Allotypic determinants of bovine immunoglobulins

by

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INTRODUCTION

Immunoglobulin allotypy, a fascinating area of immunochemistry and immunogenetics, has been attracting the attention of immunologists all over the world. This interest is due to circumstances in which studies of allotypes have provided an invaluable tool for probing the genetic control of antibody synthesis and for contributing to a better understanding of immunoglobulin structure.

Oudin (126) first discovered the phenomenon of allotypy in rabbit immunoglobulins. His description of the phenomenon is attributed to the presence of genetic variants on serum proteins in some but not all members of a species. Since then, study of allotypy has progressed rapidly in man, mice, and particularly in the rabbit. Information concerning molecular structure of immunoglobulins as well as theories and hypotheses for the genetic control of antibody synthesis have been derived from observations in these animal species.

The finding of two groups of rabbit allotypes located on heavy (H) and light (L) chains of rabbit immunoglobulin G (IgG) molecules led to the conclusion that IgG molecules are encoded by two separate genes (38). Immunochemical study of immunoglobulins (26) has demonstrated a symmetry of the multichained immunoglobulin molecules with respect to their allotype. The discovery of a variable region of heavy chain (V_H) allotype which is common to the immunoglobulin classes led to the establishment of the "two genes - one polypeptide" theory (167). From these studies, it is obvious that allotypy is a powerful tool in genetic studies of immuno-

globulins at the level of the molecules, the cells and the total organism (84).

Bovine immunoglobulins have not been studied extensively and the knowledge of immunoglobulin allotypy in cattle is still in a state of infancy. The initial observation was not made until the late 1960's when Millot (112) was first to report the existence of an allotypic specificity in cattle serum. Since then, several workers have described further antigenic specificities in cattle serum proteins but none of them has fully described the immunochemistry and genetics of these allotypic factors.

Blakeslee et al. (12) presented immunochemical and genetic analyses on two allotypes, A_1 and B_1 which are located on the H chains of the IgG class and the L chains of all immunoglobulin classes, respectively. In contrast to Blakeslee's finding, Dykstra (39) described three allotypic determinants (C_1, C_2 and D_1) on gamma G immunoglobulins of cattle. Due to their restriction to a single immunoglobulin class, these markers were assumed to be located on gamma-G H chains. No allotypic markers were detected on L chains by Dykstra (39). Whether these two groups of allotypic markers (Blakeslee and Dykstra) have any genetic relationship with one another is unknown.

In this thesis, no attempt was made to duplicate the work of detecting allotypic determinants in cattle immunoglobulins but an attempt was made to extend Dykstra's research by localizing the existing markers on the IgG molecule.

REVIEW OF LITERATURE

Immunoglobulins, like other proteins, contain variable antigenic determinant groups and, when injected into appropriate animals, will provoke the production of antibodies. Oudin (126) first described this phenomenon of variation in the antigenic structure of immunoglobulins when he observed that rabbit antibodies were immunogenic when injected into other rabbits. He coined the term "allotype" to designate a distinctive immunogenic determinant of a serum protein found in some but not all members of a particular species.

About the same time, Grubb (61) discovered a similar phenomenon for human immunoglobulins and their structure was shown to be controlled by Mendelian law. Following these discoveries, the study of allotypy in man and rabbits progressed rapidly and experimentation was extended to other animal species including pigs, mice, guinea pigs, baboons, ducks, chickens, rhesus monkeys and cattle (78b).

Since the discovery of allotypy was based on the finding of variation in antigenic structure of antibody molecules, a general review of immunoglobulin structure was considered to be important.

Immunoglobulin Structure

Immunoglobulins belonging to classes IgG, IgM, and IgA are common to several species including man, mice, rabbits, chickens and cattle. The molecules exhibit a four-polypeptide-chain structure comprised of two heavy (H) chains (molecular weight of each about 50,000) and two light (L)

chains (molecular weight of each about 25,000) (50b) which are held together mainly by a number of disulfide bonds and noncovalent forces (41). Each H and L chain is divided into a variable (V) amino acid sequence segment and a constant (C) amino acid sequence segment (73). A pair of H and L chains contribute amino acid residues which form an antigen-binding site of the immunoglobulin molecule; thus a four chain structure yields two sites.

Upon cleavage of the molecule with papain (137), three fragments are separated by ion-exchange chromatography, namely, the two Fab subunits and one Fc subunit. Fc contains only the C-terminal half of the two heavy chains which are held together by a disulfide bond. The Fab subunit contains a complete L chain and the N-terminal half of an H chain (Fd fragment, Fig. 1) which are held together by a disulfide bond and noncovalent forces.

Allotypic determinants have been detected on both the Fab and Fc subunits (H and L chains). More recently, determinants have also been detected on the variable (V) portion of heavy chains in rabbit immunoglobulins (167).

Enzymatic Cleavage and Chemical Treatment of Immunoglobulin Molecules

In early studies of immunoglobulin structure, detection of N-terminal amino acids was used to determine the number of polypeptide chains in a molecule. Although data on N-terminal amino acids has been useful for studying the structure of many proteins, such information has not been of

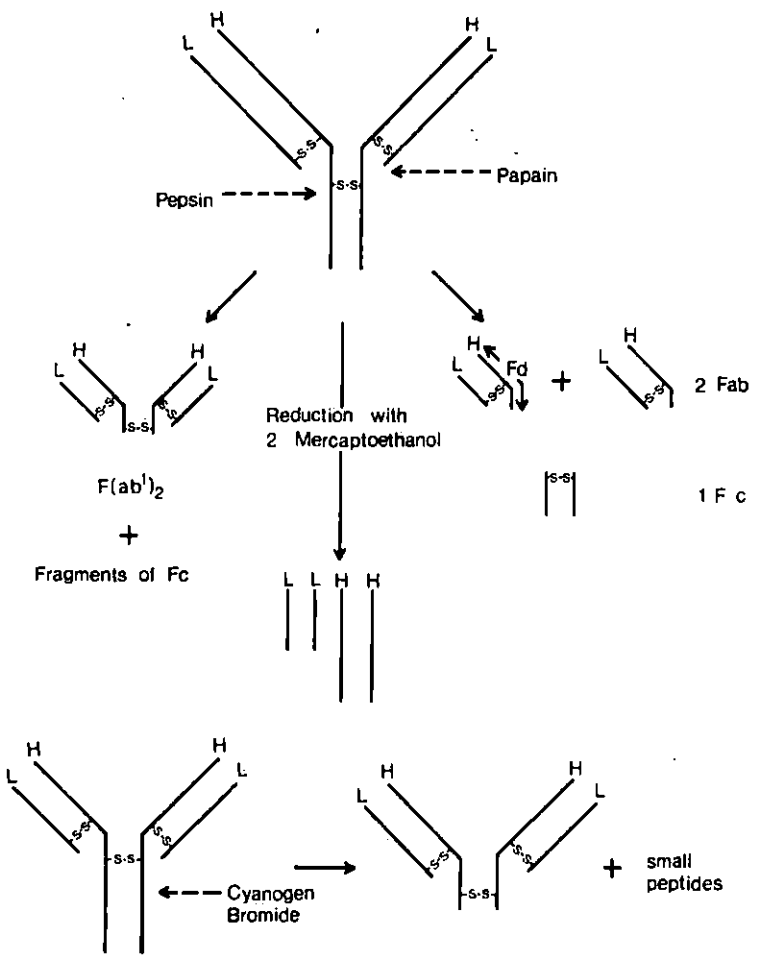

Fig. 1. A diagramatic sketch showing the structure of IgG and its sub-units resulting from different enzymatic and chemical treatments. Modified from Eisen (45).

any essential help in elucidating the complete structure of immunoglobulins owing to the fact that some of the N-terminal groups are blocked. As a result, the total number of N-terminal amino acids detected does not reflect the true number of polypeptide chains.

Resolution of immunoglobulin structure can be accomplished with various methods of proteolytic enzyme cleavage and by chemical reduction of the intact immunoglobulin molecule (138). Immunoglobulin molecules can be split into subunits by various methods, such as enzymic digestion (123, 137, 146, 169), treatment with cyanogen bromide (18), reduction and alkylation accompanied by exposure to urea and acidic pH (41, 43), detergent (169), or quanidine hydrochloride (96, 159).

Papain digestion of immunoglobulin

Porter (136) first showed that unpurified papain hydrolyzed immune rabbit globulins into fragments having a molecular weight of about 44,000 which were not precipitated by antigen (e.g., ovalbumin) but which were able to inhibit the reaction of antigen with unrefined antibody. However, a more detailed study of these fragments was difficult because of the considerable amount of contamination by enzyme protein. Nine years later, Porter (137) employed purified crystalline papain to hydrolyze rabbit gamma globulin in the presence of cysteine. With the aid of carboxymethylcellulose, the hydrolysate was divided into three main fractions which were designated as fragment I, II and III (Fig. 1). Fragment I and II (molecular weight about 45,000) had similar properties and were able to inhibit the reaction of antigen with the respective antibody. Each fragment contained a single binding site and they were later designated as the

Fab (antigen-binding) fragments. Fragment III (molecular weight about 50,000) differed sharply from fragment I and II since it crystallized spontaneously in cold neutral buffer and lacked the antigen-binding capacity. This fragment was designated as the Fc (crystallizable) fragment.

Inman and Nisonoff (75) showed that the site of action of papain is localized in the region of disulfide bonds which link the two heavy chains together. The concentration of cysteine in the digestion mixture affected the proteolytic action of the enzyme. At relatively low cysteine concentration in the medium and a relatively short hydrolysis time, peptide bonds split nearer to the N-terminals from the disulfide bridge. The resultant fragment Fc contains an inter-chain disulfide bridge after oxidation. With increases in the cysteine concentration and hydrolysis time, proteolysis is more complete and a disulfide bond can no longer be detected in Fc fragment. The pH is another factor which affects papain digestion of globulin molecules. Prahl (143) showed that at pH 7.0 papain does not further digest the fragments but at acid pH, Fc is hydrolyzable. Very recently, Stewart and Stanworth (163) showed that IgG, when pretreated with acid, was more susceptible to papain digestion.

Papin digestion products can be separated by chromatography on carboxymethylcellulose with the aid of sodium acetate solution in concentrations of 0.05M, 0.1M, and 0.5M (137). Upon separation by this method, Porter (137) obtained two univalent (Fab) fractions and one Fc fragment. Later it was found that the appearance of the two univalent fractions corresponding to the Fab fragment was due to the charge heterogeneity of

the gamma globulin molecule population, and that fragments I and II carried different charged amino acids and were present in diverse gamma globulin populations (129).

Franklin (54) used DEAE-cellulose to separate a papain hydrolysate. Three fractions, A, B and C, were obtained, of which A and C resembled fragments I and II of rabbit gamma globulin, while B was similar to fragment III. More recent experimentation showed that DEAE-cellulose yielded two fragments with step-wise gradients from 0.005M phosphate buffer pH 8.0 to 0.5M phosphate buffer pH 8.0 (43). Fab and Fc possessed different electrophoretic mobilities since the former moved toward the cathode and the latter moved toward the anode (116, 142). Olein and Edelman (124) demonstrated that Fab and Fc fragments could also be fractionated by starch zone electrophoresis according to their difference in electrophoretic mobility. Tan and Epstein (164) later showed that gel filtration is a convenient method for separating the fragments from the digest.

The immunogenicity of the proteolytic fragments made it possible to match fragments with dissociated chains. Observations included finding that goat antiserum against isolated Fc fragment of rabbit IgG could form a specific precipitate with H chain and Fc only, and that antiserum to Fab fragments reacted with both light and heavy chains. This indicated that Fc was composed primarily of H chain while Fab was composed of H as well as L chain. Putting this information together, Fleischman et al. (51) put forward a four-chain model for immunoglobulin molecules. It was Edelman and Gally (42) who finally confirmed the multichain structure of immunglobulin molecules.

Pepsin cleavage of immunoglobulin

Pepsin was the first proteolytic enzyme used to digest antibodies (120). Bridgman (13) showed that prolonged peptic hydrolysis of human gamma globulin will yield 5.8S and 3.1S fragments (Fig. 1). Nisonoff et al. (121, 122) hydrolyzed rabbit gamma globulin with pepsin at pH 4.0 and observed that the sedimentation constant of the gamma globulin fell from 7S to 5.25S with a molecular weight of about 106,000. These fragments with a sedimentation constant of about 5S were precipitable with antigen and thus were presumably bivalent. In the presence of reducing agents (0.01M cysteine, 0.01M mercaptoethanolamine), the 5S fragment was reduced to 3.5S fragments with a molecular weight of about 56,000. Antigen precipitating activity was lost but the product was able to inhibit precipitation of antigen by corresponding antibodies. Thus the 3.5S pepsin-cleaved fragments were univalent and were similar to the papain-cleaved fragments I and II.

Utsumi and Karush (168, 169) showed that the univalent pepsin-cleaved fragments were larger than papain-cleaved fragments by a peptide chain section with a molecular weight of 4000-5000. According to the data of Utsumi and Karush (168, 169), pepsin first split the gamma globulin molecule into a 5S fragment and a fragment which was similar in its antigen properties to the papain-cleaved fragment III and had a sedimentation constant of 3.3S. This latter fragment was further broken down into smaller units by continued hydrolysis with the enzyme. The bivalent pepsin cleaved fragment with molecular weight of about 100,000 was designated as the $F(ab')_2$ subunit (Fig. 1).

Cleavage of immunoglobulins by other enzymes

Many other enzymes have been used in attempts to fragment immunoglobulins. They include sulfhydryl-activated proteases such as ficin, chymopapain, and bromelin (136), proteases and peptidases of the digestive tract, lysozyme and neuraminidase (145). The sulfhydryl-activated proteases yield products similar to those obtained after papain treatment. However, enzymes such as carboxypeptidase, leucine aminopeptidase, crude hog peptidase, enterokinase, urease and lysozyme had no apparent effect.

Treatment of immunoglobulin with trypsin yields fragments resembling those of papain treated gamma-G immunoglobulin (133). The presence of cysteine in trypsin digestion increased the yield of 3.5S fragments but the time of incubation with enzyme was up to 72 hours (154). Plaut and Thomas (133) demonstrated that digestion of IgM with trypsin at 65°C for 8 minutes resulted in the formation of ten antigen-binding Fab fragments held together by the inter-subunit disulfide bonds. This pentameric unit is easily separated by gel filtration and crystallizes spontaneously.

Chemical treatment of immunoglobulins

Edelman (41) was first to show that the gamma-globulin molecule consisted of subunits linked by disulfide bonds. He used 0.1M 2-mercaptoethanol in 6M urea to sever the disulfide bonds; the sedimentation constant of the intact molecule was reduced from 7S to 2.3S with a molecular weight of about 48,000 (Fig. 1).

Many chemical reagents have been used for selective cleavage of interchain disulfide bonds including 2-mercaptoethylamine (41, 156), dithiothreitol (8, 160, 156) and 2-mercaptoethanol (44, 95, 156). It has

been reported that 0.2M 2-mercaptoethanol (43, 111) and 0.01M dithiothreitol (8, 160) are effective in dissociating or reducing immunoglobulin molecules without causing extensive conformational changes in the molecules. The reduced molecules are usually alkylated with iodoacetic acid or iodoacetamide to prevent reoxidation during subsequent processing.

Edelman and Poulik (43) further characterized the structure of gammaglobulin by separating subunits of the reduced, alkylated protein, dissolved in 6M or 8M urea, electrophoretically on starch gel at low pH and by chromatography on carboxymethyl cellulose. This work led to the identification of two types of polypeptide chains: the light (L) chains, which migrate more rapidly toward the cathode and the heavy (H) chains. The molecular weight of H and L chains was found to be about 50,000 and 25,000 respectively (43).

Separation of H and L chains from reduced and alkylated immunoglobulin molecules was best accomplished by gel filtration (50b) on Sephadex G100 with 1.0M propionic acid or acetic acid. Fleischman (50b) reported that propionic acid gave a higher recovery of both H and L chains. More recently, Tan and Epstein (165) reported that separation of H and L chains was more efficient on Sephadex G166 (two parts G200 with one part G100).

Palmer et al. (129, 130) demonstrated that on mild reduction of rabbit immunoglobulins with 0.01M 2-mercaptoethanol, half molecules consisting one complete H chain and one complete L chain can be obtained. The half molecules recombine spontaneously at neutral pH through strong noncovalent forces acting between the Fc segments of the two H chains.

Hybrid molecules of mixed allotypes were produced artificially by Seth et al. (157). Inman and Nisonoff concluded that there was no preferential recombination of half molecules (75).

Cyanogen bromide (CNBr) has been used for the selective cleavage of peptide bonds in the polypeptide chains of a number of enzymes (10, 60, 74). Cahnmann (18) was the first to treat gamma globulin with CNBr and he succeeded in isolating biologically active fragments. In 70% formic acid, CNBr specifically breaks the methionine residues in the protein. Methionine is converted to homoserine in this process. Under less drastic conditions such as in dilute HCl, methionine residues near the hinge region may be preferentially attacked producing a bivalent fragment designated $F(ab")_2$ which is somewhat larger than the $F(ab')_2$ resulting from peptic digestion. Subsequent reduction of the single interheavy chain disulfide bond yields monovalent Fab" fragments which are similar to the papain Fab piece. Cyanogen bromide has also been used to cleave the heavy chains of rabbit gamma G globulin in amino acid sequence studies (58).

Fougereau and Edelman (52) and Fleischman et al. (51) demonstrated that when Fab fragments from papain digestion were reduced in the absence of urea and placed in dissociating solvents, the light chain dissociated from the heavy chain segment. This N-terminal half of the heavy chain was designated as the Fd fragment (Fig. 1) and could be separated by gel filtration on a Sephadex G100 in 1N propionic acid.

Nomenclature for immunoglobulins and their subunits is shown in Table 1.

Table 1. Nomenclature for immunoglobulins and their subunits[a]

	Present nomenclature	Previous nomenclature
Immunoglobulins	IgG or γG	γ2, γ8S, 7Sγ, 6.6Sγ
	IgA or γA	γ_1A, β_2A
	IgM or γM	γ_1M, β_2M, 19Sγ
Papain fragments	Fab	I, II, A, C, S
	Fc	III, B, F
	Fd	A piece
Peptic fragments	$F(ab')_2$	5S divalent fragment
	Fab'	univalent fragment
Chains	Heavy (H)	A
	Light (L)	B
Light chain antigenic types	Kappa	I, B
	Lamda	II, A
Cyanogen-cleaved fragment	$F(ab'')_2$	--
	Fab"	--

[a]Modified from Fleischman et al. (50a).

Bovine Immunoglobulins

Three classes of immunoglobulins, IgG, IgM and IgA have been described in the bovine species where they occur in serum and in the lacteal secretions. A proposed nomenclature has been established for naming these classes of immunoglobulins by Aalund and his working group (1).

IgG designated a heterogeneous group of immunoglobulins which is divided into two subclasses; IgG1 and IgG2 comprise 85-90% of the serum

and whey immunoglobulins (90). IgG1 is the predominant immunoglobulin in lacteal and salivary secretions. In a basic agar-gel electrophoretic field it migrates slower toward the cathode than IgG2. IgG2 constitutes slightly less than 50% of the serum IgG. It migrates faster toward the cathode in electrophoresis, and can be differentiated from IgG1 by detection of antigenic determinants on the gamma heavy chains, presumably in the Fc region (81).

Kickhöfen et al. (81) reported a third subclass of IgG on the basis of its behavior on DEAE-Sephadex and on immunoelectrophoresis. They termed it IgGS and found that it had a molecular weight of 163,000. Butler (14) later demonstrated that the IgGS was antigenically identical to IgG1. Since Kickhöfen et al. (81) demonstrated only the physicochemical but not antigenic differences in this immunoglobulin, IgGS cannot be accepted as a distinct subclass.

Crude immunoglobulin G is most frequently prepared by salting out procedures with ammonium sulfate at 33% saturation (17) with Na_2SO_4 (48), with a combination of Rivanol fractionation and ammonium sulfate precipitation (81), or with alcohol precipitation (24). A more laborious method to prepare IgG is by gel filtration of ammonium sulfate-precipitated globulin on Sephadex G200 (49). In this method, both IgG subclasses are present but there is a tendency for contamination with IgA class globulins.

A complete and detailed outline for the preparation of IgG1 and IgG2 was presented by Butler (14). Column chromatographic separation on DEAE-cellulose or DEAE-Sephadex with molarity gradients and varying pH was

usually employed to isolate IgG1 and IgG2 subclasses. Colostral whey is recommended as a source of IgG1 because of the strong predominance of this protein. Bovine serum is used for IgG2 production. The IgG2 immunoglobulins are not retained on DEAE-cellulose in 0.01M phosphate buffer at pH 8.3 and are eluted in the break-through peak. The second major peak from a continuous gradient with DEAE-cellulose is composed primarily of IgG1.

IgM generally is a 19S immunoglobulin with a molecular weight of 900,000 but a 7S form (MW = 200,000) occurs in adult bovine serum (17). Bovine IgM comprises less than 10% of the serum and colostral immunoglobulins (90). It possesses physico-chemical and biological (59) properties similar to the IgM of other species. Immunoelectrophoretic analysis demonstrated that IgM has a migration mobility distinct from that of IgG and IgA (5). IgM is eluted in the first peak from Sephadex G-200 (17) or in a third major peak from a continuous gradient on DEAE cellulose or DEAE Sephadex fractionation when the $(NH_4)SO_4$ precipitate of milk or colostral whey is used as the starting material (2).

Contamination by IgA and alpha 2 macroglobulin is a major problem in the purification of IgM. Technical procedures such as sucrose density gradient ultracentrifugation and acrylamide gel electrophoresis were employed to separate IgM from contaminants of IgG and IgA respectively (76). Contaminating alpha 2 macroglobulin can be removed from IgM preparations by Pevikon block electrophoresis (89).

