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Mycoplasma Species of Man

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INTRODUCTION

Until recently, one of the most neglected areas of research in microbiology has been the study of the order Mycoplasmatales. A renaissance of interest in these microorganisms has occurred as a result of three major observations: (i) the recognition by molecular biologists and biophysicists that the mycoplasmas are the smallest free-living microorganisms; (ii) the discovery that the etiological agent of cold agglutinin positive atypical pneumonia of man is a mycoplasma (this represents the first unequivocal demonstration of pathogenicity of a mycoplasma for man); and (iii) the detection of mycoplasmas as common contaminants of tissue cultures where they may produce changes in cellular growth and cell susceptibility to virus infection.

Since the mycoplasmas are widely dispersed in nature and relatively simple methods exist by which they can be isolated, it is all the more surprising that they have been so neglected. In addition to their isolation from man, mycoplasmas have been isolated from chickens (and their eggs), turkeys, pigeons, parakeets, ducks, mice, rats, cattle, sheep, goats, dogs, cats, horses, swine, and guinea pigs. An animal species may harbor many kinds of mycoplasmas, but, with few exceptions, the types are species specific. Rabbits and hamsters appear to be the only common laboratory animals from which mycoplasmas have not yet been isolated. Mycoplasmas have also been isolated from sewage and from decomposing organic materials. Some of the mycoplasmas are known to be etiologically responsible for a number of animal diseases, in particular bovine pleuropneumonia (*Mycoplasma mycoides*) which is still of great economic importance although confined to the Iberian Peninsula, India, and the African and Australian continents. Importation of *M. mycoides* into the U.S.A. is prohibited by law. The wide distribution of the mycoplasmas in laboratory and domestic animals and their association with disease has accounted for a greater interest in these microorganisms by veterinarians than by medical microbiologists.

Contrary to the view that the mycoplasmas are a newly recognized group of microorganisms, and in support of the contention that current interest is, indeed, a renaissance, is the fact that the etiological agent of bovine pleuropneumonia was first recognized by Louis Pasteur, although he did not observe it microscopically nor could he grow it in nutrient broth. In 1898 Nocard and Roux (192) succeeded in growing the agent in cell-free medium, and in 1910 its morphology was described by others (17, 19). Filterability through Berkefeld V filters was soon demonstrated, and, in 1929, Gradocol membrane filtration studies indicated that the size of the minimal reproductive units was in the range of 125 to 150 mμ (90). *M. mycoides* was, therefore, in the unique position of being considered as a virus for about 25 years even though it was known to multiply in a cell-free medium. Curiously, scientific history has now repeated itself, and the etiological agent of cold agglutinin positive primary atypical pneumonia in man, which was considered for many years to be a virus, has now also been identified as a mycoplasma (38).

In 1941, following the isolation of mycoplasmas from other animals and from man and sewage, it was recognized that there existed in nature a new group of parasitic and saprophytic microorganisms with unusual properties that distinguished them from the bacteria, rickettsiae, and viruses. The first review of the field by Sabin should be consulted for details of the earlier work (227). A comprehensive treatment of the entire field can also be found in the books of Klieneberger-Nobel (147) and Freundt (101), in a symposium publication (83), and in other more specialized reviews (2, 18, 46, 68, 80, 82, 144, 145, 178, 244, 269, 270).

It is the intention of the present reviewers to draw attention to the role of the mycoplasmas as part of the microbial flora of man and, in particular, to those observations made during the past decade.

GENERAL PROPERTIES OF THE GENUS MYCOPLASMA

Definition

The mycoplasmas are a group of microorganisms with the following properties. (i) They can reproduce in a cell-free medium. On agar they exhibit a characteristic colonial morphology with the center or the whole colony embedded beneath the surface. (ii) The smallest reproductive forms have a size range of 125 to 150 mμ. Thus, they are the smallest free-living organisms, with a size similar to that of the myxoviruses. (iii) They are highly pleomorphic because they lack a rigid cell wall and instead are bounded by a triple-layered "unit membrane." The inner and outer layers are electron-dense and the middle layer is less dense. (iv) Excepting the saprophytic species (*M. laidlawii*), most species require sterol and native protein for growth. (v) All species exhibit an absolute resistance to penicillin, whereas most strains are inhibited by low concentration of tetracyclines. (vi) The growth of the mycoplasmas is inhibited by specific antibody. (vii) Mycoplasmas show no history of reversion to or from a bacterial parental form.

Taxonomy

The organism causing contagious bovine pleuropneumonia is the obvious choice for the prototype species, because it was the first of the mycoplasmas to be discovered and, in consequence of its virulence and economic importance, has commanded the most attention.

Although first recognized as a microbial entity by Pasteur, it was first isolated by Nocard and Roux in 1898 (192) and designated the "microbe de la peripneumonie." Microorganisms from other sources, having properties similar to the pleuropneumonia organism of cattle (PPO), soon came to be known as pleuropneumonia-like organisms (PPLO). This term, although in wide use until recently, has now been superseded by general acceptance of the nomenclature and classification scheme of Edward and Freundt (88). Although originally designated in the Linnean system as *Asterococcus mycoides* in 1910 (19), the generic name is invalid, having been used first for a genus of algae in 1908 (229). Two subsequent generic terms, *Coccobacillus* (170) and *Micromyces* (104), must be discarded for the same reason; thus, the term *Mycoplasma*, introduced in 1929 (193), becomes the first valid generic term. Since taxonomic rules do not invalidate the use of the species designation even if it has been used in other genera, the correct name of the prototype species is *Mycoplasma mycoides*. The recognition of only a single genus is justification for invoking the taxonomic rule that the family name be derived from the generic name, thus becoming Mycoplasmataceae. The question of whether to assign the mycoplasmas to a separate order has not been fully settled, although a number of arguments have been raised in its favor (101).

A cursory consideration of the properties of the order Actinomycetales suggests it as the proper order in which to place the mycoplasmas (153). Characteristically, the colonies of both groups penetrate solid medium. However, those features which distinguish these two groups are of such a magnitude that the placing of the family Mycoplasmataceae in the order Actinomycetales seems unjustified. The minimal reproductive units of the mycoplasma do not seem to be related at all to the spores of actinomycetes, morphologically or in respect to the latter having greater resistance than mycelial filaments (101). In addition, the mycoplasmas are gram-negative and penicillin-resistant, whereas the actinomycetes are opposite in these properties. Thus, not only are the mycoplasmas unrelated to the actinomycetes but they are quite obviously unrelated to other schizomycetes, if only because they lack a well-defined cell wall. They differ also in other properties, such as complete resistance to penicillin, great resistance to thallium acetate, and dependence upon sterols as a growth factor. Furthermore, with the exception of the leptospires, the mycoplasmas are unlike all bacteria in that their growth is inhibited by homologous antiserum (87).

In fact, a literal consideration of the aforementioned properties of the mycoplasmas would make a good case for the notion that they are not only unrelated to the bacteria, but to the plant kingdom itself. A requirement for sterols and the absence of a rigid cell wall would, strictly speaking, place the mycoplasmas in the animal kingdom where they could be considered for classification with the protozoa.

Reasonable arguments have also been made for designating the mycoplasmas as a separate class between the Schizomycetes and Microtatobiotes (Rickettsiales and Virales). These arguments have been largely based on size range, absence of cell wall, growth inhibition in the presence of specific antiserum, and requirement for growth of preformed materials such as sterols and proteins. Additional support for at least a separate class for the mycoplasmas may be found when one reflects upon their position in biological evolution. The requirement of viruses for more highly evolved hosts in order for them to replicate precludes their being placed at the base of the evolutionary tree. However, a consideration of the properties of the mycoplasmas places them in the position of being the most primitive microorganisms known. They are the smallest organisms possessing the necessary metabolic machinery to assemble themselves from a limited number of nonliving, pre-existing parts. That they are, indeed, the most primitive microorganisms on the evolutionary scale is realized when one considers that they contain all of the integrative mechanisms and, in addition, exhibit unusually exacting nutritional requirements (203).

Nevertheless, the majority of investigators would probably favor the intermediate position adopted by the editors of the seventh edition of *Bergey's Manual* (20) wherein the mycoplasmas are considered as a separate order (Mycoplasmatales) in the class Schizomycetes.

Division I. Protophyta Sachs, 1874, emend. Krassilnikov
Class II. Schizomycetes von Naegeli, 1857
Order X. Mycoplasmatales Freundt, 1955
Family I. Mycoplasmataceae Freundt, 1955
Genus I. Mycoplasma, Nowak, 1929

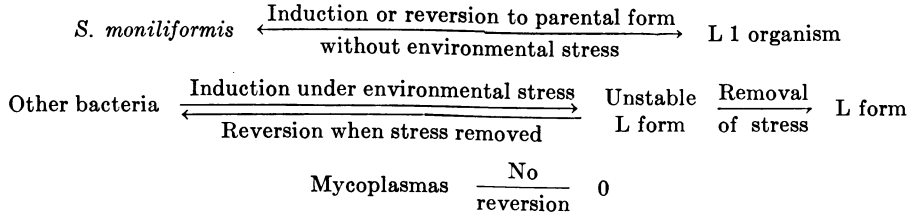
Relationship of Mycoplasmas to L Forms

Although the L-form variants of bacteria are outside the scope of this review, it is important to briefly consider them because they have properties similar to the mycoplasmas. Disagreements regarding the relationship of the mycoplasmas to the L forms have for many years been one of the major obstacles to a meaningful taxonomic classification of the former organisms. The bases for these divergent views are fundamental to an understanding of the biology of the mycoplasmas.

The original L form ("the L 1 organism") was isolated by Klieneberger in 1935 (142, 143) from a rat and regarded as a symbiont of *Streptobacillus moniliformis*. Subsequent studies indicated that the L form (named for the Lister Institute, London, England) of *S. moniliformis* was a variant phase of the parental form. Thus, the L 1 organism was found to grow under ordinary conditions for cultivation of *S. moniliformis*. The subsequent demonstration that organisms of most bacterial genera could be induced by various environmental stresses to give rise to an L-form variant led to much speculation concerning their relationship to the mycoplasmas.

The L forms of bacteria are remarkably similar to mycoplasmas in that both lack a cell wall. Many similarities also exist in colonial morphology. In addition, the minimal reproductive units are morphologically and physically similar, as is the mode of reproduction, resistance to penicillin, and some aspects of metabolism. The antigenic structure of the L-form cell membrane is similar to that of the unit membrane of the parental bacterium. The biochemical reactions of the L forms are generally similar to those of the parental bacterium; however, their metabolism is often more sluggish. Unlike the parasitic mycoplasmas, L forms do not have a sterol requirement; thus, they are similar to the saprophytic mycoplasmas in this respect. L forms are insensitive to surface-active agents, such as digitonin, whereas the mycoplasmas which have been studied are not (244).

Despite the fact that many workers claim to be able to distinguish L forms from mycoplasmas on the basis of subtle differences in their colonial growth on agar and the morphology of the minimal reproductive units, an equal number of well-qualified investigators have aligned themselves against this view. In fact, the notion has been advanced that the mycoplasmas are, in fact, L forms which have lost their capacity to revert to the bacterial parent (68, 269). In practice, however, the L forms are products of laboratory manipulation, appearing when bacteria are grown under adverse environmental conditions. Penicillin, glycine, and high salt concentrations are the most common agents used for the conversion of bacteria to their L forms. L forms have never been cultured from disease processes of man or animals in the absence of the parental bacterium, and those L forms derived from bacterial pathogens are not pathogenic. Other than the L form of *S. moniliformis*, no L forms have been found free in nature. It has been suggested that the L form capable of reverting to the parental bacterium be called the "transition form" or "unstable L form," and that the "L form" designation be reserved for the growth form that no longer reverts (147). The resulting semantic difficulties are, of course, apparent, although acceptance of the fact that L forms are ultimately derived from bacteria is not in dispute. Perhaps the simplest and most conservative view would be the following: if it is known that the organisms were derived from, or could revert to, the bacterial parent, then the organisms are L forms. Lacking such information, one would conclude that he was dealing with a mycoplasma. A schematic representation of these relationships is represented by the following:



Size, Morphology, and Mode of Replication

The curious morphology and uncertain mode of replication of the mycoplasmas is reflected by the fact that no fewer than 40 papers on these topics have appeared. In this area, it is probable that few other microorganisms have attracted as much attention. Consequently, it is against a voluminous background of divergent views and conflicting evidence that the morphology and mode of replication of the mycoplasmas must be considered.

The mycoplasmas go through a cycle of morphological changes during growth, commenc-

ing with a small particle usually referred to as an elementary body or a minimal reproductive unit. The minimal reproductive unit is probably spherical, with a diameter ranging from 125 to 250 mμ, thus making the mycoplasmas smaller than the larger viruses and the rickettsiae.

Although few attempts have been made to describe the morphology of the human mycoplasmas, *per se*, the literature is replete with studies of other mycoplasmas, particularly the prototype species *M. mycoides*. Thus, what follows is largely descriptive of *M. mycoides* but, in all probability, holds for at least *M. hominis* and *M. fermentans* as well (102).

In any discourse on the morphology of the mycoplasmas, cognizance must be taken of the extreme plasticity of these microorganisms. Indeed, the very name of the family, Mycoplasmataceae, was chosen (147) to emphasize this feature. Thus, the very existence of so many studies concerned with the morphology of the mycoplasmas reflects not only their pleomorphism, but also the varying interpretations by workers confronted with the differing manifestations of such pleomorphism.

The morphology and growth pattern of the mycoplasmas must be considered in the light of essentially two schools of workers who represent divergent and, at times, completely opposing views. The first group to be considered is that championed by Freundt (10, 13, 102, 153, 193, 210, 262, 278, 280). The second is that of Klieneberger-Nobel (145, 147, 148), Dienes (62, 63), Liebermeister (158, 159), and others (135, 180, 227).

According to the first group, very thin filaments emerge from the minimal reproductive unit, terminating in a spherical body similar to the minimal reproductive unit (102). The terminal body may develop new filaments, giving rise to arborized branching structures. True lateral branching with probable coenocytic outgrowth of lateral branches from the main filament also occurs. Thus, during the first 18 hr of growth, a mycelial structure develops. Then, within the filaments, regularly spaced spheres of uniform size and shape can be seen. No septa can be found, and the filament retains its cylindrical caliber. Subsequently, constrictions develop between the spheres, dividing the filament into a chain of coccoid elements. This maneuver may occur simultaneously throughout the entire filament length or regionally. The spherical elements are considered to be new minimal reproductive units and are subsequently liberated by disintegration of the chain. This cycle is repeated many times, and is presumably responsible for growth of the colony.

The "large bodies" described by a number of investigators are interpreted to be swollen elementary bodies or fat droplets. These large bodies are very pleomorphic, and they contain tiny granules. The tendency to produce large bodies is thought to vary from strain to strain. These structures are most frequently found in older cultures or in cultures in which the medium is deficient. They are thus regarded largely as abnormal or involutionary forms by this group of workers.

On a solid medium, the organisms grow down into the agar. Some growth also occurs over the surface around the central embedded area, giving rise to a lighter zone. Thus, the characteristic "fried-egg" appearance is produced.

Although the mycelial theory of growth is based primarily on observation of organisms grown in broth, it is argued (102) that, in spite of the great difficulty in observing these events in agar-grown colonies, a modification in the usual technique of observation of colonies on agar allows for the detection of many of these stages (197, 198). It has been emphasized that the mycelial stage, which is at the crux of the divergent views, cannot always be demonstrated in every colony and is dependent on medium composition and other environmental conditions. The extension of these observations on morphology from the prototype, *M. mycoides*, to other mycoplasmas is of extreme importance and, although the view is held (102) that this is justified, it is difficult to detect some of the aforementioned stages in many mycoplasma strains. For example, shorter filaments may characterize other strains, and the mycelial stage may be transitory or unstable with a rapid breakdown to the minimal reproductive units. It has been pointed out that such a spectrum of variation regarding degree of development of mycelia is found in the Actinomycetales.

The second group of morphologists reject the interpretation of the branching filaments as mycelia, claiming that these are artifacts which develop during the preparation of specimens. They point to the fact that mycoplasmas, because they lack a cell wall, are very plastic, and, consequently, extrusions develop in response to osmotic shock and various other external physical forces. In fact, they claim that this feature results in almost any shape being assumed by the organism as a consequence of the topological features of the surface on which it rests.

This group of investigators would, perhaps, concede the presence of a filamentous stage in *M. mycoides* and *M. mycoides* var. *capri*, but they do not accept this as a feature common to all mycoplasma species. Indeed, they claim that

under adverse environmental conditions many bacteria can be induced to grow filamentously. They would interpret their observations, generally, as follows.

The minimal reproductive unit increases in size, becoming a disclike or irregular-shaped particle of protoplasm lacking a cell wall. Internal changes take place, giving rise to more concentrated areas which develop into the minimal reproductive units. This "cell" can assume many shapes, but finally divides by segmentation into a number of smaller "cells" which eventually produce minimal reproductive units.

The number of minimal reproductive units which develop is proportional to the size of the "cell." Although these events are similar in broth and on agar, short filamentous bodies are observed and occur more often in broth. This difference, however, should be de-emphasized as a major stage in the growth of mycoplasma, since it is due to the physical environment of the liquid in which the "cell" finds itself and in no way is to be taken as a significant morphological form. Thus, the famous aster form, from which the early name "*Asterococcus*" for *M. mycoides* was derived, is explained. This group holds, then, that the pleomorphism of the mycoplasmas, from which so much controversy has developed, is nothing more than a variation on a theme, and that a shift in emphasis to observations on development and not on individual forms is in order. Thus, the second group emphasizes the effect of cultural conditions on morphological variations.

Although electron microscopy has not been helpful in resolving sequential events in the growth of mycoplasmas, it has provided important information on their ultrastructure. Comparative studies indicate that organisms of all species examined possess a triple-layered limiting membrane, 75 to 100 A wide (70, 129, 272*a*). In addition, ribosomes and "strands of nuclear material" have been observed in all strains examined (70, 89, 129).

Mycoplasma colonies range in size from 10 to 600 μ in diameter. They generally have an embedded center which, together with a layer of surface growth, leads to the analogy with a "fried egg." Many colonies, however, lack the peripheral zone. Some colonies, which exhibit a characteristic dark center and transparent peripheral zone, have in the peripheral zone numerous lacelike or highly vacuolated structures. These vacuolated areas may, at higher magnification, show tiny granules in Brownian motion.

There are no reports which describe the development of a single minimal reproductive unit into a mature mycoplasma colony. Thus, all of the accounts of the mycoplasma growth cycle have been based on sequential studies by phase contrast, dark-field and bright-field microscopy, and electron microscopy. Subjective interpretation has thus played a major role because of the need to piece together isolated events to present a continuous account. In any case, either method of multiplication, as outlined above, is peculiar to the mycoplasmas and appears to distinguish them further from all other microorganisms.

Growth Requirements

The first necessity for the cultivation of mycoplasmas in vitro is a rich medium. Currently, the most widely used basal media are those utilizing a beef-heart infusion with the addition of 1% peptone (78, 182). The basic ingredient is prepared from an infusion of 500 g of fresh beef-heart muscle per liter of water or from 50 g of dehydrated beef heart for infusion, such as Beef Heart for Infusion (Difco; 182). Because some preparations have been found to be inhibitory (182), the choice of the peptone appears to be important. Difco peptone and tryptose have been recommended (182). This formula supplemented with 5 g per liter of NaCl and 14 g per liter of agar is commercially available as Difco PPLO Agar (dehydrated), and conforms to published specifications (182). The characteristic growth of most mycoplasmas, in which the central portion of the colony is embedded in the agar, and their requirement for a moist environment, have dictated the use of a softer agar than that used in routine bacteriology.

The crucial component to be added to any basal medium formulation is the proteinaceous enrichment which must be incorporated into all formulas to support the growth of the nonsaprophytic mycoplasmas. Justifiably, therefore, more attention has been given to the choice of this supplement than to the basal medium itself. The following supplements have been used singly or in concert:

(i) Human ascitic fluid in a concentration of 20 to 30% of the total volume of final medium. Variations in composition and availability of a constant, sterile supply and the potential presence of antibodies represent serious disadvantages in the use of this material.

