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APPLICATION OF ENZYME-LINKED IMMUNOASSAY AND A MODIFIED GROWTH MEDIUM FOR AVIAN MYCOPLASMA

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APPLICATION OF ENZYME-LINKED IMMUNOASSAY AND A MODIFIED GROWTH MEDIUM FOR AVIAN MYCOPLASMA

By

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ABSTRACT

APPLICATION OF ENZYME-LINKED IMMUNOASSAY AND A MODIFIED GROWTH MEDIUM FOR AVIAN MYCOPLASMA

By

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A medium was developed for growth and antigen production of avian Mycoplasma. This modified Mycoplasma medium was named MSU-Mycoplasma medium (MSU-medium). The medium contains Gibco Mycoplasma media, maltose (or dextrose), L-histidine, cystine HCl, Waymouth medium, tris buffer, penicillin, thallous acetate, phenol red and horse serum. Nicotinamide adenine dinucleotide was added only to the media for the growth of M. synoviae (Ms). This medium was used for Mycoplasma gallisepticum (Mg) antigen production for hemagglutination-inhibition (HI), tube agglutination test and Mycoplasma-Enzyme-Linked Immunosorbent Assay (MCYO-ELISA). Comparative studies were conducted between MSU-medium and brain-heart infusion medium, PPLO medium, tryptose phosphate broth, B-medium of Fabricant and modified Frey medium. All media were inoculated with Mg organisms, and samples were taken every 4 hours for the first 56 hours and at 3, 4, 5, 6, 7, 9, 30 and 60 days post-inoculation (PI) to determine the pH and the total bacterial counts. A significant correlation between bacterial counts and pH was demonstrated. The Mycoplasma organisms were isolated from MSU-medium 30 and 60 days PI and the pH readings were 6.5 and 6.5 at 30 and 60 days PI. According to the results, the MSU-medium

is superior to other media which were compared for the growth of avian Mycoplasma based on bacterial yield and the survival time of the microorganisms in the media.

The identification of avian Mycoplasma species has been very difficult because of the nature of the organisms. Cross reactions in serological tests have been reported by many authors. Fluorescence antibody technique was used for identification of avian Mycoplasma in broth culture by direct and indirect methods. However, FA has some limitations for expedient identifications of Mycoplasma organisms, especially in mixed cultures. More research work is required to prepare the specific labeled antisera to avoid non-specific reactions.

For this reason, protein A-Sepharose chromatography was employed to isolate and purify IgG from normal and hyperimmune chicken and rabbit sera. IgG molecules of subclasses 1, 2 and 4 of mammals and their fragments containing the Fc region have been reported to have the affinity to bind to protein A. This activity to the IgG Fc fragments has been shown to be correlated with IgG H-chain type. The two fractions obtained by washing protein A-Sepharose 4 B column chromatography sensitized by normal and hyperimmune chicken sera were identified by using Ouchterlony immunodiffusion. The 0.1 M sodium phosphate buffer, pH 7.0, eluate contained all immunoglobulin fractions including IgG antibodies. The 1 M acetic acid, pH 7.0, eluate did not contain any chicken IgG. It is believed that the non-binding properties of Fc region of chicken IgG are because of the absence of some of the C-terminal amino acids which are responsible for binding process in mammals. Another possible reason for the non-binding properties was that other protein is present

in chicken serum which binds with protein A molecule and, therefore, the binding process with IgG molecule is inhibited.

The enzyme immunoassay technique for the detection of avian Mycoplasma antibodies of chicken was first adopted in the MSU Avian Microbiology Laboratory. The Mycoplasma-Enzyme-Linked Immunosorbent Assay (MYCO-ELISA) antigen was prepared from Mg whole bacterial cell or its disrupted cell suspension. The affinity of antigen for binding or adsorption to the surface of polystyrene walls of microtiter plates was demonstrated by using 0.1 M carbonate bicarbonate buffer, pH 9.6. Ninety micrograms of antigen protein per well and 1:50 serum dilution were used to give high absorbance reading of 405 nm (using spectrophotometer) as compared to the reading of control serum. The conjugate consisted of IgG fraction of rabbit anti-serum and chicken IgG labelled with horseradish peroxidase enzyme (HRP). 2,2' Azino di {3 ethyl-benzthiazolin-sulfonate (6)} was used as a substrate. A temperature of 22 C (room temperature) for 2 hours was selected for routine incubation of serum and conjugate. The results indicated that there was no cross reaction between serum of Mycoplasma and serum of other bacterial or viral disease infection. However, cross reactions on a large scale between positive serum of Mg and Ms were observed.

A comparison study between HI and MYCO-ELISA test was conducted by using sera obtained from naturally and experimentally Mg infected chickens. The results indicated that the absorbance reading of positive sera gave much higher ELISA titer than the HI titer. It is believed that enzyme immunoassay can be the future method used to achieve a successful eradication program against Mycoplasma infections because of its simplicity, reproducibility, sensitivity and the automation potential for a large scale diagnosis in the laboratory or in the field.

Dedicated to the two men who have
contributed to my existence in
this life. My father, "Ibrahim"
the wise, the kind and the saint.
My uncle, "Abdel-Daiem" the ambitious,
the disciplinary and the rich.

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INTRODUCTION

Chronic respiratory disease (CRD) had been an important flock problem in chicken and turkey production in all areas of the United States (Yoder, 1978 a). It appears to be world wide in distribution. A drop in egg production of 10 to 20% over a period of two or four months or longer, retarded growth and carcass condemnation constitute the biggest loss to the poultry industry.

Isolation and identification of the causative agent from birds infected with Mycoplasma is not always successfully achieved. Serological tests routinely used to detect subclinical and chronic mycoplasmosis among susceptible flocks are not satisfactory. Opinions have differed considerably about the specificity of the antigen used. Cross reaction with other antibodies from related (either pathogenic or non-pathogenic) microbes has been frequently reported. Furthermore, Mycoplasma infections can develop slowly and may not be detected for several weeks or months by currently and routinely used serological tests. Therefore, a rapid and specific serologic test is needed for diagnosis, since the National Poultry Improvement Plan for Eradiation of mycoplasmosis in breeder flocks has been approved.

The first objective of this dissertation study was to develop a culture medium for isolation and maintenance of avian Mycoplasma. This medium was also used for Mg antigen production for tube agglutination test, hemagglutination-inhibition test and Mycoplasma-Enzyme-Linked Immunosorbent Assay (MYCO-ELISA Test).

The second objective of this study was to establish a diagnostic procedure for Mg infections in chickens.

1. Develop immunofluorescence method to detect and differentiate Mg from other species of Mycoplasma.

   This method has the distinct advantage of rapid identification and determination of Mycoplasma. Pure culture of Mycoplasma organism is not required.

2. Immunochemical studies to isolate and purify IgG fraction from chicken serum using protein A-Sepharose column chromatography.

   Protein A is a protein isolated from the cell wall of Staphylococcus aureus. This protein A has the ability to interact with a wide variety of IgG molecules to the Fc region in mammals. No research has been recorded in the literature to isolate chicken IgG by protein A chromatography. This purified IgG may be used in preparation of the conjugate in immunofluorescence methods.

3. Apply the enzyme-linked immunosorbent assay method for serological diagnosis of Mycoplasma infections in chickens.

Enzyme immunoassays are believed to be the simplest and a promising serological technique that can be successfully applied in a wide range of diagnosis and epidemiological investigations. This assay depends on the assumption that either an antigen or antibody can be linked to an enzyme and the complex will have both immunological and enzymatic activities.

LITERATURE REVIEW

It is generally believed that Louis Pasteur first recognized that a specific and very small microorganism caused contagious bovine pleuropneumonia, although he was unable to isolate or to see the organism. However, it was not until 1898 that Nocard and Roux accomplished the first isolation of the microbe in a very interesting and ingenious way. They filled collodion sacs with sterile broth media inoculated with lung fluid from infected animals. The sacs were then placed in the peritoneal cavity of a rabbit and left for several days. They soon were able to grow the organisms in serum enriched broth *in vitro*, but it was not until 1900 that Dujardin-Beaumet described colonial growth on a solid medium. He showed that colonies were characterized by a dark center, as the result of penetration of the organism into the medium, and a light periphery. Later on, Bordet (1910) and Borrel *et al*. (1910) described in detail the morphological appearance of these pleomorphic organisms and later Elford (1931) showed the existence of viable forms 125 to 150 mμ in size, using gradacol filter. For this reason, as well as many others, the organisms were thought by many to be a virus and, for 25 years, occupied this anomalous position.

A. Pleuropneumonia-like Organism (PPLO)

The accumulation of large amounts of serous fluid in the lungs and pleural cavities as a result of the infection by filterable organisms gave rise to call those organisms Pleuropneumonia-like Organisms (PPLO), which was primarly caused contagious bovine pleuropneumonia (Sabin, 1934,

Foster, 1934).

Infectious sinusitis of turkeys was first recorded by Dodds (1905), which appeared to be the first disease described in poultry caused by what is known now as Mycoplasma. Graham Smith (1907) stated that the sinus exudate from those birds was infectious, although the organisms ordinarily isolated from it failed to produce sinusitis. These observations indicated the possibility that an ultramicroscopic agent was the cause of the disease. Tyzzer (1926) was the first scientist to report sinus infections in turkeys in the USA and he experimentally used argyrocol for treatment.

During the following decade, the majority of researchers believed that a virus (filterable agent) was the etiological agent responsible for respiratory infections in turkeys and chickens. The organism was first described by Nelson (1935), who referred to the organisms seen in embryonated eggs as coccobacilliform bodies. Also, Van Herick and Eaton (1945) encountered filterable organisms in chicken embryos by cultivation of the organisms on an enriched agar and demonstrated for the first time that avian PPLO organisms agglutinated chicken red blood cells and that the immune system specifically inhibited the hemagglutination. Markam and Wong (1952) demonstrated that PPLO organisms that did grow in all-free media induced typical swelling and exudate from turkey sinus and air sac infection and chronic respiratory disease (CRD) in chickens. Numerous strains of coccobacilliform organisms (PPLO) isolated from chickens and turkeys of different serotypes were subsequently described by Adler et al. (1957). Adler et al. (1958) isolated two species of PPLO from chickens and turkeys. One of these PPLO organisms did not affect chickens

and has not been found in that species. However, PPLO in turkeys was not taken seriously until Yamamoto (1965, 1966) demonstrated conclusively that what was called at that time PPLO turkey sinusitis agent caused air sacculitis in turkeys and he reported as Adler et al. (1958) had, the egg transmission cycle of the microbes. The researcher named this organism M. meleagridis (Mm) (Yamamoto et al., 1965).

Another PPLO infection was observed by Olson et al. (1954) and Wills (1954) in broiler flocks in West Virginia and Texas. The symptoms were described as swellings of the joints, caseous exudate accumulations (arthritis) and, frequently, hepatosplenomegaly. The agent grew in chicken embryos and produced lesions similar to those produced by CRD agent. Later on, the organism was first cultivated by Lecce (1960) as satellite colonies around a Staphylococcus. Chalquest and Fabricant (1960) were able to grow PPLO in pure culture by replacing the Staphylococcus with diphosphopyridine nucleotide or nicotinamide adenine dinucleotide (NAD). They confirmed that the etiological agent for the disease was PPLO organism after several in vitro culture passages. A typical joint lesion resulted after inoculation of the organisms into chickens foot pads (Olsen et al., 1964). The name M. synoviae (Ms) was proposed for the infectious synovitis agent.

B. L-phase Organisms and Mycoplasma

L-phase was defined as independent growth variants of bacteria that lack a rigid cell wall and have a potential reversibility to the original form (Klieneberger, 1931). Also, he reported the growth of an organism resembling PPLO in a pure culture of Streptobacillus moniliformis. Klieneberger (1931) first considered this to be a mixed culture of the

bacterium and a contaminant PPLO, but Diens and Edsall (1937) established that these colonies which were called L-forms were derived from the bacterium and not related to PPLO organisms. McKay and Taylor (1954) and Kelton and Gentry (1957) were the first people to postulate that PPLO organisms isolated from chronic respiratory diseases in chicken were actually L-form bacteria. During that period, the PPLO organisms were included in order Mycoplasmatales (Freund, 1955) family Mycoplasmataceae on the basis that all PPLO organisms require sterol for growth (Freund, 1955). Rogul et al. (1965) stated that avian Mycoplasma (Avian PPLO) were not derived from bacteria and the bases of DNA compositions (the molecular percent of guanine plus cystine (G + C) in the DNA) and genetic compatability or molecular hybridization indicated no relationship between the two strains of avian Mycoplasma (M. gallisepticum and M. gallinarum) and L-form of Haemophilus gallinarum. Also, the L-form could be formed by many other species when wall synthesis was impaired by penicillin and high salt concentration (Thomas, 1973). Hijmans et al. (1969) published a list that contained 37 different bacterial species which had been reported in the literature to grow as L-phase. They indicated that L-phase was generally not considered to be pathogenic, even when it derives from a pathogenic bacterial phase. However, McKay et al. (1966) and Hijmans et al. (1969) reported that L-phase Haemophilus parainfluenza, Salmonella urium and Vibrio cholera were pathogenic.

C. Classification of Mycoplasma

Mycoplasmas appear to be ubiquitous in nature, occurring in both animal and plant kingdoms. Mycoplasma was recorded to be under class Mollicutes (Edward and Freundt, 1967). The old name for this class

was Paramycetes, by Sabin (1941). Order Mycoplasmatales (Freundt, 1955) was divided into two families, Mycoplasmataceae, which required sterol for growth, and Acholeplasmataceae, which didn't require sterol for growth. Family Mycoplasmataceae contains 37 known and documented species (Freundt, 1973). This class was recorded to be prokaryotic organisms bounded by a single triple-layered membrane; they lack cell wall precursors such as muramic and diaminopimelic acids.

D. Avian Mycoplasma

The first clinical case describing an infraorbital sinusitis of turkeys by Dodd (1905) was apparently caused by Mycoplasma sp. The viral etiology remained unchallenged for years while research in this disease continued. In 1952, the so-called "air-sac infection" and chronic respiratory desease emerged as a serious problem, particularly in broiler chickens and turkeys. The incredible amount of research done by Nelson (1935) on coccobacilliform coryza was completely overlooked for many years. He succeeded in isolation of coccobacillary bodies from chickens and showed upper respiratory disease signs of unknown etiology. The organisms were propagated in tissue culture, embryonating chicken eggs and on cell-free media. It was classified by Smith et al. (1948) on the basis of electron micrographic morphology and cultural characteristics. The organism was placed in what was at that time called PPLO organism, according to Sabin's (1939) definition of PPLO organisms. During the last 20 years, many aspects of Mycoplasma infections in poultry have been investigated and published.

Avian Mycoplasma Serotyping

Many investigators attempted to classify various avian Mycoplasma isolates by serologic techniques. Yamamoto and Adler (1958 a,b) characterized 5 serotypes. Kleckner (1960) described 8 serotypes designated A through H including the previous 5 serotypes described by Yamamoto and Adler. Yoder and Hofstad (1964) characterized 12 serotypes (A through L) and Dierks et al. (1967) characterized 19 serotypes (A through S). However, the three pathogenic serotypes A, H, and S remained separated and distinct from the other serotypes, which represent M. gallisepticum (Mg), M. meleagridis (Mm) and M. synoviae (Ms). Different serotypes of avian Mycoplasma by different authors are summarized in Tables 5, 6, and 7 in the Appendix. The combination of serotypes I, J, K, N, Q and R into one antigenically related group was in agreement among all authors. However, the fact that the metabolic inhibition test used by Barber and Fabricant (1971a) was more reliable than complement fixation made their classification more accurate than other tests, indicated by Frey et al. (1972). In addition to the species and serotypes in Table 5, practically every investigator has added additional unclassified avian Mycoplasma, which represented a problem in identification. Serotyping, origin and pathogenicity of avian Mycoplasma are summarized by Yoder (1978), as shown in Table 6 in the Appendix.

Characterization of Avian Mycoplasma

Classification and characterization of avian Mycoplasma was an obvious problem to many researchers for many reasons, as summarized by Fabricant (1969).

(1) Primary isolation of avian Mycoplasma often yields more than one species from infected birds (Fabricant, 1960; Yoder and Hofstad, 1964).

(2) Definite identification of different Mycoplasma sp. depends on the proper biochemical and serological tests applied and standardized testing procedures (Fabricant and Freund, 1967).

(3) Serologic tests performed using serum containing antibodies against different species of Mycoplasma (pathogenic and nonpathogenic) are of very limited value for identification of Mycoplasma sp. isolates.

Characterization of avian Mycoplasma was mainly based on serotyping and biochemical reactions, as presented in Table 7 (Appendix). These biochemical characterization studies were carried out by many authors (Fabricant, 1969; Yoder, 1975, 1978a; Freundt, 1974).

These procedures of identification were built on:

(1) Arginine decarboxylation: Indicated by alkaline change (deep red color) in liquid basal medium with 1% arginine and phenol red at a pH of 7.0.

(2) Dextrose fermentation: The production of acid in liquid basal medium supplemented with 0.5% dextrose (glucose) and phenol red at pH of 7.6.

(3) Tetrazolium reaction: Indicated by changes from colorless to red in liquid basal medium containing 1 to 10,000 tetrazolium red (2, 3, 5-triphenyl-2H-tetrazolium choloride). Some avian Mycoplasma species will reduce the tetrazolium chloride which acts as a growth indicator.

(4) Hemagglutination: Using chicken or turkey red blood cells

depends on the source of the serum.

(5) Diphospho-pyridine nucleotide (DPN) or nicotinamide adenine dinucleotide (NAD) requirements: Ms was the only avian Mycoplasma requiring NAD for growth (Chalquest and Fabricant, 1960).

(6) Serum requirements: The addition of 10-15% heat inactivated serum (horse, swine, turkey or chickens) in the medium was required by all avian Mycoplasma species. M. anatis (Roberts, 1964) and M. laidlawii var inocuum (Adler et. al., 1961) did grow on serum enriched media. But whether or not they required serum needs more investigation.

Mycoplasmas

1. Mycoplasma gallisepticum (Mg)

Avian serotype A (Kleckner, 1960), avian serotype S_6, A-5969, 801, X95 and others (Adler et al., 1958) were described extensively in the literature. This organism was the primary etiological agent of chronic respiratory disease of chickens and turkeys. The disease in turkeys had also been commonly called infectious sinusitis of turkeys. However, the field condition popularly known as "air sac" disease or complicated CRD was not due to a Mg infection alone. Complicating infections which were designated as airsacculitis and which produced fibrinous or fibrinopurulent pericarditis and perihepatitis along with the massive airsacculitis were responsible for the severe mortality. The economic impact of the disease, represented in reduced feed conversion and decreased egg production, weight loss, increased medication costs and downgrading of carcasses and excessive condemnation rate at slaughter, made this disease

one of the major disease problems of the American broiler industry.

a. Chemical Compositions:

(1) Whole cell: Except for some variations, the Mycoplasmas resembled other bacteria in their growth compositions. They lacked the bacterial cell wall components such as muramic acid and diamino-pimelic acid (Razin, 1969). Morowitz et al. (1962) and Razin et al. (1963) studied the chemical composition and the microsopic morphology of Mg (Avian PPLO 5510:5969). A packed cell suspension contained approximately 80% water, which meant that the cell contains 20% dry weight. Protein constituted about 80% of the dry weight. Total lipid determination uniformly ran at between 10% and 12% of the dry weight. Carbohydrate was usually less than 1%; RNA and DNA were 8.3% and 4% from the dry weight of the whole cell, respectively (Morowitz et al., 1962). Generally, there was no great variation in the chemical composition of the organisms of different strains except that most pathogenic strains had a somewhat higher lipid content than the saprophytic strains. The Mg contained a smaller amount of total carbohydrates than other Mycoplasmas (Rauin et al., 1963).

(2) The Genome: Electron microscopy of the Mycoplasma gallisepticum showed the absence of nuclear membrane (Morowitz et al., 1967). The genome size of two Mg was 1200 x 10^6 daltons (Morowitz et al., 1967). Other Mycoplasma species had a similar result. However, the Mycoplasma genome was much smaller that that of the average bacteria; the genome size of E. coli, for instance, was 2500 x 10^6 daltons. On the other hand, the Mycoplasma genome was far larger than the genome of the virus, but may be comparable to that of the bedsonia organisms (PLT group), or the

trachoma agent, the size of which was estimated at 600 x 10^6 daltons (Razin, 1969).

(3) The Ribosomes: The ribosomes were similar to bacterial ribosomes, but showed some difference in base composition and sedimentation behavior. Five percent of the total RNA in the cell was messenger fractions (Morowitz et al., 1967). The RNA protein ratio was about 60:40 compared with about 40:60 in eukaryotic cell ribosomes. Electrophoresis in acidic polyacrylamide gels separated 20 protein bands compared with 28 bands in E. coli ribosomes and the sedimentation of the ribosomal RNA was approximately 16^6 (Kirk, 1966). The ribosomes were arranged in cylindrical or helical corncob structures of over 50 ribosomes each (Maniloff et al., 1965, and Domermuth et al., 1966). The biophysical similarity between the Mycoplasma and prokaryotic cell ribosomes was also apparent. Chloramphenicol and erythromycin, which specifically inhibited protein synthesis on the 70 S microbial ribosomes, effectively inhibited protein synthesis in many Mycoplasma including Mg (Tourtellotte et al., 1967). RNA was synthesized by a DNA dependent RNA polymerase, and its synthesis can be effectively inhibited by actinomycin D.

(4) Cell membrane: Mg, as well as other Mycoplasma, does not have a cell wall or intracytoplasmic membrane; it possesses only plasma membrane. In thin section, the plasma membrane seems to be composed of two electron - dense layers with a less dense layer in between (Van Iterson and Ruys, 1960). The three-layered structure corresponds to the unit membrane which covers cells and organelle of various origins.

The plasma membrane can be separated from the cell, either by osmotic lysis (Rottem et al., 1968) or by mechanical pressure using sonic or

ultrasonic oscillators (Pollack, et al., 1965). However, Mg resisted the osmotic lysis by the usual procedures (Rottem et al., 1968, and Robrish and Marr, 1962). The washed Mycoplasma cells were suspended in a 2 M glycerol solution, incubated for 10 minutes at 37 C and then rapidly injected into deionized water. Glycerol freely penetrated through the cell membrane and increased the internal osmotic pressure. The rapid injection of glycerol-loaded cells into deinoized water caused rapid penetration of water molecules, resulting in the sudden swelling of the cell, rupture of the cell membrane, and releasing of the cell contents into the medium.

The membrane consisted of 50-60% protein, 30-40% lipid and a very little amount of carbohydrate (Razin et al., 1963). The major portion of the lipids (60%) was phospholipids. Cholesterol and cholesterol esters made up to about 18.8% of the total lipids. The controversial issue of what is the structure and the organization of the protein and lipids in the membrane still exists. The two main theories were that the membrane is built of a bimolecular leaflet of lipid coated on both sides with protein (Danielli and Davson, 1935) or lipoprotein subunits (Green et al., 1967). Chu and Horne (1967) described a surface projection, resembling those of myxoviruses on negatively stained Mg. Mycoplasma membranes were recently found to be solubilized by a variety of detergents, anionic, cationic, nonionic and sodium dodecyl sulfate (SDS) with SDS being the most effective (Rottem et al., 1968). SDS bound to different molecular weight of protein to form protein SDS complex and to lipid to form fat-SDS complex. However, a striking feature was discovered by Razin et al. (1965), in that the detergent solubilized membrane material had the ability to reaggregate spontaneously to membrane-like structures

upon the removal of the detergent. The reformed membrane in this section was shown to consist of typical triple-layered unit membrane about as thick as the original one (Terry et al., 1967). Also, Magnesium (Mg^{++}) or some other divalent or polyvalent agents, but not the monovalent, were essential in this aggregation.

b. Growth Requirements:

Mg and the rest of the Mycoplasma group were of special interest, because they were the smallest organisms capable of growing in cell free media. Mg required a rather complex enriched media with 10-15% heat inactivated horse, swine or avian serum. The culture media available at present are not entirely satisfactory. Most workers would agree that more avian Mycoplasma sp. may be discovered if the quality of the present media can be improved. Perhaps the most distinctive nutritional requirements of Mycoplasma are lipids and lipid precursors, especially cholesterol, which incorporate in forming the plasma membrane. Serum, Tween 80 and TEM-4T (diacetyl tartaric acid ester of tallow monoglycerides), which contain fatty acids in various forms (saturated and unsaturated), are found to be suitable for the growth of avian Mycoplasma (Lund and Shorb, 1966). Amino acid requirements have been found to be absolute for all except glutamic and aspartic acids and cystine. Carbohydrate requirements as a source of carbon and energy had been provided in monosaccharide form as dextrose (Rodwell, 1969).

Vitamin requirement had been determined for a few strains of avian Mycoplasma. Nicotinic acids, thiamine, nicotinamide adenine dinucleotide (NAD or Coenzyme I, formerly DPN) and riboflavin are required for the group of Mycoplasma (Tourtellotte et al., 1964). Ms is unable to synthesize NAD from nicotinic acid or nicotinamide either because of the poor

penetration of the NAD into the cells or the absence of a nicotinamide adenine dinucleotidase (Chalquest, 1962). The role of urea in metabolism is still under investigation. Comparison of different media used for isolation and antigen production has been recorded (Adler et al., 1957; Taylor et al., 1957; Fabricant, 1958; Ferey et al., 1968). Media for isolation and maintaining have been developed by Adler et al. (1954); Yamamoto and Bigland (1964); Yoder and Hofstad (1964); Frey et al. (1968); and Yoder (1975).

c. General Morphology and Staining

The organisms in general were described by electron microscopy as being spherical or elongated (tear drop) in shape with a diameter of 0.15μ - 0.5μ (Morowitz et al., 1962). Maniloff and Morowitz (1967) studied the ultrastructure and ribosomes of Mg. They indicated that a Mycoplasma cell is composed of 3 organelles: the cell membrane, the ribosomes and the nucleoid.

(1) Membrane: The triple 3 layered plasma membrane had the thickness of 110 A structure.

(2) Ribosomes and ribosomal structure: The cylindrical ribosomal arrays, showing irregular packing, filled the intracytoplasmic space between the nuclear area and the unit membrane. The RNA-protein ratio of 0.68 was within the 0.6 to 1.7 range found for isolated ribosomes and detached microsomal RNA particle.

(3) The nuclear material, DNA: It was in the center part of the posterior section and had a fibrillar appearance.

(4) The bleb and infra-bleb regions: The bleb was described as one of the very characteristic features of Mg and so far this region

has never been found in any other Mycoplasma species. The bleb, shaped approximately like an oblate ellipsoid, measured about 800 A by 1.300 A, excluding the bounding membrane. The bleb region was reported to contain high amounts of lipids.

In summary, Mg differed from other Mycoplasma in several morphological properties: a) the cell possessed a bleb which contained no nucleic material, but protein and lipid and the function of the bleb are not yet clear (infectious role), b) the ribosomes were arranged in helical (polysome-like) arrangement, corncob structure; c) they were not elongated or coccid, but they resembled a tear drop or a coca-cola bottle, with the bleb in the top. The Giemsa stained preparations of isolates showed that the cells were coccoid forms and were approximately 0.4 μ in diameter.

d. Modes of Replication: Mycoplasma, in general, have been reported to have more than one mode of cell replication, depending on the various cell and the environmental conditions. Kelton (1962) and Morowitz and Maniloff (1966) showed that Mg multiplied by simple binary fission (formation of two identical cells). Domermuth et al. (1964) indicated that Mg produced elementary bodies at one locus of the Mycoplasma filaments. Several authors have suggested that Mycoplasma reproduced by budding (Liebermeister, 1960; Dutta et al., 1965).

e. Colonial Morphology: Mg can grow either on serum-enriched agar or broth medium. The initial isolates of Mycoplasma have been described to grow better in broth than on agar media. Colonies of different serotypes are different in sizes and heights of their centers. In general

the size of Mycoplasma is approximately 0.2-0.4 mm; however, it can be as large as 0.8 mm such as serotype F (Yoder and Hofstad, 1964). The colonies appeared to be tiny, smooth, circular, translucent masses of dense raised central area with greater optical density (fried egg colonies).

f. Biochemical and Biological Properties: Mg ferments glucose, maltose and mannose with the production of acid, but not gas. It does not ferment lactose, mannitol, dulcital, salicin, arabinose, xylose, trehalose, rhamnose, sorbitol, abonitol or raffinose. Fermentation results are varied with sucrose, galactose, fructose, and inulin (Dierks et al., 1967; Yoder and Hofstad, 1964). The S_6 isolate utilizes sucrose, whereas strain A 5969 does not (Adler, 1970). Mg reduces 2, 3, 5, triphenyltetrazolium chloride; therefore, it is incorporated into medium as a growth indicator. All strains of Mg hemagglutinate with red blood cells of chicken and turkey as well as other mammalian and avian erythrocytes. The hemagglutinating activity of Mg is attributed to neuraminidase and neuraminidase-like enzyme (Gesner and Thomas, 1966; Roberts, 1967). They presented evidence that N-acetyl-neuraminic acid or a closely related sialic acid provided binding sites for Mg at the erythrocyte surface. The situation appeared analogous to hemagglutination by influenza A virus and Myxoviridae group, where neuraminic acid provides sites for virus attachment. Furthermore, the pathogenicity of Mg may be attributed to the function of a neuraminidase-like enzyme. Mg was shown to be hemolytic, which meant that after attachment to the erythrocyte, the organisms produced peroxide, changed the hemoglobin to methemoglobin and led to lysis of the red blood cells (Thomas and Bitensky, 1966).

g. Physical Properties: The Mycoplasma are readily disrupted by mechanical means such as sonic and ultrasonic disintegrators and French press. Razin et al. (1964) found that pathogenic Mg cultures are resistant to osmotic effects while the nonpathogenic Mycoplasma are susceptible. The osmotic lysis is heat-dependent. Mg can stand glycerol osmotic shock, and has been stored for years in 50% buffered glycerol at -40C to -60C without loss of viability. Most strains of Mg can be kept for long periods of time in the frozen state at -60C in yolk, or broth media, but not in albumen, serum, saline, feces, feather meal, chicken muscle and muslin (Chandiramini et al., 1976). Yodi et al. (1973) indicated that adding sucrose was beneficial for lyophilization. Furthermore, no changes in antigenicity or pathogenicity took place during storage for a month at -20C. Adler (1970) reported that lyophilization in either milk, yolk, or serum broth was the most satisfactory method for storage. Yoder and Hofstad (1964) indicated that the organisms remained viable for fourteen years. They also found that stored broth cultures remained viable for 2-4 years. Fabricant (1973) recovered the organism from 60%, 35% and 13% of infected materials stored at 25 C for 1, 2 and 3 years, respectively. Olesivk and Van Roekel (1952) reported that infective allantoic fluid remained infective 4 days in the incubator, 6 days at room temperature and 32-60 days in the refrigerator.

No work has been done with the effectiveness of disinfectants or other chemicals on Mycoplasma. However, it has been known for some time that the Mycoplasma can be killed by the disinfectants. Mycoplasma has been reported to be resistant to penicillin and thallium acetate (Yoder, 1978).

h. Pathogenicity

The pathogenicity of Mg in different hosts varies according to the nature of isolate, method of propagation, and number of passages before it is inoculated into the host (Yoder, 1978). Also, the route of infections, the number of organisms, the presence of secondary infections, the type of host, and other environmental factors, including stress and vaccinations, affected the pathogenicity of the microbe. Roberts (1964) indicated that the pathogenicity of Mg could be attributed to the neuraminidase-like enzyme. However, this enzyme was reported to be in nonpathogenic strains of Mycoplasma as well.