The antigenic distinctiveness of bovine IgM that can be demonstrated by immunodiffusion appears to reside in the Fc fragment which shares no antigenic determinants with bovine IgG (59).

Mach et al. (100) pioneered experimentation on IgA which is the principal immunoglobulin synthesized in salivary glands, the gut and the respiratory tract. Bovine IgA is an 11S protein containing a secretory component (molecular weight = 50,000); free secretory component which is glycoprotein in nature can be easily demonstrated in normal milk (15). Recently, Komar et al. (93) described a 15S and a 12S IgA from bovine nasal secretions. The small quantity of IgA present in bovine serum (0.3 ± 0.2 mg/ml) was reported by Fey et al. (49); therefore, isolation of IgA from this source was difficult. Isolation of IgA was usually conducted on secretions such as saliva, nasal secretions and milk or colostral whey (49) with many steps of purification being necessary. Gel filtration of serum on Sephadex G200 is usually employed but commonly there is contamination with IgG. IgA obtained from gel filtration is usually recycled on DEAE cellulose (134, 135). Recently, Fey et al. (49) developed a method for the isolation of bovine IgA. Saliva was used as the IgA source and after dialysis against 0.02M phosphate buffer pH 7.2, the protein solution was added to a batch of DEAE cellulose DE-52 equilibrated with the same buffer. Adsorption was carried out with gentle stirring at 4°C overnight. The slurry was then washed twice with one volume of 0.02M phosphate buffer pH 7.2. This step eliminated most of the IgG1 and IgG2 if present. The cellulose was densely suspended in the same buffer,

poured into a column and the IgA-containing peak was eluted by 0.06M phosphate pH 7.0.

Enzymatic Cleavage of Bovine Immunoglobulin

Papain and pepsin have normally been used to cleave bovine IgG into its subunits. Fab and Fc fragments were obtained from papain digestion and the $F(ab')_2$ fragment obtained from pepsin cleavage. $F(ab')_2$ fragments differ from the Fab fragments in that the $F(ab')_2$ consisted of the two Fab pieces bound together by the S-S bridge (Fig. 1).

The original method of Porter (137) utilizing papain to digest immunoglobulins in neutral buffer in the presence of cysteine has been widely used to digest bovine immunoglobulins and the digest was normally fractionated by ion-exchange on CM-cellulose at pH 5.5 using a molarity gradient. Fab fragments were obtained at the break-through peak while the Fc fragments were eluted as the molarity gradient increased. Papain digest was also fractionated on a DEAE cellulose or Sephadex column. This yielded two major peaks corresponding to the Fab and Fc fragments (117). Further experiments revealed these products were not pure. Kickhöfen et al. (82) filtered their mildly papainized IgG through Sephadex G100 and obtained a 3.5S Fab/Fc mixture in the second peak. This was further purified by zone electrophoresis on Geon Resin X-427. This procedure, however, depends on an apparatus which is not readily available.

Recently, Fey (48) developed a simple procedure for the preparation of Fab from bovine IgG. The papain digest was chromatographed on DEAE cellulose equilibrated with 0.01M phosphate buffer pH 8.0, rechromato-

graphed on DEAE-cellulose with 0.01M Tris-HCl pH 8.6 and 0.05M NaCl, and then precipitated with ammonium sulfate at 60% saturation. The supernatant fluid contained Fab which is free of the Fc piece and of intact IgG.

Payne (131) reported that when bovine IgG was digested with pepsin in 0.1M acetate buffer, pH 4.5, an immediate precipitate appeared which redissolved when digestion was stopped after 18 hours by adjusting the mixture to pH 7.0. Chromatography of the whole digested mixture, including the redissolved precipitate, on Sephadex G200 separated two peaks. Immunoelectrophoresis of the protein of the first peak against antiserum to bovine IgG gave a single precipitation arc with a mobility slightly slower than that of the intact IgG. The second peak contained a mixture of peptides. Ouchterlony plate studies showed that the digested protein was antigenically deficient with respect to bovine IgG, but gave a reaction of identity with bovine Fab. The protein eluted from first peak of pepsin-digested bovine IgG was found to be identical to the human $(Fab')_2$ fragment, with the conclusion that the material was bovine $F(ab')_2$ fragments.

Butler (16) reported different results from his studies with pepsin digestion. When total IgG was hydrolyzed with crystallized pepsin, five fractions were separated by gel filtration and these corresponded in order of descending size to aggregated IgG, 7S-IgG, $F(ab')_2$, traces of Fab and Fc and finally a mixture of peptides.

Chemical Treatment of Bovine Immunoglobulin

It has been reported that 2-mercaptoethanol and dithiothreitol (8, 160) were effective in dissociating or reducing immunoglobulin molecules without causing extensive conformational changes in the molecules. Bovine IgG has been reduced to H and L polypeptide chains by treatment with 2-mercaptoethanol (113), by sulfitolysis (12, 99), and dithiothreitol (99). H and L chains were separated by gel filtration on G75 or G100 Sephadex columns and eluted with 1M acetic or propionic acid.

Human and rabbit immunoglobulins were treated with cyanogen bromide for immunoglobulin structure studies (175) but no reports have been published on cyanogen bromide treatment of bovine Ig.

Immunoglobulin Allotypy of Experimental Animals

Immunoglobulins have been found throughout the vertebrate kingdom and allotypy of immunoglobulins has been reported in many animal species including pigs, guinea pigs, baboons, fowls, cats, ducks, men, rabbits (78b) and recently cattle (11). A notation for allotype was established by Dray et al. (28). In general, allotypes are denoted by a capital A, the genetic locus involved is denoted by a smaller letter (a, b, c, etc.) and an arabic number is used to denote specificities. Therefore Aa1 is allotype A with specificity 1 at locus a.

Rabbit allotypes

Immunogenicity of rabbit antibodies was first observed by Oudin (126) when he found that immunoglobulins of an individual rabbit emulsified with

paraffin oil and injected into recipient animals elicited antibodies that reacted with immunoglobulins from the donor and some other rabbits. Oudin (126) termed this phenomenon "allotypy." Using isoimmunization methods, Oudin (127) detected seven allotypic determinants in rabbits and named them allotypes a, b, c, d, e, f, and g. Genetic studies on rabbit allotypes by Dubiski (36) and Oudin (128) demonstrated that these allotypic determinants were controlled separately by two loci (a and b) with three alleles at each locus. Dray and Nisonoff (27) as well as Dray et al. (29, 30) confirmed that these determinants followed Mendelian inheritance.

Group a (a locus) allotypes Three allelic genes at locus a that code for three allotypes, a1, a2, and a3 respectively, were first identified by Oudin (126) and later confirmed by Kelus (77), Dubiski et al. (38, 37), and Dray et al. (29). These three allotypic determinants have been found on the H chain of several immunoglobulin classes including IgG, IgA, IgM and IgE (Table 2). It was shown that these group a allotypes were located on the Fd region of the H chain since they were absent on L chains (160) and present on the Fab piece (78b, 78a, 80, 110). Recently, Mage et al. (103) and Mole et al. as cited in Kindt (84) further confined the localization of these specificities to the V_H region of the heavy chain.

Hamers and Hamers-Casterman (64) and Hamers et al. (65) identified two other allotypes, A8 and A10, on the Fc fragments of rabbit Ig molecules. These specificities were present only on the H chain which contained the allotype a1. Hamers (65) suggested that A8 or A10 may be genetically linked to locus a.

Table 2. Location of allotypic specificities in rabbit Ig[a]

Locus	Known specificities (allotypes)	Ig classes	Location	Allelism
Aa	a1,a2,a3	IgG,M&A	Fd portion	Yes
Unamed	A8,A10	IgG	Fc portion	--
Ab	b4,b5,b6,b9	IgG,M&A	Constant region of K Light chain	Yes
Ac	c7,c21	IgG,M&A	Lamda Light chain	Probably pseudoalleles
Ad	d11,d12 may be d13	IgG	Hinge region position 226 (Met/Thr)	Yes
Ae	e14,e15	IgG	Fc, position 309, (Thr/Ala)	Yes
Af	f69,f70,f71 f72,f73	IgA	Fc and Fab[b]	Yes
Ag	g74,g75,g76 g77	IgA	Fc and Fab	Yes
An	n81,n82	IgM	Constant region of Heavy chains	Yes
Unamed	Ms1,Ms2,Ms3 Ms4,Ms5,Ms6	IgM	Heavy chain	Unknown
Ax	x32	IgG	Variable region of Heavy chains	--
Ay	y33	IgG		

[a]Data from Nisonoff et al. (123).

[b]Not conclusive.

Group b (b locus) allotypes Three allotypic markers, b4, b5, and b6, were originally detected by Oudin (127) and shown to be under allelic control at the b locus. Leskowitz (97) showed that b4 and b5 were located on the antibody combining fragment I and II obtained by papain digestion. Stemke (162) further showed that these markers were located on the L chain. Since all classes of Ig share the kappa and lamda light chains, these allotypic specificities are expected to be found on all classes of Ig (19, 85, 155b).

An additional allotype b9 located on L chain was later added to this group (35).

Reisfeld et al. (148) and Appella et al. (4) further demonstrated that the b locus allotypic markers are localized on K light chains. Rejnek et al. (150, 149), deVries et al. (25), and Zikan et al. (181) showed that both K light chain subtypes (K_A and K_B) carry the b locus allotypes.

Group c (c locus) allotypes Rabbit serum contains only a small percentage of molecules with L chains of the λ type. These do not carry group b allotypes. However, two markers designated c7 and c21 controlled by the c locus were found on the λ chain type (102). Initially these were thought to be alleles, but breeding experiments yielded results inconsistent with this view. This led Gilman-Sachs et al. (57) to suggest c7 and c21 are pseudoalleles, that is, they are closely linked but not encoded at homologous DNA regions. Therefore, they segregate as if they were allelic in some, but not all breeding colonies.

Group d (d locus) allotypes An allotype designated Ad11 detected by hemagglutination assay was described by Mandy and Todd (105). This specificity was shown to be present on the $F(ab')_2$ IgG fragment but not on the Fab fragment (106). This indicated that the determinant is located on the hinge region of the molecule. Additional d locus allotypes, Ad12 and Ad13, were discovered soon afterwards (107). Kindt et al. (86, 87) showed that allotype Ad11 is associated with a methionine residue at position 226 of the gamma chain while d12 has threonine at this position. In fact, this position 226 is in the hinge region (144). Combinations of a and d allotypes have been observed on the same heavy chain by Kindt, Mandy and Todd (86, 87, 107).

Group e (e locus) allotypes Two IgG allotypic markers designated as Ae14 and Ae15 were found to be located in the Fc region (33a, 33b). They are governed by a pair of allelic genes and are inherited in Mendelian fashion. Like the d locus, there is linkage between the e locus and the a locus so a and e locus allotypes can be found on the same molecule (33a, 33b).

Group f and g (f and g locus) allotypes (IgA allotypes) Conway et al. (23) and Knight et al. (92) demonstrated the existence of two allotypic groups on IgA molecules. They are group f [69, 70, 71, 72, 73] and group g [74, 75, 76, 77] allotypic markers which are controlled by two loci, f and g linked closely together (23, 67, 66, 92). Group f allotypes are present on α chains that are resistant to papain digestion while group g markers are on α chains that are sensitive to papain (67).

Since f and g loci are closely linked, the potential combination of the five group f and four group g allotypes provides twenty possible combinations of allotypic phenogroups such as $f^{71}g^{75}$, $f^{72}g^{74}$, $f^{73}g^{74}$, $f^{70}g^{76}$, or $f^{69}g^{77}$ (32). Though f and g loci have been shown to be linked closely to one another, no recombinants have been observed (66). Like the d and e loci, f amd g loci are also linked closely to the a locus which controls the V_H region of the several Ig classes (66).

Very recently, other allotypes have been found on the secretory component of IgA (92). These allotypes were designated as At61 and At62 and they are not linked to any allotypes of the H or L chains. Masuda et al. (108) reported that an allotype, c1, was detected on the Fc fragment of rabbit colostral IgA. Whether allotype c1 is identical to other f and g markers is unknown.

The exact location of the f locus specificities has not been reported. Hanly et al. (67) showed that g^{74} antiserum reacted with a high percentage of the Fc and Fab fragment molecules. If f and g are closely linked genes as reported by Conway et al. (23), f locus specificities possibly also exist on the Fc and Fab portion of IgA molecules.

IgM allotypes Allotypes have been described for rabbit IgM by Kelus and Gell (78a) and Sell (155a). Five allotypes designated as Ms1 through Ms5 were detected only by precipitation methods, and a sixth one, Ms6, could be detected only by immunofluorescence techniques. It was shown that Ms1 marker is always found in association with a3 and Ms3 associates with b4 (78a). These findings indicated that Ms3 appears to be associated with L chains in IgM rather than μ chains (80), but there is

some uncertainty relative to the Ms1 marker. Whether it could be a C_μ marker which is expressed in association with an a3 V_H region or a V_H marker expressed only when the C_μ marker is present is unknown. Kelus and Gell (78a) believed that the Ms system is controlled by at least three loci. Detailed genetic investigations have been hampered by the difficulty in isolating pure IgM owing to its low concentration in normal rabbit serum.

Gilman-Sachs and Dray (56) identified two allotypic specificities (n81 and n82) in rabbit IgM. Their relationship to determinants described by Kelus and his collaborators (78a) is not yet known. Determinants n81 and n82 are controlled by allelic genes at the locus which is closely linked to the a locus; it has been suggested that these antigenc determinants are present in the C_H region of the μ chain (56).

a-negative allotypes Dray and Nisonoff (26) and Stemke (162) described an a locus a-negative IgG molecule in rabbit serum. Kindt et al. (88) found that when rabbits were immunized with streptococcal carbohydrate, they produced large amounts of homogeneous antibodies lacking all a locus markers. Kim and Dray (83) distinguished two antigenic determinants designated x32 and y33, although x and y are two undefined new loci. These two allotypic specificities were shown to be located on the V_H region of the molecules but differed from those which carry the group a determinants. The x and y loci are closely linked to the a locus and to all other genes controlling rabbit H chains but no two of the allotypic specificities, a2, x32, or y33, are located on the same molecule (83).

Other rabbit allotypes Using a radioimmunoassay technique, Mandy and Rodkey (104) showed the presence of molecules bearing a2, a3, and b5 specificities in hares. Albers and Dray (3) reported four allotypes of rabbit low-density lipoprotein which are inherited as phenogroups controlled by three codominant alleles. Other rabbit allotypes have been reported in α2 macroglobulin (91) and haptoglobin (20).

Mouse allotypes

Mouse allotypy was first described by Kelus and Moor-Jankowski (79). They immunized inbred strains of mice with Proteus vulgaris; The anti-Proteus antibody was then complexed with Proteus cells and injected into a second strain of mice. A precipitating antibody response specifically to an antigenic determinant present on the immunoglobulin of the donor but absent in the recipient mice was detected, and was designated as allotype gamma-B^A. Soon afterwards, more allotypic determinants were identified in mice (21, 34, 180). Almost all studies of allotypy in mice have been carried out with homologous antisera, prepared by injecting immune precipitates, immune agglutinates or myeloma proteins from one strain into another inbred strain of mice (101, 139).

Allotypic determinants have been detected in IgG1, IgG2a, IgG2b, and IgA classes but none of the determinants have been reported in IgG3 or IgM classes (139, 72). Four loci, Ig-1, Ig-2, Ig-3, and Ig-4, each with several alleles, controlling the allotypic determinants in the mouse were identified (71, 174). The loci are closely linked since no recombinant allotypes were found (71, 174).

Ig-1 locus allotypes The Ig-1 locus is regulated by eight alleles ($Ig\text{-}1^a$ to $Ig\text{-}1^h$) controlling a total of eleven antigenic specificities on IgG2a molecules (70, 172, 177, 174). Each allotype controlled by the Ig-1 locus carries a group of allotypic specificities (phenogroup) which are inherited as a single unit. For example, one of the alleles of the Ig-1 locus, $Ig\text{-}1^a$, will be inherited as a phenogroup consisting of six antigenic specificities, 1.1, 1.2, 1.6, 1.8, 1.10, 1.12 (70).

Early investigation demonstrated that allotypes controlled by Ig-1 locus were located exclusively on the H chain Fc region of the IgG2a molecules (46, 116). Later, Herzenberg and Warner (70), Minna (115) and Potter et al. (140) showed the same molecular localization of other Ig-1 locus specificities on IgG2a molecules. However, with a more sensitive method, using radioimmunoassay method, Spring and Nisonoff (160) detected Ig-1 locus specificities on the Fab fragments of mouse IgG2a molecules. Since the specificities were detected on the H chain of the Fab fragment, specificities must localize on the Fd fragments of the IgG2a molecules.

Ig-2 locus allotypes The Ig-2 locus is regulated by four alleles controlling four antigenic specificities on the Fc portion of IgA molecules (69). Herzenberg demonstrated linkage between the Ig-1 and Ig-2 locus. Later, Lieberman and Porter (98a) also reported linkage of genes controlling IgG2a and IgA allotypes.

Ig-3 locus allotypes The Ig-3 locus is also regulated by eight alleles controlling a total of eight antigenic determinants (30, 98a, 141, 172, 173, 174). All allotypic determinants of the Ig-3 locus are found on IgG2b heavy chain. Three determinants, Ig-1.9, Ig-3.5, and Ig-3.6, were

shown to be present on both IgG2a and IgG2b heavy chains and were designated as IgG2 common antigenic determinants (173). Linkage between the Ig-1 and Ig-3 locus has been shown (70, 98b, 180).

Ig-4 locus allotypes Mage and Lieberman (101) demonstrated that the two determinants F8 and F19 controlled by the Ig-4 locus are present on the IgG1 immunoglobulin molecules of some but not all mice. Later, Minna et al. (115) and Coe (22) demonstrated the same genetic polymorphism in IgG1 through a physico-chemical approach. They discovered that inbred strains of mice can be divided into two groups on the basis of the electrophoretic mobility of the Fc fragments of their IgG1 molecules. The IgG1 of all strains that carry the F8 specificity have Fc fragments of relatively fast (anodal) electrophoretic mobility at pH 8.2 while the Fc fragments of all strains lacking F8 specificity migrate more slowly. Minna (115) named these two electrophoretic mobilities of IgG as Em1 and Em2, and the locus governing them consists of two alleles, $Ig\text{-}4^a$ and $Ig\text{-}4^b$, respectively.

Allotypic markers on the Fab fragments of mouse IgG In general allotypic markers studied in the mouse system were detected by a direct precipitation method, and all markers were found present in the Fc segments of mouse IgG or IgA molecules (70, 139). With a more sensitive method using ^{125}I labeling, Spring and Nisonoff (160) detected allotypic determinants on the Fab (Fd portion) fragments of mouse IgG. It is interesting to note that allotypic markers have not been identified in the L chain of mouse immunoglobulins.

Table 3. Location of allotypic specificities in mouse immunoglobulins[1]

Subclass	Locus	Allele	Allotypic specificities	Location
IgG2a	Ig-1	Ig-1^{a} to Ig-1^{h}	1.1 to 1.8 1.10 to 1.12	Fd and Fc region of H chains
IgA	Ig-2	Ig-2^{a,h} Ig-2^{c,g} Ig-2^{d,e} Ig-2^{f}	2.2, 2.3, 2.4 2.1 2.3 2.4	Fc region of heavy chains
IgG2b	Ig-3	Ig-3^{a} to Ig-3^{h}	3.1 to 3.8	Heavy chain
IgG1	Ig-4	Ig-4^{a} Ig-4^{b}	Fast Fc (F8, 19) Slow Fc	Fc region of H chains

[1]Data from Herzenberg et al. (72) and Mage and Lieberman (101). Table modified from Nisonoff et al. (123).

Human allotypes

About the same time that Oudin (126) described allotypy in rabbits, Grubb (61, 63) showed that sera from certain patients with rheumatoid arthritis reacted with the anti-Rh antibodies of some individuals but not others. They also showed that certain rheumatoid sera agglutinated group O Rh-positive red cells coated with selected incomplete anti-Rh antibodies. Human serum groups were then classified by their ability to inhibit the rheumatoid agglutination. Those sera capable of inhibiting

agglutination were designated as Gm(a+) (new terminology as Gm(a) or Gm(1)) as recommended by World Health Organization (178).

Grubb (61) identified four human serum groups and named then as Gm, Inv, Am and Isf allotypic systems. The Gm allotypic system consists of a total of 25 antigenic determinants designated by numbers as established by the World Health Organization (178). Family studies indicated that Gm allotypes are inherited according to the Mendelian law of independent assortment (61). Gm allotypic determinants have only been found in IgG1, IgG2, and IgG3 subclasses; none of the Gm allotypic markers have been found located on IgG4 molecules although genetic variants of IgG4 have been reported (94).

Unlike rabbit and mouse allotypes, no phenogroups have ever been found in the human allotypic system. Thus, an allotypic determinant can be used as a marker for identifying a particular immunoglobulin class or subclass (63). For example, the Gm factors are present only on IgG populations, and not found in the IgA or IgM fractions. Within the Gm system, certain allotypic specificities are strictly limited to only a single subclass (IgG1, IgG2 or IgG3) (Table 4). Nevertheless, there are certain determinants which are genetic variants in one subclass of IgG but which are present also in all molecules of at least one other subclass. An example is the Gm(-1) determinant which is an allotypic marker for IgG1 but is present also in all molecules of IgG2 or IgG3 (119). Frangione et al. (53) described these as "nonmarkers." Two nonmarkers designated 4a and 4b are shown to be under the control of allelic genes in the IgG4 of Caucasians (93). Recently Natvig and Kunkel (118) suggested

Table 4. Allotypic markers on human immunoglobulin G molecules[a]

System	Immunoglobulin classes	Allotypic specificities	Location
Gm	IgG1	1, (-1), 2, 3, 4, 7, 17, 18, 20	Heavy chains
	IgG2	23	Heavy chains
	IgG3	5, (-5), 6, 10, 11, (-11) 13, 14, 15, 16, 21, (-21) 24, 25	Heavy chains
	IgG4	4a, 4b	Heavy chains
Inv	IgG, IgA and IgM	Inv(1), Inv(2) and Inv(3)	Kappa Light chains
Isf	IgG	Isf(1)	Heavy chains
Am	IgA1 and IgA2	1, 2	Heavy chains

[a]Data from Grubb (61).

that the nonmarkers Gm(-1) may be the same as Gm(8), Gm(9), or Gm(22) markers.