(ii) Blood serum from various animal species, usually human, calf, or horse, in a concentration of 10 to 20%. Since some sera from individual animals or entire species may be toxic or contain antibodies, this source is not without disadvantages (78, 100, 249). However, these objections

can be met, in part, by using large pooled batches of sera that have been tested for their capacity to support growth of known fastidious strains of mycoplasmas.

(iii) A blood serum fraction, commercially available (Difco PPLO Serum Fraction), used at a level of 1% instead of 10 to 20% whole serum. This supplement, although supporting the growth of many mycoplasmas, is unable to support the growth of all. Widespread use of this material in lieu of whole serum may have contributed to previous failures to cultivate *M. pneumoniae* on agar, because the serum fraction does not support the growth of this organism (116, 119).

(iv) Whole blood. This has been used for the isolation of some mycoplasma strains (61, 201) but may reflect a deficient basal medium. The addition of intact red blood cells should be avoided whenever possible, because the resultant opaque medium makes detection of colonies by transmitted light very difficult. (v) Liver extract (61). (vi) Yeast extract (38, 78, 116, 119). (vii) Deoxyribonucleic acid (DNA; 82). (viii) Egg yolk (81, 85). (ix) Rabbit infusion (147, 266).

The wide range of choice of basal medium supplements merely represents ignorance of the exact nutritional requirements of these microorganisms. Perhaps the most widely used medium is that developed by Hayflick (38, 116, 119), which utilizes the basal PPLO medium of Morton, Smith, and Leberman (182; Difco PPLO Agar, dehydrated) plus supplementation with 200 ml per liter of pooled horse serum and 100 ml per liter of a 25% extract of fresh baker's yeast. This formula was employed by us in the first successful agar propagation of *M. pneumoniae* (38), and also supports the growth of all other recognized human strains. *M. pneumoniae* and *M. orale* require yeast extract supplements, particularly for the isolation of naturally occurring strains. Since *M. pneumoniae*, which is probably the most fastidious human mycoplasma, grows well on this medium, it is, at present, the medium of choice for the growth of the human mycoplasmas. This medium has a final agar concentration of 1%.

The *p*H of most media is adjusted to the alkaline range between 7.6 and 8.0 (79, 183). Petri dishes containing agar should be incubated in a humid atmosphere and at a temperature of 33 to 37 C. For growth of mycoplasmas in fluid media, the agar portion of the formula is omitted. It is essential that workers using agar formulas containing high concentrations of animal serum be able to recognize "pseudo-colonies" (22, 119). These "colonies," which resemble colonies of mycoplasmas, are composed of calcium and magnesium soaps. Their crystalline structure creates the impression of "growth" when observed over a period of time, and they can be "transferred" to fresh agar plates creating niduses for new crystal formation, thus giving the impression of serial passage.

Nutrition and Metabolism

The reader is referred to the recent comprehensive review by Smith (244) for a detailed treatment of the nutrition and metabolism of the mycoplasmas. We will, therefore, concern ourselves here with a consideration of only the main points of these aspects of the biology of the human mycoplasmas.

Protein and lipid requirements. A major area of interest in the study of the mycoplasmas has been the attempt to identify the growth factors in different protein supplements. The replacement of the chemically undefined supplement by simpler chemically characterized materials would be a major contribution to an understanding of the physiology of these microorganisms. Early attempts (246, 249, 250) resulted in the separation and characterization of a protein component from serum capable of supporting the growth of many mycoplasma species. This protein contains eight different amino acids with lysine predominating, and has an isoelectric point and sedimentation constant similar to the α-1 lipoprotein of serum. It also contains esterified cholesterol and phospholipid, and has a molecular weight of 1.35×10^6. This protein, when lipid-extracted, was inactive in lipid-free media. When the lipid was extracted from horse serum, neither the lipid fraction alone nor the lipid-free fraction supported growth (81, 85). When both fractions were recombined, mycoplasma growth occurred (81, 85). The protein growth factor probably acts by regulating the uptake of cholesterol or other sterols essential for growth. It is likely that the differences in the relative concentrations of cholesterol, phospholipid, and protein in the serum supplement from different animal species may explain differences in their growth-promoting ability. Thus, rabbit serum, known to contain relatively little cholesterol, fails to support the growth of some mycoplasmas; however, when this serum is supplemented with cholesterol, mycoplasma growth occurs (82).

The cholesterol requirement of *M. hominis* type 2 can be partially or completely satisfied by several other sterols, for example, β-sitosterol, stigmasterol, ergosterol, and cholestanol (239, 245, 247). The configurational requirements of the sterol molecule necessary to support the growth of *M. hominis* type 2 have been critically evaluated (244). In studies with *M. mycoides*, it was concluded that cholesterol was required for

the synthesis of some cell component necessary for structural integrity (219). Others maintain that sterol is essential for cell-membrane formation where it is interspersed with other lipids in the central lipid layer. It is also necessary for the transport of substrates and end products across the cell membrane (242).

Cholesterol is required by some protozoa, for example, *Trichomonas columbiae* (28), and by the myxothallophyte *Labyrinthula vitellina* var. *pacifica* (273). Cholesterol is not thought to be an essential nutrient for any bacterial species, and its requirement by most mycoplasmas is, therefore, of great interest.

It is known that factors other than the aforementioned protein and lipids are required for the growth of certain mycoplasma species. Hog gastric mucin is necessary for the cultivation of certain fastidious bovine strains. This mucin is thought to contribute DNA which cannot be synthesized by the organisms (82, 86). As mentioned previously, *M. pneumoniae* requires nutrients present in yeast extract for optimal growth. It is possible that other as yet unrecognized mycoplasmas exist, and that they have not been isolated because certain unknown growth factors are lacking in the media currently used. It is, however, fallacious to argue for the presence of a mycoplasma in any system on the basis that a mycoplasma is present but that the proper media is unavailable for its recognition. Such circuitous reasoning is contrary to the rules of logic, since the definition of the Mycoplasmatales includes the absolute demonstration of their characteristic growth on agar.

In summary, the conclusions of Smith (244) relevant to the role of serum as a growth factor for the mycoplasma are pertinent:

> "Mycoplasma strains requiring serum for growth possess a requirement for nonsaponifiable lipid and for saturated and unsaturated fatty acids which they are unable to synthesize. The other components of the serum, phospholipid and protein, are necessary only to permit incorporation of the required lipids into the cell: the protein for regulation of sterol and possible fatty acid uptake and the phospholipid for solubilization of lipid in aqueous media. Protein also acts secondarily in reducing the toxicity of some lipids. Those mycoplasma strains having no serum requirement, and also L organisms, both of which appear capable of *de novo* lipid synthesis, possess a requirement for protein under certain cultural conditions for purposes of detoxification only. These latter two groups of organisms are similar to the lipid-requiring mycoplasmas in that exogenous lipid is incorporated, thereby sparing *de novo* lipid synthesis."

Amino acids and carbohydrates. Analysis of the amino acid requirements of the mycoplasmas has been difficult because of the lack of a chemically defined medium. Only *M. hominis* type 2 has been studied, and this species, which does not ferment carbohydrates, has a requirement for L-arginine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, DL-isoleucine, L-methionine, DL-phenylalanine, and DL-tryptophan (238, 240). These amino acids do not serve as the carbon or energy source.

M. hominis type 2 has a requirement for the pentose sugars of nucleic acids either in the form of the free sugar or as nucleoside (164). Other carbohydrates are not essential for this human species (238). Three other human species resemble *M. hominis* type 2 in that they are unable to utilize glucose or a series of related carbohydrates (82). Two recognized human species, *M. fermentans* and *M. pneumoniae*, differ in that they can utilize glucose (82, 103, 257). Certain avian strains of *Mycoplasma* (105), *M. mycoides* (219), and *M. laidlawii* (213, 222, 243) can also utilize glucose as a carbon and energy source. Glucose also appears to satisfy these requirements for the L forms (1, 209, 214).

Thus, all mycoplasmas can be divided into two groups—fermenters and nonfermenters. It is in this latter group that we find most of the human strains.

Nucelic acid requirements. M. hominis type 2 has been demonstrated to require ribonucleic acid (RNA), DNA, guanine, and hypoxanthine (238). Subsequently, it was found that the nucleic acids could be replaced by guanine, uracil, cytosine, ribose, and deoxyribose (164).

Vitamin requirements. Very little information is available concerning the vitamin requirements of the mycoplasmas. What little is known indicates that the requirements of most strains are similar. Human strains of mycoplasma apparently require choline, inositol, biotin, folic acid, pantothenate, pyridoxine, and thiamine (238).

Chemical Composition

There are no reports on the elemental or ash composition of any of the mycoplasmas or L forms. Studies of chemical composition have been directed primarily toward the constituents of the mycoplasma membrane. In this connection, α,ϵ-diaminopimelic acid, a typical constituent of the cell wall of some bacteria, is absent from all mycoplasmas and most L forms (136, 200, 204

253). Small amounts of this material, however, have been detected in the *Proteus* L form, L 9 (276, 277). The membrane of cholesterol-requiring mycoplasmas contains this sterol, whereas bacterial membranes do not. There are no other essential differences between the chemical composition of the mycoplasma strains tested and bacteria, except for a lower nucleic acid content of the former (134, 166, 167). Deoxyribose nucleic acid has been found in the range of 1.5 to 4.0% of the dry weight and remains relatively constant throughout the growth cycle, whereas RNA is found to range from 3 to 10%. The latter increases rapidly during the logarithmic phase and is at its lowest level during the stationary phase (137, 166, 177, 275). The base content and ratios have been determined for only two human species, *M. hominis* type 2 and *M. pneumoniae* (166, 187*a*).

The carbohydrate content of the human mycoplasmas has not been reported. Variable values of carbohydrate, ranging from 0.1 to 10% of the dry weight, have been found for other mycoplasmas (27, 177, 204, 205). The carbohydrate constituents of the bacterial cell wall are missing from the mycoplasmas and L forms.

The sterol composition of the mycoplasmas has been investigated more thoroughly than have other chemical constituents, undoubtedly because of the unique requirement for these compounds. A large proportion of the dry weight of *M. hominis* type 2 consists of both free and esterified sterol. The sterol-nonrequiring saprophytes, *M. laidlawii* strains B and B 15, were found to contain no sterols (167). The incorporation of cholesterol solely into the nonsaponifiable lipid fraction of *M. hominis* type 2 was found in both growing and resting cells (252). Parasitic human mycoplasma strains contain 10 to 20% total lipid, of which 50 to 65% is nonsaponifiable, whereas sterol-nonrequiring strains contain 8 to 9% total lipid with the same proportion nonsaponifiable (254). *M. hominis* type 2 contains nonsaponifiable sterol identical with the sterol in which it is grown (222, 241). *M. hominis* type 2 contains approximately 0.13 to 0.29% of lipid phosphorus by dry weight (166).

Antibiotic and Chemical Sensitivity

Sulfadiazine and sulfanilamide were the first bacteriostatic agents to which the mycoplasmas were found to be resistant (13, 21). Subsequently, mycoplasmas were also found to be resistant to penicillin (14). Mycoplasmas as a group are generally inhibited by low concentrations of tetracycline compounds. They are also sensitive in many instances to kanamycin; however, a number of resistant mycoplasma strains have recently been found (116, 119, 257). An extensive study of the antibiotic sensitivity of the mycoplasmas was reported by Newnham and Chu (190). The resistance of the mycoplasmas to potassium tellurite, brilliant green, crystal violet, basic fuchsin Nile blue A, sodium azide, and thionin is somewhat greater than that of bacteria, but not enough to justify their use in any selective media (25, 78). All mycoplasmas tested thus far, except for the T strains, are resistant to 1:1,000 thallium acetate, which is highly bacteriostatic for aerobic spore-formers and gram-negative bacteria (78, 179).

Conversely, certain agents and environmental conditions which are harmless to most bacteria have been found to be harmful to the mycoplasmas. Distilled water and physiological saline have been reported to cause rapid loss of viability, as has broth free from serum (251, 255). The human genital mycoplasmas have also been found to be very susceptible to ordinary hand soap (140), and certain brands or batches of agar have been found to be deleterious (165, 251).

HUMAN SPECIES OF MYCOPLASMAS

Well-Characterized Species

Nomenclature. The first human mycoplasma strain to be recognized was recovered from a Bartholin's gland abscess (65). Subsequently, many strains were recovered from the oropharynx (23, 183, 237, 248), urogenital tract, lower intestinal tract, brain (199), and blood. Unfortunately, most of these isolates were not characterized antigenically or stored for subsequent analysis. Thus, the relationship of most of these isolates to each other and to contemporary prototype species remains unknown.

In 1953, several strains recovered from the mouth were shown to differ antigenically from isolates obtained from the genital tract (66). The following year, a more extended serological analysis of a representative collection of human isolates indicated that these organisms could be separated into four antigenically distinct species —*M. hominis* type 1, *M. hominis* type 2, *M. salivarium*, and *M. fermentans* (191). In 1962, the etiological agent of cold agglutinin positive atypical pneumonia was shown to be a mycoplasma. This organism, designated *M. pneumoniae*, was found to be distinct from the four prototype species (38, 43, 266).

Another mycoplasma, first isolated from tissue cultures (116, 141*a*, 154, 155, 156) has recently been found to be a common inhabitant of the oropharynx (50, 124, 263) and has been demonstrated to be a separate species (50, 124, 155). This mycoplasma has been named *M. orale* by

TABLE 1. *Recovery of Mycoplasma species from different sites in healthy (H) and ill (I) individuals*

Site	*M. hominis* type 1	*M. hominis* type 2	*M. salivarium*	*M. orale*	*M. fermentans*	*M. pneumoniae*	T strain
Oropharynx	H, I		H, I	H, I		H, I	
Lung						I	
Pleural fluid	I						
Blood	I						
Ovary or fallopian tube	I						
Cervix or vagina	H, I	H, I (?)			I		H, I
Urethra	H, I	H, I (?)					H, I
Glans penis					I		
Anal canal	H, I						

TABLE 2. *Frequency of recovery of Mycoplasma species from oropharynx of healthy individuals*

Location	Age	No. tested	Per cent from whom indicated species was recovered								Reference
			M. hominis type 1	*M. hominis* type 2	*M. salivarium*	*M. orale*	*M. fermentans*	*M. pneumoniae*	Unidentified	Total	
Amsterdam, The Netherlands	Young adults	27	0	0	85	0	0	0	0	85	194
Chapel Hill, N.C. and Kansas City, Kan.	Not specified	93	1	0	16	72	0	0	0	89	50
Washington, D.C.	Infants and young children	206	2	0	9	8	0	0	1	20	29
	Adults	423	4	0	18*	22*	0	0	2	46	184

* In these instances, 0.5% yielded both *M. salivarium* and *M. orale*.

TABLE 3. *Recovery of mycoplasmas from males with and without genitourinary-tract disease during controlled studies*

Syndrome	Ill		Not Ill		Identity of isolate	Reference
	No. tested	Per cent positive	No. tested	Per cent positive		
Nonspecific urethritis	45	7	28	14	Not identified*	228
	70	20	67	0	Not identified	15
	839	17	50	0	Not identified	114, 115
	61	18	60	16	Not identified	175
	140	26	90	13	*Mycoplasma hominis* type 1	191
	75	11	20	0	Not identified	274
	38	53	57	33	Not identified	234
	109	30	28	54	Not identified	101
	120	27	115	19	Not identified	218
	631	14	28	18	Not identified	72
	65	48	100	3	*M. hominis* type 1	146
	75	44	51	10	Not identified	279
	44	43	80	14	Not identified	126
	45	60	55	22	T strain	99
	45	18	55	16	"Large colony"†	
	100	79	200	34	T strain	96
	100	27	200	24	"Large colony"	
Cystitis and other urinary-tract infections	88	52	98	35	Not identified	12

* Not identified serologically.

† Large-colony strains as distinct from T strains.

some (263) and *M. pharyngis* by others (50). It would appear that at least three additional human species have been isolated and that more probably exist.

Doubt has been expressed concerning the human origin of *M. hominis* type 2 (156). Initially, this organism was described as a common genital strain in the U.S.A. (66). Workers in Europe could not confirm this relationship nor were they able to recover the organism from the genital tract (191).

Recent studies indicate that *M. hominis* type 2 is antigenically indistinguishable from *M. arthritidis*, a species which is indigenous to rats. For this reason, it has been suggested that the organism is indigenous to the rat and on occasion can be found in man as a commensal or saprophyte (156).

Ecology. M. hominis type 1 has been recovered from many different sites, ranging from the oropharynx to the genitourinary tract (Table 1). It is of interest that all the identified isolates from blood and localized abscesses have been *M. hominis* type 1 (260, 261). The distribution of the other species appears to be more restricted; however, this may only reflect the limited sampling of areas other than the oropharynx and genital tract. Possibly the greater ease of isolation of *M. hominis* type 1 may also play a role.

Frequency of isolation. The frequency of recovery of the human species from the oropharynx is shown in Table 2. *M. salivarium* and *M. orale* are the organisms most frequently found in the mouth and throat of healthy individuals, and *M. hominis* type 1 is found less often (29, 50, 184, 215). *M. pneumoniae* has been recovered on a few occasions from healthy individuals (111).

The frequency of recovery of human species of mycoplasmas from the genitourinary tract of healthy individuals is shown in Tables 3 and 4. Most of the strains recovered have not been identified serologically. Those strains which were identified were *M. hominis* type 1 (146, 191).

Properties. The mycoplasmas recovered from man have the same basic structure as those species which infect birds and animals. Populations of infective organisms contain cells of varying sizes, the smallest of which have a diameter of 120 to 150 mμ (70). The organisms are bounded by a triple-layered limiting membrane 75 to 100 A thick (70, 129, 212). The inner and outer layers of the membrane are electron-dense, and the middle layer is considerably less dense. The cytoplasm contains ribosomes which are generally peripheral in location, and thin filaments which are centrally located and presumably consist of nucleoprotein (70, 89, 129).

The fundamental biochemical properties of the human species of mycoplasma have not been as thoroughly investigated as have those of certain animal and saprophytic strains. The most thoroughly studied of the human species is *M. hominis* type 2 (244), although there is a general belief that this species should now be classified as a rat strain. This organism requires cholesterol for growth. Cholesterol is incorporated into the limiting membrane where it functions structurally and for physiological transport. Glucose and other sugars are not metabolized; however, it appears that a portion of the tricarboxylic acid cycle is utilized for energy (271). The respiratory chain of this organism is relatively complex and involves quinones, flavoproteins, and cytochromes (271). Arginine, glutamine, glutamic acid, and aspartic acid are utilized rapidly by the organism, whereas histidine, leucine, and threonine are utilized slowly aerobically, and tyrosine and tryptophan are utilized slowly anaerobically. Both *M. hominis* types 1 and 2 convert arginine to ornithine via a three-enzyme system (244). Little is known of the metabolism of the other human species of mycoplasmas.

Although the intermediary metabolism of species other than *M. hominis* type 2 has not

TABLE 4. *Recovery of mycoplasmas from females with and without genitourinary-tract disease[a] during controlled studies*

Ill		Not Ill		Identity of isolate	Reference
No. tested	Per cent positive	No. tested	Per cent positive		
18	44	17	6	Not identified[b]	228
11	27	101	17	Not identified	15
46	26	15	0	Not identified	115
35	48	40	22	*Mycoplasma hominis* type 1	191
50	84	75	19	Not identified	176
27	30	12	0	Not identified	181
27	56	224	17	Not identified	211
187	62	156	33	Not identified	101
11	82	17	0	Not identified	146
80	35	45	7	*M. hominis*[c]	11
298	62	31	22	Not identified	72
571	40	134	37	Not identified	189
20[d]	30	59[d]	14	Not identified	196

[a] Nonspecific cervicitis or vaginitis, or both.
[b] Not identified serologically.
[c] All strains antigenically similar but not compared with recognized prototypes. Presumably these strains were *M. hominis* type 1.
[d] Children 3 to 11 years old.

been examined extensively, a number of biological properties have been defined for those organisms (Table 5). The composite picture of these properties presented by certain of the human species is sufficiently distinctive to be helpful in presumptive identification of isolates.

