (18, 33, 34).

Human IgG is reported to have a sedimentation constant of 7S (24, 40, 63, 108), and a molecular weight of approximately 160,000. However, values ranging from 6.3 to 7.2S (16, 18, 32, 112) for sedimentation constant, and 130,000 to 170,000 (18, 34, 40) for molecular weight have been reported. It has a carbohydrate content of 2 to 4% (40) predominantly hexoses (18).

Reductive cleavage of disulfide bonds of IgG results in a drop in molecular weight from about 160,000 to 50,000 confirming a multiple chain structure linked through S-S bonds (34). Both papain and pepsin break the IgG molecule into the smaller fragments, Fab and Fc (34).

IgA. IgA is the principal immunoglobulin of the exocrine secretions of man (69). It is also present in blood sera (70) and in some endocrine secretions (93, 148, 155) but only as a minor component. Chodriker and Tomasi (28) showed by quantitative measurements that IgA was the predominant immunoglobulin of human colostrum. Similar results
have been reported for other external secretions (18, 69, 181, 182).

IgA isolated from blood serum has a molecular weight of 160,000 (93), whereas IgA from external secretions have molecular weights ranging from 150,000 to 600,000 (155), the predominant molecular weight being 390,000 (18, 171). These values suggest that IgA may occur as a monomer, dimer or even a trimer. Immunelectrophoretic studies by Tomasi and Bienenstock (181) revealed that IgA from external secretions contains an extra protein (secretory) piece bonded to two monomers of IgA. The secretory piece which has a molecular weight of approximately 50,000 has also been found in free form (18, 33, 34).

IgA resembles other immunoglobulins in being composed of heavy (\(\alpha\)) and light (\(\kappa\) or \(\lambda\)) chains in the same relative proportion (34). Human IgA has a higher carbohydrate content than IgG. Reported values range from 8 to 14\% (18, 34). Nagasawa et al. (128) analyzed the carbohydrate moiety and found 3.43\% hexose; 3.74\% hexosamine; 2.72\% fucose; and 3.22\% sialic acid. Most species have only one class of IgA (18). However, two subclasses IgA1 and IgA2 have been reported for the human species (191).

**IgM.** One of the earliest reports on IgM was that of Kabat and Pederson (81) in 1939 on the presence of 18S antibodies in the human sera. On further characterization,
Kabat (80) noted that these antibodies had an average $S_{20}$ value of 19.8 corresponding to a molecular weight of 990,000. More recent studies have shown that IgM appears in the serum as a primary response to antigenic stimulation (2, 32, 53, 54, 68, 73, 88, 109). In the newborn baby, the first immunoglobulins synthesized belong almost exclusively to the IgM class (40). However, in normal adult human, IgM comprises only about 5 to 10% of total serum immunoglobulins (93).

Deutsch and Morton (42) isolated a number of high molecular weight immunoglobulins ranging from 17.8S to 33.0S with about 80% of these having a sedimentation constant of 18.0S. The term macroglobulins has been used to designate this group of high molecular weight immunoglobulins. The isoelectric points of the macroglobulins range between 6.8 to 7.65. Sulfhydryl reagents such as mercaptoethanol convert the macroglobulins to subunits of 6.3S. Morton and Deustch (125) demonstrated that the native immunoglobulins as well as the subunits have identical antigenic determinants suggesting that the native immunoglobulin is a polymer and sulfhydryl reagents bring about its monomerization.

Physico-chemical characteristics of human IgM were investigated in depth by Caputo and Appella (23). They observed the presence of three macroglobulins with molecular weights of 610,000, 1,360,000 and 1,780,000, and proposed a phenomenon of reversible association of IgM molecules to account for very high molecular weight. Addition of 0.1M
merceptoethanol resulted in the formation of 6S subunits. The isoelectric point for IgM was reported to be 6.4. Kovacs and Daune (90) studied the molecular weight and size of IgM reporting values of 750,000 and 100 Å radius, respectively.

The chemical nature of IgM obtained from the sera of patients suffering from Waldenstrom's macroglobulinemia was investigated by Miller and Metzger (117, 118, 119). The protein showed a sedimentation constant of 17.9S corresponding to a molecular weight of 890,000. Reduction of the disulfide linkages yielded subunits corresponding to a molecular weight of 185,000 supporting their proposal that IgM is composed of five subunits. Lamm and Small (94) isolated IgM from rabbits stimulated with endotoxins. The native macroglobulins had molecular weights ranging from 850,000 to 900,000 with heavy and light chains of 70,000 and 27,000, respectively. Based upon the 4-chain structure of monomers, their findings support the five-monomer structure proposed for IgM. Micheli and Isliker (114) used reducing agents and reducing enzyme systems to split the IgM molecule into 4-chain units of 180,000 molecular weight. The present status of knowledge, thus, indicates that IgM is probably made up of five 7S units linked by inter 4-chain disulfide linkages (26, 119, 124, 132) probably formed between the C-terminal cysteine residues (46). A schematic diagram of the proposed structure of IgM is shown in Figure 2.
Figure 2. Diagrammatic representation of a proposed model of IgM molecule (10).
Like other immunoglobulins, IgM is a glycoprotein (11, 64, 106, 117). Heimburger et al. (64) analyzed the carbohydrate composition of IgM and reported a total carbohydrate content of 11.8% composed of 5.4% hexose; 4.4% acetylated hexosamines; 1.3% sialic acid; and 0.7% fucose. Miller and Metzger (117) estimated the carbohydrate concentration at approximately 10.2%. The carbohydrate moiety was 3.3% mannose, 1.6% galactose, 0.7% fucose, 3.3% N-acetylated hexosamines, and 1.3% sialic acid.

Studies by Lindqvist and Bauer (102) and Onoue et al. (132) indicate that IgM antibodies may carry five or six combining sites. If IgM is composed of five 7S subunits (26, 119) this would indicate that every subunit of IgM carries one combining site: an assumption which has been substantiated by Hill and Cebra (71) and further confirmed by other workers (26, 46, 64, 73). Kaplan and Kabat (82) presented direct evidence that the combining sites of IgM monomers are significantly smaller than those of IgG with similar specificities. However, it is not known whether the IgM monomer inherently has only one combining site or if only one combining site is available stereochmically.

The presence of a low molecular weight immunoglobulin antigenically related to IgM has been reported by several workers (17, 31, 38, 73, 160, 176, 179). Rothfield et al. (160) found that sera from patients with systemic lupus erythematosus contained a 7S immunoglobulin analogous to
IgM. Stobo and Tomasi (176) isolated a 7S IgM and showed that reduction and alkylation had no effect on this fraction. Clem and Small (31) observed that lemon sharks contain both 19S and 7S IgM having identical peptide fingerprints and carbohydrate content. Hunter et al. (73) studied the response of infants to antigenic stimulation by casein. The serum contained both 19S and 7S IgM fractions. The 7S fraction was found to be more sensitive to variations in temperature than the polymeric form. Dammacco et al. (38) obtained sera from normal persons with hypergammaglobulinemia and from patients with Waldenstrom's macroglobulinemia. None of the hypergammaglobulinemic persons had 7S IgM, whereas 11 of 18 patients with macroglobulinemia contained 7S IgM in concentrations as high as 45% of total IgM. Swedlund et al. (179) isolated naturally occurring 7S IgM and compared its properties with the subunit of IgM obtained by reduction. These were found to be identical chemically and antigenically leading to the conclusion that 7S IgM may be a product of incomplete synthesis of 19S IgM.

Immunoglobulins of Bovine Milk and Colostrum

The status of the knowledge of immunoglobulins in milk and colostrum is rather primitive as compared to that of human or rabbit immunoglobulins. In 1892, Ehrlich (cited in 106) realized that bovine colostrum contained immune bodies which provided passive immunity to the calf. However,
it was not until the extensive work of Smith (169) in 1948 that a systematic attempt was made to isolate and characterize the bovine immune proteins. By salt precipitation, Smith (168) resolved the immune fraction of milk and colostrum into two components designated "Euglobulin" and "Pseudoglobulin". The primary difference between the two fractions was their water solubility: the true globulin (euglobulin) being water insoluble and the false globulin (pseudoglobulin) being water soluble. However, electrophoretic analysis as well as the analytical ultracentrifugation data revealed that the two fractions are not necessarily homogeneous (168). Pseudoglobulin from milk and colostrum was observed to have two components with sedimentation constants of 7S and 10S, whereas the euglobulin was found to be composed of three components with $S_{20}$ values of 7S, 10S and approximately 20S (168). The 7S fraction was reported (168) to be the major component of euglobulin ranging between 76 to 92%. The 18-20S component was found only in the euglobulin fraction and constituted about 10-12% of the fraction (168).

Bovine colostrum has been shown to be rich in immunoglobulins. Popovici et al. (138) reported that 70 to 75% of whey proteins in the first milking are immunoglobulins. Zhadanova et al. (193) found that immunoglobulins constituted about 80% of the colostral whey proteins. In normal milk, the immunoglobulins made up approximately 15% of the
total whey proteins. Similar results were reported by Larson and Kendall (96), who followed the change in whey proteins throughout the lactation period. Porter and Conrad (141) noted that the concentration of immunoglobulins in colostrum was 3.56% and decreased logarithmically during the 60 hours after parturition. The absolute concentration of these proteins diminished to 0.05% within ten days after which it remained relatively constant.

An excellent review on bovine immunoglobulins has been recently published by Butler (18). Three classes of immunoglobulins have been recognized to be present in bovine sera and in lacteal secretions. Based upon their similarities to known classes of other species, these are designated as IgG, IgA, and IgM (157).

IgG. IgG has been shown to be present in serum, milk and colostrum constituting the major part of the bovine immunoglobulins. About 85 to 90% of the immunoglobulins present in serum or whey belong to this class. Bovine IgG is comprised of two subclasses IgG1 and IgG2 which can be separated by anion exchange chromatography (127) or by ethanol fractionation (18) and have different electrophoretic (18) and immunoelectrophoretic (127) patterns. Apparently, these differences are due to the amino acid composition (64) and the antigenicity (18) of the two subclasses.
IgG1 is the principal immunoglobulin in milk and colostrum, whereas IgG2 constitutes the major portion of immunoglobulins in bovine sera. Findings of Murphy et al. (126) suggest that there is a selective transport of IgG1 from bovine sera to lacteal secretions. IgG1 has a sedimentation constant of 6.6S (127) as opposed to 6.3S (127) for IgG2 and is the less basic of the two subclasses (157). However, both have identical Fab fragments (33, 157) indicating that the differences reside in the Fc fragment (18).

IgA. IgA has been shown to be the predominant immunoglobulin of human colostrum (69). In bovine colostrum, the presence of a 10S immunoglobulin has been reported by Smith (168) and has also been confirmed by other workers (60, 61, 86, 87). IgA isolated from bovine colostrum and other secretions has a molecular weight of approximately 390,000 (18, 157); a sedimentation constant of 10 to 12S; a carbohydrate content of 8-9% (18); and is sensitive to 2-mercaptoethanol (18). The evidence suggests that IgA of colostrum is a dimer possibly linked to the secretory piece (18, 157). The function of the secretory piece has not been established.

IgM. A macroglobulin identical to human IgM in physico-chemical and biological properties has been reported as present in bovine serum (3, 18, 59, 60, 74, 87, 88, 126, 137) and colostrum (18, 60, 88, 127, 137). Based primarily upon
the similarities in properties with human IgM, this protein has been designated as bovine IgM (157).

Early work by Hanson (60) with bovine serum and colostral immunoglobulins revealed the presence of three to four different fractions. The immunoelectrophoretic patterns, although not identified, show a definite arc characteristic for IgM. Murphy et al. (127), in their extensive work on the identification and characterization of immunoglobulins from bovine milk and colostrum, have demonstrated the presence of a 19S immunoglobulin. This globulin was found to have properties quite similar to those of the analogous protein in human serum as determined by gel filtration, immunoelectrophoresis, anion exchange chromatography, ultracentrifugation, and reduction with mercaptoethanol (127). Similar observations were made by Gough (59) and Anderson et al. (3) who isolated Brucella-agglutinating antibodies from bovine colostrum.

Klaus et al. (87) studied the distribution of IgM in bovine sera, colostra, and calf sera. Bovine sera and colostra contained approximately 3.0 mg IgM/ml. and constituted about 10% of the total immunoglobulins. The calf sera contained very little IgM at the time of birth but the level increased about ten-folds within the first 24 hours after which it gradually decreased. They concluded that the newborn calf is capable of absorbing colostral IgM. This is in sharp contrast to the findings that the human baby can
not absorb IgM (28, 32, 34, 56, 95).

Bovine IgM has $\beta_2$ electrophoretic mobility and a sedimentation constant of 19S corresponding to a molecular weight of 900,000 (157). The carbohydrate content has been reported by Gough (59) to be 12.3%. It is insoluble in water and thus forms a part of the euglobulin fraction of Smith (169). Because of its high molecular weight, IgM diffuses slowly in agar gels, and is identified on immunoelectrophoresis against specific anti-sera as the arc appearing nearest to the sample well (127, 157). In disc gel electrophoresis at pH 4.3, IgM forms a band at the separating gel/stacking gel interface (18).

**Antimicrobial Activity of Milk**

The presence of antimicrobial substances in milk was recognized as early as in 1891 when Freudenereich (55) reported the presence of bacterial inhibitory substances in bovine milk. In 1892, Ehrlich's (cited in 106) classic paper reported the presence of immune bodies in human colostrum and their role in transmission of immunity from mother to offspring.

Stocking (177) inoculated mixed-herd raw milk with a number of different bacterial species. By incubating the samples at 20°C, and conducting plate counts at different time intervals, he found that a significant reduction in the number of microorganisms occurred. However, Heinemann
(66) observed that milk inhibits the growth of some microorganisms, and is ineffective against others. Later Heinemann and Glenn (67) suggested that milk agglutinins were, in part, responsible for the observed inhibition. The selectivity of the inhibitory action of milk was further supported by Evans (50). The bactericidal action of milk was effective against *Streptococcus pyogenes* and *E. coli* but not against *Bacillus subtilis*.

Rosenau (159) studied the phenomenon of inhibition in considerable detail. He demonstrated that the number of *Bacillus* (*Salmonella*) *typhosus* and *Vibrio cholerae* cells decreased during the first 8-10 hours of incubation in raw milk and then rose rapidly. The decrease was attributed largely to agglutination with the additional possibility of phagocytosis by the leucocytes. Hanssen (62) infected fresh raw milk with *B. typhosus* and *Bacillus* (*Salmonella*) *paratyphosus* and observed a considerable reduction in the number of these microorganisms within the first 1 to 4 hours at 37°C. This effect was lost when milk was heated at 75°C for 15 min. He proposed the theory that the bactericidal property was closely related to the oxidizing enzymes of the milk.

Jones and his associates (75, 76, 77, 78, 79), in a series of experiments on the antimicrobial property of milk, observed that: (a) skimmilk had an inhibitory effect on non-hemolytic streptococci; (b) inhibition was the strongest
during the first 2-4 hours of incubation and did not appear to be due to agglutination; (c) artificial immunization with killed culture did not increase the bactericidal property of milk indicating that the substance was naturally present; (d) boiled milk was a perfect medium for the growth of microorganisms which were inhibited by raw milk; (e) the lag phase of mastitic streptococci increased significantly when the organisms were cultured in raw milk; and (f) decreases in the lag phase occurred upon repeated reculturing in raw milk suggesting that the organisms adapted or overcame the inhibitory agents. Jones and Simms (78, 79) termed the inhibitory agent as "Lactenin" and found it was associated with the whey proteins.

Wilson and Rosenblum (186, 187, 188) investigated the antistreptococcal property of milk observing a considerable variation in the susceptibility of the different groups of streptococci. Auclair and coworkers (7, 8, 9, 10) conducted extensive inhibition studies using *Streptococcus pyogenes* and demonstrated the presence of two inhibitory substances "Lactenin 1" and "Lactenin 2". Both inhibitory agents were shown to retard the rate of growth of microorganisms and to have no effect on the lag growth phase. Lactenin 1 was associated mainly with colostrum, and Lactenin 2 with normal milk (7). Both these inhibitors were found in the serum portion.