Ropartz et al. (152) used the same method as Grubb to detect genetic factors in patients with rheumatoid arthritis. He observed that instead of 60% of the sera from normal donors being Gm(a+) as found by Grubb (61), only 19% are Gm(a+). In addition, family studies showed that the genetic factor was inherited independently of the Gm factor and was therefore named as InV (now Inv) by Ropartz et al. (152).

The Inv determinants have been demonstrated to be located on the light chains of IgG, IgA, and IgM molecules and on Bence-Jones proteins

(55, 68, 96). Terry et al. (166) finally confined the determimants exclusively to Kappa light chains of IgG, IgA and IgM immunoglobulin molecules. A single substitution of an amino acid residue (leucine or valine) at position 191 on Kappa light chain was demonstrated by Baglioni (6), Easley and Putnam (40) and Milstein (114).

Vyas and Fudenberg (170) reported the first genetic marker for human IgA. Allotype Am(1) is located on IgA molecules, showed simple Mendelian segregation and is independent of the Gm and Inv system. More recently, Wang et al. (171) described a second marker Am(2) on IgA2 molecules. The reagent used for detection was an antiserum from an IgA deficient patient which reacted with IgA lacking Am(1). The marker is found to be located on the $F(ab')_2$ (presumably Fd) fragment of Am(2) immunoglobulins. Family studies demonstrated that the gene controlling Am(2) marker is closely linked to the Gm locus and behaves as an allele of Am(1).

The Isf marker described by Grubb (62) is found only on some γ chains of IgG1 and is inherited independently of the Gm and Inv allotypes.

Bovine allotypes

Three antigenically distinct classes of bovine immunoglobulins have been described (14). They all occur in serum and in lacteal secretions and are designated IgG, IgM and IgA. IgG consists of two subclasses, namely IgG1 and IgG2.

The existence of allotypic specificity of immunoglobulins of cattle was first reported by Millot (112) who detected two genetically controlled antigens. Millot found naturally occurring anti-globulin in some cattle can agglutinate sensitized cattle erythrocytes and certain normal cattle

serum could inhibit this reaction. Millot (112) described this inhibiting factor as allotypic factor A. Using antiserum prepared in goats against cattle globulins, Millot subsequently described another factor and named it allotypic factor B (112).

Rapacz et al. (147) reported a γ M allotype in cattle. This marker was called Mc1 but later was proved not to be valid (12).

More recently, Blakeslee and Stone (11) described two γ G allotypes in cattle, namely A1 and A2 (later called B1). More detailed immunochemical and genetic analyses have been made on these two allotypes by Blakeslee et al. (12). They showed that A1 is a marker of IgG heavy chain while B1 marker is located on the light chains of all the immunoglobulin classes (12). Each of these two markers is controlled by an independent gene bva^1 and bvb^1 respectively. The two genes are autosomal dominants and are inherited independently.

In contrast to Blakeslee et al.'s findings (12), Dykstra (39) described three different allotypic determinants (C1, C2, and D1) on γ G molecules of cattle. He showed that these markers were detected exclusively on a single immunoglobulin class and therefore assumed that the markers were located on the gamma G H chains. No allotypic markers were detected on the light chains. The relationship of these two groups of allotypic markers described by Blakeslee and Dykstra respectively has not been determined.

MATERIALS AND METHODS

Bovine Allotypic Sera

Serum from donor cattle L27, L8, L11 and A5 carrying one or more allotypic determinants were used in this thesis research. Fractionated bovine sera, containing primarily gamma-globulins were used for enzymatic and chemical treatments to yield various antigenic fragments for determination of the molecular location of allotypic markers.

Preparation of Gamma-Globulin (IgG) from Normal Bovine Serum

Bovine IgG was prepared from normal bovine serum. A batch method modified from that of Stanworth (161) and Baumstark (7) was used for fractionation. Diethyl-aminoethyl (DEAE) Sephadex A-50[1] was equilibrated in 0.01M phosphate buffer, pH 7.5 for 24 hours. The fine suspension of DEAE A-50 was decanted off and the excess buffer was removed from the Sephadex by vacuum filtration in a buchner funnel. Normal bovine serum which had been dialyed overnight against the same buffer was added to the moist DEAE Sephadex in a ratio of 1:2. The serum-Sephadex mixture was allowed to stand at room temperature for two hours with occasional stirring. After two hours, the serum-Sephadex mixture was placed in a buchner funnel and filtered by vacuum filtration. The moist Sephadex was washed in the filter with 0.01M phosphate buffer. The filtrates were combined and exposed to a second batch of DEAE Sephadex A-50. The final filtrate

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[1]Pharmacia, Uppsala, Sweden.

collected was concentrated by pressure dialysis using a Diaflo apparatus.[1] This gamma-globulin preparation was used immediately for enzyme digestion and chemical treatments or stored at -20°C.

Bovine Allotypic Antiserums

Twelve allotypic antisera (L14, L24, L12, L32, L25, B55, B905, B10, B19, B29, 059 and 070) prepared by Dykstra (39) were used. Fractions of bovine sera containing primarily gamma-globulin were used as antigens for production of allotypic antibodies. The purified IgG was coated onto E. coli cells and the mixture was injected subcutaneously or intravenously via the jugular vein into recipient cattle. All calves were bled from the jugular vein. The blood was allowed to clot, the clotted blood was centrifuged at 1,000 g for 10 minutes, and the serum was removed and stored at -20°C.

Determination of Serum Protein (IgG) Concentration

The protein concentration of IgG preparations was determined by the Biuret method as described by Wuchselbaum (179). Biuret reagent was prepared from Biuret Reagent Tablets[2] and a 50% Biuret solution was used for the protein determination. A standard curve for protein concentration was established from a known concentration of bovine serum albumin. To 0.1 ml gamma-globulin solution, 4.3 ml Biuret solution was added. The mixture

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[1]Amicon Corporation, Lexington, Massachusetts.

[2]Scientific Products, Evanston, Illinois.

was incubated in a 37°C water bath for 30 minutes and standards (blanks) and the test samples were read in a spectrophotometer[1] at a wavelength of 540 nm. Concentration of gamma-globulin (mg/ml) in each sample was calulated from the standard protein curve.

Immunoelectrophoretic Analysis

Immunoelectrophoresis was employed to determine the purity of gamma-globulin preparations and for identification and detection of products of enzymatic digestion and chemical treatment. The method essentially followed the procedures established by Scheidegger (153).

Kodak projector slide cover glasses (4 x 3 inch)[2] were washed, dried and coated with a 0.1% agarose solution[3] containing 0.5% glycerol and 0.1% Tween 80.[4] The coated slides were dried in an oven at 70°C. Approximately 13 ml of hot 1% agarose-Noble agar solution in 0.05M barbital buffer, pH 8.6[5] was pipetted onto the slides. The slides were cooled, placed in a moist chamber and stored at 4°C for 24 hours before use. Several wells and troughs were cut with a template as a guide. The agar in the wells was removed and 5-10 ul of sample to be analyzed were cautiously pipetted into the wells. The sample-loaded slide was placed in a

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[1]Spectronic 20, Bausch and Lomb, Rochester, New York.

[2]Eastman Kodak Company, Rochester, New York.

[3]Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, New York.

[4]Difco Laboratories, Inc., Detroit, Michigan.

[5]Corning ACL, Palo Alto, California.

Gelman electrophoresis apparatus[1] filled with 0.05M sodium barbital buffer, pH 8.6. The samples were electrophoresed for 60-80 minutes at 150 volts. When electrophoresis was terminated, the agar from the troughs was removed and the troughs filled with rabbit anti-bovine serum. Immuno-electrophoretic patterns were allowed to develop at room temperature in a moist chamber and resulting patterns were observed after 24 to 48 hours. Slides were washed with normal saline solution and distilled water, dried and stained with amido black stain.

Ouchterlony Analysis

Fragments resulting from papain or pepsin digestion, cyanogen bromide treatment and 2-mercaptoethanol treatment were tested for purity and antigenic relationship by double diffusion precipitation in agar gel (125). Microscope slides were washed, dried and coated with 0.1% agarose solution. After drying in an oven at 70°C, 4.0 ml of hot agar solution were pipetted onto the agarose coated microscope slide. When the agar had solidified, the slides were stored at 4°C for at least 24 hours before use.

Six wells were punched in the agar slide by an immunodiffusion cutter and template kit[2] and the agar in the wells was removed by aspiration. Antiserum was placed in the large center well while antigens (IgG and fragments of IgG) were placed in the peripheral wells. Diffusion was

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[1]Gelman Instrument Company, Ann Arbor, Michigan.

[2]Miles Laboratories, Inc., Elkart, Indiana.

allowed to proceed at room temperature in a moist chamber. Slides were examined after 24 and 48 hours.

Chromatography Methods

Gel filtration on Sephadex column

Sephadex columns of G-100 and G-200[1] were used for separation of bovine IgG fragments and polypeptide chains. Sephadex G-100 or G-200 was allowed to swell in distilled water for 3 days (132) at room temperature. The gel was then equilibrated with appropriate buffers, allowed to settle and excess supernatant containing fine particles was decanted off leaving a slurry. A column[2] of 2.5 x 90 cm was used for separation. The column was mounted vertically and 3/4 filled with eluent buffer. Air bubbles were eliminated from the column by gently tapping the column. The Sephadex slurry was poured carefully down a glass rod into the column while the flow was started. The slurry addition was continued as the Sephadex settled until the column was filled. An eluent reservoir was connected to the column and buffer was allowed to flow for several hours to pack the column. A sample of 2-3 ml of protein solution (60-70 mg/ml) was loaded onto the column and a flow rate of about 20 ml per hour was established. The effluent was monitored with an UV analyzer[3] at 280 nm wavelength. Aliquots of 3-4 ml were collected in a fraction collector.[3]

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[1]Pharmacia Fine Chemicals Inc., New Jersey.

[2]Pharmacia, Uppsala, Sweden.

[3]Instrumentation Specialties Co., Lincoln, Nebraska.

Fractionation on ion-exchange column

Papain digest (Fab and Fc fragments) was fractionated on a DEAE (diethylaminoethyl) cellulose[1] column. Approximately 20 grams of the dry DEAE-cellulose ion-exchanger was used to pack a column of 2.5 x 35 cm. The cellulose was suspended in about 600 ml of a 0.5N NaOH-0.5N NaCl solution (177). The suspension was stirred until free of trapped air bubbles. After settling for 30 minutes, the cloudy supernatant was decanted off and the cellulose was resuspended in 600 ml of 1M NaCl. The suspension was again allowed to settle and decanted. The thick slurry was filtered dry by suction on a buchner funnel through two pieces of filterpaper.[2] The dry DEAE-cellulose was washed with one liter 1M NaCl, filtered dry by suction and resuspended in 600 ml 1N HCl. The suspension of cellulose was filtered dry immediately by suction and was washed with distilled water until the wash solution (filtrate) was near neutral pH. The washed DEAE-cellulose was then brought to the desired pH by three cycles of decantation and resuspended in one liter concentrated solution of the starting buffer. After decantation of the excess concentrated buffer, the cellulose was rinsed several times with the starting buffer and resuspended in one liter of starting buffer.

In packing the column, the cellulose-buffer mixture was first thoroughly dispersed with magnetic stirring and the uniform suspension was poured into the column (half filled with starting buffer). The

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[1]Bio-Rad Laboratories, Richmond, California.

[2]Whatman #31, W & R Balston Limited, England.

cellulose was allowed to settle as more cellulose was added until the column was filled. Air pressure of about 10 psi was applied to pack the cellulose. An eluent reservoir was connected to the column. Samples to be fractionated were dialyzed against the starting buffer overnight, and a maximum of 13 ml papain digest (200-300 mg protein) was fractionated on the column. A step wise buffer gradient was applied to fractionate Fab and Fc fragments from the papain digest. The flow rate was maintained at about 1 ml per minute and the effluent was passed through an UV analyzer at 280 nm. Aliquots of 3-4 ml were collected in an automatic fraction collector.[1]

Enzymatic Cleavage of Bovine IgG

Papain cleavage of bovine Immunoglobulin G

Bovine IgG preparations were digested with mercuripapain[2] by the method of Porter (137). IgG obtained by the batch chromatographic method was dialyzed against 0.1M sodium phosphate buffer pH 7.0 containing 0.01M cysteine[2] and 0.002M EDTA[3] for 24 hours. Protein concentration was determined by Biuret analysis after dialysis. Mercuripapain was added to the dialyzed IgG preparation in an enzyme:substrate ratio of 1:100. The mixture was incubated in a 37°C water bath for 16-18 hours. Digestion was stopped by the addition of iodoacetamide[2] to a final concentration of

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[1] Instrumentation Specialties Co., Lincoln, Nebraska.

[2] Sigma Chemical Co., St. Louis, Missouri.

[3] Eastman Organic Chemicals, Rochester, New York.

0.022M. The digest was dialyzed against several changes of 0.005M sodium phosphate buffer, pH 8.0. Fab and Fc fragments were then separated on a DEAE-cellulose column with elution by three successive changes of sodium phosphate buffer (0.005M, 0.01M and 0.5M), pH 8.0. Fractions containing the Fab and Fc fragments were identified by immunoelectrophoresis and agar gel diffusion.

Purification of Fab and Fc fragments by starch block electrophoresis

Fab and Fc fragments from papain digestion were purified by starch block electrophoresis. Potato starch[1] was washed in distilled water followed by several washes with sodium borate buffer, pH 8.6. Two wicks soaked in borate buffer were placed at each end of the starch tray and the washed starch slurry was poured carefully and evenly into the tray until the tray was completely filled. Excess moisture in the starch was eluted by blotting the wicks extending from both ends of the tray. A trough was cut at the center of the starch block and one ml (70 mg/ml) of the Fab or Fc fragments was transferred into the trough. A small drop of RBY reference dye[2] was placed at the center of the starch block and the sample was electrophoresed at 150 volts for 12 hours in an electrophoresis apparatus[3] placed in a cold room at 4°C.

When electrophoresis was terminated (the red dye had migrated almost to one end of the starch block), 1 cm wide starch slices were cut and each

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[1]Fisher Scientific Co., Fair Lawn, New Jersey.

[2]Gelman, Ann Arbor, Michigan.

[3]P.E.A., Arthur H. Thomas Co., Philadelphia, Pennsylvania.

slice was transferred into a 12 cc plastic syringe barrel. The protein was eluted from the starch with phosphate buffer. Eluents were concentrated by vacuum dialysis or by dialysis against PVP solution.[1] Fractions containing corresponding Fab or Fc fragments were tested for purity by immunoelectrophoresis and gel diffusion precipitation.

Pepsin cleavage of bovine Immunoglobulin G

Bovine IgG was digested with 2X crystallized pepsin[2] by the method of Utsumi and Karush (169) with minor modification. Total IgG obtained by the chromatographic method was dialyzed against 0.1M sodium acetate buffer, pH 4.3 overnight. IgG concentration was determined by the Biuret method. Pepsin was added to the IgG preparation in a ratio of 1:20. Digestion was carried out in a 37°C water bath for 24 hours. Digestion was stopped by adjusting the mixture to pH 7.0 with 1N NaOH. The digest was then dialyzed overnight against 0.02M Tris-HCl buffer containing 0.01% (w/v) sodium azide, pH 8.4. The digest was fractionated on Sephadex G-200 which had been equilibrated with the same buffer. Fractions containing the $F(ab')_2$ fragments were pooled, concentrated by vacuum dialysis and tested for purity by immunoelectrophoresis and agar gel diffusion precipitation.

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[1]Polyvinylpyrrolidone, Pope Scientific, Inc., Menomonee Falls, Wisconsin.

[2]Sigma Chemical Co., St. Louis, Missouri.

Chemical Treatment of Bovine IgG

Reduction of bovine IgG with 2-mercaptoethanol

Bovine IgG preparation was reduced to its heavy and light polypeptide chains by the method of Fleischman et al. (50b). A 2% (w/v) solution of IgG was prepared and dialyzed against 0.55M Tris-HCl buffer, pH 8.2 overnight. The preparation was first degassed and then nitrogen gas was bubbled slowly through the preparation for 5-10 minutes. 2-mercaptoethanol[1] was added to the IgG solution to a final concentration of 0.75M. The mixture was allowed to stand at room temperature for one hour. When reduction was completed, the mixture was cooled in ice water, an equal volume of 0.75M iodoacetamide[1] was added to the mixture and the pH was maintained at pH 8.0 by cautiously adding Tris-HCl buffer. Alkylation was allowed to proceed at 0°C for one hour. The reaction mixture was dialyzed against cold normal saline solution overnight and then against 1M propionic acid.[2]

Heavy and light chains were separated on a Sephadex G-100 column with elution by 1M propionic acid. Fractions containing H and L chains respectively were pooled and were concentrated by vacuum dialysis against cold normal saline solution.

Cyanogen bromide treatment of bovine IgG

Bovine IgG preparations were cleaved with cyanogen bromide by the method of Cahnmann et al. (18). A 5% (w/v) IgG solution was dialyzed against 0.0175M sodium phosphate buffer, pH 6.3 for 24 hours. An equal volume of

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[1] Sigma Chemical Co., St. Louis, Missouri.

[2] J. T. Baker Chemical Co., Phillipsburg, New Jersey.

0.14M cyanogen bromide[1] (prepared in 0.6M HCl) was added to the IgG preparation. The reaction vessel was tightly closed with a glass stopper. The mixture was allowed to stand at room temperature for four hours with gentle swirling to avoid focal localization of cyanogen bromide. The mixture was then dialyzed directly against cold 1M acetic acid[2] overnight. The $F(ab")_2$ fragments were separated by passing the reaction mixture through a Sephadex G-100 column, with elution by 1M acetic acid. Fractions containing the $F(ab")_2$ fragments were pooled and concentrated by vacuum dialysis against cold normal saline solution.

Ultracentrifugation

The sedimentation coefficient of bovine IgG fragments (Fab, Fc, H, L, $F(ab')_2$ and $F(ab")_2$ were determined by sedimentation at 59,780 and 56,000 rpm at 25°C in an AN-H aluminum rotor in a Spinco analytical ultracentrifuge.[3] Samples were dialyzed against normal saline solution prior to ultracentrifugation. Photographs were taken at 4 or 8 minute intervals during the formation peaks. Ultracentrifuge patterns were measured on the photographic plates, using a microcomparator. Experimentally observed sedimentation coefficients were corrected to a water basis at 20°C($S_{20,w}$).

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[1]Eastman Kodak Co., Rochester, New York.

[2]Fisher Scientific Co., Fairlawn, New Jersey.

[3]Beckman Instruments Inc., Stanford Industrial Park, Palo Alto, California.

Molecular Weight Determination of Bovine IgG H and L Chains

The method of Weber and Osborn (176) which employs the use of sodium dodecyl sulfate (SDS) gels was used to determine the molecular weights of the bovine IgG H and L polypeptide chain preparations.

Preparation of protein solutions

1.0 ml of 1.0 mg per ml H and L chain preparations and all protein standards (pepsin[1], trypsin[1], lysozyme[1] and bovine serum albumin[1]) were treated with 100 ul of 10% SDS[2] and 10 ul of 2-mercaptoethanol at 37°C for 2 hours. After treatment, the protein solutions were dialyzed overnight at room temperature against a 10 liter volume of 0.01M sodium phosphate buffer, pH 7.2 containing 0.1% SDS and 0.1% 2-mercaptoethanol.

Preparation of acrylamide gels

A 10% acrylamide solution was made by dissolving 22.2 g of acrylamide[3] and 0.6 g of methylenebisacrylamide[3] in 100 ml of distilled water. 10 cm long glass gel tubes were used and were previously cleaned and oven-dried. For a run of 12 gels, 15 ml of gel buffer (0.1M sodium phosphate, pH 7.2 containing 0.1% SDS) was mixed with 13.5 ml of acrylamide solution, 1.5 ml of freshly prepared ammonium persulfate[4] (15 mg per ml) and 0.045 ml of N,N,N',N'-tetramethlethylenediamine.[3] After mixing, each

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[1]Sigma Chemical Co., St. Louis, Missouri.

[2]Bio Rad Laboratories, Richmond, California.

[3]Eastman Kodak Co., Rochester, New York.

[4]Fisher Scientific Co., Fair Lawn, New Jersey.

tube was filled with 2 ml of the solution. A few drops of water were layered carefully on top of the gel solution before the gel hardened. When the gel solidified after 30 minutes, the water layer was sucked off and the tubes were placed into the disc electrophoresis apparatus.[1]

Preparation of samples for gel-electrophoresis

For each 50 ul-100 ul of sample, 3 ul of 0.05% Bromphenol blue[2] in water (tracking dye) and 1 drop of glycerol was added. The two compartments of the electrophoresis apparatus were filled with gel buffer. 50 ul-100 ul of sample was applied per gel. The gel buffer was carefully layered on top of each sample to fill the tubes. Electrophoresis was performed at a constant current of 5-8 ma per gel for 5-6 hours with the positive electrode in the lower chamber. Following electrophoresis, gels were removed from the tubes with a syringe and water pressure. The migration distance of the dye was measured and recorded.