(1) Embryonated chicken eggs. Inoculation of broth cultures with exudate containing Mg in seven-day-old embryonated chicken eggs via the yolk sac route usually resulted in embryo deaths within five to 10 days (Yoder and Hofstad, 1964). Stunting of the embryos, subcutaneous hemorrhages, and generalized edema were frequently noted. Hepatitis and splenomegaly were the most obvious lesions (Van Roekel et al., 1952). The periarticular abscesses were creamy-white to yellow nodules which occurred in the subcutaneous areas adjacent to the mandible, wing, hip, stifle, hock, and toe joints (Chute, 1960; Yoder and Hofstad, 1964). However, Yoder and Hofstad (1964) stated that such lesions were produced by serotypes of Mycoplasma other than Mg. Also, it is interesting that isolates from trachea of chickens inoculated into chicken embryos produced "joint abscess" (Calnek and Levine, 1957; Yoder and Hofstad, 1964). However, they failed to demonstrate that known pure isolates of Mg (serotype A) were capable of producing subcutaneous periarticular granulomas in chicken embryo.

(2) Chickens. Mg produced a severe extensive airsacculitis and caseated exudate in the air sac (Dierks et al., 1967). However, Yoder and Hofstad (1964) indicated that moderate airsacculitis was produced only occasionally, and that moderate to severe tendo-vaginitis was produced two to three weeks post inoculations. Also, they reported that seven birds out of 19 developed airsacculitis lesions and 14 out of 22 developed tendo-vaginitis lesions. Yoder and Hofstad (1964) reported high titer of antibody and formation of severe lesions after inoculation of the tendo-vaginal cavity in the region of the hock and foot pad of chickens while inoculated air sacs in the same chickens showed little or no gross lesions and low antibody titer.

(3) Turkeys. The disease affects most of the turkeys in a flock, although some of them may not exhibit sinusitis. Inoculated turkeys or poults developed more severe airsacculitis. Only Mg (isolate serotype A) produced sinusitis in turkeys. Yoder and Hofstad (1964) reported that from 45 turkeys inoculated with serotype A, 31 birds showed sinusitis, 23 showed airsacculitis, 10 showed tendo-vaginitis and 39 birds showed high antibody titer. Neurologic and arthritis syndromes of turkey poults inoculated intravenously with Mg (strain 6) have been investigated by Clyde and Thomas (1973).

(4) Game birds. Yoder and Hofstad (1964) failed experimentally to introduce Mg infections into bobwhite quail (Colinus virginianus) by inoculation of first yolk passage of isolate 894 of serotype A, which was pathogenic for chicken embryos, chickens, and turkeys. However, Madden et al. (1967a) isolated pathogenic Mg from bobwhite quail.

(5) Pigeons. The significance of Mycoplasma in pigeon nasal

cavities has not been evaluated. Yoder and Hofstad (1964) inoculated three different isolates of Mg into four groups of seven pigeons each with no visible clinical signs or lesions. Winterfield (1953) isolated the "turkey sinusitis agent" from pigeons via chicken embryo inoculation. Gianforte et al. (1955) and Mathey et al. (1956) isolated unclassified strains of Mycoplasma from pigeons.

i. Epizootiology

(1) Natural and experimental hosts. Mg infection occurs naturally in chickens and turkeys. It has also been isolated naturally from other birds such as chukar partridges (Alectoris graeca) (Wichmann, 1957), bobwhite quail (Colinus virginianus) (Madden, et al., 1967a), peacocks (Pavo cristatus) (Willis, 1955) and pheasants (Osborn and Pomeroy, 1958a). The organisms have been isolated from pigeons (Winterfield, 1953; Gianforte, et al. 1955). Van Roekel and Olesiuk (1953) reported that guinea fowls and pheasants were readily infected.

(2) Spread and transmission. Mg infections were reported as an egg-borne disease in chickens (Van Roekel et al., 1952; Fahey and Crawley, 1954b) and in turkeys (Jerstad et al., 1949; Hofstad, 1957a). Yoder and Hofstad (1964) isolated the organism from the oviduct of infected chickens and semen of infected roosters, which indicated the congenital or vertical transmission pattern. Under the modern pattern of the poultry industry, where the breeder company provides the chicks to the grower in flocks of 10,000 to 20,000 birds, under crowded conditions and confined housing, a small number of newly hatched chicks will serve as a nucleus for contact infections (horizontal transmission). The infections are usually accelerated by vaccination programs or other stress factors.

Airborne infections by dust or droplets have been recorded (Fahey and Crawley, 1955a). The role of wild birds or other animal vector in transmission did not appear to be significant in chickens, but since turkeys were usually raised on range, airborne transmission had been observed and animal vectors were potentially more important. Field evidence suggested that the infection can be carried from one flock to another by human visitors or employees (Yoder, 1978).

(3) Incubation period. Under natural conditions, it is very difficult to determine the exact data of exposure because many factors seem to influence the onset and extent of the infections. Experimentally, Delaplane and Stuart (1943) and Van Roekel et al. (1952) found the incubation period to vary from four to 21 days. Sixty-five percent of 233 chicks had symptoms of nasal discharge between 11 to 18 days following inoculation of infective turbinate material (Hofstad, 1952). Yoder (1978) stated that numerous chicken and turkey flocks developed clinical signs near the onset of egg production, suggesting a low level of inherent infection (probably due to egg transmission) due to a series of stressful events.

(4) Morbidity, mortality, and the role of secondary factors. Chickens were susceptible to the infection at all ages, but they varied in their susceptibility and the duration of the disease. Young chickens and turkeys were more susceptible to disease outbreak than older birds. However, starting about 1950 to 1951, extensive outbreaks of a severe respiratory disease were reported, especially in broiler-growing areas. As early as 1954, Wasserman et al. demonstrated that Escherichia coli was the most frequent complicating organism (especially O-group 2).

Other researchers reproduced a severe air sac infection experimentally by infecting the chicken with a combination of E. coli, Mg, and viruses of infectious bronchitis or Newcastle disease (Gross, 1961a, 1962; Adler et al., 1962). These relationships were further studied by Fabricant and Levine (1962), confirming the observation of Gross (1961 a,b), who determined that experimental lesions were indistinguishable from the typical field cases. They concluded that E. coli did not readily invade the lower respiratory tract unless it was previously infected with Mg or other respiratory viral or bacterial infections.

The role of viruses in establishing CRD such as reovirus and infectious bursal disease needs more investigation. Mortality was very low in adult chickens, but in four- to eight-week-old broilers it was higher than in laying hens. In complicated cases, mortality reached about 30 percent.

j. Clinical Signs

(1) Chickens: Fabricant (1969) and Yoder (1979) summarized the description of the disease in chickens and turkeys. The most characteristic symptoms of the disease were tracheal rales, nasal discharge and coughing. The duration of the clinical symptoms was recorded to be as long as one to two months in experimentally infected chickens. However, because of the long term duration of the disease and the long incubation period, clinical signs may continue for a much longer time. In laying hens, egg production declined but maintained at lowered levels. Food efficiency decreased in broilers and significant levels of lowered hatchability were reported due to embryo mortality caused by Mg. However, antibody titer against Mycoplasma reached high levels in some flocks

without any evidence of clinical signs, especially if the infection occurred when they were young (Yoder, 1979).

(2) Turkeys: In turkeys, the disease has been described as prolonged, progressive and severe, and manifested by swelling and distention of the infraorbital sinuses and/or air sac infection. Subclinical infections were rare (Fabricant, 1969). Tracheal rales, coughing and labored breathing sometimes became evident if tracheitis or airsacculitis were present. Lowered egg production in breeder flocks was reported.

K. Gross Lesions

The most consistent gross lesion associated with this disease was the presence of mucoid to mucopurulent exudate in the trachea, bronchi, air sacs, and nasal passages. Sinusitis was usually more prominent in turkeys, but it has also been observed in chickens and other avian species. The severe purulent, fibrinopurulent, or caseous exudates seen in the pericardium, around the liver, and in the air sacs were due to secondary E. coli infections (Gross, 1961a, 1962). Domermuth and Gross (1962) reported that salpingitis in chickens and turkeys occurred occasionally due to the mycoplasmosis.

L. Histopathology

The microscopic lesions of CRD have been studied by Jungherr et al. (1953) and Van Roekel et al. (1957). Thickening of the mucous membrane of the affected tissues due to infiltration with mononuclear cells and hyperplasia of the mucous gland were recorded as typical microscopic descriptions of CRD. Focal areas of lymphoid hyperplasia, so-called "lymphofollicular reaction", were commonly found in submucosa of the

respiratory tract. Barber (1962) recorded similar lesions in apparently normal turkeys, and suggested that the presence of lymphofollicular lesions might be of limited value. It is not clear whether the occasional granulomatous lesions seen in the lungs were due to Mg or to secondary bacterial infections (Van Roekel et al., 1957).

m. Laboratory Diagnosis

(1) Specimen collection. Since Mycoplasma infections tended to be respiratory and involved most birds in the flocks, five or ten tracheal swabs were sufficient. Air sac swabs were cultured directly into the media or from a suspension prepared in a mortar and pestle with a small amount of nutrient broth. Sinus fluid was usually obtained from the infraorbital sinus with a syringe and 28- or 25-gauge needle. Specimens of brain, heart, liver, kidney, spleen, oviduct, and cloaca were used for cultures (Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens, National Academy of Science, Subcommittee on Avian Diseases, 1971).

(2) Identification of the organism

(a) Broth and agar media. Broth passage prior to plating on agar increased the number of primary isolates. If there was no evidence of growth after 96 hours, the cultures were incubated for another seven days. Serial passages to a new broth tube every two to three days enhanced the multiplication of the organisms. Mg reduced phenol red when it was present in the medium to yellow color as the medium became acid. None of the available media which have been published were suitable for both isolation and antigen production of various avian Mycoplasmas (Olson et al., 1965; Yoder and Hofstad, 1964; Yoder et al., 1972; Yoder, 1975).

The plates should be incubated in a moist atmosphere (sealed container with a damp towel) at 37 C. Serotyping was required to achieve accurate classification.

(b) Embryonated chicken eggs. Inoculation of seven-day-embryonated chicken eggs via the yolk sac with the original exudate was a very effective means to enhance the growth of Mg (Yoder, 1978). Embryo death has been recorded within five to ten days post inoculations. Lesions such as hepatitis, splenomegaly, pericarditis, pneumonia and airsacculitis were reported (Yoder and Hofstad, 1964).

(c) Staining. Cultures were further identified by preparing Giemsa - stained smears that revealed small coccoid organisms often in clumps when examined through the oil immersion lens of a microscope (Yoder, 1979).

(d) Fluorescent technique. Fluorescent antibody (FA) was used for differential diagnosis by applying FA directly to colonies on agar. Corstvet and Sadler (1964) used high-titer FA conjugate prepared from antiserum produced in rabbits.

(e) Biochemical reactions. Most of the laboratories working with diagnosis of Mycoplasma did not require biochemical reaction studies for differentiation of different Mycoplasma species. However, as mentioned before and summarized in Table 7 (Appendix), different avian Mycoplasma species had different biochemical reactions. Arginine decarboxylase, dextrose fermentation, tetrazolium reaction, and DPN requirements were the basis for diagnosis by biochemical reaction.

n. Serological Diagnosis

A positive serologic test, together with history and typical symptoms

of the disease, constitute a presumptive diagnosis of the disease. Isolation and identification of the organism constitute the very important diagnostic steps for definite Mycoplasma diagnosis (Yoder, 1975). Several serologic procedures are currently in use.

(1) Rapid serum plate test (RSP). The test was used primarily by Adler (1954). Antigen was prepared according to the procedure of Adler and Yamamoto (1956a). The test was performed by placing a drop of serum on a rotating glass plate at room temperature and mixed with a drop of stained antigen to make a spot about two cm. in diameter. The results were read after two minutes. A positive reaction was characterized by the formation of definite clumps, usually starting at the periphery of the mixture. Positive and negative control sera were included whenever the test was run. Yoder (1979) reported that turkey serum reacted to confirm the presence of Mycoplasma in the flock more slowly than chicken serum when RSP test was used. The main advantage to the test is that it can be used in the field to give preliminary diagnosis and relatively easy, fast results. Many common human errors occurred, such as improper temperatures of equipment, room, and antigen, improper light for reading results, carelessness in placing the proper amount of antigen and serum on the plate, or dirty plate and other equipment, causing inaccurate readings. Other disadvantages included cross reactions; this feature will be discussed in more detail later.

(2) Tube agglutination test (TAT). TAT was found to be more reliable than the plate test using chicken serum by Jungherr et al. (1955). It was also found more reliable than the plate test in testing turkey serum (Hofstad, 1957b). The antigen prepared for the plate test could be used

in the tube test when diluted 1:20 in phenolized (0.25%) buffered saline (pH 7.0). The tube test was conducted by mixing 0.02 to 0.08 ml of test serum with 1.0 ml of diluted (1:20) antigen in a glass tube and allowing the mixture to react for 18 to 24 hours at 37 C. A detailed review of the recommended procedures of TAT is cited by The Methods for Examining Poultry Biologics and for Identifying and Quantifying Avian Pathogens, Subcommittee on Avian Diseases, National Academy of Science (1971). The test usually was read after overnight incubation at 37 C. Clear supernatant fluid with clumps of antigen covering the entire bottom of the tube was indication of a positive test. The advantage of this test was that it detected IgM and IgG antibody of Mg (Kleven and Pomeroy, 1971). Yoder (1975) indicated that RSP and TAT are a sample screening procedure for flocks, but some nonspecific reactions may result that could not be confirmed by HI test.

(3) Hemagglutination inhibition test (HI). The HI test has been used routinely to confirm various rapid serum plate or tube agglutination tests and to differentiate Mg from Ms. It has been recorded that the HI test detected IgG antibody which appeared in the circulation several weeks post infection (Kleven, 1975). The test was conducted by the constant-antigen decreasing-serum method (Beta procedure) or vice versa (Alpha method). This method required the use of a 4-unit antigen system. Differences in the number of hemagglutination (HA) antigen units changed the result markedly. The detailed procedure for HI test was presented in the Isolation and Identification of Avian Pathogens (Yoder, 1975).

Jungherr et al. (1953) compared the HI test with slide and tube agglutination tests and found that the HI test was the most reliable.

Many investigators arrived arbitrarily at the number of HA units to be used in the test, which accounts for many unreliable results. Leach and Blaxland (1966) reported wide variations in HI results from laboratories when a series of Mg antisera were evaluated for HI. They attributed this variation to the kind of HA antigen used. Kuniyasu and Ando (1966) found that Mg-HA antigen activity in broth developed to its peak on the fifth day of incubation. HA activity of Mg antigen was not related to the number of viable organisms, and various chemical treatments had no adverse effect on the HA. In their studies, formalin destroyed the HA activity of the antigen. Adler and DaMassa (1968) found that adding dextrose to the culture medium for propagating Mg markedly reduced the sensitivity of plate agglutination antigens, but they did not observe any effect on HI test. The sensitivity and the specificity of the antigen used varied from one laboratory to another. The requirements of red blood cells, buffers, and reagents made the test more expensive than the other two serological methods.

(4) Complement fixation test (CFT). Frey and Hanson (1969) and Marquardt and Newman (1971) described a direct complement fixation test to detect antibody of Mg infections in chickens and turkeys. They used guinea pig complement with normal unheated chicken or turkey serum directly to quantitate antibodies against Mg. The technique showed as much specificity and sensitivity as the HI test.

(5) Metabolic inhibition test (MIT). Barber and Fabricant (1971) used this technique to classify Mg. They used microtiter equipment and a constant antigen dilution in all wells (1000 units of metabolic activity of the antigen/well). Hyperimmune rabbit antisera to serotype A (Mg) and

other serotypes were used. The test has been reported to be very sensitive but modification is needed, especially for antigen production, before it could be applied on a wide range.

(6) Growth inhibition test (GIT). For this test, small plates (100 x 15 mm) containing agar medium and grid marks were used (Aycardi et al., 1971). The plates were placed in an incubator at 37° C for one hour to dry the surface. The Mycoplasma culture to be tested was diluted serially in sterile broth medium, and 0.2 ml of each dilution was spread over the dry surface with a bent glass rod. When the inoculum had dried (approximately 1/2 hour), filter paper discs of 5 mm diameter soaked in hyperimmune sera were placed gently on the agar surface with sterile forceps. The plates were incubated right-side up in a moist chamber at 37° C for 5 to 7 days, and then read. The zone of inhibition was measured in mm. This test was used only in laboratory classification of different serotypes of avian Mycoplasma, but it can be adapted for diagnostic purposes.

(7) Agar-gel diffusion test (AGD). Aycardi et al., 1971) described the procedures of preparing the diffusion antigen used in this test as well as the methodology of the test. The diffusion medium consisted of 0.8 g agarose, 5 ml borate buffer (pH 8.6), 95 ml physiological saline, one ml sodium azide (10% stock solution) to prevent contamination, and one ml trypan blue (0.5% stock) to provide contrast. The test was performed in 50 x 12 mm plastic plates with tight lids. Five ml of diffusion medium was deposited in each plate, and wells were made (5 mm in diameter) and filled with approximately 0.05 ml of antiserum. Rabbits were used only for serotyping of different Mycoplasma, but not for diagnostic purposes.

(8) Fluorescent antibody technique (FA) (Immunofluorescent technique (IF). The capacity of a substance to absorb light energy and emit this energy at a longer wavelength is called fluorescence. Antibodies complexed to a fluorescent dye (usually fluorescein isothiocyanate) became highly fluorescent and retained their antigen specificity.

Several variations of the FA technique have been successfully utilized, but those most commonly used for diagnostic purposes were the following:

(a) Direct methods, in which fluorescein-labeled antibody was applied to a preparation containing the corresponding antigen. A smear made of the antigen (microorganisms, tissue, etc.) and the labeled antibody was placed over it. Complexing by homologous antigen and labeled antibody was revealed by fluorescent organisms when viewed by fluorescence microscope.

(b) Indirect method consisted of a two-step procedure. The first step allowed the reaction of antigen with specific antibody. If the antigen and antibody were homologous, the antibody was retained through complexing with the antigen. The second step entailed the addition of antiglobulin (an "antibody to the antibody") labeled with fluorescent dye. The antiglobulin complexing with specific antibody resulted in fluorescing the organisms.

The manipulation of the FA made this test one of the most important diagnostic techniques in modern medicine. For instance, tissue sections, tissue culture, smears from various organs, exudates, or frozen tissue

can be used to diagnose the presence of such organisms (Goldman, 1968). FA has been used in diagnosis of bacterial, viral, parasitic, and mycotic infections, as well as different strains of Mycoplasma (Goldman, 1968). Noel et al. (1964) demonstrated that indirect FA could be used to detect Mg in infected tissues. Corstvet and Sadler (1964) used indirect FA to detect Mg is tissue sections, tissue impressions and Mycoplasma colony imprints. Identification of different avian Mycoplasma colonies was accomplished by using FA (Kleven, personal communication, 1979).

σ. Cross Reactions and Nonspecific Agglutination in Serological Tests

The cross reaction between different Mycoplasma positive serum and Mycoplasma antigen was first recorded by Olson et al. (1965). When using the serum plate test, they found definite cross reaction between Ms positive serum samples and Mg antigen. Roberts and Olesiuk (1967) and Vardaman and Yoder (1969) reported similar results when they employed serum plate tests. Later on, Roberts (1970) reported non-specific agglutination reactions with Mg antigen. This cross reaction was sometimes due to partial cross reaction of Mg antigen with sera from chickens or turkeys during the early stages of Ms infection, or after erysipelas vaccination (Boyer et al., 1960; Olson et al., 1965). Various dead virus vaccines produced rheumatoid factor-like antiglobulins and induced agglutinins to Mg antigen (Roberts, 1970). Infections of chickens with Streptococcus faecalis or Staphylococcus aureus had similar reactions (Thornton, 1973). Other factors which have been suggested to cause non-specific agglutination include contaminated serum (Roberts et al., 1967), storage of serum at 4° C (Windsor and Thornton, 1973), freezing and thawing of serum (Thornton, 1965) and inoculation of the birds with sterile media (Windsor and Thornton,

1973). Kleven (1975) summarized many different attempts that have been used to eliminate the non-specific reaction.

p. Differential Diagnosis

HI test for identification of antibodies against Mg, Ms, and Mm is used routinely in the voluntary control program for chickens and turkeys within various states and as a part of the National Poultry Improvement Plan (1976).

Chicken: Mg was found to be the primary cause of CRD; other organisms frequently caused complications (Biddle and Cover, 1957). Newcastle disease and infectious bronchitis virus and/or antibodies may be present as separate entities or as part of the complicated CRD problem. Infectious coryza, fowl cholera and other Mycoplasma can be identified by cultural and serological methods (Yoder, 1978).

Turkey: Repiratory disease and sinusitis in turkeys may be due to fowl cholera, ornithosis, Ms infections, vitamin A deficiency, avian influenza A, as well as Mg (Yoder, 1978).

q. Host Immunological Response

After natural or artificial infections with Mg the antibody response was detected by RSP or TAT tests as early as a few days post infection (Adler and Wiggins, 1973; Kuniyasu, 1969). Positive results from the HI test can not be expected until approximately 2 weeks post infection (Adler and Wiggins, 1973). Therefore, most of the diagnostic laboratories employed RSP for screening and the HI for confirmation. Antibodies produced in response to Mycoplasma infections belonged to the 19S or 7S class of immunoglobulins, depending upon the nature of the antigen eliciting

their formation, the route of infection, and the homogenicity or hetrogenicity of the infections. It has been reported that immunoglobulin M (IgM) (19S, agglutinating antibody) was the first antibody to appear in chicken blood after infection with Mg regardless of the route of infection (Kuniyasu, 1969; Adler and Wiggins, 1973). The persistence of IgM in the blood varied according to the route of the infection. Antibody can be detected up to 32 days after intravenous inoculation and 77 days after infranasal inoculation (Kuniyasu, 1969). After the disappearance or declining of the initial IgM antibodies, IgG 7S immunoglobulin population started to rise and stayed high for a much longer time (Kleven, 1975). This antibody was found to be less efficient than IgM in the agglutination reactions. Roberts (1969) and Roberts and Olesiuk (1966) found that RSP were mercaptoethanol sensitive and associated only with IgM, not with IgG. The HI test was more sensitive in detecting IgG antibodies and was not able to detect IgM, the initial antibody (Roberts, 1969 and Kuniyasu, 1969). This observation led Adler and Wiggins (1973) to conclude the HI test was dependent on IgG activity exclusively. The tube agglutination test was more sensitive to detect initial antibody than the HI test. It was observed that TAT reacted with both IgG and IgM. Therefore, the RPT was able to detect the early antibody response (IgM) and the HI detected the late antibody response (IgG). Similarly, turkeys infected with Mg had an early IgM response followed by IgG (Kleven, 1975).

Roberts and Olesiuk (1966) studied, in detail, the immunologic tolerance in chicken embryos inoculated with Mg. Their results showed that chick embryos infected with Mg during early embryonic life start

producing agglutinins (IgM) at 4 weeks of age. After subsequent challenge with Mg, all chicks became serologically positive. Chicks infected at 1 day of age were all positive at 7 weeks. In summary, immunologic tolerance was not detected under these conditions. Adler et al. (1973) described the function of the bursa of Fabricius in the development of resistance and serologic response to Mg.

r. Treatment

Effective prophylactic and therapeutic drugs have been developed against avian Mycoplasma: streptomycin, oxytetracycline, chlortetracyline tylosin, erythromycin (Domermuth and Johnson, 1955; Yamamoto and Adler, 1956; Hamdy et al., 1957; Kiser et al., 1961; Yoder et al., 1961). Some strains of Mg are resistant to streptomycin, spiromycin, erythromycin, and tylosin. As described by the above mentioned researchers Tylosin is one of the most common antibodies used for treatment of mycoplasmosis in chickens and turkeys. However, constant results cannot be expected. It can be injected subcutaneously at 3-5 mg per pound body weight or administered at 2.3 g per gal. of drinking water for 3-5 days. Also, antibiotic injection of infected breeding stock to control egg transmission has been used.

s. Prevention and Control

There are several methods which have been used to establish an effective control program:

(1) Medication of breeder flock

(2) Egg dipping

(3) Egg heating

(4) Vaccination

(5) General hygienic measures

A detailed review of those control measures was cited by Yoder (1978).

2. Mycoplasma meleagridis (Mm)

This infection has only been found in turkeys. The organism was designated "N" type (N strain PPLO, H serotype) and was recognized by Adler et al. (1958) in several groups of newly hatched turkey poults with air sac lesions which were free of Mg. In contrast to Mg infected poults, the lesions tended to decrease in severity and disappear as the poults grew older. Mm was the causative agent of egg-transmitted disease of turkeys in which the primary lesion was airsacculitis in the progeny. Other clinical signs were decreased hatchability, skeletal abnormalities and poor growth performance. The organism is known to be a specific pathogen of turkeys and has no public health significance. It has been found generally that the organism is usually eliminated from the vagina of naturally infected hens within 4 to 14 weeks following removal of the source of infection (i.e., contaminated semen), while hens continuously inseminated with contaminated semen maintain a high incidence of infection (Kleven and Pomeroy. 1971b). The organism persisted for 55 to 344 days in the male phallus (Yamamoto et al., 1968). Passive antibodies (agglutinins) were detected in a high percentage of poults from infected dams and persisted for approximately 2 weeks after hatching. However, these antibodies appeared not to protect against development of air sac lesions in infected embryos (Yamamoto et al., 1966b; Mohamed and Bohl, 1968).

Hamdy et al. (1969) found that spectinomycin-lincomycin combination (2 g per gal. for 10 days) was effective in reducing airsacculitis incidence

in turkeys experimentally infected with Mm. High temperature and dipping eggs using tylosin or gentamycin was reported as a successful procedure to control the transmission cycle of Mm infections (Saif et al., 1970b and 1971). The treatment of turkey semen with antibiotics to eliminate Mm was not successful when tylosin (Kleven et al., 1971) or gentamycin (Saif and Brown, 1972) was used.

3. Mycoplasma synoviae (Ms)

This organism was first described by Olson et al. (1954, 1956) and Wills (1954) as the primary etiological agent of infectious synovitis in chickens and turkeys. The infections occurred most frequently as a subclinical upper respiratory infection. Airsacculitis developed after secondary infections occurred when combined with Newcastle disease, infectious bronchitis, or both. Ms became systemic and resulted in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys involving primarily the synovial membranes of joints and tendon sheaths and producing an exudative synovitis, tenosynovitis, or bursitis (Olson, 1978).

It has been recorded that the disease can be transmitted by eggs (vertical) or by contact (horizontal) (Olson et al., 1965). No significant attempts have yet been made to control the disease. Extensive studies on antibiotic treatment of clinically affected flocks indicated that chlortetracycline (50 to 100 g per ton of feed) given continuously will provide satisfactory control of infectious synovitis. A higher concentration of 200 g per ton of feed was needed to control synovitis after infection had occurred in the flock.

The disease has been observed primarily in growing birds 4 to 12

weeks of age in the broiler-growing regions of the United States (Olson, 1956). It has been observed in turkeys at 10 to 20 weeks of age and has been reported to be a greater problem in turkeys than in chickens (Olson, 1978). The synovial lesions were most frequent and severe in the hock region, the foot pads, and the keel bursa. In the early stages of the disease, a viscous, creamy-to-gray exudate, involving the synovial membranes of the joints, tended to become caseous, or purulent exudate developed as the disease progressed.

E. Protein A Chromotography

1. Introduction

Protein A was isolated from the cell wall of Staphylococcus aureus. It was first described in 1940 by Verwey, named protein A by Grov et al. (1964) Sjöquist et al. (1972) reported that protein A covalently linked to the peptide part of the cell wall. Later on, Movitz (1974) found that this linkage takes place within a very short time after synthesis of protein A. Protein A from Staphylococcus aureus binds specifically to the Fc region of human and other mammal immunoglobulin G (IgG) of subclasses 1, 2 and 4 (Forsejren and Sjōquist, 1966). This information has been an invaluable tool for investigating cell surface antigens and immunological reactions in vitro. Early isolation methods involved extraction of protein A from the bacteria by boiling, followed by acid and ethanol precipitation (Jensen, 1959) or by mechanical disintegration, followed by precipitation, gel filtration and electrophoresis (Löfkvist and Sjöquist, 1963, 1964). Following these procedures heterogeneous products resulted. Recently, protein A preparation method

has been improved by the introduction of lysotaphin digestion of the bacteria followed by ion-exchange chromatography on DEAE-sephadex and gel filtration on Sephadex G-100 (Sjöquist et al., 1972) and by the use of affinity chromatography on immunoglobulin G (IgG) coupled to Sepharose (Kronvall, 1973).

2. Physical and Chemical Properties

Protein A consists of a single polypeptide chain of molecular weight 42,000 containing several regions of internal homology (Sjöquist et al., 1972; Bjork et al., 1972), but no significant amounts of carbohydrate (Sjöquist et al., 1972). Sjöquist et al. (1972) indicated that protein A contains no tryptophan or cystine residues but has 4 residues of tyrosine per molecule. These tyrosines are responsible for the biological activity of protein A.

The C-terminal amino acid of the protein chain was reported to be lysine, but the amino terminal residue was "blocked" to Edman degradation and no clear identification had been reported (Sjöquist et al., 1972). The native structure of the molecule was recorded to be very stable and was spontaneously reformed after removal of denaturing agents such as 6M guanidine hydrochloride (Hjelm et al., 1975) and about 50% of the molecule was found to have an α-helical configuration.

3. Biological Properties

The most important biological property of protein A is its ability to interact with many subclasses of IgG molecules from several species, mostly mammals, such as humans, mice, guinea pigs, rabbits, and other domestic and wild animals. Kronvall and Frommel (1970) studied protein A-IgG

reaction in serum obtained from different species representing seven classes and 30 orders of living vertebrates. However, he obtained negative reactions with sera of most birds, such as penguins, ostriches, ducks, chickens, and turkeys, except paleognathus species (the primitive flightless bird, rhea) Rhea americana. Kronvall and Williams (1969) found that the precipitation reaction was specific for human subclasses of IgG 1, 2, and 4, whereas IgG 3 was non-reactive. Sjöquist et al. (1970) reported that pure protein A was able to bind 2 molecules of IgG per molecule. No other human serum proteins combined with protein A, including IgA, IgM, IgD, and IgE (Kronvall and Frommel, 1970). The protein A content of the swollen gel was reported to be 2 mg/ml and the binding capacity for human IgG was approximately 25 mg IgG/ml gel. Later on, Forsgren and Sjöquist (1966) indicated that protein A reacted with Fc H-chain structure of IgG globulin. In addition to that, since protein A reacts with Fc structures, it is capable of blocking phagocytosis by competing with polymorphonuclear leukocytes for Fc sites on the IgG molecule (Dosselt et al., 1969).

4. Applications

The specific interaction with the Fc region of IgG made protein A a powerful tool in cytochemical and immunochemical studies and in the investigation of the surface structure of cells. Protein A labeled with fluorescein isothiocyanate (FITC) has been used for immunofluorescence microscopy to demonstrate the presence of specific antigens in tissue sections. FITC-labeled protein A was found to show less non-specific reactivity with tissues and cells than FITC-labeled anti-IgG antibodies (Pharmacia, 1975).

F. Enzyme-linked Immunosorbent Assay

1. Introduction

The enzyme-linked immunosorbant assay (ELISA) was developed independently by Engvall and Perlmann (1971) and van Weeman and Schurrs (1971). Since then, many publications concerning the use of ELISA in serodiagnosis of infectious diseases - pioneered mainly by Carlsson and collaborators in Stockholm (1971) and by Voller and his associates (1971) in London - have been reported.

