THE FILTRABLE MICROÖRGANISMS OF THE PLEUROPNEUMONIA GROUP

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CONTENTS

In 1898, when our knowledge of filtrable viruses was but a few years old, the then ultramicroscopic etiological agent of bovine pleuropneumonia was cultivated in a cell-free medium by Nocard, *et al.* (63). The morphologic studies of Bordet (3) and of Borrel, *et al.* (4) in 1910, and of others later on established the remarkable polymorphic character of the microörganism; and the filtration experiments through graded collodion membranes carried out by Elford (32) in 1929 established that the cultures contained "particles," 125 to 150 mμ in size, which were capable of reproducing not only themselves but also the larger and more complex structures. For 25 years after its cultivation in serum broth it was unique among infectious agents, but was practically always grouped with the filtrable viruses. In 1923, Bridré and Dona-

tien (5) demonstrated not only that the filtrable etiological agent of agalactia of sheep and goats could be cultivated *in vitro* in the same manner as the microörganism, or virus as it was still called, of bovine pleuropneumonia but also that, excepting the pathogenic and immunologic properties, there was the closest morphological and biological resemblance between these two agents (6). Although in the ensuing period many communications appeared dealing with the morphology and mode of reproduction of the bovine pleuropneumonia and agalactia microörganisms (43, 53, 65, 66, 103), another 11 years passed before a new member of this group was discovered. In 1934, Shoetensack (86) reported the cultivation in cell-free media of morphologically and biologically similar microörganisms from dogs suffering of distemper, and although insufficient work was done on the relation of these microörganisms to the virus of canine distemper, it was nevertheless established that members of the pleuropneumonia group occurred in dogs.

In 1935, Klieneberger (44) reported the remarkable observation that a pleuropneumonia-like microörganism could be demonstrated in all available strains of *Streptobacillus moniliformis*, a gram-negative pleomorphic bacillus which is a normal inhabitant of the nasopharynx of rats and the cause of at least one type of rat-bite fever in man. Although she has been able to isolate this microörganism (L_1) in pure culture and maintain it in continuous subculture without reversion to *Streptobacillus moniliformis*, other investigators (21, 24) have challenged her hypothesis that such symbiosis existed and suggested that the L_1 microörganism is a variant of the bacillus. These studies, however, have led to the isolation of other pleuropneumonia-like, pathogenic microörganisms from rats, unassociated with bacteria and distinct from L_1 (47, 48).

The next important contribution in this field came in 1936, when Laidlaw and Elford (52) reported on a new group of filtrable microörganisms which they isolated from raw sewage. These resembled the other members of the pleuropneumonia group in that minute particles, 125 to 175 mμ in size, reproduced the same

type of polymorphic structures, and gave rise to similar microscopic colonies on solid media, but differed in not requiring protein for their growth; also they possessed no pathogenic properties. Seiffert (84) confirmed these observations when he reported the isolation of similar microörganisms from filtrates of soil, compost, decomposing leaves, and manure.

Late in 1938, Sabin (75) and Findlay, *et al.* (35) simultaneously described the isolation of a new pleuropneumonia-like microörganism from mice which developed a peculiar nervous disease in the course of routine passage of toxoplasma or lymphocytic choriomeningitis virus. The studies on this group received a new orientation when it was demonstrated (74, 75, 76) that this filtrable microörganism of the mouse, although capable of multiplying in a cell-free medium *in vitro*, was an intracellular parasite *in vivo*, with a special affinity for the mesenchymal cells of the pleura, peritoneum, and joints, and that during the course of its multiplication a typical neurotropic exotoxin was produced which gave rise to choreiform nervous signs. Furthermore, early in 1939 Sabin (76) isolated from normal mice another such microorganism, immunologically distinct from the first, and with such limited cellular affinities that it could multiply only in the mesenchymal cells of the joints in which it produced a proliferative, progressive, and chronic ankylosing arthritis. Subsequent studies (77, 80) revealed that normal mice are carriers of these pathogenic microörganisms, especially in their conjunctiva and nasal mucosa, and at least five immunologically distinct types have already been described (80). Although attempts to demonstrate members of the pleuropneumonia group in pathological material from patients with rheumatic fever and rheumatoid arthritis have met with no success, studies are being continued on human beings (80); and Dienes (28) has already brought forth more than suggestive evidence of their existence in the female genital tract.

At the present time our knowledge has progressed far enough to indicate that there exists in nature a distinct group of filtrable, saprophytic and parasitic microörganisms, of which the etiological

agent of bovine pleuropneumonia is the prototype, and which possess properties that clearly distinguish them from the ordinary bacteria, the filtrable viruses, and the rickettsiae.