Staining and destaining of the gel

Gels were stained and destained by the method of Fairbanks et al. (47). Gels were first stained overnight in mixture of 25% isopropylalcohol, 10% acetic acid and 0.05% Coomassie blue.[2] Destaining of gels was achieved by placing gels in the following mixture of solutions: (1) 6-9 hours in mixtures of 10% isopropylalcohol[3], 10% acetic acid and 0.0025% Coomassie blue; (2) overnight in 10% acetic acid with 0.0025% or

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[1]Instrumentation Specialties Co., Lincoln, Nebraska.

[2]Eastman Kodak Co., Rochester, New York.

[3]Mallinckrodt, St. Louis, Missouri.

less Coomassie blue; (3) several hours in 10% acetic acid. All staining and destaining procedures were performed at room temperature with moderate stirring in 50 ml or more staining or destaining solution.

Determination of molecular weight

The relative mobility of the protein standards and the H and L chains were calculated by the following formula:

$$\text{Relative mobility} = \frac{\text{Distance of protein migration}}{\text{Distance of dye migration}}$$

The mobilities were plotted against the known molecular weights on a semi-logarithmic scale. Molecular weights of H and L chains were determined from the protein standard plot.

Tests for Presence of Allotypic Determinants

Gel diffusion precipitation method

The Ouchterlony method (125) was used to detect antigen-antibody reactions. The procedure for this test was exactly the same as described in Ouchterlony analysis on page 37. Immunoglobulin subunits were placed in adjacent wells and the allotype antibodies were placed at center wells. Diffusion was allowed to proceed at room temperature. Positive reaction of the immunoglobulin units with allotype antibodies confirmed the presence of allotypic determinants on that fragment of the IgG molecule.

Inhibition precipitation tests

This test procedure was used to measure the capacity of Ig subunits to inhibit the reaction of allotypic antiserums with corresponding fragments or intact IgG molecules. The reaction of allotypic antiserum with

the intact IgG can be blocked when allotypic antiserum is absorbed with a fragment that carries the allotypic determinants. By this method, allotypic determinants on the IgG molecules could be located.

Diffusion slides were prepared as described in the previous section. The center well was first filled with the particular fragment preparations to be tested for inhibition capacity. Time was allowed for it to diffuse completely. The well was then refilled with allotype antiserum and the peripheral wells were filled with corresponding fragments and intact IgG. Fragments were considered to possess allotypic determinants if they blocked the isoantiserum from reacting with fragment preparation and the intact IgG molecules in the adjacent wells.

Radioimmunoassay for the Detection of Allotypic Determinants on Heavy and Light Chain of Bovine IgG

Preparation of specific anti-Fab and anti-Fc antiserum

Antisera directed specifically against the Fab and Fc portions of the bovine IgG molecule were prepared by absorption of rabbit anti-bovine IgG antiserum with purified Fab and Fc fragments respectively. Two ml of rabbit anti-bovine IgG2 antiserum were absorbed with small quantities (drop-wise) of Fab or Fc fragments. Absorption was allowed to take place in a 37°C water bath with occasional shaking. Precipitates were removed by centrifugation, and the supernatant fluid was exposed to a second absorption with Fab or Fc. The process was repeated until no precipitate was detected. The absorbed serum was held at 4°C overnight, any precipitate was removed by centrifugation and the anti-Fab or anti-Fc

antiserum was tested for reactivity with Fab, Fc, heavy and light chains by agar gel diffusion.

Labeling of heavy and light chains with I^{125}

McConahey and Dixon's (109) method was employed to label heavy and light chains with I^{125}[1]. A total of two mg of heavy or light chains in 1.5 ml normal saline solution were prepared in a 15 ml screw cap test tube with a polyethylene-coated magnetic stirring bar. The test tube was placed in a beaker of crushed ice to keep the reactants cold. With gentle stirring, 5 mCi I^{125}[1] was added to the chains. While the protein-iodine mixture was stirred, 100 µg of Chloramine-T[2] dissolved in normal saline solution was added by means of a syringe and 26 gauge hypodermic needle. The mixture was allowed to react for five minutes and 100 µg sodium metabisulfite[3] in 0.1 ml normal saline solution was added to stop the reaction. The I^{125} labeled heavy and light chains were then passed through a Sephadex G25 column previously equilibrated with 1% bovine serum albumin. Elution of I^{125} from the column was monitored by a radioisotope activity detector. I^{125} labeled chains were dialyzed against several changes of normal saline solution and kept refrigerated until use.

Since heavy chains tended to aggregate in phosphate buffer, all labeling procedures were conducted in 0.85% saline solution. One drop of

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[1]New England Nuclear, Boston, Massachusetts.

[2]Eastman Organic Chemicals, Rochester, New York.

[3]Sigma Chemical Co., St. Louis, Missouri.

0.55M Tris-HCl pH 8.2 was added to the chain preparations before I^{125} labeling to stabilize the pH throughout the labeling process.

Detection of allotypic determinants on H and L chains

20 µl (10,000-30,000 cpm) I^{125} labeled H or L chains were allowed to incubate with 1.0 ml anti-allotype antiserum or normal bovine serum in 0.05M Tris-HCl buffer, pH 7.0 with 10% NaCl in a 37°C water bath for one hour with occasional shaking. Six drops (0.2 ml) anti-Fab or anti-Fc serum was added to I^{125}-H and I^{125}-L chain reacting mixture respectively. After standing overnight in the refrigerator, the reacting mixture was centrifuged, the supernatant was separated and both precipitate and supernatant was counted in a gamma counter.[1] The percentage of radioactivity precipitated was calculated.

[1]Picker Nuclear, White Plains, New York.

RESULTS

Isolation of Gamma-Globulin (IgG) from Normal Bovine Serum

Purified bovine IgG was successfully prepared from normal bovine serum by the batch method of anion exchange chromatography on DEAE-Sephadex A-50. The two-stage batch absorption proved to be fast and efficient.

First batch exposure to DEAE-Sephadex A50 removed all serum proteins except IgG and traces of IgM and IgA. Upon exposure to a second batch of DEAE-Sephadex, the contaminating IgM and IgA were removed. Immunoelectrophoretic analysis of the purified IgG preparations revealed a single distinct band residing in the gamma-globulin region (Fig. 2a) indicating a protein of the IgG class. The splitting ends of the single band indicated IgG1 and IgG2 subclasses were present. Ouchterlony analysis revealed the purified IgG contained no contaminating proteins (Fig. 2b). This IgG preparation was used for subsequent enzymatic and chemical cleavage studies.

Enzymatic Cleavage of Bovine IgG

Cleavage of bovine IgG with papain

When purified bovine IgG was cleaved with mercuri-papain and the papain digest was fractionated on a DEAE-cellulose column with stepwise elution gradients, two major protein peaks corresponding to the Fab and Fc fragments were obtained (Fig. 3). The Fab fragments were eluted in the first peak with 0.005M phosphate buffer, pH 8.0. After passing 0.01M

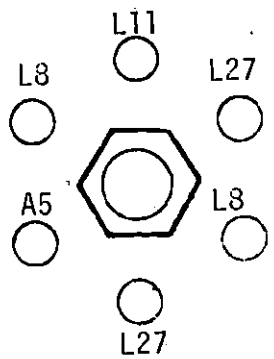
Fig. 2a. Immunoelectrophoretic analysis of bovine IgG preparations. Photograph showing the reaction of rabbit anti-bovine serum with bovine IgG preparations. Antigens were placed in the wells and electrophoresed following which rabbit anti-bovine serum was placed in the troughs. Antigens placed in the wells from top to bottom respectively:

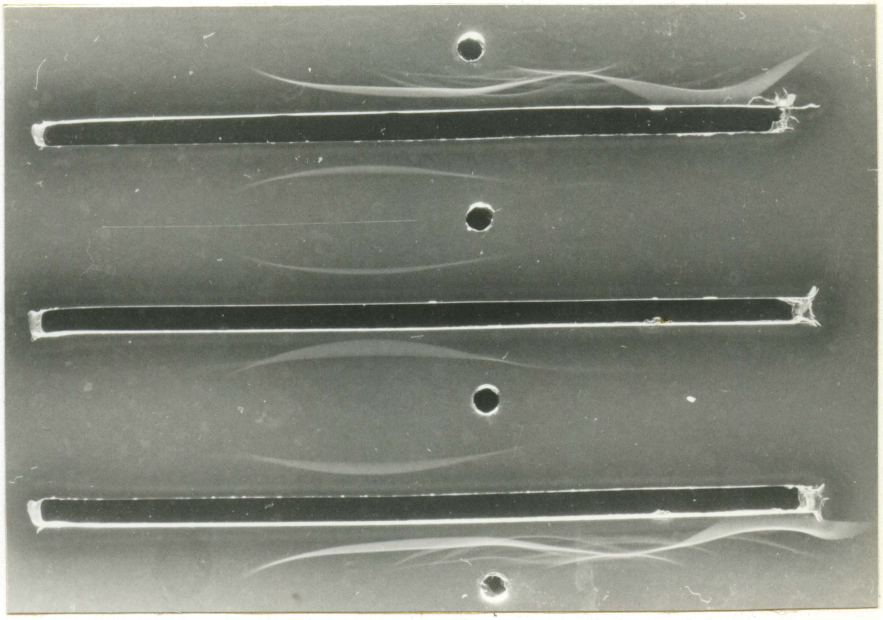
Normal bovine serum (NBS)

NBS exposed to 1st batch of DEAE-Sephadex
NBS exposed to 2nd batch of DEAE-Sephadex
NBS

The cathode is toward the left.

Fig. 2b. Ouchterlony analysis of bovine IgG preparations. Photograph showing reactions of rabbit anti-bovine serum with different purified bovine IgG preparations. Rabbit anti-bovine serum was placed in the center well. Purified bovine IgG preparations were put in the peripheral wells. Diffusion was allowed to proceed at room temperature. The slide was examined at 24 to 48 hours for precipitation bands.





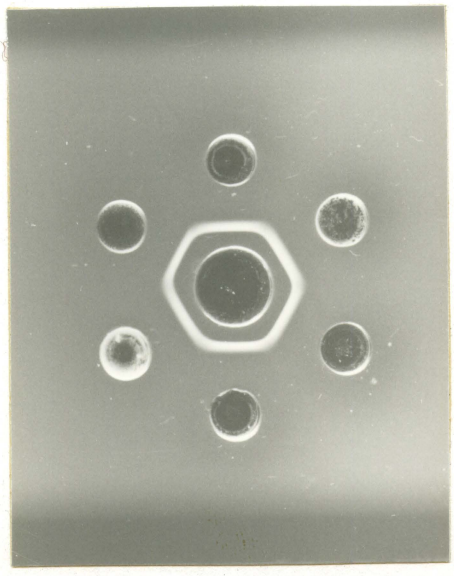
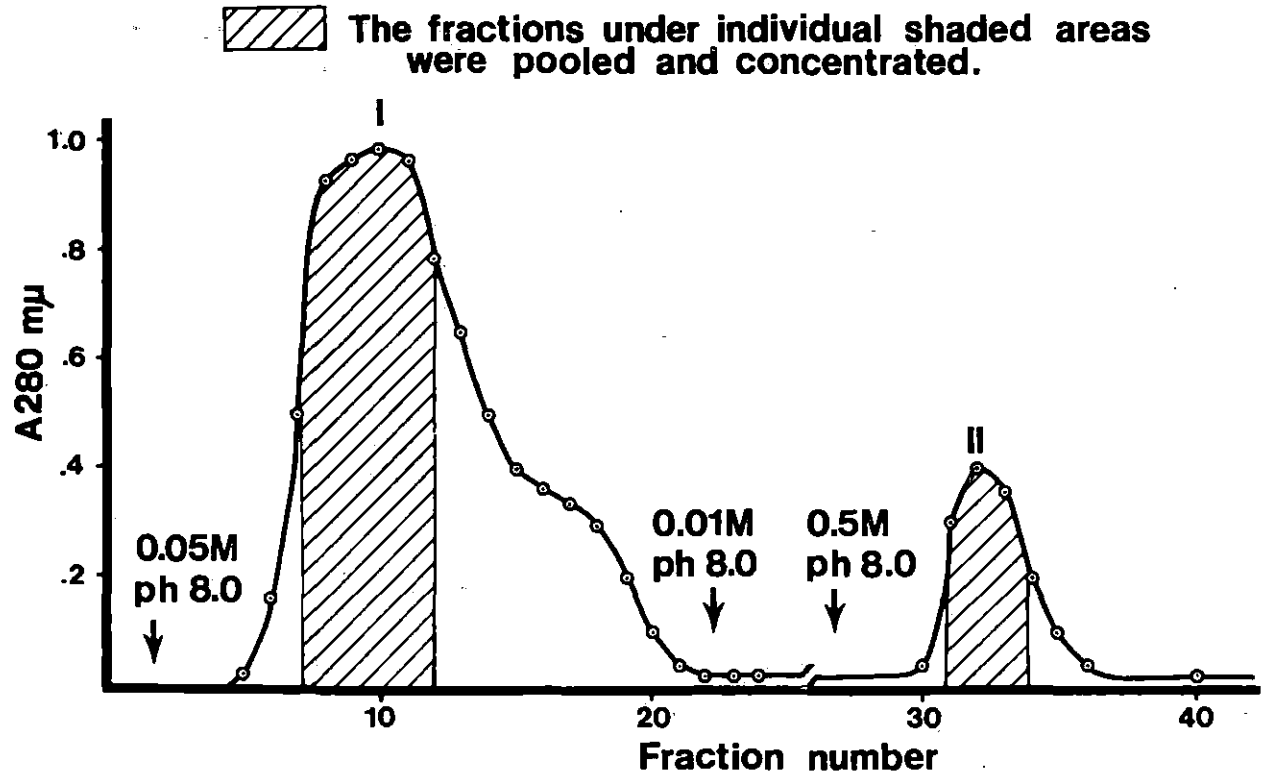


Fig. 3. Chromatography of papain digest on DEAE-cellulose column (2.5 x 35 cm). 13 ml (200-300 mg) papain digest was loaded on the column and separated with stepwise elution gradients of 0.005M phosphate buffer, pH 8.0; 0.1M phosphate buffer, pH 8.0 and 0.5M phosphate buffer, pH 8.0. Fractions under the shaded area were pooled and concentrated by vacuum dialysis.

1st peak = Fab
2nd peak = Fc


The fractions under individual shaded areas were pooled and concentrated.
I
II
1.0
.8
.6
.4
.2
A280 mμ
0.05M
ph 8.0
0.01M
ph 8.0
0.5M
ph 8.0
10
20
30
40
Fraction number

phosphate buffer, pH 8.0 through the column, Fc fragments were obtained by elution with 0.5M phosphate of the same pH. Fab and Fc fragments so obtained were found to be impure. The Fab fraction eluted in the breakthrough peak contained a high concentration of Fab but was contaminated with traces of undigested IgG and Fc fragments. Similarly, the Fc fraction was contaminated with a small amount of Fab materials.

After purification of both fragments with Sephadex G200 filtration and by starch block electrophoresis, Fab and Fc fragments were obtained in a high degree of purity. In immunodiffusion tests, both fragments gave a single precipitation band (Fig. 4b). They gave lines of nonidentity with rabbit anti-bovine IgG and showed partial identity with intact IgG molecules.

By immunoelectrophoretic analysis, the Fab fragment exhibited greater mobility toward the cathode than intact IgG while the Fc fragments migrated toward the anode (Fig. 4a).

Pepsin cleavage of bovine IgG

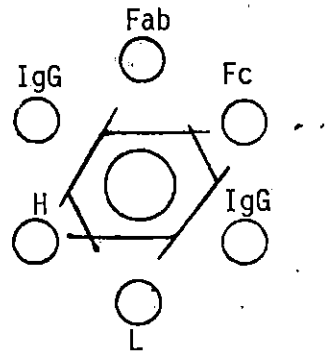
When bovine IgG was digested with pepsin in 0.1M acetate buffer, pH 4.5, precipitate appeared immediately but disappeared when digestion was terminated after 18 hours by adjusting the mixture to pH 7.0. Chromatography of the whole digestion mixture on Sephadex G200 separated four major peaks (Fig. 5). Immunoelectrophoretic analysis of the protein from the first peak demonstrated a precipitin arc with a mobility identical to that of the intact IgG. Analysis of the second peak revealed a mixture of undigested IgG and $F(ab')_2$ fragments. Peak three gave a single precipitin arc with a mobility slightly faster than that of the IgG molecules. The

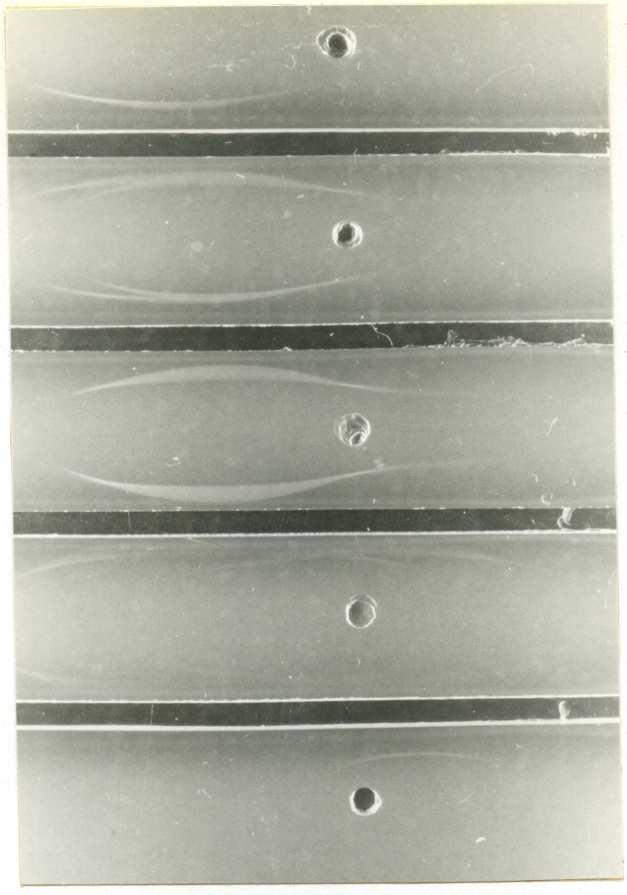
Fig. 4a. Immunoelectrophoretic patterns of Fab and Fc fragments from papain digestion of IgG. Photograph showing the reactions of various papain digestion fragments of bovine IgG with rabbit anti-bovine IgG. Antigens were placed in the wells and were electrophoresed following which rabbit anti-bovine IgG was placed in the troughs. Antigens placed in the wells from top to bottom respectively:

Fab preparations
Fab + purified IgG preparation
Purified IgG preparation
Whole papain digest
Fc fragments

The cathode is toward the left.

Fig. 4b. Ouchterlony analysis of antigenic relationship between papain digestion fragments and H and L chains. Photograph showing the gel diffusion precipitation reactions of various bovine IgG fragments with rabbit anti-bovine IgG. Rabbit anti-bovine IgG was placed in the center well. Bovine IgG fragments were placed in the peripheral wells. Fab and Fc fragments showed reaction of nonidentity with each other and both showed reaction of partial identity with intact IgG. H and L chains showed reaction of nonidentity with each other but showed antigenic deficiency in respect to intact IgG.





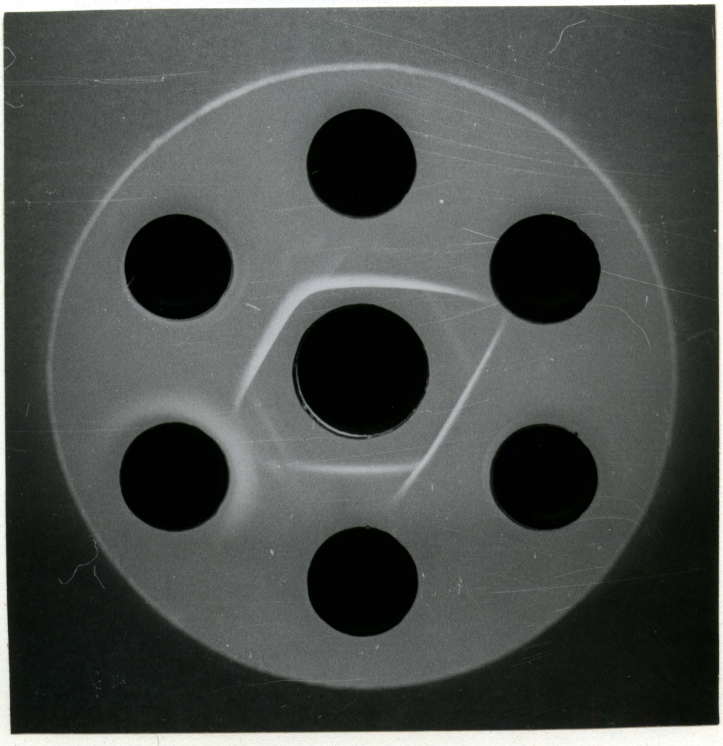
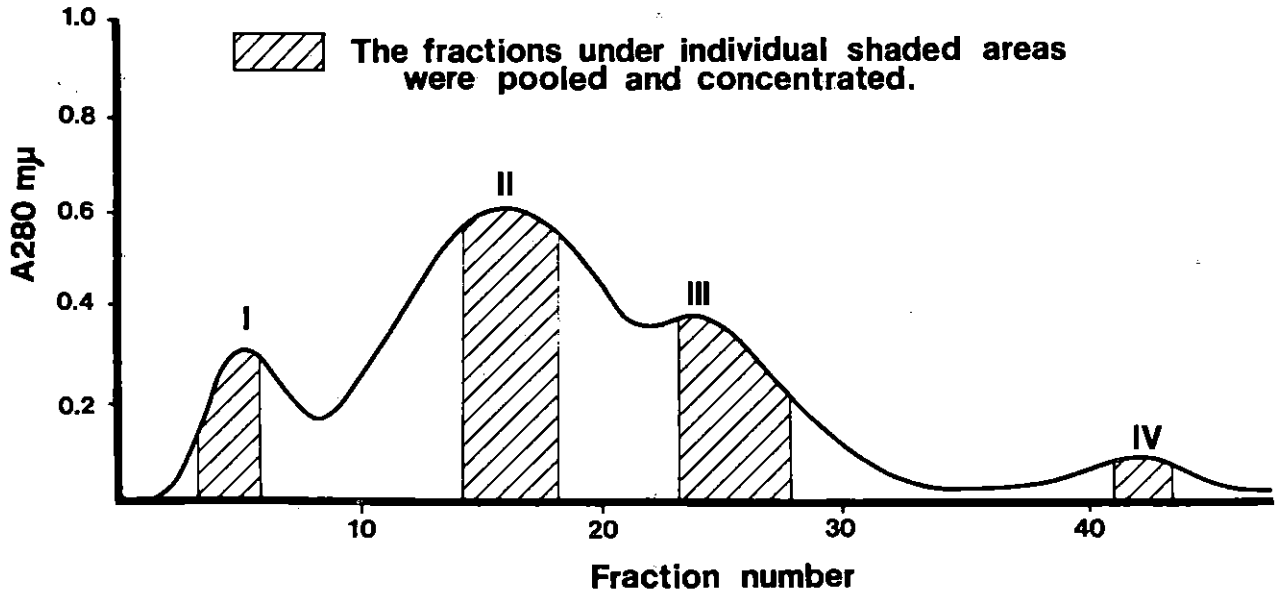


Fig. 5. Elution profile from Sephadex G200 of pepsin digest of bovine IgG. Fragments were eluted with 0.02M Tris-HCl, pH 8.4. A column of 2.5 x 90 cm was used for fractionation. Two ml of the pepsin digest preparation and a flow rate of about 20 ml per hour were used. Peak #3 contained exclusively the $F(ab')_2$ fragments was pooled and concentrated by vacuum dialysis.