Naturally occurring strains of several species either fail to grow or grow very poorly under aerobic conditions. Such organisms grow well when incubated in nitrogen and 5 to 10% CO_2. Other species replicate equally well under aerobic or anaerobic conditions.

Most of the human species grow well without added yeast extract; however, two of the species (*M. orale* and *M. pneumoniae*) have a requirement for this supplement and grow poorly in its absence (38, 119, 124, 263). The nutrient(s) supplied by yeast extract is heat-stable, water-soluble, and nonprotein in nature (116, 119, 258). Differences in growth rates on agar or in broth exist among the species that infect man, but these species differences are only relative and tend to decrease as isolates become "adapted" to growth on laboratory medium.

Most strains of human origin produce the classic "fried-egg" colonies on agar medium. Certain generalizations concerning the ratio of the central to peripheral zones can be made; however, colonial morphology is a labile property which is influenced by many variables, such as medium composition, agar concentration, and other environmental conditions. Colonies of *M. orale* tend to have a small central dense zone, whereas colonies of *M. hominis* type 1 or *M. salivarium* usually have a wider central zone. *M. pneumoniae* usually grows as a dense spherical "mulberry" colony without a light peripheral zone.

Only two of the species, *M. pneumoniae* and *M. fermentans*, ferment glucose and a series of other sugars including xylose, mannose, maltose, dextrin, and starch (82, 103, 259). One of these species, *M. pneumoniae*, is unique among the human species in producing complete lysis of certain mammalian red cells within 24 to 48 hr (48, 103, 259). The other human species lyse red cells, but at a slower rate, and generally lysis is incomplete. When red cells are added to an agar plate containing colonies of *M. pneumoniae*, they are adsorbed directly onto the mycoplasma colonies (60). *M. pneumoniae* is the only human species which hemadsorbs erythrocytes. Subsequently, the absorbed erythrocytes lyse. *M. pneumoniae* is also unique among the human species in reducing tetrazolium under aerobic conditions (151).

TABLE 5. *Biological properties of human Mycoplasma species*

Species	Growth				Colonial morphology	Fermentation of glucose	Hemolysis of guinea pig red cells	Hemadsorption	Aerobic reduction of tetrazolium
	Anaerobic	Aerobic	Requirement for yeast extract	Rate					
M. hominis type 1	+	+	0	Rapid	"Fried egg," smooth or foamy periphery	0	Slow	0	0
M. hominis type 2	+	+	0	Rapid	"Fried egg," smooth periphery	0	Slow	0	0
M. salivarium	+	0	0	Rapid	"Fried egg," smooth periphery	0	Slow	0	0
M. orale	+	±	+	Moderate	"Fried egg," small periphery	0	Slow	0	0
M. fermentans	+	±	0	Moderate	Granular, often without periphery	+	Slow	0	0
M. pneumoniae	+	+	+	Slow	Granular, usually without periphery	+	Rapid	+	+

Antigenic relationships. A number of techniques have been used to define the antigenic relationships among the human species. Complement fixation (CF) has been used most often for this purpose. Agglutination of mycoplasma suspensions has also been quite useful; however, the problem of instability of antigen preparations has limited the use of this method (30). Growth inhibition tests in which antiserum is applied to the agar surface have yielded highly specific results; however, the sensitivity of this method for measurement of antibody is quite low (50, 87). Indirect hemagglutination (IHA) of sensitized tanned red cells has proved to be both a highly specific and sensitive assay technique (71, 263, 265). Interpretation of the antigenic relation-

ships of the mycoplasmas is often impaired by the existence of mixed cultures of different strains. It is important to point out that all mycoplasma cultures should be cloned, and isolated colonies should be used for the preparation of antigens.

The six human prototypes (*M. hominis* type 1, *M. hominis* type 2, *M. salivarium*, *M. orale*, *M. fermentans*, and *M. pneumoniae*) have been compared by CF, IHA, growth inhibition, and gel diffusion (50, 124, 154, 155, 156, 191, 263, 265, 266). These species were antigenically distinct when tested by each of these techniques. The most specific results were observed in growth-inhibition tests (50, 124, 263). There was no evidence of antigenic similarity among the human prototypes when they were examined by this method. However, these organisms share related antigens which are demonstrable by other serological techniques such as CF, IHA, and gel diffusion (50, 154, 155, 156, 263, 265, 266). Examples of antigenic relatedness are shown in Tables 6 and 7 (263). Related antigens were more evident when prototype strains were tested by CF than by IHA (263). In the test shown in Table 6, *M. orale* antiserum reacted in CF with *M. salivarium* antigen to almost the same titer as with the homologous strain. In another study, this relationship was less striking (50). These differences probably reflect the total antigenic mass used for testing: eight units in the test shown in Table 6 and two units in the other study. A series of related antigens has been identified by gel diffusion, particularly among *M. hominis* types 1 and 2, *M. salivarium*, and *M. orale* (263, 265, 266).

Strains within a given species may exhibit some differences in antigenic composition. This was first noted for genital strains of *M. hominis* type 1 examined by agglutination when one strain was not agglutinated by antisera to other strains, whereas its antiserum agglutinated both itself and the other strains (191). Subsequently, a gel-diffusion and immunofluorescent analysis of the prototype *M. hominis* type 1 strain (recovered from the genital tract) and two *M. hominis* type 1 isolates from the oropharynx indicated that the former was deficient in an antigen which was prominent in the oral strains (266).

TABLE 6. *Antigenic relationships of human Mycoplasma species by complement fixation**

Rabbit antiserum	*M. hominis* type 1	*M. hominis* type 2	*M. salivarium*	*M. orale*	*M. fermentans*	*M. pneumoniae*
M. hominis type 1	*1,280*	20	160	160	<20	<20
M. hominis type 2	40	*2,560*	160	<20	<20	<20
M. salivarium	<20	40	*2,560*	40	<20	<20
M. orale	160	160	1,280	*2,560*	<20	<20
M. fermentans	<20	<20	<20	<20	*80*	<20
M. pneumoniae	<20	<20	80	<20	<20	*640*

* Reference 263. Eight units of antigen used in this test. Results are expressed as the reciprocal of the complement-fixation antibody titer with the indicated antigen. Homologous titers italicized.

TABLE 7. *Antigenic relationships of human Mycoplasma species by indirect hemagglutination**

Rabbit antiserum	*M. hominis* type 1	*M. hominis* type 2	*M. salivarium*	*M. orale*	*M. fermentans*	*M. pneumoniae*
M. hominis type 1	*10,240 or >*	<20	20	40	80	40
M. hominis type 2	40	*10,240 or >*	20	40	20	40
M. salivarium	80	20	*10,240*	<20	40	40
M. orale	40	<20	80	*10,240*	40	20
M. fermentans	40	80	40	40	*640*	80
M. pneumoniae	20	<20	<20	<20	<20	*5,120*

* Reference 263. Results are expressed as the reciprocal of the serum titer with sheep erythrocytes sensitized with the indicated antigen.

Partially Characterized Species

In addition to the six well-characterized species, four groups of strains have been described which appear to represent distinct species.

New isolates from the oropharynx. The first group comprises a series of four isolates from the oropharynx (265). These strains grow aerobically and anaerobically, and produce incomplete lysis of guinea pig erythrocytes, but they do not ferment glucose. They form a homogeneous antigenic group which is distinct from the six recog-

nized human species. Antigenic distinctness was demonstrated by growth inhibition, IHA, CF, and gel diffusion. However, one or more of the antigens of the newly recognized strains are related to antigens of the six recognized species, particularly *M. orale* and *M. hominis* type 2.

The designation *M. orale* type 2 has been suggested for these strains. It is probable that this term will be used only temporarily, because it is now clear that a numerical system of nomenclature is required for the human mycoplasma species.

The new oral strains appear to be infrequent inhabitants of the mouth and throat. Such strains were recovered from 1% of 629 persons sampled in the Washington, D.C. area (29, 184).

Navel strain. The navel strain was recovered from a skin lesion of the umbilicus (225). Other similar strains have not been recognized. The organism, which is nonfermentive, is antigenically distinct from the six recognized species and from the new oral species (156, 265). The significance of the navel strain cannot be assessed at present, since it has not been determined whether the organism is pathogenic or saprophytic.

T strains. T strains have been recovered with some frequency from the urogenital tract (96, 99, 236). These strains are characterized by the unique property of producing on agar very small colonies which rarely exceed 15 to 25 μ. On primary isolation, the organism is usually found in close association with urethral epithelial cells which contain cytoplasmic inclusions (235, 236). The T strains grow best on tryptic-heart digest, ascitic fluid-agar, or on the medium of Hayflick (38, 119) in 80% nitrogen and 20% CO_2 (94). The organisms grow rapidly in broth, reach maximal numbers in 16 hr, and then rapidly lose viability (94). The morphology of the small colonies which form on agar is similar to that of the recognized mycoplasma species, except for size (98). Unfortunately, it has not been possible to prepare sufficient quantities of the organisms for immunization of animals. Thus, the T strains have not been compared among themselves or with the recognized mycoplasma species. For this reason it is not known whether the T strains represent one or a number of serological types.

Isolates from tissue cultures inoculated with human material. A number of mycoplasma strains have recently been recovered from tissue cultures inoculated with materials from human sources (3, 9, 107, 186, 186*a*, 226). In each instance, serial "blind passages" were performed with cultures free from contaminating mycoplasmas. Many of the strains were not recognized until a cytopathic effect (CPE) developed during subpassage. This effect was then transmissible in series. Initially, many of these agents were thought to be viruses (187); however, subsequent studies indicated that they were mycoplasmas (107, 111*a*). In several instances, after propagation in broth medium, the mycoplasma strains were shown to produce a CPE in tissue culture which was identical to that observed during the "blind subpassage" series.

The sequence of events just described has occurred after inoculation into tissue culture of organ suspensions from patients with leukemia (107, 186, 186*a*, 187), a variety of solid tumors (3, 226), lupus erythematosis (9), Reiter's syndrome (9), and rheumatoid arthritis (9). These strains ferment glucose and are antigenically unlike the usual tissue culture contaminants, *M. hominis* type 1 and *M. orale*, as well as the other recognized human mycoplasma species. Preliminary analysis suggests that the new strains comprise at least two antigenic groups (3, 107, 257, 264).

The origin and significance of these strains is not clear. Their absence from parallel passage series of cultures not inoculated with human material suggests that the organisms were present in the original specimens. The possibility that they represent contaminants, however, has not been ruled out in all cases. Recently, mycoplasmas having similar properties were recovered in independent laboratories from uninoculated tissue cultures (26, 106, 257). These strains produce CPE in tissue cultures, ferment glucose, and are antigenically related to one of the groups of strains recovered from cultures inoculated with human tumor material. The relationships and significance of these mycoplasmas recovered from tissue cultures inoculated with human tumor tissue suspensions remain to be assessed.

Isolate from human leukemia. The direct isolation on agar of mycoplasmas from three bone marrow samples obtained from a child with acute lymphoblastic leukemia has been reported (119*a*). This isolate, called N-1, is the first mycoplasma obtained directly from a human malignancy without the use of tissue culture. Of the three children whose leukemic marrows were tested, the only positive isolation was made from an untreated, recently diagnosed case. Growth-inhibition studies indicated that the isolate was a strain of *M. orale* type 1 or one of its subspecies with the unusual property of remaining viable at high concentration for a period of time in excess of 3 months at 37 C. The relationship of N-1 to human leukemia is unknown, and the possibility that N-1 is a laboratory contaminant cannot be abso-

lutely excluded. However, the circumstances surrounding the isolation make such an explanation unlikely (119*a*). The view that mycoplasmas may sometimes be found even secondarily to the leukemic process makes it interesting to speculate on their effect upon myelopoiesis.

At the very least, the isolation of mycoplasmas either directly or indirectly from human malignancies implies a serious potential pitfall for virologists. Some mycoplasmas share all or some of the following properties with certain viruses: size, filterability, ether sensitivity, ability to hemagglutinate and to cause hemadsorption, interference with virus replication in vitro, lack of inhibition by many antibiotics, neutralization by homologous antisera, and production of CPE. Thus, investigations directed toward elucidating the role of viruses in malignant disease are now made even more difficult by the possible presence of mycoplasmas. It should, therefore, be of paramount importance to include a test for the presence of mycoplasmas in the description of any new virus isolate. The salient criterion must be the demonstration of characteristic colonial growth on agar.

ROLE OF MYCOPLASMAS IN HUMAN DISEASE

Respiratory Disease Caused by M. pneumoniae

Primary atypical pneumonia. In the late 1930's and early 1940's, a broad group of nonbacterial pneumonias was first recognized and given the name primary atypical pneumonia (PAP; 69, 73). The name was applied to pneumonia which was unlike the typical lobar disease caused by *Diplococcus pneumoniae*. A description of PAP as a clinical syndrome was necessary at the time, because the etiological agents involved were not known. In 1943, it was first noted that many patients with the atypical pneumonia syndrome developed cold agglutinins during the course of illness (202). Since that time, a test for such antibody has been widely used as a nonspecific serodiagnostic procedure.

During the past 10 years, it has become clear that atypical pneumonia is a syndrome of multiple etiology. In certain age groups and ecological settings, adenoviruses, influenza A and B viruses, parainfluenza type 3 virus, and respiratory syncytial virus have been defined as important causes of this syndrome (40, 44). These viral agents, however, have not been implicated in that portion of the atypical pneumonia syndrome characterized by a cold agglutinin response.

Studies during and shortly after World War II indicated that cold agglutinin positive atypical pneumonia was a distinct epidemiological entity. Individual illnesses, however, could not be distinguished clinically from pneumonias not associated with a cold agglutinin response, although, as a group, the former were found to be more severe and prolonged than the latter. Careful epidemiological investigation indicated that the average incubation period was 2 weeks, an unusually long interval for an acute respiratory illness (133). Human volunteer studies performed by the Commission on Acute Respiratory Disease revealed that the etiological agent was filterable (55).

History of the agent. During the late 1930's and early 1940's, a series of agents was recovered from different animal species inoculated with specimens from patients with PAP (73). Most of these organisms were probably indigenous to the animal species used and were activated by the inoculation procedure. One organism, the Eaton agent, was an exception, and the circumstances surrounding its recovery suggested that it came from patients with PAP (77). All subsequent studies have confirmed this relationship.

In the original Eaton agent study, embryonated eggs were inoculated with filtered sputum from patients with PAP. The inoculated eggs did not show any definite pathological change. Tissue suspensions and extra-embryonic fluids from these eggs, however, produced pneumonitis when given intranasally to hamsters or cotton rats (77). The agent was successfully propagated serially in eggs, and material from successive passages produced pneumonitis in cotton rats and hamsters. When filtered sputum from PAP patients was administered directly to cotton rats and hamsters, pneumonitis also developed; however, serial passage of this effect in these animals was unsuccessful (73, 77). Early studies were hindered by the fact that lung lesions were observed in only a portion of inoculated animals, but not in all. In addition, cotton rats and hamsters were subject to pneumonia resulting from activation of their own latent viruses (73).

Gradocol membrane filtration of infected chick-embryo material indicated that the organism was between 180 and 250 mμ in diameter (73). In later experiments, both chlortetracycline and streptomycin were shown to inhibit infectivity (74, 76).

In 1957, it was shown that the Eaton agent could be observed in the chick embryo lung by immunofluorescence (160). Specific antigen was localized in the area of the bronchial epithelium. Adaptation of immunofluorescence for recognition of infection in eggs represented a major

advance, because it made possible quantitation of the organism and its antibody. Subsequently, it was shown that the agent grew in primary tissue cultures prepared from chick-embryo yolk sac or monkey kidney (37).

Etiological role in respiratory-tract disease. Data obtained during the earliest Eaton agent studies suggested that this organism was related to the PAP syndrome. Thus, the agent was recovered most often during the early phase of illness and from individuals who developed cold agglutinins (73). In addition, neutralization tests performed in hamsters, and, subsequently, indirect immunofluorescence studies, provided serological confirmation that cold agglutinin positive PAP patients were infected with the Eaton agent. In spite of these findings, some reservations were expressed concerning the relationship of the organism to the PAP syndrome. In part, these reservations were based on the finding that PAP patients develop antibodies to a number of "bizarre" antigens (normal tissue suspensions from mice, chick embryos, etc.; 267). In addition, infectivity and serum antibody end points were not always clear because, generally, only a portion of animals inoculated with infectious material developed lung lesions. However, the 1941–1945 studies clearly demonstrated that a serological response to the Eaton agent occurred most often among patients with atypical pneumonia (Table 8; 77*a*).

During the past 5 years, controlled epidemiological investigations of naturally occurring disease have provided convincing evidence that the Eaton agent is a human respiratory-tract pathogen (35, 41, 43, 56, 162). Thus, in two separate populations, infection with the organism was detected significantly more often among patients with moderate to severe respiratory diseases than among comparable individuals free from such illness (Table 8; 35, 41). The specificity of the fluorescent-antibody response was indicated by recovery of the organism from 26 of 30 serologically positive military recruits (41, 42).

Final proof that the Eaton agent can produce respiratory disease in man was provided during a series of studies in volunteers (45, 58, 216). In these studies, 69 individuals without fluorescent antibodies and 25 individuals with naturally acquired fluorescent antibody for *M. pneumoniae* were challenged experimentally. Of the 69 volunteers free from fluorescent antibodies, 27 were infected with *M. pneumoniae* grown in tissue culture. Three of these volunteers developed pneumonia, and 7 developed febrile respiratory disease without pneumonia; all 27 acquired fluorescent antibody for *M. pneumoniae*, and 12 developed cold agglutinins. The other 42 volunteers free from fluorescent antibodies were infected with *M. pneumoniae* propagated in cell-free agar. Of these, none developed pneumonia, but eight developed febrile respiratory disease without pneumonia; 38 of the 42 acquired fluorescent antibody for *M. pneumoniae*, and 31 developed cold agglutinins. Thus, 18 of the 69 volunteers free from fluorescent antibodies de-

TABLE 8. *Evidence for etiological role of Mycoplasma pneumoniae in human respiratory-tract illness*

Study (reference)	Method of investigation	Group	Time	Diagnosis	No. tested	Per cent positive for *M. pneumoniae*
1 (77*a*)	Neutralization test in rodents	Respiratory disease patients, civilian adults	1941–45	Atypical pneumonia	84	62
				Bacterial or viral pneumonia	25	0
				Influenza A	27	3
2 (41)	Fluorescent antibody with chick-embryo lung sections	Marine recruits	1959–60	Pneumonia	238	68*
				Febrile respiratory illness	144	28
				No respiratory-tract disease	262	6
3 (35)	Fluorescent antibody with chick-embryo lung sections	Infants and children, hospitalized	1957–59	Undiagnosed lower respiratory-tract disease	110	16
				Diagnosed viral lower respiratory-tract disease	42	2
				No respiratory-tract disease	64	0

* *M. pneumoniae* was recovered from 26 to 30 individuals who developed a rise in fluorescent antibody.

veloped a febrile respiratory illness, with or without pneumonia. None of the 25 volunteers with naturally acquired fluorescent antibody (1:10 to 1:80) developed pneumonia, febrile respiratory disease, or cold agglutinins when challenged with the *M. pneumoniae* grown in tissue culture.

Recognition as a mycoplasma. For a number of years, the Eaton agent was considered to be a virus. Doubts arose when the organism was found to be inhibited by chlortetracycline and streptomycin (74, 76). Subsequently, several observations suggested that the agent might be a mycoplasma. First, small coccobacillary bodies were visualized on the mucous layer covering the bronchial epithelium of infected chick embryos, and the distribution of these bodies corresponded with the localization of the agent as determined by immunofluorescence (108, 168*a*). Second, extracellular "colony-like" structures were seen in stained preparations of infected tissue cultures, and these structures corresponded to the areas of specific immunofluorescence (49, 49*a*).

The nature of the Eaton agent was finally established when it was successfully cultivated by us on a cell-free agar medium (38, 119). This medium, developed by Hayflick, was a modification of a formula described by Edward (78). Colonies developed 6 to 7 days after a tissue culture-adapted strain was inoculated onto an agar medium composed of seven parts Difco PPLO Agar, two parts unheated horse serum, and one part 25% fresh yeast extract (38, 119). The colonies were circular and partially embedded in the agar, and they had a homogeneous granular appearance like a mulberry. Characteristically, these colonies lacked the light peripheral zone typical of many mycoplasma species. Growth also occurred in broth medium identical in composition to the agar medium except for the omission of agar. The organism did not grow when horse serum was omitted from the medium. When studied with the Dienes stain (64, 67, 68), the colonies were seen to contain many blue densely staining small granules which resembled those observed with other mycoplasma species.