GENERAL CHARACTERISTICS OF THE GROUP AND METHODS OF STUDY

Criteria for Identification. The criteria which admit a microorganism into the pleuropneumonia group are: (1) growth in cell-free culture media with the development of polymorphic structures including, "rings," globules, filaments, and minute, filtrable elementary bodies, usually 125 to 250 mμ in size, which are the minimal reproductive units; and (2) the development on suitable solid media of characteristic minute colonies which may be as small as 10 to 20μ and as a rule not larger than 600μ. These characteristics are shared by the saprophytic as well as the parasitic members of the group, but the latter are further distinguished by their inability to grow in cultures that do not contain a high concentration of serum protein.

Cultivation from Infected Tissues and Exudates. When smears, made with animal tissues or exudates in which a pathogenic member of the group has multiplied, are stained with the ordinary aniline dyes or by Gram's method one can find no formed elements suggesting the presence of a microörganism. Furthermore, when such tissues or exudates are cultured on ordinary solid or fluid media or on media containing less than 5 to 10 per cent of blood or serum, there is usually no growth. It is for this reason that a number of filtrable infectious agents were believed to be viruses until cultivation on suitable media revealed that they were members of the pleuropneumonia group. However, even when suitable media are employed the primary growth, because it can be so unlike that which occurs with the familiar bacteria, may fail to be recognized by the uninitiated. The media usually considered suitable consist of heart-muscle infusion peptone broth or agar (2 per cent) having a pH of 7.6 to 8.0, to which is added 10, 20, 30, or even 40 per cent of various animal serums (horse, bovine, rabbit) or human ascitic fluid. Boiled blood has been incorporated in the basic medium by some (24, 45, 86) and glucose by others (75) because primary isolation of certain strains is thus

facilitated. The use of dried meat extracts instead of fresh meat infusion was enough on one occasion in my experience to make the difference between growth and no growth.

When a suitable fluid medium is inoculated with infected tissue or exudate which in itself gives rise to appreciable turbidity, the best procedure to follow is subculture of 0.1 to 0.2 ml. into fresh medium on the 4th day and again on the 7th day. If the initial culture is not clouded by the inoculum the first sign of growth may be the development of very slight diffuse turbidity, which can be appreciated only by comparison with an uninoculated tube of medium, or by the appearance of a slight granular sediment. An uninoculated tube of the culture medium is, therefore, always incubated along with the inoculated ones. In primary cultures the first turbidity or other evidence of growth may not appear for 3 to 14 days. Subculture should be carried out as soon as growth is suspected, or on the 4th and 7th days if the medium remains clear even if a Giemsa-stained film reveals no formed elements of any kind. I have referred to the latter type of subculture as "blind passage" (75, 76), because good growth developed on a number of occasions in such subcultures even when the primary culture itself remained negative over a period of weeks. After several serial passages have been carried out growth may become apparent as early as 24 or 48 hours. That the transmissible turbidity which appears in fluid cultures is due to the growth of a member of the pleuropneumonia group can be proved in the following ways: (a) gram-stained smears reveal no ordinary bacteria and indeed may show no distinct forms of any kind; (in smears of centrifuged sediments suspended in saline, the microörganisms, though faintly stained are gram-negative); (b) Giemsa-stained smears reveal characteristic polymorphic structures; (c) demonstration of *large numbers* of similar polymorphic structures in the dark-field; and most important of all, (d) development of characteristic microscopic colonies when some of the fluid culture is seeded on solid medium containing the same kind of protein. In doubtful cases, the dark-field examination may be most misleading since structures which morphologically are remarkably similar to these microörganisms may be

found in uninoculated tubes especially those incubated at 37° for a long time. These "pseudo" structures, however, fail to show up in Giemsa-stained films and no colonies appear on solid media.

The use of 30 per cent serum or ascitic fluid agar for primary isolation of these microörganisms from infected tissues or exudate often gives decisive results more quickly, and when the material to be cultured is contaminated with ordinary bacteria, it is the method of choice. The tissue is minced to expose a greater surface which is brought into contact with the soft agar in many different places; if the material to be cultured is fluid, 0.1 ml. of it is poured on and allowed to spread over the agar. The optimum colonial development occurs when evaporation of the medium is prevented, which is accomplished by inserting a piece of filter paper in the cover and sealing the Petri dish with parafilm (a procedure suggested to me by Dr. Homer Swift). Macroscopic examination of such an agar plate may reveal nothing but the dried inoculum to the uninitiated and oftentimes to the experienced as well. With the aid of a hand lens, however, and sometimes with the naked eye it is possible to discern the minute colonies which may require as little as 2 days' or as much as 7 days' incubation to become apparent. These colonies are best examined under the microscope, with the substage condenser removed, using the 10× ocular and 16 mm. objective with oblique illumination obtained from a blue light by the concave mirror. Where the growth is not confluent, the isolated colonies appear distinctly outlined and slightly elevated, with a nipple-like darker center or surface vacuolar meshwork, and 10 to 600μ in size depending on the species and conditions of growth. When such colonies are present or suspected, the piece of agar on which they occur is cut out and streaked on another agar plate which is incubated as before. Numerous minute colonies usually appear in a few days along the streaks if microörganisms of the pleuropneumonia group are present. The "pseudo colonies" occurring on certain kinds of serum agar described by Brown, Swift, and Watson (8) are sufficiently different from those of the pleuropneumonia group not to cause confusion when their possible occurrence is appreciated. Once colonial growth is established on