2. Assays, Principles, and Methods

a. Reaction mechanism and methodology.

The basic ELISA test depends on two assumptions: (1) that antigens or antibodies can be attached to a solid phase support yet retain immunological activity, and (2) that either antigens or antibodies can be linked to an enzyme and the complex retain both immunological and enzymatic activities. Antibodies or antigens were shown to be readily attached to paper discs or to plastic surfaces, such as polyvinyl or polystyrene, either chemically or by passive absorption, and still retain their activities. Voller et al. (1976) summarized the methodological aspects of ELISA, which are described as follows:

(1) Competitive method (as seen in Figure 1).

This system was described for detection and measurement of antigens and it was carried out as follows:

(a) Specific antibody was attached to solid phase, which was then washed by phosphate buffer.

(b) Test solution (fluid thought to contain antigenic

components) was then added. It was either mixed with enzyme labeled antigen, or enzyme labeled antigen was added after the starting time. Plates were then incubated.

(c) Enzyme substrate was added. The reference wells containing only enzyme-labeled antigen (step 2) showed coloration. The inhibition of that color change in the wells with test samples was proportional to the amount of antigen in the test samples.

(2) The sandwich method (Figure 2). This system was described for the detection and measurement of antigens and it was carried out as follows:

(a) Specific antibody (to the antigen to be measured) was attached to the solid phase, which was then washed.

(b) The test solution was then incubated with the sensitized solid phase for a given length of time, then was washed.

(c) Enzyme labeled specific antibody was then incubated with the solid phase, followed by washing.

(d) Enzyme substrate was added. The color change was proportional to the amount of antigen in the test solution in step 2.

The technique has been described as analogous to the immuno-radio-metric assays of Miles and Hales (1968), and has the the same sensitivity and specificity for high molecular weight antigens.

(3) Indirect method (Figure 3). This system has been used for the detection and measurement of antibodies. It was carried

FIGURE 1
The competitive method of ELISA for assaying antigen

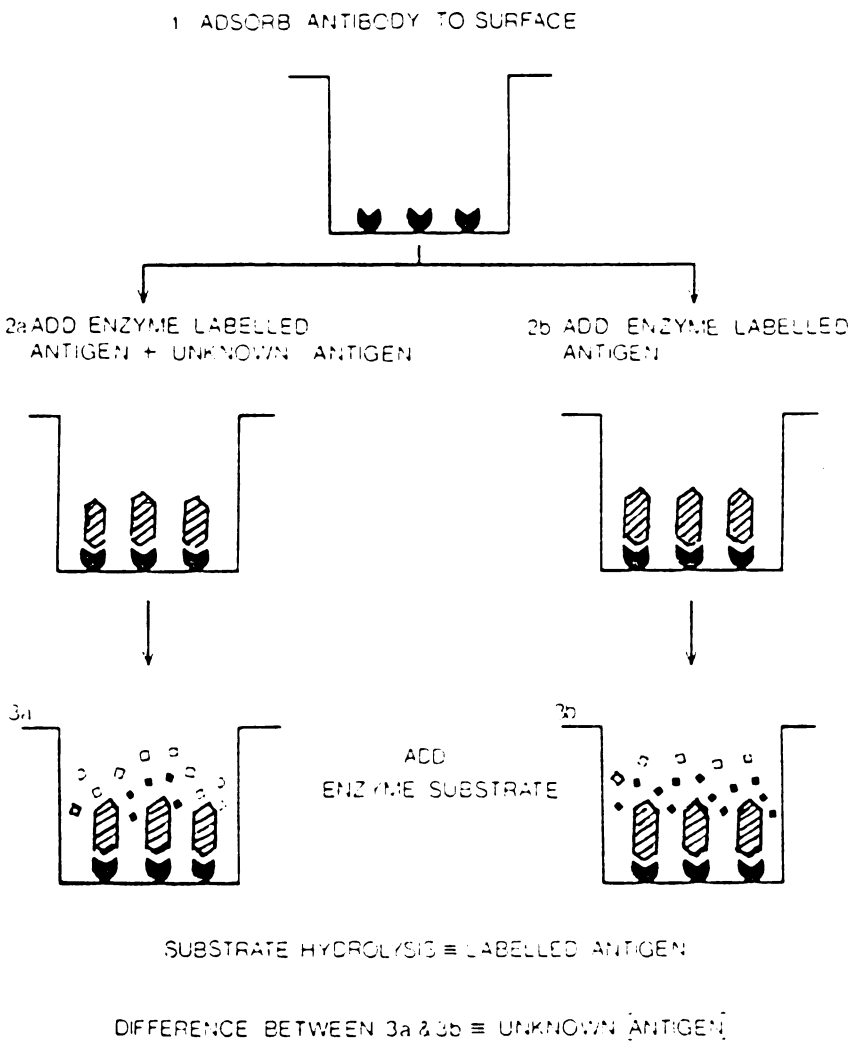


FIGURE 2
The Double Antibody Sandwich ELISA for measuring antigen

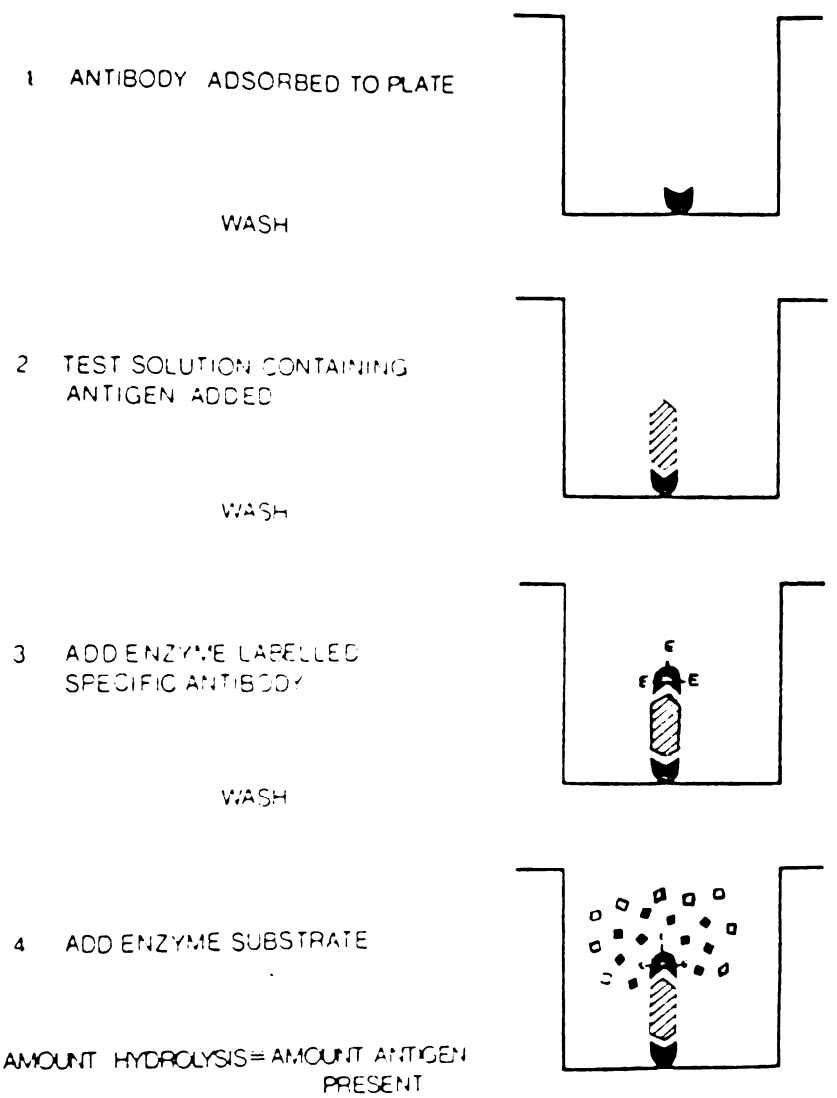
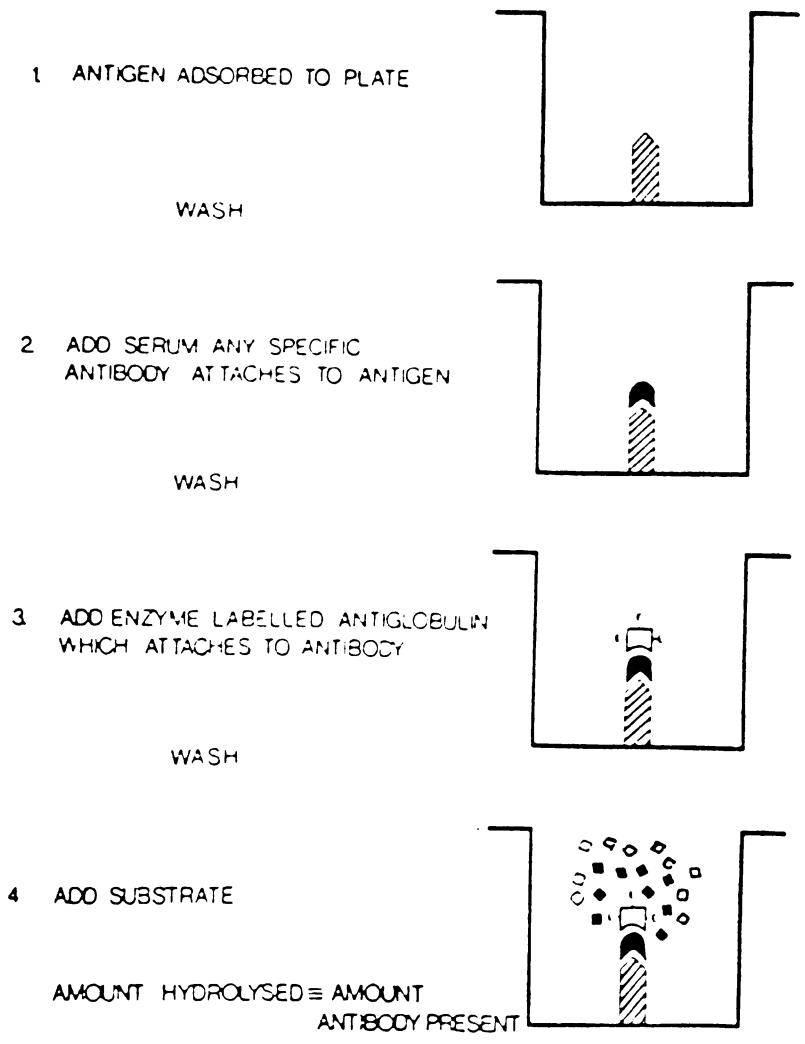


FIGURE 3
The Indirect ELISA for measuring antibody



out as follows:

(a) The relevant antigen was attached to the solid phase which was then washed.

(b) The diluted test serum was added and incubated, followed by washing.

(c) An enzyme labeled anti-species-globulin was added and allowed to react for a given time before washing.

(d) A specific enzyme substrate was added. Degradation of substrate resulted in a color change. The amount and rate of color change were related to the amount of antibody in the test serum.

b. Solid phase support

During the last few years a variety of solid phases have been used in enzyme-immunoassay particulate materials, such as cellulose, agarose, or polyacrylamide with covalently bound antigen or antibody (van Weemen, 1974). The use of a solid phase such as test tube, glass beads, or discs simplifies handling procedures. Antigen can be passively absorbed to such carriers, made of polyvinyl, polypropylene (Engvall and Perlmann, 1971), polycarbonate, glass, or silicone rubber (Hamaguchi et al., 1976a, 1976b). For large-scale enzyme immunoassays, disposable micro-titration plates were particularly convenient (Voller et al., 1974). Dipstick tests were also tried by Felgner (1977). Although various carrier materials can be used, it was essential that each new type was thoroughly tested to find the amount and reproducibility of uptake of the antigen or antibody since these variables influence the results of a test (Bidwell et al., 1977).

c. Equipment and mechanization (automation).

ELISA was carried out with only sensitized plates or tubes with antigens or antibodies. The result was detected either visually or using a spectrophotometer. Also, the technique consists of a series of additions of reagents separated by washing steps. The reagent additions were accomplished by using any of the commercially available dispensers. The mechanization of practically all steps of the assay was accomplished for macro-ELISA by Tuitenberg et al. (1977), and for micro-ELISA (microplate assays) by Ruitenberg and Brosi (1978). The automated line system included dispenser, a washer, and a spectrophotometer. These authors indicated that by using line system for macro-ELISA, as many as 4000 serum samples were processed daily with two persons. Many companies offered equipment for macro- and micro-ELISA, such as LKB Ultralab System (Stockholm, Sweden), Finnpipette (Helsinki, Finland), Olli (Kivenlahti, Finland), Organon Technika (Oss, The Netherlands) and Dynatech Companies (Alexandria, Virginia, United States). In general, reliable qualitative and quantitative determinations were achieved by macro- and micro-ELISA.

3. Preparation and Characterization of ELISA Reagents

a. Choice of the enzyme.

Schurrs and van Weeman (1977) indicated that there was no single enzyme ideal for use as a label in every conceivable ELISA. Instead, the researchers had to determine which enzyme for a particular assay system could be used. It has been recorded that factors such as turn-over number of the pure enzyme (the number of substrate molecules converted to product per enzyme site per unit of time), purity of the enzyme

preparation, and a substrate which produces stable, soluble, easily measured products would affect the sensitivity and specificity of ELISA (Ishikawa and Kato, 1978; Rubenstein, 1978). The enzyme must be reasonably cheap, available in a highly purified form, and suitable for linkage with protein. The smaller the amount of enzyme label which could be detected, the more sensitive the resulting assay would be.

b. Coupling procedures

Coupling enzymes (or protein in general) to other substances has been carried out using several different reagents. The coupling has been satisfactorily carried out using glutaraldehyde in either one step (Avrameas, 1969) or two steps (Avrameas and Ternyck, 1971) or by using periodate (Nakane and Kawaoi, 1974). The one-step method has yielded satisfactory conjugates with alkaline phosphatase and horseradish peroxidase although the ratio of enzyme to antibody varies somewhat (Avrameas, 1969). The two-step glutaraldehyde method gave conjugates in which enzyme and antibody were present in equal ratio and this led to more sensitive assays (van Weemen and Schuurs, 1974). The periodate method (Nakane and Kawaoi, 1974) produced a good yield of high molecular weight conjugates, which worked well in ELISA, as did the dimaleimide linked conjugates of β-galactosidase, which had small complexes (Kato et al., 1976b). Figure 4 shows possible mechanisms of cross-linking reactions using sodium periodate and glutaraldehyde.

c. Purification of the conjugates

Enzyme-labeled protein preparations obtained by any one of the above procedures contained not only enzyme-protein conjugates but also other

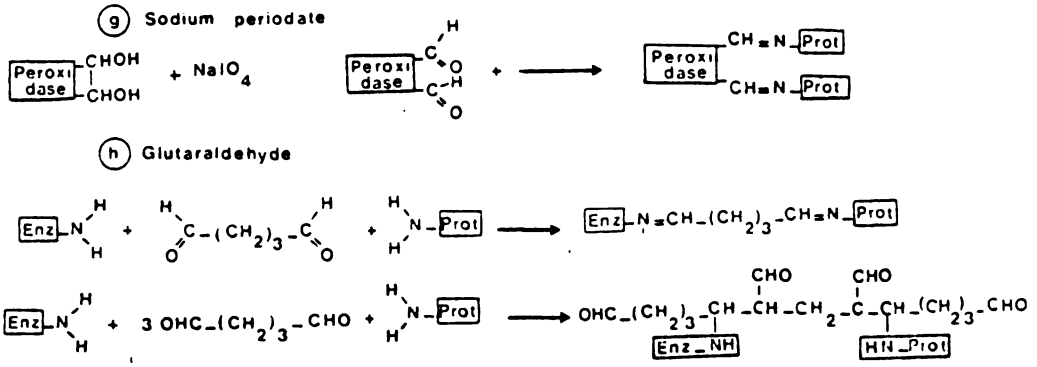


Figure 4. Possible mechanisms of cross-linking reactions

protein derivatives and reagents. Isolated conjugates gave less background and had greater specific activity than unpurified preparations. Sephadex G-200 (Avrameas and Ternyck, 1971) and Ultrogel (Polyacrylamide-agarose gel) (Boorsma and Stearfkerk, 1976) were shown to be very efficient materials for purification of the conjugate. Others, such as Sepharose (Herrmann and Morse, 1974) and Bio-Gel (Kato et al., 1976a,b) columns, gave highly purified conjugate depending on the molecular weight of both protein and enzyme. Purification of density gradient centrifugation was also performed to purify horseradish peroxidase conjugated to Fab fragments of IgG molecules (Mannick and Downey, 1973). Gel filtration permits the separation of peroxidase antibody (or Fab) conjugate from free antibody (of Fab) and free peroxidase. Ammonium sulphate precipitation allows the elimination of free peroxidase, but not of unconjugated antibody or Fab (Avrameas and Ternynck, 1971;

Avrameas et al., 1978).

d. Properties of the conjugates.

The properties of the conjugates have two parts: (1) immunological properties, and (2) enzymatic properties. The immunological properties of the immune reactant might be changed following conjugation to enzymes. This reaction resulted in loss of antigenic determinants or binding sites, or in a decreased affinity for the binding partner. In the end, this might have affected the specificity, as well as sensitivity, of the ELISA. Fab fragments of IgG molecule have been labeled with β-galactosidase to yield more sensitive ELISA than a conjugate prepared from whole IgG molecule (Kato et al., 1976a). Like the immune reactant, the enzyme might have suffered from the conjugation reaction. However, little has been known about the degree of influence on the enzymatic acitivity of the conjugate. Binding of 0.6 molecules of IgG (Kato et al., 1975a) or 4 molecules of Fab fragment (Kato et al., 1975b) to one molecule of β-galactosidase did not affect the enzyme activity after measuring the Michaelis-Menton constant (K_m) and the maximal reaction velocity (V_{max}).

e. Substrates

Substrates were usually chosen to give a colored product following enzymic degradation. For visually red ELISA tests an insoluble or partially insoluble product was acceptable, for example, 5-amino-salicylic acid (5AS) or diaminobenzidine (DAB) for peroxidase conjugates. However, for all quantitative ELISA tests, the substrate products must be soluble. For alkaline phosphatase conjugates, para-nitrophenyl

phosphate was proven to be an excellent substrate. The chromogen ABTS 2.2'-Azino-di-(3-ethyl benzothiazolin sulfonate-6) diammonium salt has been used for peroxidase (Childs and Bardclay, 1975), but this compound was expensive and its reaction products, being free radical, were unstable.

4. Characteristics of Enzyme-immunoassay

Four factors are characteristic of ELISA, as well as other enzyme-immunoassays. These factors are accuracy, specificity, sensitivity and precision. Schuurs and van Weemen (1977) explained in detail the characteristics of enzyme-immunoassays which cover ELISA. They indicated that comparisons between sensitivity of different assays were difficult because of the many variables involved. However, most authors compared EIA with other tests, such as FA, HA, HI, or RIA, and found that EIA was as sensitive (Farag et al., 1975; Ruitenberg and van Knapen, 1977; and Huldt et al., 1975) or more sensitive (Engvall and Ljungstrom, 1975; Ruitenberg et al., 1976; Ruitenberg et al., 1977).

5. Practical Application of ELISA

a. Endocrinology.

Several attempts have been made to replace current radioimmunoassay (RIA) with ELISA, but it has not yet become a generally acceptable test. Van Weemen and Schuurs (1971, 1972, 1974, 1976) set up ELISA for measuring human choriogonadotropin (HCG) and more recently Yorde et al. (1976) described a slightly different type of competitive enzyme immunoassay for measuring HCG which gave results identical to those obtained by RIA. Both insulin and thyrotropin have also been measured by enzyme-immunoassay

(Miedema et al., 1972, and Miyai et al., 1976). Smaller molecular weight hormones have also been measured, with a high sensitivity, including progesterone (Dray et al., 1975, and Gross et al., 1976), oestriol (van Hell et al., 1976) and cortisol (Comoglis and Celada, 1976).

b. Serum protein.

Immunoglobulin G was first measured using competitive ELISA methods by Engvall and Perlmann (1971), Engvall et al. (1971) and Avrameas and Guilbert (1971). Kato et al. (1975a, 1976a) used the double antibody sandwich method, which together with a fluorogenic substrate resulted in an exceedingly sensitive assay. However, other techniques such as radial gel diffusion or the more rapid nephelometric assays had adequate sensitivity to detect IgG without applying FA. ELISA could be considered a practical tool to measure IgE while other assays were not sensitive enough to quantitate the antibody (Hoffmann, 1973). Other applications of ELISA were measuring clotting factor VIII levels in the blood (Bartlett et al. 1976) and detection of snake venoms and antibodies to such venom in persons suffering from snake bite (Theakston, 1977). Measuring of C1q by ELISA was achieved by Ahlstedt et al. (1976).

c. Detection and measurement of antigens of infectious agents.

As early as 1972, ELISA was used to measure the toxins on Vibrio cholerae (Holmgren and Svennerholm, 1973), but it was not followed up for bacterial antigens until recently, when Carlsson et al. (1976) assayed antigens from Brucella, Yersinia, and Salmonella and, more recently, by Yolken et al. (1977b), who detected Escherichia coli toxins by ELISA.

More attention has been paid to viral antigen detection, and the first practical microplate ELISA system was developed by Wolters et al. (1976, 1977) for the detection of hepatitis B surface antigen. Duermeyer et al. (1977) had developed an ELISA for hepatitis A, and it appeared to be suitable for detection of the virus in fecal materials. Yoklen et al. (1977a) and Scherrer and Bernard (1971) used ELISA in detection of infantile gastroenteritis caused by rotavirus. Fungal antigen detection was achieved by Warren et al. (1977) in detection of Candida albicans antigen in infected patients.

d. Determination and assays of antibodies in infectious diseases.

The published literature indicated that the main impact of ELISA has been the measurement of antibodies. Engvall et al. (1971) first showed that antibodies were measured by indirect ELISA using enzyme-labeled antiglobulins as the indicator. Such enzyme-labeled anti-human IgG, IgM, or IgA became available commercially to be used for ELISA, so that it was not necessary for the investigator to carry out his own enzyme-antibody conjugates. Initially, Carlsson et al. (1972) set up ELISA to measure antibodies to Salmonella antigens and they found that ELISA was more sensitive than passive hemagglutination or the Widal test. Other bacterial diseases, such as Brucella, Yersinia, Vibrio cholera (Holmgren and Svennerholm, 1973), rickettsia (Halle et al. 1977) and Treponema (Veldkamp and Visser, 1975), were diagnosed by ELISA. The first large-scale use of ELISA for antibody quantitation was in the area of viral diagnosis and of epidemiological viral screening. Using ELISA in viral diagnosis in humans started in 1974, when Vollner and Bidwell described a simple microplate ELISA for detection of antibodies against

rubella infection and the results correlated with HI.

The high sensitivity of ELISA has been found to be advantageous in the assay of antibody to herpesviruses adenoviruses, coxsackievirus, cytomegalovirus, Epstein Barr viruses (Voller et al., 1976; Wallen et al., 1977) and rotaviruses (Yolken et al., 1977a), and other parasitic agents, such as Plasmodium malaria, trypanosomes, amoebae, schistosomes, Toxoplasma (Voller et al., 1976) and Trichinella (Saunders et al., 1977). Fungal diseases caused by Aspergillus, Candida (Hommel et al., 1976) and Cryptococcus (Desgeorges et al., 1979) were diagnosed by detection of specific antibodies in the patients' sera.

6. Uses of ELISA in Veterinary Medicine

ELISA has been reported to be used in diagnosis of some animal diseases using anti-animal species globulin conjugate. Saunders et al. (1977) have shown that the ELISA can be very successfully used for diagnosis of Trichinella spiralis in pigs, Babesia divergens in cattle (Purnell et al., 1976), and trypanosomiasis (Voller et al., 1976) can also be diagnosed by the ELISA method. Other diseases such as brucellosis, hog cholera (Saunders et al., 1977), calf rotavirus (Scherrer and Bernard, 1977) and Mycoplasma superpneumoniae in pigs (Armstrong et al., 1979) were also diagnosed by ELISA.

7. Uses of ELISA in Avian Medicine

ELISA has been reported to be used experimentally for detecting antibodies in chickens infected with Mycobacterium avium serotype 2 (Thoen et al., 1978) and reovirus (Slaght et al., 1978). Direct assays for detection of avian leukosis-sarcoma virus were developed by Smith et al.

(1979). There are no published reports about diagnosis of Mycoplasma infections in birds by using either direct or indirect ELISA methods.

MATERIALS AND METHODS

A. *Mycoplasma* Organisms

1. Source of the Organisms

M. gallisepticum (Mg), Adler S_6-F_{16} strain, and *M. synoviae* (Ms), WVU 853-T22 strain, were received in unknown passages in serum-enriched brain-heart infusion medium from Dr. M.Y. Seif, Ohio Agriculture Research and Development Center, Wooster, Ohio. *M. gallinarum* culture #2032 was received in lyophilized form from Dr. E.E. Ose, Eli-Lilly Research Laboratories, Greenfield, Indiana. Also, Mg was obtained in frozen form, 11th broth passage and 2nd embryo yolk culture (R#980), from Dr. H.W. Yoder, USDA, Southeastern Poultry Regional Laboratory, Athens, Georgia. All organisms were transferred and maintained in broth media, agar media and chicken egg embryos.

2. Growth and Maintenance Procedures

a. Broth and agar media: *Mycoplasma* organisms were maintained by serial subcultures in modified Frey and MSU-*Mycoplasma* media. They were subcultured in broth media twice a week. Broth to broth transfers were usually done by transferring 0.1 - 1 ml (after shaking the broth tube) with a 1.0 ml sterile pipette. These media were incubated aerobically and anaerobically with 10% CO_2 at 37° C. Subculturing broth to agar was done by streaking the agar with calibrated loops (*vide supra*) from the sediment in the broth media and plates were incubated aerobically in moist atmosphere at 37° C. Agar to agar transfers were made by the

"push block" technique. A block of agar, 1 cm^2, was cut out with a sterilized scalpel. The block of colonies was placed face down on the fresh medium and moved over the surface of the agar with the aid of a sterile forceps. Subculturing agar to broth was done by transferring a strip of agar containing colonies directly into broth media. Plates were examined for Mycoplasma colonies over a 7-day period using a microscope with oblique indirect lighting or a regular microscope at 20x or 40x magnification lens.

b. Embryonating chicken eggs: Fertile chicken eggs were obtained from the Department of Poultry Science, Michigan State University. Seven-day-old embryos were inoculated with 0.1 ml of broth culture containing Mycoplasma suspensions by using a 27-gauge, 1/2 inch needle. In most cases embryo-death occurred after 5-7 days and the yolk materials were harvested by aspiration procedures with 20-gauge, 1 1/2 inch needle.

3. Storage of the Organisms

Broth cultures containing Mycoplasma organisms were centrifuged at 35,000 xg for 30 minutes under sterile conditions (Sorvall, RC-5, super-speed refrigerated centrifuge). The sediments were diluted with 5 ml of broth media and were frozen at -20 C in 100 x 13 mm screwcap tubes. Some cultures were stored in the frozen egg yolk after the passage of organisms was lyophilized and held at 5 C. The Mycoplasma culture sediment to be lyophilized was mixed with 2 ml of broth media or stablizing solutions (SPGA) as described for rickettsia (Bovarnick, et al., 1950), see Appendix. The lyophilized cultures were sealed under vacuum. Titer and biochemical characteristics were determined for the lyophilized cultures to ascertain viability and purity at certain time intervals.

B. Culture Media

The medium which was selected for antigen production or for isolation of Mycoplasma depended on two factors: the adaptability of the Mycoplasma strain to the medium and the availability of the serum and other enriched factors. All media used required the addition of serum. Serum was inactivated for 30 minutes at 56° C before use. All media (broth or agar base) were sterilized by autoclaving before adding the serum. The other components of the medium were sterilized by filtration (see the sterilization procedures below).

1. Media for Antigen Production

Three media were commonly used for large-volume antigen production: brain-heart infusion medium (Vardmann, 1967), PPLO broth medium (Morton and Lecce, 1954) and tryptose phosphate broth medium (Hall, 1962). These media were used also in the primary isolations and for routine serial passages of seed stock.

2. Media for Isolation, Growth and Maintenance

Many kinds of media for isolation of avian Mycoplasma were described by several authors. Two media were used in our experiments for comparative studies: B-media of Fabricant (Fabricant, 1959), which was prepared with Difco beef-heart broth enriched with 10-15% horse or swine serum, and a modified Frey medium (Yoder, 1975), which was used in broth and agar forms with the addition of β-nicotinamide adenine dinucleotide (NAD).

3. A Modified Medium for Isolation and Antigen Production (MSU-Mycoplasma Medium)

This modified medium was used in isolation of Mycoplasma organisms and for antigen production as well. The Mycoplasma broth base (Gibco) was used as a base medium. The MSU-Mycoplasma formula is as follows:

Ingredients	Per Liter
Mycoplasma broth base (Gibco)	24.0 g
Maltose or Dextrose	2.0 g
L-histidine	2.48 g
Cystine HCl	0.1 g
Nicotinamide adenine dinucleotide (NAD)	0.1 g
Waymouth media	100.0 g
Tris (hydroxymethyl) aminomethane Hydrochloride	3.0 g
Penicillin	1000.0 IU/ml
Thallous acetate	0.1 g
Phenol red	0.025 g
Horse serum	50-100 ml
Water	to 1000 ml

Adjust pH to 7.8 with 20% NaOH

a. Procedures used for mixing MSU-Mycoplasma medium:

(1) Mycoplasma broth base (Gibco), maltose or dextrose (Difco), and tris buffer base (Sigma) were dissolved in 700 ml deionized distilled water (DDW) to make the base medium. Adjust the pH to 7.9 - 8.0. Addition of 3 grams of yeast autolysate has been recommended but it was not necessary because the Mycoplasma broth base contained yeast extract.

(2) Thallous acetate (Fisher) was added to the base media before

or after autoclaving. One percent solution was prepared by dissolving 0.5 gram in 5 ml DDW and sterilized by filtration

(4) Phenol red solution was prepared by dissolving 0.3 g in 300 ml DDW (dissolving by heat) and sterilized by filtration; 25 ml was added to 1000 ml medium. Phenol red wasn't added to the media for antigen production.

(5) L -histidine 1% solution was prepared by dissolving 2.48 gm in 10 ml DDW and sterilized by filtration.

(6) Horse or swine or calf sera (Gibco) were used in the preparation of different media for isolation and for antigen production. All sera were heat inactivated at 56 C for 30 minutes and centrifuged for 30 minutes at 10,000 rpm to clarify. They were then sterilized by filtration.

(7) Waymouth's medium (MD 705/1, Gibco) was added as a source of amino acids and vitamins.

(8) Penicillin G (potassium salt) was reconstituted in sterile distilled water and stored frozen: 100,000 IU/100 ml of medium was added to the medium after mixing all other ingredients. The final pH of the medium was adjusted to 7.7 - 7.8 with sterile 10% NaOH.

b. Sterilization procedures:

(1) Autoclaving referred to heating for 15 minutes at 120 C and 15 pounds of steam pressure.

(2) Filtration usually was achieved by successive passages through 20, 5.0, 3.0, 1.2, 0.45, 0.1 and 0.025 µm Millipore filters. Serum and phenol red were filtered first through No. 1 and 50

Whatman filter paper discs before passage through Millipore filters.