The agglutination of a number of strains of *E. coli*,
Aerobacter aerogenes, Staphylococcus aureus, and Streptococcus faecalis by raw milk was studied by Hobbs (72). Morris (121) reported reductions in the number of coliform organisms upon incubation in sterile raw milk. In contrast, tremendous growth of the organisms was observed in the same milk heated at 70°C for one hour. He concluded that milk contains both a specific bactericidal agent and a growth inhibitory factor. Morris and Edwards (122) observed that the morphological appearance of Streptococcus lactis organisms grown in raw and heated milk was considerably different: the cultures grown in raw milk had longer chains than those grown in heated milk. They (122) suggested that agglutination was the cause of the inhibition, and denaturation of proteins was a possible mechanism for the loss of agglutinating power of milk. Further studies (123) indicated that the inhibitory substance was not specific for any particular strain, with the organisms varying considerably in their response. The inhibitory property of raw fresh milk was completely destroyed by heat treatment of 55°C for 30 min. The inhibitory agent failed to lyse the cells but changed the surface characteristics of the organisms so that they failed to reflect light.

Sasaki and Aibara (162) reported that the inhibitory substances of milk passed into whey irrespective of how the whey was obtained. They fractionated the whey proteins by use of acetone concentrations of 10, 20, 30, 40, and 50%.
The fraction obtained at 30% acetone exhibited highest inhibition and was found to contain β-lactoglobulin and two other proteins precipitable at 25 and 40% ammonium sulfate. Portmann (144) demonstrated the agglutination of *E. coli* by raw milk and whey. He attributed the inhibition of *E. coli* to the action of agglutinins. Keogh (84) prepared an acetone extract from colostrum and found that it contained both agglutinins and peroxidase. The acetone extract when added to heat-treated milk caused inhibition of the same cultures inhibited by the raw milk.

Randolph (149) and Randolph and Gould (150, 151) demonstrated that all milk contains inhibitory substances: the inhibition ranged from 30 to 80%, depending upon the milk and the sensitivity of the culture. The inhibitory property was not altered when the raw milk was stored at 40°F (4.4°C) for up to 48 hours. However vat pasteurization of the milk significantly decreased the inhibitory effect. Acid and rennet wheys exhibited inhibition similar to the skimmilk. By several fractionation methods, the inhibitory factor was found to be associated with immune globulin fraction.

Singh and Mikolajcik (167) reported that milk was inhibitory to the growth of selected lactic and enteropathogenic microorganisms. The growth patterns of *S. lactis*, *Streptococcus cremoris*, *Salmonella typhimurium*, *S. aureus* and *E. coli* revealed a depressed growth rate in the presence
of raw milk. Heat-treatment of milk at temperatures above 80°C destroyed the inhibitory properties of milk. Reiter and Oram (153) have reviewed the literature on bacterial inhibitors in milk and colostrum, and associated inhibition with one or more of the following: xanthine oxidase, lactotransferrin, lactoperoxidase/thiocyanate/peroxide system, complements, and antibodies. Lactotransferrin was inhibitory to the growth of *Bacillus subtilis* and *Bacillus stearothermophilus*. Complements and antibodies appeared to be responsible for the bactericidal effect on Gram-negative organisms and for the agglutination of Gram-positive organisms.

Stadhouders (174) studied the distribution and inhibitory action of milk agglutinins. Agglutinins were found to be associated with whey proteins and were also present on the fat globule membrane. Homogenization of milk was reported to inactivate the agglutinins. Stadhouders reasoned that increased adsorption of agglutinins on the fat globule membrane was responsible for the loss of inhibitory activity of milk. He obtained pseudoglobulin and euglobulin fractions from milk by the classical procedure of Smith (168), and demonstrated that only euglobulin was capable of inhibiting lactic acid bacteria because of its agglutinating property.

Mikolajcik and Choudhery (115) studied the germination and outgrowth of *Bacillus cereus* spores in skimmilk and
model systems supplemented with immune globulins. Raw skimmilk was found to be most inhibitory. Pasteurization of milk seemed to destroy its inhibitory property. Immune globulins exerted a significant inhibitory effect but not equivalent to that by raw milk. Pasteurization inactivated the immune globulins with respect to their effect on the germination and outgrowth of the spores.

**Antimicrobial Activity of Immunoglobulins**

Sera of normal animals contain substances which react with toxins, viruses, and bacteria to produce neutralization, agglutination and bactericidal effects (40). These substances have been shown to be immunoglobulins in nature and because they appear in the serum without apparent stimulation by exogenous antigens are called natural antibodies (161). Voluminous literature exists on the distribution of these antibodies among various animals and man, and the remarkable range of bacterial species with which they react (189).

Landy and Weidanz (95) found that serum obtained from all mammals exhibited bactericidal activity against *E. coli*, *A. aerogenes*, *Salmonella typhosa*, and *Shigella shiga*. Fink et al. (54) fractionated the serum antibodies by DEAE-cellulose column chromatography and demonstrated that the natural antibodies against *S. typhosa*, *Salmonella paratyphi* and *E. coli* were 19S macroglobulins. These findings were supported by Smith (172) and Gitlin et al. (57) who isolated
IgM by sucrose density gradient ultracentrifugation and found it to be bactericidal to enterobacterial species. In contrast, these workers failed to show any bactericidal effect by IgG or IgA. Killander and Hogman (86) fractionated the serum proteins by Sephadex G-200 filtration into a 7S macroglobulin and an albumin fraction. The bactericidal activity was limited to first macroglobulin fraction (86). Immunoelectrophoretic and sedimentation velocity data revealed the macroglobulin to be predominantly IgM. Michael and Rosen (111) determined the concentrations of IgM required for 50% killing of S. typhosa and E. coli cultures and found these to be 0.0072 and 0.0018 mg respectively. IgM absorbed with S. typhosa lost any further bactericidal activity against additional S. typhosa, but remained effective against E. coli indicating that the IgM molecule may have different binding sites for different organisms. In order to demonstrate the bactericidal effect of IgM in vitro, Michael and Rosen (112) injected varying amounts of IgM into mice and infected them with S. typhosa. All mice injected with 0.5 mg or more of IgM survived whereas all those with less than 0.15 mg IgM died. Rosen (158) obtained sera from several patients with dysgammaglobulinemia, a disease characterized by a very high concentration of IgM and virtually no 7S immunoglobulin, and found extraordinarily high titres of bactericidal activity. His findings suggest that the antibodies to Gram-negative bacteria are present
principally in the 19S immunoglobulin (158).

Cohen and Norins (32) challenged the hypothesis that natural antibodies to somatic antigens of Gram-negative bacteria are located principally in the IgM class of immunoglobulins. They studied the immunoglobulins active against Neisseria gonorrhoea, E. coli, and S. typhosa by the indirect fluorescent antibody technique, a procedure much more sensitive than the widely used agglutination test, and found that IgG, IgA, and IgM were all capable of reacting with the somatic antigens of the organisms studied. However, it has been reported (32, 40, 59) that purified IgG is many times less efficient than purified IgM in bactericidal, agglutinating, complement fixing, and hemolytic activity.

Beernink and Steward (12) observed that sera from normal guinea pigs were bactericidal for E. coli and Salmonella enteritidis. They exposed the sensitized cells of E. coli to guinea pig sera and isolated the proteins adhering to the cells. The proteins were found to be immunoglobulins, primarily IgM and IgG. Further work (13) revealed that IgM attached to S. enteritidis without any bactericidin, whereas IgG required normal bactericidins for attachment and hence for bactericidal effect. Normal bactericidins are substances found in blood serum and act as complements.

Sharpe et al. (166) isolated IgM-type agglutinating antibodies to strains of anaerobic bacteria from bovine rumen fluid and bovine colostrum. Similar agglutinating
antibodies were found in goats and sheep but not in sera of pigs, rabbits, rats, guinea pigs or man.

Porter and Noakes (140) obtained IgG, IgA, and IgM from sows' serum and colostrum and demonstrated that anti-\textit{E. coli} activity was associated with both IgM and IgA.

It has been well established that immunoglobulins are transferred from blood to colostrum and milk (87, 97, 99). It may, therefore, be expected that immunoglobulins isolated from lacteal secretions would exhibit antimicrobial properties similar to blood serum immunoglobulins. Several authors (149, 167, 174) have reported that agglutinins are one of the major natural bacterial inhibitors present in milk. Stadhouders (174) reported that the inhibitory property of milk affecting lactic acid bacteria resided in euglobulin fraction which has been postulated to contain IgG, IgA, and IgM. Reiter and Oram (153) examined the effect of milk immunoglobulins on several species of streptococcus and \textit{E. coli} and found them to be inhibitory.

\textit{Origin of Immunoglobulins in Milk and Colostrum}

The site of synthesis of immunoglobulins present in milk and colostrum has been a subject of much controversy. Until about 1950, it was generally accepted that immunoglobulins of milk and colostrum are derived from the blood (99). However in 1950, Campbell \textit{et al.} (22) suggested
that immunoglobulins of lacteal secretions were synthesized locally in the mammary gland. Extensive reviews favoring (21), and opposing (98) the theory of local synthesis of immunoglobulins have been published. Irrespective of the site of synthesis, however, it is generally recognized that production of immunoglobulins can be stimulated under the influence of antigens. Immunoglobulins, so produced, specifically react with the antigens that caused stimulation. A review of the literature on whether or not the specific immunoglobulins can pass from the blood into milk and colostrum and on the site of synthesis of immunoglobulins will be presented in this section.

Klemperer (89) immunized a lactating goat with S. typhosa using intraperitoneal or subcutaneous injections. The milk obtained from the goat protected mice infected with S. typhosa. An almost identical experiment was carried out by Dieudorre (44) who immunized guinea pigs against cholera but was unable to protect young guinea pigs by feeding milk from immunized animals.

The presence of specific immune bodies effective against E. coli was demonstrated by Reymann (154) in the colostrum of goats infected with E. coli. Little and Orcutt (103) injected Brucella abortus in cows, and were able to show the presence of B. abortus agglutinins in the colostrum. Pattison and Holman (135, 136), in a series of studies, examined the effect of injection of pathogenic strains of
streptococci into the teat canal of goat. The milk from experimental goats was found to contain antibodies against the specific organisms.

Smith and Holm (170) performed hyperimmunization of cows prior to parturition using a mixture of diphtheria toxin and vaccinia virus, and reported the transfer of diphtheria antitoxin and protective antibodies to vaccinia through colostrum to the calf. They concluded that the calf may acquire a high level of passive immunity through colostrum. Antibodies against tetanus were found to be present in bovine milk obtained from animals immunized against the disease.

From a cow infused with a phenol-killed suspension of *S. pullorum*, Greene et al. (58) obtained colostrum which showed high titres of agglutinins specific against *S. pullorum*. Heat treatment of colostral whey at 155°F (68.3°C) for more than 25 minutes decreased the titres. They further demonstrated that the agglutinins had heat sensitivity curves identical to those for immunoglobulins. Lassila et al. (100) injected killed cells of *S. pullorum*, *Salmonella gallinarum*, and *Salmonella anatum* into the mammary gland of cows. The milk obtained from the cows specifically agglutinated the cells of the challenging organism.

Portmann et al. (146) immunized cows against *S. cremoris* and *S. lactis* by subcutaneous injections of heat-killed bacterial cells. Samples of blood and milk were
examined for inhibitory activity against injected organisms. Inhibitory titer of blood and milk increased upon immunization with *S. cremoris*. With *S. lactis*, however, only the inhibitory titer of blood was increased. Randolph (149) immunized rabbits and cows against *S. cremoris* and *S. lactis*, and reported specific increases in the agglutination titers for the homologous strain in the blood serum of both animals. Increases in the inhibition and agglutination titers of the skim milk were observed only in the case of cows immunized with *S. cremoris*. Udder infusions with *S. lactis* or *S. cremoris* did not affect the agglutination titer.

Campbell, Petersen and their associates (21, 22, 141), who have been strong advocates of the hypothesis that mammary gland is capable of synthesizing antibodies, have published several papers reporting the presence of immunoglobulins specific against the microorganisms injected in cow's udder. On the other hand, Lascelles and coworkers (98, 99) have reported evidence which refuted the hypothesis of immunoglobulin synthesis in the mammary gland. They believe that antibodies are transferred to milk or colostrum from blood. This contention has been supported by numerous workers (5, 24, 32, 34, 54, 87, 93, 97). Larson and Gillespie (97), using isotope tracers to study the origin of the milk proteins, reported that immunoglobulins were synthesized at a site other than the mammary gland and passed unchanged from serum to lacteal secretions. Through detailed character-
ization of serum and lacteal immunoglobulins, Askonas et al. (5) demonstrated that immunoglobulins of colostrum are qualitatively identical to those of serum and, thus apparently, are derived from blood serum without any degradation or resynthesis.

Pierce and Feinstein (137) observed that immunoglobulins from bovine sera and colostrum were identical qualitatively but differed with respect to the concentrations of individual immunoglobulins. Similar results were reported by Murphy et al. (126) who demonstrated a selective transport of IgG over other immunoglobulins from bovine serum to colostrum. They further found that transport of IgG1 far exceeded the transport of IgG2 during the formation of colostrum.

Very recently, in a symposium on the bovine immunoglobulins, several workers (20, 85, 129) have reported on studies of the synthesis of immunoglobulins by various tissues of the cow. IgA was found to be the principal immunoglobulin synthesized by the ileum, lungs, lacrimal and thymus glands. The uterus and the mammary gland were reported to be capable of synthesizing IgA and IgG, whereas spleen and lymph nodes synthesized primarily IgG and IgM. Using a direct fluorescent technique, they detected the presence of IgA producing plasma cells in mammary, lung and intestinal tissues, and IgG cells in spleen and lymph node tissues.
From the current knowledge, thus, it appears that: (a) under normal circumstances, immunoglobulins of lacteal secretions are derived from the blood serum; (b) the immunoglobulins are transported from blood to milk or colostrum without any significant change; (c) some selectivity for immunoglobulin transport from serum to colostrum exists; and (d) the mammary gland is capable of synthesizing immunoglobulins, but whether or not this does occur under normal conditions is questionable.
OBJECTIVES

The major objectives of the study are -

1. To evolve a suitable procedure(s) for the isolation of immunoglobulins from bovine milk and colostrum;

2. To isolate and purify the IgM fraction from bovine lacteal secretions;

3. To determine the physical-chemical and chemical characteristics of the isolated IgM; and

4. To ascertain the antimicrobial activity of IgM.
EXPERIMENTAL PROCEDURE

Isolation of Crude Immunoglobulins

Fresh colostrum from an individual cow or raw mixed herd milk was obtained from the Ohio State University Dairy farm. The fat was removed by centrifugation (2-4 C) at 2,000 X G for 15 min in an International High Speed Centrifuge Model HR-1. The pH of the skimmilk or defatted colostrum was adjusted to 4.6 with 10% acetic acid in a 1M sodium acetate solution. The sample was re-centrifuged to obtain a clear supernatant. The precipitated caseins were discarded and the pH of the whey was adjusted to 6.5 with 0.5N NaOH. The fine precipitate formed was removed by centrifugation at 2,000 X G for 20 min. A mixture of the crude immunoglobulins was obtained by the ammonium sulfate precipitation method of Smith (168). A flow diagram is shown on page 37.

Fraction D from this procedure consisted of a mixture of immunoglobulins and was used for further fractionation and characterization.

Fractionation of Immunoglobulins

For the fractionation of the immunoglobulins into
Figure 3. Flow diagram of Smith's Procedure.
individual classes, the following procedures were evaluated
(a) Smith's ammonium sulfate treatment (168), (b) ultra-
filtration, (c) gel filtration alone or in combination with
ion exchange column chromatography, and (d) density gradient.

The efficacy of the methods and the identification of
the various classes of immunoglobulins were made by either
or both of the following: immunoelectrophoresis and poly-
acrylamide disc gel electrophoresis. For immunoelectro-
phoresis, the procedure followed was that of Kumar and
Mikolajcik (92) and Arquembourg et al. (4). All preparative
reagents and equipment were purchased from Colab Laboratories,
Inc., Chicago Heights, Illinois. For polyacrylamide disc
gel electrophoresis at alkaline pH, the procedure of Davis
(39) was used with Tris-glycine buffer, pH 8.9, and gel
strength of 7.5% and at acid pH, acetic acid-β-alanine
buffer, pH 4.5, and 7.5% gel strength were used as outlined
by Reisfeld et al. (152).