The morphological and nutritional properties of the agar-grown organism indicated that it was a member of the genus *Mycoplasma* (38, 88). The identity of this organism was then established by immunofluorescence with the use of specific rabbit sera and paired sera from patients with Eaton agent pneumonia. In a series of tests with 15 sets of paired sera from patients with Eaton agent pneumonia, a 1:10 dilution of each acute-phase serum failed to stain the colonies, whereas intense fluorescence was observed with a 1:20 to 1:80 dilution of convalescent serum (38). In addition, rabbit antiserum prepared against the egg-grown FH strain of Eaton agent produced intense fluorescence of the mycoplasma colonies, whereas it failed to stain other mycoplasma species of human, bovine, or avian origin. These findings indicated that the agar-grown mycoplasma was indeed the same as the Eaton agent. Recently, the organism was designated *M. pneumoniae* by us and other workers in the field (36). However, in view of the valid criticisms of multiple authorship in taxonomic papers (172), the authority for this new microorganism would be *M. pneumoniae* Chanock and Hayflick, 1963.

Retrospectively, the claim by Grünholz (113) in 1950 to have isolated mycoplasmas from 100 cases of "virus pneumonia" in infants may have had some validity, despite criticisms by others that these were artifacts (16).

Growth and nutritional properties. Yeast extract-horse serum-agar supports the growth of naturally occurring *M. pneumoniae* as well as of laboratory strains (4, 42, 38, 119). By use of agar prepared according to this formula, it is possible to recover the organism from most naturally infected individuals (4, 42, 111). *M. pneumoniae* grows equally well under aerobic and anaerobic conditions.

Serum from the pig, the cow, or the rabbit can be successfully substituted for that of the horse. Egg yolk can also be substituted for horse serum (130). Serum or egg yolk provides cholesterol plus other as yet undefined growth factors.

Yeast extract supplies water-soluble growth factors of low molecular weight (258). These compounds will pass through a dialysis membrane, and dialysates of yeast extract are as active in promoting growth as the extract itself. When yeast is omitted from the medium, growth either is limited or fails to occur. However, it is possible to select variants which grow to moderate titer under such conditions by repeated passage on yeast-free agar (34, 116). In several instances, naturally occurring strains of *M. pneumoniae* have been recovered on yeast-free agar plates (34). The most actively proliferating "yeast-nonrequiring" variants still require yeast, however, for optimal growth; a 100- to 1,000-fold increase in level of growth occurs when yeast is added to the medium (257). In broth or agar medium, growth of *M. pneumoniae* is slower than that of other human mycoplasma species. The mean generation time for a well-adapted strain in liquid medium is approximately 6 hr (51, 116). Generally, naturally occurring strains do not produce detectable colonies on agar before the 6th to 20th day of incubation. When such isolates are serially propagated, growth becomes more

rapid; however, the most rapidly growing strains do not produce colonies in less than 60 to 72 hr.

The typical *M. pneumoniae* colony measures 10 to 150 μ in diameter, has a coarse "mulberry" surface, and lacks a peripheral halo. The last property is not fixed, since colonies on sparsely populated plates or on swine serum-agar may develop a peripheral halo (34, 103, 116). Spherical "mulberry" colonies measuring 100 to 200 μ characteristically develop in liquid medium. These colonies stain intensely with neutral red and are easily observed by this method (34).

Biochemical properties. M. pneumoniae ferments a variety of sugars, including glucose, xylose, mannose, maltose, dextrin, and starch (103, 259). This serves as a useful property for distinguishing this organism from most other human mycoplasma species, since only *M. fermentans* also ferments glucose and other sugars (82). Utilization of sugars is accompanied by an accumulation of acids. In liquid medium containing 1% glucose, a rapid fall in *p*H occurs when growth reaches maximal levels. Incorporation of phenol red in the liquid medium permits observation of this phenomenon and serves as a useful guide to growth of the organism during experimental and diagnostic studies (111, 162*a*).

Hemolysis and hemadsorption. M. pneumoniae differs from other human mycoplasmas in producing a hemolysin which completely lyses guinea pig and certain other mammalian red cells within 24 to 48 hr (48, 259). Circular areas of complete (β) hemolysis develop when agar plates containing colonies are overlaid with a 3% suspension of guinea pig red cells in agar. A mycoplasma colony is present in the center of each hemolytic plaque, but the peripheral area is free from colonies, indicating that the hemolysin is freely diffusible in agar (259).

Under optimal nutritional conditions, each colony manufactures sufficient hemolysin to produce a hemolytic plaque (259). Hemolytic plaque formation is inhibited when colony density becomes too high, thus suggesting that nutrients essential for hemolysin production are depleted when overcrowding occurs. Yeast extract supplies one or more nutrients essential to hemolysin production, since colonies on "yeast-free" agar are unable to form hemolytic plaques (258). An oxidative step is essential to the production and /or action of the hemolysin because anaerobiosis inhibits hemolysis (258). When colonies are removed from the agar by micromanipulation, hemolytic plaques do not form at that site. This finding suggests that the hemolysin is labile and is continuously produced by the colony. The hemolysin has a low molecular weight, as it is small enough to pass through a dialysis membrane. Recent studies suggest that the hemolysin is peroxide (257). It has been suggested that cold agglutinins, which commonly develop during *M. pneumoniae* infection, represent an autoimmune response to red cells antigenically altered by the action of hemolysin (43).

Guinea pig red cells are also altered by other species of mycoplasma which infect man, but hemolysis occurs at a slow rate (259). Small greenish (α) zones of partial clearing develop around colonies of these organisms, but complete lysis does not occur. For this reason, the property of β, or complete, hemolysis provides a valid basis for presumptive identification of *M. pneumoniae* isolates (48, 259).

Erythrocytes from a number of animal species can adsorb to surface colonies of *M. pneumoniae* (60). Subsequently, the adsorbed red cells lyse. Other human mycoplasma species do not hemadsorb. It is likely that the hemolysin and hemadsorption-lysis phenomena are related, but this has not been investigated.

M. pneumoniae differs from other human mycoplasma species in two additional properties. The organism reduces 2,3,5-triphenyltetrazolium chloride aerobically and is not inhibited by methylene blue at a concentration of 0.2% (151).

Host range and pathogenicity. M. pneumoniae grows in tissue cultures of avian, simian, and human origin, usually without producing cell destruction (37, 49). However, a delayed cytopathic effect has been reported in a human heteroploid amnion cell line (75). In infected simian renal cultures, the organism produces extracellular mycoplasma colonies which can be visualized by Giemsa stain or immunofluorescence (49). Serum-containing cell culture media alone does not support the growth of *M. pneumoniae*, and, although this point has not been clarified, the cells may be providing nutrients ordinarily supplied by the yeast extract in cell-free media.

In embryonated eggs, the organism produces an inapparent infection which is limited in most instances to the bronchial epithelium (160). Growth occurs primarily in the mucous layer covering the epithelium rather than intracellularly. Eggs 12 to 13 days old are most susceptible to infection, presumably because inoculated organisms are denied access to the lungs until the tracheal plug is absorbed on the 12th to 13th day (160).

Cotton rats and hamsters are susceptible to infection and develop microscopic or macroscopic lung lesions 10 to 14 days after inoculation (59, 77). The lesions are peribronchiolar and consist primarily of an infiltrate of mononuclear cells.

In man, the effects of *M. pneumoniae* range from inapparent infection to upper respiratory-tract disease to bronchopneumonia (43, 45, 58, 216). This broad spectrum of effects has been observed for both experimental and naturally acquired infections. The majority of human infections do not progress to a clinically evident pneumonia. In two studies, it was estimated that only 3 to 10% of infected individuals developed recognizable bronchopneumonia (41, 45, 216).

In addition to respiratory-tract involvement, *M. pneumoniae* has also produced ear disease in experimentally infected volunteers (45, 216). Of 27 fluorescent-antibody-free individuals, 12 developed myringitis, often bullous, after infection with a strain grown in tissue culture. *M. pneumoniae* infection has been detected in a number of patients with naturally occurring myringitis; however, an etiological role in this condition cannot be assumed until data are available from controlled studies (256).

M. pneumoniae can be recovered from the oropharynx of most naturally or experimentally infected individuals (42). The duration of excretion has not been studied extensively; however, several findings suggest that the organism may persist in the throat of convalescent individuals for a prolonged period despite the presence of high levels of serum antibody. A number of volunteers were still shedding the organism when last tested 4 weeks after infection, i.e., at a time when high levels of fluorescent antibody were present in the serum (58). *M. pneumoniae* has been recovered from patients with high levels of antibody in their acute-phase serum. Presumably, these individuals were sampled late in the course of illness (42). Finally, the organism was recovered from four patients 20 to 45 days after onset of symptoms (111).

Whether variations in virulence for man exist among natural strains of *M. pneumoniae* is not known. A preliminary investigation involving 50 volunteers suggests that cultivation of the organism on a cell-free medium, either agar or broth, results in attenuation of *M. pneumoniae* for man (58). Volunteers administered agar- or broth-grown organisms developed significantly less febrile illness than individuals given an early-passage tissue-culture isolate. The former material stimulated lower levels of fluorescent antibody than the tissue-culture suspension; however, these levels were in the range which is known to confer protection against illness. These experiments suggest that it may be possible to develop a living attenuated vaccine for prevention of *M. pneumoniae* respiratory disease.

Antigenic relationships. Antibodies for *M. pneumoniae* can be measured by five different serological techniques: immunofluorescence (162), CF (39, 131, 266), IHA with sensitized tanned sheep red cells (71, 263, 265), precipitation in agar gel (266), and growth inhibition (50, 87, 124, 263). Each of these methods has been used to characterize the antigenic composition of the organism and to compare it with other mycoplasma species.

At least two and possibly three antigens have been demonstrated for *M. pneumoniae*. Two and probably three precipitation lines developed when a concentrated suspension of the organism and a potent rabbit hyperimmune serum were tested by the gel-diffusion technique (264). The chemical nature of the precipitating antigens is not known. Possibly one of the antigens is the "lipid" complement-fixing antigen which can be extracted from the organism by ether or chloroform (138). When inoculated into rabbits, the "lipid" antigen stimulates the development of complement-fixing antibody (138).

M. pneumoniae has been compared by a number of different serological techniques with the other mycoplasma species which infect man (38, 43, 156, 263, 266). In each instance, it has been found to be distinct from the other species (Tables 6 and 7). Those antigenic relationships which have been demonstrated have been minor. The most specific results have been observed in immunofluorescence and growth-inhibition studies; in these tests, *M. pneumoniae* did not exhibit antigens related to those of other species. It should be noted that paper-disc growth inhibition and fluorescent-antibody staining of mycoplasma colonies are relatively insensitive methods of measuring antibody. *M. pneumoniae* exhibited a minor degree of relatedness to other organisms when tested by the sensitive IHA technique; however, there was no question of its specific antigenic composition (Table 7; 263). Similar results were observed when the various mycoplasmas of man were tested by the CF technique (Table 6; 263). In different studies, *M. pneumoniae* antiserum has exhibited low-level heterotypic reactions with *M. salivarium* and *M. hominis* types 1 and 2 (263, 266). The existence of these shared heterotypic antigens was also observed in gel-diffusion experiments. It is probable that the low-level heterotypic reactions exhibited by *M. pneumoniae* represent true minor antigenic relationships, since precautions were taken to minimize the possibility of immunized animals developing antibody for growth-medium constituents. Immune sera were prepared by inoculation of rabbits with antigens grown in rabbit muscle infusion and rabbit serum (266).

Diagnosis of infection and antibody response. Until recently, indirect immunofluorescence was

the standard technique for serodiagnosis of *M. pneumoniae* infection. This procedure is a sensitive method for detection of antibody and for diagnosis of infection; however, it is laborious because frozen sections of infected chick-embryo lung are used as the source of antigen (160). The difficulties inherent in performing this test have limited its application to serodiagnostic and epidemiological studies. Since the identification of the organism as a mycoplasma, a number of new approaches to the problem became possible. Several of these approaches, all based upon growth of the organism in cell-free medium, have proved successful, and have resulted in the development of simple specific diagnostic procedures.

The yeast extract-horse serum-agar medium used for the first successful cultivation of *M. pneumoniae* was found to be an efficient system for recovery of the organism from individuals with natural infection (42). *M. pneumoniae* was recovered from 12 of 13 pneumonia patients who developed fluorescent antibody during convalescence, whereas it was not recovered from 14 seronegative pneumonia patients. The organism was also recovered on the agar medium from 81% of 27 experimentally infected volunteers (58).

Before specific identification is made by serological techniques, it is possible to recognize most *M. pneumoniae* isolates by their slow growth on agar and the homogeneous, granular "mulberry" appearance of the colonies (38). Other mycoplasma strains found in the oropharynx characteristically produce "fried-egg" or "nippled" colonies on agar. The property of rapid β hemolysis of guinea pig red cells can be used for presumptive identification of *M. pneumoniae* isolates (48, 259), as can hemadsorption (60).

Several serological procedures have recently been developed which use antigens prepared from organisms grown in broth. In the tetrazolium reduction inhibition (TRI) test, a live suspension of organisms is used to measure growth-inhibition antibody; inhibition of growth is indicated by failure of tetrazolium to be reduced (130*a*). In the IHA test, sonically disrupted organisms are employed as antigen to sensitize tanned sheep erythrocytes (71, 263). Broth-grown mycoplasmas can also be used as complement-fixing antigen (39). In infected broth suspensions, a considerable portion of the complement-fixing antigen is apparently smaller or less dense than the organism; i.e., it can not be sedimented with the organism (39). The antigen(s) involved in complement fixation appears to be lipid in nature, because it can be extracted from broth concentrates by ether or chloroform (138). Untreated or washed suspensions of *M. pneumoniae* are usually anticomplementary; however, this difficulty can be overcome by phenol treatment or lipid extraction of antigen (39, 138).

The highest antibody titers are usually obtained in the IHA test (71, 264). Immunofluorescence with frozen chick-embryo lung sections, CF, and TRI are relatively sensitive methods for measurement of *M. pneumoniae* antibody, but they are generally less sensitive than the IHA technique. The least sensitive methods for detection of antibody are immunofluorescence with the use of mycoplasma colonies transferred from the agar surface to glass slides and growth inhibition with the use of serum-impregnated paper discs placed on the agar surface (50, 263).

Immunofluorescence (with chick-embryo lung antigen) remains the most effective technique for serodiagnosis of infection (39). The IHA and CF methods are approximately 80% as efficient as immunofluorescence (39, 264).

The IHA technique, although it measures higher levels of antibody than does immunofluorescence, is less effective for diagnosis because a proportion of naturally infected individuals develop high levels of IHA antibody by the onset of symptoms, whereas this occurs less often with fluorescent antibody.

Antibodies measured by immunofluorescence with chick-lung sections appear to confer protection against illness caused by *M. pneumoniae* (45, 216). None of the 25 volunteers who possessed such antibody developed febrile illness when challenged with a strain of the organism grown in tissue culture, whereas 10 of 27 men who lacked this antibody developed febrile disease, in 3 instances accompanied by pneumonia. In a similar fashion, TRI antibody, and to a lesser extent IHA antibody, also appear to confer resistance to illness caused by *M. pneumoniae* (232, 264).

Cold agglutinins and streptococcus MG agglutinins. In six separate studies, a significant relationship has been observed between *M. pneumoniae* respiratory disease and the development of cold agglutinins (Table 9; 41, 52, 56, 111, 162, 272). Of pneumonia patients who developed cold agglutinins, 72 to 92% developed specific antibody for *M. pneumoniae*, suggesting that most cold agglutinin positive pneumonia is caused by the organism (43). The reciprocal relationship is less significant, and many patients with *M. pneumoniae* infection fail to develop cold agglutinins (41). The cold agglutinin response is directly related to the severity of illness;

however, a fair proportion of individuals with mild illness develop this antibody (41, 54).

Patients with atypical pneumonia also develop agglutinins for the MG strain of nonhemolytic streptococci (268). These antibodies develop less often than cold agglutinins. Like the latter, streptococcus MG agglutinins are associated with *M. pneumoniae* infection.

By use of cross-absorption techniques, it was shown that the specific fluorescent-stainable antibody was distinct from cold agglutinins and streptococcus MG agglutinins (161, 162). The question of a relationship of *M. pneumoniae* to the L form of streptococcus MG has been raised; however, there is no evidence to support this claim. A careful serological comparison indicated that *M. pneumoniae* did not share antigens with the L form of streptococcus MG (169).

During 1941–1945, *M. pneumoniae* was associated with 62% of atypical pneumonia illnesses studied (77*a*). In a recent study, *M. pneumoniae* was recovered from 20% of 215 patients of all ages in the Seattle area during 1962–1963 (111). In addition, the organism was implicated in 10 to 16% of pneumonia illnesses which were investigated in Great Britain and Finland (109, 132).

Military recruit training appears to offer one type of environment which favors dissemination of *M. pneumoniae* infection (41). Probably important in this regard are prolonged close personal contact and a large population of susceptibles into which new susceptibles are regularly introduced. These conditions also characterize prison populations, and it is interesting to note that 87% of federal prisoners

TABLE 9. *Cold agglutinins and Mycoplasma pneumoniae illness*

Location	Time	Diagnosis	No. tested	Per cent positive for *M. pneumoniae*[a]	Per cent positive for cold agglutinins[b]	Reference
U.S.A.	1947–59	Pneumonia	95	86	45	56
Cleveland	1947–49	Pneumonia	112	72	72	52
Boston	1952–55	Pneumonia	38	92	NT[c]	162
S. Carolina	1959–61	Pneumonia	290	85	47	41
	1959–61	URI[d]	31	NT	33	
Holland	1962–63	URI	26	NT	42	272
Seattle	1962–63	Pneumonia	94	76 (73)[e]	52 (76)[e]	111

[a] Percentage of patients with a rise in cold agglutinins who developed specific antibody for *M. pneumoniae*.
[b] Percentage of patients with *M. pneumoniae* infection who developed a rise in cold agglutinins.
[c] Not tested.
[d] Upper Respiratory Infection.
[e] Includes also individuals with high unchanging levels of cold agglutinins.

Importance in disease. At present, the overall importance of *M. pneumoniae* in human respiratory disease can only be estimated in a preliminary fashion (Table 10). The agent was associated with 8 to 40% of the pneumonias which occurred in three naval and marine recruit populations (41, 43, 99*a*). Other evidence suggests that the organism may be important in other military populations; thus, serological evidence of infection was found in 26% of 69 cold agglutinin negative pneumonia illnesses which occurred among soldiers and dependents at various army camps over a 12-year period (56). The findings from the University of Wisconsin study suggest that college populations may also offer special conditions which favor dissemination of *M. pneumoniae*. In this 8-year study, 22% of students with pneumonia developed antibody for the organism (91).

In the general civilian population, the agent appears to be an important cause of pneumonia. were found to have fluorescent-stainable antibody (58).

In the general population, *M. pneumoniae* appears to spread quite slowly from person to person. Fluorescent antibody is usually not detectable during infancy. Thereafter, acquisition of this antibody occurs at a slow rate with increasing age (56). The highest proportion of seropositive individuals is found in the third decade.

M. pneumoniae does not produce sharp epidemics as the influenza and respiratory syncytial viruses do. Instead, infection occurs throughout the year, generally with peak activity during the fall and early winter. This pattern is characteristic of both civilian and military infections (5, 41, 43, 99*a*, 111, 132).