All liquid broth media were incubated at 37 C overnight after preparation to ascertain their sterility. They were then held at 5 C until used. Agar plates were left at room temperature overnight and then stored at 5 C.

4. Experimental Studies on the Effect of pH of Different Mycoplasma Media on the Survival of the Organisms.

a. Culture media: The broth media used to evaluate growth were brain-heart infusion medium, PPLO broth medium, tryptose phosphate broth medium, B-media of Fabricant medium, modified Frey broth medium and MSU-Mycoplasma medium. Exact formulation of each medium is detailed in the Appendix. The broth media were used to support the growth of Mg. Agar (Difco) was added at 1.5% to each broth medium to make the agar plate and that was used to determine the viable organism counts.

b. pH: The initial pH of all media was measured before inoculating the broth tube with Mg organisms. The pH of each medium was determined at selected time intervals as follows: 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52 and 56 hours and on the 3rd, 4th, 5th, 6th, 9th, 30th and 60th days post inoculation. The hydrogen ion concentration was measured in an expanded scale pH meter (Model 7415, Leeds and Northrup).

c. Determination of numbers of viable organisms: Viable bacterial count was determined by using dilution plate method (i.e., counting the total number of colony forming units in 1 ml media (CFU/ml) from each dilution). This was conducted by making a series of 10-fold dilutions of the original from 10^{-1} to 10^{-10} in sterile distilled water. One-tenth ml

from each of these dilutions was placed on a Mycoplasma agar plate (divided into two, 0.1 ml/half plate). The plates were incubated at 37 C for 7 days. The highest dilution showing any growth was recorded as the plate titer. The number of colonies presented in a plate at a given dilution was multiplied by the reciprocal of the volume of the original sample presented in a plate. For example, a plate containing a $1:10^3$ dilution contained 10^{-3} ml of the original sample, so the number of colonies was presented, multiplied by $1/10^{-3}$ or 10^3 to estimate the number of colonies per ml of the original sample.

C. Mycoplasma Antigen Production and Standardization

1. Preparation of Mycoplasma Cells

a. Cell production: MSU-Mycoplasma medium (MSU) was used for the growth of Mycoplasma organisms and for the preparation of antigen. The medium was stored at 4 C in 3 different quantities as follows:

(1) Propagation medium tube: 16 x 150 mm screw cap tubes contain 9 ml of MSU. These tubes were used for serial transfers.

(2) Seeding medium flask: 300 ml Erlenmeyer flasks contain 90 ml of broth medium.

(3) Antigen production medium flask: 2800 ml Erlenmeyer wide bottom flasks contain 900 ml of broth medium.

The medium from the storage was warmed to incubator temperature (37 C) before being inoculated. The original culture of Mg was serially transferred in the broth medium (propagation medium tube) several times before streaking on Mycoplasma agar plates. The inoculated agar plates were incubated for 7-10 days at 37 C aerobically in a moisture-rich

Atmosphere to avoid dryness of the media. Examining the colonies under a dissecting microscope with oblique indirect lighting or 20x or 40x lens power of a regular microscope showed typical 0.1-1 mm diameter fried egg colonies. Under aseptic conditions, a small block of agar containing a colony was excised and transferred into propagation tube media. After 24 hours at 37 C, the contents of the tubes were transferred into the seeding flasks, which contained 90 ml of the medium. After 24 hours at 37 C, the contents of the seed flasks were poured into 900 ml of fresh medium for antigen production. Antigen production flasks were agitated, some for 2 hours at room temperature, then incubated for 4 hours at 37 C. Other flasks were incubated at 37 C for 15 hours and then agitated for another 6 hours at room temperature. However, all flasks were agitated on a variable speed shaker oscillating in a horizontal plane approximately 92 times per minute either in a room temperature or in a walk-in incubator at 37 C. The incubation period was stopped when the pH of the medium reached 6.8, which is the optimum pH to obtain the log phase of growth. Sterility tests were routinely carried out with each passage of the growing organisms to eliminate the possibility of bacterial contamination. Cultures in which any contamination was observed were immediately discarded.

b. Cell harvesting: The antigen production media containing the organisms were centrifuged at 35,000 xg for 30-45 minutes at 5 C using Sorvall RC-5 super speed refrigerated centrifuge. The sediments were resuspended in small quantities in phosphate buffered saline (PBS) at pH 7.2 (see Appendix). The Mycoplasma cell paste sediment was homogenized in a sterile Ten Broeck tissue grinder with PBS or agitated with glass

beads and the final volume brought up to 10 ml per 1000 ml of the original production medium, thus accomplishing a 100-fold concentration of Mycoplasma cells. This stock suspension was either kept frozen at -20 C until needed or lyophilized using fresh broth media or sucrose phosphate glutamate albumin (SPGA) as preservative stabilizing solution (Bovarnic, Miller and Snyder, 1950) or proceeding to another step of standardization. The suspended antigen was diluted to a final concentration equivalent to 2X of No. 10 McFarland tube (60 x 10^8 microorganisms). This was accomplished by using a dilution procedure to establish the concentration that would provide a given percent transmission in an exciter and a galvanometer lamp. The McFarland standard tube (2X a No. 10) was diluted 1:20, which gave a 54% transmission (O.D. = 0.25 $\pm$ 0.01) reading at wavelength of 540 millimicrons (μm) using a Coleman Junior II A spectrophotometer, Model 6/20 A. The spectrophotometer was adjusted to 100% transmission with a phosphate buffer as a blank. This was matched by a series of antigen dilutions using PBS buffer to provide a density of cells to be read directly in the spectrophotometer. The antigen dilution that gave a reading of 54% $\pm$ 2 transmission was then used as equivalent to 2X No. 10 McFarland nephelometer tube. The stock antigen solution was kept frozen at -20 C.

c. Sterility test: During the process of antigen production and after thawing the antigen for further standardization, a sterility check-up proceeded for bacterial and fungal contamination. A few milliliters of the culture medium at different stages of the antigen production was inoculated into blood agar plates with brain-heart infusion and Sabouraud's dextrose agar. It was noticed that the broth media in case of contamination

changes its color from rose red to a deep red.

2. Antigen for Immunization

Mycoplasma whole cell antigen has been described in the literature as a particulate antigen. For the specific production of precipitating antisera against Mg in rabbits, Mycoplasma broth medium was enriched with rabbit serum instead of swine or horse serum. Organisms were harvested, washed twice with PBS, then homogenized as previously described. The final volume of the antigen suspension was 1/100 fold concentration of the growth medium volume (antigen production media). No preservative was added to the concentrated antigen suspension. The antigen was diluted to give 2X No 10 McFarland tube (60 x 10^8 microorganisms) using a spectrophotometer before it was injected into the rabbits.

3. Antigen for Hemagglutination Inhibition Test

Mycoplasma whole cell paste was resuspended in nonphenolized phosphate buffer at pH 7.0. Highly dense cell suspension with an absorbance reading of 0.25 at a wavelength of 540 μm at 1:10 dilution was referred to as standard hemagglutination antigen (HA antigen). An equal volume of glycerine was added and thoroughly mixed. This antigen was stored at -50 to -70 C.

4. Antigen for Enzyme-Linked Immunosorbent Assay

Preparation of soluble antigen in the form of disrupted cell suspension was used for enzyme-linked immunosorbent assay. The preparation of Mycoplasma cells was basically the same as described before. The Mycoplasma cell paste was harvested and washed twice with PBS, pH 7.2. The harvested cells were then resuspended in enough PBS to reach a 1/100 fold

concentration of the original volume. The total protein concentration in the antigen suspension was determined by using Biuret method (Goznall et al., 1949) and crystalline bovine albumin as a standard protein solution. Absorbance reading was recorded on a Coleman Junior IIA spectrophotometer Model 6/20 A at 560 μm. The total protein content of the antigen averaged 5.7 mg/ml. The antigen was solubilized by using sodium dodecyl sulfate (SDS) (Sigma Chemical Co.). The antigen was diluted with PBS, pH 7.2, to make a 3 mg/ml suspension and then incubated with SDS (1 mg of detergent/mg of protein) for one hour at 37 C. The antigen was then dialyzed against PBS, pH 7.2, containing 1 mM ethylenediaminetetraacetate (EDTA) (Fisher Chemical Co.) or other suitable chelating agent (to prevent reaggregation of the solubilized antigen on removal of SDA). The extract was centrifuged (20,000 xg for 30 minutes at 4 C). The supernatant fluid was removed and protein concentration was determined using Biuret method. The antigen was diluted and coated with 0.1 M carbonate-bicarbonate buffer, pH 9.6.

D. Preparation of Fluorescent Antibody Reagents

1. Production of Rabbit Antisera

New Zealand rabbits were obtained from the Laboratory Animal Care Service (LACS), Michigan State University, East Lansing, Michigan. The weight of each rabbit was approximately 6 lbs. (2.7 kg) and the age was 3 months. They were fed Purina Rabbit Chow (Purina Company) and were kept individually in a metal cage in the isolation room. The cage size was as recommended by the LACS, 3.5 square feet per rabbit for floor and 14 inches high. Serum was collected from rabbits prior to immunization

and examined for the presence of agglutinating and precipitating antibodies. Three rabbits were inoculated according to the immunization schedule as shown in Table 1. One rabbit was kept uninoculated as a control.

TABLE 1. Immunization schedule for rabbits

Days	Route and Amount of Injection		
	Rabbit I	Rabbit II	Rabbit III
1	SC 1.0 ml	IV 0.5 ml	SC .25 ml + 0.25 IFA
7	IM 0.5 ml	SC 1.0 ml	SC 0.5 ml + 0.5 IFA
14	FP 2.0 ml	IV 2.0 ml	IV 2.0 ml
21	IV 3.0 ml	IV 3.0 ml	IV 3.0 ml
28 and every other week for 3 weeks	IP 5.0 ml	IP 5.0 ml	IP 5.0 ml

SC = subcutaneously
IM = intramuscularly (hindquarter)
FP = intrafoot pad
IV = intravenously
IP = intraperitoneally
IFA = incomplete Freund adjuvant

Rabbits I and II were injected with Mycoplasma bacterial suspension without adjuvant. One ml of the bacterial inoculum contained 60 x 10^8 microorganisms. Rabbit III was injected with combined Mycoplasma and incomplete Freund adjuvant (Difco). Rabbits were bled (25-40 ml each bleeding) one week after the third injection from the marginal ear vein and every other week for 8 weeks after the first IP injection. The rabbits were terminally bled by exsanguination. The antiserum was dispensed in 2 ml amounts into

screwcap vials and stored at -20 C. The antibody titer in serum was determined in the three inoculated rabbits as well as the control one by the HI and TA tests.

2. Production of Chicken Antisera

Two hundred male 1-day-old SCWL from a Mycoplasma-free breeder flock, unvaccinated for Marek's disease, were obtained from the Rainbow Trail Hatchery, Inc., St. Louis, Michigan. The chickens were maintained in metal cages and isolated in separate rooms at the Poultry Teaching Research Center, Department of Poultry Science, Michigan State University, East Lansing, Michigan. At 4 weeks of age, the birds were divided into 5 groups, 40 birds each group, according to the route of infections. Birds were infected with live Mg organisms prepared at the Avian Microbiology Laboratory, Michigan State University; each 1 ml of the Mycoplasma suspensions contained 4×10^{10} organisms. The dose of the infections was designated as follows: 0.2 ml intravenous, 0.2 ml subcutaneous, one drop intranasal, 0.5 ml intraperitoneal inoculation. The last group of birds was either sharing the room with the infected birds in separate cages or sharing the same cages with the infected birds. Two to four inoculations were administered to the chickens at weekly intervals. Serum samples were collected from some of the chickens before inoculation and specific antibody titers for Mg and Ms were determined by HI and TA tests. Also, the mortality was recorded. The chickens were bled from the wing vein 4 weeks postinoculation and every other week for another 4 weeks. They were terminally bled by exsanguination. Tissue samples were taken for histopathology and isolation of Mycoplasma organisms was achieved by using modified Frey Mycoplasma and MSU-Mycoplasma medium.

3. Conjugation of Rabbit IgG Fraction with Fluorescein Isothiocyanate

a. Fractionation of rabbit antisera with ammonium sulfate: fractionation of rabbit antiserum with ammonium sulfate was conducted by Herbert method (1971) with some modification. The method is summarized as follows:

(1) Five ml of 70% saturated ammonium sulfate $(NH_4)_2SO_4$ was slowly added (dropwise) to a 5 ml serum sample with constant stirring. Ajust the pH of the suspension to 8.0 with 1N NaOH.

(2) The suspension was agitated on an automatic shaker for 2 hours in order to avoid mechanical trapping of the serum components other than gamma globulin and the mixture was allowed to stand at 4 C for 24 hours. The precipitated globulin was packed by centrifuging at 4 C for 30 minutes at 1440 xg using a Sorval refrigerated superspeed centrifuge.

(3) The supernatant fluid was poured off and the precipitate was redissolved in 5 ml of borate buffered saline (BBS), see Appendix.

(4) The globulin was reprecipitated as described in steps 1 and 2 and centrifuged immediately as before. The supernatant was discarded and resuspended as in step 3.

(5) The globulin fraction was precipitated for the third time and resuspended as in step 4.

(6) The globulin fraction was dialyzed against frequent changes of 0.85% NaCl solution, pH 8.0, at 4 C until sulfate was no longer detected in the dialysate after overnight use. The presence of sulfate was determined by adding a small volume of saturated barium chloride ($BaCl_2$) solution to an equal volume of the well mixed saline dialysate. No cloudiness resulted, which meant that the globulin solution was considered

to be substantially free of sulfate. This gamma globulin fraction was considered to contain mostly IgG.

The isolation of immunoglobulin G (IgG) from the rest of gamma globulin was experimentally approached by employing protein A (Pharmacia) instead of ammonium sulfate.

b. Protein determination of gamma globulin fraction: The nitrogen content of the crude gamma globulin and the total mg protein/ml of globulin fraction were determined by applying the Biuret method (Goznall et al., 1949). Also, Bio Rad protein assay (Bio Rad Laboratories, Richmond, California) was used. Standard solution of bovine gamma globulin was used for preparation of the standard curve for both methods.

c. Conjugation of globulin with fluorescein isothiocyanate: Rabbit gamma globulin fraction was conjugated with fluorescein isothiocyanate (FITC) (BBL, Baltimore, Maryland) according to the methods described by Riggs et al. (1958) and Herbert et al. (1977).

The procedures used were as follows:

(1) Protein concentration of the globulin fraction was determined. Rabbit I, II serum contained 38 and 44 mg protein/ml serum simultaneously Rabbit II gamma globulin fraction was used in most of the experiments. Ten ml of globulin was used and the total protein content of the globulin was 10 x 44 = 440 mg.

(2) Ten ml of globulin fraction was placed in a flask and 5 ml of 0.2 M Na_2HPO_4 was added slowly with gentle mixing. The pH was brought to 9.5 by dropwise addition of 0.1 M Na_3PO_4. The final volume was brought to 20 ml by using 0.85% NaCl.

(3) The fluorescein protein ration used in the experiments was

17.6 ratio (FITC ug/mg protein). So the weight of FITC used was 440 x 17.6 = 7.74 mg. FITC solution was prepared by using 10 ml dye solution to 1 ml of globulin (10 ml x 10 ml globulin = 100 ml FITC solution). FITC (7.74 mg) was dissolved in a volume of 0.1 M Na_2NPO_4 equal to 3/4 of the final volume desired (75 ml). The pH was adjusted to 9.5 with 0.1 M Na_3PO_4 and the final volume was brought up to 100 ml.

(4) The globulin fraction was placed in a dialysis tube (Union Carbide) and the sac was tied so that there were no air bubbles. The dialysis tube was placed in the FITC dye solution so that the globulin was completely submerged. The reaction was allowed to proceed at 25 C for 6 hours.

(5) The dialysis tube was removed and rinsed briefly under cool tap water to remove all exterior dye.

(6) To remove the unreacted fluorescent material (UFM) the labeled globulin was dialyzed against phosphate buffered saline (PBS), pH 7.6, at 5 C for 24 hours. The low temperature of the buffer was to reduce the labeling rate of the unreacting dye in the dialysis tube. Dialysate PBS was used in a large volume and changed frequently. The removal of UFM was completed when no fluorescence was visible in an aliquot of the dialysate observed in the dark under a Wood's Lamp (ultraviolet). The labeled globulin was centrifuged and the precipitate was removed.

(7) Merthiolate was added as a preservative in a final concentration of 1:10,000 to some of the prepared conjugate. The conjugate was stored in 1 ml quantities in small screw cap tubes and kept in liquid form at 5 C.

4. Fixation Procedures for Direct and Indirect Methods

The avian Mycoplasmas used in this study were Mg, Ms, Mm. These organisms were grown on Mycoplasma broth and agar media as mentioned before. The rabbit antiserum was prepared from Mg only. The Mycoplasma antigens for the three different species mentioned above used in these experiments were broth culture sediments and their agar colonies. A thin smear of bacteria was prepared on microscopic slide (clean slide by acetone) from broth culture sediments or bacterial colonies. The smear was air dried at 37 C on slides prior to being subjected to various fixing agents and conditions. Dry heat fixation for 1 minute or exposure to 95% ethanol for 10 minutes were used in this study. Also, agar blocks about cm^2 with colonies of Mycoplasma were cut from the agar plates with a sterile scalpel blade. The agar blocks were placed on a microscope slide with colonies up. The colonies were fixed by using the hot water fixation technique of Clark et al. (1963). Hot distilled water fixed the colonies on the slide as it dissolved away the surrounding agar, thus leaving colonies relatively free of agar substances and thereby the nonspecific fluorescence was reduced.

5. Direct Staining

The basics of the staining procedure used in this study were those of Coons and Kaplan (1950) and Marshal et al. (1958). Rabbit antisera conjugated to FITC was freshly diluted 1:10 and 1:20 with 0.85% NaCl, pH 7.0. The area of fixed antigen on the slide preparation was covered with a few drops of the conjugate and the slide was placed on a moist towel in a covered tray and kept at 37 C for 20 and 40 minutes. The slide was then washed with PBS twice and was stored in fresh PBS for

2-3 minutes. Excess fluid was drained and the slides were mounted in 10% buffered glycerol, pH 7.2 (glycerol 9 parts, PBS 1 part), and a cover slip was added. The specificity of the reactions was determined by using 2 control systems labeled normal rabbit serum and homologous and heterologous labeled anti-serum positive for Mg. Smears of Ms and Mm were stained by the direct method to show the specificity of labeled antibody against Mg organisms.

6. Microsocopy

Fluorescence observations were made with Carl Zeiss photomicroscope G 40 - 430 (W. Germany) by using a 40 X high dry Neofluor lens and an epi-illuminator Fluorescein. Fluorescence was excited with a super pressure mercury lamp HBO 200 w/4 which served as a radiation source. The transmission curve for the light source is shown in Figure 5 (Appendix). Permanent and exciter filters, combinations BG 12 and BF 004100 respectively, were used as ideal filter sets in these experiments. The transmission curve for the exciter and barrier filters is shown in Figure 6. Photography was performed with Kodak (Ektachrome, 100 Tungsten filter using the Leica Camera.

7. Indirect Staining

A thin smear of Mg organism was prepared and fixed on a microscopic slide as described before. A few drops of 1:10 rabbit unconjugated antiserum for Mycoplasma was added to the slide and allowed to stand in a moist atmosphere at 37 C for 20 minutes, then washed with PBS. The slide was stained with FITC - labeled goat anti-rabbit IgG. The stained slide was kept in a moist chamber at 37 C for 20 minutes, then was rinsed

with PBS, pH 7.2. The slide was mounted with a coverslip after the addition of 10% buffered glycerol. Control slides were prepared as in direct staining methods.

E. Immunochemical Studies on Purification of Chicken and Rabbit IgG Using Protein A-Sepharose Chromatography

1. Source of Protein A

Protein-A-Sepharose CL-4B for chromatography was purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey. Protein A initially was isolated from the cell wall of Staphylococcus aureus and covalently coupled to Sepharose CL-4B by the cyanogen bromide method. One g of freeze-dried powder was reported to be equivalent to approximately 3.5 ml of swollen gel (Pharmacia Fine Chemical Publication, 1978).

2. Isolation and Purification of Rabbit IgG

Normal and immune serum were obtained from the same group of rabbits which had been used in the immunization experiments. Protein concentration was determined by Biuret method. Immune serum from rabbit I (immunized with Mg) containing 38 mg protein/ml and normal serum from rabbit IV containing 26 mg protein/ml were used in these experiments. Part of the serum (normal and immune) was used to recover IgG fractions by successive precipitations with ammonium sulfate and recycling through Sephadex G-200 with phosphate buffer saline pH 7.2. The IgG fraction recovered from the column was dialyzed extensively for 3 days at 4 C with 0.85% NaCl pH 8.0.

Furthermore, rabbit IgG was isolated from both normal and immune

sera using column chromatography on Protein-A-Sepharose (Kessler, 1975). Serum (10 ml) was applied to a column chromatography (1.1 x 4 cm) equilibrated at 4 C with 0.1 M sodium phosphate, pH 7.0. The column was washed with approximately 10 volumes of equilibration buffer. The IgG fraction (35-70 mg) was then eluted with 1 M acetic acid and the pH was adjusted with concentrated NH_4OH to be 7.0. The neutralized IgG fraction was dialyzed overnight at 4 C against 500 ml of 0.1 M sodium phosphate, pH 7.0. The buffer was changed twice during the period of the dialysis. The absorbance of the eluates of 0.1 M sodium phosphate and 1 M acetic acid were measured at 280 nm using Hitachi Perkin Elmer 139-spectrophotometer (Coleman Instrument Division).

3. Immunodiffusion Tests for Rabbit Serum

The method of immunodiffusion (Ouchterlony) was used in these experiments as described basically by Ouchterlony (1968). Lines of precipitations were formed as a result of antigen-antibody reactions. The numbers of the precipitating band and the reaction of identity represented the minimum numbers of homologous antigen-antibody systems. The two experimental studies were conducted to compare the efficiency of protein A to purify and isolate rabbit IgG to that of the ammonium sulfate precipitation method.

First study: The center well contained 0.1 ml of goat anti-rabbit serum and the surrounding wells contained eluates washed by 0.1 M sodium phosphate, 1 M acetic acid and ammonium sulfate precipitates as shown in Figure 16 (a,b).

Second study: 0.1 ml of goat anti-rabbit IgG was put in the center well and 0.1 ml of different eluates washed by 1 M acetic acid, which has higher absorbance reading than 2.0 at 280 nm was added to the other

surrounding wells. Also, 0.1 ml of ammonium sulfate precipitates was added to several wells surrounding the center, as shown in Figure 17.

4. Isolation and Purification of Chicken IgG

Chicken normal and immune sera were obtained from the same group of chickens used in the immunization experiments. The sera were delipidated by centrifugation at high speed as 2700 xg at 4 C for 20-30 minutes and the lipid on the surface was removed by wooden stick or by Pasteur type pipette. Also, precipitation of ß-lipoprotein in the serum by certain bivalent cations such as managanese chloride, cobalt chloride and potassium bromide (Burstein and Praverman, 1957), was applied in these experiments for the purpose of delipidation.

The IgG fraction was recovered by successive precipitation with Na_2SO_4 and recycling through Sephadex G-200 with borate buffered saline (BBS), pH 8.2. The eluate fraction was dialyzed extensively against PBS for 2 days. Protein concentration was estimated by measuring the absorbance at 280 nm and using bovine gamma globulin to draw the standard curve. Protein A Sepharose (Pharmacia Fine Chemicals) was used to isolate IgG fraction by the same method which has been described previously with rabbit serum. The absorbance readings of 0.1 M sodium phosphate and 1 M acetic acid elutes were measured at 280 nm.

5. Immunodiffusion Tests for Chicken Serum

Immunodiffusion experiments were conducted employing Ouchterlony methods (Ouchterlony, 1968). Several studies were designed to determine the specificity of the eluates.

First study (Figure 19 a,b): 0.1 ml of rabbit anti-chicken globulin

(Rb-glb) was put in the center well, as shown in Figure 19a. One-tenth ml of different eluates washed by 0.1 M sodium phosphate (A-NP) which has higher absorbance readings than 2.0 by spectrophotometer at 280 nm, as shown in Figure 18. In the same study (Figure 19, b) 0.1 ml of Rb-glb was put in the center well against 0.1 ml of the following: IgG fraction purified by ammonium sulfate (AS), 1 M acetic acid washed eluate using protein A-Sepharose (A) and control well contained chicken IgG fraction (C-IgG).

Second study (Figure 20 a,b): One-tenth ml of rabbit anti-chicken IgG (Rb-G) was placed in the center well. One-tenth ml of 0.1 M sodium phosphate washed eluate (A-ND), which has a higher absorbance reading than 2.0, and control well contained 0.1 ml of chicken IgG fraction (C-IgG), were placed in the surrounding wells, as shown in Figure 20a. Furthermore, another Ouchterlony agar plate was designed in which the center well contained 0.1 ml of Rb-G and the surrounding wells contained the following: 0.1 ml of 1 M acetic acid washed eluate of protein A-Separose (A), 0.1 ml of IgG fraction purified by ammonium sulfate (AS) and 0.1 ml of chicken IgG fraction (c-IgG) as a control well, as shown in Figure 20b.

Third study (Figure 21 a,b): This study was designed to compare the activity of the contents of the eluates of protein A-Sepharose column chromatography washed by 1 M acetic acid for rabbit and chicken serum.

a. One-tenth ml of protein A-Sepharose, 1 M acetic acid washed eluate of rabbit serum (Prt. A-Rb IgG-frct) was placed in the center well. One-tenth ml of normal rabbit serum (NRS), goat anti-rabbit serum (Gt-ant RS) and goat anti-rabbit IgG (Gt-ant RIgG) were placed in the surrounding wells, as shown in Figure 21a.

b. One-tenth ml of protein A-Sepharose, 1 M acetic acid washed eluate of chicken serum (Pr A Ck IgG-frct) were placed in the center well and 0.1 ml of the following were in the surrounding wells: normal chicken serum (NCS), rabbit anti-chicken globulin (Rb-ant Cglb) and rabbit anti-chicken IgG (Rb-ant CIgG), as shown in Figure 21b.

6. Plasminogen and Their Affinity to Protein A

One-tenth ml of rabbit anti-chicken plasminogen (Rb-PL) was placed in the center well. One-tenth ml of different eluates washed by 0.1 M sodium phosphate (A-NP), which has a higher absorbance reading than 2.0. The control well contained 0.1 ml of chicken plasminogen (PL), as shown in Figure 21a. The Rb-PL and PL were obtained from Dr. E. Smith, USDA Regional Poultry Research Laboratories, East Lansing, Michigan. In another experiment, as shown in Figure 21b, 0.1 ml of Rb-PL was added to the center well and 0.1 ml of the following was added to the surrounding wells: different eluates of protein A-Sepharose washed by 1 M acetic acid (A), chicken IgG fraction purified by ammonium sulfate method (AS) and chicken plasminogen (PL) as a control.

F. Mycoplasma-Enzyme-Linked Immunosorbent Assay (MYCO-ELISA)

1. Antigen Production, Harvesting and Standardization

Mg antigen was prepared from the whole bacterial cell and from disrupted cell suspension, as described before in the chapter on antigen production and standardization. The procedures of antigen preparation are summarized as follows: Mg organisms were grown in MSU-medium and the

cell paste was harvested, washed and protein concentration of the cell suspension was determined. The antigen (cell suspension) was solubilized by SDS to disrupt the plasma membrane, then the suspension was dialyzed against PBS containing 1 mM ethylenediaminetetraacetate (EDTA). The protein concentration of the antigen was determined by spectrophotometer. The antigen was diluted and coated by 0.1 M carbonate-bicarbonate buffer, pH 9.6.

2. Source of Serum Samples

Blood from naturally and experimentally infected chickens was collected and sera were separated. Some sera were inactivated by heat at 56 C for 30 minutes and stored at 5 C until used and other sera were kept frozen at -20 C. Positive and negative serum references for avian Mycoplasma were obtained from the National Veterinary Service Laboratory (NVSL), Ames, Iowa. All serum samples were tested for antibody titer against Mg and Ms by the HI method before using for MYCO-ELISA test.

3. Preparation of Horseradish Peroxidase Conjugate

The conjugate used was IgG fraction of rabbit antiserum to chicken IgG labeled with horseradish peroxidase enzyme (HRP) Type VI, Sigma Chemical Co., St. Louis, Missouri. The conjugate was prepared by the two-step glutaraldehyde method (Avrameas et al., 1978) with modification. Ten ml of HRP was dissolved in 0.2 ml 1% glutaraldehyde solution in 0.1 M phosphate buffer, pH 6.8, and the preparation was incubated for 24 hours at room temperature. Five mg of rabbit anti-chicken IgG (5 mg/ml) was added to the peroxidase solution. Two-tenths ml of 0.5 M carbonate-bicarbonate buffer, pH 9.5, was added and kept at 4 C for 24 hours.

0.1 ml of 1 M lysine solution, pH 7.0, was added and kept at room temperature for 2 hours. The mixture was dialyzed overnight against PBS, pH 7.0, at 4 C. The conjugate was filtered through a sterile Millipore membrane (0.22 µm). An equal volume of glycerol and bovine serum albumin (BSA) (5 mg BSA/ml) were added. The stock conjugate was stored in the dark at 4 C until used. The conjugate remained viable for 10 weeks when preserved in this manner. The use of optimum dilution of rabbit anti-chicken IgG conjugated to HRP is essential for minimizing nonspecific absorption to the control wells uncoated with antigen.

Initial experiments were conducted to standaradize the conjugate. Conjugate dilutions of 1:50, 1:00, 1:200, 1:300, 1:400, 1:600, 1:800 and 1:1000 were used with serum dilution of 1:50 and 1:100 and antigen concentration of 90 mg/ml and 180 mg/ml.

4. Preparation of the Substrate

2,2'-Azino-di-3-ethyl-benzthiazolin-sulfonate (ABTS), $(c_{18}H_{16}N_4O_6S_4\text{-}NH_4)_2$, MG 5487, substrate was obtained from Boehringer Mannheim GMBH, Indianapolis, Indiana. Preparation of the substrate was done as follows: 100 µl of ABTS Stock solution (22 mg/ml) was added to 20 ml of 0.1 citrate - 0.2 M phosphate buffer, pH 4.0, containing 20 µl of freshly prepared 0.3% hydrogen peroxide (Saunders et al., 1977). The substrate was prepared just before adding it to the wells. The ABTS stock solution should be kept at 4 C in the dark at all times.

5. MYCO-ELISA Protocol

The indirect microtiter ELISA was performed as described by Engval and Perlmann (1971) and Voller et al. (1976). This procedure is

summarized in Figure 7. The MYCO-ELISA procedure is illustrated step by step in Figure 8. Two hundred µl volumes of ELISA-Mg antigen diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6, were added to each well of the polystyrene substrate plates. The plates were incubated overnight at 4 C. The plates were washed three times in PBS, pH 7.4, containing 0.05% Tween 20. The buffer consisted of NaCl, 8.0 g; KH_2PO_4, 0.2 g; $Na_2HPO_4 \cdot 12H\ O$, 2.9 g; KCl, 0.2 g; and Tween 20, 0.5 ml, as a detergent. The buffer may be stored at 5 C for 4 weeks. The procedure for washing the plates consisted of shaking out the buffer in the wells, refilling the wells with buffer, and then allowing this to stand for 1 minute.