(a) **Smith's ammonium sulfate treatment (168).** The D
Fraction was subjected to further purification to obtain
euglobulins and pseudoglobulins as outlined below:

\[
\begin{align*}
\text{D} \\
(\text{i}) & \text{Dissolve in water at } 1^\circ\text{C} \\
(\text{ii}) & \text{Adjust pH to 4.5} \\
(\text{iii}) & \text{Filter}
\end{align*}
\]
(b) Ultrafiltration. Isolation and fractionation of immunoglobulins were undertaken by ultrafiltration of whey or Smith's D Fraction. The ultrafiltration was carried-out using an Amicon Ultrafiltration apparatus, Model TCF-10, and a Diaflow XM-300 membrane. The equipment and membrane were purchased from the Amicon Corporation, Lexington, Massachusetts.

In order to find optimum conditions for satisfactory separation of immunoglobulins, two different starting materials, whey or Fraction D; and three different eluting
solutions, distilled water, 0.15M NaCl, or 0.1M Tris-HCl buffer (pH 8.0) containing 0.15M NaCl were tried. The following procedure was used: A solution of Fraction D was prepared in the eluting solution to an approximate concentration of 0.1%. In the case of whey, the sample was diluted with an equal volume of the eluting solution. The ultrafiltration cell was assembled for recirculation. The cell was charged with 100 ml of the sample and the nitrogen gas pressure was regulated at 5-10 psi. The sample was constantly recirculated to avoid deposition of proteins on the membrane surface. The filtrate was collected in a flask. When the level of the sample in the cell chamber was reduced to 50 ml, 50 ml of the eluting solution was added and the filtration step was repeated. The filtration and recharging steps were repeated for a total of three times. The filtrates were collected as one fraction and the retained material constituted the second fraction. The two fractions were dialyzed against distilled water for 24 hours, freeze-dried, and then stored at 2-4 C.

(c) Gel filtration/ion exchange column chromatography. DEAE-cellulose. Twenty-five grams of DEAE-cellulose was suspended in approximately one liter of distilled water, allowed to settle for 15-20 min, and the supernatant was discarded. The slurry was washed for two additional times with distilled water to remove the fine particles and soluble impurities. In a similar fashion, three washings
were made with 0.5N NaOH. Finally, the slurry was washed with distilled water to remove alkali and then was neutralized to pH 7.0 with 0.5N phosphoric acid. The slurry was then suspended in 0.005M phosphate buffer, pH 7.0. A column (2.5 X 40 cm) was packed with the DEAE-cellulose using air pressure of 5-6 psi. The phosphate buffer was passed through the column continuously until the effluent was at pH 7.0.

One milliter of an approximate 1% solution of Fraction D was applied to the column and eluted: first, with one column volume of 0.005M phosphate buffer, pH 7.0, and then, by the a gradient elution system. The gradient elution system was achieved by using 250 ml of 0.005M phosphate buffer, pH 7.0, in one flask and in the second flask, 0.1M phosphate buffer (pH 7.0) containing 1M NaCl. The flasks were connected through a glass siphon arrangement so that the stronger molar solution flowed into the weaker solution and then to the column.

The effluent was monitored at 280 nm with an LKB Uvicord II UV Spectrophotometer with attached recorder. The fractions exhibiting activity at 280 nm were collected. For each peak, the fraction was pooled, dialyzed against distilled water at 4 C, concentrated by ultrafiltration through a DiaFlo UM-10 membrane, freeze-dried, and stored at 2-4 C.

**DEAE-Sephadex.** For this phase of the study, DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) of fine grade was suspended in 0.02M phosphate buffer, pH 8.0, and a column of
1.5 X 30 cm was charged according to the procedure of Murphy et al. (127). The column was equilibrated with 0.02M phosphate buffer, pH 8.0. One milliter of the Fraction D solution of known protein concentration was applied to the column and eluted with 0.02M phosphate buffer, pH 8.0. The protein in the eluant was monitored at 280 nm with the Uvicord II apparatus. The elution was continued until the eluant demonstrated no absorbance at 280 nm for one hour. At this point, a 0.3M phosphate ion gradient was applied. All fractions showing absorbance at 280 nm were collected, dialyzed against distilled water, lyophilized, and stored at 2-4 C. The conductivity of selected fractions was measured with an LKB Conductolyzer Type 5300B in order to follow the phosphate concentration during elution.

Bio-Gel. Biol-gel A-5m, 100-200 mesh, was purchased from Bio-Rad Laboratories, Richmond, California. The gel was selected for its wide operating range, i.e. 10,000 to 5,000,000 molecular weight. The gel was suspended in 0.1M Tris-HCl buffer (pH 8.0) containing 0.15M NaCl to a thin slurry, warmed to 50 C, and deaerated using a diffusion pump. A column of 2.5 X 40 cm was packed and the Tris-HCl-NaCl buffer was passed through the column until the pH of the eluant stabilized at 8.0 and a constant flow rate was achieved.

A measured volume of Fraction D was applied to the column and eluted with the Tris-HCl-NaCl buffer. The
absorbance at 280 nm of the eluant was monitored continuously with an LKB Uvicord II. Fractions corresponding to individual absorbance peaks were collected, dialyzed, and concentrated using an Amicon ultrafiltration apparatus with a Diaflo XM-100A membrane. The samples were either lyophilized for storage at 2-4°C or used directly for further identification of the fractions.

(d) Sucrose density gradient. Fraction D was dissolved in 0.1M Tris-HCl buffer (pH 8.0) containing 0.15M NaCl to an approximate protein concentration of 1%. Sucrose solutions containing 10, 20, 30, and 40% (w/w) were prepared in the buffer. Into a series of Beckman No. 303934 cellulose nitrate tubes was added carefully by means of a 2.5 ml syringe and a 23 gauge 5 cm blunt end needle, 2.2 ml of each sucrose solution as suggested by Gough (59). Alternately, a gradient-making cell (59) was used for preparing the 10 to 40% sucrose gradient tubes. For gradient equilibration, the tubes were stored at 2-4°C for 24 hours. Just prior to centrifugation, 0.5 ml of the Fraction D solution was layered on the surface of each tube excepting the blank tube to which was added 0.5 ml buffer. The tubes were then centrifuged in an Beckman Model L preparatory ultracentrifuge at 78,000 X G for 24 hours using a type 50 angle rotor.

At the end of the run, the tubes were punctured at the bottom and measured fractions were collected. Alternately, measured portions were withdrawn through a capillary tube
arrangement in which one end of the capillary tube reached the bottom of the gradient tube and the other end was connected to a suction pump through a graduated centrifuge tube which served as the collection flask. Care was taken not to disturb the precipitate, if any, in the gradient tube. Upon withdrawing the liquid portion of the gradient, the precipitate was dissolved in the Tris-HCl-NaCl buffer and this constituted an additional fraction. Identical fractions from each gradient tube were pooled, dialyzed against running tap water at approximately 10 C, concentrated by ultrafiltration through a Diaflo UM-10 membrane, freeze-dried, and stored at 2-4 C.

The blank tube was used for determination of the gradient. Eighteen 0.5 ml and one 0.3 ml fraction were withdrawn from the blank tube and the sucrose concentration of each fraction was determined by the method of Dubois et al. (47).

Chemical and Physical-Chemical Characterization of IgM

For chemical and physical-chemical characterization of the fractionated IgM, analyses were made of (a) sedimentation constant, (b) extinction coefficient, (c) hexose content, (d) sialic acid, (e) fucose, (f) amino sugars, and (g) sulfhydryl groups and disulfide bonds.
Figure 4. Standard curve for the determination of sucrose.
(a) Sedimentation constants. A Beckman Model E analytical ultracentrifuge equipped with Schlieren optics was used to determine the sedimentation constants. All experiments were carried out at a speed of 40,000 rpm (116,272 X G) in a pH 8.0, 0.1M Tris-HCl buffer containing 0.15M NaCl at a temperature of 20 C. The peak mobilities were measured with a Nikon Profile Projector Model 6C. The sedimentation constants were calculated with a Olivetti-Underwood Programma (0) computer using the computer program outlined in Fig. 43, Appendix.

(b) Extinction coefficient (E 280 nm). The immunoglobulin IgM was dissolved in 0.1M Tris-HCl buffer (pH 8.0) containing 0.15M NaCl to obtain solutions of approximate protein concentrations of 0.8, 0.6, 0.4, and 0.2%. The protein concentration of the solutions was determined by the micro-Kjeldahl method (6). Based upon the nitrogen content of 14.5% reported for human IgM (117), a conversion factor of 6.90 was used. One of the solutions of known protein concentration was then serially diluted with the Tris-HCl-NaCl buffer to obtain the desired range of protein concentrations. The absorbance of the solutions was measured at 280 nm in a Hitachi Perkins-Elmer Model 134 UV-VIS spectrophotometer and the extinction calculated for 1% protein.

(c) Hexose content. The hexose content of IgM was determined as follows: solutions containing 0.1 - 1.0% IgM were prepared in 0.15M NaCl. To one milliliter of the IgM
solution, one milliliter of 2N H$_2$SO$_4$ was added and the mixture was heated at 100 C for one hour to hydrolyze the protein. The hydrolysate was neutralized with saturated Ba(OH)$_2$ and the precipitate was removed by centrifugation. The supernatant was deionized by passing through Amberlite MB-3 resin. The eluant was subjected to paper chromatography in a pyridine-ethyl acetate-water (1.0:3.6:1.15) system. Simultaneously, standard solutions of glucose, galactose, and mannose were run on the same paper. The color of the standards was developed with aniline-acid-oxalate reagent (101). The paper with the unknowns was cut at points with mobilities corresponding to the standards and eluted with water. The hexose content of the individual points was determined by the method of Park and Johnson (134). From these values, the ratio of the hexoses present in IgM was calculated.

Standard solutions containing different concentrations of hexoses in a ratio identical to that determined for IgM were prepared. A standard curve was prepared by the method of Mokrasch (120). The total hexose content was determined for the deionized eluant of the hydrolyzed IgM by the procedure of Mokrasch (120).

The concentration of individual hexoses was calculated by using the known ratio of individual hexoses.

(d) Sialic acid. The sialic acid content was determined by the thiobarbituric acid method of Warren (184). Bovine serum albumin served as a blank. A standard curve
Figure 5. Standard curve for hexose determination.
Figure 6. Standard curve for the determination of sialic acid.
for the determination is shown in Fig. 6.

(e) **Fucose.** Fucose was determined by the procedure of Dische and Shettles (45).

Solutions containing 0.5, 1.0, 2.0, 3.0, and 5.0 mg% of fucose (Nutritional Biochemicals Corp, Cleveland) were prepared in water. The standard curve shown in Fig. 7 was obtained.

Two stock solutions were prepared: one containing 5 mg% of fucose and the other 1% IgM. For the determination of fucose in IgM, a series of test solutions were prepared according to the following scheme:

<table>
<thead>
<tr>
<th>Fucose Stock Solution</th>
<th>IgM Stock Solution</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

The solutions were mixed well, and 1 ml samples were subjected to reaction with sulfuric acid for a period of 10 minutes. The absorbance of the reaction mixtures was measured at 396 nm and 430 nm after the addition of cyteine hydrochloride (Nutritional Biochemi Corp.). \( \Delta \text{OD} (E_{396} - E_{430}) \) values were calculated for each individual solution. Similarly \( \Delta \text{OD} \) values were obtained for solutions containing only fucose and no IgM. \( \Delta \text{OD} \) values for IgM were then calculated by difference and the fucose concentration determined
Figure 7. Standard curve for the determination of fucose.

\[ Y = 0.165X - 0.012 \]

\[ \Delta E \]

CONC. (mg x 10^{-3})

0.0 0.2 0.4 0.6 0.8

1.0 2.0 3.0 4.0 5.0
from the standard curve.

(f) Amino sugars. Total hexosamines (free and acetylated) were determined as recommended by Boas (14) under the following conditions: from various preparations of IgM of known protein content as determined by the micro-Kjeldahl method, solutions were prepared containing from 5–10 mg protein/ml. The protein was hydrolyzed by adding 2 ml of 1.5N HCl to 1 ml of the sample and heating at 100 C for 15 hrs. The volume was then made up to 10 ml with water. Five milliliters of the hydrolyzate was passed through a Dowex-50 column, followed by a washing with 10 ml of distilled water. The washing was discarded. The hexosamines were eluted from the resin with 2N HCl and a total volume of 5 ml was collected. One milliliter of the eluant was used for the determination of total hexosamines. Glucosamine-HCl (Nutritional Biochemicals) was used as a standard.

N-acetyl hexosamines were determined by the procedure of Kumar and Hansen (91) using N-acetyl-D-glucosamine (Calbiochem, Los Angeles) as standard.

(g) Sulfhydryl groups and disulfide bonds. Free -SH groups were determined by Ellman's method (48). \( \beta \) -lactoglobulin served as the standard. 5,5' dithio-bis-(2-nitrobenzoic acid), DTNB, was purchased from Nutritional Biochemicals Corp.

The disulfide bonds were reduced at pH 8.6 in 0.2M Tris-HCl buffer at 25 C for one hour using 1% protein and
0.001M dithiothreitol (Nutritional Biochem). The total -SH content was determined by the procedure of Miller and Metzger (118) using 5M guanidine and DTNB. The disulfide bonds were calculated as:

\[ S-S \text{ bonds} = \frac{\text{Total SH} - \text{Free SH}}{2} \]

Subunits of Bovine IgM

Subunits of IgM were prepared by reduction and alkylation of the IgM molecule. Two agents, 2-mercaptoethanol or cysteine, were used to achieve reduction. The reduction was performed in pH 8.6, 0.2M Tris-HCl buffer at room temperature.

To two milliliters of a 1% solution of IgM in Tris-HCl buffer was added two milliliters of 0.2M 2-mercaptoethanol. The mixture was incubated at room temperature (22 - 25 C) for two hours, after which, the reaction was stopped with a 10% excess of iodoacetamide. In the case of cysteine, 0.1M cysteine was used in place of 0.2M 2-mercaptoethanol and the reaction time was 10 minutes.

The reduced IgM was subjected to column chromatography using Sephadex G-200.

Preparation of Anti-Sera

Commercial anti-bovine sera, prepared by injecting
bovine blood serum into rabbits, were purchased from three different sources: Difco Laboratories, Detroit, Pentex Biochemicals, Kankakee, Illinois, and Dexter Biologicals Co., Dexter, Michigan.

Anti-bovine gamma globulins and 7S fractions prepared in rabbits were obtained from the above sources as well as from Nutritional Biochemicals Corp., Cleveland.

Polyvalent anti-sera to bovine immunoglobulins and monospecific anti-IgM were kindly supplied by Dr. John E. Butler, U. S. Department of Agriculture, Washington, D. C.

Polyvalent anti-sera to immunoglobulins of bovine colostrum (Fraction D) were also prepared in the laboratory by immunizing a rabbit against Fraction D according to the following schedule: five milligrams of Fraction D dissolved in 2 ml of normal saline was emulsified with 2 ml of Freund's incomplete adjuvant. The rabbit was injected with 0.5 ml each at four interdermal sites and at one subcutaneous site. Three weeks later, the above procedure was repeated with the D Fraction being suspended in Freund's complete adjuvant. At approximately ten days later, three milligrams of a fraction containing predominantly IgM was dissolved in 2.5 ml of normal saline and injected into the four intradermal sites and the one subcutaneous site. At one week later, the process was again repeated but this time Fraction D was used. Seven days after the last injection, the rabbit was bled directly from the heart and
the serum was separated and freeze-dried. The lyophilized preparation constituted anti-Fraction D sera and was used in the various immunological assays.

Assay of Antimicrobial Activity

Four cultures, *Streptococcus cremoris* R-1, *Streptococcus lactis* C-2, *Streptococcus agalactiae* OARDC, and *Escherichia coli* OSU 66, were examined for their susceptibility to IgM.

The procedure of Mikolajcik and Sinha (116) was used for *S. lactis* and *S. cremoris* to determine if agglutination was involved.

For the inhibitory effect, growth of the various organisms was followed with and without added IgM according to the following scheme: Lyophilized cultures were activated in Elliker's broth (Difco, 0974) and incubated at 30 C for *S. lactis* and *S. cremoris* and at 37 C for *S. agalactiae* and *E. coli*. After 48 hours, 0.5 ml of the culture was transferred into 10 ml of fresh broth and incubated for 4 hours. One milliliter of the 4-hr active culture was transferred into 99 ml of Elliker's broth.