The fractions under individual shaded areas were pooled and concentrated.
A280 mμ
1.0
0.8
0.6
0.4
0.2
I
II
III
IV
10
20
30
40
Fraction number

last peak gave a very faint band with rabbit anti-bovine serum and was assumed to contain a mixture of peptides (Fig. 6a).

Ouchterlony analysis showed that the $F(ab')_2$ from peak three was antigenically deficient with respect to bovine IgG but gave a reaction of identity with bovine Fab fragments (Fig. 6b).

Chemical Treatment of Bovine IgG

Cyanogen bromide cleavage of bovine IgG

Fractionation of CNBr-treated IgG on a Sephadex G100 column provided an elution profile containing two major peaks that corresponded to the $F(ab")_2$ fragment and smaller peptides (Fig. 7).

Immunodiffusion analysis showed that the $F(ab")_2$ gave a fairly strong reaction with rabbit anti-bovine IgG antiserum. It gave a reaction of identity with bovine Fab and $F(ab')_2$ fragments but showed antigenic deficiency with respect to intact bovine IgG (Fig. 8b). In comparison to the electrophoretic mobility of other immunoglobulin subunits, $F(ab")_2$ behaved almost identically with that of intact bovine IgG (Fig. 8a).

Treatment of bovine IgG with 2-mercaptoethanol

Three major peaks were obtained from the fractionation (Fig. 9). The first peak probably contained aggregates of heavy chains. The second peak contained primarily heavy chains and the third peak mainly light chains. Fractions containing H or L chains were collected in such a way as to avoid possible contamination of one with the other.

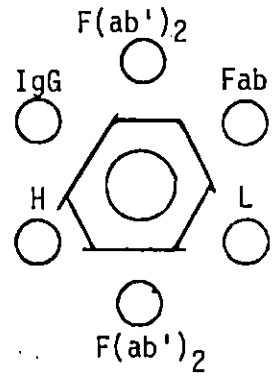
Ouchterlony analysis revealed both H and L chains were pure fractions by the fact that they gave a single precipitation line indicating

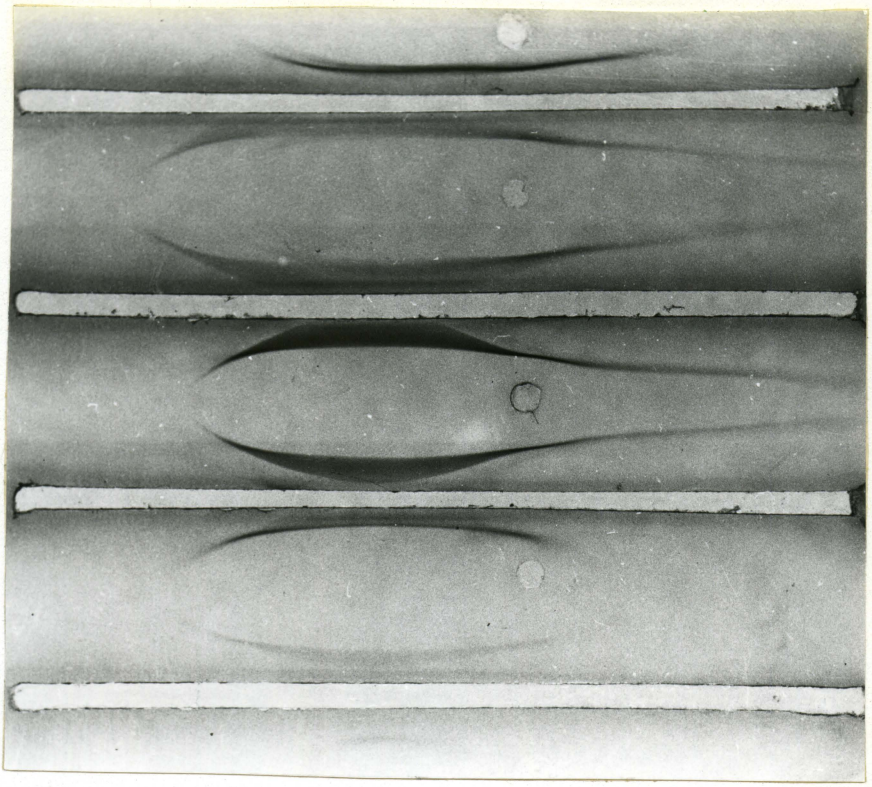
Fig. 6a. Immunoelectrophoretic patterns of various fragments from pepsin digestion of bovine IgG molecules. Photograph showing the reaction pattern of various pepsin digestion fragments with rabbit anti-bovine IgG serum. Antigens were placed in the wells and were electrophoresed following which rabbit anti-bovine IgG was placed in the troughs. Antigens placed in the wells from top to bottom respectively:

1st protein peak eluted from Sephadex G200
2nd protein peak eluted from Sephadex G200
3rd protein peak eluted from Sephadex G200
Purified bovine IgG preparation
4th protein peak eluted from Sephadex G200

The cathode is toward the left.

Fig. 6b. Ouchterlony analysis of antigenic relationship between papain and pepsin digestion fragments. Photograph showing the gel diffusion precipitation reaction between various bovine IgG subunits with rabbit anti-bovine IgG. Bovine IgG fragments were placed in the peripheral wells and reacted with rabbit anti-bovine IgG in center well. $F(ab')_2$ and Fab fragments showed reaction of identity with each other, but showed partial identity with intact IgG. Both H and L chains showed partial identity with the $F(ab')_2$ fragments.





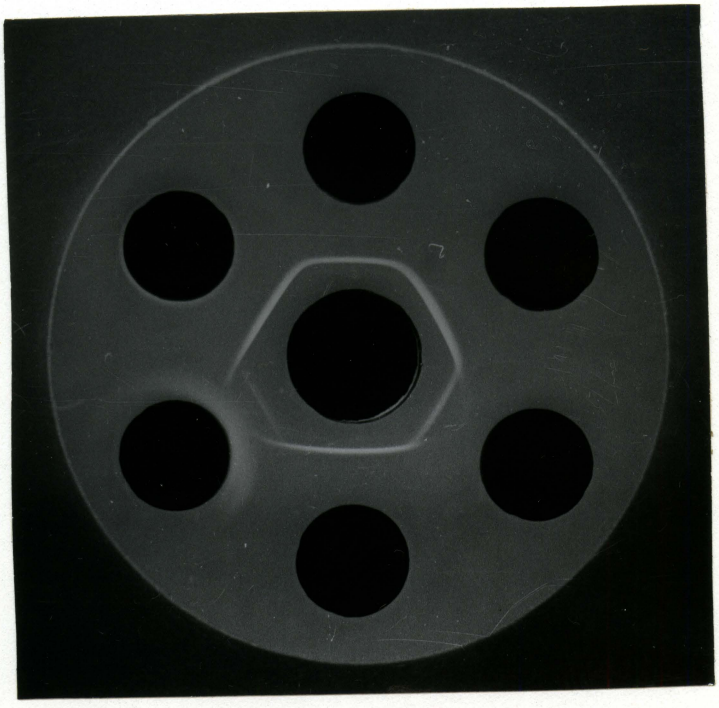


Fig. 7. Fractionation of cyanogen bromide (CNBr) treated bovine IgG on a Sephadex G100 column (2.5 x 90 cm). The column was equilibrated with 1M acetic acid and CNBr treated bovine IgG was dialyzed against the same solvent before fractionation on the column. The fractions in the shaded area were pooled and represent the $F(ab")_2$ fragments.

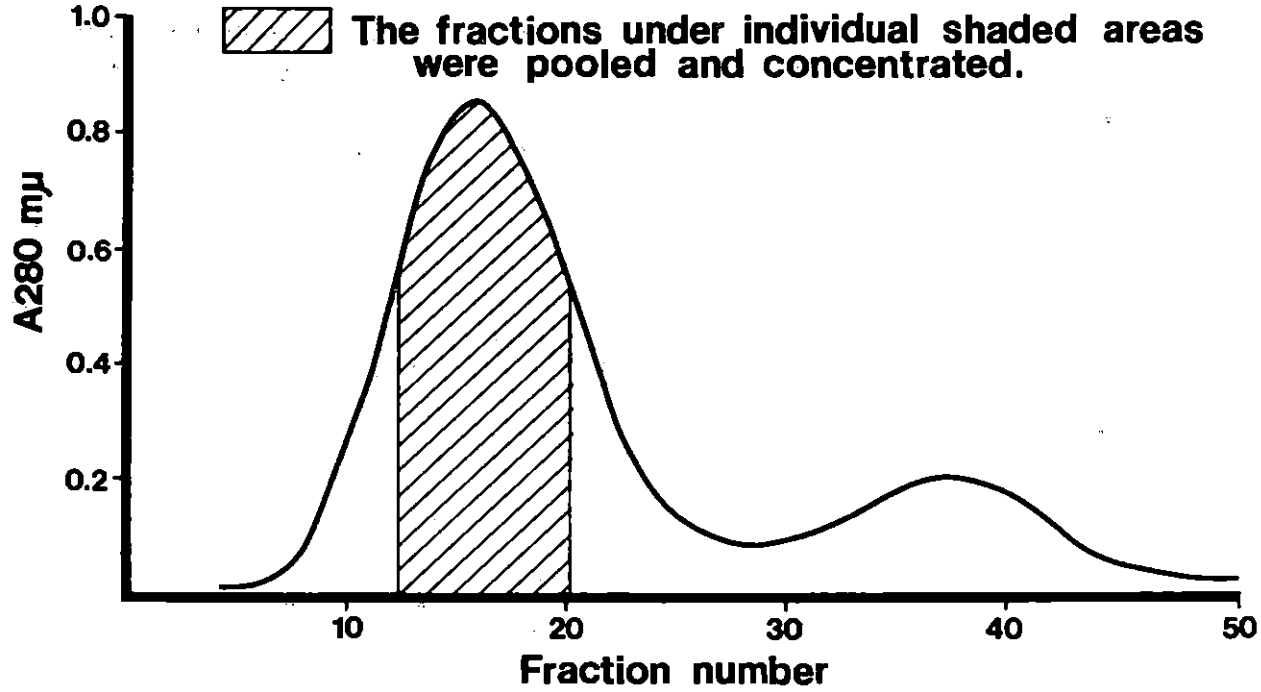
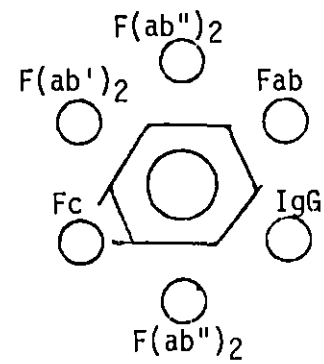

The fractions under individual shaded areas were pooled and concentrated.
A280 mμ
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
Fraction number

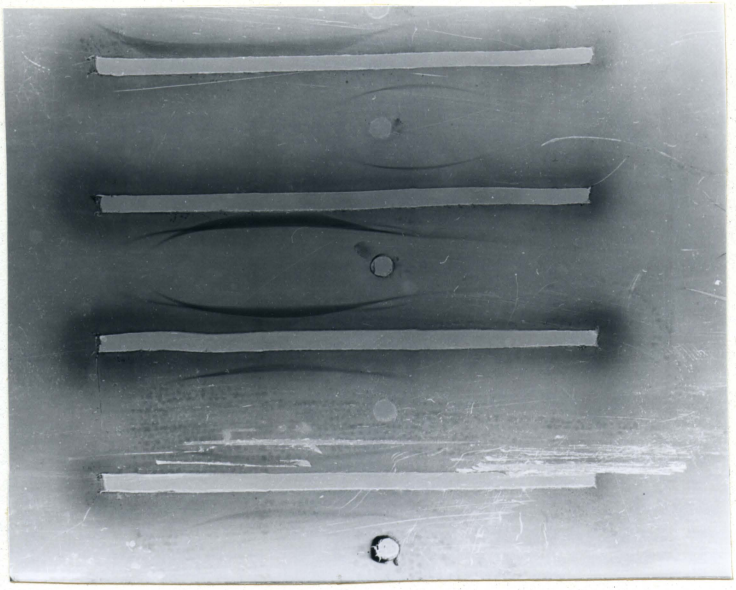
Fig. 8a. Immunoelectrophoretic patterns of fragments resulting from CNBr treatment of bovine IgG. Comparison with papain and pepsin digested fragments. Photograph showing the immunoelectrophoretic patterns of various bovine IgG fragments when reacted with anti-bovine IgG. Antigens were placed in the wells and were electrophoresed following which rabbit anti-bovine IgG was placed in the troughs. IgG fragments placed in the wells from top to bottom respectively:

Fab fragments
Fc fragments
Purified bovine IgG preparation
$F(ab')_2$ fragments
$F(ab'')_2$ fragments

The cathode is toward the left.

Fig. 8b. Ouchterlony analysis of antigenic relationship between papain and pepsin digested fragments and CNBr-treated bovine IgG fragments. Photograph showing the gel diffusion reaction of various bovine IgG fragments with rabbit anti-bovine IgG. Rabbit anti-bovine IgG was placed in the center well. Bovine IgG fragments were placed in the peripheral wells. $F(ab'')_2$ showed reaction of identity with $F(ab')_2$ and intact IgG but showed reactions of partial identity with Fab and Fc fragments.





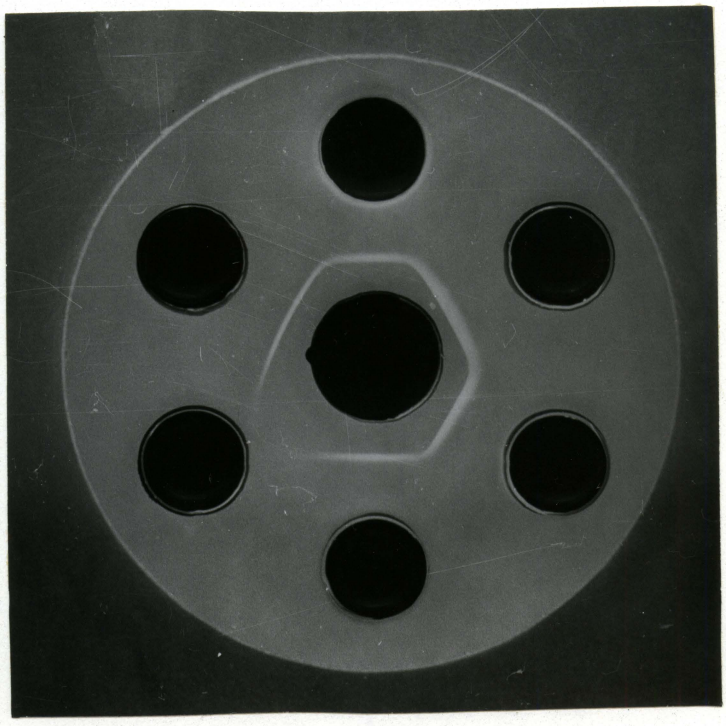
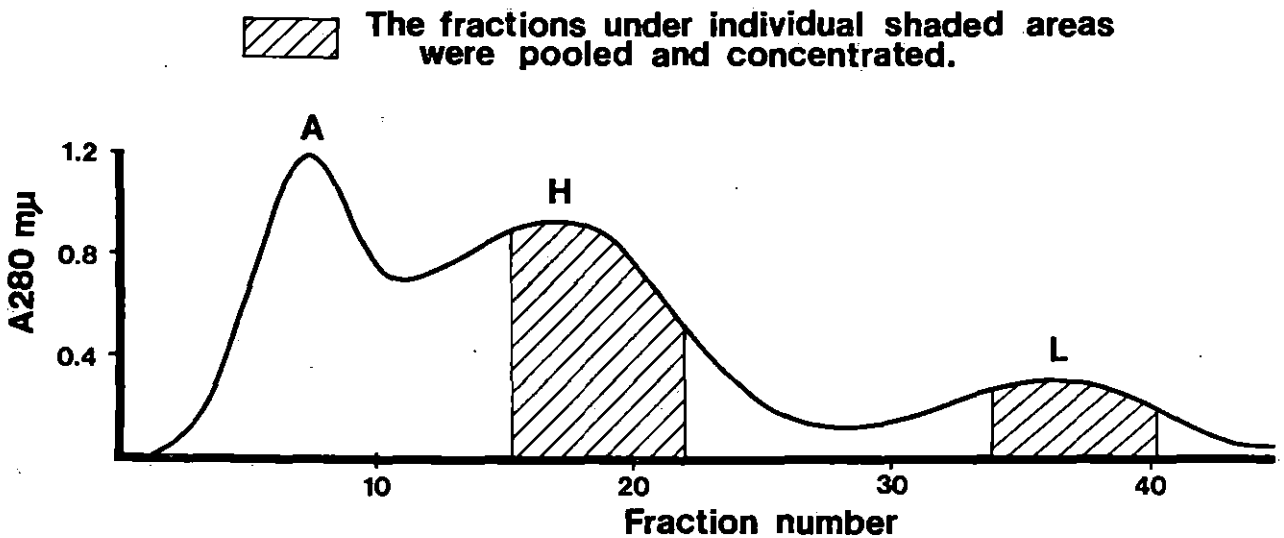


Fig. 9. Separation of Heavy (H) and Light (L) chains from 30 mg of reduced alkylated bovine IgG by gel filtration on Sephadex G100 in 1M propionic acid.

A. 1st protein fraction, containing aggregates of H chains.
B. Heavy chains.
C. Light chains.

Fractions from shaded areas were pooled, concentrated by vacuum dialysis and dialyzed against cold normal saline solution.


The fractions under individual shaded areas were pooled and concentrated.
A
H
L
1.2
0.8
0.4
A280 mμ
10
20
30
40
Fraction number

homogeneity. Spurring between precipitation lines with H and L chains indicated a lack of identity (Fig. 4b, 10, 13a). H chains reacted only with specific rabbit anti-bovine Fc antiserum (Fig. 13b) while L chains reacted only with rabbit anti-bovine Fab antiserum (Fig. 14b). H and L chains gave a reaction of identity with bovine Fc and Fab respectively but both showed partial identity with the intact IgG molecule when reacted with rabbit anti-bovine IgG (Fig. 10).

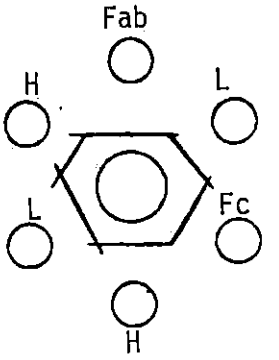
Molecular Weight Determination

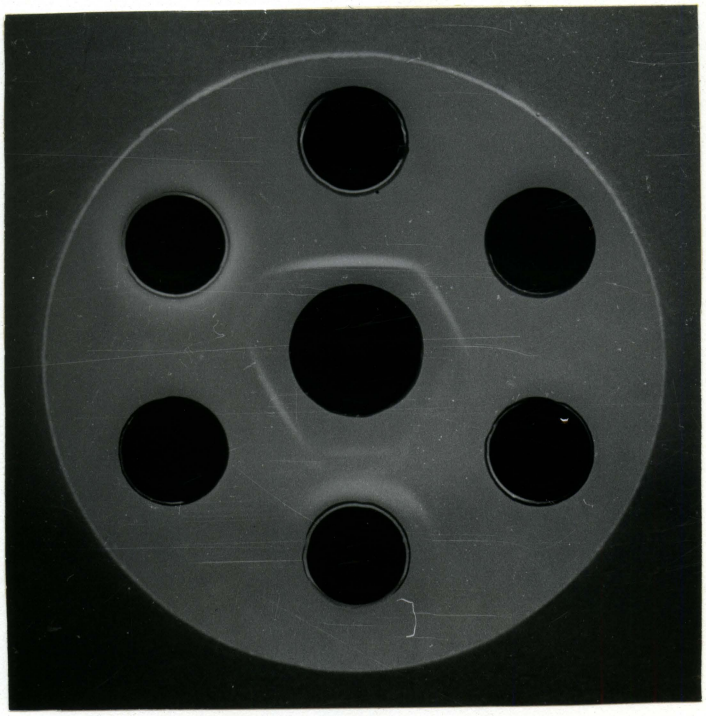
Molecular weights of bovine IgG H and L chain preparations were determined by the method of Weber and Osborn (176). The results are indicated in Table 5 and Fig. 12. Gel electrophoresis of H chains in SDS buffer, pH 7.2 revealed a band corresponding to a molecular weight of 55,000 and minor bands corresponding to molecular weights of 86,000 and 111,000 respectively. Three bands were obtained in gel electrophoresis of L chain preparations. The heaviest band was found to have a molecular weight of 27,000. Two minor bands had molecular weights of 48,000 and 65,000 respectively (Fig. 12). Several bands were also observed in the marker protein gels except for pepsin which gave a single band. Only the band corresponding to the known molecular weight of individual marker protein was used for plotting a molecular weight standard curve (Fig. 11).