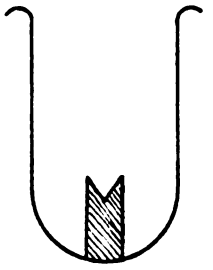
The process was repeated two more times for 1 minute per washing. Two hundred µl of serum sample, diluted with PBS, was placed in the appropriate well. Each serum sample was tested in duplicate. The plates were incubated for 2 hours at room temperature (22-24 C) in a moist chamber or incubated at 37 C for 1 hour. The plates were then washed 3 times with PBS-Tween 20 washing buffer, as described previously.

To each well, 200 µl of freshly prepared diluted conjugate HRP-rabbit anti-chicken IgG was added and incubated at room temperature for 2 hours in a moist chamber. The plates were again washed three times and 200 µl of ABTS substrate (freshly prepared) was added. After 30-45 minutes, or after the formation of green color in the positive serum control, 50 µl of 0.45% hydrofluoric acid (HF) was added to each well to stop the hydrolysis. The plates were then agitated well for 1-2 minutes. The positive result is indicated by the formation of green color.

For quantitative measurement of the antibody level, the intensity

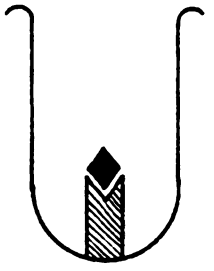
Figure 1 PRINCIPLE OF MYCO-ELISA

1. Mycoplasma antigen adsorbed to wells.



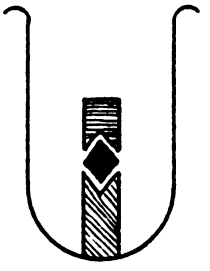
WASH

2. Serum sample is added, specific antibody, if present, will bind to the antigen (Ag-Ab).



WASH

3. Enzyme-labeled antiglobulin is added, it will attach to Ag-Ab.



WASH

4. Substrate is added

Amount substrate hydrolyzed (color change) = amount of antibody in serum sample.

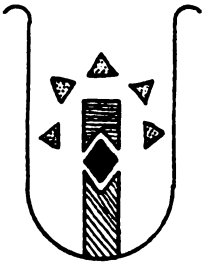
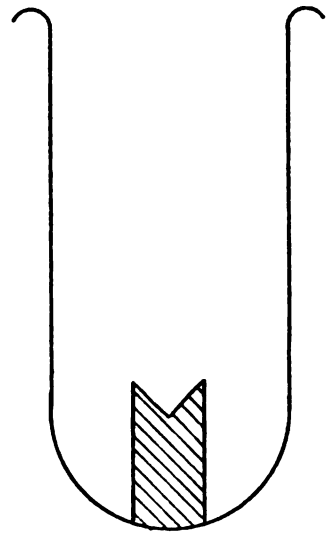


Figure 8. MYCO-ELISA PROCEDURE

1. ADD 200 µl OF MG ANTIGEN (Ag) INTO EACH WELL.
   ANTIGEN IS ADSORBED TO THE WELL SURFACE.

   WASH WITH PBS + 0.05% TWEEN 20
   THREE TIMES, THEN SHAKE DRY.



2. 200 µl OF DILUTED TEST SERUM IS ADDED INTO THE APPROPRIATE WELL. EACH SERUM IS TESTED IN DUPLICATE. INCUBATE AT ROOM TEMPERATURE FOR 2 HOURS.

WASH WITH PBS + 0.05% TWEEN 20 THREE TIMES, THEN SHAKE DRY.

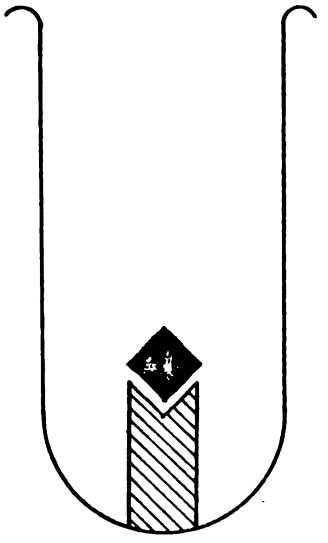


Figure 8 continued

3. 200 µl OF FRESHLY PREPARED HORSERADISH PEROXIDASE CONJUGATED TO IgG FRACTION OF RABBIT ANTI-CHICKEN IgG IS ADDED TO EACH WELL. INCUBATE AT ROOM TEMPERATURE FOR 2 HOURS.

WASH WITH PBS + 0.05% TWEEN 20 THREE TIMES, THEN SHAKE DRY.

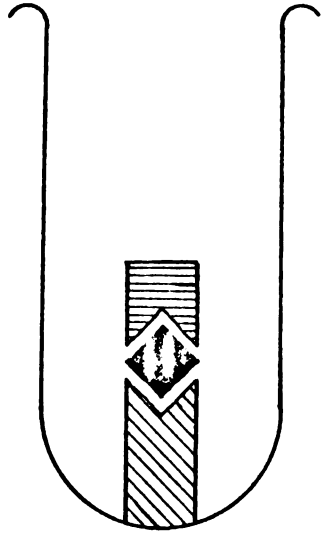


Figure 8 continued

4. 200 µl OF FRESHLY PREPARED ABTS SUBSTRATE [2,2' AZINO-DI-(3-ETHYL-BENZTHIAZOLINE SULFONATE)] IS ADDED TO EACH WELL.

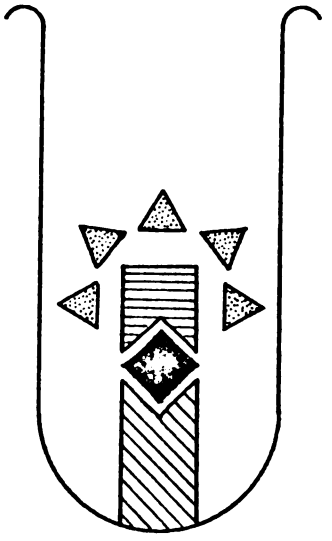


Figure 8 continued

5. AFTER 30 MINUTES OR AFTER THE POSITIVE REFERENCE SERUM CONTROL HAS COMPLETED COLOR CHANGE, 25 µl OF 0.49% HYDROFLOURIC ACID (HF) IS ADDED TO EACH WELL TO STOP THE HYDROLYSIS.

SUBSTRATE HYDROLYSIS (GREEN COLOR DEVELOPS) IS PROPORTIONAL TO THE AMOUNT OF ANTIBODY IN THE SERUM SAMPLE.

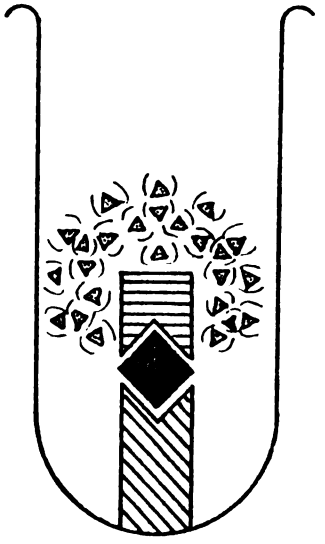


Figure 8 continued

of the reaction of the serum-control antigen reaction, measured by absorbance reading, is subtracted from the absorbance-value of the test serum sample. The blank is the antigen control wells, which do not contain serum.

RESULTS

A. Cultural Media

1. Growth and Survival of Mg in Different Broth Media

Six different Mycoplasma media were used either for antigen production or for isolation of the Mg organisms. Brain-heart infusion medium (BHI), PPLO medium (PPLO), tryptose phosphate broth medium (TPB), B-medium of Fabricant (BMF) and modified Frey media (MFM) were compared to the new formula, MSU-Mycoplasma media (MSU). The growth curve of Mg in four different medium formulas by hours or day post-inoculation (PI) are illustrated in Figures 9 and 10. The total bacterial count was calculated in the $\log_{10}$ number of colony forming units per ml (log No CFU/ml) of the culture. MSU-Mycoplasma medium gave the highest CFU/ml after 24 hours PI, which was 3.9×10^{12} CFU/ml. The lowest bacterial count was recorded for PPLO and TPB medium, which was 3×10^{6} and 2×10^{6}, respectively, at 24 hours PI. The total CFU/ml for modified Frey medium at 24 hours PI was 1.2×10^{10} CFU/ml. The PPLO medium and TPB medium gave similar bacterial counts in the first 56 hours PI at each reading. They gave the highest bacterial count at 32 hours PI, which was 0.3×10^{7} and 0.4×10^{7} CFU/ml for PPLO anad TPB media, respectively. The total bacterial count for all media declined after 32 hours PI, as shown in Table 3 and illustrated in Figures 9 and 10. The bacterial count at 30 and 60 days was 0.0 for PPLO and TPB media. Modified Frey medium gave total bacterial count of 0.1×10^{1} CFU/ml at 30 days

TABLE 2. Comparative study on the total bacterial count and the pH of different Mycoplasma media

MEDIA		Before Moalite	4 hours	8 hours	12 hours	16 hours	20 hours	24 hours
MSU	pH	7.8	7.8	7.8	7.7	7.6	7.4	7.3
	CFU	-	$2x10^3$	$1.7x10^6$	$3.0x10^8$	$2.0x10^{10}$	$3.4x10^{11}$	$3.9x10^{12}$
Modified Frey Medium	pH	7.8	7.8	7.6	7.5	7.2	6.6	6.3
	CFU	-	$1.8x10^3$	$1.4x10^4$	$0.9x10^7$	$1.3x10^9$	$1.6x10^{10}$	$1.2x10^{10}$
PPLO	pH	7.6	7.5	7.3	7.1	7.0	6.4	6.4
	CFU	-	$.5x10^2$	$.4x10^3$	$1.8x10^5$	$.3x10^6$	$1.1x10^6$	$3x10^6$
Tryptose Phosphate Broth Medium	pH	7.5	7.4	7.3	7.2	7.0	6.5	6.4
	CFU	-	$.5x10^3$	$.9x10^3$	$.8x10^4$	$.6x10^5$	$.7x10^6$	$2x10^6$

TABLE 3. Comparative study on the total bacterial count and the pH of different Mycoplasma media.

MEDIA		28 hours	32 hours	36 hours	40 hours	44 hours	48 hours	52 hours	56 hours
MSU	pH	7.3	7.0	6.8	6.8	6.8	6.7	6.6	6.6
	CFU	$1.0x10^{12}$	$1.8x10^{11}$	$1.2x10^{11}$	$0.8x10^{11}$	$1.0x10^{11}$	$0.6x10^{11}$	$1.2x10^{10}$	$1.0x10^{10}$
Modified Frey Medium	pH	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.6
	CFU	$2.0x10^{11}$	$1.4x.0^{10}$	$0.7x10^{9}$	$0.9x10^{9}$	$1.0x10^{9}$	$0.9x10^{9}$	$1.6x10^{8}$	$1.0x10^{10}$
PPLO	pH	5.8	5.8	5.6	5.5	5.3	5.3	5.3	5.2
	CFU	$2.8x10^{6}$	$0.3x10^{7}$	$1.0x10^{5}$	$0.6x10^{5}$	$0.4x10^{5}$	$0.2x10^{5}$	$1.1x10^{5}$	$1.2x10^{5}$
Tryptose Phosphate Broth Medium	pH	6.1	6.0	5.5	5.4	5.3	5.1	5.1	5.0
	CFU	$2.2x10^{6}$	$0.4x10^{7}$	$2.0x10^{5}$	$1.2x10^{5}$	$0.2x10^{5}$	$0.1x10^{5}$	$1.0x10^{5}$	$0.9x10^{5}$

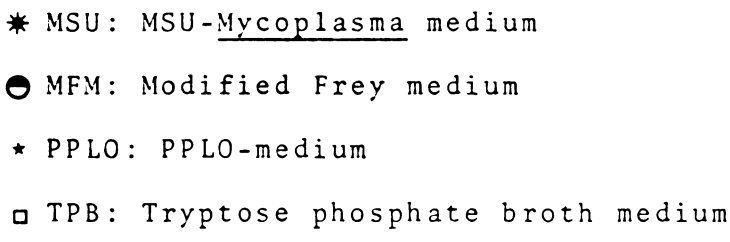


Figure 9. The total bacterial count (Log No CFU/ml) for Mg in different media recorded in hours post-inoculation

Figure 9

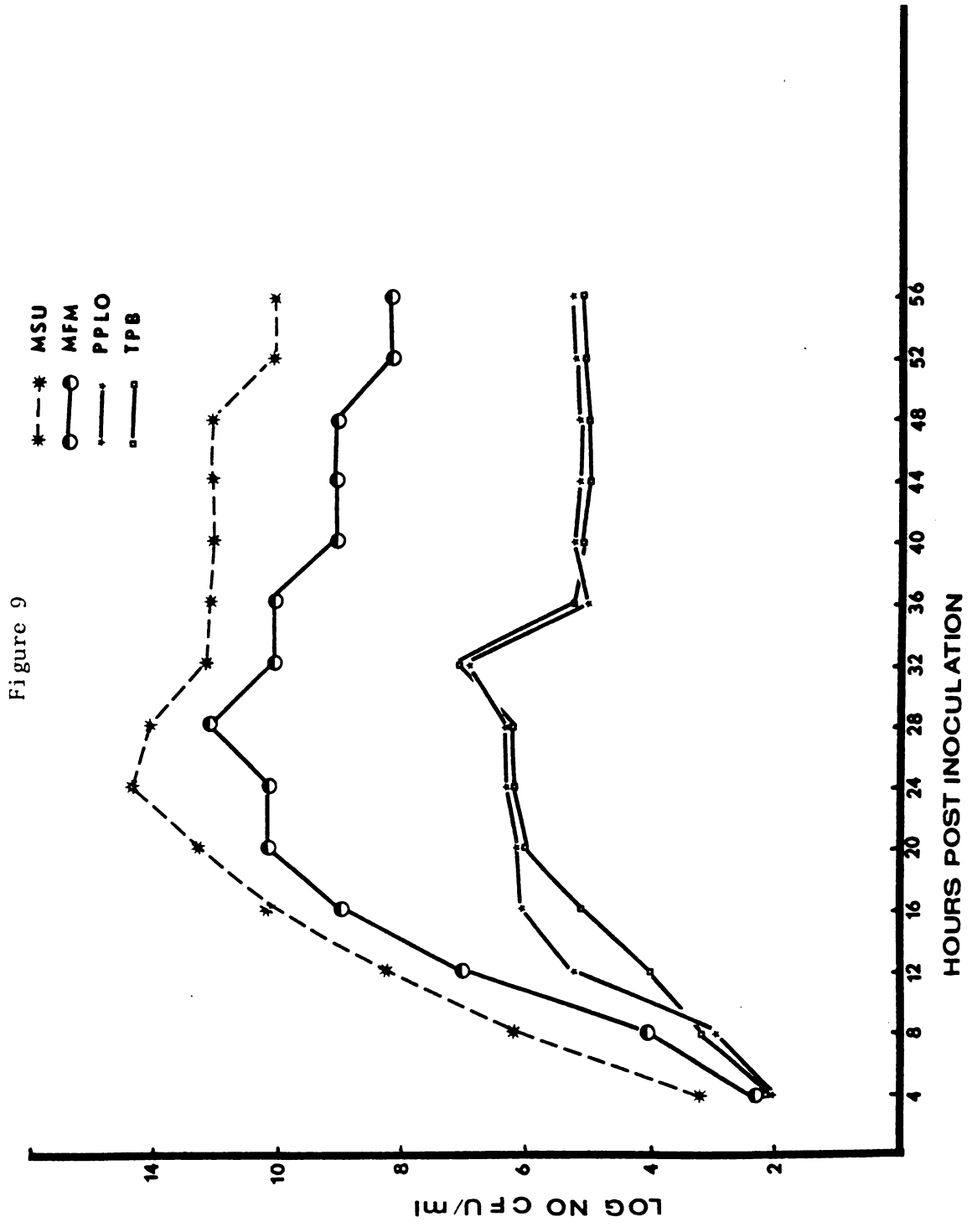

MSU
MFM
PPLO
TPB
LOG NO CFU/ml
2
4
6
8
10
14
4
8
12
16
20
24
28
32
36
40
44
48
52
56
HOURS POST INOCULATION

Figure 10. The total bacterial count (Log No CFU/ml) for Mg in different media recorded in days post-inoculation

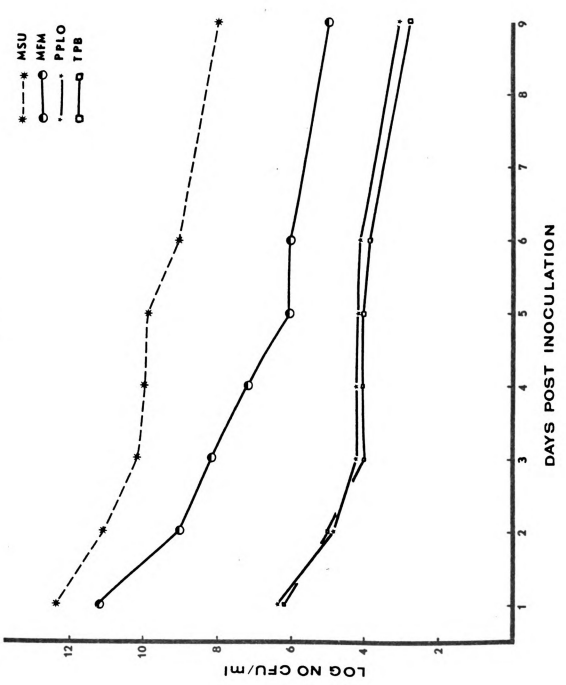
✱ MSU: MSU-Mycoplasma medium

◓ MFM: Modified Frey medium

⋆ PPLO: PPLO-medium

□ TPB: Tryptose phosphate broth medium

Figure 10


MSU
MPM
PPLO
TPB
LOG NO CFU/ml
12
10
8
6
4
2
1
2
3
4
5
6
7
8
9
DAYS POST INOCULATION

and 0.0 CFU/ml at 60 days, as shown in Table 4. MSU-Mycoplasma media gave a reading of 0.3×10^5 CFU/ml and 0.1×10^3 CFU/ml at 30 and 60 days PI, respectively.

2. Changes of the pH of Different Mycoplasma Media

The changes of the pH of MSU-medium, MFM-medium PPLO-medium and TPB medium were measured and recorded in Tables 2, 3 and 4 and illustrated in Figures 11 and 12. The initial pH (before inoculation) of MSU-medium, MFM medium, PPLO medium and TPB broth medium were 7.8, 7.8, 7.6 and 7.5, respectively. The pH of all media dropped as a result of the growth of Mg in the media after 6 days PI to reach 6.5, 6.1, 5.1 and 4.8, respectively. The reduction in the pH value after 24-32 hours was accompanied by a rapid loss of the viable Mycoplasma organisms. The pH readings of MSU-medium stayed much higher (basic) than the pH of MFM at 24, 28, 32, and 36 hours PI. Furthermore, MSU-media gave a higher pH reading (basic) as compared to the other media at all times.

3. Preservation of Mycoplasma Organisms

Mg antigen was harvested by centrifugation procedures and stored with or without the addition of preservatives. The main function of preservative was to prevent the growth of any contaminating microorganisms and to kill the Mycoplasma organisms. Merthiolate solution 1% was added to the antigen suspension in a final concentration of 1:1000 and 1:5000. The merthiolate in both concentrations did not inhibit the contaminating microorganisms from growing in the antigen suspension. Phenol was added to phosphate buffer in a concentration of 0.25%, which was used for washing and as a diluent for Mycoplasma antigen. Phenol was proven to be very

TABLE 4. Comparative study on the total bacterial count and the pH of different Mycoplasma media

MEDIA		3rd Day	4th Day	5th Day	6th Day	9th Day	30th Day	60th Day
MSU	pH	6.6	6.5	6.5	6.5	6.5	6.5	6.5
	CFU	1.6×10^{10}	0.3×10^{10}	0.4×10^{10}	0.8×10^{9}	0.6×10^{8}	0.3×10^{5}	0.1×10^{3}
Modified Frey Medium	pH	6.1	6.1	6.1	6.1	6.1	6.1	6.1
	CFU	2.0×10^{8}	2.1×10^{7}	1.4×10^{6}	0.6×10^{6}	1.0×10^{5}	0.1×10^{1}	-
PPLO	pH	5.2	5.1	5.1	5.1	5.1	5.0	5.0
	CFU	2.0×10^{4}	1.3×10^{4}	0.6×10^{4}	0.4×10^{4}	0.3×10^{3}	-	-
Tryptose Phosphate Broth Medium	pH	4.9	4.9	4.9	4.8	4.8	4.7	4.7
	CFU	1.2×10^{4}	0.6×10^{4}	0.9×10^{4}	0.3×10^{4}	0.2×10^{3}	-	-

Figure 11. The changes of the pH of different media for Mg by hours post-inoculation

✷ MSU: MSU-Mycoplasma medium

◓ MFM: Modified Frey medium

⋆ PPLO: PPLO-medium

□ TPB: Tryptose phosphate broth medium

Figure 11

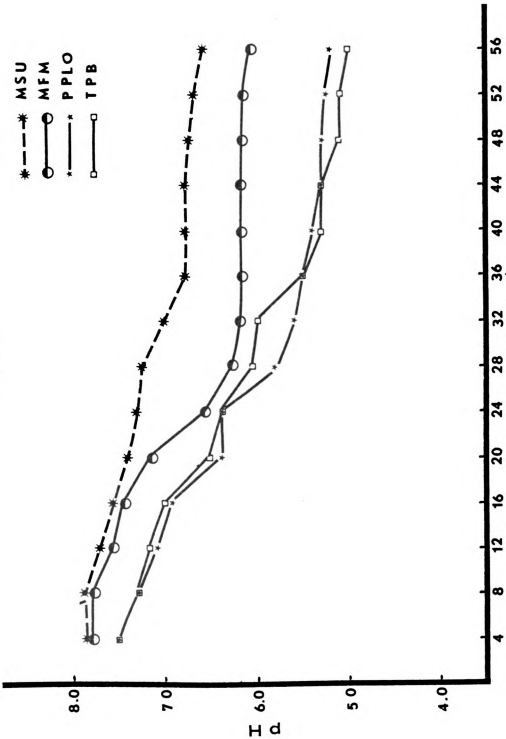

MSU
MFM
PPLO
TPB
pH
8.0
7.0
6.0
5.0
4.0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
HOURS POST INOCULATION

Figure 12. The changes of the pH of different media for Mg by days post-inoculation

✱ MSU: MSU-Mycoplasma medium

◓ MFM: Modified Frey medium

★ PPLO: PPLO-medium

□ TPB: Tryptose phosphate broth medium

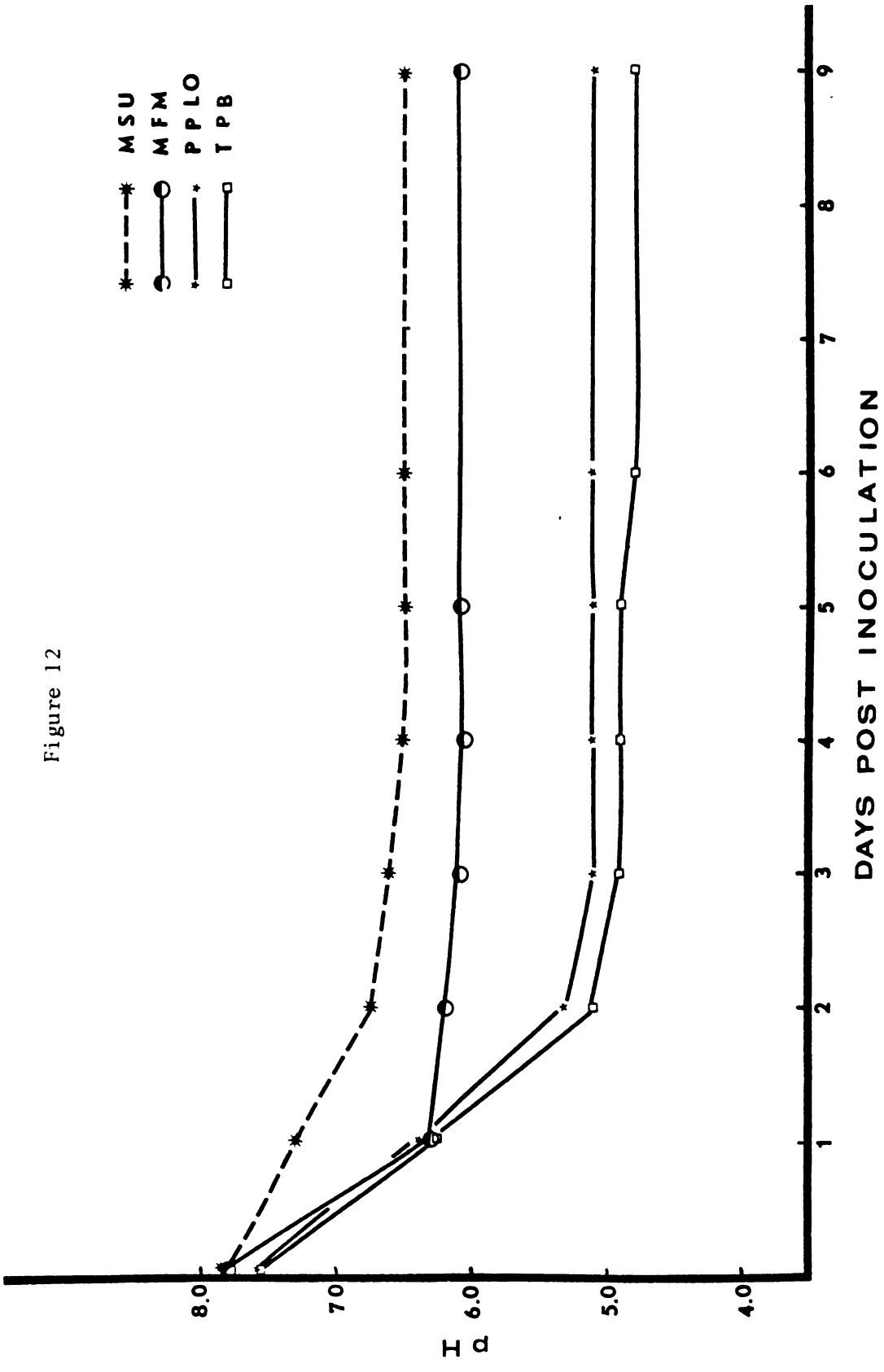

MSU
MFM
PPLO
TPB
8.0
7.0
6.0
5.0
4.0
pH
1
2
3
4
5
6
7
8
9
DAYS POST INOCULATION

Figure 12

satisfactory as a preservative. Phenolized (0.25%) phosphate buffer at pH 7.0 was used to standardize and to homogenize tube Mg antigen but not the hemagglutination (HA) antigen. Samples from Mycoplasma antigen were taken for culturing on blood agar, thioglycollate and MSU-Mycoplasma media to isolate either Mycoplasma organisms or any other contaminants. No growth was observed. The HA antigen was resuspended in phosphate buffered saline and then homogenized. Glycerol (Difco) was added as a preservative to the antigen and also to prevent denaturation of the antigen by frequent freezing and thawing. No preservative was added to MYCO-ELISA antigen.

4. Standardization of Mg Antigen

The Mg tube antigen was standardized to a concentration equivalent to the density of 2 x No. 10 McFarland nephelometer tube with phenolized phosphate buffer, pH 7.0. This antigen concentration was found to give comparable results to the USDA (Ames, Iowa) Mg tube antigen. No dye was used in the preparation of the tube antigen and the antigen was allowed to age for several days before checking the sensitivity and again before use.

The acitivjty of HA antigen was checked out and the final product was diluted using equal quantities of buffer and glycerin, which gave HA titer of 1:320 or 1:640. High titer concentration of the antigen was found to be unnecessary. No preservative other than glycerol was added. Antigen in quantities of 5 ml was stored for future use at -40 - -70 C.

The Mycoplasma-Enzyme-Linked Immunosorbent Assay (MCYO-ELISA) antigen was prepared as described in the Materials and Methods. The harvested

Mycoplasma cell paste was homogenized with PBS at pH 7.2. An equal amount of glycerin was added to make up suitable volume of the antigen. A total protein content of the antigen had an average of 5-7 mg/ml with no preservative added. Detergent solution, sodium dodecyl sulfate (SDS), was used to dissolve the lipoprotein content of the plasma membrane of Mycoplasma. A final concentration of 3-5 mg protein/ml was achieved.

B. Immunofluorescence Identification of Mycoplasma Isolates

1. Antibody Titer of Rabbit and Chicken Antisera

Antibody titer in the serum was determined by HI method. Rabbit red blood cell suspensions in 2 and 3% were used in the HI test and 0.5% chicken red blood cell was used in the same test. The HI titer of rabbit antisera ranged from 1:160 to 1:640 and the agglutination titer ranged from 1:640 to 1:2560 for immunized rabbits. The antibody titer for chicken antiserum ranged from 1:80 to 1:1280.

2. Direct Fluorescence Method

The Mycoplasma organisms stained with homologous conjugate displayed typical yellow-green fluorescence, as shown in Figure 13. Control stained slides showed no specific yellow-green fluorescence, as shown in Figure 14. No significant differences in staining reaction were noticed with 1:10 or 1:20 diluted FITC conjugated rabbit antisera or in the time of antigen-conjugate reaction at 37 C.

3. Indirect Fluorescence Method

Specific green fluorescence organisms were observed after adding the labeled anti-globulin, as shown in Figure 13. No specific fluorescence

Figure 13. Direct immunofluorescence staining of Mg organisms showed a specific yellow-green fluorescence

Figure 14. Control (negative) fluorescence for Mg organisms stained with labeled heterologous antibody showed no specific fluorescence

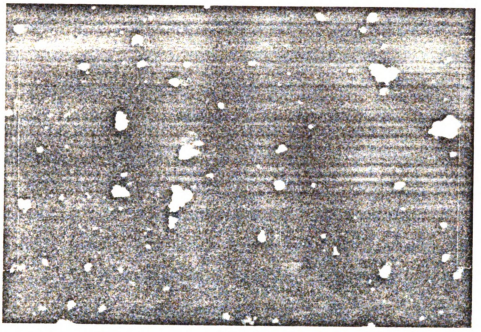


Figure 13

Figure 14

was observed in the control slide labeled with heterolcgous antisera or using other Mycoplasma antigen as Ms or Mm, as shown in Figure 14.

Both methods (direct and indirect) were able to identify Mg organisms in media culture and differentiate Mg from other species of avian Mycoplasma. The dull yellow to yellowish green autofluorescence which varied in intensity was common with the same old culture of Mycoplasma when reacted with heterologous antisera. Care was exercised so that autofluorescence would not be confused with minor cross-reactions or poor staining from dilute antiserum. Autofluorescence tended to fade much more quickly upon exposure to ultraviolet light and caused difficulty in obtaining a good image. These characteristics were helpful in distinguishing positive fluorescence.

C. Purification and Assaying IgG by Protein A-Sepharose Chromatography

1. Purification of Rabbit IgG by Protein A

The rabbit IgG was isolated from normal and immune serum by chromatography fractions of the serum on a column of protein A coupled to Sepharose 4B (2 x 10 cm), as illustrated in Figure 15. The flow rate of the eluate was 60 ml/hour. The fraction numbers from 0-20 were the eluate wash of the serum by 0.1 M sodium phosphate, pH 7.0. The high peak and the large volume of the eluate, which has higher absorbance than 2.0, indicated that the amount of immunoglobulin washed by sodium phosphate buffer was greater than the amount of immunoglobulin washed by acetic acid. The arrows in Figure 15 indicate where elution with 1M acetic acid was started to release the bound IgG fraction. The initial protein

Figure 15. Chromotography of hyperimmune rabbit serum on a protein A-Sepharose column

0.1M sodium phosphate buffer was used as a washing buffer. The arrow indicates where 1M acetic acid was added to release the bound IgG.