Into each of four sterile Bausch and Lomb Spectronic 20 colorimeter tubes, was placed 0.1 ml of a sterile IgM solution prepared in saline. Four IgM solutions were prepared ranging in protein concentration from 50 to 200 mg. The solutions were sterilized by passage through a Gelman membrane filtration apparatus a using 0.2 m membrane. Into
a fifth tube, 0.1 ml of sterile saline was added in place of the IgM. This served as the control. Into each tube, 5 ml of the 4-hr culture previously diluted with 99 ml Elliker's broth was added. The blank consisted of 0.1 ml of the IgM solution and 5 ml of sterile uninoculated Elliker's broth.

All tubes were incubated in a waterbath at the optimum temperature for the organism. The growth of the culture was determined by reading the change in percent transmission for each tube in a Bausch and Lomb Spectronic 20 colorimeter at 610 nm and(or) by withdrawing 0.1 ml of the culture from the colorimeter tubes and plating on Standard Methods Agar (Fisher, B11637).
RESULTS

Evaluation of Anti-sera

For the identification and characterization of immunoglobulins and their classes, immunoelectrophoresis is the single most important technique because of its sensitivity and specificity. Interpretation of immunoelectrophoretic patterns is dependent upon the specificity and potency of the anti-sera employed. Therefore, an examination was made of commercially purchased anti-sera, anti-sera prepared in our laboratory, and anti-sera furnished by Dr. John E. Butler, Dairy Products Laboratory, U. S. Department of Agriculture, Washington, D. C. 20250.

Immunoelectrophoresis with the various anti-sera was carried-out as described by Kumar and Mikolajcik (92).

Commercial Anti-sera

The immunoelectrophoretic patterns of fresh bovine blood serum developed with anti-bovine sera from three different commercial outlets are shown in Figure 8. The three slides shown on the left were developed with 10 \( \lambda \) of anti-sera and a 60 min immunoelectrophoretic run. For the three slides on the right, 20 \( \lambda \) of anti-sera was used with a 70 min run. The three different suppliers of anti-bovine
Figure 8. Immunoelectrophoretic patterns of fresh bovine serum developed with anti-bovine sera (ABS) from three different commercial sources (a, b, and c). The patterns shown on the left were run for 60 min and developed with 10 \( \lambda \) ABS, whereas, those on the right were run for 70 min and developed with 20 \( \lambda \) ABS.
serum (ABS) are designated as a, b, and c.

It is evident that ABS from b was far more potent than that from a or c. At the 10 A concentration, b showed characteristic arcs for IgG, bovine serum albumin, and an additional arc tentatively identified as IgA. ABS-a showed only one major arc corresponding to serum albumin, whereas ABS-c failed to exhibit any protein precipitin bands. For the three patterns on the right, Slide 1 again reveals the high potency of ABS-b with a large number of precipitin bands.

Based upon reported patterns for human blood serum (40), the major band developed by ABS-a was serum albumin with faint arcs characteristic for IgG, transferrin, and probably IgA. ABS-c gave rise to only a faint arc for albumin and transferrin. However, it did display anti-gamma globulin activity as evidenced by arcs for IgG and possibly IgM.

While not shown, identical results were obtained when reconstituted bovine blood serum was used in place of fresh blood serum. Anti-sera from b being the most potent and c the least.

A comparison of the potency and specificity of anti-bovine gamma globulins (AGG) obtained from three different sources is presented in Figure 9. The preparations exhibited differences in potency: c being the most potent and b the least. Only with the preparation, AGG-c, characteristic patterns for IgG1 and IgG2 were evident. AGG-d
Figure 9. Immunoelectrophoretic patterns of fresh bovine serum developed with three different (b, c, and d) commercial preparations of anti-bovine gamma globulins (AGG). Preparations b and c were described by the supplier as anti-bovine gamma globulins, whereas preparation d was sold as anti-bovine serum IgG (7S fraction).
which was labelled by the supplier as anti-bovine serum IgG (7S) exhibited only one arc. Interestingly, outlet b which supplied the most potent ABS dispensed relatively poor quality AGG.

These findings suggest that evaluation should be made of every lot of anti-serum before undertaking any experimentation.

Laboratory and USDA Anti-sera

In Figure 10 are shown the immunoelectrophoretic patterns of immunoglobulins isolated from colostrum or milk (Fraction D) and developed with USDA anti-sera or the anti-sera prepared in our laboratory.

Slide 1 shows the patterns for Fraction D developed with USDA mono-specific anti-IgM serum (Trough A) and with USDA anti-bovine immunoglobulins serum (Trough B). Only one arc was observed with the anti-IgM serum indicating that this preparation was specific for IgM. In contrast and as would be expected, the pattern developed with the anti-bovine immunoglobulin serum revealed the presence of three arcs corresponding to IgG1, IgG2, and IgM. As seen from Slide 2-B, similar patterns were observed when Fraction D was subjected to immunoelectrophoresis and developed with our preparation of anti-sera. The pattern in Slide 2-A was produced with USDA anti-IgM serum.

A comparison (Slide 3) was made of the USDA anti-bovine immunoglobulin serum (Trough A) with our anti-serum (Trough
Figure 10. Immunoelectrophoretic patterns of Fraction D developed with USDA and our lab anti-sera. Slide 1 was developed with USDA anti-IgM (A) and USDA anti-bovine immunoglobulin serum (B); Slide 2 with USDA anti-IgM (A) and our lab anti-serum (B); Slide 3 and 4 with USDA anti-bovine immunoglobulin serum (A) and our lab anti-serum (B). Fraction D in Slide 4 was different from that used in other slides.
B) to ascertain the potency and specificity of the two antisera. The patterns were identical excepting that our preparation yielded a fainter IgM arc than did the USDA antisera.

For Slide 4, the Fraction D preparation differed from the others in that it had been found by ultracentrifugation to contain an 11S fraction (IgA). The patterns developed with the USDA anti-gamma globulin serum (Trough A) had four arcs corresponding to IgG1, IgG2, IgM, and IgA, whereas our preparation (Trough B) failed to reveal the presence of IgA indicating that our anti-serum had been prepared with serum lacking or deficient in IgA. However, it should be emphasized that for our preparation of anti-sera, immunoglobulins isolated from bovine milk or colostrum were used, whereas most commercial houses use immunoglobulins from blood serum or unfractionated bovine blood serum as the challenging agent.

It may be concluded that (a) the USDA anti-IgM preparation is mono-specific, (b) USDA anti-bovine immunoglobulin serum exhibits specificity against IgG1, IgG2, IgM, and IgA, (c) our anti-sera contain antibodies for IgG1, IgG2, and IgM but not for IgA, and (d) some Fraction D preparations are devoid of IgA.

Evaluation of Immunoglobulin Fractionation Procedures
A number of techniques have been reported for the isolation of immunoglobulins from human blood serum and to a lesser degree from other body fluids. Only meager information is available on the applicability of these methods to the bovine species.

At the outset, evaluation and where needed modification was made of procedures for the isolation of immunoglobulins from bovine milk or colostrum. Recently developed analytical tools, polyacrylamide gel disc electrophoresis (PAGE), immunoelectrophoresis (IME), and analytical ultracentrifugation, were used for appraisal of the methods.

Ammonium Sulfate Fractionation

In 1946, Smith (168) reported on the isolation of bovine immunoglobulins from milk and colostrum by salting-out with varying concentrations of ammonium sulfate. A step-wise examination of Smith's fractions was undertaken using PAGE in alkaline and acid systems, IME, and analytical ultracentrifugation.

Polyacrylamide gel disc electrophoresis. In Figure 11 are shown the PAGE patterns at pH 9.5 of the various fractions obtained by Smith's procedure. Tube A is of the whey at pH 4.5 separated from mixed herd milk and reveals the presence of the major whey proteins, i.e. immunoglobulins, proteose-peptone, blood serum albumin, \( \alpha \)-lactalbumin, and \( \beta \)-lactoglobulin. When the pH of the whey was adjusted to 6.5 (Tube C), the pattern was similar to that obtained with
Figure 11. Polyacrylamide gel electrophoretic patterns of (A) pH 4.5 whey, (B) precipitate formed in whey upon adjustment of pH to 6.5, and (C) pH 6.5 whey. Bands identified as IM, immunoglobulins; P/P, proteose-peptone; BSA, blood serum albumin; $\alpha$-Lac, $\alpha$-lactalbumin; and $\beta$-Lg, $\beta$-Lactoglobulin.
pH 4.5 whey. Some reduction in the concentration of the immunoglobulin fraction is evident. However, it is apparent from the pattern shown in Tube B that the fine precipitate formed upon pH adjustment did not contain immunoglobulins and only trace amounts of the other serum proteins.

The PAGE patterns of Smith's fractions are shown in Figure 12. Starting with the neutral whey (Tube A), the first fraction separated at 50% saturation with ammonium sulfate was A (Tube B) and this contained all of the immunoglobulins plus most of the other serum proteins. The supernatant of this fraction (Tube C) was devoid of any immunoglobulins containing mainly $\beta$-lactoglobulin, $\alpha$-lactalbumin, and blood serum albumin.

Fraction C (Tube D) obtained by adjusting the pH of Fraction A to 4.6 and 25% saturation with ammonium sulfate contained all of the casein contaminants leaving the immunoglobulins and other serum proteins in the supernatant (Tube E). At this point, the supernatant was adjusted to pH 6.0 and 40% saturation with ammonium sulfate. The resulting precipitate (Tube F) called Fraction D or Smith's Fraction D showed only one band with a slow mobility and D contained the immunoglobulins. It is apparent that alkaline PAGE failed to resolve the immunoglobulins into individual classes.

The separation of Fraction D on PAGE run in the acid system was undertaken. The patterns obtained with three
Figure 12. Polyacrylamide gel electrophoretic patterns of (A) neutral whey, (B) Fraction A, (C) supernatant from Fraction A, (D) Fraction C, (E) supernatant from Fraction C, (F) Fraction D, and (G) supernatant from Fraction D. The whey was separated from mixed herd milk. Both runs were at pH 9.5, 4 mamp/tube for 40 minutes, and gel strength of 7.5%.
different run times are shown in Figure 13. The amount of sample electrophoresed was not the same for all tubes. One major band and two minor bands are evident in all systems. With increased run times, additional minor bands appeared in the gels as the migration of the proteins increased. There is some evidence that a protein is retained in the spacer gel. The immunoglobulins present in each band were not identified. Considering the nature and concentration of the individual immunoglobulin classes in bovine milk, it is postulated that the major band with greatest mobility is IgG. The protein band in the spacer gel or just below likely is IgM. Because of the low net charge and high molecular weight of the immunoglobulins, one would expect them to migrate slowly as a single component in an alkaline PAGE system and, if present, the individual classes of immunoglobulins would move as separate components in an acid system because of differences in the net charge.

Polyacrylamide gel electrophoresis in an alkaline environment was used for an evaluation of Smith's procedure with bovine colostrum. The PAGE patterns (Figure 14) for pH 4.6 whey (serum) (Tube A), the precipitate formed on readjustment of the pH to 6.5 (Tube B), pH 6.5 whey (Tube C), Fraction A (Tube D), and the supernatant from Fraction A (Tube E) were similar to those observed with mixed herd milk (Figure 12). However, there were some differences in that colostrum was a much richer source of immunoglobulins
Figure 13. Polyacrylamide gel disc electrophoretic patterns of Fraction D obtained from mixed herd milk and run at pH 4.3, 7.5% gel strength, 7 mamp/tube with run times of (A) 45 minutes, (B) 90 minutes, and (C) 120 minutes. The protein concentration was not the same for all tubes.
Figure 14. Polyacrylamide gel disc electrophoretic patterns of (A) colostral whey, pH 4.6; (B) precipitate at pH 6.5; (C) whey, pH 6.5; (D) Fraction A; (E) supernatant from Fraction A; (F) Fraction C; (G) supernatant from Fraction C; (H) Fraction D; and (I) Supernatant from Fraction D. The whey was separated from the colostrum of a single cow. Run was at pH 9.5, 4 mamp/tube for 40 minutes and gel strength of 7.5%.
than normal milk. Furthermore, there was some loss of immunoglobulins during readjustment of the pH to 6.5 (Tube B). Difficulty was also experienced in the separation of the serum (whey) from the casein fraction of colostrum at pH 4.6. This was overcome by the use of a mixture of 10% acetic acid and 1M sodium acetate for pH adjustment.

The major difference between fractions obtained from milk and those from colostrum is revealed by patterns shown in Tube F (Fraction C) and Tube G (supernatant from Fraction C) in that Fraction C of mixed herd milk contained little or no immunoglobulins whereas, this fraction from colostrum was rich in immunoglobulins. Thus, considerable immunoglobulins are lost when this fraction is discarded. However, Fraction D (Tube H) moves as a single band and is relatively free of non-immunoglobulin proteins which remained in the supernatant (Tube I).

In Figure 15 are shown the PAGE patterns obtained when Fraction D from colostrum was fractionated as suggested by Smith into Fractions E and F with further separation into water-soluble pseudoglobulins and water-insoluble euglobulins. It is apparent that PAGE at alkaline pH failed to resolve the immunoglobulins into individual distinct bands, thus, limiting this procedure as an effective tool for characterization of the individual immunoglobulins. However, the PAGE method does reveal the presence of contaminating non-immunoglobulin proteins as shown in Tube E where the
Figure 15. Polyacrylamide gel disc electrophoretic patterns obtained upon further fractionation of the D Fraction by Smith's procedure. Tube A, Fraction D; Tube B, pseudoglobulin from Fraction E; Tube C, euglobulin from Fraction E; Tube D, pseudoglobulin from Fraction F; and Tube E, euglobulin from Fraction F. Fraction D was prepared from colostrum. Run was at pH 9.5, 4 mamp/tube for 40 minutes and gel strength of 7.5%.
water insoluble euglobulins from Fraction F still contained some β-lactoglobulin and α-lactalbumin.

**Immunoelectrophoresis.** Examination of wheys from colostrum of different cows and of Smith's fractions was undertaken using IME.

In Figure 16, Slide 1, the colostral whey from a normal cow shows primarily two arcs identifiable as IgG and blood serum albumin (BSA). If present, other classes of immunoglobulins are not revealed. This does indicate that IgG is the predominant immunoglobulin in colostral whey. Likely, the other classes are in too low concentration to yield their characteristic immunological patterns.

Slide 2 in this figure is the IME pattern of colostral whey from a cow suffering from milk fever. The number of arcs is exceedingly larger than that from a normal cow. When these patterns are compared with those of bovine blood serum (Slide 3), it is evident that whey from a cow with milk fever contains almost all of the proteins present in bovine blood serum. The concentration of BSA in the whey from the diseased cow was significantly greater than that in whey of a normal cow. Similar results were found with wheys obtained from several cows with milk fever. Irrespective of the medical interpretation of these findings, the immuno-chemist should determine beforehand that his source of colostrum is truly representative and not one obtained from a pathological mammary gland.
Figure 16. Immunelectrophoretic patterns of colostral wheys compared with bovine blood serum. Patterns developed with anti-bovine serum. Slide 1, normal colostral whey from a single cow; Slide 2, colostral whey from a cow suffering from milk fever; and Slide 3, bovine blood serum.
A systematic examination by IME was made of the various Smith's fractions and these are shown in Figure 17. Normal colostral whey is shown in Slide 1. Fraction A (Slide 2), the precipitate obtained by 50% saturation with ammonium sulfate yielded a mixture of proteins with electrophoretic behavior corresponding to IgG, IgM, α2M, and BSA. Fraction C (Slide 3) separated from colostrum contained some IgG. However, Fraction D (Slide 4) prepared from the supernatant of Fraction C was rich in immunoglobulins showing characteristic arcs for IgG1, IgG2, and IgM. Fraction D (Slide 5) from the colostrum of a different cow exhibited an additional band tentatively identified as IgA. The euglobulin fraction obtained from both Fractions E and F exhibited IME patterns corresponding to IgM and IgG1 (Slide 6), whereas the pseudoglobulins had either a single IgG arc (Slide 7) or an arc with a spur indicating both sub-classes of IgG. The euglobulins (Slide 8) from Fraction D containing IgA (Slide 5) had IME patterns characteristic for IgG, IgM, and IgA.