Ultracentrifugation Studies

Bovine IgG fragments were ultracentrifuged at 59,780 and 56,000 rpm in a Spinco analytical ultracentrifuge. When the peaks started to form,

Fig. 10. Ouchterlony analysis of antigenic relationship between H and L chains with papain digested fragments. Photograph showing the gel diffusion reaction of rabbit anti-bovine IgG with Fab, Fc H and L chains. Bovine IgG fragments were placed in the peripheral wells and reacted against rabbit anti-bovine IgG serum in the center well. Fab showed reaction of partial identity with H and L chains while Fc fragment showed reaction of identity with H chains. H and L chains showed reaction of nonidentity with each other.





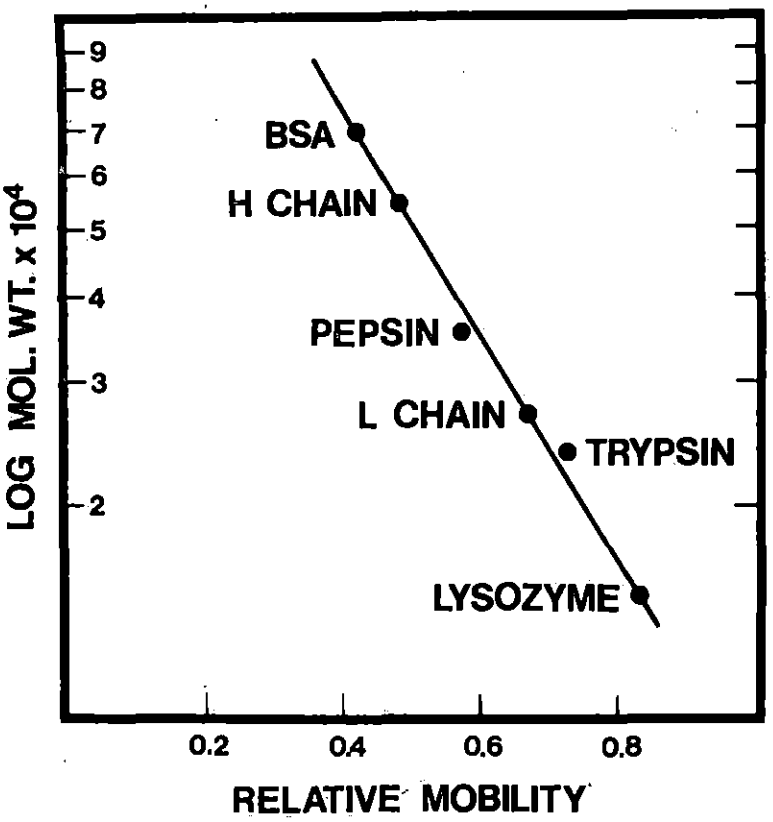


Fig. 11. Determination of the molecular weight of the bovine IgG H and L polypeptide chains from a set of 4 individual protein standards. The 4 protein standards used were lysozyme (MW = 14,000), trypsin (MW = 24,000), pepsin (MW = 35,000), and BSA (MW = 68,000). All protein standards and the H and L chain preparations were run on SDS polyacrylamide gel in SDS phosphate buffer pH 7.2 for 6 hours as described in materials and method. The extrapolated value of the molecular weight of the H and L chain is 55,000 and 27,000 respectively.

Fig. 12. Disc gel electrophoresis of bovine IgG H and L chain preparation and 4 protein standards. All protein samples were electrophoresed at room temperature in 0.1% SDS-sodium phosphate buffer pH 7.2 for 6 hours. All gels were stained and destained by the method of Fairbanks et al. (46). The heaviest protein band in H and L chain gels were used for molecular weight determination.

1. Bovine serum albumin
2. Pepsin
3. Trypsin
4. Lysozyme
5. H chain
6. L chain

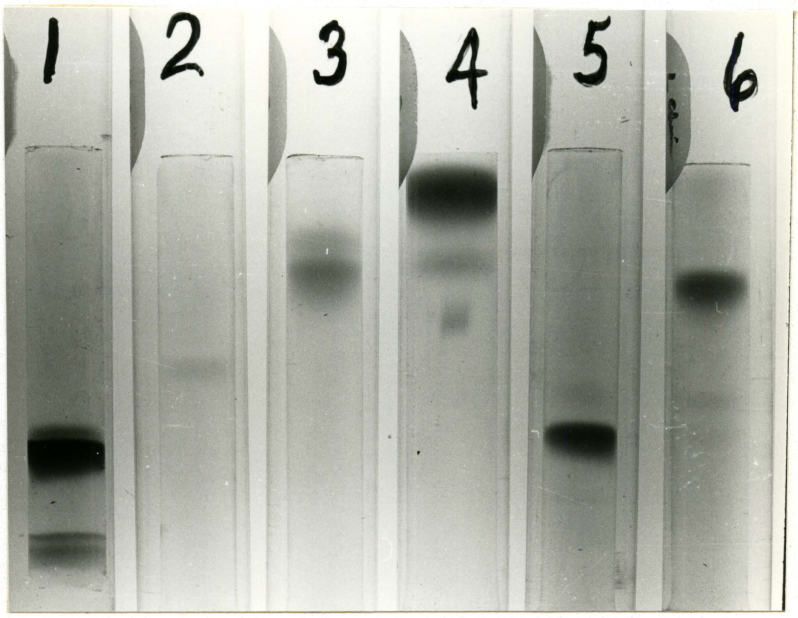
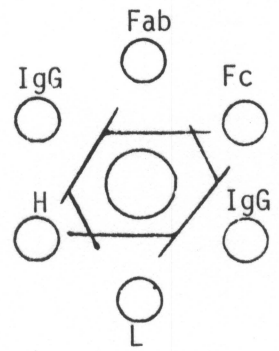
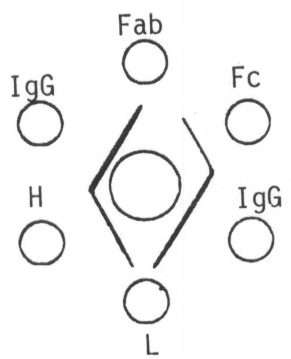

1
2
3
4
5
6

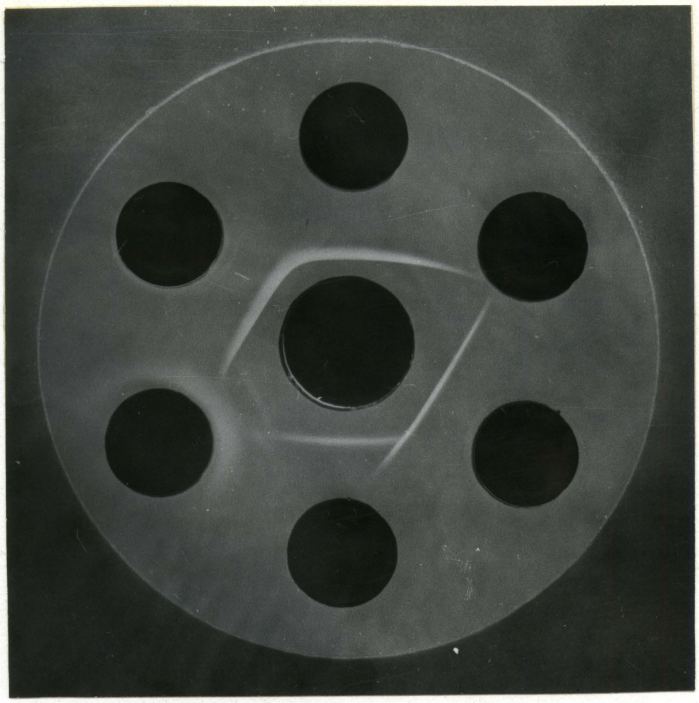
Fig. 13. Production of rabbit anti-bovine Fc antiserum by absorption of rabbit anti-bovine IgG antiserum with bovine Fab fragments. Photograph showing reactions of rabbit anti-bovine IgG with bovine IgG and its subunits prior and after the absorption with Fab or Fc preparation. Rabbit anti-bovine IgG/Fc was placed in the center wells. Fab, Fc, H, L and IgG preparations were placed in the peripheral wells as shown in the diagrams.

a. Reaction of rabbit anti-bovine IgG with IgG, Fab, Fc, H and L chains prior to absorption with Fab fragments.



b. Reaction of rabbit anti-bovine IgG serum with IgG, Fab, Fc, H and L chains after absorption with Fab fragments.





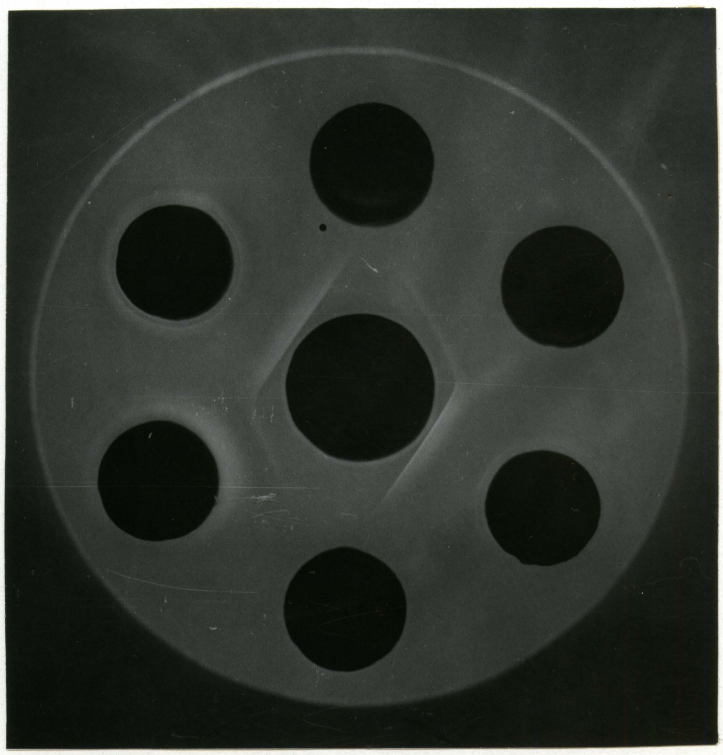
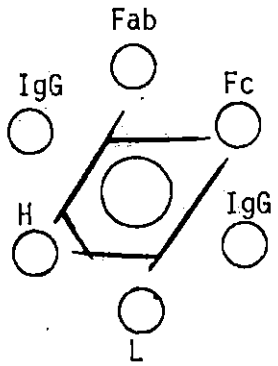
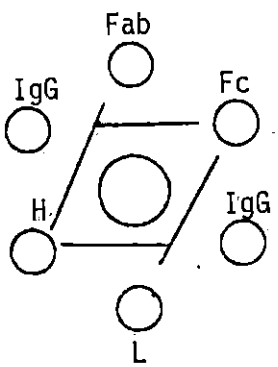


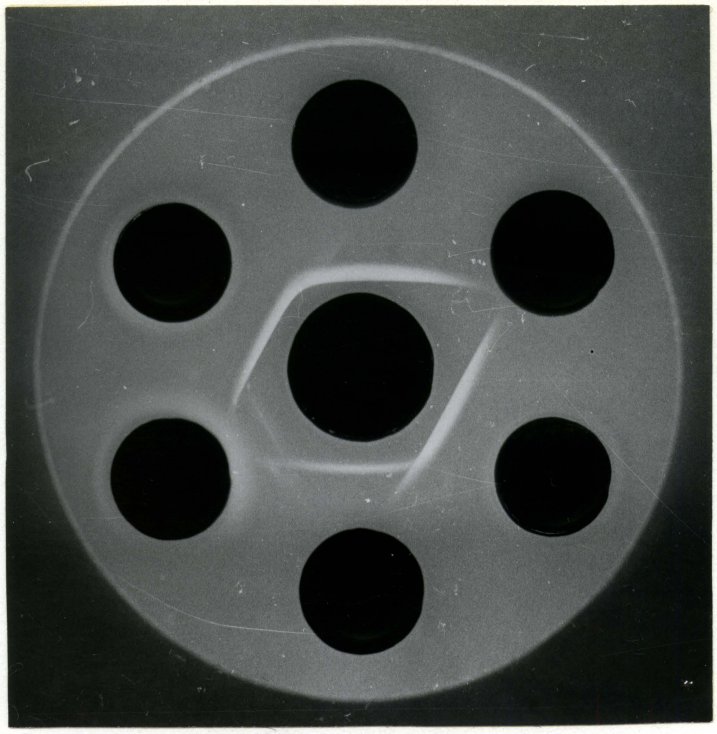
Fig. 14. Production of rabbit anti-bovine Fab antiserum by absorption of the rabbit anti-bovine IgG antiserum with bovine Fc and H chain preparations. Photograph showing reaction of rabbit anti-bovine IgG serum with bovine IgG and its subunits after absorption with Fc fragments.

a. Reactions of anti-bovine IgG with IgG, Fab, Fc, H and L chains after absorption with Fc fragments.



b. Reaction of rabbit anti-bovine IgG with IgG, Fab, Fc, H and L chains after absorption with Fc and H chains. Specific antiserum was developed to Fab and L chains.





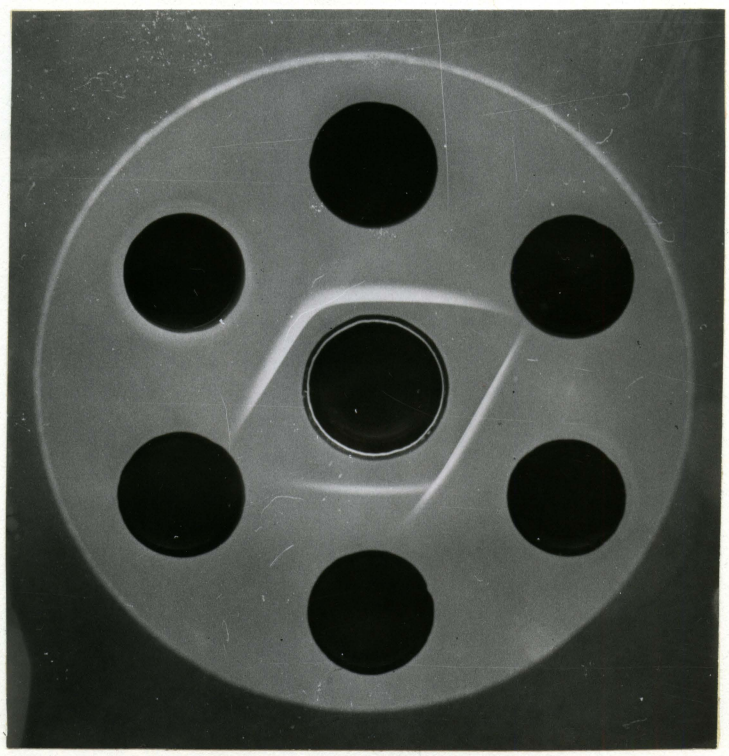


Table 5. Results showing the migration patterns of individual marker proteins and the H and L chains. Relative mobilities of the marker proteins were calculated. From this, the molecular weights of bovine IgG H and L chains were determined

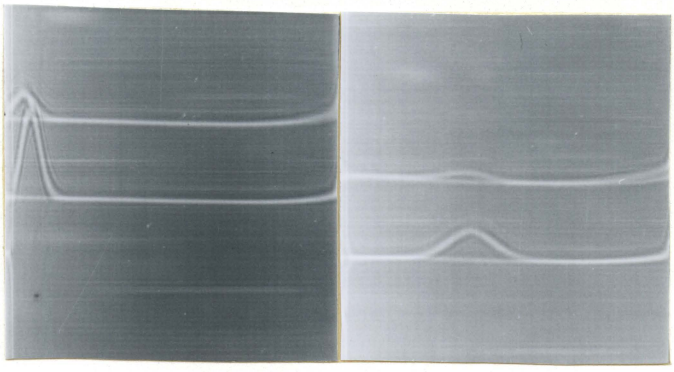
Proteins	Total distance of dye migration (cm)	Total distance of protein migration (cm)	Relative mobility	Molecular weight
BSA	6.70	2.90	0.433	68,000
Pepsin	6.70	3.85	0.575	35,000
Trypsin	6.30	4.60	0.730	24,000
Lysozyme	6.60	5.40	0.818	14,600
H chains	6.45	3.10	0.481	55,000[a]
L chains	6.70	4.45	0.664	27,000[a]

[a]Calculated values in this research.

photographs were taken at 4 or 8 minute intervals (Fig. 15). Sedimentation coefficient ($S_{20,w}$) of each fragment are shown in Table 6. The step wise calculation performed to obtain the sedimentation coefficient of the Fab fragment is shown in the Appendix.

Production of Rabbit Anti-Bovine Fc and Fab Antiserum

Complete absorption of rabbit anti-bovine IgG antiserum with either bovine Fab or Fc fragments yielded specific anti-Fc and anti-Fab antiserums, respectively. The anti-Fc reacted strongly with Fc fragment, heavy chains and the intact IgG but not with Fab fragments or L chains (Fig. 13b). The anti-Fab reacted strongly with Fab fragments, Light



Upper peak - Fc fragment
Lower peak - Fab fragment

Fig. 15. Schlieren pattern of purified Fab and Fc fragments of bovine IgG in the analytical ultracentrifuge. Conditions for centrifugation are described in Materials and Methods. The pattern is photographed at 32 and 112 mins.

Table 6. Experimental values of sedimentation coefficient and molecular weights of bovine IgG and its subunits

Ig fragments	$S_{20,w}$	Mol. Wt.
IgG	6.52S	Not done
Fab	3.78S	Not done
Fc	3.98S	Not done
$F(ab')_2$	5.48S	Not done
$F(ab'')_2$	5.3S	Not done
H	9.42S	55,000
L	4.45S	27,000
H^a	1.78S	55,000
L^a	1.67S	27,000

[a]H and L chains were ultracentrifuged in propionic acid.

chains, intact IgG and surprisingly also with heavy chains but not with Fc fragments (Fig. 14a). Further absorption of anti-Fab with H chains eliminated the reaction with H chains but retained those for Fab and L chains (Fig. 14b).

Detection of Allotypic Determinants by Method of Immunodiffusion Precipitation

IgG from four cattle serums (L27, L8, L11 and A5) was fragmented by the previously described procedures to yield immunoglobulin subunits of

Fab, Fc, $F(ab')_2$, $F(ab")_2$, H and L chains. These immunoglobulin fragments were tested for reactivity with several allotypic antisera.

Reaction of L27 IgG subunits with allotypic antisera

As shown in Table 7, the five allotypic antisera reacted positively with L27 IgG. Among these five allotypic antisera, four of which, L32, B29, L12 and 070, gave positive reaction with all immunoglobulin units except the Fab fragment and the L chains. B905 antiserum gave a slightly different reaction pattern in that it did not react with the $F(ab')_2$ fragments. The negative reaction of the Fab and L chain with all allotypic antisera indicated the absence of allotypic determinants on the L chains. Positive reactions indicated that the determinants resided in the Fc region or in regions somewhere towards the carboxy-terminal end of the H chains.

Reaction of L11 IgG subunits with allotypic antisera

Eleven allotypic antisera were reacted with L11 IgG and its subunits. All allotypic antisera except L25, B10 and 059 reacted weakly with individual immunoglobulin fragments. Precipitation bands appeared only after 24 to 36 hours, and the precipitation lines formed were diffuse and vague. Four allotypic antisera (L25, B10, 059 and B19) did not react with the Fc fragments, the Fab fragments nor the L chains (Table 8a).

Inhibition precipitation tests were conducted with five stronger allotypic antisera (L14, L24, B55, B29, and B19) and each was absorbed exhaustively with either Fab, Fc, H or L chains respectively. Data in Table 8b showed that upon absorption of the five allotypic antisera with H or Fc fragments, specificities to the Fc and intact IgG molecules were

Table 7. Ouchterlony tests[a]

L27 IgG fragments	Allotypic antiserum				
	L32	B29	L12	070	B905
Fab	-	-	-	-	-
Fc	+	+	+	+	+
$F(ab')_2$	+	+	+	+	-
$F(ab")_2$	+	+	+	+	+
H	+	+	+	+	+
L	-	-	-	-	-
IgG	+	+	+	+	+

[a]Immunodiffusion precipitation analysis of bovine L27 IgG and its subunits with bovine allotypic antisera. Five bovine allotypic antisera were used to react with bovine L27 IgG subunits. Ouchterlony plates were set up in such a way that allotypic antiserum was placed in the center well and was allowed to diffuse for two hours before IgG subunits were placed in the peripheral wells. Diffusion was allowed to proceed at room temperature in a moist chamber. Plates were examined at 24 and 48 hours. The presence of allotypic determinants on a particular fragment was indicated by the appearance of positive precipitation band.

eliminated. Absorption with Fab fragments or L chains still retained specificities to the Fc and intact IgG.