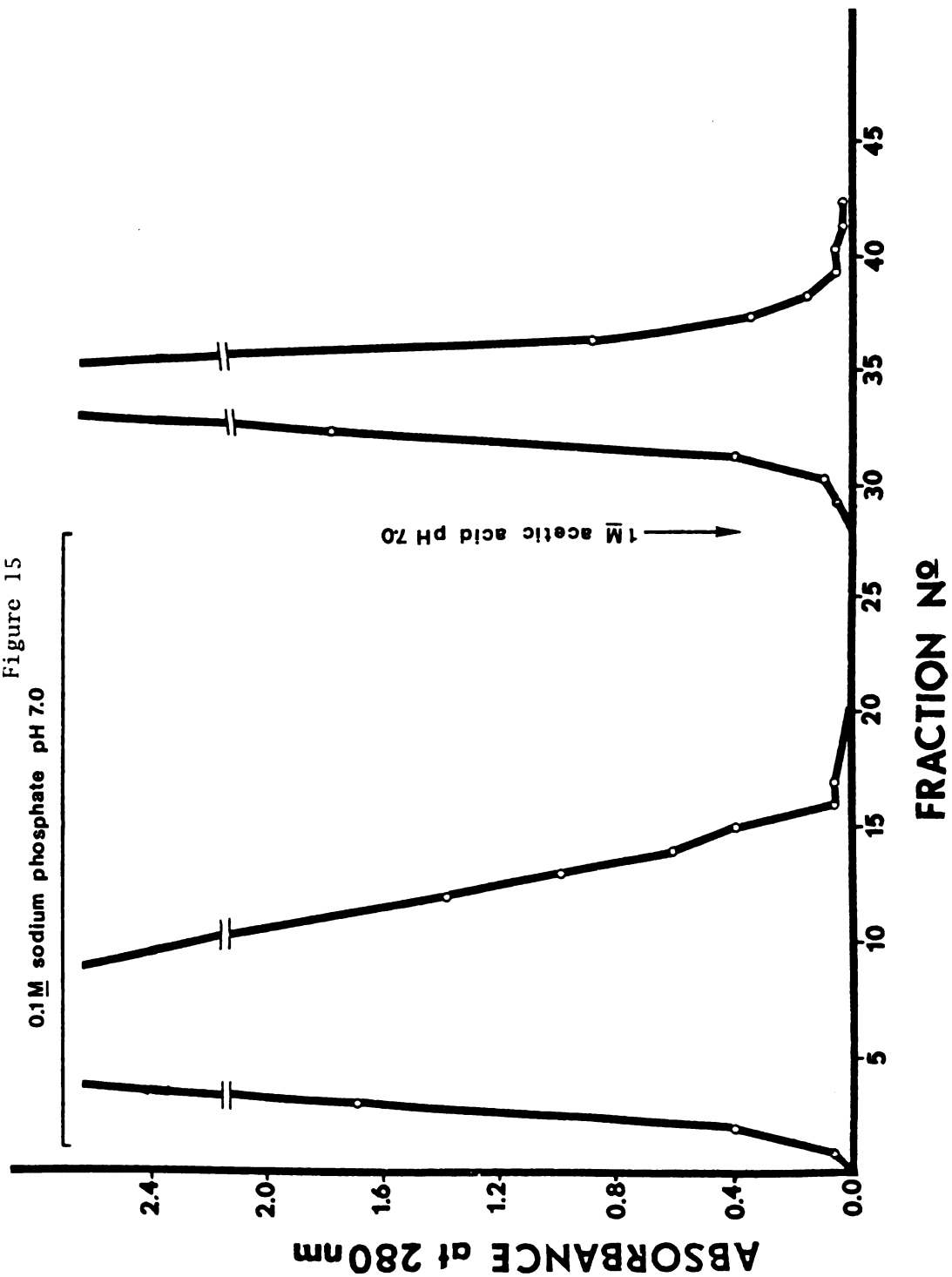

ABSORBANCE at 280nm
FRACTION Nº
0.1M sodium phosphate pH 7.0
1M acetic acid pH 7.0

Figure 15

concentration of the immune serum was 44 mg/ml and the IgG fraction was 7 mg/ml. The two major peaks were obtained; one was the eluate of sodium phosphate buffer wash, which apparently contained all immunoglobulin except IgG, and the second was the eluate of acetic acid wash, which contained only IgG fraction.

2. Immunodiffuison Analysis for Rabbit Serum

Agar immunodiffusion was used to study the reactions of different protein A-Sepharose chromatography eluates against goat anti-rabbit serum and goat anti-rabbit IgG. The results are illustrated in Figures 16 and 17.

First study (Figures 16a,b): The ammonium sulfate precipitating fraction (AS) formed a multiple precipitating band against goat anti-rabbit serum (Gt-s), as shown in Figure 16a. The reaction of identity (converging arc) was very clear among AS and A fractions. The identity band between AS and A fraction against Gt-s was very clear, as illustrated in Figure 16b. Protein A 0.1 M sodium phosphate eluate (A-NP) and AS fractions had multiple precipitating bands and two identical bands. Furthermore, A-NP did not form a reaction of identity with both A and AS as one group.

Second study (Figure 17): The fractions of A and AS formed a reaction of identity against goat anti-rabbit IgG fraction (Gt-G), as illustrated in Figure 17. This reaction indicated that fractions A as well as AS contain rabbit IgG.

3. Purification of Chicken IgG by Protein A

Protein A coupled to Sepharose 4B in a column (2x10 cm) was used to fractionate chicken IgG from normal and hyperimmune chicken sera. A

Figure 16 (a&b). Immunodiffusion of rabbit immunoglobulin fractions

Gt-s: Goat anti-rabbit serum
A: Protein A, 1M acetic acid eluate
AS: IgG fraction by ammonium sulfate
A-NP: Protein A, 0.1M sodium phosphate eluate

Figure 16

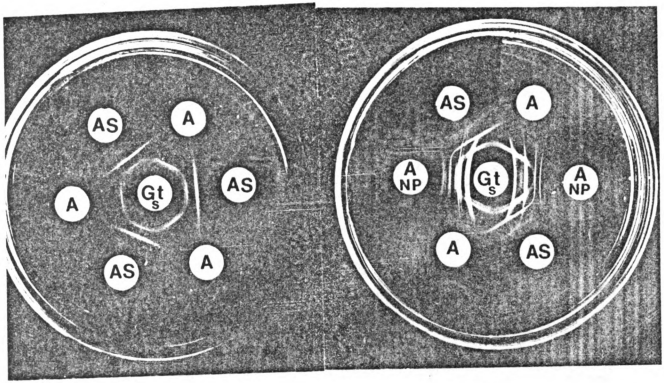
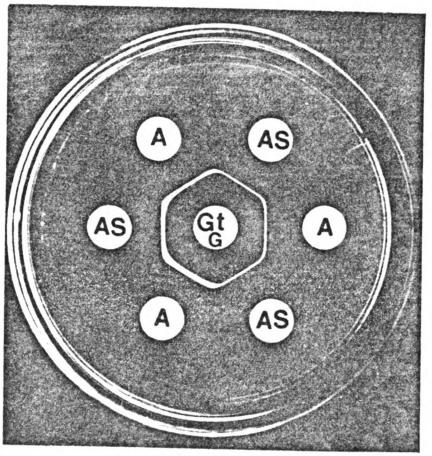


Figure 17. Immunodiffusion of different rabbit IgG fractions

Gt-G: Goat anti-rabbit IgG
A: Protein A, 1M acetic acid eluate
AS: IgG fraction by ammonium sulfate

Figure 17



typical elution pattern was illustrated in Figure 18. The flow rate was 60 ml/hour. The 0.1 M sodium phosphate buffer, pH 7.0, eluate gave the highest peak, which was similar to the peak of phosphate buffer eluate of rabbit serum. Furthermore, this fraction (from 0-15) was very rich with immunoglobulin because of the high absorbance reading (higher than 2.0). The arrow in the same figure indicated the addition of 1 M acetic acid, pH 7.0, to the column. A very small peak which had absorbance less than 2.0 at 280 nm appeared after adding the acetic acid. These results indicated that chicken IgG did not bind to the protein A as rabbit IgG fraction did.

4. Immunodiffusion Analysis for Chicken Serum

First study (Figures 19 a,b): Several identity bands were formed from all protein A, 0.1 M sodium phosphate eluate (A-NP) against rabbit anti-chicken globulin (Rb-glb), as shown in Figure 19a. Protein A, 1 M acetic acid eluate (A) did not contain any immunoglobulin and did not form any precipitating band against(Rb-glb), as illustrated in Figure 15b. However, IgG fraction precipitated by ammonium sulfate (AS) showed two precipitating bands against Rb-glb and c-IgG showed very clear bands against Rb-glb.

Second study (Figures 20 a,b): These figures illustrate that all A-NP fraction contained IgG. The control well, which contained chicken IgG (c-IgG), gave a band of identity with the two neighboring wells A-NP. Protein A, 1 M acetic acid eluate did not contain IgG, as illustrated in Figure 20b. There was no band of identity between A, AS and c-IgG as fraction did form one precipitating bard against anti-chicken IgG (Rb-G).

Figure 18. Chromotography of chicken hyperimmune serum on a protein A-Sepharose column

0.1M sodium phosphate was used as a washing buffer. The arrow indicates where 1M acetic acid was added to release the bound IgG

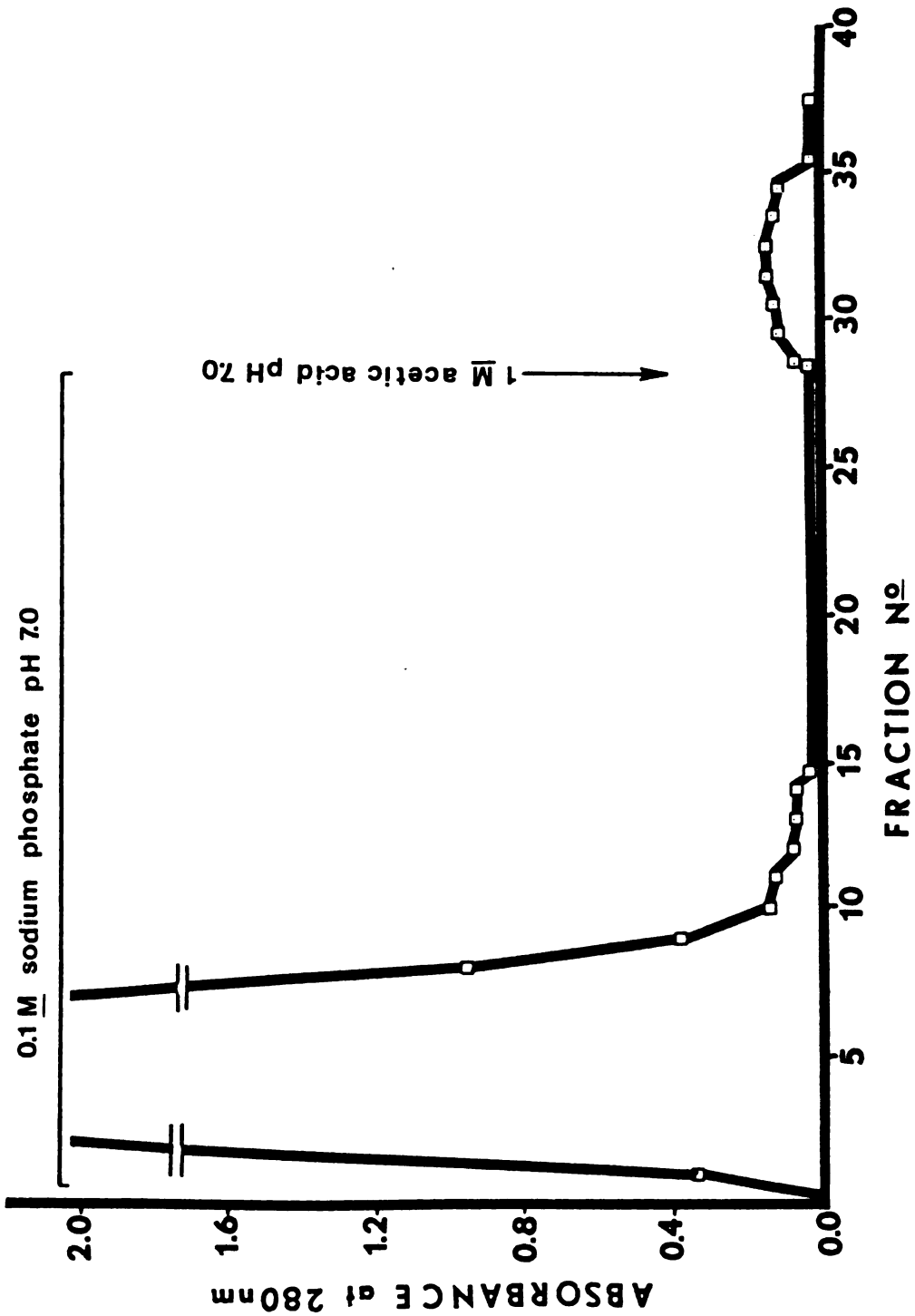
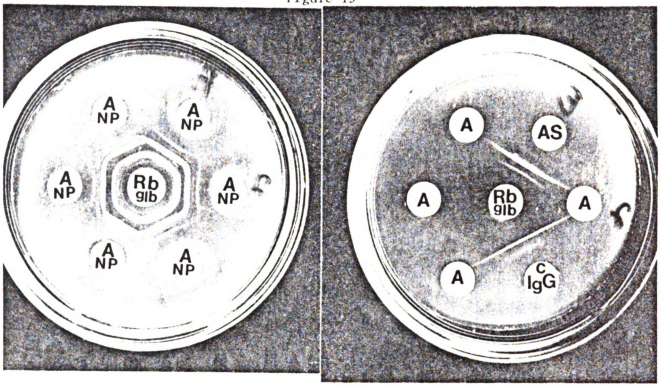

ABSORBANCE at 280nm
2.0
1.6
1.2
0.8
0.4
0.0
5
10
15
20
25
30
35
40
FRACTION Nº
0.1 M sodium phosphate pH 7.0
1 M acetic acid pH 2.0

Figure 18

Figure 19 (a&b). Immunodiffusion of chicken immunoglobulin fractions

Rb-glb: Rabbit anti-chicken globulin
A-NP: Protein A, 0.1M sodium phosphate eluate
A: Protein A, 1M acetic acid eluate
AS: IgG fraction by ammonium sulfate
C-IgG: Control, normal chicken IgG fraction

Figure 19

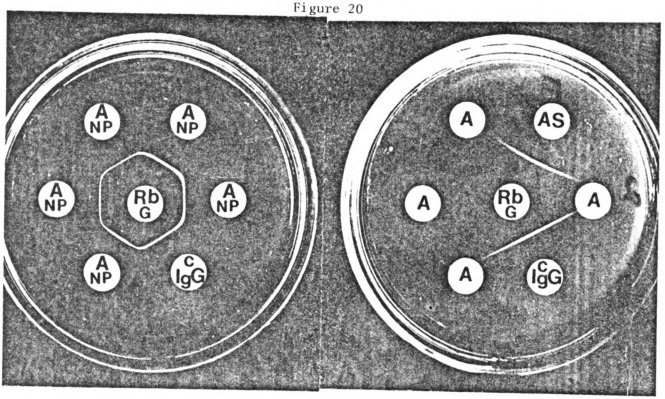


a b

Figure 20 (a&b). Immunodiffusion of different chicken IgG fractions

Rb-G: Rabbit anti-chicken IgG
A-NP: Protein A, 0.1M sodium phosphate eluate
c-IgG: Control normal chicken IgG
A: Protein A, 0.1M fraction by ammonium sulfate

Figure 20



Third study (Figures 21 a,b): This immunodiffusion study was designed to compare the content of the eluate of protein A-Sepharose column chromotography washed by 1 M acetic acid for rabbit and chicken serum. Band of identity was formed between protein A, 1 M acetic acid eluate (Prt A-Rb IgG); goat anti-rabbit serum (Gt-ant RS) and goat anti-rabbit IgG (Gt ant-RIgG frct), as shown in Figure 21a. These results indicated that 1 M acetic acid eluate of rabbit serum on protein A-Sepharose chromatography contained IgG fraction. Normal rabbit serum (NRS) formed several bands against Gt-ant RS which corresponds to the numbers of homologous system between both wells. Chicken serum, 1 M acetic acid eluate on protein A-Sepharose chromatography did not contain IgG fraction, as shown in Figure 21b. The only precipitating band formed was between normal chicken serum (NCS) and rabbit anti-chicken globulin (Rb-ant Cglb).

5. The Effect of Chicken Plasminogen on Isolation of Chicken IgG by Protein A-Sepharose Chromatography

Immunodiffusion studies were conducted to study the effects of chicken plasminogen on isolation and purification of chicken IgG by protein A chromatography. Rabbit anti-chicken plasminogen (Rb-PL) was put in the center well against A-NP, A and AS, as shown in Figures 22a and b. A precipitating band was formed between all A-NP eluates and Rb-PL, as illustrated in Figure 22a. Also, the band of identity was formed between the central well which contained chicken plasminogen (PL) and the two neighboring wells which contained A-NP. However, A and AS fractions did not react against Rb-PL, as shown in Figure 22b. These two figures (Figure 22a and b) indicated that plasminogen present

Figure 21. a. Immunodiffusion of rabbit IgG fractio

Prt A-Rb IgG-frct: Protein A,1M acetic acid eluate
NRS: Normal rabbit serum
Gt-ant RS: Goat anti-rabbit serum
Gt-ant R IgG: Goat anti-rabbit IgG

b. Immunodiffusion of protein A,1M acetic acid eluate of chicken serum

Prt A-Ck IgG-frct: Protein A,1M acetic acid eluate
NCS: Normal chicken serum
Rb-ant Cglb: Rabbit anti-chicken globulin
Rb-ant CIgG: Rabbit anti-chicken IgG

Figure 21

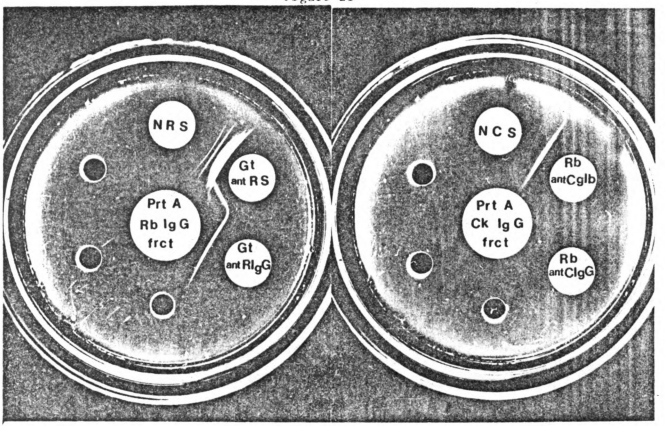
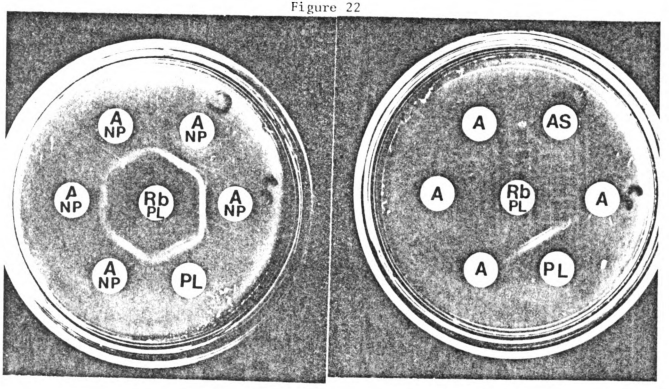

NRS
Gt ant RS
Prt A Rb Ig G frct
Gt ant RIgG
NCS
Rb antCgIb
Prt A Ck Ig G frct
Rb antCIgG
a
b

Figure 22 (a&b). Immunodiffusion of chicken immunoglobulin against rabbit anti-chicken plasminogen

Rb-PL: Rabbit anti-chicken plasminogen
A-NP: Protein A, 0.1M sodium phosphate eluate
PL: Chicken plasminogen
A: Protein A, 1M acetic acid eluate
AS: Chicken IgG precipitate by ammonium sulphate

Figure 22



in chicken serum did not bind to protein A-Sepharose molecule; instead it got washed by 0.1 M sodium phosphate buffer.

D. Standardization of MYCO-ELISA

The use of MYCO-ELISA, as any immunologic assay, depends on the strict standardization of all reagents and procedures used. Without standardization, the performance of the test will be varied from one laboratory to another. Thus, preliminary experiments were performed to determine the conditions and reagents which should be used for the detection of antibody to Mg.

1. Determination of the Optimum Antigen Concentration and Serum Dilution

Sera positive and negative for Mg (various dilutions) were titrated against various concentrations of antigen using checkerboard design experiments. Mg antigen was tested at 720, 360, 180, 90, 45, 21.5 and 10.75 µg protein concentration per well against 1:50, 1:100, 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000 and 1:64000 serum dilutions. The test showed that 90 and 180 µg protein concentrations gave similar absorbance reading at 405 nm after 30 minutes of conjugate substrate reaction with 1:50 positive serum dilutions, as shown in Figure 23. The absorbance readings were 1.29 and 1.33 using 90 and 180 mg antigen protein per well and 1:50 positive serum dilution. Furthermore, the result indicated that the optimum serum dilutions using 1:200 conjugate dilution and 90 µg antigen protein per well were 1:50, 1:100 and 1:250, as shown in Figure 24. The readings were 1.29, 1.15 and 0.99 at 1:50,

Figure 23. Determination of Mg antigen concentration for MYCO-ELISA using standard dilution of 1:200 HRP conjugate and 1:50 reference control serum

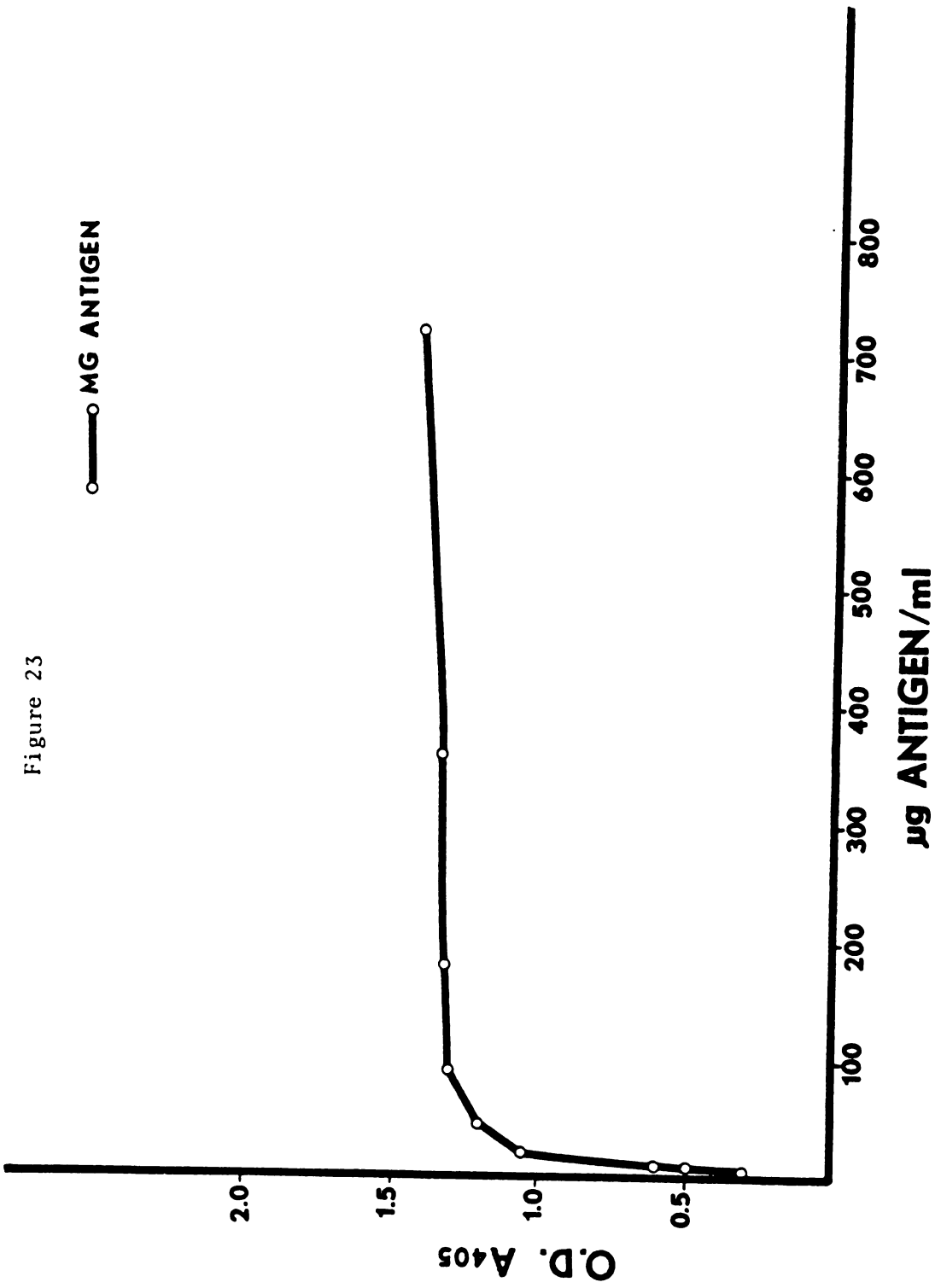

MG ANTIGEN
O.D. A405
2.0
1.5
1.0
0.5
100
200
300
400
500
600
700
800
µg ANTIGEN/ml

Figure 23

Figure 24. Determination of the optimum serum dilution in MYCO-ELISA, using standard dilution of 1:200 HRP conjugate and 90 ug antigen protein

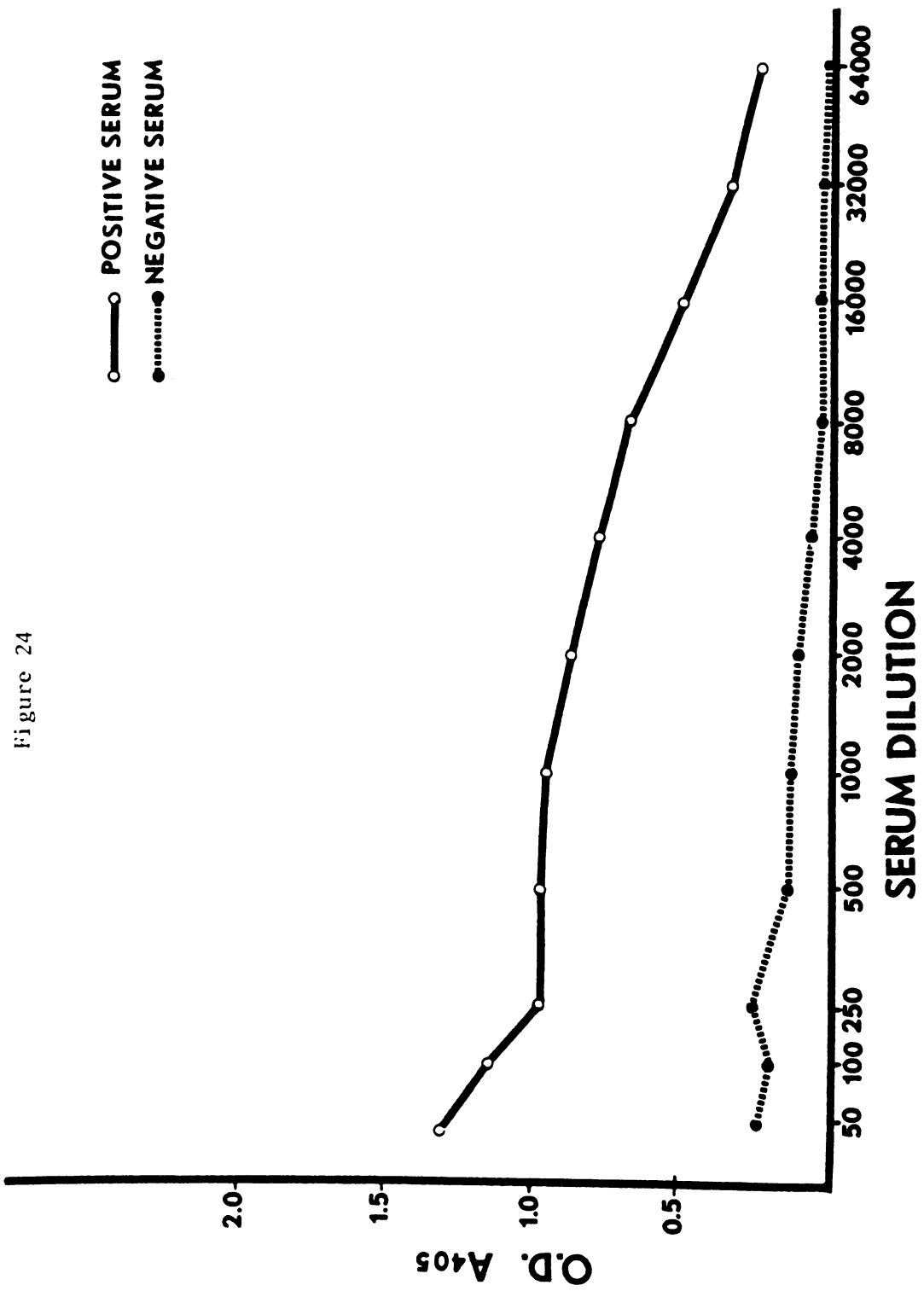

POSITIVE SERUM
NEGATIVE SERUM
O.D. A405
2.0
1.5
1.0
0.5
50 100 250 500 1000 2000 4000 8000 16000 32000 64000
SERUM DILUTION

Figure 24

1:100 and 1:250 serum dilutions, respectively. It should be pointed out that even with high serum dilution of 1:32,000, the absorbance reading at 405 nm (0.304) was higher than the negative serum at all dilutions (lower than 0.3). However, serum dilutions of 1:50 or 1:100 were considered the dilutions of choice for diagnostic purposes.