These results indicate that (a) bovine colostrum may contain from one to three classes of immunoglobulins, (b) immunoelectrophoresis when run with high potency anti-sera is an excellent tool for determination of the immunoglobulin classes present, (c) some loss of immunoglobulins may occur during Smith's fractionation procedure, and (d) the pseudoglobulins and euglobulins are heterogeneous with respect to immunoglobulin classes.
Figure 17. Immunoelectrophoretic patterns of 1, normal colostral whey; 2, Smith's Fraction A; 3, Fraction C from colostrum; 4, Fraction D; 5, Fraction D containing IgA; 6, euglobulins from Fraction D shown in Slide 4; 7, pseudoglobulins from Fraction D shown in Slide 4; and 8, euglobulins from Fraction D shown in Slide 5. Patterns were developed with anti-bovine serum.
Analytical ultracentrifugation. For further confirmation, the D Fraction obtained from colostrum showing IgG and IgM or IgG, IgA, and IgM upon IME was subjected to analytical ultracentrifugation studies. The results are presented in Figure 18. The D Fraction showing only IgG and IgM patterns resolved into two distinct peaks (Cow B) corresponding to sedimentation coefficients of 6.5S and 19.1S, whereas the D Fraction with IgG, IgA, and IgM contained three distinct peaks (Cow A) with sedimentation coefficients of 6.4S, 10.6S, and 18.8S. This is additional evidence of the heterogeneity of bovine colostral immunoglobulins and of the presence in some samples of IgA.

Isolation and Purification of IgM

Smith's ammonium sulfate fractionation procedure when carried to the D fraction provides a rapid means of attaining a reasonably pure and concentrated preparation of bovine lacteal immunoglobulins. Therefore, it was found expedient to fractionate all milk or colostral sample to the D fraction step with further treatment by ultrafiltration, gel filtration, ion exchange column chromatography, and(or) density gradient separation in order to isolate electrophoretically pure IgM.

Ultrafiltration

When Fraction D was suspended in distilled water and passed through an Amicon XM-300 membrane, cloudiness
Figure 18. Schlieren patterns of Fraction D prepared from colostral whey secured from two different cows. The sedimentation coefficients of the peaks are shown. For Cow A, the patterns were darkened for photographic reproduction.
appeared in the retained portion indicating possible dena-
turation or precipitation. A similar observation was made
of the retained portion from whey which had been diluted
with distilled water prior to filtration. NaCl solutions
(0.15M) appeared to prevent cloudiness of the D fraction
solutions but were not effective with whey. Both systems
generally remained clear when pH 8.0, 0.1M Tris-HCl buffer
with 0.15M NaCl was used. The ultrafiltration experiments
were, therefore, carried out using this latter buffer sys-
tem.

In Figure 19 are shown the IME patterns for Fraction D
(Slide 1), the material retained after addition of three
volumes of the Tris buffer and passage through the filter
(Slide 2), and the ultrafiltrate obtained (Slide 3). Fra-
tion D contained IgG1, IgG2, and IgM, the retained portion
had some IgG and IgM, whereas the filtrate revealed the
presence of only IgG. However, the IgG in the retained por-
tion and in the filtrate exhibited only one arc without a
characteristic spur. This may be due to a low protein con-
centration.

The whey (Figure 19, Slide 4) demonstrated an IME pat-
tern characteristic for immunoglobulins and blood serum
albumin (BSA). Upon passage through the XM-300 membrane,
IgG, IgM, and BSA were retained (Slide 5) and the filtrate
(Slide 6) contained IgG1, IgG2, and BSA. Bovine blood serum
albumin could not be effectively removed even after repeated
Figure 19. Immunoelectrophoretic patterns of fractions obtained upon ultrafiltration of Fraction D or whey through an Amicon XM-300 membrane. Upper left: 1, Fraction D; 2, retained fraction; 3, filtrate. Upper right: 4, whey; 5, retained fraction; 6, filtrate. Patterns developed with anti­bovine blood serum.
passage through the membrane. Because the reported molecular weight of BSA is only 69,000, much lower than the exclusion molecular weight (up to 300,000) of the XM-300 membrane, it is evident that retention of this protein by the filter would only be possible through inter- or intra-species molecular association i.e. either the BSA polymerizes or interacts with the immunoglobulins.

Gel Filtration

IgM has a much higher molecular weight (approximately 900,000) than the other immunoglobulin classes. Therefore, an attempt was made to isolate IgM from Fraction D using gel filtration. Sephadex G-200 and Bio-Gel A-5 were selected because they reportedly are operative in the desired molecular weight range.

In Figure 20 are shown the tracings of elution patterns obtained with Sephadex G-200 and Bio-Gel A-5. Identical patterns were obtained: a minor fraction eluting in the void volume with the major fraction following.

Immunoelectrophoretic characterization of these fractions (Figure 21) revealed that the minor peak effluent did not demonstrate any immunoglobulin activity (Slides 1 and 3), whereas the major peak effluent (Slides 2 and 4) in both cases contained primarily IgG with smaller amounts of IgM.

Hence, attempts to separate IgG from IgM in Fraction D by gel filtration were not successful. However, a workable procedure may be possible. This requires further study.
Figure 20. Fractionation of immunoglobulins by molecular sieving techniques. Elution patterns obtained by chromatography on Sephadex G-200 (upper left), and on Bio-gel A-5 (upper right).
Figure 21. Immunoelectrophoretic patterns of fractions obtained upon Sephadex G-200 (Slide 1, minor peak and 2, major peak) and Bio-Gel A-5 (Slide 3, minor peak and 4, major peak) column chromatography of Fraction D. Patterns developed with anti-bovine blood serum.
DEAE-Cellulose Chromatography

The elution pattern of Fraction D upon passage through a DEAE-cellulose column (Figure 22) reveals the presence of three protein fractions. These were examined by IME (Figure 23). Peak 1 apparently contained no immunoglobulins and was discarded. Peak 2 (Slide 1) contained a mixture of IgG1 and IgG2 and some IgM, whereas Peak 3 (Slide 2) yielded a single arc which had a mobility corresponding to IgM.

When the effluent comprising Peak 3 was dialyzed, concentrated, and rechromatographed on DEAE-cellulose, two minor and one major peak were observed (Figure 22). The major peak, labelled 6, had an IME pattern (Figure 23, Slide 3) similar to that of IgM and was tentatively identified as IgM. Sedimentation velocity analysis, not presented here, showed the presence of two components corresponding to 7.2S and 18.8S. These components may be IgG and IgM or monomeric and polymeric forms of IgM. Since the IME patterns indicated the presence of only IgM, the latter hypothesis has more merit.

DEAE-Sephadex Chromatography

A typical elution pattern obtained by chromatography of Fraction D on DEAE-Sephadex A-50 is shown in Figure 24. The conductivity of the effluent is plotted in a dotted line as a means of estimating the phosphate buffer concentration.

Four protein fractions were obtained upon initial chromatography (Figure 24). The IME patterns of these
Figure 22. Fractionation of immunoglobulins by DEAE-Cellulose column chromatography. Top: Elution pattern from Fraction D; Bottom: Fraction corresponding to Peak 3, dialyzed, concentrated, and chromatographed. The gradient was 0 to 1M NaCl.
Figure 23. Immunoelectrophoretic patterns of fractions obtained upon DEAE-Cellulose column chromatography of Fraction D. Slide 1, Peak 2; Slide 2, Peak 3; and Slide 3 is of Peak 6 obtained upon rechromatography of Peak 3. Patterns developed with anti-bovine immunoglobulin serum from USDA.
Figure 24. Elution patterns obtained upon DEAE-Sephadex column chromatography of Fraction D. Top: Pattern obtained by first chromatography; Bottom: Rechromatography of Peak 4. The gradient was 0.02M to 0.3M phosphate buffer (pH 7.0). The discontinuous line shows the conductivity of the eluting buffer.
fractions were developed with USDA anti-bovine immunoglobulin serum and are presented in Figure 25. Peak 1 yielded no IME pattern indicating that this fraction was either a non-immunoglobulin protein or consisted of denatured immunoglobulins. Peaks 2 and 3 were electrophoresed together and were found to consist of a mixture of IgG1 and IgG2 (Slide 1). Peak 4 (Slide 2) yielded an IME pattern corresponding to IgM. When this fraction was rechromatographed on DEAE-Sephadex, two UV absorbing peaks were observed as shown in Figure 24. These fractions, labelled as Peaks 5 and 6 were identified as IgG (Slide 3) and IgM (Slide 4), respectively.

The results suggest that DEAE-Sephadex chromatography of Fraction D can be used effectively to isolate individual immunoglobulin classes. Immunoelectrophoretically pure IgM was isolated successfully.

Sucrose Density Gradient

Separation of Fraction D in a density gradient system of 10 to 40% sucrose was undertaken in a Beckman Model L preparatory centrifuge with a fixed angle type 50 rotor. The linearity of the sucrose gradient was ascertained by analysis of the sucrose concentration in each of the eighteen 0.5 ml and the one 0.3 ml fraction withdrawn from the gradient tube.

The sucrose concentration of each of the 19 fractions is presented in Table 8, Appendix. A plot of the data is presented in Figure 26 and reveals that the gradient was
Figure 25. Immunoelectrophoretic patterns obtained upon DEAE-Sephadex column chromatography of Fraction D. Slide 1, Peaks 2 and 3 mixed together; 2, Peak 4; 3, Peak 5 upon rechromatography of Peak 4; and 4, Peak 6 upon rechromatography of Peak 4. Peaks are identified in Figure 24. Patterns were developed with USDA anti-bovine immunoglobulin serum.
Figure 26. Sucrose concentration of the fractions withdrawn after centrifugation from the density gradient tubes.
linear between 10 and 40% sucrose. The slope of the curve was -1.70 with a correlation coefficient of -0.99 and an intercept point of 41.9.

A protein profile upon density gradient separation of Fraction D (Figure 27) reveals that this fraction was resolved into two distinct peaks. The relative peak areas indicate that the higher molecular weight proteins were present in lesser concentration than the lower molecular weight proteins. Two separate peaks do not necessarily indicate that Fraction D is composed of two homogeneous proteins because the substances comprising these peaks may contain more than one protein of similar particle size and (or) density either inherently or through the phenomenon of association-dissociation.

Examination of the five fractions obtained by withdrawing, starting with the bottom portion, four 2.0 ml lots and a final 1.3 ml lot from the gradient tubes was made by IME and analytical ultracentrifugation. When Fraction D in protein concentration of 2.5% or greater was used, a precipitate was formed in the gradient tube and upon resuspension this constituted the sixth fraction examined.

The IME findings for each fraction were compared with the analytical ultracentrifugation data. The immunoelectrophoretic slides are shown in Figure 28 and selected Schlieren patterns in Figure 29.

Slide One (Figure 28) represents the IME pattern of
Figure 27. Protein profile obtained upon density gradient separation of Fraction D.
Figure 28. Immunoelectrophoretic patterns of fractions obtained upon sucrose density gradient separation of Fraction D. Upper left: Slide 1, Fraction D; 2, bottom 2 ml portion from gradient tube; 3, 2-4 ml portion; 4, 4-6 ml portion; Upper right: Slide 5, 6-8 ml portion; 6, 8-9.3 ml portion (top fraction); and 7, redissolved precipitate. Patterns for Slide 1-6 were developed with anti-bovine blood serum and Slide 7 was with USDA anti-bovine immunoglobulin serum.
Fraction D. Only two precipitin arcs characteristic of IgG and IgM are noted. The D fraction had sedimentation constants of 6.5S and 18.8S confirming the presence of IgG and IgM (Figure 29, a). The IME patterns (Slides 2 to 6) are for the five fractions from the gradient tubes with Slide 2 representing the bottom 2 ml portion progressively to Slide 6 which is for the top 1.3 ml fraction. Slide 7 is for the precipitate.

Evidently, the bottom 2 ml portion in the gradient tube consisted of a mixture of IgG and IgM as shown by the two distinct arcs (Slide 2) and two peaks (Figure 29, b) on analytical ultracentrifugation with sedimentation constants corresponding to 7.0S and 17.8S. The protein in the 2-4 ml fraction (Slide 3) yielded a single precipitin arc characteristic of IgG and was also homogeneous on analytical ultracentrifugation (Figure 29, c) with a sedimentation constant of 7.1S. Surprisingly, the 4-6 ml portion yielded two distinct arcs on IME (Slide 4) and these were identical to IgG and IgM. On ultracentrifugation of this fraction, only one peak (Figure 29, d) was observed with a sedimentation constant of 6.5S indicating one of two possibilities: (a) the fraction contains IgG and a monomer of IgM or (b) IgM may be occurring in the colostrum at least in part as a 6.5S protein. Slides five and six reveal the presence of IgG-like proteins but with sedimentation constants of 5.5S and 5.1S, respectively.
Figure 29. Schlieren patterns of fractions obtained by density gradient separation of Fraction D. a; Fraction D, b; bottom 2 ml portion from the density gradient tube, c; 2-4 ml fraction at 48 min and 134 min, d; 4-6 ml fraction at 86 min and 216 min, e; precipitate obtained during density gradient separation at 18 min and 53 min. All runs were made at 40,000 rpm in pH 8.0, 0.1M Tris-HCl buffer containing 0.15M NaCl.
The IME pattern (Slide 7) of the precipitate showed the presence of proteins identical in mobility to IgM. In the ultracentrifuge, the protein moved as a single peak (Figure 29, e) with a sedimentation constant of 19.1. The results suggest that the precipitate is rich in the immunoglobulin class, IgM, and is relatively free of other classes.

The sucrose density gradient method, while demonstrating limited separation of the bovine lacteal immunoglobulins into individual classes, was not an entirely satisfactory method under the experimental conditions used. Further study is needed to perfect the method for colostral or milk immunoglobulins.

**Physical-Chemical and Chemical Characterization of IgM**

For chemical and physical-chemical characterization, the IgM fraction separated by density gradient or DEAE-Sephadex was extensively dialyzed against cold distilled water. The protein suspension in the dialysis tubing was precipitated with 50% saturated ammonium sulfate, washed twice with 2-4 C distilled water, and resuspended in 0.15M NaCl. It was again dialyzed against cold distilled water. The dialysate was freeze dried and stored at 0-2 C until used. Unless specified, the protein concentration of IgM solutions was determined by UV absorption at 280 nm.
In the following section, presentation is made of the results obtained when the IgM preparation was characterized with respect to (a) sedimentation coefficient, (b) extinction coefficient, (c) hexose sugars, (d) sialic acid, (e) fucose, (f) amino sugars, (g) sulfhydryl groups and disulfide bonds, and (h) subunits.

Physical-Chemical

**Sedimentation coefficient.** The sedimentation constant of IgM was determined in 0.1M Tris-HCl buffer, pH 8.0, containing 0.15M NaCl. The Schlieren patterns in Figure 30 are for one experiment and reveal that IgM moved as a single peak indicating the preparation to be relatively pure. $S_{20}$ values for three different concentrations of IgM were calculated from the peak mobilities as a function of time. A plot of these values is shown in Figure 31 in order to indicate the concentration dependence of sedimentation of IgM. When the $S_{20}$ values for the various concentrations were extrapolated to infinite dilution, an $S_{20}$ value of 19.3 was obtained. It should be emphasized that these results are of a single preparation of IgM. For other preparations of IgM, $S_{20}$ values ranged from 18.2-19.8.

**Extinction coefficient.** A stock solution (0.15M NaCl) of 0.17% IgM-protein, as determined by the micro-Kjeldahl method, was further diluted with 0.15 M NaCl to obtain IgM solutions of protein concentrations ranging from 0.0085-0.0850%.
Figure 30. Schlieren patterns for IgM, a; 16 min, and b; 50 min after achieving full speed of 40,000 rpm.
Figure 31. The concentration dependence of sedimentation of IgM at pH 8.6, 0.1M Tris-HCl buffer containing 0.15M NaCl.
The absorbance value corresponding to each protein concentration was determined and the extinction coefficient, 
\[ \varepsilon_{280 \text{ nm}} \], was calculated as absorbance at 280 nm/\% protein.

The extinction coefficient obtained at each protein concentration is shown in Table 9, Appendix. The values ranged from 11.70 to 12.12 with an average value of 11.87.

A plot of the protein concentration versus absorbance at 280 nm (Figure 32) was found to be linear indicating that the solutions obeyed Boyle's law.