M. pneumoniae can cause pneumonia in persons of any age; however, the majority of such illnesses occur during late childhood and during the second and third decades (111, 132). This

TABLE 10. *Importance of Mycoplasma pneumoniae in severe respiratory illness in different locations*

Age	Respiratory disease	Location	Time	No. tested	Per cent positive	Reference
Adults	Atypical pneumonia	U.S.A.	1941–45	84	62[a]	77[a]
Adult	Pneumonia	U.S.A.	1947–59	97	37[b]	56
Adolescents	Pneumonia	Massachusetts	1952–56	68[c]	79[b]	162
Infants and children	Lower tract	District of Columbia	1957–59	152	10[b]	35
College students	Lower tract	Wisconsin	1953–60	119	22[b]	91
Naval or marine recruits	Pneumonia	S. Carolina	1959–63	1,095	40[d]	41
		N. Carolina	1959–62	163	20[e]	43, 99*a*
		Illinois	1959–61	294	8[e]	43
All ages	Lower tract	Great Britain	1962	112	10[e]	109
All ages	Pneumonia	Finland	1962–63	246	16[e]	132
All ages	Pneumonia	Washington	1962–63	215	20[f]	111

[a] Diagnosis established by neutralization test in rodents.
[b] Diagnosis established by fluorescent-antibody technique.
[c] Mostly cold agglutinin-positive.
[d] Diagnosis established by fluorescent-antibody and complement-fixation techniques.
[e] Diagnosis established by complement-fixation technique.
[f] Diagnosis established by recovery of the organism.

characteristic pattern has been observed in a number of different localities (Table 11).

One of the most interesting aspects of the ecology of *M. pneumoniae* concerns the wide fluctuations in prevalence which can occur in a given locality or population (Table 12). Over the past 20 years, a number of physicians have commented upon the cyclic occurrence of cold agglutinin pneumonia. Recently, with the aid of specific serological techniques, it has been possible to document wide fluctuations in the importance of *M. pneumoniae* in lower respiratory-tract infections in two separate localities. During the years 1957–1959, 10% of pediatric lower respiratory-tract illness in Washington, D.C., was associated with *M. pneumoniae* infection, whereas during 1962–1963 only 1% of patients with such illness developed antibody for the organism (34, 35). Similarly, in 1959, 67% of marine recruits with atypical pneumonia at Parris Island, S.C., had serological evidence of infection, and during subsequent years the proportion of *M. pneumoniae* pneumonias has decreased, until in 1963 only 7% of patients were serologically positive (34, 41, 43). This marked change in importance of *M. pneumoniae* infection at Parris Island could not be correlated with any recognizable change in population size, method of recruit training, or physical environment.

The presence of high-titer complement-fixing antibody to *M. pneumoniae* has been reported in cases of Stevens-Johnson syndrome (163). Since paired sera were not tested nor were attempts made to isolate *M. pneumoniae*, any relationship is unclear at this time.

Inhibitors of M. pneumoniae. M. pneumoniae

TABLE 11. *Importance of Mycoplasma pneumoniae in pneumonia by age*[a]

Age (years)	Infection detected by: Complement fixation — Washington, D.C.[b] No. tested	Complement fixation — Washington, D.C.[b] Per cent positive	Complement fixation — Helsinki, Finland[c] No. tested	Complement fixation — Helsinki, Finland[c] Per cent positive	Recovery of the organism — Seattle, Wash.[d] No. tested	Recovery of the organism — Seattle, Wash.[d] Per cent positive
0–4	355	1	56	4	37	3
5–9	—		42	29	23	13
10–29	28	14	72	27	76	40
30–49	129	2	35	9	47	13
50+	92	7	52	8	23	9

[a] Studies were performed during 1962–1963.
[b] References 34 and 184.
[c] Reference 132.
[d] Reference 111.

TABLE 12. *Fluctuation in importance of Mycoplasma pneumoniae infection in lower respiratory-tract illness*

Time	Infants and children,* Washington, D.C. No. tested	Infants and children,* Washington, D.C. Per cent positive‡	Marine recruits,† Parris Island, S.C. No. tested	Marine recruits,† Parris Island, S.C. Per cent positive‡
1957–59	152	10		
1959			382	67
1960			314	35
1961			113	45
1962			142	12
1963			123	7
1962–63	355	1		

* Pneumonia, bronchiolitis, and bronchitis.
† Pneumonia.
‡ Fourfold or greater rise in fluorescent or complement-fixing antibody.

is inhibited by tetracycline and related compounds in vitro, in ovo, and in vivo (49, 74, 141). The organism can also be inhibited by streptomycin and an organic gold salt (76, 108). Thallium acetate and penicillin do not inhibit *M. pneumoniae*, and these compounds can be incorporated in agar or broth medium for recovery of the organism to inhibit bacterial contamination (38, 42, 116, 119).

Treatment. The efficacy of tetracycline drugs in the therapy of atypical pneumonia has been a controversial subject during the past decade (92). A beneficial effect was observed in studies which included a variable proportion of cold agglutinin positive cases (173). Other workers, however, failed to observe a therapeutic effect (92). It seems probable that the variable results of tetracycline treatment can be ascribed to differences in the proportion of patients infected with *M. pneumoniae* in the various studies (92, 141). In a recent investigation, this difficulty was resolved by evaluating the effect of treatment in a group of 109 serologically diagnosed *M. pneumoniae* pneumonia illnesses (141). Patients with pneumonia were randomized, and approximately equal groups received either 0.9 g of demethylchlortetracycline or a placebo. Administration of the drug for 6 days significantly reduced the duration of fever, cough, rales, and malaise. In addition, treatment markedly accelerated the clearing of pulmonary infiltration. These findings are compatible with the known inhibitory effect of tetracycline derivatives on *M. pneumoniae* and mycoplasmas in general (49, 74, 119, 141, 190).

Respiratory Disease Caused by M. hominis Type 1

Strains of *M. hominis* type 1 have been recovered from the oropharynx of patients with pneumonia (185). The significance of these observations is not completely clear, because similar strains have also been isolated from healthy individuals, although less frequently than from respiratory disease patients (185). Oral isolates of *M. hominis* type 1 grow equally well aerobically and anaerobically. The biological properties of such strains are identical to those of the prototype strain originally recovered from the genital tract. Antigenically, the recent oral isolates closely resemble the prototype strain; however, the latter is deficient in one antigen which is prominent in the former strains (266).

Volunteer studies were performed to determine the pathogenic potential of the oral isolates. Unexpectedly, a proportion of the first group of experimentally infected individuals developed exudative pharyngitis and tonsillitis (185). These findings were extended in a subsequent study in which volunteers without detectable IHA antibody and individuals with low or high levels of antibody were inoculated intranasally with the organism. Again, a proportion of the volunteers developed exudative pharyngitis and tonsillitis. These changes were observed most frequently in the antibody-free group and least often in the high antibody group (185). This inverse relationship was statistically significant and indicated that the *M. hominis* type 1 oral isolate was responsible for the observed pharyngeal changes. The role and importance of *M. hominis* type 1 strains in naturally occurring exudative pharyngitis and tonsillitis remains to be determined.

Genitourinary Tract Disease

At present, the role of organisms of the genus *Mycoplasma* in genitourinary-tract disease remains equivocal. A number of findings suggest that these organisms may play a role in certain genitourinary-tract illnesses (15, 114, 115, 175, 228). However, equally compelling evidence from other studies indicates that mycoplasmas act as commensals in the genitourinary tract (96, 99, 101, 191).

In early studies, mycoplasma strains which produce large colonies were recovered from a number of patients with genitourinary-tract disease—urethritis, cervicitis, or vaginitis. Subsequently, these organisms were also recovered from individuals free from genitourinary-tract symptoms, but less often, as a rule, than from patients with disease (Tables 3 and 4; 15, 114, 115, 175, 228). In later investigations, there has been disagreement as regards the rate of mycoplasma infection among genitourinary disease patients and comparable healthy individuals. Indeed, several recent studies indicate that the rates for strains which produce large colonies may be similar (96, 99, 101, 191).

One difficulty in evaluating the conflicting results presented by different workers concerns the comparability of healthy control individuals to patients with genitourinary-tract disease. Matching the two groups for age, sex, location, and socioeconomic status is not enough, because the incidence of mycoplasma infection appears to be directly related to the extent of sexual promiscuity (234). It is not clear from several of the published reports whether or not this factor was carefully evaluated in selecting the control group.

In the female, an additional difficulty exists regarding interpretation of mycoplasma infection of the genital tract. The growth of mycoplasmas in the vagina is favored with the *p*H shifts from the normal acid range to alkaline (11, 101). Such an alkaline shift in *p*H occurs during infection by

a number of genitourinary-tract pathogens. This effect results from a replacement of the normal bacterial flora, which includes Döderlein's bacilli, by pathogenic organisms (101). A high proportion (59 to 92%) of females with syphilis, gonorrhea, or trichomonas vaginitis also have mycoplasma organisms in their vaginas (115, 126, 191). Although a mycoplasma may be the only organism recovered from a patient with genitourinary-tract disease, the possibility cannot be excluded that another pathogenic organism is responsible for the disease process while incidentally providing conditions which favor mycoplasma growth.

Most mycoplasma strains recovered from patients with genitourinary-tract disease have not been identified serologically. Most of the strains which have been identified have been *M. hominis* type 1 (146, 191). It is probable that the untyped strains from the studies listed in Tables 3 and 4 are also of this serotype, except where biological properties indicate otherwise, i.e., the T strains. In future studies, it is possible that new variations in culture technique may lead to the recovery of heretofore unknown strains. If such hypothetical strains are to be recognized as new serotypes and evaluated for their role in disease, it is essential that all isolates from each investigation be characterized antigenically.

A relationship of T strains to urethritis of males was suggested by the results of a recent study (Table 3; 96, 99). Organisms of this group were recovered twice as often from patients as from controls. In addition, the isolation rate from patients was 79%. However, the reservations expressed concerning the role of "large-colony" organisms in urethritis also apply to the T strains. It should be emphasized that the T strains are grouped together because of a common biological property, i.e., small colony size. The number of antigenically distinct species within the group is not known. Until this has been determined, it may be difficult to define the natural history and potential pathogenicity of these organisms.

Four different serological studies with *M. hominis* type 1 complement-fixing antigen have provided evidence that this species may, under certain circumstances, be associated with genitourinary-tract disease (30, 157, 174, 194). Complement-fixing antibodies were found significantly more often and in higher titer in patients with genital-tract disease than in healthy controls (Table 13; 30, 157, 174, 194). This difference was observed for patients with salpingitis as well as

TABLE 13. *Complement-fixing antibody for Mycoplasma hominis type 1 in persons with and without genital-tract diseases*

Location	Diagnosis	Age	Male		Female		Reference
			No. tested	Per cent positive*	No. tested	Per cent positive*	
Great Britain	Venereal disease†	Adult	284	19	416	44	30
	No venereal disease	Adult	299	2	336	8	
	No venereal disease	Children	72	3	32	0	
Great Britain	Salpingitis	Adult	—		51	55	157
	No venereal disease	Adult	—		109	4	
Sweden	Gonococcal salpingo-oophoritis	Adult	—		6	33	174
	Nongonococcal salpingo-oophoritis	Adult	—		21	33‡	
	Other pelvic inflammatory disease	Adult	—		26	11	
	No inflammatory pelvic disease	Adult	—		52	6	
Great Britain	Active Reiter's syndrome	Adult	31	51	—		194
	Inactive Reiter's syndrome	Adult	23	9	—		

* Complement fixation at 1:8 to 1:16 serum dilution.

† Urethritis, cervicitis, vaginitis, gonorrhea, syphilis, yaws, or trichomonas vaginalis; approximately one-third of the patients had urethritis, cervicitis, or vaginitis. The proportion of the latter patients with antibody did not differ from that of the group as a whole.

‡ Five of seven positive individuals had complement-fixation antibody titer 1:32 to 1:256; positive individuals in other groups of this study did not exceed 1:16.

those with less severe forms of genital-tract inflammation (174). However, patients with gonorrhea, syphilis, or trichomonas infection also had a higher frequency of positive findings for *M. hominis* type 1 than did healthy controls (174). Extension of the complement-fixing antibody approach may prove helpful in evaluating the role of mycoplasmas in disease, since it offers the possibility of differentiating infections in which organisms are invasive and those in which they are predominantly commensal.

In five instances, mycoplasma strains have been recovered directly from fallopian tube or ovarian abscesses (110, 211, 260, 261). All but one of these patients were treated with penicillin or chlortetracycline. Recovery of a mycoplasma from treated patients does not preclude the possibility that another antibiotic-sensitive agent was responsible for the pyogenic process. However, a mycoplasma was the only agent recovered from the one untreated patient (110). In addition, one of the patients treated with penicillin and chlortetracycline developed a high level of complement-fixing antibody for *M. hominis* type 1 during convalescence (110). *M. hominis* type 1 has also been recovered on two occasions from the blood of septic patients (260, 261). These findings suggest that genital-tract organisms, ordinarily nonpathogenic, may under special circumstances produce suppurative lesions.

Arthritis

Mycoplasmas cause arthritis in a number of avian and animal species (95, 233). Joint changes occur with varying frequency in sheep and goats infected with *M. agalactiae*, cows infected with *M. mycoides*, rats infected with *M. arthritidis*, and fowl infected with *M. gallisepticum*. In addition, *M. hyorhinis* is thought to cause arthritis in pigs. These findings have suggested to many workers the possibility that mycoplasmas may also cause arthritis in man. The best-studied of the animal arthritides is that produced by *M. arthritidis* in rats. The joint lesions are predominantly septic, and for this reason this condition cannot be considered as analogous to rheumatoid arthritis (233).

Recovery of mycoplasmas from synovial fluid has been reported for 10 patients with Reiter's syndrome and 7 patients with rheumatic fever or rheumatoid arthritis (9, 24, 67, 152). However, most attempts at isolation of mycoplasmas from joint fluid have been unsuccessful (97, 233). The strains of mycoplasmas recovered from synovial fluid have not been characterized antigenically nor have they been compared with the recognized human species. This must be done, the possibility of laboratory contamination must be ruled out, and, finally, normal synovial fluid must be studied before serious consideration can be given to the role of mycoplasmas in human arthritis (Reiter's syndrome, rheumatoid arthritis, etc.).

MYCOPLASMA CONTAMINATION OF TISSUE CULTURES

Contamination of cell cultures by mycoplasmas was first reported in 1956 (217), although it was established somewhat earlier that mycoplasmas could grow in purposely infected tissue cultures (120, 121). In quick succession, other reports appeared which confirmed these findings (31, 33, 53, 57, 122, 123, 207, 220, 221). Interest in mycoplasma contamination of cell cultures was soon aroused owing to the almost exponential increase in numbers of investigators using the tissue-culture methodology during the decade 1955–1965. As many as 60% of the cultures examined were found to be contaminated with mycoplasmas. The major questions posed by these findings have been related to the source of the contaminants, detection and prevention of contamination, and the question of why most are human species.

The contamination of cell cultures is, generally, unrecognizable macroscopically or microscopically. Most cell cultures appear to behave equally well when contaminated with 10^7 minimal reproductive units per milliliter or when sterile. Thus, the insidious nature of the contamination has resulted in a serious pitfall for the unwary investigator using tissue cultures.

The almost universal use in tissue cultures of penicillin, to which all mycoplasmas are resistant, and of streptomycin, to which most mycoplasmas are resistant, has resulted in a general relaxation of aseptic technique. More recently, and less commonly, mycoplasmas producing overt CPE in cell cultures have been described (16, 26, 32, 106, 107, 112, 150, 208, 224). These strains are probably unrelated to the known human species, and their biological importance is currently being assessed. Their discovery, however, means that the description of virus activity in vitro, based solely on a transmissible CPE, is done at great risk (107, 187). In those cases that have been studied, mycoplasma contamination of tissue cultures depletes arginine from the fluid medium (139, 149, 171, 208, 223, 230, 231). In the absence of this essential nutrient, cell metabolism is depressed but can be reversed by supplementation with arginine. The bizarre behavior of viruses or other biological materials added to contaminated cell cultures can often be traced to this reaction (223). It is likely that those mycoplasmas causing CPE in vitro operate in this manner and, unlike cytopathic viruses, do not

directly cause overt cell destruction (149). The replication of mycoplasmas appears to occur extracellularly. Consistent with this view is the finding that contamination of tissue cultures can be cured by treatment with antiserum (125, 206). In certain instances, a heat-labile serum accessory factor is required in addition to specific antibody.

Source and Species Identification

The majority of tissue-culture contaminants have been identified as *M. hominis* type 1 (6, 53, 57, 84, 125). *M. orale* type 1, first isolated as a tissue-culture contaminant (116, 119, 141*a*, 154, 155, 156) and now known to be a common resident of the human oropharynx (50, 124, 263), has recently been frequently detected in cell cultures. *M. hominis* type 2 was described as a contaminant on one occasion (6) as was the avian species *M. gallisepticum* (84). Although the preponderance of contaminants are human mycoplasma species, a number of reports describe the origin of at least some tissue culture contaminants as L forms. It has been postulated and, in fact, demonstrated that the use of penicillin in cell culture media can give rise to the induction of L forms when media are contaminated with airborne bacteria (7, 117, 168, 221). That contamination of tissue cultures may sometimes be due to the reversion of bacterial contaminants to L forms cannot be denied; however, the weight of evidence is that most contaminants are mycoplasmas and, moreover, are human species.

There is no published evidence that the reagents used in tissue-culture media, including serum and embryo extract, are the usual source of the contaminants (31, 116, 117, 119, 123, 125, 168, 221). There are also no confirmed reports of contamination of primary tissue grown in vitro. Furthermore, since most contaminants are human species, it would be unreasonable to expect animal sera or tissues to give rise to these strains. The findings that primary tissue cultures are usually free from mycoplasma contamination and that heteroploid cell lines are frequently contaminated imply a direct relationship between the number of subcultivations or manipulations of a cell culture and the expectation that it will be found to be contaminated. When cell cultures have been monitored for mycoplasma contamination from the primary culture and through many subsequent passages, the frequency of contamination is correlated directly with the number of subcultivations (116, 119). Although it has been suggested that contaminants may arise from the primary tissue (188), this theoretical possibility is not universally applicable, especially since the mycoplasmas found in contaminated animal-tissue cultures are almost always human species.

It is probable that mycoplasma contamination of tissue cultures can occur via droplet infection in consequence of faulty aseptic technique (125, 195). Although mycoplasmas have never been found as ordinary airborne contaminants, it is probable that faulty aseptic technique within the confines of a tissue-culture "sterile room" could account for their spread. A recent awareness of the contamination of cell cultures with cells of other cultures lends credence to the probability that gross carelessness when subcultivating cell cultures may be a leading cause of the spread of mycoplasma contamination. The initial contaminant in the majority of cases probably came from the oropharynx of cell culturists.

Careless aseptic technique has continued because workers using penicillin and streptomycin, impressed by the lack of bacterial contamination, have assumed that asepsis was maintained. Penicillin is completely ineffective against the mycoplasmas, and streptomycin is rarely effective. In support of this contention is the finding that, in those laboratories not using antibiotics, mycoplasma-contaminated cell cultures are rarely found. Workers in such laboratories would be expected to be the most skillful in utilizing strict aseptic techniques when handling cell cultures.

Detection of Contamination

Although two enzyme assay methods have been proposed for the detection of mycoplasmas in cell cultures (8, 127, 230), these approaches cannot replace the direct demonstration of the contaminant by its growth on agar. The definition of the genus *Mycoplasma* presupposes a demonstration of characteristic growth on agar, and any method circumventing this absolute requirement begs the question. Furthermore, although these enzyme systems may be present in the mycoplasmas and absent in mammalian cells, they do not discriminate between the presence of mycoplasmas or other microbial contaminants in cell cultures. Finally, the limit of sensitivity of these methods is such that only relatively heavy contamination can be detected. The detection of mycoplasmas by any other method, including electron microscopy, visualization by staining of cells, or fluorescent antibody techniques, is by itself insufficient. Therefore, without the demonstration of characteristic growth on agar, the presence of mycoplasmas in any material cannot be conclusively established (20, 101, 147).

Prevention of Contamination

In view of the almost total absence of mycoplasma contamination in cell cultures carried in laboratories not using antibiotics, this approach is probably the most effective. It is likely that the absence of antibiotics from culture media does not *per se* contribute to the absence of mycoplasmas, but that the greater skill necessary for maintaining asepsis in antibiotic-free cultures prevents mycoplasma contamination from the worker himself or from already contaminated cultures received from other laboratories.