Reaction of L8 and A5 IgG subunits with allotypic antisera

Identical reaction patterns were obtained when L8 IgG subunits and A5 IgG subunits were reacted with eight positive allotypic antiserum. Though some fragments gave weaker reactions and somewhat diffuse precipitation bands with some allotypic antisera, the overall reaction pattern as shown in Tables 9a and 10a showed that all allotypic antiserum reacted positively with all L8 and A5 IgG subunits except those of Fab and L chains. Both L8 and A5 $F(ab")_2$ fragments gave a very weak and diffused precipitation band with antiserum B905, and the precipitation bands appeared only at 36 and 48 hours. Weak reactions between A5 $F(ab")_2$ fragments and allotypic antiserums L12 and L32 were also observed. Five allotypic antisera (L14, L24, B55, L12, and B29) were used for the testing of the capacity of the H, L, Fab, and Fc fragments to inhibit the reaction of allotypic antiserum with intact IgG molecules. Identical results were obtained in the tests for L8 and A5 IgG subunits. Experimental results in Tables 9b and 10b showed that upon absorption of the five allotypic antisera with H chains or Fc fragments, specificities to the Fc and intact IgG molecules were totally removed. Upon absorption with Fab fragments or L chains, specificities to the Fc and intact IgG molecules were retained. The results clearly showed the allotypic determinants were located only on the heavy chains, particularly on the Fc portion of the IgG molecules.

Table 8a. Ouchterlony tests[a]

Ll1 bovine IgG fragments	Allotypic antiserums										
	L14	L24	B55	L12	B29	L32	B905	B19	B10	L25	059
Fab	-	-	-	-	-	-	-	-	-	-	-
Fc	+	+	+	+	+	+	+	-	-	-	-
$F(ab')_2$	+	+	+	+	+	+	+	+	+	+	+
$F(ab")_2$	+	+	+	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+	+	+	+
L	-	-	-	-	-	-	-	-	-	-	-
IgG	+	+	+	+	+	+	+	+	+	+	+

[a]Immunodiffusion analysis of bovine Ll1 IgG subunits with bovine allotypic antisera. Allotypic antiserum was placed in the center well. Diffusion of antiserum was allowed to proceed for 2 hours before antigens were placed around the peripheral wells. Positive precipitation band indicated the presence of allotypic determinant on that fragment.

Table 8b. Inhibition precipitation tests[a]

	Fragments	Absorption with L	H	Fab	Fc
L14	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
L24	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B55	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B29	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B19	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-

[a]Inhibition precipitation tests with IgG fragments. The capacity of various IgG fragments to inhibit the reaction of allotypic antiserum with intact IgG was tested. IgG fragments were first placed in the center well. Time was allowed for fragments to diffuse out completely. The center well was again filled with allotypic antiserum and was allowed to diffuse for another 2 hours. IgG fragments were placed in the peripheral wells. Fragments were considered to possess allotypic determinants if they blocked the allotypic antiserum from reacting with the intact IgG in the adjacent wells.

Table 9a. Ouchterlony tests[a]

L8 IgG fragments	Allotypic antiserums							
	L14	L24	B55	B29	L12	L32	B905	070
Fab	-	-	-	-	-	-	-	-
Fc	+	+	+	+	+	+	+	+
$F(ab')_2$	+	+	+	+	+	+	+	+
$F(ab'')_2$	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+
L	-	-	-	-	-	-	-	-
IgG	+	+	+	+	+	+	+	+

[a]Immunodiffusion analysis of bovine L8 IgG subunits with bovine allotypic antisera. Allotypic antiserum was placed in the center well. Diffusion of antiserum was allowed to proceed for 2 hours before antigens were placed around the peripheral wells. Positive precipitation band indicated the presence of allotypic determinant on that fragment.

Table 9b. Inhibition precipitation tests[a]

	Fragments	Absorption with L	H	Fab	Fc
L14	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
L24	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B55	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
L12	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B29	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-

[a]Inhibition precipitation tests with IgG fragments. The capacity of various IgG fragments to inhibit the reaction of allotypic antiserum with intact IgG was tested. IgG fragments were first placed in the center well. Time was allowed for fragments to diffuse out completely. The center well was again filled with allotypic antiserum and was allowed to diffuse for another 2 hours. IgG fragments were placed in the peripheral wells. Fragments were considered to possess allotypic determinants if they blocked the allotypic antiserum from reacting with the intact IgG in the adjacent wells.

Table 10a. Ouchterlony tests[a]

A5 IgG fragments	Allotypic antiserums							
	L14	L24	B55	B29	L12	L32	B905	070
Fab	-	-	-	-	-	-	-	-
Fc	+	+	+	+	+	+	+	+
$F(ab')_2$	+	+	+	+	+	+	+	+
$F(ab'')_2$	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+
L	-	-	-	-	-	-	-	-
IgG	+	+	+	+	+	+	+	+

[a]Immunodiffusion analysis of bovine A5 IgG subunits with bovine allotypic antisera. Allotypic antiserum was placed in the center well. Diffusion of antiserum was allowed to proceed for 2 hours before antigens were placed around the peripheral wells. Positive precipitation band indicated the presence of allotypic determinant on that fragment.

Table 10b. Inhibition precipitation tests[a]

			Absorption with		
	Fragments	L	H	Fab	Fc
L14	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
L24	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B55	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
L12	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-
B29	Fab	-	-	-	-
	Fc	+	-	+	-
	IgG	+	-	+	-

[a]Inhibition precipitation tests with IgG fragments. The capacity of various IgG fragments to inhibit the reaction of allotypic antiserum with intact IgG was tested. IgG fragments were first placed in the center well. Time was allowed for fragments to diffuse out completely. The center well was again filled with allotypic antiserum and was allowed to diffuse for another 2 hours. IgG fragments were placed in the peripheral wells. Fragments were considered to possess allotypic determinants if they blocked the allotypic antiserum from reacting with the intact IgG in the adjacent wells.

Detection of Allotypic Determinants on Bovine H and L Chains of Bovine IgG by Radioimmunoassay

No precipitation was observed when I^{125} labeled H or L chains were incubated with allotypic antiserum in a 37°C water bath for one hour with occasional shaking. No precipitation formed either when rabbit anti-bovine Fab or rabbit anti-bovine Fc was added to I^{125}-H chain and I^{125}-L chain reacting mixture respectively. After standing overnight in the refrigerator, precipitates were observed when reacting mixtures were centrifuged. Results in Table 11 showed that I^{125}-H chains were bound to allotypic antiserum and the complex was precipitated with rabbit anti-bovine Fab. Since rabbit anti-bovine Fab serum did not react with H chains in the gel diffusion tests, precipitation must be due to specific reaction between I^{125}-H chain bound allotypic antiserum and rabbit anti-bovine Fab only. The radioactivity precipitated by the normal bovine serum control suggested a nonspecific binding of I^{125}-H chains with the normal bovine serum. Approximately twice the amount of radioactivity to that of the controls was precipitated by specific allotypic antiserum indicating that H chains carried allotypic determinants.

I^{125}-labeled L chains were also reacted with allotypic antiserums. No precipitation was observed following incubation with allotypic antiserums. After rabbit anti-bovine Fc was added and allowed to stand overnight in the refrigerator, little or no precipitate was formed. Results in Table 12 showed a low level of radioactivity detected when the precipitates were counted in a gamma counter. Results suggested that I^{125}-labeled L chains did

not bind or react with allotypic antiserum. The percent of radioactivity precipitated by the control serum showed little difference from that of the allotypic antiserum. This indicated that the L chains did not carry allotypic determinants.

Table 11. Radioimmunoassay to detect binding of I^{125}-H chains of bovine allotypic IgG to anti-allotypic antisera

Anti-allotypic antisera (1.0 ml)	Amount of I^{125}-H chains added (µl)	Amount of rabbit anti-bovine Fab added (ml)	Total cpm	Average cpm of precipitates	Percent (%) of radioactivity precipitated
L14	20	0.2	29,892	873	2.9
L24	20	0.2	28,206	500	2.1
L25	20	0.2	27,311	495	1.8
L12	20	0.2	26,052	729	2.8
L32	20	0.2	27,356	775	2.8
B905	20	0.2	27,300	844	3.1
B55	20	0.2	27,790	1270	4.6
Normal bovine serum (control)	20	0.2	27,226	370	1.3

Background counts = 40 cpm

Table 12. Radioimmunoassay to detect binding of I^{125}-L chains of bovine allotypic IgG to anti-allotypic antisera

Anti-allotypic antisera (1.0 ml)	Amount of I^{125}- L chains added (µl)	Amount of rabbit anti-bovine Fc added (ml)	Total cpm	Average cpm of precipitates	Percent (%) of radioactivity precipitated
L14	20	0.2	10,604	221	2.1
L24	20	0.2	11,003	196	1.8
L25	20	0.2	9,815	143	1.5
L12	20	0.2	10,971	248	2.3
L32	20	0.2	11,029	201	1.8
B905	20	0.2	10,222	139	1.4
B55	20	0.2	10,238	187	1.8
Normal bovine serum (control)	20	0.2	10,713	214	2.0

Background counts = 40 cpm

DISCUSSION

Unlike human immunoglobulins and immunoglobulins of other animal species such as the rabbit and mouse, bovine immunoglobulins have not been studied extensively. This is due in part to a lesser interest of immunologists in this species, the nonavailability of homogeneous immunoglobulins and to the fact that reagents required for detection and identification of bovine immunoglobulins are not available from commercial sources.

In man, production of large quantities of homogeneous immunoglobulins of a given class, subclass and light chain type results from neoplastic diseases. The occurrence of these paraproteins in association with neoplastic diseases facilitates the isolation, identification and chemical and biological studies of human immunoglobulins. In particular, the myeloma globulins and Bence-Jones proteins have yielded a vast quantity of information. In some animal species such as the mouse, neoplastic disease can be artificially induced resulting in the production of large quantities of immunoglobulins of a particular class or subclass (139). Discovery of neoplastic diseases in the bovine species leading to the isolation of large quantities of homogeneous immunoglobulins of a particular class or subclass may be helpful in providing new insights into the exact physico-chemical properties of the bovine immunoglobulins. In 1971, immunoglobulin light (L) chains (Bence-Jones proteins) in the urine of a cow which had unusual cutaneous and subcutaneous nodules distributed on the lateral aspects of the thorax and abdomen from the scapulae to the tuber coxae was reported by Rodkey and Kimmel (151). The proteins

isolated from this cow were subjected to physical, biochemical and immunological studies. Their test results suggested that the proteins isolated from this cow were similar to the widely studied human and mouse Bence-Jones proteins. Although this report by Rodkey and Kimmel (151) may be very valuable to a better understanding of the structure of bovine immunoglobulins, the validity of the finding has not been confirmed. Until the existence of myeloma-type neoplasms in the bovine species is confirmed or the disease is inducible in the bovine system, the study of allotypy of bovine immunoglobulin at its molecular level will be difficult. Immunoglobulin classes of cattle are known to be heterogeneous (117); this may delay the investigation of amino acid sequences of both H and L polypeptide chains of bovine immunoglobulins which could well serve to elucidate molecular structure and help to determine the exact location and chemical nature of the allotypic markers on immunoglobulin molecules.

Several allotypic specificities have been reported in cattle. The majority, however, have not been fully described. Detailed immunochemical and genetic analyses have been made on two allotypes by Blakeslee et al. (12), namely A1 which is a marker of IgG heavy chains and B1 which occurs on the light chains of all the immunoglobulin classes (12). Each of these allotypes is controlled by a separate locus. A third allotype, A2, is determined by a gene which is allelic to the gene controlling the allotypic specificity A1 (12). Although Rapacz (147) reported an allotypic specificity on IgM molecules, it later proved to be invalid (11).

Dykstra (39) demonstrated three different allotypic determinants (C1, C2 and D1) on γG molecules of cattle. The exact relationship of this

group of allotypic markers and those reported by Blakeslee has not been determined although the genetic information and occurrence would indicate that they are different.

Instead of trying to look for new allotypic markers on bovine IgG molecules, this study attempted to extend Dykstra's research by localizing the existing markers on the IgG molecule since this would lead to a better understanding of the structure of bovine immunoglobulins. The investigation started with the preparation of pure bovine IgG from whole bovine serum. Only IgG preparations without contamination by other serum proteins were used throughout the experimentation. A wide variety of methods have been used by researchers to fractionate serum. Methods such as salt (ammonium and sodium sulfate) precipitation (17, 48), alcohol fractionation (24), gel filtration (49), and ion-exchange chromatography (14) are frequently used. Some of these have advantages over others in terms of purity and efficiency with respect to the various serum proteins. A modified method from Baumstark (7) and Stanworth (161) was employed in this research for the preparation of pure IgG from whole bovine serum. A two-step batch method utilizing DEAE-Sephadex A-50 proved to be efficient in preparing IgG in a highly purified form. Immunoelectrophoretic and Ouchterlony analysis of the product revealed no contamination of the IgG preparation by other serum proteins. The batch method was easy to conduct, reproducible and had the advantage over a column chromatographic preparation in that a large quantity of serum could be fractionated within a short period of time. The recovery or yield of IgG from whole serum was over 85%.

Purified IgG was subjected to enzymatic and chemical treatment to yield fragments of different size (Fig. 1). Papain was used to cleave purified IgG at neutral pH and in the presence of cysteine. When human or rabbit IgG were cleaved by papain under these same conditions, digestion reached completion after two hours (131). However, a considerable amount of undigested IgG was detectable during fractionation of papain digest of the bovine immunoglobulin. Payne (131) reported that bovine IgG contained more carbohydrate (hexose) in the hinge region and the Fc portion; this may be the reason why bovine IgG was digested somewhat more slowly than human or rabbit IgG.

Upon separation of the papain digest on a DEAE-cellulose column, two protein peaks corresponding to the Fab and Fc fragments were obtained. Ouchterlony analysis showed that neither of the two fragment preparations were pure. Since Fab and Fc fragments migrated differently in an electric field, Fab toward the cathode and Fc toward the anode, starch block electrophoresis was employed to purify each of the fragments. Contaminating IgG may not be separated from Fab fragments by starch block electrophoresis as in the separation of Fc from the Fab preparation because both intact IgG and the Fab migrate toward the cathode, although the Fab fragment migrates faster than the intact molecule. In lieu of using Fab preparations directly from DEAE-cellulose fractionation, the Fab preparations from ion-exchange chromatography were first purified by passage through a G200 Sephadex column. Intact IgG having a higher molecular weight was eluted off first. The IgG-free Fab preparation was subsequently purified by starch block electrophoresis. Fey (48) demonstrated

a simpler method to prepare pure Fab from bovine IgG molecules by adding saturated ammonium sulfate to a final concentration of 60% to the crude Fab preparation which had been chromatographed twice on DEAE-cellulose. They demonstrated that, at this concentration, all undigested IgG will be precipitated and the Fab fragment is free of undigested IgG. Upon repeating Fey's procedure in this research, traces of undigested IgG were still detectable in the Fab preparation and possibly some Fab fragment was lost due to co-precipitation with the intact molecules.

Rabbit and human Fc fragment crystallized upon dialysis against neutral buffer (168, 169) and so was bovine Fc. Crystallization usually renders homogeneity to a substance. Nevertheless, bovine Fc crystals obtained upon dialysis against neutral buffer still carried contaminants of Fab and intact IgG. This may be due to incorporation of the two substances into the Fc crystals during the process of crystallization.

Ouchterlony analysis of Fab and Fc fragments purified from starch block electrophoresis revealed a single entity of either fragment. As shown in Fig. 4b there was reaction of nonidentity between Fab and Fc and each fragment showed reactions of partial identity with the intact IgG molecules. This is due to the fact the Fab fragment is composed of intact light chains linked to the N-terminal portion of the H chain through an inter-chain disulfide bond, while Fc is composed of the C-terminal halves of two heavy chains linked together by a disulfide bond (Fig. 1). Upon reaction with anti-Fab and anti-Fc antisera, Fab and Fc fragments reacted only with the respective antiserum; this further indicated that the two fragments were free of contamination with each other. Ultracentrifugation analysis revealed the sedimentation coefficients (S value) of Fab and Fc

fragments were 3.78S and 3.89S respectively. This value compares well with the 3.5S value for rabbit Fab fragments reported by Putnam et al. (146). Sedimentation patterns of the Fab and Fc fragments revealed a single peak; this further indicated the homogeneiety of the two preparations (Fig. 14).

Payne (131) reported that when pepsin was added to bovine IgG preparations, the preparation became cloudy. The cloudiness persisted throughout the course of digestion (18 hours) but disappeared when the enzymatic reaction was stopped by adjusting the pH of the mixture to 7.0. The same phenomenon was observed during pepsin digestion of bovine IgG in this research. Payne did not explain the cause of the cloudiness, but possibly it was due to continued hydrolysis of the Fc portion of the molecule with release of small insoluble peptides (Utsumi and Karush (168, 169).

The elution profile from gel filtration of the pepsin digest revealed four major peaks. Immunoelectrophoresis identified the four peaks as undigested IgG, $F(ab')_2$ + intact IgG, $F(ab')_2$ and presumably some small peptides of unknown properties, respectively. These findings contradicted those of Payne (131) who reported two protein fractions and of Butler (16) who reported five protein peaks were obtained by gel filtration on a G200 Sephadex column (Table 13). The difference in the number of protein peaks obtained in this research with those obtained by Payne and Butler may be due to the enzymatic digestion conducted under different conditions or to different pretreatments of the pepsin digest before fractionation on the Sephadex column (Table 13).

Table 13. Comparison of the protein peaks obtained upon fractionation of pepsin digest on G200 Sephadex column. Data from Payne (131), Butler (16) and this research

	Data from Payne (131)	Data from Butler (16)	Data from this research
Duration of peptic digestion (hours)	18	20	24
Enzyme: protein ratio	3:100	1:100	3:100
Sephadex column for fractionation	G200	G200	G200
Pretreatment of pepsin digest before fractionation	Precipitation with 10% trichloroacetic acid	None	None
Number of protein peaks obtained from fractionation	1. $F(ab')_2$ 2. Mixture of peptides	1. IgG aggregates 2. 7S IgG 3. $F(ab')_2$ 4. Traces of Fab and Fc 5. Mixture of peptides	1. IgG 2. IgG+$F(ab')_2$ 3. $F(ab')_2$ 4. Mixture of peptides

It is surprising to see a considerable amount of IgG unaffected by the enzyme after digestion has proceeded for 24 hours. In this experiment, the enzyme to protein ratio (3:100) was 3 times that of Utsumi and Karush (169) who used a ratio of 1:100. Very likely bovine IgG is somewhat resistant to peptic digestion. Whether the resistance is due to the presence of carbohydrate (hexose) as it is in the case of resistance to papain digestion is unknown (131).

Recovery of $F(ab')_2$ fragments in the third peak from a Sephadex G200 column, its antigenic behavior in Ouchterlony analysis, and the migration patterns of the $F(ab')_2$ fragments in immunoelectrophoresis revealed similarities to the $F(ab')_2$ fragment reported by Butler (16). The finding of a 5.48S sedimentation coefficient for the $F(ab')_2$ fragment also confirmed its resemblance to the $F(ab')_2$ reported by Butler (16). Since $F(ab')_2$ and intact IgG migrate electrophoretically approximately at the same rate toward the cathode (Fig. 8a), this rendered the purification of $F(ab')_2$ by starch block electrophoresis difficult. Recycling of $F(ab')_2$ preparation on G200 Sephadex column was performed in order to eliminate undigested IgG and other contaminants. A single band observed in Ouchterlony analysis of purified $F(ab')_2$ confirmed that the preparation was pure (Fig. 8b).

Free H and L chains of immunoglobulin molecules can be obtained by reduction of inter-chain disulfide bonds by a number of chemicals such as dithiothreitol (dTT) (8), 2-mercaptoethanol (50b, 113) and sulfites (12). H and L chains are normally separated on G75 or G100 Sephadex columns equilibrated with acetic or propionic acids (50b). Tan and Epstein (165) claimed that a better separation of H and L chains can be achieved on a G166 Sephadex (two parts G200 + one part G100) column; however, the method has not been widely employed simply due to the fact that G166 Sephadex is not commercially available.

In this research, bovine IgG was treated with 0.75M 2-mercaptoethanol and H and L chains were separated on a G100 Sephadex column after alkylation with iodoacetamide. To obtain pure products, both chain preparations

were recycled once on the same column. Upon dialysis of H chains against phosphate buffer at neutral pH, aggregation of the chain preparation was observed. The same phenomenon was reported by Fleischman (50b) and Fey (49). No aggregation of H chains was detected except after storage of H chain preparations in normal saline solution at 4°C for several weeks. To avoid aggregation, H and L chain preparations were used within 1-2 weeks.

Contamination of H chains with L chains has frequently been observed (113). Fey et al. (49) demonstrated a simple method to isolate L chains from H chains. The reduced and alkylated IgG mixture was dialyzed against neutral buffer; the H chains aggregate and can be removed by centrifugation. The supernatant fluid contained pure L chains. They did not demonstrate the process to recover the aggregates of H chain; however, one might expect conformational changes may be induced due to aggregation.

In this research, it was important that the H and L chain preparations were free of contamination with each other to avoid inaccurate findings. Ouchterlony analysis demonstrated that H and L chains reacted only with respective rabbit anti-bovine Fc and anti-bovine Fab antiserums and gave a single precipitation band. By Weber and Osborn's method of molecular weight determination (176) H chains and L chains were found to have molecular weights of 55,000 and 27,000 respectively. Although several protein bands were observed when the H and L chains were electrophoresed in polyacrylamide gel, none of the bands in the H chain preparation corresponded to the molecular weight of L chains nor did any minor bands in the L chain gel correspond to the molecular weight of H chain. This further confirmed their purity relative to the other type of chain.