solid medium, and a Gram stain of a film of such colonies reveals no ordinary bacteria, it is advisable to establish growth in fluid media by dropping a piece of agar with many such colonies into a tube of fluid medium containing the same kind of serum or ascitic fluid, whichever may have been present in the solid medium. It may take a few days before growth appears and when it does it is usually in the form of granules or flakes close to the piece of agar, and only rarely as a diffuse turbidity. After a number of rapid subcultures (sometimes as many as 6 to 10 are required) a culture often changes from granular suspension to diffuse turbidity. When all colonies do not appear the same, it is advisable to subculture single colonies on solid media. In this manner Sabin and Johnson (80) were able to demonstrate three distinct immunological types in a culture from the nasal mucosa of a single mouse.

When one has thus obtained characteristic growth on fluid and solid media, it is desirable to demonstrate filtrability preferably through suitable gradocol membranes before finally classifying a microörganism as a member of the pleuropneumonia group. Filtration through Berkefeld filters, impervious to *Serratia marcescens*, is significant only when it is shown by plating or dilution that a relatively large number of reproductive units, although not necessarily a large proportion of the total, have passed through.

General Remarks about Conditions of Growth. The saprophytic members of the pleuropneumonia group differ from the parasitic ones in that they do not require protein for growth and can multiply at 22°. Although Nocard, *et al.* (63) first cultivated the microörganism of bovine pleuropneumonia in a medium containing 4 to 5 per cent of serum, practically all the other members of this group require 10 per cent or more of serum for primary isolation. After adaptation to growth *in vitro*, multiplication also occurs with smaller concentrations of serum protein. Experiments with the microörganism of agalactia (6) revealed that when the concentration of serum is increased to 80 or 90 per cent growth is retarded, and in pure serum it is apparently completely inhibited; no growth whatever occurred in serum diluted with physi-

ologic salt solution. In the case of bovine pleuropneumonia growth was reported to be entirely arrested when 50 per cent of the medium consisted of horse serum (94). Although the function of the protein is still unknown I have observed in working with the microörganisms isolated from mice that some strains and types can become so thoroughly adapted to the protein of one species that they fail to grow or will grow very poorly when transferred to a culture medium containing the serum of another species.

Growth occurs both aerobically and anaerobically, but, with the exception of the microörganisms isolated from dogs, it is less abundant under anaerobic conditions. Whenever the influence of pH has been studied it was found that pH 7.8 to 8.0 is optimum for growth. The addition of various sugars improves growth in some instances and not in others. In the case of agalactia, the addition of glucose, levulose, galactose, raffinose, arabinose, xylose, sucrose and maltose in concentrations of 1 to 2 per cent is reported (6) as exerting a retarding effect while lactose and mannitol, for example, have a stimulating effect. Fermentation often occurs with the production of acid, and when a pH of 7.0 or less is reached growth usually ceases. With the mouse microorganisms I found that in the presence of 0.5 per cent glucose, subculture is no longer possible on the 3rd day of fully adapted cultures, while without the added sugar positive subcultures can be obtained even at the end of a month at 37°. This amount of glucose, however, did not have the same effect on at least one rat strain (L_4) and not quite as rapid an effect on the microörganism of bovine pleuropneumonia.

Reduction of hemoglobin has been observed in cultures of the pleuropneumonia microörganisms, and in tests carried out with Doctor Joel Warren both hemolysis and reduction of hemoglobin were observed on solid media with the pleuropneumonia, L_3, and Types A, B, and C mouse microörganisms but not with L_1 and L_4. I have also observed that with some strains the yellow pigment of the added serum disappears with the first signs of growth. Certain metabolic studies on the microörganisms of the group have been reported (41, 71, 72, 98).