An alternative to cell cultivation in antibiotic-free medium is cultivation in the presence of antibiotics known to be effective against the mycoplasmas. These include kanamycin (93, 207) and the tetracyclines (31, 118, 119, 123). Although kanamycin-resistant mycoplasmas have been found (116, 119), fewer strains resistant to chlortetracycline (Aureomycin, Lederle Laboratories, Pearl River, N.Y.; 116, 118, 119) have been encountered. In addition to their effectiveness prophylactically, kanamycin and chlortetracycline have been successfully used to cure contaminated cell cultures (93, 116, 119, 207). Alternatively, some mycoplasma contaminants can be eliminated by exposure to high temperatures, which differentially kills the contaminant while sparing the cells (117). Successful decontamination of cell cultures has also been achieved by incorporating specific antisera into the culture medium (125, 206).

SUMMATION

At present, six distinct species of mycoplasmas are known to infect man. Certain biological and ecological properties of these agents have been defined. One species, *M. pneumoniae*, has been definitely shown to be an important respiratory-tract pathogen, and another species, *M. hominis* type 1, may play a role in respiratory-tract and genital-tract disease.

Certainly, much remains to be learned concerning the fundamental biology as well as the ecological properties of the known human mycoplasma species. It is probable that additional species from humans will be recognized as epidemiological studies of mycoplasma infections are extended and as modifications of existing nutrient media are evaluated in such studies.

Questions of potential pathogenicity of the mycoplasmas may also involve their behavior in concert with viruses or bacteria. Such a relationship may be important in nongonococcal urethritis whose etiology is still obscure. The possible loss of pathogenicity upon passage on culture media may further complicate attempts to determine etiology. Indeed, the laboratory strain of the Eaton agent from which *M. pneumoniae* was first isolated proved, upon agar passage, to be relatively avirulent.

With the availability of a medium upon which *M. pneumoniae* can be isolated and with the existence of simple differential tests, the detection of this species is possible in most diagnostic laboratories. Rapid detection of *M. pneumoniae* infection should be of obvious value in the diagnosis and therapy of the group of atypical pneumonias in man. A search for other mycoplasma species involved in that segment of human respiratory disease for which no etiological agent has been found is an important area for exploration.

In view of the number of properties shared by viruses and mycoplasmas, the identification of any new virus isolate, especially in tissue cultures, should always include tests for mycoplasmas.

Currently, the attention of a number of molecular biologists is being directed toward the mycoplasmas. As the smallest free-living microorganisms, mycoplasmas have raised questions relevant to the smallest dimensions compatible with a free-living existence. Is the complete range of biochemical activities found in larger microorganisms also present in the mycoplasmas? If not, how are the fundamental life processes performed? Finally, do the mycoplasmas represent the lowest possible size limit compatible with life as an independent organism?

In view of the relative ease and frequency with which mycoplasmas can be isolated from various sources in nature, it is essential that quicker and more sensitive methods be found by which species and strains can be identified. Frequently, isolation of mycoplasmas has been reported without any attempt to subcultivate the original growth, much less to identify the isolate with known species. It is often found that strains of mycoplasmas can be easily isolated but are very difficult to subcultivate. A broad area of research also exists on studies of the antigenic relationships of all of the mycoplasmas.

Knowledge of the mycoplasmas should increase at an exponential rate during the next few years, as microbiologists become more aware of these microorganisms which are as small as most viruses and are frequently pathogenic, yet can be cultivated on agar medium.

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LITERATURE CITED

1. ABRAMS, R. Y. 1955. A method for the cultivation of L forms in liquid media. J. Bacteriol. **70**:251.
2. ADLER, H. E., AND M. SHIFRINE. 1960. Nutrition, metabolism, and pathogenicity of mycoplasmas. Ann. Rev. Microbiol. **14**: 141–160.
3. ARMSTRONG, D., G. HENLE, N. L. SOMERSON, AND L. HAYFLICK. Cytopathic mycoplasmas associated with two human tumors. I. Isolation and biological aspects. (in preparation).
4. BAERNSTEIN, H. D., JR., AND J. J. QUILLIGAN, JR. 1963. Cultivation of *Mycoplasma pneumoniae* (Eaton agent). J. Bacteriol. **86**:339.
5. BAERNSTEIN, H. D., JR., E. TREVISANI, S. AXTELL, AND J. J. QUILLIGAN, JR. *Mycoplasma pneumoniae* (Eaton atypical pneumonia agent) in children's respiratory infections. J. Pediat. (in press).
6. BAILEY, J. S., H. W. CLARK, W. R. FELTS, R. C. FOWLER, AND T. McP. BROWN. 1961. Antigenic properties of pleuropneumonia-like organisms from tissue cell cultures and the human genital area. J. Bacteriol. **82**:542–547.
7. BARILE, M. F., W. F. MALIZIA, AND D. B. RIGGS. 1962. Incidence and detection of pleuropneumonia-like organisms in cell cultures by fluorescent antibody and cultural procedures. J. Bacteriol. **84**:130–136.
8. BARILE, M. F., AND R. T. SCHIMKE. 1963. A rapid chemical method for detecting PPLO contamination of tissue cell cultures. Proc. Soc. Exptl. Biol. Med. **114**:676–679.
9. BARTHOLOMEW, L. E., AND J. HIMES. 1964. Isolation of mycoplasma (PPLO) from patients with rheumatoid arthritis, systemic lupus erythematosus and Reiter's syndrome. Am. Rheumat. Assoc. Ann. Meet., June, p. 291.
10. BARTMANN, K., AND W. HÖPKEN. 1955. Phasenkontrast-mikroskopische beobachtungen zur morphologie der peripneumonieähnlichen organismen PPLO. Zentr. Bakteriol. Parasitenk. Abt I Orig. **163**: 319–332.
11. BERCOVICI, B., S. PERSKY, R. ROZANSKY, AND S. RAZIN. 1962. Mycoplasma (pleuropneumonia-like organisms) in vaginitis. Am. J. Obstet. Gynecol. **84**:687–691.
12. BERG, R. L., H. WEINBERGER, AND L. DIENES. 1957. Acute hemorrhagic cystitis. Am. J. Med. **22**:848–864.
13. BEVERIDGE, W. I. B. 1943. Isolation of pleuropneumonia-like organisms from the male urethra. Med. J. Australia **2**:479–481.
14. BEVERIDGE, W. I. B., A. D. CAMPBELL, AND P. E. LIND. 1946. Pleuropneumonia-like organisms in cases of non-gonococcal urethritis in man and in normal female genitalia. Med. J. Australia **1**:179–180.
15. BOCK, M., R. GÖNNERT, L. GRÜN, W. KIKUTH, AND G. B. ROEMER. 1951. Pleuropneumonieartige mikroorganismen bei "Viruspneumonien." Klin. Wochschr. **29**: 87–88.
16. BÖGEL, K., M. BERCHTHOLD, A. BRUNNER, AND L. KLINGER. 1962. Die eignung der gewebekulturtechnik zur isolierung und charakterisierung eines zytopathogenen mycoplasmastammes vom bullen. Zentr. Bakteriol. Parasitenk. Abt. I Orig. **185**: 423–439.
17. BORDET, J. 1910. La morphologie du microbe de la péripneumonie des bovides. Ann Inst. Pasteur **24**:161–167.
18. BOREL, L. J. 1952. Pleuropneumonia et bacteriés en phase "L." Étude des problèmes bactériologiques et cliniques. Biol. Med. (Paris) **41**:379–449.
19. BORREL, A., E. DUJARDIN-BEAUMETZ, JEANTET, AND JOUAN. 1910. Le microbe de lá peripneumonie. Ann. Inst. Pasteur **24**:168–179.
20. BREED, R. S., E. G. D. MURRAY, AND N. R. SMITH. 1957. Bergey's manual of determinative bacteriology, 7th ed. The Williams & Wilkins Co., Baltimore.
21. BROWN, T. McP., AND G. S. HAYES. 1942. Isolation of microorganisms of the pleuropneumonia group from apparently pure cultures of the gonococcus (Neisseria gonorrhoeae). J. Bacteriol. **43**:82.
22. BROWN, T. M., H. F. SWIFT, AND R. F. WATSON. 1940. Pseudo-colonies simulating those of pleuropneumonia-like microörganisms. J. Bacteriol. **40**:857–866.
23. BURNETT, G. W., AND E. GILMORE. 1959. Incidence and distribution of oral pleuropneumonia-like and L organisms. J. Dental Res. **38**:632.
24. BUTAS, C. A. 1957. The isolation of pleuropneumonia-like organisms from two cases of polyarthritis. Can. J. Microbiol. **3**:419–426.
25. BUTLER, M., AND B. C. J. G. KNIGHT. 1960. The survival of washed suspensions of mycoplasma. J. Gen. Microbiol. **22**:470–477.
26. BUTLER, M., AND R. H. LEACH. 1964. A mycoplasma which induces acidity and cytopathic effect in tissue culture. J. Gen. Microbiol. **34**:285–294.
27. BUTTERY, S. H., AND P. PLACKETT. 1960. A specific polysaccharide from *Mycoplasma mycoides*. J. Gen. Microbiol. **23**:357–368.
28. CAILLEAU, R. 1937. La nutrition des flagellés tétramitidés, les stérols, facteurs de croissance pour les trichomonades. Ann. Inst. Pasteur **59**:293–328.
29. CANCHOLA, J. G., H. W. KIM, D. TAYLOR-ROBINSON, R. M. CHANOCK, AND R. H. PARROTT. 1964. Mycoplasma species in respiratory tract infections of childhood. Am. J. Diseases Children (in press).

30. CARD, D. H. 1959. PPLO of human genital origin; serological classification of strains and antibody distribution in man. Brit. J. Venereal Diseases **35**:27-34.
31. CARSKI, T. R., AND C. C. SHEPARD. 1961. Pleuropneumonia-like (mycoplasma) infections of tissue culture. J. Bacteriol. **81**:626-635.
32. CASTREJON-DIEZ, J., T. N. FISHER, AND E. FISHER, JR. 1963. Experimental infection of tissue cultures with certain mycoplasma (PPLO). Proc. Soc. Exptl. Biol. Med. **112**: 643-647.
33. CHANG, R. S. 1958. Differences in inositol requirements of several strains of HeLa, conjunctival and amnion cells. Proc. Soc. Exptl. Biol. Med. **99**:99-102.
34. CHANOCK, R. M. Unpublished results.
35. CHANOCK, R. M., M. K. COOK, H. H. FOX, R. H. PARROTT, AND R. J. HUEBNER. 1960. Serologic evidence of infection with Eaton agent in lower respiratory illness in childhood. N. Engl. J. Med. **262**:648-654.
36. CHANOCK, R. M., L. DIENES, M. D. EATON, D. G. FF. EDWARD, E. A. FREUNDT, L. HAYFLICK, J. F. PH. HERS, K. E. JENSEN, C. LIU, B. P. MARMION, H. E. MORTON, M. A. MUFSON, P. F. SMITH, N. L. SOMERSON, AND D. TAYLOR-ROBINSON. 1963. *Mycoplasma pneumoniae:* proposed nomenclature for atypical pneumonia organism (Eaton agent). Science **140**:662.
37. CHANOCK, R. M., H. H. FOX, W. D. JAMES, H. H. BLOOM, AND M. A. MUFSON. 1960. Growth of laboratory and naturally occurring strains of Eaton agent in monkey kidney tissue culture. Proc. Soc. Exptl. Biol. Med. **105**:371-376.
38. CHANOCK, R. M., L. HAYFLICK, AND M. F. BARILE. 1962. Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. Proc. Natl. Acad. Sci. **48**:41-49.
39. CHANOCK, R. M., W. D. JAMES, H. H. FOX, H. C. TURNER, M. A. MUFSON, AND L. HAYFLICK. 1962. Growth of Eaton PPLO in broth and preparation of complement fixing antigen. Proc. Soc. Exptl. Biol. Med. **110**:884-889.
40. CHANOCK, R. M., AND K. M. JOHNSON. 1961. Infectious disease: respiratory viruses. Ann. Rev. Med. **12**:1-18.
41. CHANOCK, R. M., J. A. MUFSON, H. H. BLOOM, W. D. JAMES, H. H. FOX, AND J. R. KINGSTON. 1961. Eaton agent pneumonia. J. Am. Med. Assoc. **175**:213-220.
42. CHANOCK, R. M., M. A. MUFSON, W. D. JAMES, H. H. FOX, H. H. BLOOM, AND B. FORSYTH. 1962. Recovery of PPLO of atypical pneumonia on artificial agar medium. Proc. Soc. Exptl. Biol. Med. **110**:543-547.
43. CHANOCK, R. M., M. A. MUFSON, N. L. SOMERSON, AND R. B. COUCH. 1963. Role of mycoplasma (PPLO) in human respiratory disease. Am. Rev. Respirat. Diseases **88**:218-231.
44. CHANOCK, R. M., R. H. PARROTT, K. M. JOHNSON, M. A. MUFSON, AND V. KNIGHT. 1963. Biology and ecology of two major lower respiratory tract pathogens-RS virus and Eaton PPLO. *In* Morris Pollard [ed.], Perspectives in virology, vol. 3. Hoeber Medical Division, Harper & Row, Publishers, Inc., New York.
45. CHANOCK, R. M., D. RIFKIND, H. M. KRAVETZ, V. KNIGHT, AND K. M. JOHNSON. 1961. Respiratory disease in volunteers infected with Eaton agent; a preliminary report. Proc. Natl. Acad. Sci. U.S. **47**: 887-890.
46. CHU, H. P. 1959. Organisms of the pleuropneumonia group. Vet. Ann., p. 1-16.
47. CLARK, H. W., J. S. BAILEY, R. C. FOWLER, AND T. MCP. BROWN. 1963. Identification of *Mycoplasmataceae* by the fluorescent antibody method. J. Bacteriol. **85**:111-118.
48. CLYDE, W. A. 1963. Hemolysis in identifying Eaton's pleuropneumonia-like organism. Science **139**:55.
49. CLYDE, W. A., JR. 1961. Demonstration of Eaton's agent in tissue culture. Proc. Soc. Expt. Biol. Med. **107**:715-718.
49*a*. CLYDE, W. A., JR. 1963. Studies on growth of Eaton's agent in tissue culture. Proc. Soc. Exp. Biol. Med. **112**:905-909.
50. CLYDE, W. A., JR. 1964. Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol. **92**:958-965.
51. CLYDE, W. A., JR. 1964. Studies on the nature and properties of Eaton's agent (*Mycoplasma pneumoniae*). Bacteriol. Proc., p. 54.
52. CLYDE, W. A., JR., AND F. W. DENNEY, JR. 1963. The etiology and therapy of atypical pneumonia. Med. Clin. N. Am. **47**:1201-1217.
53. COLLIER, L. H. 1957. Contamination of stock lines of human carcinoma cells by pleuropneumonia-like organisms. Nature **180**:757-758.
54. COMMISSION ON ACUTE RESPIRATORY DISEASES. 1944. Cold hemagglutinins in primary atypical pneumonia and other respiratory infections. Am. J. Med. Sci. **208**: 742-750.
55. COMMISSION ON ACUTE RESPIRATORY DISEASES. 1946. The transmission of primary atypical pneumonia to human volunteers. I. Experimental methods. II. Results of inoculation. III. Clinical features. IV. Laboratory studies. Bull. Johns Hopkins Hosp. **79**:97-167.
56. COOK, M. K., R. M. CHANOCK, H. H. FOX, R. J. HUEBNER, E. L. BUESCHER, AND R. T. JOHNSON. 1960. Role of Eaton agent in disease of lower respiratory tract. Evidence for infection in adults. Brit. Med. J. **1**:905-911.

57. CORIELL, L. L., D. P. FABRIZIO, AND S. R. WILSON. 1960. Comparison of pleuropneumonia-like organism strains from tissue culture by complement fixation. Ann. N.Y. Acad. Sci. **79**:574–580.
58. COUCH, T. R., M. D. CATE, AND R. M. CHANOCK. 1964. Infection with artificially propagated Eaton agent. (*Mycoplasma pneumoniae*). J. Am. Med. Assoc. **187**:146–151.
59. DAJANI, A. S., W. A. CLYDE, AND F. W. DENNY. 1964. Pathogenesis of M. pneumoniae infection in hamsters. Federation Proc. **23**:192.
60. DEL GUIDICE, R. A., AND R. PAVIA. 1964. Hemadsorption by *Mycoplasma pneumoniae* and its inhibition with sera from patients with primary atypical pneumonia. Bacteriol. Proc., p. 71.
61. DIENES, L. 1939. "L" organism of Klieneberger and Streptobacillus moniliformis. J. Infect. Diseases **65**:24–42.
62. DIENES, L. 1945. Morphology and nature of the pleuropneumonia group of organisms. J. Bacteriol. **50**:441–458.
63. DIENES, L. 1953. Electron micrographs made from L forms of Proteus and two human strains of pleuropneumonia-like organisms. J. Bacteriol. **66**:280–283.
64. DIENES, L. 1960. Controversial aspects of the morphology of PPLO. Ann. N.Y. Acad. Sci. **79**:356–368.
65. DIENES, L., AND J. EDSALL. 1937. Observations on L-organisms of Klieneberger. Proc. Soc. Exptl. Biol. Med. **36**:740–744.
66. DIENES, L., AND S. MADOFF. 1953. Differences between oral and genital strains of human pleuropneumonia-like organisms. Proc. Soc. Exptl. Biol. Med. **82**:36–38.
67. DIENES, L., M. W. ROPES, W. E. SMITH, S. MADOFF, AND W. BAUER. 1948. The role of pleuropneumonia-like organisms in genito-urinary and joint diseases. N. Engl. J. Med. **238**:509–515, 563–567.
68. DIENES, L., AND H. J. WEINBERGER. 1951. The L forms of bacteria. Bacteriol. Rev. **15**:245–288.
69. DINGLE, J. H., AND M. FINLAND. 1942. Virus pneumonias. II. Primary atypical pneumonias of unknown etiology. N. Engl. J. Med. **227**:378–385.
70. DOMERMUTH, C. H., M. H. NIELSEN, E. A. FREUNDT, AND A. BIRCH-ANDERSEN. 1964. Ultrastructure of *Mycoplasma* species. J. Bacteriol. **88**:727–744.
71. DOWDLE, W. R., AND R. Q. ROBINSON. 1964. An indirect hemagglutination test for diagnosis of *Mycoplasma pneumoniae* infections. Proc. Soc. Exptl. Biol. Med. **116**: 947–950.
72. DUREL, P., AND SIBOULET. 1957. *In* Les uretrites non gonoccocique, vol. 1, p. 57. Maisson, Paris, France.
73. EATON, M. D. 1950. Virus pneumonia and pneumonitis viruses of man and animals. Handbuch der Virusforschung II Erganizugsband **87**:140.
74. EATON, M. D. 1950. Action of aureomycin and chloromycetin on the viruses of primary atypical pneumonia. Proc. Soc. Exptl. Biol. Med. **73**:24–26.
75. EATON, M. D., A. E. FARNHAM, J. D. LEVINTHAL, AND A. R. SCALA. 1962. Cytopathic effect of the atypical pneumonia organism in cultures of human tissue. J. Bacteriol. **84**:1330–1337.
76. EATON, M. D., AND C. LIU. 1957. Studies on sensitivity to streptomycin of the atypical pneumonia agent. J. Bacteriol. **74**:784–787.
77. EATON, M. D., G. MEIKLEJOHN, AND W. VAN HERICK. 1944. Studies on the etiology of primary atypical pneumonia. A filterable agent transmissible to cotton rats, hamsters, and chick embryos. J. Exptl. Med. **79**:649–668.
77*a*. EATON, M. D., AND W. VAN HERICK. 1947. Serological and epidemiological studies on primary atypical pneumonia and related acute upper respiratory disease. Am. J. Hyg. **45**:82–95.
78. EDWARD, D. G. FF. 1947. A selective medium for pleuropneumonia-like organisms. J. Gen. Microbiol. **1**:238–243.
79. EDWARD, D. G. FF. 1950. An investigation of the biological properties of organisms of the pleuropneumonia group, with suggestions regarding the identification of strains. J. Gen. Microbiol. **4**:311–329.
80. EDWARD, D. G. FF. 1952. The pleuropneumonia group of organisms and their significance in genital infection. Brit. J. Venereal Diseases **28**:89–96.
81. EDWARD, D. G. FF. 1953. A difference in growth requirements between bacteria in the L-phase and organisms of the pleuropneumonia group. J. Gen. Microbiol. **8**:256–262.
82. EDWARD, D. G. FF. 1954. The pleuropneumonia group of organisms: a review, together with some observations. J. Gen. Microbiol. **10**:27–64.
83. EDWARD, D. G. FF. 1960. Biology of the pleuropneumonialike organisms. Ann. N.Y. Acad. Sci. **79**:305–758.
84. EDWARD, D. G. FF. 1960. Biology of the pleuropneumonia-like organisms. Discussion of part V. Ann. N.Y. Acad. Sci. **79**: 608–609.
85. EDWARD, D. G. FF., AND W. A. FITZGERALD. 1951. Cholesterol in the growth of organisms of the pleuropneumonia group. J. Gen. Microbiol. **5**:576–586.
86. EDWARD, D. G. FF., AND W. A. FITZGERALD. 1952. A growth factor needed to isolate organisms of the pleuropneumonia group from the genital tract of cattle. Vet. Rec. **64**:395.
87. EDWARD, D. G. FF., AND W. A. FITZGERALD. 1954. Inhibition of growth of pleuropneu-