Chemical

**Hexose sugars.** The hydrolyzed and neutralized samples of IgM yielded two spots upon paper chromatography in a pyridine-ethyl acetate-water system. The unknown spots corresponded to those obtained with known solutions of mannose and galactose run on the same chromatogram. Glucose was not evident. The ratio of mannose to galactose was found to be 1.93 to 1. This was taken for practical purposes to be 2 to 1.

In Table 2 are shown the results of hexose determinations on various concentrations of IgM. The values for mannose and galactose were calculated from the total hexose content:

\[
\text{Mannose} = \frac{2}{3} \times \text{total hexose}
\]
\[
\text{Galactose} = \frac{1}{3} \times \text{total hexose}.
\]

The values for the total hexose content ranged from
Figure 32. Effect of IgM Concentration on UV absorption at 280 nm.

\[ y = 0.1166X + 0.006 \]
<table>
<thead>
<tr>
<th>IgM (ug)</th>
<th>Total Hexose (ug)</th>
<th>% Hexose</th>
<th>% Mannose</th>
<th>% Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>6.3</td>
<td>5.25</td>
<td>3.50</td>
<td>1.75</td>
</tr>
<tr>
<td>240</td>
<td>12.2</td>
<td>5.08</td>
<td>3.39</td>
<td>1.69</td>
</tr>
<tr>
<td>360</td>
<td>18.0</td>
<td>5.02</td>
<td>3.35</td>
<td>1.67</td>
</tr>
<tr>
<td>480</td>
<td>24.7</td>
<td>5.16</td>
<td>3.44</td>
<td>1.72</td>
</tr>
</tbody>
</table>

**TABLE 2**

MANNOSE AND GALACTOSE CONTENT OF IgM
5.02 to 5.25% consisting of 3.35-3.50% mannose and 1.67-1.75% galactose.

**Sialic acid.** The thiobarbituric acid method of Warren (184) was used to determine the sialic acid content of IgM. The sialic acid content of IgM was calculated from the linear equation for the standard curve:

\[
\% \text{sialic acid} = \frac{\text{absorbance} + 0.0061}{41880} \times \frac{100}{\text{grams IgM}}
\]

The data presented in Table 3 reveal that the sialic acid content of bovine lacteal IgM ranged from 1.34-1.45% with an average value of 1.40%.

**Fucose.** For the determination of the fucose content of IgM, the procedure of Dische and Shettles (45) was used. As shown in Table 4, samples were prepared containing from 0.005-0.030 mg fucose with and without one milligram protein as IgM. The absorbance readings were taken at 396 nm and 430 nm. The difference, \( \Delta \text{E}_{396} - \text{E}_{430} \), was the value for fucose alone without other interfering carbohydrates. The fucose content of the protein was calculated from the contribution of IgM to the absorbance reading. The milligrams fucose were read directly from the standard curve. The data (Table 4) revealed the fucose content of IgM to be quite consistent being in the range of 1.2-1.3%.

**Amino sugars.** Analyses for the total hexosamine content of IgM as determined by the procedure of Boas (14) are
TABLE 3

SIALIC ACID CONTENT OF IgM

<table>
<thead>
<tr>
<th>IgM</th>
<th>Absorbance at 549 nm</th>
<th>Sialic Acid</th>
<th>% Sialic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0.067</td>
<td>1.74</td>
<td>1.45</td>
</tr>
<tr>
<td>240</td>
<td>0.133</td>
<td>3.34</td>
<td>1.39</td>
</tr>
<tr>
<td>360</td>
<td>0.206</td>
<td>5.06</td>
<td>1.41</td>
</tr>
<tr>
<td>480</td>
<td>0.263</td>
<td>6.42</td>
<td>1.34</td>
</tr>
</tbody>
</table>

*By the method of Warren (184).*
### TABLE 4

**FUCOSE CONTENT OF IgM**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$E_{396}^a$</th>
<th>$E_{430}^b$</th>
<th>$\Delta E^c_{\text{Sample}}$</th>
<th>$\Delta E^c_{\text{Fucose Alone}}$</th>
<th>$\Delta E^d_{\text{Fucose in IgM}}$</th>
<th>$% \text{ Fucose}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005 mg Fucose + 1 mg Protein</td>
<td>0.372</td>
<td>0.120</td>
<td>0.252</td>
<td>0.081</td>
<td>0.171</td>
<td>0.012</td>
</tr>
<tr>
<td>0.010 mg Fucose + 1 mg Protein</td>
<td>0.464</td>
<td>0.139</td>
<td>0.325</td>
<td>0.149</td>
<td>0.176</td>
<td>0.012</td>
</tr>
<tr>
<td>0.020 mg Fucose + 1 mg Protein</td>
<td>0.658</td>
<td>0.154</td>
<td>0.504</td>
<td>0.317</td>
<td>0.187</td>
<td>0.013</td>
</tr>
<tr>
<td>0.030 mg Fucose + 1 mg Protein</td>
<td>0.819</td>
<td>0.157</td>
<td>0.662</td>
<td>0.468</td>
<td>0.194</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*a$_{E_{396}}$ = Absorbance at 396 nm.*

*b$_{E_{430}}$ = Absorbance at 430 nm.*

$c \Delta E = E_{396} - E_{430}$

$d$ By difference between $\Delta E$ of sample and $\Delta E$ of fucose.
The protein content of IgM ranged from 0.58-0.92 mg as determined by UV absorbance. A standard solution containing 0.02 mg glucosamine-HCl was also run in each case. In order to express the results in terms of free glucosamine, a factor of 0.829 was used and the concentration of hexosamine was calculated as:

$$\text{Total hexosamine (mg)} = \frac{\text{absorbance of glucosamine-HCl}}{\text{absorbance of the sample}} \times 0.02 \times 0.829.$$ 

Thus, the percent hexosamine present in the protein was equal to

$$\frac{\text{Hexosamine (mg)}}{\text{Protein (mg)}} \times 100$$

This was found to range from 2.81-3.00% (Table 5) with an average value of 2.9%.

The N-acetylhexosamines expressed as N-acetylglucosamine were calculated from the least squares equation for the standard curve:

$$\text{Absorbance} = 0.00674 \times \mu g \text{ N-acetylglucosamine/ml} + 0.011.$$ 

The acetylated hexosamines constituted approximately 2.18-2.36% of IgM (Table 6) indicating that the amino sugars present in IgM exist primarily in the acetylated form.

**Sulfhydryl groups and disulfide bonds.** Results for the free -SH groups and the total -SH groups are presented in
<table>
<thead>
<tr>
<th>Protein Conc. (mg)</th>
<th>Absorbance of the Sample (A)</th>
<th>Absorbance of Standard (B)</th>
<th>Total Hexosamine ( \frac{A}{B} \times 0.020 \times 0.829 ) (mg)</th>
<th>% Hexosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.58</td>
<td>0.108</td>
<td>0.103</td>
<td>0.0174</td>
<td>3.00</td>
</tr>
<tr>
<td>0.73</td>
<td>0.132</td>
<td>0.107</td>
<td>0.0205</td>
<td>2.81</td>
</tr>
<tr>
<td>0.80</td>
<td>0.141</td>
<td>0.108</td>
<td>0.0217</td>
<td>2.94</td>
</tr>
<tr>
<td>0.92</td>
<td>0.158</td>
<td>0.104</td>
<td>0.0262</td>
<td>2.96</td>
</tr>
<tr>
<td><strong>Mean % hexosamine</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>2.93</strong></td>
</tr>
</tbody>
</table>

^a Average of 4 separate trials.

^b 0.02 mg of glucosamine hydrochloride.

^c Expressed as glucosamine.
### TABLE 6

**N-ACETYLHEXOSAMINE CONTENT OF IgM**

<table>
<thead>
<tr>
<th>Protein Conc. (mg/ml)</th>
<th>Absorbance at 545 μm</th>
<th>N-acetylhexosamines (mg/ml)</th>
<th>% N-acetylhexosamines</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.58</td>
<td>0.104</td>
<td>0.0137</td>
<td>2.36</td>
</tr>
<tr>
<td>0.73</td>
<td>0.125</td>
<td>0.0172</td>
<td>2.33</td>
</tr>
<tr>
<td>0.80</td>
<td>0.129</td>
<td>0.0175</td>
<td>2.18</td>
</tr>
<tr>
<td>0.92</td>
<td>0.149</td>
<td>0.0204</td>
<td>2.22</td>
</tr>
</tbody>
</table>

*a*Absorbance = 0.00674 X μg N-acetylhexosamine/ml + 0.011.
Table 7. The total -SH groups were determined after reduction of the protein with dithiothreitol in 0.2M Tris-HCl buffer, pH 8.6. For comparison, determination was made of β-lactoglobulin.

β-lactoglobulin was found to contain 1.8 moles of free -SH groups and 5.7 moles of total -SH groups per mole of protein based on a molecular weight of 36,000. The number of -SH groups evolved from S-S bonds was 3.9 corresponding to 2 S-S bonds/mole of β-lactoglobulin. Thus, β-lactoglobulin has 1.8 moles of free -SH groups and 2 S-S bonds. The reported values are 2 -SH groups and 2 to 3 S-S bonds.

IgM contained only 0.9 moles of -SH groups/mole of IgM (molecular weight of 900,000) indicating that free -SH are almost absent. However, the total number of -SH groups was 43.4/mole of IgM. Thus, the number of S-S bonds would approximate 21/mole of IgM. These findings would indicate that almost all the sulfur in IgM is present in disulfide linkage.

Subunits. The IgM preparation was subjected to reduction with 2-mercaptoethanol and cysteine and alkylated with iodoacetamide. The native and reduced proteins were chromatographed on Sephadex G-200. The elution patterns are shown in Figure 33.

The native IgM eluted as a single major peak (A) in the void volume of the column. A very minor peak followed
TABLE 7
-\(\text{SH}\) GROUPS AND S-S LINKAGES IN IgM

<table>
<thead>
<tr>
<th>Protein</th>
<th>Free -(\text{SH}) Groups(^a)</th>
<th>Total -(\text{SH}) Groups(^b)</th>
<th>No. of S-S Linkages</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta)-Lactoglobulin (Standard)</td>
<td>1.8</td>
<td>5.7</td>
<td>2</td>
</tr>
<tr>
<td>IgM (Unknown)</td>
<td>0.9</td>
<td>43.4</td>
<td>21</td>
</tr>
</tbody>
</table>

\(^a\)By the method of Ellman (48).

\(^b\)By the method of Miller and Metzger (118).
Figure 33. Sephadex G-200 elution patterns of A, native IgM; B, IgM reduced with cysteine; and C, IgM reduced with 2-mercaptoethanol.
which may either be a contaminating protein or monomers of IgM present in the preparation. The IgM reduced with cysteine (B) showed two fractions: one eluting in the void volume, identified as IgM, and the second fraction eluting somewhat later which was identified as subunits of IgM. A tailing of the first peak was observed which may be due to intermediate subunits of IgM. Reductions of IgM with 2-mercaptoethanol (B) resulted in almost complete monomerization as evidenced by the elution patterns.

The results indicate that IgM is composed of smaller units which are linked through S-S bonds. Reduction with sulfhydryl reducing agents caused monomerization of the IgM molecule into smaller units. Apparently under the conditions employed, cysteine failed to produce complete monomerization of IgM, whereas 2-mercaptoethanol did.

**Antimicrobial Activity of IgM**

To ascertain the in vivo antimicrobial activity of IgM against *Streptococcus cremoris* R-1, *Streptococcus lactis* C-2, *Streptococcus agalactiae* OARDC, and *Escherichia coli* OSU 66 (serotype 055 B5), one or more of the following methods were used (a) dye reduction, (b) agar pour-plate, and (c) turbidimetry.

The results are presented for each organism studied. **Streptococcus cremoris R-1**

The dye reduction assay procedure of Mikolajcik and
Sinha (116) was used for the determination of agglutination of *S. cremoris* R-1 by IgM in a milk system. Briefly, this involved measuring the depth of surface color development during incubation at 30°C of the several milk systems cultured with the organisms and containing 0 to 40 µg IgM protein/ml plus TTC indicator.

The data shown in Table 10 of the Appendix were plotted (Figure 34) and these demonstrated that an inverse relationship exists between the depth of surface color and the concentration of IgM in the milk. The slope of the graph between 0 and 10 µg was much greater than between 10 and 40 µg indicating that increasing the concentration of IgM above 10 µg/ml resulted in a decreasing rate of surface color development. Apparently, there is an optimum concentration of IgM above which further agglutination of the organisms occurs at only a limited rate.

The distribution of *S. cremoris* cells was determined in the 4-hr incubated dye reduction assay mixture by withdrawing successive one milliliter portions from the top of the tube and determining the viable cell count by the pour-plate technique. The histogram (Figure 35) reveals that in the top 3-ml portion of the mixture, the number of organisms/ml was greater in the case of samples containing 40 µg IgM than in the control lots containing no IgM. In the portions withdrawn between 4 and 9 milliliters, the control contained higher numbers of organisms than did the IgM samples. The
Figure 34. Effect of IgM concentration on growth of *S. cremoris* R-1 as measured by the procedure of Mikolajcik and Sinha (116).
Figure 35. Effect of IgM on *S. cremoris* R-l cell distribution in the assay system of Mikolajcik and Sinha (116).
total number of organisms in the control and test samples was approximately the same indicating that IgM displayed an agglutinating effect on *S. cremoris* R-1 with no evident bactericidal effect. A similar effect was reported by Mikolajcik and Sinha (116) for crude immunoglobulins isolated from milk or colostrum.

The growth patterns of *S. cremoris* in Elliker's broth with and without added IgM were determined turbidimetrically at 610 nm. The data tabulated in Table 11, Appendix are expressed as change in optical density, $\Delta$OD at 610 nm, of the inoculated broth between the OD at time, $t$, and the OD at zero time. Plots prepared of $\Delta$OD versus incubation time (Figure 36) reveal that the growth rate of the organism was unaffected by the presence of up to 40 $\mu$g IgM/ml: further confirmation that IgM had no apparent bactericidal activity against *S. cremoris* R-1.

**Streptococcus lactis** C-2

Results of the effect of IgM on *S. lactis* C-2 (Table 12, Appendix and Figure 37) were similar to those observed for *S. cremoris* R-1 in that 10 $\mu$g IgM/ml exerted the most rapid depression of surface color development. At higher concentrations, the plot of IgM concentration and depth of color development was linear with a more gradual slope than at 10 $\mu$g IgM/ml.

The distribution of *S. lactis* cells in the dye reduction assay system (Figure 38 and Table 13, Appendix) also
Figure 36. Outgrowth of *S. cremoris* R-1 in Elliker's broth with or without IgM.
Figure 37. Effect of IgM concentration on growth of *S. lactis* C-2 as measured by the procedure of Mikolajcik and Sinha (116).
Figure 38. Effect of IgM on S. lactis C-2 cell distribution in the assay system of Mikolajcik and Sinha (116).
followed the pattern observed for \textit{S. cremoris} R-1. IgM exhibited only an agglutinating effect on the organisms and no demonstrable bactericidal effect. This was further confirmed by growth studies of \textit{S. lactis} C-2 in Elliker's broth (Table 14, Appendix). The plot of these data in Figure 39 shows that the rate of growth was unaffected by IgM up to concentrations of 40 \( \mu \text{g/ml} \).

\textit{Streptococcus agalactiae} OARDC

The growth rate, as measured turbidimetrically, of \textit{S. agalactiae} in Elliker's broth was found to be depressed in the broth containing IgM (Figure 40 and Table 15, Appendix). The effect was evident during the first 120 minutes of incubation. After this period, the \( \Delta \text{OD} \) values for the systems with IgM increased at a faster rate than those for the control lot without IgM: an indication that after the initial lag period, the cultures incubated with IgM grew more rapidly than those without IgM. The graph also reveals that the log growth phase for the organisms began at approximately 120 minutes of incubation. This would suggest that IgM affected the lag phase of \textit{S. agalactiae} with no detrimental effect on the exponential growth phase. A possible explanation of this phenomenon may be that the initial injury of the cells by IgM is reversible upon further incubation. The cells apparently repair or otherwise overcome the damage and subsequently reproduce at a faster rate than the cells cultured in broth without IgM. Another
Figure 39. Outgrowth of *S. lactis* C-2 in Elliker's broth with or without IgM.
Figure 40. Outgrowth of *S. agalactiae* in Elliker's broth with or without IgM.
explanation may be that IgM might change the surface characteristics of the cells so that optical density would be an inaccurate measure of cell numbers and thus of growth rate.