One protein band in the H chain gel had a molecular weight twice that of the H chain and suggested the presence of H chain dimers although aggregation of protein in SDS buffer is very unlikely. Sjöquist and Vaughan (158) observed heterogeneity of the H and L chains as demonstrated by starch gel electrophoresis at pH 7 to 9. Reduced and alkylated H chains from normal human gamma G globulins formed up to eighteen tightly spaced bands. The H chains of individual myelomal gamma G globulins showed similar heterogeneity but to a lesser degree. Banding of H and L chains in disc gel electrophoresis was possibly not a result of contamination or aggregation of chain materials but might be possibly be due to heterogeneity of the H and L chain populations in the preparations.

Since H and L chains were prepared from IgG preparations of bovine whole serum, the presence of proteolytic enzymes in the chain preparations may cause degradation of the H or L chains during storage. This may account for the detection of minor proteins that had smaller molecular weights (14,000 and 30,000) in H and L chain preparations by SDS-gel electrophoresis.

Bovine H and L chains were found to have sedimentation coefficients of 9.42S and 4.45S respectively when chains were suspended in normal saline solution. Contrasting results were obtained when chains were suspended in 1M propionic acid. The H chains were found to have a $s_{20,w}$ value of 1.78S and the L chains a value of 1.67S. The molecular weight for H and L chains obtained from SDS-gel electrophoresis were considerably lower than would be expected for a particle of 9 or 4S. This discrepancy proabably indicates that the values might correspond to aggregates of the

H and L chains. Fleischman et al.(50b) reported 2.4S and 1.8S values for human H and L chains respectively. The coefficients of 9.42S and 4.45S observed in this experiment might indicate values of trimers or tetramers of H and L chains. Björk and Tanford (9a, 9b) reported dimers of rabbit H and L chains at pH 7.0 had sedimentation values of 5.7S and 3.29S respectively, while the values for monomers were 2.9S and 2.23S respectively. Their chain preparations were suspended in acetate buffers while normal saline solution or 1M propionic acid were used as the solvent in this research. In 1M propionic acid, H and L chains tend to dissociate to give free H and L chains. A substantial drop in $S_{20,w}$ values was found as compared to those obtained in normal saline solution. Compared to the findings of Fleischman et al.(50b) and Björk and Tanford (9a, 9b), the values of 1.78S and 1.67S for respective H and L chains are considerably lower (Table 14). The following explanations may help to account for this discrepancy: (1) Since H and L chain preparations were suspended in propionic acid, conformational changes might have occurred that would affect the actual sedimentation value of both the H and L chains. Since the rate of migration of protein molecules through a particular solvent depends on the shape of the protein molecules, a globular-shaped protein may have different rate of migration than those with a different configuration. If the shape of H and L chains was changed from a globular molecule to a rod-shaped one in propionic acid, a lower sedimentation value would be expected. (2) A type of solvent which had a different density or viscosity may also affect the rate of migration of the protein molecule. At a particular temperature the density or viscosity of propionic acid may

Table 14. A comparison of sedimentation coefficient values of H and L chains

Chain preparations	Data from Fleischman (50)	Data from Björk and Tanford (9a, 9b)	Data from this research
H	2.4S	H (dimer) 5.7S H (monomers) 2.9S	9.42S, 1.78S
L	1.8S	L (dimer) 3.29S L (monomers) 2.23S	4.45S, 1.67S
Buffers used in ultracentrifugation	8M urea (pH 7.5)	Sodium acetate buffer (pH 7.0)	Normal saline solution 1M propionic acid
Temperature used during ultra-centrifugation	25°C	20°C	20°C

be different from that of phosphate buffer or acetate buffer; as a result, a different sedimentation value would be obtained. Since the values were calculated at 20°C in water, the influence of the organic acids is unknown.

Although the sedimentation coefficients obtained in this research did not totally agree with values reported in the literature, molecular weight determination by SDS-gel electrophoresis did confirm the polypeptide chains resulting from 2-mercaptoethanol treatment of intact IgG were H and L chains.

Allotypic antisera used in this research were lyophilized and reconstituted in distilled water so that approximately a 5X concentration of the antiserum was obtained. Although they were in concentrated form, some

allotypic antisera reacted more weakly than others. For this reason, in gel diffusion precipitation tests, the allotypic antiserum was placed in the center well of the agar gel slide and was allowed to diffuse for two hours or more before test IgG fragments were placed in the adjacent wells. This allowed the viscous allotypic antiserum to diffuse out and avoid precipitate bands from forming too close to the antiserum well. Variation in the strength of reactivity of the allotypic antiserum may be due to variation in types of adjuvants, routes of inoculation, immunization schedules and the antigenicity of donor γ-globulins used for the production of allotypic antiserum (39). The strength of the reaction may also be due to the concentration of allotypic globulins in the test serums.

Allotypic antiserums L14, L24, B55 and L12 produced strong, well-defined precipitation bands with immunoglobulin fragments indicating the presence of a high titer of precipitating antibodies and the reactive level of the corresponding allotypic globulin.

Upon analyzing the Ouchterlony reaction patterns of L27, L11, L8 and A5 globulin and their fragments (Table 7, 8, 9, 10) allotypic determinants were demonstrated to be present exclusively on the H chains since the H chains reacted distinctively with all allotypic antisera tested. The lack of reactions with Fab and L chains but with $F(ab')_2$, $F(ab")_2$, Fc and H chains indicated the allotypic markers were possibly located in the Fc or the hinge region of the IgG molecules.

To confirm the absence of allotypic markers on Fab and L chains but presence only on Fc and H chains, five stronger allotypic antiserums (L14, L24, L12, B55, B29) were used for inhibition precipitation studies. This

test was used to detect the capacity of Fab, Fc, H and L chains to inhibit the allotypic antiserum from reacting with intact IgG. Fragments were considered to possess allotypic determinants if they blocked the allotypic antiserum from reacting with intact IgG. Absorption of allotypic antiserum with respective IgG fragments was done both in agar-gel plates and in agglutination tubes before reacting with IgG, Fab, Fc, H and L fragments. As shown in Tables 8b, 9b, and 10b, identical reaction patterns were obtained with L11, L8, and A5 serums. Reactivity of allotypic antiserum with intact IgG was retained after absorption with Fab or L chain fragments indicating the absence of allotypic markers on the Fab or L chains. Upon absorption of allotypic antiserum with Fc or H chains, specificity to the intact IgG was no longer retained. This indicated that allotypic markers were located primarily on Fc or at least on the posterior half of the H polypeptide chains of the IgG molecule.

Four allotypic antisera, namely B10, B19, 059 and L25 showed reactivity only with L11 IgG and its subunits. However, the reactions were weak. The precipitation bands that developed only after 24-36 hours were diffuse and vague. Possibly, this was due to the presence of weakly precipitating antibodies, antibodies with low affinity for IgG fragments, or a deficiency of reactive determinant in the preparations. The presence of reactivity of these four allotypic antiserums with $F(ab')_2$, $F(ab")_2$, H chains but not Fc fragments indicated that the four antisera contained antibodies for allotypic determinants residing on the hinge region of the IgG molecules. Similar observations with the rabbit allotype system were reported by Mandy and Todd (105, 106, 107). They demonstrated that, by

hemagglutination studies, the allotypic specificities A11 and A12 were detected on the $F(ab')_2$ fragments but not on the Fab or Fc fragments. This indicated that the determinant must lie in the hinge region of the IgG molecules.

Blakeslee et al. (12) reported L chain specificities in bovine IgG. Surprisingly no specificities belonging to the L chain were detected in this research by gel diffusion tests. Radioimmunoassay, a more sensitive technique, was employed to confirm the presence of allotypic determinants exclusively on the H chain.

^{125}I-labeled H and L chains were incubated with different allotypic antiserums. Radiolabeled chains carrying allotypic determinants were expected to react with the allotypic antiserum forming soluble antigen-antibody complexes since the chains may not be large enough to form a lattice structure and precipitate out of the solution. Rabbit anti-bovine Fab and rabbit anti-bovine Fc were used to precipitate the ^{125}I-H-allotypic antiserum complex and the ^{125}I-L-allotypic antiserum complex respectively. Since anti-Fab did not react with H chains nor the anti-Fc with L chains as determined by gel diffusion tests, any radioactivity precipitated would suggest binding or reaction of ^{125}I labeled chains with the allotypic antiserum.

Nonspecific binding of H and L chains to normal bovine serum (control) was encountered in this thesis research. The binding may be due to hydrophobic interaction or ionic bonding. When allotypic antiserum was mixed with 0.05M Tris-HCl buffer, pH 7.0 + 10% NaCl, the nonspecific binding was lowered but could not be completely eliminated. As shown by the

data in Table 11, a difference was observed in the amount of radioactivity precipitated by an allotypic antiserum and the control serum. This supported the finding of allotypic markers on H chains by Ouchterlony analysis. In the radioimmunoassay with I^{125}-L chains, percent radioactivity precipitated in the control serum showed no difference with that of the test serums. This suggested that L chains did not carry any allotypic determinants.

Dykstra (39) demonstrated the existence of three allotypic markers C1, C2 and D1 on bovine IgG molecules, where C1 and C2 were found located on the IgG2 subclass and D1 on IgG1 subclass. He also reported that allotypic antiserum L14 carried the anti-C1 specificity, L32 carried the anti-C2 specificity, L25 carried the anti-D1 specificity and L24 carried both the anti-C1 and anti-D1 specificities. In addition to these four allotypic antiserums, 8 more allotypic antiserums (L12, B55, B19, B905, B10,B29, 059 and 070) were used in this research for the molecular location of allotypic markers. Genetic studies (including the screening of the reactivity of these allotypic antiserums with a hundred or more normal bovine serums) indicated a relationship between several of the serums. From this analysis, it was found that allotypic antiserums L14, L24, B55 and L12 possessed the same specificity, Dykstra's C1. B29 and L32 possessed a similar specificity, Dykstra's C2. B19, L24, L25 and 059 carried a third specificity, Dykstra's D1. Three other antisera B10, B905 and 070 are individual specificities unrelated to those already designated (Dr. M. L. Kaeberle, Department of Veterinary Microbiology, Iowa State University, personal communication, March, 1977).

Reaction of the allotypic antiserums with individual normal bovine IgG preparations and subunits of L11, L8, L27 and A5 demonstrated reaction patterns of the six different allotypic antiserum specificities (Table 15). The first group comprised 4 antiserums (L14, L24, L12 and B55). When reacted with L11, L8 and A5 IgG and their subunits, a reaction pattern was obtained which revealed the localization of the C1 allotypic determinant in the Fc region of the IgG molecule. The second group of allotypic antiserum comprised 2 antiserums (L32 and B29). When reacted with all four (L27, L11, L8 and A5) normal serum IgG and their subunits, a reaction pattern was obtained which revealed the localization of the C2 allotypic determinant in the Fc region of the IgG molecule. The third group comprised 4 allotypic antiserums (L24, L25, 059 and B19) which reacted only with L11 IgG and its subunits and the reaction pattern revealed the localization of the D1 determinant in the hinge region. The other three groups (specificities unrelated) comprising a single allotypic antiserum revealed unknown, probably new allotypic specificities on the Fc and hinge region of the IgG molecules (Table 15). It would be interesting to further identify and characterize these unknown specificities.

Although gel diffusion precipitation tests and the radioimmunoassay method failed to detect L chain determinants on all four (L11, L8, L27 and A5) bovine IgG preparations in this study, this does not preclude the occurrence of L chain specificities on bovine IgG molecules. Obviously, anti-L chain allotypic specificities were absent in all allotypic antiserums used in this research. Other possibilities may also account for the negative finding: (1) The L chain allotypic marker may consist of

Table 15. A summary of the anti-allotypic specificities in different allotypic antiserums and the localization of allotypic specificities on bovine IgG molecules

Allotypic antiserums	Reactions with normal bovine IgG preparations	Allotypic specificities	Location
L14, L24, L12, B55	L11, L8, A5	C1	Fc region (posterior half of the H chain)
L32, B29	L11, L8, L27, A5	C2	Fc region (posterior half of the H chain)
L24, L25, 059, B19	L11	D1	Hinge region
B905	L8, L27, A5	Unknown	Fc region
070	L8, L27, A5	Unknown	Fc region
B10	L11	Unknown	Hinge region

several antigenic determinants. Thus, only the donor light chain allotypes possessing all these determinants would show detectable reaction with allotypic antiserum while others possessing some of them do not react at all. Actually, this hypothesis was first proposed by Blakeslee et al. (12) when he observed that not all L chain specificities reacted strongly or distinctively with the anti-L chain allotypic antiserum. (2) In the radioimmunoassay studies, L chains were labeled with I^{125}. Possibly, only a small fraction of the L chains carrying the L chain allotypic determinants have taken up the I^{125} labeling and, as a result, only a small percentage of radioactivities were precipitated by the allotypic anti-

serum. However, the radioactivities measured did not show marked differences due to nonspecific binding in the control serum.

All allotypic determinants described by Dykstra (39) and the A1 and A2 markers described by Blakeslee et al. (12) were found exclusively on the Fc (C-terminal half of the H chain) region of the IgG molecules. Pure Fc fragments which carry a singe allotype can be identified and subsequently prepared by repeating recrystallization. Amino acid sequencing of this Fc fragment may help to elucidate the exact location and the amino acid composition of the allotypic determinant.

The exact number of allotypic determinants on bovine IgG molecules is still unknown. There may be ten or more allotypic specificities associated with this particular Ig class as observed in the rabbit allotypic system. The finding of new allotypic markers would depend on the availability of well-characterized allotypic antiserum. Future experimentation on bovine allotypy would be the detection, identification and defining more determinants not only on IgG class but also on IgA and IgM classes. Until then, the elucidation of the immunochemistry and genetics of the entire bovine allotypic system cannot be achieved.

SUMMARY

Bovine IgG preparations were subjected to enzymatic (papain and pepsin) and chemical (2-mercaptoethanol and cyanogen bromide) treatments to yield fragments of Fab, Fc, $F(ab')_2$, $F(ab")_2$, H and L chains. Each fragment was purified by various methods and partial characterization of the fragments was performed. Sedimentation coefficients of Fab, Fc, $F(ab')_2$ and $F(ab")_2$ fragments were found to be 3.78S, 3.98S, 5.48S and 5.3S respectively. Aggregation of H and L chains in neutral buffers created problems in determination of their exact sedimentation values. Values of 9.42S and 4.45S for H and L chains respectively were found when chain preparations were suspended in normal saline solution. Values of 1.78S and 1.67S were found respectively when chain preparations were dispersed in 1M propionic acid. By Weber and Osborn's method of molecular determination in SDS polyacrylamide gel (176) H and L chains were found to have molecular weights of 55,000 and 27,000 respectively which agreed well with the values reported in the literature.

Four allotypic antiserum, L14, L24, L12 and B55 revealed the localization of Dykstra's C1 determinant on the Fc region of IgG molecules. Two allotypic antiserum, B29 and L32 revealed the localization of Dykstra's C2 allotypic determinant on the Fc region of IgG molecules. Four allotypic antiserums, B19, L25, L24 and 059, revealed the localization of Dykstra's D1 allotypic determinant on the hinge region of the IgG molecules. Unknown allotypic determinants were revealed by three allotypic antiserums, namely B905, B10 and 070 in the Fc and hinge region.

This study failed to detect L chain allotypic specificities on the IgG molecule. Obviously the anti-L chain allotypic specificities were absent in all allotypic antiserums used in this research.

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APPENDIX:

STEP WISE CALCULATION OF THE SEDIMENTATION COEFFICIENT ($S_{20,w}$) OF THE BOVINE Fab FRAGMENT

Step 1. Measurements from microcomparator

Picture No.	Reference line (A)	Distance of peak from reference line (B)
1.	4.914	35.753
2.	4.940	34.310
3.	6.225	34.391
4.	6.084	32.939
5.	7.491	33.082
6.	7.688	31.838
7.	9.273	32.101
8.	9.255	30.729
9.	10.670	30.843
10.	11.303	30.047

Step 2

Picture No.	(B) - (A)	log (x)[1]
1.	30.839	0.77025
2.	29.370	0.77525
3.	28.166	0.77929
4.	26.855	0.78367

---

[1](x) = (B-A)(0.046404) - 7.325; magnification factor = 0.046404; distance from outer ference = 7.325.

5.	25.591	0.78784
6.	24.150	0.79253
7.	22.828	0.79679
8.	21.474	0.80113
9.	20.173	0.80525
10.	18.744	0.80973

Step 4. Find slope by extrapolation.

$$\frac{\log x_2 - \log x_1}{t_2 - t_1} = \frac{0.8107 - 0.7731}{144 - 0} = 2.6111 \times 10^{-4}$$

Step 5. Multiply results in step 4 by K factor.

K factor = 0.979725 for 60,000 rpm

$S_{observed} = 2.6111 \times 10^{-4} \times 0.979725 = 2.56 \times 10^{-4}$ or 2.56S

Experimentally observed sedimentation coefficient were corrected to a water basis at 20°C ($S_{20,w}$) by the following formula.

$$S_{20,w} = S_{obs.}\left(\frac{n_t}{n_{20}}\right)\left(\frac{1 - \bar{V}\rho_{20,w}}{1 - \bar{V}\rho t,sol}\right)$$

$n_{H_2O,6} = 1.472$

$n_{H_2O,20} = 1.002$

$\rho_{H_2O,6} = 0.999941$

$\rho_{H_2O,20} = 0.998203$

$\bar{V} = 0.73$

$$S_{20,w} = 2.56\left(\frac{1.472}{1.002}\right)\left(\frac{1-(0.73)(0.998203)}{1-(0.73)(0.999941)}\right)$$

$= 3.78$

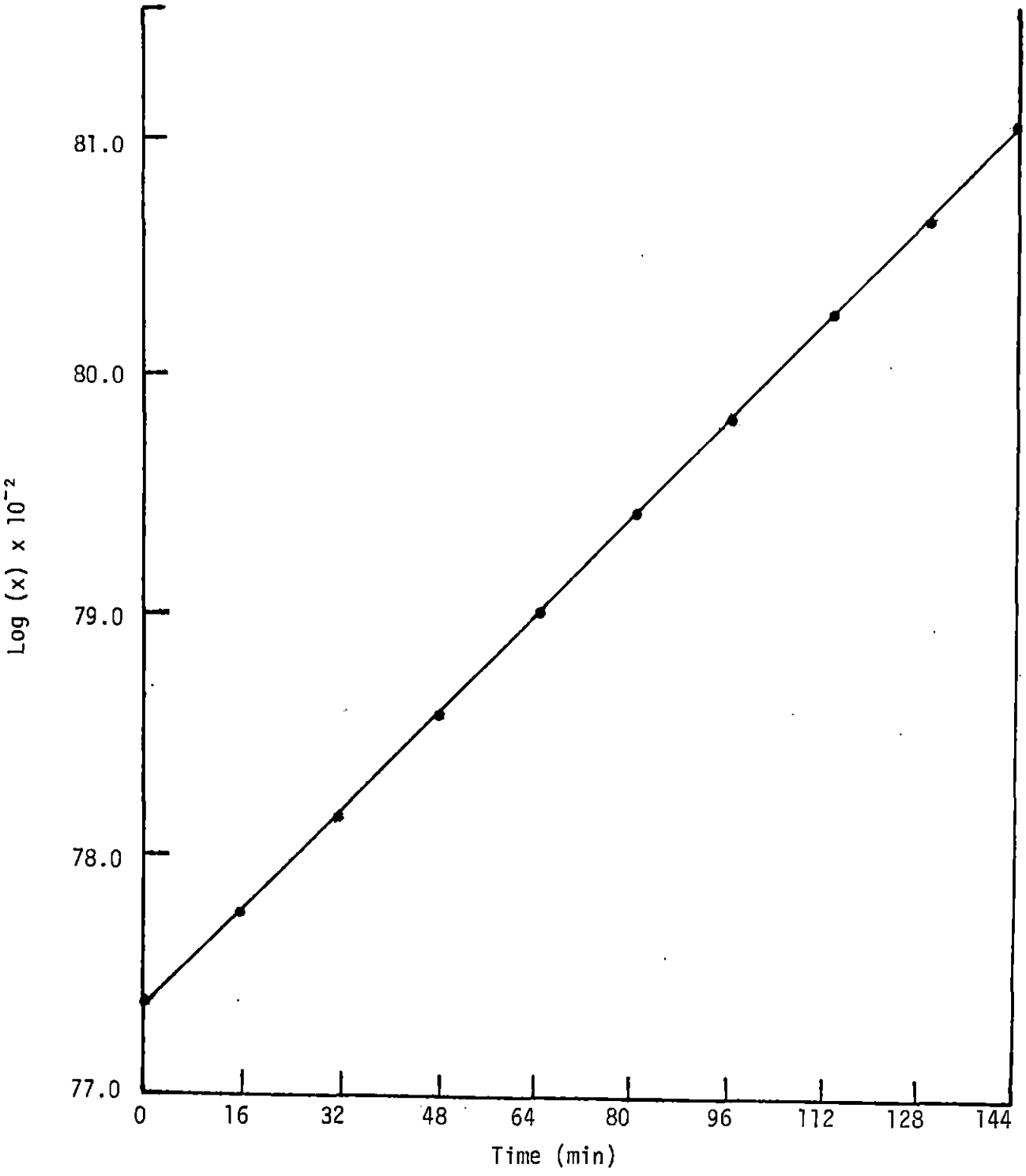

Fig. 16. Step 3 of the calculation of sedimentation coefficient of Fab fragment.