monia-like organisms by antibody. J. Pathol. Bacteriol. **68**:23–30.
88. EDWARD, D. G. FF., AND E. A. FREUNDT. 1956. The classification and nomenclature of organisms of the pleuropneumonia group. J. Gen. Microbiol. **14**:197–207.
89. EDWARDS, G. A., AND J. FOGH. 1960. Fine structure of pleuropneumonialike organisms in pure culture and in infected tissue culture cells. J. Bacteriol. **79**:267–276.
90. ELFORD, W. J. 1929. Ultrafiltration methods and their application in bacteriological and pathological studies. Brit. J. Exptl. Pathol. **10**:126–144.
91. EVANS, A. S., AND M. BROBST. 1961. Bronchitis, pneumonitis and pneumonia in University of Wisconsin Students. N. Engl. J. Med. **265**:401–409.
92. FINLAND, M. 1952. Antimicrobial treatment for viral and related infections. I. Antibiotic treatment of primary atypical pneumonia. New Engl. J. Med. **247**:317–325.
93. FOGH, J., AND C. HACKER. 1960. Elimination of pleuropneumonia-like organisms from cell cultures. Exptl. Cell Res. **21**:242–244.
94. FORD, D. K. 1962. Culture of human genital "T-strain" pleuropneumonia-like organisms. J. Bacteriol. **84**:1028–1034.
95. FORD, D. K. 1963. Rheumatoid arthritis–an infection? Arthritis Rheumat. **6**:159–165.
96. FORD, D. K., AND M. DU VERNET. 1963. Genital strains of human pleuropneumonia-like organisms. Brit. J. Venereal Diseases **39**:18–20.
97. FORD, D. K. 1960. The relationship of human genital pleuropneumonia-like organisms to arthritis complicating urethritis. Arthritis Rheumat. **3**:395–402.
98. FORD, D. K., AND J. MACDONALD. 1963. Morphology of human genital "T-strain" pleuropneumonia-like organisms. J. Bacteriol. **85**:649–653.
99. FORD, D. K., G. RASMUSSEN, AND J. MINKEN. 1962. T-strain pleuropneumonia-like organisms as one cause of non-gonococcal urethritis. Brit. J. Venereal Diseases **38**: 22–25.
99*a*. FORSYTH, E. R., H. H. BLOOM, K. M. JOHNSON, AND R. M. CHANOCK. 1965. Etiology of primary atypical pneumonia. J. Am. Med. Assoc. **191**:364–368.
100. FREUNDT, E. A. 1952. Morphological studies of the peripneumonia organism (Micromyces peripneumonia bovis). Acta Pathol. Microbiol. Scand. **31**:508–529.
101. FREUNDT, E. A. 1958. The mycoplasmataceae. Munksgaard, Copenhagen.
102. FREUNDT, E. A. 1960. Morphology and classification of PPLO. Ann. N.Y. Acad. Sci. **79**:312–325.
103. FREUNDT, E. A. 1962. Personal communication.
104. FROSCH, P. 1923. Zur Morphologie des lungenseucheerregers. II. Mitteilung. Arch. Wiss. Prakt. Tierheilk. **49**:273.
105. GILL, J. W. 1962. Culture and metabolism of *Mycoplasma gallisepticum*. J. Bacteriol. **83**:213–218.
106. GIRARDI, A. J., V. V. HAMPARIAN, N. SOMERSON, AND L. HAYFLICK. In preparation.
107. GIRARDI, A. J., L. HAYFLICK, A. M. LEWIS, AND N. L. SOMERSON. 1965. Recovery of mycoplasmas in the study of human leukemia and other malignancies. Nature **205**: 188–189.
108. GOODBURN, G., AND B. P. MARMION. 1962. A study of the properties of Eaton's primary atypical pneumonia organism. J. Gen. Microbiol. **29**:271–290.
109. GOODBURN, G. M., B. P. MARMION, AND E. J. C. KENDALL. 1963. Infection with Eaton's primary atypical pneumonia agent in England. Brit. Med. J. **1**:1266–1270.
110. GOTTHARDSON, A., AND B. MELEN. 1953. Isolation of pleuropneumonia-like organisms from ovarian abscesses. Acta Pathol. Microbiol. Scand. **33**:291–293.
111. GRAYSTON, J. T., E. R. ALEXANDER, G. E. KENNY, AND E. R. CLARKE. 1965. *Mycoplasma pneumoniae* (Eaton agent) infections: clinical and epidemiological studies. J. Am. Med. Assoc. **191**:369–374.
111*a*. GRIST, N. R., AND R. J. FALLON. 1964. Isolation of viruses from leukemic patients. Brit. Med. J. **1**:1263.
112. GRUMBLES, L. C., C. F. HALL, AND G. CUMMINGS. 1964. Characteristics of avian mycoplasma (PPLO) in tissue cultures of human and avian cells. Avian Diseases **8**:274–280.
113. GRÜNHOLZ, G. 1950. Pleuropneumonieartige mikroorganismen bei "Viruspneumonien." Klin. Wochschr. **28**:480.
114. HARKNESS, A. H. 1950. Non-gonococcal urethritis. Livingstone Co. Edinburgh, Scotland.
115. HARKNESS, A. H., AND A. HENDERSON-BEGG. 1948. The significance of pleuropneumonia-like or "L" organisms in non-gonococcal urethritis, Reiter's disease and abacterial pyuria. Brit. J. Venereal Diseases **24**:50–58.
116. HAYFLICK, L. Unpublished results.
117. HAYFLICK, L. 1960. Decontaminating tissue cultures infected with pleuropneumonia-like organisms. Nature **185**:783–784.
118. HAYFLICK, L. 1965. The limited *in vitro* lifetime of human diploid cell strains. Exptl. Cell Res. **37**:614–636.
119. HAYFLICK, L. 1965. Cell cultures and mycoplasmas. Texas Rept. Biol. Med. **23** (suppl. 1):285–303.
119*a*. HAYFLICK, L., AND H. KOPROWSKI. 1965. Direct agar isolation of mycoplasmas from human leukemic bone marrow. Nature **205**: 713–714.
120. HAYFLICK, L., AND W. R. STINEBRING. 1955. Intracellular growth of pleuropneumonia-like organisms. Anat. Record **121**:447.
121. HAYFLICK, L., W. R. STINEBRING, F. C. BRECKENRIDGE, AND C. M. POMERAT. 1956. Some effects of human pleuropneu-

monia-like organisms on tissue cultures of human synovial cells. Bacteriol. Proc., p. 83.

122. HAYFLICK, L., AND W. R. STINEBRING. 1960. Intracellular growth of pleuropneumonia-like organisms (PPLO) in tissue culture and *in ovo*. Ann. N.Y. Acad. Sci. **79**:433–447.

123. HEARN, H. J., JR., J. E. OFFICER, V. ELSNER, AND A. BROWN. 1959. Detection, elimination, and prevention of contamination of cell cultures with pleuropneumonialike organisms. J. Bacteriol. **78**:575–582.

124. HERDERSCHEÊ, D., A. C. RUYS, AND G. R. VAN RHIJN. 1963. A new oral mycoplasma isolated from the tonsils of two patients suffering from scarlatina. Antonie van Leeuwenhoek J. Microbiol. Serol. **29**:157–162.

125. HERDERSCHEÊ, D., A. C. RUYS, AND G. R. VAN RHIJN. 1963. Pleuropneumonia-like organisms in tissue cultures. Antonie van Leeuwenhoek J. Microbiol. Serol. **29**:368–376.

126. HOROSZEWICZ, J. 1961. Incidence of pleuropneumonia-like organisms (PPLO) in man. Brit. J. Venereal Diseases **37**:183–186.

127. HOROSZEWICZ, J. S., AND J. T. GRACE, JR. 1964. PPLO detection in cell culture by thymidine cleavage. Bacteriol. Proc., p. 131.

128. HUIJSMANS-EVERS, AND A. C. RUYS. 1956. Microorganisms of the *pleuropneumonia* group (family of *mycoplasmataceae*) in man. Antonie van Leeuwenhoek J. Microbiol. Serol. **22**:371–376, 377–384.

129. HUMMELER, K., N. TOMASSINI, AND L. HAYFLICK. Ultrastructure of the Negroni Mycoplasma. J. Bacteriol., *in press*.

130. JENSEN, K. E. 1963. Measurement of growth-inhibiting antibody for *Mycoplasma pneumoniae*. J. Bacteriol. **86**:1349–1350.

130*a*. JENSEN, K. E. 1964. Antibodies to *Mycoplasma pneumoniae* measured by inhibition of tetrazolium reduction. Bacteriol. Proc., p. 70–71.

131. JANSSON, E. 1964. Preparation of complement-fixing antigen for routine use in diagnosis of Eaton pneumonia. J. Clin. Pathol. **17**:458–460.

132. JANSSON, E., O. WAGER, R. STENSTROM, E. KLEMOLA, AND P. FORSSELL. 1964. Studies on Eaton PPLO pneumonia. Brit. Med. J. **1**:142–145.

133. JORDAN, W. S. 1949. The infectiousness and incubation period of primary atypical pneumonia. Am. J. Hyg. **50**:315–330.

134. KANDLER, O. 1956. Vergleichende untersuchungen über den nukleinsäure und atmugsstoffwechsel von proteus vulgaris, dessen stabiler L-phase und den pleuropneumonie-ähnlichen organismen. Zentr. Bakteriol. Parasitenk. Abt. II **109**:335.

135. KANDLER, G., AND O. KANDLER. 1954. Untersuchungen über die morphologie der pleuropneumonia-ähnlichen organismen und der L-phase der bakterien. I. Lichtmikroskopische untersuchungen. Arch. Mikrobiol. **21**:178–201.

136. KANDLER, O., AND C. ZEHENDER. 1957. Über das vorkommen von a-E diaminopimelinsäure bei verschiedenen L-phasentypen von proteus vulgaris und bei den pleuropneumonie-ähnlichen organismen. Z. Naturforsch. **12b**:725–728.

137. KANDLER, O., C. ZEHENDER, AND J. MULLER. 1956. Vergleichende untersuchungen über den nucleinsäuren und atmugsstoffwechsel von proteus vulgaris, dessen stabil L-phase und den pleuropneumonie-ähnlichen organismen. Arch Mikrobiol. **24**:219–249.

138. KENNY, G. E. 1964. Eaton PPLO (*Mycoplasma pneumoniae*) antigen. Federation Proc. **23**:581.

139. KENNY, G. E., AND M. E. POLLOCK. 1963. Mammalian cell cultures contaminated with pleuropneumonia-like organisms. J. Infect. Diseases **112**:7–16.

140. KELLER, R., P. F. SMITH, AND H. E. MORTON. 1952. Susceptibility of pleuropneumonia-like organisms from human genital tract to the action of soaps. J. Gen. Microbiol. **7**:313–319.

141. KINGSTON, J. R., R. M. CHANOCK, M. A. MUFSON, L. P. HELLMAN, W. D. JAMES, H. H. FOX, M. A. MANKO, AND J. BOYERS. 1961. Eaton agent pneumonia. J. Am. Med. Assoc. **176**:118–123.

141*a*. KLIENEBERGER-NOBEL, E. 1963. Some current trends in the field of PPLO. Recent Progr. Microbiol. **8**:504–510.

142. KLIENEBERGER, E. 1935. The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with *Streptobacillus moniliformis* and other bacteria. J. Pathol. Bacteriol. **40**:93–105.

143. KLIENEBERGER, E. 1936. Further studies on streptobacillus moniliformis and its symbiont. J. Pathol. Bacteriol. **42**:587–598.

144. KLIENEBERGER-NOBEL, E. 1951. Filterable forms of bacteria. Bacteriol. Rev. **15**:77–103.

145. KLIENEBERGER-NOBEL, E. 1954. Microorganisms of the pleuropneumonia group. Biol. Rev. **29**:154–184.

146. KLIENEBERGER-NOBEL, E. 1960. Pathogenicity and immunology of organisms of the pleuropneumonia group. Ann. N.Y. Acad. Sci. **79**:615–625.

147. KLIENEBERGER-NOBEL, E. 1962. Pleuropneumonia-like organisms (PPLO): Mycoplasmataceae. Academic Press, Inc., New York.

148. KLIENEBERGER, E., AND J. SMILES. 1942. Some observations on the developmental cycle of the organism of bovine pleuropneumonia and related microbes. J. Hyg. **42**:110–123.

149. KRAEMER, P. M. 1964. Interaction of mycoplasma (PPLO) and murine lymphoma cell cultures: prevention of cell lysis by ar-

ginine. Proc. Soc. Exptl. Biol. Med. **115:** 206–212.

150. KRAEMER, P. M., V. DEFENDI, L. HAYFLICK, AND L. A. MANSON. 1963. Mycoplasma (PPLO) strains with lytic activity for murine lymphoma cells *in vitro*. Proc. Soc. Exptl. Biol. Med. **112**:381–387.
151. KRAYBILL, W. H., AND Y. E. CRAWFORD. 1964. Tetrazolium and methylene blue for isolation and recognition of *Mycoplasma pneumoniae*. Bacteriol. Proc., p. 70.
152. KUZELL, W. C., AND E. A. MANKLE. 1950. Cortisone acetate and terramycin in polyarthritis of rats. Proc. Soc. Exptl. Biol. Med. **74**:677–681.
153. LEDINGHAM, J. C. G. 1933. The growth phases of pleuropneumonia and agalactia on liquid and solid media. J. Pathol. Bacteriol. **37:** 393–410.
154. LEMCKE, R. 1965. A serological comparison of various species of Mycoplasma by an agar gel double-diffusion technique. J. Gen. Microbiol. **38**:91–100.
155. LEMCKE, R. M. 1964. The relationship of a type of *Mycoplasma* isolated from tissue cultures to a new human oral Mycoplasma. J. Hyg. **62**:351–352.
156. LEMCKE, R. M. 1964. The serological differentiation of mycoplasma strains (pleuropneumonia-like organisms) from various sources. J. Hyg. **62**:199–219.
157. LEMCKE, R., AND G. W. CSONKA. 1962. Antibodies against pleuropneumonia-like organisms in patients with salpingitis. Brit. J. Venereal Diseases **38**:212–217.
158. LIEBERMEISTER, K. 1953. Untersuchungen zur Morphologie der pleuropneumonia (PPLO) Gruppe. Z. Naturforsch **8b**:757–766.
159. LIEBERMEISTER, K. 1960. Morphology of the PPLO and L-forms of proteus. Ann. N.Y. Acad. Sci. **79**:326–343.
160. LIU, C. 1957. Studies on primary atypical pneumonia. I. Localization, isolation, and cultivation of a virus in chick embryos. J. Exptl. Med. **106**:455–467.
161. LIU, C. 1961. Studies on primary atypical pneumonia. III. A factor in normal serum which enhances the reaction between PAP virus and convalescent serum. J. Exptl. Med. **113**:111–123.
162. LIU, C., M. D. EATON, AND J. T. HEYL. 1959. Studies on primary atypical pneumonia. II. Observations concerning the development and immunological characteristics of antibody in patients. J. Exptl. Med. **109**:545–556.
162*a*. LOW, I. E., AND M. D. EATON. 1965. Replication of *Mycoplasma pneumoniae* in broth culture. J. Bacteriol. **89**:725–728.
163. LUDLAM, G. B., J. B. BRIDGES, AND E. C. BENN. 1964. Association of Stevens-Johnson syndrome with antibody for *Mycoplasma pneumoniae*. Lancet **1**:958–959.
164. LYNN, R. J. 1956. Nucleic acid metabolism of pleuropneumonia-like organisms. Ph.D. Thesis, University of Pennsylvania, Philadelphia.
165. LYNN, R. J., AND H. E. MORTON. 1956. The inhibitory action of agar on certain strains of pleuropneumonia-like organisms. Appl. Microbiol. **4**:339–341.
166. LYNN, R. J., AND P. F. SMITH. 1957. Nucleic acid content of pleuropneumonia-like organisms from human sources. J. Bacteriol. **74**:811–817.
167. LYNN, R. J., AND P. F. SMITH. 1960. Chemical composition of PPLO. Ann. N.Y. Acad. Sci. **79**:493–498.
168. MACPHERSON, L. A., AND K. ALLNER. 1960. L-forms of bacteria as contaminants in tissue culture. Nature **186**:992.
168*a*. MARMION, B. P., AND G. M. GOODBURN. 1961. Effect of an organic gold salt on Eaton's primary atypical pneumonia agent and other observations. Nature **189**:247–248.
169. MARMION, B. P., AND J. F. PH. HERS. 1963. Observations on Eaton primary atypical pneumonia agent and analogous problems in animals. Am. Rev. Respirat. Diseases **88**:198–211.
170. MARTZINOVSKI, E.-J. 1911. De l'etiologie de la péripneumonie. Ann. Inst. Pasteur **25:** 914–917.
171. MCCARTY, K. S., B. WOODSON, M. AMSTEY, AND O. BROWN. 1964. Arginine as a precursor of pyrimidines in strain L-929 fibroblasts infected with pleuropneumonia-like organisms. J. Biol. Chem. **239**:544–549.
172. MCCAULEY, J. E. 1963. Multiple authorship. Science **141**:579.
173. MEIKLEJOHN, G., W. G. THALMAN, D. J. WALIGORA, C. H. KEMPE, AND E. H. LENNETTE. 1954. Chemotherapy of primary atypical pneumonia. J. Am. Med. Assoc. **154**:553–557.
174. MÉLEN, B., AND A. GOTTHARDSON. 1955. Complement fixation with human pleuropneumonia-like organisms. Acta Pathol. Microbiol. Scand. **37**:196–200.
175. MÉLEN, B., AND B. LINNROS. 1952. Pleuropneumonia-like organisms in cases of nongonococcal urethritis in man. Acta Dermatol. Venereol. **32**:77–85.
176. MÉLEN, B., AND E. ODEBLAD. 1951. Pleuropneumonia-like microorganisms in the female genitourinary tract. Scand. J. Clin. Lab. Invest. **3**:47–51.
177. MOROWITZ, H. J., M. E. TOURTELLOTTE, W. R. GUILD, E. CASTO, AND C. WOESE. 1962. The chemical composition and submicroscopic morphology of Mycoplasma gallisepticum, avian PPLO 5969. J. Mol. Biol. **4**:93–103.
178. MORTON, H. E. 1958. Pleuropneumonia and pleuropneumonia-like organisms, p. 563–581. *In* R. T. Dubos [ed.], Bacterial and mycotic infections of man. J. B. Lippincott Co. Philadelphia.