2. Determination of the Optimum Conjugate Concentration

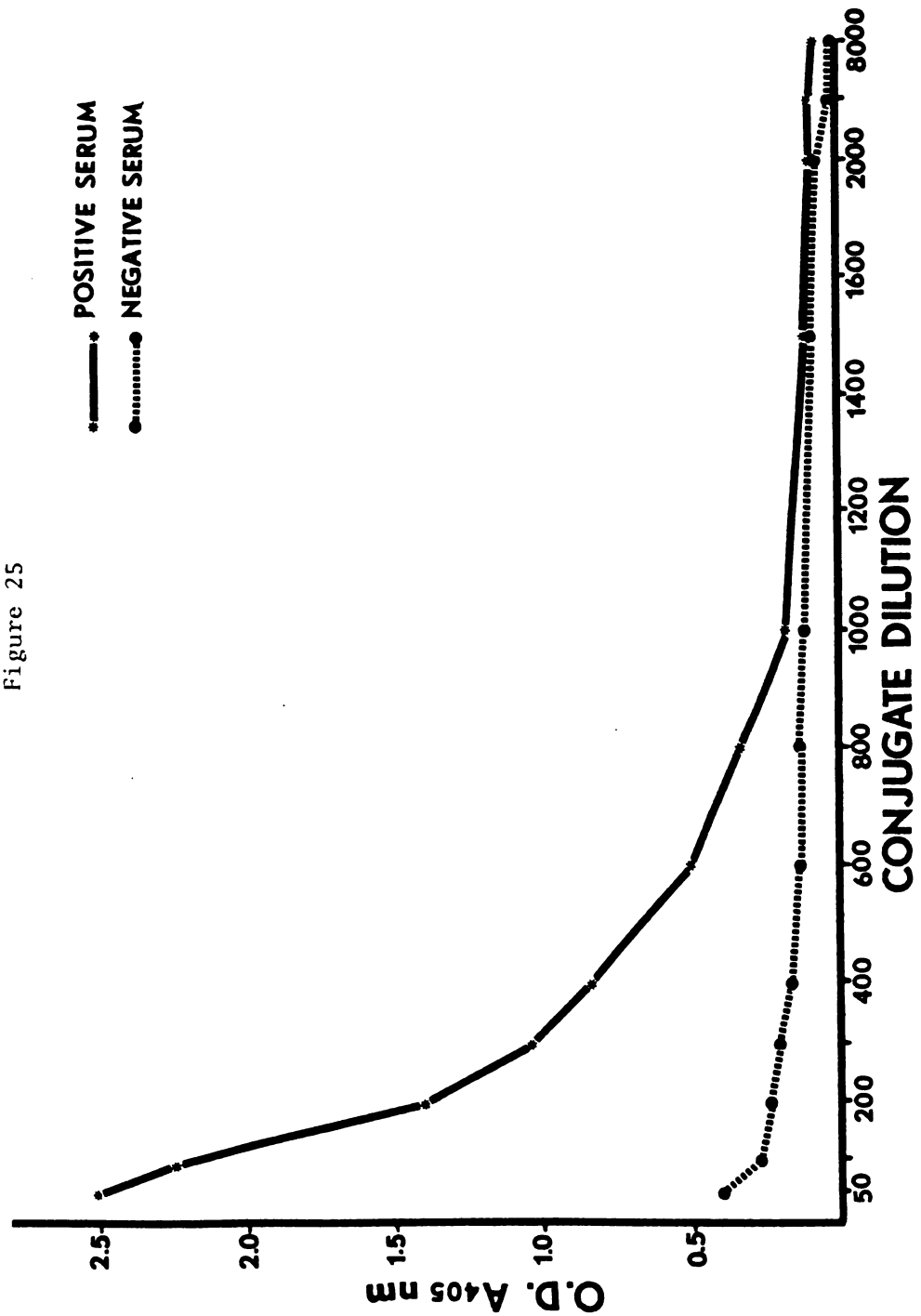
The optimum concentration of horseradish peroxidase enzyme (HRP) conjugated to rabbit IgG fraction anti-chicken IgG was determined. Antiserum was obtained from two different sources, Miles Laboratories, Inc., and Cappel Laboratories. The two antisera are essentially the same except that the antiserum from Miles contains 0.1% sodium azide as preservative. Furthermore, a labeled HRP with IgG fraction rabbit anti-chicken IgG (heavy and light chain) was obtained from Cappel Laboratories for comparison purposes (Cappel conjugate).

Both conjugates (MSU and Cappel conjugates) were titrated at various dilutions (1:50, 1:100, 1:200, 1:300, 1:400, 1:600, 1:800 and 1:1000). Antigen concentrations of 90 and 180 µg protein were coated to each well and 1:50 and 1:100 dilutions of known positive serum were titrated against each conjugate dilution.

MSU-conjugate dilutions of 1:50 and 1:100 gave the highest absorbance readings, as shown in Figure 25. The average absorbance readings was 2.49 and 2.25 for conjugate dilutions of 1:50 and 1:100, respectively. The absorbance readings for the negative serum control using 1:50 and 1:100 conjugate dilutions were 0.623 and 0.595. MSU-conjugate dilutions of 1:200 and 1:300 gave absorbance readings higher than 1.0 Other conjugate dilutions of 1:400 and 1:600 gave considerably higher absorbance readings

Figure 25. The effect of different concentrations of MSU-HRP conjugate on the specific activity of MYCO-ELISA using standard 1:50 reference control serum and 180 ug antigen protein per well

Figure 25


POSITIVE SERUM
NEGATIVE SERUM
O.D. A405 nm
2.5
2.0
1.5
1.0
0.5
50
200
400
600
800
1000
1200
1400
1600
2000
8000
CONJUGATE DILUTION

than the controls. Cappel Laboratories conjugate, as shown in Figure 26, gave the optimum absorbance readings at 1:200, 1:300, and 1:400 conjugate dilutions, which were 2.18, 2.06 and 2.01, respectively, at antigen concentration of 180 µg per well and 1:50 serum dilution. Furthermore, the absorbance readings of 1:600, 1:800 and 1:1000 conjugate dilutions were 1.74, 1.44 and 1.44, using the same antigen concentration and serum dilution, which were much higher than the control absorbance reading. The negative serum control gave absorbance readings of 0.718, 0.723, 0.718, 0.701, 0.623, 0.595, 0.443 and 0.404 at 1:50, 1:100, 1:200, 1:300, 1:400, 1:600, 1:800 and 1:1000 Cappel conjugate dilutions, as illustrated in Figure 26.

3. Determination of the Optimum Temperature and the Length of the Incubation Period

Maximal binding of serum antibody and conjugate antibody to the antigen or to the antigen-antibody complex is essential to attain high levels of sensitivity and to avoid non-specific reactions. Replicate assays of different dilutions of positive sera for Mg, 1:50, 1:500, 1:5000 and 1:50000, were incubated at various temperatures, 5 C, 22 C, 37 C, 50 C for different lengths of time, 1, 2 and 3 hours, both the serum and conjugate incubation periods. Two different antigen concentrations were used, 90 and 180 µg protein per well. Also, two different conjugate dilutions (1:100 and 1:200) were used in this experiment. The effect of the temperature of incubation of serum and conjugate on the specific activity of MYCO-ELISA using standard dilution of 1:100 and 2 hours of incubation is illustrated in Figure 27. The effect of various time of incubations at room temperature (22 C) on the absorbance reading of

Figure 26. The effect of different concentrations of Cappel -HRP conjugate on the specific activity of MYCO-ELISA using standard 1:50 reference control serum and 180 ug antigen protein per well

Figure 26

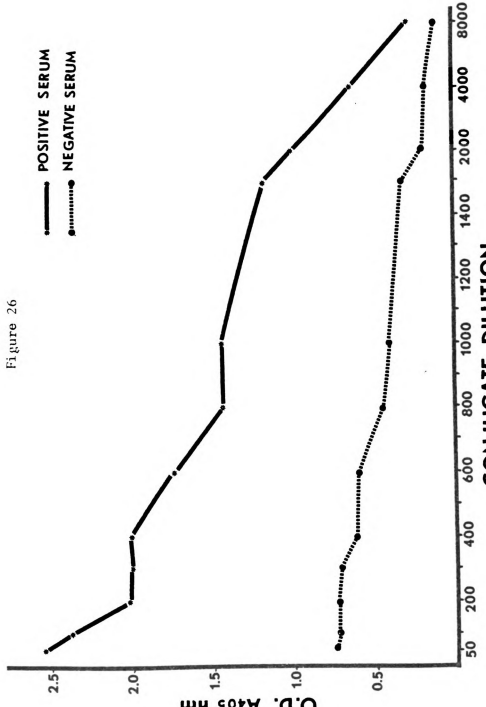

POSITIVE SERUM
NEGATIVE SERUM
O.D. A405 nm
2.5
2.0
1.5
1.0
0.5
50
200
400
600
800
1000
1200
1400
2000
4000
8000
CONJUGATE DILUTION

Figure 27. The effect of different temperature of incubation of serum and conjugate on the specific activity of MYCO-ELISA using standard dilution of 1:50 reference control serum dilution, HRP-conjugate dilution of 1:100 and 2 hours of incubation

Figure 27

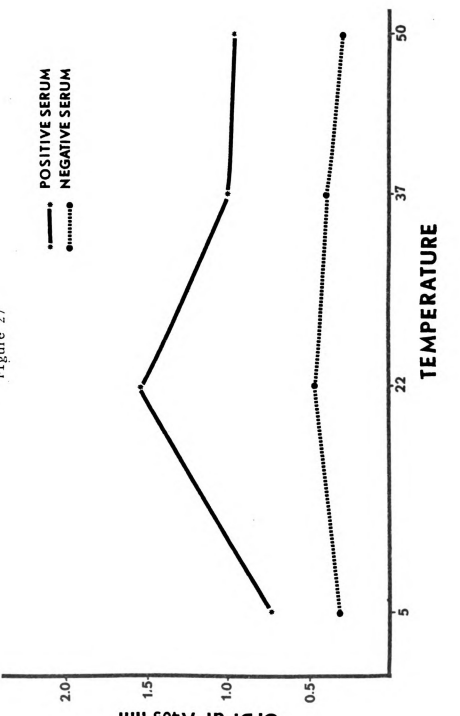
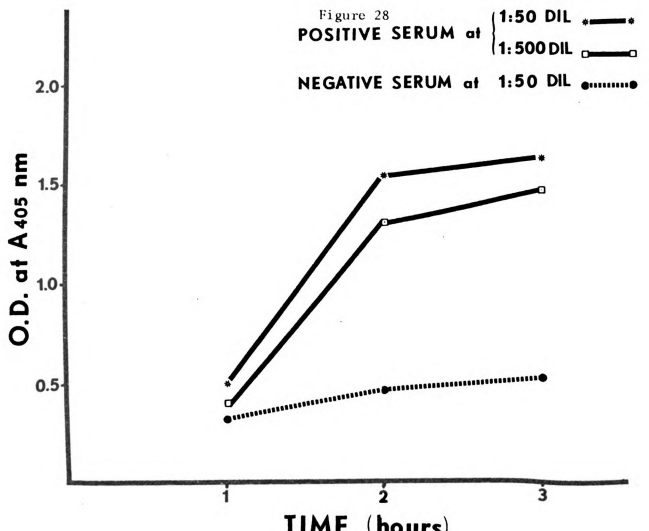

POSITIVE SERUM
NEGATIVE SERUM
O.D. at A405 nm
2.0
1.5
1.0
0.5
5
22
37
50
TEMPERATURE

Figure 28. The effect of various time of incubation at room temperature (22C) on the absorbance readings of MYCO-ELISA using 1:50 and 1:500 positive serum control dilution at MSU-HRP dilution of 1:100

Figure 28


POSITIVE SERUM at 1:50 DIL
1:500 DIL
NEGATIVE SERUM at 1:50 DIL
O.D. at A405 nm
2.0
1.5
1.0
0.5
1
2
3
TIME (hours)

MYCO-ELISA using 1:50 and 1:500 positive serum dilution and 1:50 negative serum control dilution with HRP-conjugate dilution of 1:100 is shown in Figure 28. The highest absorbance reading for MYCO-ELISA was 1.54 at 22 C. The readings at 4 C, 37 C and 50 C were 0.73, 1.09 and 1.05, respectively, as illustrated in Figure 28.

The specific activity increased with increasing time of the incubation. The absorbance readings of positive Mg control serum at 1:50 dilution at 1, 2 and 3 hours of incubation were 0.498, 1.462 and 1.623. These readings were much higher than the readings of the negative serum control, which were .321, 0.462 and 0.523 at 1, 2 and 3 hours of incubation, respectively. The absorbance readings for positive serum control at 1:500 dilution were a little lower than 1:50 serum dilution, as illustrated in Figure 28.

4. Determination of the Specificity of MYCO-ELISA

Positive known serum for 4 different subgroups of lymphoid leukosis, 2 different subgroups of Marek's disease and infectious bursal disease were obtained from USDA Regional Poultry Research Laboratory, East Lansing, Michigan. Positive and negative known chicken and turkey sera for Mg, Ms, Mm and M. gallinarum were obtained from Veterinary Service Laboratories, USDA, Ames, Iowa, and Southeastern Poultry Research Laboratory, USDA, Athens, Georgia. Also, positive serum with high antibody titer for Pasteurella multocida and Mycobacterium avium were obtained from other sources to conduct the sensitivity test. The Mg antigen (MYCO-ELISA antigen) reacted strongly with positive sera of Mg and Ms. The HI titers for both sera used in the test were 1:100 and the MYCO-ELISA was 1:8000. Positive sera for diseases other than Mg and Ms showed no reactions to Mg antigen and very low absorbance readings similar to

that of control serum.

5. Determination of the Sensitivity of MYCO-ELISA

A comparison study between HI test and MYCO-ELISA test was made by using sera obtained from naturally and experimentally Mg infected chickens. The HI titersof 8 sera compared to those of MYCO-ELISA titer are shown in Table 5. The data indicated that MYCO-ELISA is far more sensitive than the HI test. The absorbance readings at 405 nm for all positive sera were higher than 2.5 times the absorbance reading of the negative serum at the same dilution. The absorbance reading of 1:50 dilution of the serum with HI of 1:640 was 1.555, HI of 1:320 was 1.329, HI of 1:160 was 1:302 and HI of 1:80 was 0.991 in comparison to the negative reference control serum, which was 0.295 at 1:50 dilution, and it was much lower at higher dilutions, as shown in Figure 29. The absorbance reading of the end point dilution for those positive serum gave much higher ELISA titer than the HI. The ELISA titers for serum number 8 (Table 5), which has HI of 1:640, was >32,000, HI of serum number 1 and number 5 was 1:320 and the ELISA titers were 1:32000 and 1:16000, respectively. Again, the results recorded in Table 5, as well as results illustrated in Figure 29, showed that correlation between the absorbance readings of MYCO-ELISA and the HI antibody titer is obvious.

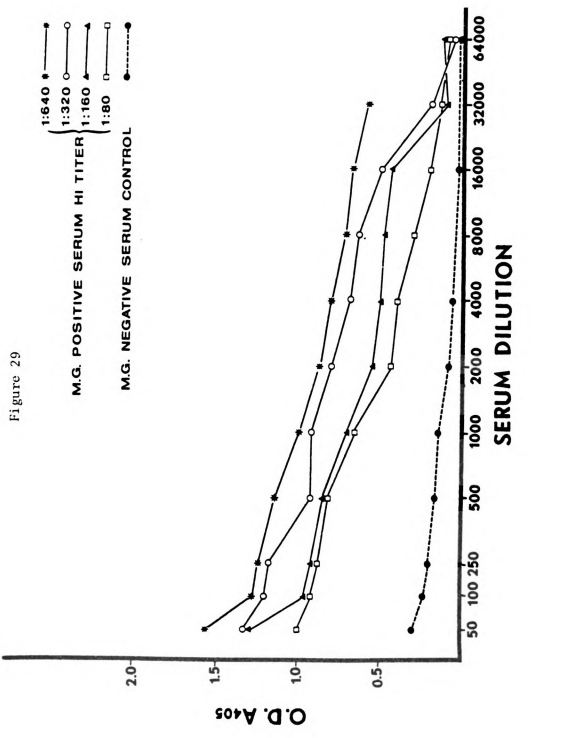
Table 5. Comparison of antibody titer for Mg by HI and MYCO-ELISA

Serum No.	Reciprocal HI titer	MYCO-ELISA			
		O.D. at A405* at 1:50 serum dilution	Reciprocal ELISA titer	O.D. of the end point dilution	O.D. of negative serum
1	320	1.427	32.000	.561	.179
2	160	1.425	16.000	.448	.104
3	320	1.329	16.000	.494	.046
4	80	.991	8.000	.314	<.089
5	320	1.188	16.000	.363	.026
6	640	1.555	>32.000	>.592	.019
7	320	1.07	16.000	.359	.026
8	160	1.302	16.000	.448	.016

*Absorbance reading of 1:50 serum dilution.

Figure 29. The absorbance readings for MYCO-ELISA at 405nm for positive and negative sera for Mg at different dilutions using standard 180 µg antigen concentration and 1:100 conjugate dilution

Figure 29


M.G. POSITIVE SERUM HI TITER
1:640
1:320
1:160
1:80
M.G. NEGATIVE SERUM CONTROL
O.D. A405
2.0
1.5
1.0
0.5
50 100 250 500 1000 2000 4000 8000 16000 32000 64000
SERUM DILUTION

DISCUSSION

A. Cultural Media

Six different culture media for Mycoplasma were used in these experiments. Brain-heart infusion medium, PPLO medium, tryptose phosphate broth, B-medium of Fabricant and modified Frey medium were compared to the MSU-Mycoplasma medium. Brain-heart infusion medium and B-medium of Fabricant were not reported in the results because of the difficulty of dealing with the small size colonies and lower numbers of CFU/ml of the original media. The Mycoplasma broth base formula (Frey et al., 1968), manufactured and marketed by Gibco, contained the following: peptone 140 (pancreatic digest of casein) 7.5 gm, peptone 110 (papaic digest of soy protein) 2.5 gm, yeast extract 5.0 gm, sodium phosphate dibasic 1.6 gm, potassium phosphate monobasic 0.1 gm. However, the contents of the base medium were not enough to produce reliable numbers of the organisms. The MSU-Mycoplasma medium (MSU-medium) contains the basic nutrient requirements for Mycoplasma organisms obtained from Mycoplasma broth base (Gibco) plus other additives. Maltose (2 gm) or dextrose (3 gm) were added to the medium to enhance the yield of viable organisms, which is in agreement with Adler and DaMassa (1968). Many investigators studied the production of Mg antigen by using basal medium containing high levels of dextrose as a carbohydrate substrate. Modified Frey medium and PPLO medium which contains 10 gm of dextrose are still commonly used media for Mycoplasma isolation and antigen production. However, this may have accounted for their failure also because the increase in

acidity of the medium will retard the growth of viable organisms.

The addition of 0.3% Tris buffer (Trihydroxy methyl aminomethane hydrochloride) in MSU-medium was benificial to maintain the pH of the medium around 7.3 for the first 48 hours of growth. The experimental results showed that the pH of the culture medium fell to about 6.8 if dextrose alone was added. These findings are in agreement with Vardamann (1967) and Gill (1962). The addition of dextrose or maltose to the basal medium would increase the total yield of the organisms. The result of the sugar fermentation increased the media acidity, which decreased bacterial growth after 24 hours. The addition of Tris buffer to the MSU-medium maintained the pH of the medium in the range of 6.5 $\pm$ 0.5. The broth base (Gibco) contains 0.5% yeast extract, which acts as a source of nitrogen and also provides maltase, the enzyme for maltose fermentation. Dierks et al. (1967) used autoclaved yeast autolysate and had no problems with nonspecific enzyme activity except with avian serotype F. However, Yoder and Hofstad (1964) earlier reported difficulty to grow Mg in PPLO medium due to the presence of enzyme sucrose of yeast origin. In our experiments, addition of 2-5 gm of yeast autolysate presented no problems in growing the organisms.

L -histidine was added to the MSU-medium (0.25%) to prolong the stationary phase and the survival of the microorganisms. It will also delay the rise of the acidity of the media, which is in agreement with Ajello and Romano (1975). It has been reported that L-histidine does not interfere with urease activity of Mycoplasma (Ajello and Romano, 1975). Also, cystine hydrochloride was added to the MSU-medium as a stabilizing agent. Coenzyme I (NAD) was added to the medium in 1%

solution for the growing of Mg and Ms. Waymouth medium MAB 87/3 (#78-027, Gibco), 50 ml, was used in the MSU-medium as a source of amino acids and vitamins, which was substituted for 70% of the serum requirements.

All avian Mycoplasma species required cholesterol and long chain fatty acids for growth and morphological and osmotic stability. Therefore, horse or swine serum, 5-10%, was added to the medium for Mg isolations and for antigen production. Thallium acetate was added in 0.01% quantity to the medium to inhibit Gram-negative bacteria. Penicillin potassium G in a final concentration of 1000 units per ml was added routinely to MSU-medium to inhibit Gram-positive bacteria. Phenol red was added in quantity of 0.025 gm/liter as a pH indicator only to the medium for isolation and not for antigen production. The closest medium formula to MSU-medium is modified Frey medium. However, maltose, L-histidine, yeast autolysate, tris buffer and Waymouth medium were not included in the modified Frey medium formula. Both media used broth medium (Gibco) as a basic ingredient.

The decline of the pH of the four media by hours and days PI was demonstrated in Figures 9 and 10. The pH of MSU-medium stayed in the basic side much longer than the modified Frey medium. This probably was due to the presence of Tris buffer and reducing the amount of dextrose in the medium. There was not much difference in the pH reading of the MSU-medium when either maltose or dextrose was used. The PPLO and tryptose phosphate media gave the lower pH (acidic) reading than the other media at all times. The lowest count of viable organisms was obtained from PPLO and tryptose phosphate media throughout the experiment as compared to MSU and modified Frey Media. The correlation between the pH of different media and viable bacterial counts was observed.

When the pH declined (acidic), the viable bacterial count decreased. However, DaMassa and Adler (1969) reported that Mycoplasma organisms grew over a wide pH range, which is in agreement with our research results. It was also shown that Mg grew at low pH (4.8) but not for a long time. After 9 days PI, Mg organisms were isolated from all media. The pH range of all media was 4.8 - 6.5. After 30 days PI, the organisms were only isolated from MSU- and modified Frey media and the pH of these media was 6.5 and 6.1, respectively. At 60 days PI, the organisms were isolated from MSU-medium only (0.1×10^3 CFU/ml) and not from the other media and the pH was 6.6. The reason for this probably was due to the presence of Waymouth's media, which provided enough nutrient support for the growth of the organism for a much longer period of time than did media containing serum only. The pH of MSU-medium stayed close to the neutral pH reading for a long time. The MSU-medium seems to be superior to other media that are currently used to isolate avian Mycoplasma regarding bacterial yield and the survival time of the microganisms in the media.

The reaquirements of growth for avian Mycoplasma were studied in detail for the three most common strains Mg, Ms and Mm (Yoder and Hofstad, 1964; Kerr and Olson, 1967 and Anderson, 1968). However, no data have been published to characterize the requirements of the growth for non-pathogenic strains such as M. gallinarum, M. anatis, and M. laidlawii var inocuum. More growth and maintenance media should be formulated which would be able to differeniate between the pathogenic and non-pathogenic Mycoplasma isolates. This can be accomplished by finding the requirements for growth for the nonpathogenic strain and

developing a medium which allows the growth of the pathogenic strains.

B. Standardization of Mg Antigen

In this study, Mg serotype A (S_6) was the only antigen prepared. However, there is no reason to doubt that the same procedures cannot be used to prepare different Mycoplasma antigen by the same methods (Sabry, 1968; Fabricant, 1969). In order to obtain a high yield of antigen, Mycoplasma organisms were added to the culture medium in the log phase of growth and also when passed to another culture medium (Sabry, 1968). The volume of culture was increased through these passages at 24-hour intervals until a one liter volume had been inoculated. The agitation was very helpful to prevent clumping the organisms. Also, using glass Ten Broeck tissue grinder or glass beads was very beneficial to obtain homogenized antigen suspension. Glycerol proved to be a good preservative for HA antigen. The antigen was thawed and frozen a number of times with very little loss in hemagglutinating activity. Without glycerol, the freezing and thawing process was very detrimental to the HA antigen. These results seem to agree with the research results of Dierks (1964). No preservative was added to MYCO-ELISA antigen to avoid any interference with the enzymatic activity of the conjugate.

C. Identification of Mycoplasma Isolate by Immunofluorescence Methods

The fluorescence antibody technique (FA) has been used in diagnosis of a wide variety of diseases.

1. Antisera Production

The purpose of these experiments was not to determine the effectiveness of such vaccination program or different route of vaccination. All vaccinated rabbits and chickens produced a high titer of antibody against Mg antigen. This hyperimmune serum was used in both immunofluorescence and MYCO-ELISA experiments. The antibody titer in both hyperimmune sera was higher than 1:80 in chickens and 1:60 in rabbits using HI test at all times of the immunization program. The response of the rabbits in developing specific antibodies for Mg was influenced by the route of vaccinations. The production of agglutinin antibodies was very high following the injection of small amounts of antigen with incomplete Freund adjuvant subcutaneously (SC) (Rabbit No. 3) one week after the second injection. The antibody titer was 1:1280 using HI test for Rabbit No. 3. The SC and intramuscular (IM) injections (Rabbit No. 1) gave higher antibody titer one week after the second injection in comparison to intravenous (IV) and SC injection programs (Rabbit No. 2). The antibody titer of Rabbit No. 1 was 1:640 and for Rabbit No. 2 was 1:320 using HI method.

Also, the HI titers in Rabbits No. 1 and 3 were very similar in the long run for 8 weeks. Thus, SC injections alone or SC with adjuvant followed by several IV injections or the combination of IM, intrafoot pad FP and IV injections for several weeks were very effective to produce high antibody titer. These results are in agreement with Morton and Roberts (1967).

2. Immunofluorescence Diagnosis for Mycoplasma

The use of direct FA for the identification of avian Mycoplasma in broth culture has the distinct advantage of rapid identification

of Mycoplasma growth without having to plate and incubate for 5-10 days until typical Mycoplasma colonies develop. The observation of fluorescence indicated the presence of specific Mycoplasma growth without having to depend on colony growth and morphology criteria. The identification of pathogenic from nonpathogenic Mycoplasma species has been very difficult because of the unavailability of specific serological tests for each Mycoplasma species. Furthermore, the identification of Mg, Ms and Mm depends primarly on serological tests such as rapid plate test (RSP), tube agglutination (TAT) and hemagglutination inhibition test (HI). Cross reactions with serological tests had been reported by Olson et al. (1965), Roberts and Olesiuk (1967) and Vardeman and Yoder (1969). Vaccines such as erysipelas bacterin (Boyer et al., 1969; Olson et al., 1965, and Roberts, 1970), contaminated serum (Roberts et al., 1967) and frequent freezing and thawing of the serum (Thornton, 1969) have been reported to cause cross reactions in serological diagnosis of avian Mycoplasma. Also, Streptococcus faecalis and Staphylococcus aureus infections in chickens had been demonstrated to cause non-specific agglutinations. Direct FA can identify different species of Mycoplasma organisms obtained from broth media if the primary identification of the organism from the host failed. Noel et al. (1964) used direct and indirect fluorescent methods to diagnose avian Mycoplasma organisms from air sac lesions and turkey sinus exudate. However, Noel et al. (1964) and Barile et al. (1962) showed that tissue exhibited a high degree of green fluorescence because of non-specific uptake of the labeled globulin. The fluorescent Mycoplasma organisms which took up the labeled antibodies were not readily differentiated from the backgrounds, which were having similar color. Clark et al. (163), Costvet and Sadler (1964) and Stewart

(1967) methods showed nonspecific reactions in identifying mixed cultures of Mycoplasma growth on an agar medium. However, each method has some limitations for expedient identifications of Mycoplasma organisms and mixed Mycoplasma cultures. The identification of specific avian Mycoplasma organisms by direct FA method by fixing the organisms on a slide from both media was satisfactory and not as time consuming as the agar block method for colony identification (Figures 13 and 14).

It has been postulated that different PPLO contamination in tissue culture was L-forms of different bacteria. This transformation to L-form occurred in the presence of specific antibodies (Rothblat and Morton, 1958). Whether the nonpathogenic avian Mycoplasma are L-forms of the pathogenic strains or are entirely different strain of Mycoplasma is not clear and needs more investigation. The cross reaction in serological tests made the identifications more difficult. Also, the preparation of different non-pathogenic Mycoplasma antigen is needed to confirm such a positive serological test for Mg, Ms and Mm. Until this can be accomplished, FA has the potential advantage for characterization of different Mycoplasma organisms isolated.

Another advantage is that small numbers of organisms can be identified when large numbersof contaminants are present. The disadvantage of the test is the cost of initial fluorescent microscopy equipment and necessity of adequately trained personnel to avoid non-specific reactions, auto-fluorescence and over-labeling of the conjugate.

D. Purification of IgG by Protein A

The IgG binding properties of protein A made affinity chromatography with protein A-Sepharose CL-4B molecules the method of choice for

isolation of IgG antibodies in mammals. Recent studies have indicated that the protein A reactivity to the IgG Fc fragments was correlated with the IgG H-chain type. Also, the activity shown by Fc fragment is not found in Fc' subfragment nor in pepsin components II or III (Kronvall et al., 1970). The IgG molecules of subclasses 1, 2 and 4 and their fragments containing the Fc region have the affinity to bind to protein A but not IgG-3 (Hjelm et al., 1975). Rabbit IgG was isolated by chromatography fractionation of the serum on a column protein A, coupled to Sepharose. Two major peaks were obtained (Figure 15). The first peak was the eluate of 0.1 M sodium phosphate, pH 7.0 which apparently contained all immunoglobulin and IgG_3 molecules. The second peak was the eluate of 1 M acetic acid, pH 7.0, which contained only IgG 1, 2 and 4 fraction, as shown in the Ouchterlony agar immuno-diffusion test (Figures 16 and 17). The same protein A-Sepharose column was used to purify IgG fraction from normal and hyperimmune chicken sera. The acetic acid eluate wash gave a very small peak which had a spectrophotometer absorbance reading at 280 nm less than 2.0, as shown in Figure 18. Immunodiffusion studies for the eluates indicated that chicken IgG does not bind to protein A molecules, as illustrated in Figures 19, 20 and 21. No reactions were noticed with any of the acetic acid eluates against rabbit anti-chicken IgG (Rb-ani CIgG). The significant difference between rabbit and chicken IgG is that acetic acid eluate did not form any precipitating band against either Rb-ant Cglb or Rb-ant CIgG, as illustrated in Figures 21a and b. The reasons for the non-binding properties of chicken IgG to protein A-Sepharose molecule may be due to: 1) the presence of other protein which binds to the protein A molecule and inhibits the binding

of protein A to the IgG molecule; 2) the amino acid configuration of the Fc region of chicken IgG is different from mammal's IgG and subsequently possesses different binding properties and antigenic determinants.

Recent studies on IgG molecules of chickens and other birds such as pheasants and quail indicated that they are closely related in respect to a number of properties that are not usually associated with mammalian IgG (Leslie and Benedict, 1970). The differences between avian and mammalian IgG were summarized as follows: the IgG of these birds dissociated when reduced in the absence of a dispersing agent (Leslie and Benedict, 1969), it aggregates especially Fc but not Fab fragments in high salt concentration (1.5 M Nacl) solution where rabbit IgG does not (Kuba and Benedict, 1969), and has a molecular weight of about 1.7×10^5 daltons (Hersh et al., 1969). Also, Leslie and Benedict (1970) indicated that the IgG molecules of these birds share certain antigenic determinants. Leslie and Benedict (1970) digested rabbit and chicken IgG by papain and other reducing agents to produce products that had a sedimentation coefficient of 3.4 S. Two antigenic components were identified by immunoelectrophoresis from chicken IgG, Fab-fragment (antigen binding) designated S' and S'' and Fc fragment (crystallizable) which was designated as F. According to their preliminary analysis, chicken IgG contained greater amounts of serine, glycine and alanine and lesser amounts of glutamine and tyrosine amino acids than were found in rabbit, human or horse IgG. We suggest that the nonbinding properties of the Fc region of chicken IgG are because of the absence of some of the C terminal amino acids which are responsible for binding process in mammals. Investigations to date have not clarified why the Fc region of rabbit and other mammalian

IgG binds to protein A molecules; conversely, the lack of binding of chicken IgG to protein A is equally vague.

A possible reason is that the chicken Fc-H chain of IgG did not bind covalently to protein A-Sepharose molecule because other protein molecules present in the chicken serum did bind to protein A molecules and did inhibit the binding process with IgG molecule. Quigley et al. (1974) described an assay system to purify chicken and mammalian plasminogen on lysine-sepharose column. For this reason, immunodiffusion study was conducted to study the effects of chicken plasminogen on purification of chicken IgG by protein A-Sepharose chromatography. The chicken plasminogen did not bind covalently to protein A molecules. Different sodium phosphate eluates gave an identical band with the control chicken plasminogen well against Rb-P1, as illustrated in Figure 22. Also, this figure showed that plasminogen did not have the affinity to bind to protein A-Sepharose molecules. The acetic acid eluate of the column as well as the ammonium sulfate did not give any precipitating band against Rb-P1.