Results of a separate experiment in which the growth rate of the organisms in the systems was determined by agar plate count method are presented in Table 16, Appendix. The counts were made at one-hour intervals of incubation at 35°C in the systems containing the organisms and 0, 10, or 40 µg IgM/ml. The data shown in Figure 41 reveal that lower counts as well as a decreased growth rate occurred upon addition of IgM to Elliker's broth. The discrepancy between the results obtained with agar plate count procedure and the optical density method may be due to either the agglutination phenomenon in which the plate counts would be lower or the inability of the injured cells to outgrow on solid medium resulting in decreased counts. On the other hand, Elliker's broth may permit the organisms to recover from injury.

**Escherichia coli OSU 66**

The growth of *E. coli* as measured by agar plate count was adversely affected by the presence of IgM in Elliker's broth (Figure 42 and Table 17, Appendix). As seen from the data, the initial counts in all samples were the same. However, within one hour of incubation, the number of *E. coli* increased twofold in the control sample and only 34% in the
Figure 41. Growth of *S. agalactiae* in Elliker's broth with or without IgM as measured by the plate count method.
Figure 42. Growth of *E. coli* in Elliker's broth with or without IgM as measured by the plate count method.
broth containing 10 μg IgM/ml. At 40 μg IgM/ml, the counts after one hour were either unchanged or slightly decreased when compared with the initial inoculum. This observation would indicate that IgM had an inhibitory effect on E. coli. At two hours of incubation, the rate of growth and the total number of cells were the highest in the control sample. Between two and three hours of incubation, the growth rate but not the total number of E. coli cells was higher in the presence than the absence of IgM. The growth rate constants (167) during this period were 1.38, 1.54, and 1.65 for samples with 0, 10, and 40 μg/ml IgM respectively. This would suggest that IgM exerts an inhibitory effect on E. coli during the initial lag growth phase. After the initial delay, the cells appear to overcome the inhibitory effect of IgM and to outgrow at a rate equal or greater than that exhibited in the system without IgM.

In general, results for the four organisms studied indicated that IgM is effective only on the lag phase of the organisms with the apparent mechanism being one of agglutination. Injury to the cells may be involved but this will require further more careful study.
DISCUSSION

The distinguishing characteristics of a protein species are size, unique composition, amino acid sequence, and three dimensional structure. These characteristics form the basis of methods used for separation of protein mixtures into individual molecular entities. Properties generally utilized in protein separation are solubility, size, charge, density, and biological activity. The separation of discrete protein species from a complex mixture such as milk requires application of several techniques. Furthermore, the various procedures have to be tailored for the individual protein under study. For these reasons, several procedures were examined for the isolation of bovine IgM.

The ammonium sulfate fractionation procedure of Smith (168) provided an effective and relatively simple method for obtaining a crude mixture of immunoglobulins (Fraction D) from milk or colostrum. Further fractionation into pseudoglobulin and euglobulin offered only a limited advantage. None of these fractions was homogeneous: pseudoglobulin contained IgG1 and IgG2, and euglobulin consisted of IgG1 and IgM or IgG1, IgA, and IgM. When one interprets Smith's data (168) in light of newer knowledge of the
immunoglobulin classes, one finds agreement with our findings and those of others (18) that pseudoglobulin and euglobulin are indeed heterogeneous.

Immunoglobulin separation by Smith's method is based on differences in the solubilities of proteins under varying conditions of salt concentration, pH, and (or) temperature. In the absence of interaction between proteins, the individual protein species should salt-out independently based upon its degree of solubility at a particular concentration. A clear separation will not be obtained if the proteins of a mixture precipitate at approximately the same salt concentration. This would explain the failure of Smith's procedure in yielding individual immunoglobulin classes and also the presence of such contaminants as bovine serum albumin and \( \beta \)-lactoglobulin in some immunoglobulin preparations. Protein concentration is also important for effective fractionation as evidenced by the loss of immunoglobulins during fractionation from colostrum. Irrespective of these limitations, Smith's procedure is a relatively rapid method for obtaining immunoglobulins in concentrated form. In addition, ammonium sulfate does not cause denaturation of proteins and also reduces possible microbial action on the proteins.

For fractionation and purification of a protein from a mixture of proteins differing in molecular size, gel filtration or molecular-sieve chromatography is an important
tool. The three classes of immunoglobulins, IgG, IgA, and IgM, differ considerably in their molecular weights: IgG has a molecular weight of approximately 150,000 (33), IgA approximately 390,000 (61), and IgM approximately 900,000 (18). The application of gel filtration to separation of immunoglobulins was therefore explored. To overcome problems due to polymerization of immunoglobulins and possible minor ionic adsorptions on Sephadex or Bio-gel, buffer with a relatively high concentration of salt (Tris-HCl with 0.15M NaCl) was used. Despite these precautions, Sephadex G-200 or Bio-gel A-5 column failed to resolve the immunoglobulin mixture into individual components. Why this occurred is not entirely clear. Probably, recycling the effluent would have improved the separation, but the major limitation would have been the time consumed permitting possible bacterial multiplication, enzymatic degradation, and (or) structural changes in the protein.

The use of ultrafiltration as a method for separation or purification of protein has not been exploited fully at this time. This is due to the fact that membranes of uniform pore size and different molecular weight exclusion ranges were heretofore unavailable. With the recent availability of highly discriminating membranes, it was tempting to speculate that by the use of membranes with appropriate pore-size, the proteins in milk serum (whey) could be isolated individually. Furthermore, because of the vast
differences in molecular weights, theoretically IgM should be separable from IgG. In practice, however, complete separation did not occur. Why? Obviously, polymerization of proteins, clogging of the membranes, and (or) creation of artificial pore-size by the "stacking effect" were in part or wholly responsible. Based upon present knowledge of practical limitations, available instrumentation, and membrane specificity, this technique is only partially successful e. g. in separation of bovine serum albumin from immunoglobulins or of different immunoglobulin classes. A more careful study of the technique might improve separation. However, for such a study, a knowledge of theoretical limitations of the technique is sorely needed.

Ion-exchange chromatography has been widely employed in the purification of proteins. It is based upon differential ionic binding between the proteins and the ion-exchange resin followed by elution with buffers of varying pH and (or) salt concentrations. DEAE-cellulose (diethylaminoethyl cellulose), a weak anion exchanger, is generally used for immunoglobulins which possess acidic to slightly basic properties. Successful separation of individual classes of immunoglobulins on DEAE-cellulose column was achieved indicating that immunoglobulin classes differed in adsorption to the anion-exchange resin. The affinity of a protein for an ion-exchange adsorbant is a function of the number of bonds that can be established between the protein and the
adsorbant under the conditions employed. Therefore, the number of charges on the molecule and the spatial distribution of the charges are two important factors for differential adsorption. It follows that a large protein of low net charge density may have higher affinity for an ion-exchanger than a smaller protein with relatively higher net charge. Hence, higher affinity of IgM for DEAE-cellulose, as indicated by higher concentration of salt to elute it, does not necessarily mean a higher net negative charge on IgM than on IgG.

DEAE-Sephadex has both molecular sieving and ion-exchange properties. However, DEAE-Sephadex A-50 has a molecular-weight exclusion limit of less than 50,000, and therefore it is doubtful that the molecular sieving properties would be of any significant value in the fractionation of immunoglobulins. The successful separation of individual immunoglobulins on DEAE-Sephadex column is, thus, primarily attributable to the ion-exchange properties which are similar to those of DEAE-cellulose.

Density gradient systems have been applied to proteins for preparatory purposes as well as for purification. A simple system employing formation of gradient by diffusion of a series of boundaries of the stratified sucrose solutions followed by centrifugation in an ultracentrifuge fitted with a fixed-angle rotor was used successfully for separating IgM from IgG. Despite possible mixing of the
gradient in the rotor or at the time of withdrawal of fractions and inability to scan the protein bands within the density gradient tubes, the results were satisfactory. The density gradient technique has tremendous potential in separating immunoglobulins, especially as more refined density gradient separation apparatus becomes available.

Differences in the electrophoretic, sedimentation, and immunological properties of proteins form the basis of most techniques used for identification and, in some cases, even characterization of protein species. Several variations of individual techniques are available, and the selection of methods for a particular purpose will depend upon considerations such as the availability of instrumentation, applicability of the method to the problem at hand, and the extent of the sensitivity required. After careful consideration and because they met the aforementioned conditions, disc polyacrylamide gel electrophoresis, sedimentation velocity, and immunoelectrophoresis were selected.

Disc polyacrylamide gel electrophoresis is more useful than other electrophoretic systems when dilute protein fractions are to be examined. This technique served as an excellent method for following the fractionation of immunoglobulins from milk or colostral whey, because of apparent differences in the isoelectric points and molecular sizes of the whey proteins. However, at alkaline pH, disc electrophoresis failed to resolve immunoglobulins into discrete
bands. Electrophoretic mobility in polyacrylamide under fixed conditions of pH and buffer species is principally a function of the net electrostatic charge density, and to a lesser degree of size and shape of the protein molecules. Unsatisfactory resolution of immunoglobulins in alkaline polyacrylamide gel electrophoresis may reflect that there is only a slight difference in the net charge on individual immunoglobulins at the pH employed. A cationic system (pH 4.3) described by Reisfeld et al. (152) was found to be more applicable to the immunoglobulins of milk and colostrum; this observation has been supported by other workers (18, 157).

Immunoglobulins have been generally described on the basis of their sedimentation velocities, e.g. 7S fraction for IgG, 11S for IgA, and 19S for IgM. Sedimentation methods require relatively concentrated samples and exhibit low sensitivity in detecting minor contaminating proteins. However, they are invaluable when used in conjunction with other methods, and for comparison of the bovine immunoglobulins with those of other species.

For positive identification, and as a means of ascertaining the purity of immunoglobulins and their specific classes, immunoelectrophoresis is the single most important technique. In immunoelectrophoresis are combined the advantages of immunodiffusion with those of electrophoretic resolution. In addition, immunoelectrophoresis is more
sensitive as compared to other common techniques. Immunoelectrophoresis involves antigen-antibody reaction and, therefore, the validity of the results depends upon the concentration and kinds of antibodies present in the anti-serum, viz. the potency and specificity. Unfortunately, anti-sera exhibited considerable variability with respect to potency and specificity. The importance of uniformly potent anti-sera can not be overemphasized because immunoelectrophoresis is used extensively for diagnostic studies, in forensic medicine, and as an important research tool. Based on our experience, it is imperative that all supplies of anti-sera are carefully evaluated against known standards, and are used with proper controls.

Of the three major immunoglobulin classes, IgG with both subclasses IgG1 and IgG2 and IgM were found in all bovine milk and colostrum samples examined, whereas IgA was present only in certain samples. It is generally accepted that IgA is one of the major immune proteins present in external secretions. Human milk and colostrum have been reported (61) to contain IgA as the major immunoglobulin. A possible explanation for the absence of IgA in the colostrum of some cows may be related to the health of the animal. That the immunoglobulin make-up of colostrum or milk is affected by the health of the animal, finds support in the observation that colostrum from the cows suffering from milk fever contained almost all the proteins present in the blood
serum. It is important to recognize that milk fever is a metabolic disorder and not an infectious disease. Therefore, the additional proteins appearing in the abnormal colostral whey may not result from necrosis or atrophy of the milk secretory cells as might be evident with mastitis but would probably appear because of an apparent change in the permeability of the cells. It is known (171) that there is selective transport of immunoglobulins from blood serum to milk or colostrum. That blood proteins pass unaltered into colostrum in cows suffering from milk fever may indicate that this disorder affects, in some manner, the selective transport mechanism. Other pathological conditions may act in a similar manner, or may cause changes in the immunoglobulin make-up due to antigenic stimulation.

The variations in the sedimentation constants of different IgM preparations, and the presence of a 7S IgM-like fraction indicate that the immunoglobulin system of bovine milk and colostrum may be more heterogenous than hitherto suspected. The $S_{20}$ values for IgM obtained were comparable to those reported by others (18, 59, 127). The 7S IgM observed may be a monomeric form of native IgM occurring in bovine colostrum or a monomerization of the 19S fraction caused by the isolation procedure. Although the results are not conclusive for the postulated existence of a 7S IgM, there are literature reports (73, 160, 176) indicating the existence of 7S IgM in human blood serum. The presence of
a similar analog in the bovine species certainly merits further attention.

The extinction coefficient of IgM was comparable to value reported for human IgM by Miller and Metzger (117). The value was higher than that for any of the non-immunoglobulin whey proteins of milk, reflecting perhaps a higher content of tyrosine, tryptophan, and phenylalanine.

Whereas our value of 10.8% carbohydrates in bovine IgM is somewhat lower than the 12.3% reported by Gough (59), it does compare favorably with the 10.3% value reported by Miller and Metzger (117) for human IgM. Excepting the fucose content, the percentages for the individual carbohydrates of IgM are in general agreement with those of human IgM (64, 117). The fucose content of bovine IgM was 1.25% and that of human IgM 0.68%. Before drawing any conclusions with regard to the differences in the carbohydrate make-up of the bovine and human IgM, a critical examination of carbohydrate methodology is needed. The analysis of sugars in glycoproteins involves acid hydrolysis and subsequent reaction of the sugar with a chromogenic reagent. The recovery of the carbohydrate in the hydrolyzate will depend upon the ease of acid hydrolysis of the glycosidic linkages and the acid lability of the carbohydrate. Pentoses require less drastic hydrolysis conditions than hexoses, and α-D glycosides are more labile than β-D glycosides (61). It is, therefore, possible that the conditions employed by
Miller and Metzger (117) for fucose determination caused either incomplete hydrolysis of fucose from IgM or caused a destruction of part of fucose. On the other hand, the determination of fucose is not entirely free from interference by other carbohydrates, and thus high values for bovine IgM may be due to the interference. Should actual differences in the fucose content of bovine and human IgM exist, the results may be highly important in the understanding of the synthesis and biological functions of these proteins.

The chemical methods available for the determination of other carbohydrates also are not free from limitations, and any results on carbohydrate composition of a glycoprotein should be viewed with caution. In general, methods employed lack accuracy and absolute specificity. For example, in case of hexosamines or N-acetylhexosamines, results are expressed as total hexosamines, although the individual amino sugars differ in their sensitivity to the test reagents (91). The tests thus provide an approximate value of the carbohydrate content, and their major significance lies in the comparison of carbohydrates of different proteins. Information of this nature may provide an answer to differences in biological functions and (or) synthesis of various glycoproteins.

The absence of free sulfhydryl groups in IgM is consistent with the observation made for human IgM (118). The 21 disulfide bonds observed are somewhat less than the
24-25 value reported for human IgM (118). The formation of smaller units of IgM upon treatment with 2-mercaptoethanol or cysteine reveals that IgM is composed of subunits held together by disulfide linkages. Twenty of the S-S bonds can be accounted for in the following manner: if each IgM molecule is composed of five subunits and each subunit is made of a four-chain structure, 20 S-S linkages would permit one S-S linkage between the heavy and the light chains, one S-S linkage between the two heavy chains of a subunit, and one S-S bond between the two heavy chains of two different subunits (Figure 2, page 13). As the results revealed the presence of 21 S-S bonds, the data are insufficient to draw conclusions with respect to the remaining disulfide bond. This may be due to experimental error or may indicate that IgM has subclasses varying in S-S groups; a situation which has been demonstrated for the subclasses of IgG or IgA (18).