179. MORTON, H. E., AND J. G. LECCE. 1953. Selective action of thallium acetate and crystal violet for pleuropneumonialike organisms of human origin. J. Bacteriol. **66**:646–649.
180. MORTON, H. E., J. G. LECCE, J. J. OSKAY, AND N. H. COY. 1954. Electron microscope studies of pleuropneumonialike organisms isolated from man and chickens. J. Bacteriol. **68**:697–703.
181. MORTON, H. E., P. F. SMITH, AND R. KELLER. 1952. Prevalence of pleuropneumonia-like organisms and the evaluation of media and methods for their isolation from clinical material. Am. J. Public Health **42**:913–925.
182. MORTON, H. E., P. F. SMITH, AND P. R. LEBERMAN. 1951. Investigation of the cultivation of pleuropneumonia-like organisms from man. Am. J. Syphilis Gonorrhea Venereal Diseases **35**:361–369.
183. MORTON, H. E., P. F. SMITH, N. B. WILLIAMS, AND C. F. EICKENBERG. 1951. Isolation of pleuropneumonia-like organisms from human saliva: a newly detected member of the oral flora. J. Dental Res. **30**:415–422.
184. MUFSON, M. A., AND R. M. CHANOCK. Unpublished results.
185. MUFSON, M. A., W. M. LUDWIG, R. H. PURCELL, T. R. CATE, D. TAYLOR-ROBINSON, AND R. M. CHANOCK. 1965. Exudative pharyngitis following experimental *Mycoplasma hominis* type 1 infection. J. Am. Med. Assoc., *in press*.
186. MURPHY, W. H., AND D. FURTADO. 1963. Isolation of viruses from children with acute leukemia. Univ. Mich. Med. Bull. **29**:201–228.
186*a*. MURPHY, W. H., D. FURTADO, AND E. PLATA. 1965. Possible association between leukemia in children and virus-like agents. J. Am. Med. Assoc. **191**:110–115.
187. NEGRONI, G. 1964. Isolation of viruses from leukaemic patients. Brit. Med. J. **1**:927–929.
187*a*. NEIMARK, H. C., AND J. J. PÈNE. 1965. Characterization of pleuropneumonia-like organisms by deoxyribonucleic acid composition. Proc. Soc. Exptl. Biol. Med. **118**: 517–519.
188. NELSON, J. B. 1960. Biology of the pleuropneumonia-like organisms. Discussion of Part II. Ann. N.Y. Acad. Sci. **79**:458–460.
189. NERMUT, M. V., J. UHER, AND A. GERYLOVOVA. 1957. Über das vorkommen und die bedeutung der sog. L-organismen (PPLO) in weiblicher geschlechtsorganen. Z. Gynäkol. **80**:669–675.
190. NEWNHAM, A. G., AND H. P. CHU. An *in vitro* comparison of the effect of some antibacterial, antifungal and antiprotozoal agents on various strains of Mycoplasma (pleuropneumonia-like organisms: PPLO) J. Hyg. **63**:1–23.
191. NICOL, C. S., AND D. G. FF. EDWARD. 1953. Role of organisms of the pleuropneumonia group in human genital infections. Brit. J. Venereal Diseases **29**:141–150.
192. NOCARD, E., E. R. ROUX, M. M. BORREL, SALIMBENI, AND DUJARDIN-BEAUMETZ. 1898. Le microbe de la péripneumonie. Ann. Inst. Pasteur **12**:240–262.
193. NOWAK, J. 1929. Morphologie, nature et cycle évolutif du microbe de la péripneumonie des bovidés. Ann. Inst. Pasteur **43**:1330–1352.
194. OATES, J. J., M. J. WHITTINGTON, AND A. E. WILKINSON. 1959. A note on the results of cultural and serological tests for pleuropneumonia-like organisms in Reiter's disease. Brit. J. Venereal Diseases **35**:184–186.
195. O'CONNELL, R. C., R. G. WITTLER, AND J. E. FABER. 1964. Aerosols as a source of widespread *Mycoplasma* contamination of tissue cultures. Appl. Microbiol. **12**:337–342.
196. OLARTE, J. 1960. The occurrence of organisms of the pleuropneumonia group in the external genitalia of female children. Ann. N.Y. Acad. Sci. **79**:632–634.
197. ØRSKOV, J. 1927. Etude sur la mórphologie du virus péripneumonique. Ann. Inst. Pasteur **41**:473–482.
198. ØRSKOV, J. 1942. On the morphology of peripneumonia-virus, agalactia-virus and Seiffert's microbes. Acta Pathol. Microbiol. Scand. **19**:586–590.
199. PAINE, T. F., R. MURRAY, I. PERLMUTTER, AND M. FINLAND. 1950. Brain abscess and meningitis associated with a pleuropneumonia-like organism: clinical and bacteriological observations. Ann. Internal Med. **32**:554–562.
200. PANOS, C., S. S. BARKULIS, AND J. A. HAYASHI. 1959. Streptococcal L forms. II. Chemical composition. J. Bacteriol. **78**:863–867.
201. PEOPLES, D. M., H. E. MORTON, AND L. G. FEO. 1957. Unusual pleuropneumonia-like organisms isolated in a study of *Trichomonas vaginalis* from cases of chronic urethritis. J. Bacteriol. **73**:398–401.
202. PETERSON, O. L., T. H. HAM, AND M. FINLAND. 1943. Cold agglutinins in primary atypical pneumonias. Science **97**:167–168.
203. PIRIE, N. W. 1964. The size of small organisms. Proc. Royal Soc. (London) Ser. B. **160**:149–166.
204. PLACKETT, P. 1959. On the probable absence of "mucocomplex" from Mycoplasma mycoides. Biochim. Biophys. Acta **35**:260–262.
205. PLACKETT, P., S. H. BUTTERY, AND G. S. COTTEW. 1963. Carbohydrates of some Mycoplasma strains, p. 535–547. *In* N. E. Gibbons [ed.], Recent progress in microbiology. Univ. Toronto Press, Toronto, Canada.

206. POLLOCK, M. E., AND G. E. KENNY. 1963. Mammalian cell cultures contaminated with PPLO. Proc. Soc. Exptl. Biol. Med. **112**:176–181.
207. POLLOCK, M. E., G. E. KENNY, AND J. T. SYVERTON. 1960. Isolation and elimination of pleuropneumonia-like organisms from mammalian cell cultures. Proc. Soc. Exptl. Biol. Med. **105**:10–15.
208. POLLOCK, M. E., P. E. TREADWELL, AND G. E. KENNY. 1963. Mammalian cell cultures contaminated with pleuropneumonia-like organisms. Exptl. Cell. Res. **31**:321–328.
209. PORTER, J. W., AND G. ANDERSON. 1962. The biosynthesis of carotenes. Arch. Biochem Biophys. **97**:520–528.
210. PRESTON, W. S. 1942. Arthritis in rats caused by pleuropneumonia-like micro-organisms and the relationship of similar organisms to human rheumatism. J. Infect. Diseases **70**:180–184.
211. RANDALL, J. H., R. J. STEIN, AND J. C. AYRES. 1950. Pleuropneumonia-like organisms of the female genital tract. Am. J. Obstet. Gynecol. **59**:404–413.
212. RAZIN, S. 1963. Structure, composition and properties of the PPLO cell envelope, p. 526–534. *In* N. E. Gibbons [ed.], Recent progress in microbiology. Univ. Toronto Press, Toronto, Canada.
213. RAZIN, S., AND A. COHEN. 1963. Nutritional requirements and metabolism of *Mycoplasma laidlawii*. J. Gen. Microbiol. **30**:141–154.
214. RAZIN, S., AND B. C. J. G. KNIGHT. 1960. A partially defined medium for the growth of Mycoplasma. J. Gen. Microbiol. **22**:492–503.
215. RAZIN, S., J. MICHMANN, AND Z. SHIMSHONI. 1964. The occurrence of Mycoplasma (pleuropneumonia-like organisms, PPLO) in the oral cavity of dentulous and edentulous subjects. J. Dental Res. **43**:402–405.
216. RIFKIND, D., R. CHANOCK, H. KRAVETZ, K. JOHNSON, AND V. KNIGHT. 1962. Ear involvement (Myringitis) and primary atypical pneumonia following inoculation of volunteers with Eaton agent. Am. Rev. Respirat. Diseases **85**:479–489.
217. ROBINSON, L. B., R. H. WICHELHAUSEN, AND B. ROIZMAN. 1956. Contamination of human cell cultures by PPLO. Science **124**:1147.
218. RÖCKL, H., AND T. NASEMANN. 1960. The influence of bacteria, pleuropneumonia-like organisms, cysticetes, and trichomonas in the genital tract on non-gonococcal urethritis, p. 266–274. *In* Z. Gallar and L. Sylvestre [eds.], First Canadian symposium on non-gonococcal urethritis and human trichomoniasis. S. Karger, New York.
219. RODWELL, A. W., AND A. ABBOT. 1961. The function of glycerol, cholesterol and long-chain fatty acids in the nutrition of *Mycoplasma mycoides*. J. Gen. Microbiol. **25**:201–214.
220. ROTHBLAT, G. H. 1960. PPLO contamination in tissue cultures. Ann. N.Y. Acad. Sci. **79**:430–432.
221. ROTHBLAT, G. H., AND H. E. MORTON. 1959. Detection and possible source of contamination of pleuropneumonia-like organisms (PPLO) in cultures of tissue cells. Proc. Soc. Exptl. Biol. Med. **100**:87–90.
222. ROTHBLAT, G. H., AND P. F. SMITH. 1961. Nonsaponifiable lipids of representative pleuropneumonia-like organisms. J. Bacteriol. **82**:479–491.
223. ROUSE, H. C., V. H. BONIFAS, AND R. W. SCHLESINGER. 1963. Dependence of adenovirus replication on arginine and inhibition of plaque formation by pleuropneumonia-like organisms. Virology **20**:357–365.
224. ROVOZZO, G. C., R. E. LUGINBUHL, AND C. F. HELMBOLDT. 1963. A mycoplasma from a bovine causing cytopathogenic effects in calf kidney tissue culture. Cornell Vet. **53**:560–566.
225. RUITER, M., AND H. M. M. WENTHOLT. 1955. Isolation of a pleuropneumonia-like organism from a skin lesion associated with a fusospirochaetal flora. J. Invest. Dermatol. **24**:31–34.
226. SABIN, A. B. Personal communication.
227. SABIN, A. B. 1941. The filterable microörganisms of the pleuropneumonia group. Bacteriol. Rev. **5**:1–67, 331–335.
228. SALAMAN, M. H., AND COLLABORATORS. 1946. The isolation of organisms of the pleuropneumonia group from the genital tract of men and women. J. Pathol. Bacteriol. **58**:31–35.
229. SCHEREFEL, A. 1908. Asterococcus n. g. superbut (Cienk.) Scherffel und dessen angebliche beziehungen zu eremosphaera. Ber. Deut. Botan. Ges. **26A**:762.
230. SCHIMKE, R. T., AND M. F. BARILE. 1963. Arginine breakdown in mammalian cell cultures contaminated with pleuropneumonia-like organisms (PPLO). Exptl. Cell Res. **30**:593–596.
231. SCHIMKE, R. T., AND M. F. BARILE. 1963. Arginine metabolism in pleuropneumonia-like organisms isolated from mammalian cell culture. J. Bacteriol. **86**:195–206.
232. SENTERFEIT, L., AND K. E. JENSEN. Personal communication.
233. SHARP, J. T. 1964. The Mycoplasmataceae (PPLO) as causes of joint infections. Arthritis Rheumat. **7**:437–442.
234. SHEPARD, M. C. 1954. The recovery of pleuropneumonia-like organisms from negro men with and without nongonococcal urethritis. Am. J. Syphilis **38**:113–124.
235. SHEPARD, M. C. 1957. Visualization and morphology of pleuropneumonia-like organisms in clinical material. J. Bacteriol. **73**:162–171.

236. SHEPARD, M. C. 1960. Non-gonococcal urethritis in the Camp Lejeune area, p. 126–131. *In* Z. Gallar and L. Sylvestre [eds.], First Canadian symposium on non-gonococcal urethritis and human trichomoniasis. S. Karger, New York.
237. SHKLAIR, I. L., M. A. MAZZARELLA, R. R. GUTEKUNST, AND E. M. KIGGINS. 1962. Isolation and incidence of pleuropneumonia-like organisms from the human oral cavity. J. Bacteriol. **83**:785–788.
238. SMITH, P. F. 1955. Synthetic media for pleuropneumonia-like organisms. Proc. Soc. Exptl. Biol. Med. **88**:628–631.
239. SMITH, P. F. 1960. Nutritional requirements of PPLO and their relation to metabolic function. Ann. N.Y. Acad. Sci. **79**:508–520.
240. SMITH, P. F. 1960. Amino acid metabolism of PPLO. Ann. N.Y. Acad. Sci. **79**:543–550.
241. SMITH, P. F. 1962. Fate of ergosterol and cholestanol in pleuropneumonia-like organisms. J. Bacteriol. **84**:534–538.
242. SMITH, P. F. 1963. The role of sterols in the growth and physiology of pleuropneumonia-like organisms, p. 518–525. *In* N. E. Gibbons [ed.], Recent progress in microbiology. Univ. Toronto Press, Toronto, Canada.
243. SMITH, P. F. 1963. The carotenoid pigments of Mycoplasma. J. Gen. Microbiol. **32**:307–319.
244. SMITH, P. F. 1964. Physiology of pleuropneumonia-like organisms and L-type organisms. Bacteriol. Rev. **28**:97–125.
245. SMITH, P. F. 1964. Relation of sterol structure to function in pleuropneumonia-like organisms. J. Lipid Res. **5**:121–125.
246. SMITH, P. F., J. G. LECCE, AND R. J. LYNN. 1954. A lipoprotein as a growth factor for certain pleuropneumonia-like organisms. J. Bacteriol. **68**:627–633.
247. SMITH, P. F., AND R. J. LYNN. 1958. Lipid requirements for the growth of pleuropneumonia-like organisms. J. Bacteriol. **76**:264–269.
248. SMITH, P. F., AND H. E. MORTON. 1951. Isolation of pleuropneumonia-like organisms from the throats of humans. Science **113**:623–624.
249. SMITH, P. F., AND H. E. MORTON. 1951. The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain pleuropneumonia-like organisms. J. Bacteriol. **61**:395–405.
250. SMITH, P. F., AND H. E. MORTON. 1952. Further characterization of the protein factor required by certain pleuropneumonia-like organisms for growth *in vitro*. Arch. Biochem. Biophys. **38**:23–28.
251. SMITH, P. F., H. E. MORTON, AND P. R. LEBERMAN. 1950. Susceptibilities of pleuropneumonia-like organisms to some selective bacteriostatic agents. Proc. Soc. Exptl. Biol. Med. **74**:552–555.
252. SMITH, P. F., AND G. H. ROTHBLAT. 1960. Incorporation of cholesterol by pleuropneumonia-like organisms. J. Bacteriol. **80**:842–850.
253. SMITH, P. F., AND G. H. ROTHBLAT. 1960. Relation of PPLO to bacteria. Ann. N.Y. Acad. Sci. **79**:461–464.
254. SMITH, P. F., AND G. H. ROTHBLAT. 1962. Comparison of lipid composition of pleuropneumonia-like and L-type organisms. J. Bacteriol. **83**:500–506.
255. SMITH, P. F., AND S. SASAKI. 1958. Stability of pleuropneumonia-like organisms to some physical factors. Appl. Microbiol. **6**:184–189.
256. SOBESLAVSKY, O., L. SYRUCEK, M. BRUCKOVA, AND M. ABRAHAMOVIC. 1965. The etiological role of *Mycoplasma pneumoniae* in otitis media in children. Pediatrics **35**:652–657.
257. SOMERSON, N. L., AND R. M. CHANOCK. Unpublished results.
258. SOMERSON, N., R. H. PURCELL, D. TAYLOR. ROBINSON, AND R. M. CHANOCK. 1965. Hemolysin of *Mycoplasma pneumoniae*. J. Bacteriol. **89**:813–818.
259. SOMERSON, N. L., D. TAYLOR-ROBINSON, AND R. M. CHANOCK. 1963. Hemolysin production as an aid in the identification and quantitation of Eaton agent (*Mycoplasma pneumoniae*) Am. J. Hyg. **77**:122–128.
260. STOKES, E. J. 1955. Human infection with pleuropneumonia-like organisms. Lancet **1**:276–279.
261. STOKES, E. J. 1959. P.P.L.O. in genital infections. Brit. Med. J. **1**:510.
262. TANG, F. F., H. WEI, D. L. MCWHIRTER, AND J. EDGAR. 1935. An investigation of the causal agent of bovine pleuropneumonia. J. Pathol. Bacteriol. **40**:391–406.
263. TAYLOR-ROBINSON, D., J. CANCHOLA, H. FOX, AND R. M. CHANOCK. 1964. A newly identified oral mycoplasma (M. orale) and its relationship to other human mycoplasmas. Am. J. Hyg. **80**:135–148.
264. TAYLOR-ROBINSON, D., AND R. M. CHANOCK. Unpublished results.
265. TAYLOR-ROBINSON, D., H. H. FOX, AND R. M. CHANOCK. 1965. Characterization of a newly identified mycoplasma from the human oropharynx. Am. J. Hyg. **81**:180–191.
266. TAYLOR-ROBINSON, D., N. L. SOMERSON, H. C. TURNER, AND R. M. CHANOCK. 1963. Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. J. Bacteriol. **85**:1261–1273.
267. THOMAS, L., E. C. CURNEN, G. S. MIRICK, J. E. ZIEGLER, AND F. L. HORSFALL. 1943. Complement fixation with dissimilar antigens in primary atypical pneumonia. Proc. Soc. Exptl. Biol. Med. **52**:121–125.

268. THOMAS, L., G. S. MIRICK, E. C. CURNEN, J. E. ZIEGLER, AND F. L. HORSFALL. 1943. Serological reactions with an indifferent streptococcus in primary atypical pneumonia. Science **98**:566–568.

269. TULASNE, R. 1951. Les formes L bactéries. Rev. Immunol. **15**:223–251.

270. TULASNE, R. 1953. Le cycle L et les formes naines des bactéries. Symp. Publ. Inst. Superiore Sanita, Rome, Italy.

271. VAN DEMARK, P. J., AND P. F. SMITH. 1964. Respiratory pathways in the *Mycoplasma*. II. Pathway of electron transport during oxidation of reduced nicotinamide adenine dinucleotide by *Mycoplasma hominis*. J. Bacteriol. **88**:122–129.

272. VAN DER VEEN, J., AND M. C. J. VAN NUNEN. 1963. Role of mycoplasma pneumoniae in acute respiratory disease in a military population. Am. J. Hyg. **78**:293–301.

272*a*. VAN ITERSON, W., AND A. C. RUYS. 1960. The fine structure of *Mycoplasmataceae*. J. Ultrastruct. Res. **3**:282–301.

273. VISHNIAC, S., AND S. W. WATSON. 1953. The steroid requirements of *Labyrinthula vitellina* var. *pacifica*. J. Gen. Microbiol. **8**:248–255.

274. WAGNER, B. M., W. H. MORSE, AND D. M. KUHNS. 1953. Recent studies on nonspecific urethritis. Am. J. Public Health **43**:853–859.

275. WEIBULL, C. 1958. Chemical analyses elucidating the structure of bacterial L forms. Acta Pathol. Microbiol. Scand. **42**:324–332.

276. WEIBULL, C., AND H. BECKMAN. 1960. Growth of bacterial L forms and bacterial protoplasts. J. Bacteriol. **79**:638–649.

277. WEIBULL, C., AND H. BECKMAN. 1961. Chemical and metabolic properties of various elements found in cultures of a stable proteus L form. J. Gen. Microbiol. **24**:379–391.

278. WHITE, F. H., G. I. WALLACE, AND J. O. ALBERTS. 1954. Serological and electron microscope studies of chronic respiratory disease agent of chickens and of turkey sinusitis agent. Poultry Sci. **33**:500–507.

279. WILKINSON, A. E., AND M. J. WHITTINGTON cited by A. J. KING. 1959. Non-gonococcal urethritis and trichomoniasis in the male, p. 1–19. *In* Z. Gallar and L. Sylvestre [eds.], First Canadian symposium on non-gonococcal urethritis and human trichomoniasis. S. Karger, New York.

280. WROBLEWSKI, W. 1931. Morphologie et cycle évolutif des microbes de la péripneumonie des bovides et de l'agalaxie contagieuse des chèvres et des moutons. Ann. Inst. Pasteur **47**:94–114.