E. MYCO-ELISA

The enzyme immunoassay technique for the detection of Mycoplasma antibodies of chickens was first adopted in the Michigan State University Avian Microbiology Laboratory. The standaradization to determine the optimum concentration of the test reagents and procedure was performed.

The MYCO-ELISA antigen was prepared from Mg whole bacterial cell or its disrupted cell suspension. This antigen showed strong affinity for binding or adsorbing to the surface of polystyrene walls of microtiter plate (U-form, Immunolon substrate plates, Cook Laboratory,

Alexandria, VA). These disposable plates were more convenient for large scale use and very economical since only small volumns of reagents were needed. It is known that polystyrene plates (Dynatech Laboratories) can be coated and sensitized with many protein and lipoprotein antigens (Voller and Badwell, 1976). The Mycoplasma antigens retain their immunologic activity after coating the surface of the wells. The process of coating the plates with Mycoplasma protein antigen was done by passive adsorption to the polystyrene plates using alkaline solution such as 0.1 M carbonate-bicarbonate buffer, pH 9.6. This method was also used by other researchers (Engvall and Perlmann, 1971, and Hamaguchi et al., 1976a,b). The optimum concentrations of antigen used in MYCO-ELISA were 90 and 180 µg antigen protein per well. Both concentrations gave the same absorbance reading at 405 nm with 1:50 serum dilution (Figure 23). Based on the results of experiments conducted at MSU, it is concluded that serum dilutions of 1:50 or 1:100 are the dilutions of choice for diagnostic purposes (Figure 24).

Since this technique was first applied in this laboratory for diagnosis of avian Mycoplasma, materials for comparative discussion were not available. Thoen et al. (1978) used 0.5 µg of Mycobacterium avium antigen protein per well and serum dilution of 1:10 in their experiments for detection of antibodies from M. avium infections in chickens. Bruggmann et al. (1977) indicated that optimum sensitization for ELISA microtiter plates was obtained with 2.5 - 5 µg solubilized antigen protein with SDS or 20 µg when the antigen was solubilized ultrasonically for Mycoplasma suipneumoniae.

The results of HRP conjugate experiments indicated that MSU conjugate is more reliable than Cappel conjugate when it is used in high concentration. This is because of the high reading of the negative serum control with Cappel-HRP conjugate. The readings were 0.723 and 0.404 at 1:100 and 1:1000 conjugate dilution, respectively (Figure 26). MSU-HRP conjugate gave a much lower absorbance reading with the negative control serum (0.285 and 0.134 at 1:100 and 1:1000 conjugate dilution, respectively) (Figure 25). Using positive serum 1:50 dilution and 90 µg antigen protein per well with both conjugates the results showed that 1:50 dilution MSU-conjugate gave similar absorbance readings as Cappel - conjugate at the same dilution. The absorbance readings were 2.39 and 2.41, respectively. However, MSU-conjugate dilutions of 1:200 or 1:300 were the dilutions of choice to be used in MYCO-ELISA since it will give high absorbance reading with the positive serum in comparison to the control serum. Cappel-HRP conjugate can be used in 1:600, 1:800 or 1:1000 dilutions with expectation that negative serum control will give high absorbance readings in comparison to MSU-HRP conjugate. The reason for this probably is due to non-specific binding of rabbit anti-chicken IgG conjugated to HRP made by Cappel Laboratories. Smith et al. (1979) reported that 1:200 dilution of rabbit antiserum (prepared against avian Myeloblastosis virus group antigen) conjugated to HRP gave high absorbance ELISA readings. Also, Thoen et al. (1978) indicated that 1:100 rabbit anti-chicken gamma globulin labeled with HRP is sensitive enough to be used in ELISA to detect antibody for Mycobacterium avium in chickens.

The room temperature of 22 C was the best incubation temperature

for high absorbance reading and subsequently for maximum binding of the antibody and the labelled conjugate to the sensitized well with the antigen. The absorbance reading at 22 C was high, 1.462 at 1:50 serum dilution as compared to 0.734, 1.092 and 0.981 at 5, 37 and 50 C, respectively (Figure 27). The results indicated that the absorbance readings, after 2 or 3 hours incubation period at 22 C,were relatively close using 1:50 or 1:500 serum dilution, 1.542, 1.290 and 1.623, 1.464, respectively (Figure 28). However, the reading, after one hour of incubation at 22 C, was very low, 0.498 and 0.401 at 1:50 and 1:500 serum dilution, respectively. Low levels of activity at low incubation temperatures or at one hour of incubation can be attributed to incomplete binding of available antibody. The decrease in activity at higher temperature (37 C or 50 C) is presumably due to a combination of temperature denaturation effects, dissociation of the enzyme-antiglobulin complex, and release of the absorbed antigen to the surface of the wells at these temperatures. A temperature of 22 C (room temperature) for 2 hours was therefore selected for routine incubation of serum and conjugate. These results are in agreement with other research results using the same conjugate but on different disease systems (Wallen et al., 1977, and Castellano et al., 1977). However, different incubation temperature and length of incubation were suggested by other researchers (Smith et al., 1979, and Bruggmann et al., 1977).

The time which was needed for complete enzyme-substrate reaction depended on both the quantity of enzyme present and the length of the reaction period. The time for reaction of labeled enzyme with the substrate must be long enough so that one can recognize various amounts

of bound enzyme, yet short enough to avoid depletion of the substrate. The difference is the amount of enzyme bound to the polystyrene well, and those of the control groups became more apparent as the time of substrate reaction increased. A substrate reaction time of 30 min was arbitrarily chosen for high dilutions of serum because it gave a high level of sensitivity while avoiding depletion of substrate. However, the change in the color of the positive control serum is the main indicator, at that time 0.45%, HF should be added to stop the hydrolysis of the substrate.

All buffer solutions and washing buffer used in these experiments did not contain any sodium azide as a preservative bacause of its interfering effect in the enzyme-substrate reaction. Persijn and Jonder (1978) reported that addition of a lower concentration of sodium azide completely stops color development in a peroxidase-labelled enzyme immunoassay using 2.2'-azino-di(3-ethylbenzthiazoline sulphonic acid (6)) as a substrate.

To determine the specificity of MYCO-ELISA, positive chicken serum for viral and bacterial diseases as well as other species of avian Mycoplasma such as Ms and Mm were used in these experiments. There was no cross reaction between Mycoplasma and other bacterial or viral diseases. However, the result of specificity tests showed that cross reaction occurs in a large scale between positive serum of Mg and Ms. The common Mycoplasma antigen, phospholipids and glycolipids of the cell membrane are the major antigenic determinants which cross react with other positve serum for other avian Mycoplasma (Kahane and Razin, 1969). Other experiments in vivo showed that whole cell or membrane antigen of

Ms stimulated antibody formation (Williams and Taylor-Robinson, 1967). Mg as well as many other Mycoplasma are bounded by a single lipoprotein membrane (Rettem et al., 1968) and this lipoprotein can be readily isolated by osmotic lysis of the organisms (Razin, 1968, 1969). Disaggregation of Mycoplasma membrane by strong detergent such as sodium dodecyl sulfate (SDS) resulted in soluble antigen clear solution. However, use of this antigen did not increase the specificity of the test among avian Mycoplasma species. There are sufficient data on the homogeneity of the antigen solution. A striking feature was discovered by Razin et al. (1975) in that the detergent solubilized membrane material had the ability to reaggregate spontaneously to membrane like structure on the removal of the detergent. They reported that reassembly of solubilized membrane (lipid and protein) after removal of SDS by dialysis or by Sephade usually occurs if the divalent cation Magnesium (Mg^{++}) is present. The addition of 1 mM disodium ethylene diaminatetra acetate (EDTA) to MYCO-ELISA antigen to prevent the aggregation of the antigen, by binding to the calcium ions, did increase the titer but not the specificity. Mycoplasma organisms do not have a cell wall or intracytoplasmic membrane; it possesses only plasma membrane. Therefore when this plasma membrane was lysed by carbonate-bicarbonate buffer at pH 10, it was used as an antigen in serological diagnosis of Mg and Ms (Goel, 1973 and Villegas et al., 1976). They discovered that membrane antigens were somewhat more sensitive in the RSP and TAT tests whereas the HI test showed no major difference between the whole and membrane antigens. Separation of the membranes is therefore a prerequisite for the specificity of these antigens.

Several methods, including lysis by osmotic schock (Razin, 1963), detergent (Rottem et al., 1968) and mechanical pressure using sonic or ultrasonic oscillators (Pollack et al., 1965), were used to separate the plasma membrane of the organism. However, the Mycoplasma organisms were insensitive to osmotic shock when transferred from 0.25 M sodium chloride to water (Razin et al., 1964). The Mycoplasma membranes for immunological studies were obtained from Mg by injecting the bacterial suspensions in 2 M glycerol into water (Kahane and Razin, 1969, Rottem et al., 1968). The majority of the organisms remained unlysed by this method. Ultrasonic treatment leaves variable amounts of organisms unlysed and disaggregates the membrane into minute fragments. The future of ELISA as a specific test for diagnosis of different avian Mycoplasma species relies on extraction of different components of the organisms and testing the antigenic determinants for each component.

A comparison study between HI and MYCO-ELISA test was conducted using sera obtained from chickens naturally and experimentally infected with Mg. The results, in Table 4, showed the sensitivity of the ELISA method in detecting Mycoplasma antibodies. The absorbance readings at 405 nm for all positive sera were higher than 2.5 times the absorbance reading of the negative sera at the same dilution. ELISA titer for 5 serum samples out of 8 gave 100 times higher titer than the titer of HI test for the same serum (Table 4). HI titers of the sera were 8:320, 1:160, 1:80, 1:640, 1:160 and the MYCO-ELISA antibody titers for the same serum were 1:32,000, 1:16,000, 1:8000, 1:32,000 and 1:16,000, respectively. The absorbance reading of the end point dilution for positive sera in another study (Figure 29) gave much higher ELISA titer

than the HI. The ELISA titers of sera which have HI of 1:640, 1:320, 1:160 and 1:80 were >1:32000, 1:16000, 1:6000 and 1:8000. These results strongly suggest that the MYCO-ELISA is much more sensitive than the current HI test for detecting Mycoplasma antibodies. The HI test has been used as the most efficient and accurate method to differentiate Mg from Ms. Furthermore, our data would suggest that HI is a more specific means of differentiation than MYCO-ELISA.

The official blood tests for Mg and Ms are RSP test, TAT test and HI test, or a combination of two or more of these tests as recommended by the National Poultry Improvement Plan (USDA, November, 1976). The plan stated that the HI test should be used to confirm the positive results of Mg for other serological tests, namely RSP and TAT tests. Villegas et al. (1976) stated that the RSP as a screening test followed by the HI test for confirmation are the most commonly used procedures. The RSP has the advantage of speed, simplicity and sensitivity but for different causes produces occasional non-specific reactions with sera from birds (Roberts, 1967 and 1969). Mg antigen used in the RSP test has been reported to cross react with sera from chickens or turkeys that have been vaccinated within 2 weeks for erysipelas (Boyer et al., 1960, and Olson et al., 1965). The HI test has been reported to be more highly specific than the other two tests. It may not attain significant titer until several weeks later even though the RSP test could be positive, because HI measures only the IgG and not the IgM (Villegas et al., 1976). The cross reaction and non-specific reaction which had been reported with HI test indicate that HI test is not completely specific for detection of Mg antibodies (Yoder, 1978b) either. Outbreaks

of typical Mg infections in chickens were characterized by a low percentage of reactors to the RSP test, which often failed to give a meaningful result. Based on this discussion, there is a definite need for a simple method to diagnose Mycoplasma infections in chickens and turkeys. A successful national eradication program for Mycoplasma requires a dependable method for disease detection with accurate specificity and sensitivity test results.

ELISA has been reported to be more sensitive than radioimmunoassay (RIA), virus neutralization test, passive hemagglutination test, tube agglutination test and hemagglutination-inhibition test in the detection of antibodies for several viral, bacterial and parasitic agents (Gilman and Docherty, 1977; Castellano et al., 1977; Voller and Bidwell, 1975; and 1976; Saunders and Clinard, 1976).

It is believed that enzyme immunoassays can be the future method used to achieve a successful eradication program for Mycoplasma infections because of their simplicity, reproducibility, sensitivity and the automation potential for a large scale diagnosis in the laboratory or in the field. The obvious existing disadvantage is the lack of specificity; however, current sophisticated biochemical, microbiological and immunological techniques will undoubtedly lend themselves to address the problem of molecular differences present in Mycoplasma plasma membrane. Once the variants are delineated, specific MYCO-ELISA techniques applicable to all the pathogenic and non-pathogenic strains will soon be feasible.

APPENDIX

ADJUVANTS

Incomplete Freund Adjuvant (Difco)

Arlacel (Mannide monoaleate)	1.5 ml
Bayol F (paraffin oil)	8.5 ml

Sterilized by autoclaving at 121 C and 15 lb pressure for thirty minutes.

ANTICOAGULANTS

1. Citrate Solution

Sodium Citrate	2.0 gr
Distilled water	100.0 ml

2. Modified Alsever's Solution

Glucose	2.050 gr
Sodium Citrate	0.800 gr
Sodium Chloride	0.420 gr
Citric Acid	0.055 gr
Distilled water	100.000 ml

STABILIZING SOLUTION

1. Sucrose Phosphate Glutamate Albumin Solution (SPGA)

Sucrose	74.60 gr
Monopotassium phosphate	0.45 gr
Dipotassium phosphate ($3H_2O$)	1.64 gr
Sodium glutamate	0.82 gr
Bovine albumin powder (fraction V)	10.00 gr

Distilled water sufficient to make 1000 ml
Sterilize by filtration
Store at 5CC

BUFFERS

1. Phosphate buffer saline (PBS), pH 7.2

Stock solution:

Sodim chloride (NaCl)	170 gm
Potassium dihydrogen phosphate (KH_3PO_4)	13.6 gm
Sodium hydroxide (NaOH)	3.0 gm
Distilled water	95. - 1000 ml

One milliliter of stock solution is diluted 1:20 with distilled water to give a buffered saline having a pH of 7.1 to 7.2.

2. Borate buffer saline (BBS), pH 8.4

Stock solution:

Boric acid	6.184 gm
Borax ($Na_2B_4O_7 \cdot 10H_2O$)	9.536 gm
Sodium Chloride	4.384 gm
Distilled H_2O to 1 liter	

3. Phosphate buffered glycerol:

For immunofluorescence
Prepare phosphate buffer, pH 8.0

0.1 M Na_2HPO_4	94.5 ml
0.1 M $Na_2H_2PO_4$	5.5 ml

Combine and mix thoroughly nine volumes glycerol and one volume phosphate buffer, pH 8.0

4. 0.1 M phosphate buffer, pH 6.8

For preparation of glutaraldehyde solution

Solution A:

Monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$)	1.38 gr
Distilled water	100.00 ml

Solution B:

Dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$)	4.68 gr
Distilled water	100.00 ml

Mix 77 ml solution A to 33 ml solution B

5. 0.05 M carbonate - bicarbonate buffer pH 9.5

As a coating buffer

Na_2CO_3	1.59 gr
Na_2HCO_3	2.93 gr
Distilled water	1000.00 ml

Store at 4 C for not more than 2 weeks

6. Citrate - phosphate buffer, pH 4.0

Buffer for the preparation of the substrate for MYCO-ELISA

0.1 M citric acid	307 ml
0.2 M dibasic sodium phosphate	193 ml
Deionized water	500 ml

7. Phosphate buffer saline, pH 7.4

MCYO-ELISA washing buffer

Sodium chloride	8.0 gr
Monabasic potassium phosphate (KH_2PO_4)	0.2 gr
Dibasic sodium phosphate ($Na_2HPO_4 \cdot 12 H_2O$)	2.9 gr
Potassium chloride	0.2 gr
Tween 80	0.5 ml

Deionized distilled water to 1 liter
Store at 4 C for not more than 30 days

8. Phosphate buffer saline, pH 7.2 - 7.4

For serum and for conjugate dilution

Sodium chloride	37.0 gr
Potassium chloride	0.2 gr
Dibasic sodium phosphate	1.2 gr
Monobasic potassium phosphate	0.2 gr

Deionized distilled water to 1 liter
PBS for serum dilution add 0.1% bovine serum albumin (BSA)
PBS for conjugate dilution add 0.05% Tween 20.

MEDIA

1. Brain heart infusion medium (Vardaman 1967):

	per liter
Brain-heart infusion broth (Difco)	37.0 gm
Dextrose	3.0 gm
Yeast autolysate (Albimi)	5.0 gm
Thiamin HCl	0.005 gm
Tris buffer	3.0 gm
Trypticase	0.5 gm
Thallium acetate	0.1 gm
Swine serum	100.0 ml
Penicillin	100,000 units

2. B-medium of Fabricant (Fabricant and Freundt, 1976):

Brain-heart infusion broth (Difco)	37.0 gm
Cystine HCL	0.1 gm
Swine serum	100.0 ml
Thallium acetate	0.1 gm
Penicillin	100,00 units

3. Modified Frey Mycoplasma medium (Yoder, 1975):

	per liter
Mycoplasma broth base	22.5 gm
Dextrose	10.0 gm
Swine or horse serum	100-200 ml
Phenol red	25 mg
Penicillin G potassium	100,000 units
Thallous acetate (1:4000-1:2000	2.5-5.0 ml 10% Sal

4. PPLO broth medium (Morton and Leece, 1954):

	per liter
PPLO w/o CV	21.0 gm
Yeast autolysate	10.0 gm
Dextrose	5.0 gm
Thallium acetate	0.1 gr
Horse serum	100.0 ml
Penicillin	100,000 units

5. Tryptose phosphate broth (Hall, 1962):

	per liter
Tryptose phosphate broth (Difco)	29.5 gm
Maltose	2.5 gm
Horse serum	125.0 ml
Yeast autolysate	10.0 gm
Thallium acetate	0.1 gm
Penicillin	100,000 units

6. Waymouth synthetic media (Gibco, MAB 87/3 #78-027)

Amino acids: mg/l		Vitamins: mg/l	
L-Alanine	11.20	Ascorbic acid	17.00
L-Arginine HCl	75.00	Biotin	0.02
L-Asparagine	24.00	Calcium pantothenate	1.00
L-Aspartic acid	60.00	Choline Cl	250.00
Cysteine (free base)	61.00	Folic acid	.50
L-Cystine	15.00	i-Inositol	1.00
L-Glutamic acid	150.00	Nicotinamide	1.00
L-Glutamine	350.00	Pyridoxine HCl	1.00
Glycine	50.00	Riboflavin	1.00
L-Histidine (free base)	121.00	Thiamine HCl	10.00
L-Isoleucine	25.00	Vitamin B_{12}	0.20
L-Leucine	50.00		
L-Lysine HCl	240.00		
L-Methionine	50.00		
L-Phenylalanine	50.00		
L-Proline	50.00		
L-Serine	12.80		
L-Threonine	75.00		
L-Tryptophane	40.00		
L-Tyrosine	40.00		
L-Valine	65.00		

7. Special agar, 1% in PBS

Ionagar No. 2 (Oxoid) or Noble agar (Difco)	1 gr
PBS, pH 7.2	100 ml

Table 6. Serotypes of avian Mycoplasma

Aycardi et al. (1971)		Barber and Fabricant (1971a)	Frey et al. (1972)
Agar gel Diffusion	Growth inhibition	Metabolic inhibition	Micro complement fixation
A	A	A	A
BM	BM	B	BM
CDOP	C	CO	DCOP
	D	DP	
	OP		
EG	EG	EG	EG
IJKNQR	IJKNQR	IJKNQR	IJKNQR
F	F	F	F
H	H	H	H
L	L	L	L
S	S	S	S

Table 7. Avian *Mycoplasma*: serotypes, origin and pathogenicity

Serotype	Species	Isolate	Origin	Original Source	Pathogenicity chicken
A	*M. gallisepticum*	X95	Chicken trachea	Markham	+
A	*Mg*	S6	Turkey brain	Zander	+
A	*Mg*	A5969	Chicken trachea	Van Roekel	+
A	*Mg*	801	Turkey air sac	Hofstad	+
B	*Mg*	Fowl	Chicken trachea	Edward	-
B	*Mg*	54-537	Chicken trachea	Kleckner	-
C	*Mg*	C	Chicken trachea	Adler	±
C	*Mg*	DIVA	Chicken trachea	Kleckner	±
D	*Mg*	NY	Chicken trachea	Markham	E
D	*Mg*	R39A	Chicken trachea	Fabricant	E
E	*Mg*	DPR 2	Chicken trachea	Kleckner	-
E	*Mg*	640	Chicken trachea	Hofstad	E
E	*Mg*	C26	Chicken trachea	Fabricant	-
F	*Mg*	SA	Turkey trachea	Adler	-
G	*M. iners*	M	Chicken trachea	Edward	-
G	*M. iners*	O	Chicken pericardium	Adler	E
H	*M. meleagridis*	N	Turkey air sac	Adler	+
H	*M. meleagridis*	1300	Turkey air sac	Yoder	+
H	*M. meleagridis*	E 2	Turkey yolk sac	Rhoades	+
I		695	Turkey air sac	Hofstad	±

Table 7 (continued)

Serotype	Species	Isolate	Origin	Original Source	Pathogenicity
J		693	Turkey hock joint	Hofstad	±
K		1805	Chicken oviduct	Yoder	-
L		694	Pigeon turbinate	Hofstad	-
M		R49	Chicken trachea	Fabricant	±
N		PHND13	Turkey air sac	Fabricant	-
O		TC5	Chicken trachea	Moore	-
P		TC3	Chicken trachea	Moore	-
Q		LC 10	Turkey yolk sac	Fabricant	±
R		DC2497	Turkey air sac	Fabricant	±
S	*M. synoviae*	1853	Chicken hock joint	Olson	S
S	*M. synoviae*	1331	Turkey sinus	Yoder	S
---	*M. anatis*		Duck sinus	Roberts	-
---	*M. laidlawii* var. *inocuum*		Chicken sinus	Adler	-

Pathogenicity symbols: + = extensive airsacculitis in inoculated turkeys
± = slight to moderate airsacculitis in inoculated turkeys
- = nonpathogenic for chickens and turkeys
E = periarticular abscess in chicken embryo
S = synovitis in chickens and turkeys

Table 8. Classification of avian Mycoplasma on biochemical and serological bases

Sero-type	Species	Arginine decar-boxylase	Dextrose fermen-tation	Tetra-zolium reaction	Hemagglutination using species specific RBC's	DPN requests	Serum requests	Film and spots on egg yolk
A	M. gallisepticum	-	+	+	+	-	+	-
BM	M. gallisepticum	+	-	+	-	-	+	+
CO		-	+	-	-	-	+	-
DP		-	+	-	+ only P	-	+	-
EG	M. iners	+	-	-	-	-	+	+
IJKNQR		+	+	+	+ except Q and R	-	+	-
F		-	+	-	-	-	+	-
H	M. meleagridis	+	-	-	+	-	+	-
L		+	-	+	-	-	+	+
S	M. synoviae	?	+	?	+	+	+	?
-	M. anatis	-	+	+	-	-	-	+
-	M. laidlawii var. inocuum	-	+	+	-	-	-	-

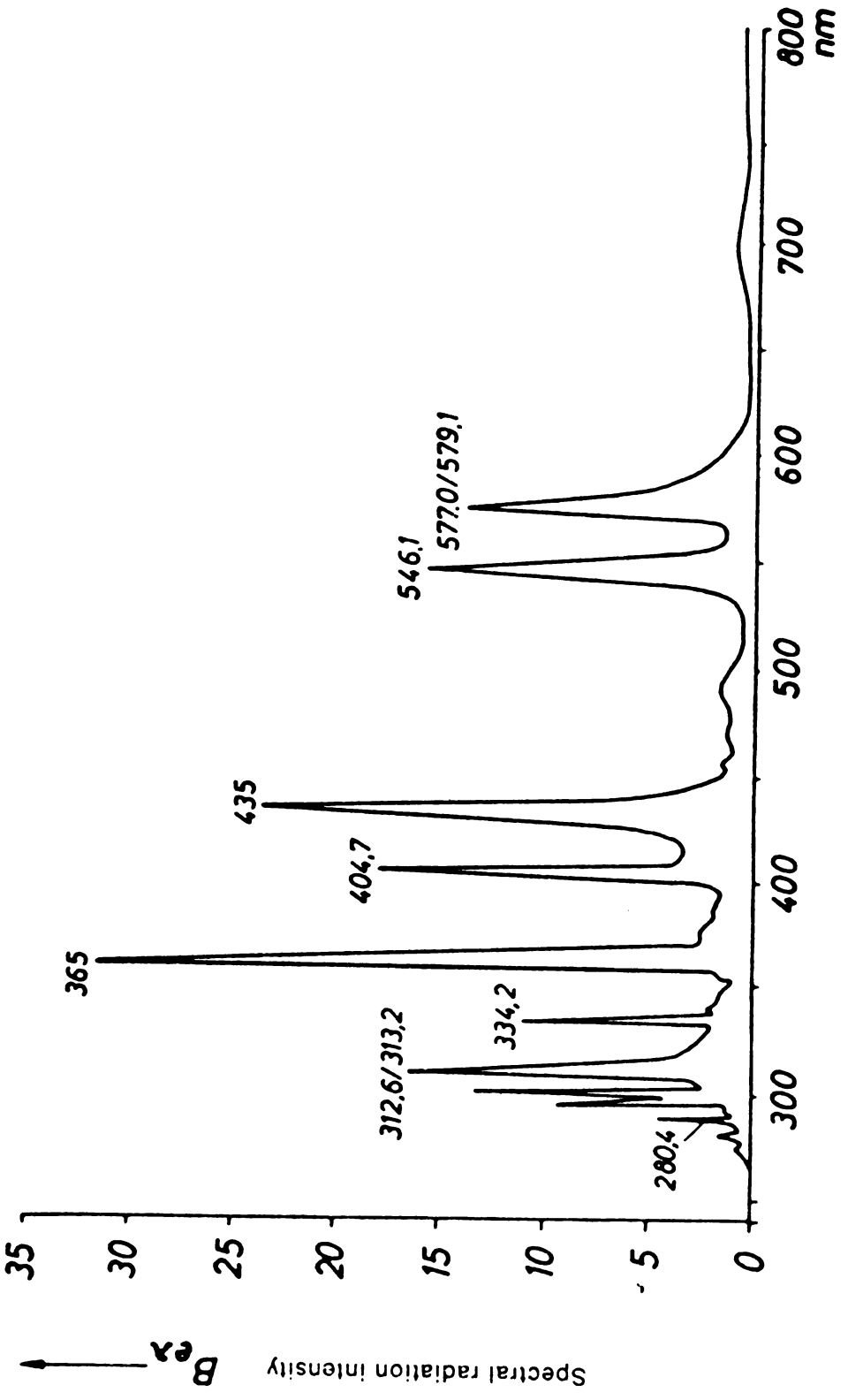


Figure 5 : Spectral emission of HBO 200 W superpressure mercury lamp (nm = mμ)

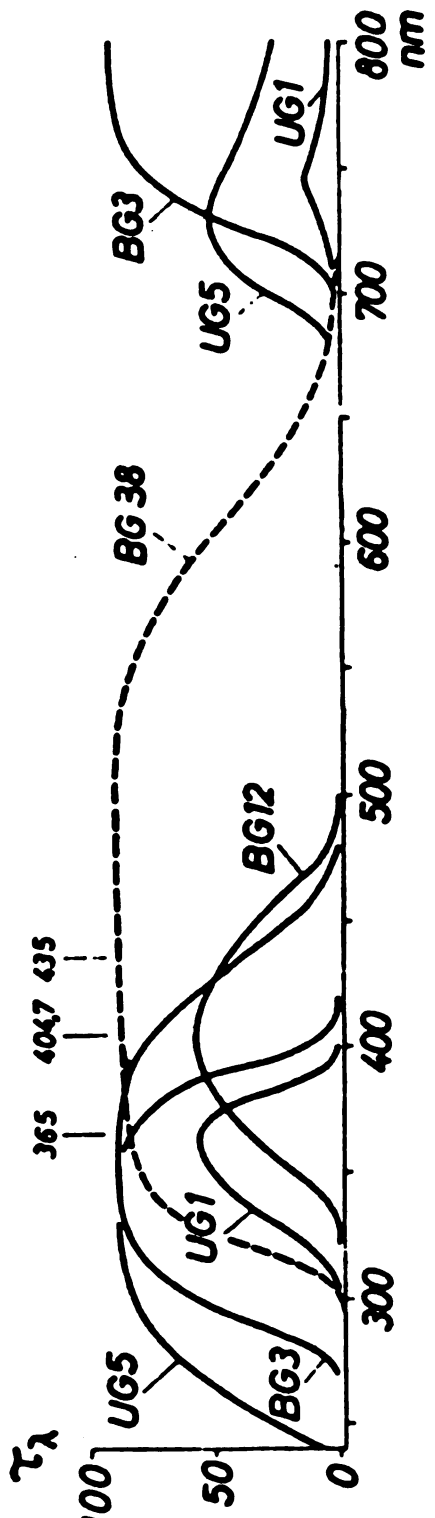


Figure 6: Spectral transmission of exciter filters

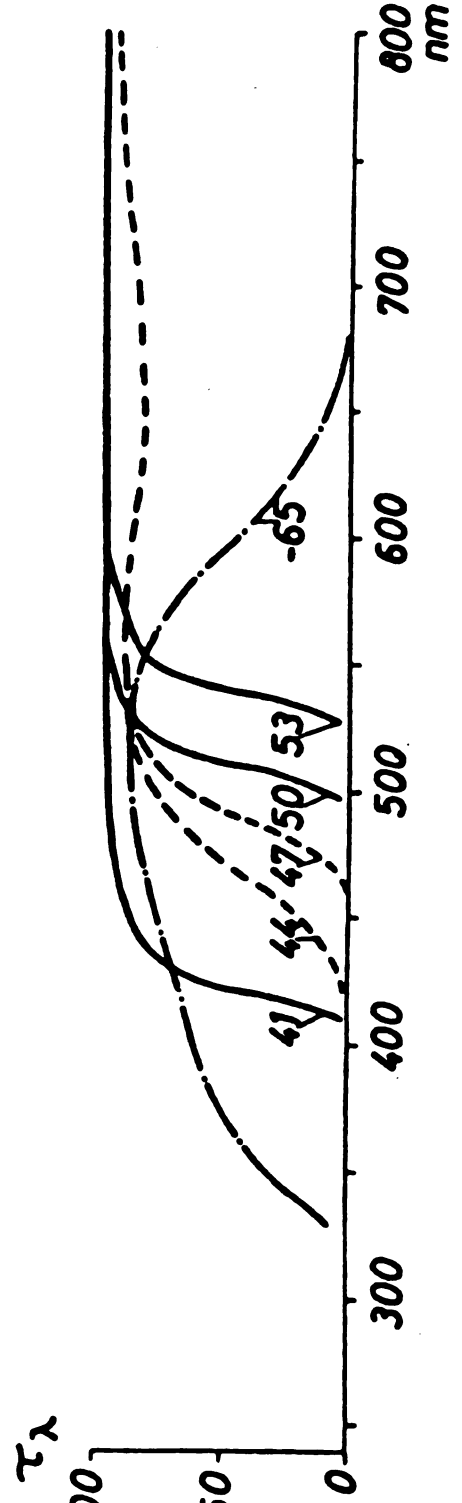


Figure 6: Spectral transmission of barrier filters

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