That immunoglobulins of milk are involved in the agglutination of lactic acid bacteria is unquestionable, and that this reaction is one of several involved in bacterial "inhibition" by raw milk is also unquestionable. Why agglutination of S. lactis and S. cremoris occurs is intriguing, especially when it is realized that these organisms are neither normal inhabitants in the cow's body nor are they infectious parasitic agents. Thus, the challenge necessary for the production of specific antibodies against these organisms by the cow is not present. It is, however,
known (40) that cross reactions due to imperfect specificity of antibodies may occur if the antibodies are produced by an antigen similar to the one under study. Therefore, it is conceivable that immunoglobulins synthesized under the influence of antigenic material similar to that of the cell wall of *S. lactis* and *S. cremoris* may cause agglutination of these organisms. The cow is a common habitat for *S. agalactiae* and *E. coli*, and thus specific antibody production may occur. These antibodies transported from blood to milk would cause inhibition of the cells which stimulated their production. The effect of IgM was found to be primarily in the lag phase of the organisms indicating that the inhibition was temporary. Similar observations have been made by other workers (76, 77) with respect to the effect of raw milk on streptococci. Studies by medical workers (95) indicate that human IgM has a specific bactericidal effect on Gram-negative bacteria such as *E. coli*. Bovine IgM does not appear to have a pronounced bactericidal effect on *E. coli*. Whether or not the complement system of milk is necessary for the bactericidal effect of immunoglobulins is a subject for future research.

A mechanism for the effect of sanitizers on certain microorganisms proposed by Scheusner et al. (163, 164) may well explain why the growth of *S. agalactiae* and *E. coli* is impaired by IgM. The immunoglobulin may cause metabolic injury to the test culture; this injury being sublethal and,
under favorable conditions, reversible. The affected organisms would thus grow slowly in the initial recuperative stages, and upon repair of the damage growth would proceed normally. The mechanism could also explain the discrepancies in the turbidimetric and plate count methods observed, in that the injured bacteria failed to grow in the solid medium but were able to repair their injury, and outgrow rapidly, in a liquid broth system.

A significant step towards the characterization of bovine immunoglobulins from milk and colostrum has been undertaken. A relatively simple method for isolation of IgM was developed. Some of the chemical characteristics of IgM and the effect of IgM on selected bacterial cultures were investigated. The knowledge gained helps explain the role of colostrum in providing immunity to calf, and is important for an understanding of the site of synthesis and the mode of action of immunoglobulins. However, several questions are still unanswered. Does IgM have subclasses and genetic variants? Is the IgM from blood serum identical in all aspects to that present in milk or colostrum? What is the effect of various diseases and metabolic disorders on the concentration and structure of IgM? Is IgM capable of agglutinating or inhibiting all pathogenic organisms? If yes, is there a selective action? What role does the complement system of milk play in the bacterial inhibition? Is IgM as effective in vivo as in vitro? What is the
effect of change in temperature, pH and other variables on the structure and functions of IgM? With the availability of relatively pure IgM, answers to these questions should be forthcoming in the near future.
SUMMARY AND CONCLUSIONS

An investigation was made of the isolation procedures, identification techniques, physical-chemical and chemical characteristics, and antimicrobial activity of IgM from bovine milk and colostrum.

By means of immunoelectrophoresis (IME), evaluation was made of anti-sera, prepared in our laboratory, secured from a government research facility, or purchased commercially, against bovine blood sera, blood gamma globulins, and immunoglobulins isolated from bovine milk or colostrum. Considerable variability in potency and specificity was encountered with commercial sources of anti-sera, whereas the government research anti-sera and anti-sera prepared in our laboratory were found to be extremely sensitive and reliable reagents for the identification of milk or colostral immunoglobulins.

A crude mixture (Fraction D) of immunoglobulins was isolated from milk and colostrum by the classical ammonium sulfate treatment. The same fraction but from different sources when examined by IME and analytical ultracentrifugation revealed consistently the presence of IgG1, IgG2, and IgM and on occasion IgA. Colostral whey from a cow suffering from milk fever when immunoelectrophoresed and developed
with anti-bovine blood sera indicated the presence of several additional proteins not observed in colostral whey from a normal cow. The abnormal whey had an IME pattern approximating bovine blood serum indicating that the proteins of blood serum and of the whey were immunologically related.

A step-wise examination of the ammonium sulfate fractionation procedure by polyacrylamide gel disc electrophoresis indicated that the method yielded relatively pure immunoglobulins moving as a single band at alkaline pH and as 2 or 3 bands in an acid system. Some loss of immunoglobulins occurred when the ammonium sulfate fractionation procedure was applied to colostrum.

The immunoglobulins were resolved into pseudoglobulins and euglobulins. IgG1 and IgG2 were present in the pseudoglobulin fraction, whereas euglobulin contained IgG1 and IgM, and IgA when present in milk or colostrum.

Isolation of IgM from Fraction D was undertaken using ultrafiltration, gel filtration with Sephadex G-200 or Bio-Gel A-5, ion-exchange chromatography with DEAE-cellulose and DEAE-Sephadex A-50, and (or) sucrose density gradient. Chromatography on DEAE-Sephadex with a gradient of 0.02M to 0.3M phosphate buffer (pH 7.0), fractionation on DEAE-cellulose with a gradient of 0.0 to 1.0M NaCl, or density gradient separation in 10 to 40% sucrose were the preferred methods of obtaining immunoelectrophoretically pure IgM from Fraction D.
Various preparations of IgM had sedimentation constant ($S_{20}$) values ranging from 18.2 to 19.8. The extinction coefficient ($E_{280}$ nm) was 11.8. The carbohydrate content of IgM was found to be $10.78 \pm 0.31\%$ consisting of $5.13\% \pm 0.12\%$ hexose, $1.25\% \pm 0.05\%$ fucose, $1.40\% \pm 0.05\%$ sialic acid, and $2.90 \pm 0.09\%$ hexosamines. The hexosamines were primarily in the N-acetyl substituted form. IgM was low in free -SH groups (0.9 mole/mole) but contained a high content of S-S groups (21 S-S linkages/mole). Reduction of the S-S linkages with cysteine or 2-mercaptoethanol resulted in the production of IgM subunits.

The influence of IgM on the growth behavior of Streptococcus cremoris R-1, Streptococcus lactis C-2, Streptococcus agalactiae OARDC, and Escherichia coli OSU 66 was studied by dye reduction, agar plate count, and (or) turbidity. An agglutinating effect but no apparent bactericidal activity was observed against S. cremoris or S. lactis. IgM exhibited inhibition of S. agalactiae and E. coli during the lag growth phase. However, the cells recovered and subsequently multiplied at a rate similar to control samples without IgM.

The results of the study support the following conclusions:

1. Fractionation of bovine milk or colostrum with ammonium sulfate yields immunoglobulins demonstrated to be free of other serum proteins by polyacrylamide gel disc
electrophoresis.

2. Ammonium sulfate treatment is simple and rapid but is not adequate for isolation of the individual immunoglobulin classes in pure form.

3. Pseudoglobulins and euglobulins are heterogeneous with respect to immunoglobulin classes: pseudoglobulin consisting primarily of IgG1 and IgG2, whereas euglobulin is composed of IgG1 and IgM and if present initially IgA.

4. IgG is the major class of immunoglobulins present in bovine milk or colostrum being composed of two subclasses, IgG1 and IgG2.

5. IgM is invariably present in lacteal secretions.

6. IgA may or may not be present.

7. Additional blood serum proteins may be transferred from the blood to colostrum as in the case of milk fever.

8. For isolation of immunoelectrophoretically pure IgM from a crude preparation of bovine immunoglobulins, DEAE-cellulose or DEAE-Sephadex A-50 ion-exchange chromatography, or density gradient in 10-40% sucrose are effective.

9. Pure IgM may exist in colostrum as the polymeric or monomeric form with the former being more abundant.

10. IgM is a glycoprotein consisting of approximately 10.8% total carbohydrate, primarily hexoses, hexosamines, sialic acid, and fucose.

11. Bovine lacteal IgM is composed of smaller subunits
linked through disulfide bonds.

12. IgM is not bactericidal to *S. cremoris* or *S. lactis* but does exhibit an agglutinating effect.

13. IgM retards the growth of *S. agalactiae* and *E. coli* during the initial lag growth phase but has no significant bactericidal effect during exponential growth phase.
### TABLE 8

**CONCENTRATION OF SUCROSE IN FRACTIONS WITHDRAWN FROM THE DENSITY GRADIENT TUBES**

<table>
<thead>
<tr>
<th>Fraction No.(^a)</th>
<th>Sucrose Content(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.2</td>
</tr>
<tr>
<td>2</td>
<td>37.5</td>
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<tr>
<td>3</td>
<td>36.9</td>
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<td>29.5</td>
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</tr>
<tr>
<td>19</td>
<td>8.1</td>
</tr>
</tbody>
</table>

\(^a\)Fractions were withdrawn from the bottom of the tube.  
\(^b\)Determined by the method of Dubois \textit{et al.} (47).
### TABLE 9

**EXTINCTION COEFFICIENT OF BOVINE IgM**

<table>
<thead>
<tr>
<th>Protein Concentration (%)</th>
<th>Absorbance at 280nm</th>
<th>Extinction Coefficient</th>
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</thead>
<tbody>
<tr>
<td>0.0850</td>
<td>0.995</td>
<td>11.70</td>
</tr>
<tr>
<td>0.0680</td>
<td>0.798</td>
<td>11.74</td>
</tr>
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<td>0.0510</td>
<td>0.605</td>
<td>11.86</td>
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<td>0.0340</td>
<td>0.405</td>
<td>11.91</td>
</tr>
<tr>
<td>0.0255</td>
<td>0.302</td>
<td>11.78</td>
</tr>
<tr>
<td>0.0170</td>
<td>0.204</td>
<td>12.00</td>
</tr>
<tr>
<td>0.0085</td>
<td>0.103</td>
<td>12.12</td>
</tr>
</tbody>
</table>
TABLE 10
AGGLUTINATION OF S. CREMORIS R-1 BY IgM

<table>
<thead>
<tr>
<th>IgM (μg/ml)</th>
<th>2 Hrs.</th>
<th>3 Hrs.</th>
<th>4 Hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.0</td>
<td>9.5</td>
<td>12.0</td>
</tr>
<tr>
<td>10</td>
<td>6.2</td>
<td>6.5</td>
<td>8.5</td>
</tr>
<tr>
<td>20</td>
<td>5.7</td>
<td>6.2</td>
<td>8.2</td>
</tr>
<tr>
<td>30</td>
<td>5.0</td>
<td>6.0</td>
<td>8.0</td>
</tr>
<tr>
<td>40</td>
<td>4.3</td>
<td>5.0</td>
<td>7.4</td>
</tr>
</tbody>
</table>

*aBased on dye reduction assay procedure of Mikolajcik and Sinha (116).*
TABLE 11

GROWTH PATTERNS OF S. CREMORIS R-1 IN ELLIKER'S BROTH IN THE PRESENCE OF IgM

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>OD&lt;sub&gt;0&lt;/sub&gt;</th>
<th>ΔOD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.014</td>
<td>0.018</td>
</tr>
<tr>
<td>40</td>
<td>0.044</td>
<td>0.045</td>
</tr>
<tr>
<td>60</td>
<td>0.086</td>
<td>0.092</td>
</tr>
<tr>
<td>80</td>
<td>0.183</td>
<td>0.178</td>
</tr>
<tr>
<td>100</td>
<td>0.315</td>
<td>0.307</td>
</tr>
<tr>
<td>120</td>
<td>0.477</td>
<td>0.486</td>
</tr>
<tr>
<td>140</td>
<td>0.616</td>
<td>0.613</td>
</tr>
</tbody>
</table>

<sup>a</sup> ΔOD<sub>610 nm</sub> = OD<sub>time, t</sub> - OD<sub>zero time</sub>
TABLE 12

AGGLUTINATION OF S. LACTIS C-2 BY IgM

<table>
<thead>
<tr>
<th>IgM (μg/ml)</th>
<th>2 Hrs.</th>
<th>3 Hrs.</th>
<th>4 Hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.5</td>
<td>7.2</td>
<td>11.7</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>6.2</td>
<td>8.1</td>
</tr>
<tr>
<td>20</td>
<td>5.7</td>
<td>6.0</td>
<td>7.3</td>
</tr>
<tr>
<td>30</td>
<td>5.0</td>
<td>5.5</td>
<td>6.9</td>
</tr>
<tr>
<td>40</td>
<td>4.8</td>
<td>5.0</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*aBased on dye reduction assay procedure reported by Mikolajcik and Sinha (116).*
**TABLE 13**

**DISTRIBUTION OF S. LACTIS C-2 CELLS IN THE DYE REDUCTION ASSAY SYSTEM**

<table>
<thead>
<tr>
<th>Fraction No.</th>
<th>IgM - µg/ml</th>
<th>SPC/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>34 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>38 x 10^7</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>36 x 10^7</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>222 x 10^6</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>97 x 10^6</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>55 x 10^6</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>41 x 10^6</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>37 x 10^6</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>34 x 10^6</td>
</tr>
</tbody>
</table>

*a One ml fractions withdrawn successively from the top of the dye reduction assay mixture.*
### TABLE 14
GROWTH PATTERN OF S. LACTIS C-2 IN ELLIKER's BROTH IN THE PRESENCE OF IgM

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>IgM - µg/ml</th>
<th>ΔOD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>0.013</td>
<td>0.005</td>
</tr>
<tr>
<td>40</td>
<td>0.027</td>
<td>0.019</td>
</tr>
<tr>
<td>60</td>
<td>0.050</td>
<td>0.066</td>
</tr>
<tr>
<td>80</td>
<td>0.080</td>
<td>0.092</td>
</tr>
<tr>
<td>100</td>
<td>0.131</td>
<td>0.142</td>
</tr>
<tr>
<td>120</td>
<td>0.235</td>
<td>0.239</td>
</tr>
<tr>
<td>140</td>
<td>0.363</td>
<td>0.359</td>
</tr>
</tbody>
</table>

<sup>a</sup>ΔOD<sub>610 nm</sub> = OD<sub>time, t</sub> - OD<sub>zero time</sub>
TABLE 15
GROWTH PATTERNS OF S. AGALACTIAE OARD IN ELLIKER'S BROTH IN THE PRESENCE OF IgM

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>IgM 1g/ml</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.013</td>
<td>0.007</td>
<td>0.004</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.020</td>
<td>0.007</td>
<td>0.009</td>
<td>0.000</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.030</td>
<td>0.013</td>
<td>0.013</td>
<td>0.009</td>
<td>0.011</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>0.039</td>
<td>0.022</td>
<td>0.030</td>
<td>0.021</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0.066</td>
<td>0.049</td>
<td>0.056</td>
<td>0.047</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.097</td>
<td>0.084</td>
<td>0.092</td>
<td>0.086</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>0.134</td>
<td>0.140</td>
<td>0.140</td>
<td>0.137</td>
<td>0.146</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>0.198</td>
<td>0.208</td>
<td>0.208</td>
<td>0.213</td>
<td>0.213</td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>0.252</td>
<td>0.272</td>
<td>0.276</td>
<td>0.283</td>
<td>0.283</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>0.420</td>
<td>0.474</td>
<td>0.495</td>
<td>0.486</td>
<td>0.486</td>
<td></td>
</tr>
</tbody>
</table>

\[ \Delta \text{OD}^{a} = \text{OD}_{t} - \text{OD}_{at zero time} \]
TABLE 16

EFFECT OF IgM CONCENTRATION ON THE GROWTH
OF S. AGALACTIAE IN ELLIKER'S BROTH

<table>
<thead>
<tr>
<th>IgM (µg/ml)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>106,000</td>
<td>180,000</td>
<td>330,000</td>
<td>820,000</td>
<td>1,100,000</td>
</tr>
<tr>
<td>10</td>
<td>94,000</td>
<td>144,000</td>
<td>286,000</td>
<td>687,000</td>
<td>990,000</td>
</tr>
<tr>
<td>40</td>
<td>98,000</td>
<td>132,000</td>
<td>250,000</td>
<td>590,000</td>
<td>960,000</td>
</tr>
</tbody>
</table>

Incubation - Hours
### TABLE 17

**EFFECT OF IgM CONCENTRATION ON THE GROWTH OF E. COLOI IN ELLIKER'S BROTH**

<table>
<thead>
<tr>
<th>IgM (μg/ml)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>600,000</td>
<td>1,380,000</td>
<td>8,950,000</td>
<td>34,000,000</td>
</tr>
<tr>
<td>10</td>
<td>620,000</td>
<td>840,000</td>
<td>6,500,000</td>
<td>29,000,000</td>
</tr>
<tr>
<td>40</td>
<td>530,000</td>
<td>480,000</td>
<td>5,000,000</td>
<td>27,000,000</td>
</tr>
</tbody>
</table>
Figure 43. Computer program for calculation of sedimentation constants. (Courtesy of C. F. Lin, Department of Dairy Technology, Ohio State University)
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