Preservation of Cultures. Bridré and Donatien (6) reported that the microörganism of agalactia under aerobic conditions at 37° lost its reproductive capacity at the end of a month (presence or absence of added sugar not indicated). Under anaerobic conditions, however, or when the fluid culture was covered with vaseline after aerobic cultivation, it was still possible to obtain positive subcultures after a sojourn of 22 months at 37°; cultures that were similarly sealed and stored at 0°, 6° to 12°, and 25° did not survive as long, failing to yield growth even after 5 months. In my own work when it was desirable to carry cultures with sugar added to the medium, they would be stored in the refrigerator and subcultures made at 10- to 14-day intervals, although with some strains it was still possible to obtain growth after 5 to 6 weeks. Cultures on solid media kept in plates or tubes sealed with parafilm may be subcultured at monthly intervals. In order to preserve various strains or types for future studies and before their pathogenic or other properties have been changed by too many subcultures, Swift's method for preserving bacteria has been applied (92). Twenty-five ml. or more of full-grown culture is spun at 4000–5000 r.p.m. for one hour in an angle centrifuge. Approximately 10 per cent of the supernatant liquid is left behind and used for resuspending the sediment. This concentrated suspension is distributed in 0.1 ml. amounts in small cotton-plugged tubes, which are then rapidly frozen with solid CO_2, and put into a chilled desiccator containing a large dish of P_2O_5. The desiccator is placed in an insulated container over a tin box containing solid CO_2 so that it remains at a temperature of approximately $-10°$ to $-20°$ during the entire period of evacuation and drying which usually does not require more than 24 hours. Suitably dried specimens appear as a white or slightly yellowish bit of foam. The tubes are sealed with picein and stored at room temperature to prevent the seal from cracking. To reconstitute, a small amount of medium is added to the dried material and transferred to a tube or flask of fresh medium. Cultures dried in this manner have yielded positive subcultures after more than a year of storage. Drying of cultures from the frozen state on the Mudd-Flosdorf

apparatus has for some reason been unsuccessful on a number of occasions; on the other hand, I have had no difficulty in preserving the same microörganisms in infected tissue dried with this apparatus.

Morphology and Mode of Reproduction. The existing descriptions of the morphology and mode of reproduction (3, 4, 25, 43, 53, 65, 66, 94, 96, 103) have varied a good deal depending on (a) whether the investigator followed the growth on solid or liquid media, (b) the type or age of culture used, and (c) the method of examination, i.e. stained films, agar fixation, or dark-field. It is remarkable how different is the impression gained of the morphology of one of these microörganisms from an examination of preparations of colonies on solid media, and of stained films or dark-field preparations of growth on liquid media. Quite aside from the recognized fact that the growth units may be so plastic as to undergo considerable distortion in films and smears, the usual preparation from a colony on solid medium presents such large discs, globules, and even amorphous masses with chromatic bodies, that the general reaction of utter confusion is not limited to the uninitiated. Near the edge of such a preparation one can find structures which are similar to those seen in liquid media. While not denying that studies of growth on solid media are essential for the ultimate concept of the true mode or modes of reproduction, I personally find it much less confusing and definitely more decisive for comparative purposes to study the growth on fluid media either stained or with the dark field. By these methods one can find morphological differences even among different types found in the same species of animal. For example, the Type A microörganism of the mouse exhibits the elementary-body-like granules, small bacilliform or spirillar forms during the early phases of growth, the rings, triangles, quadrangles, etc. with the denser bodies distributed irregularly through these structures; but at no time have streaming filaments attached to these structures been found such as are regularly seen in the Type B microörganism and in certain other members of the group.

While various investigators have described many distinct types

of reproduction, the following synthesis by Ledingham (53) for the microörganisms of pleuropneumonia bovis and agalactia corresponds most to my own observations on many other members of this group with certain allowances for individual differences:

"Commencing with the filterable viable element we note its spore-like capacity to pullulate to filamentous and ramifying elements. The protoplasmic substance of these filaments, whether at their extremities or in their course, retains the power to elaborate the more deeply stained chromatic and consolidated nodes from which further moniliform growth proceeds. The term 'moniliform' I use only for convenience to express the beaded character of the growing filament. Unlike those of a moniliform streptobacillus the "beads" exhibit the greatest variety in size and shape, particularly during the early stages in their development. In early colonial growths, as I have described and figured, these chromatic condensations may assume considerable dimensions, bizarre shapes and a quite characteristic differential reaction of their outer and inner parts to the Giemsa stain, and their further pullulation by a unipolar or multipolar pseudopodial budding process furnishes the great mass of polymorphic units, rings, spheres, filaments, etc. present in cultures at the period of maximal growth."

Growth on Chorioallantoic Membrane and in Tissue Culture. Tang, *et al.* (95) inoculated 9- to 11-day-old eggs with a culture of pleuropneumonia bovis. "Oedema and sometimes white spots" were found throughout the chorioallantoic membrane. Although the embryos were usually dead 3 or 4 days after inoculation, positive cultures were obtained only from the chorioallantoic membrane and from the surface of the embryo but not from the internal organs, yolk or amniotic fluid. While they saw all the usual forms of the microörganism on dark-field examination, they found no cytoplasmic or intranuclear inclusions in Giemsa-stained scrapings from affected areas of the chorioallantoic membrane. Sullivan and Dienes (91) working with microörganisms isolated from mice (some of which I identified as Type A) reported that they were unsuccessful in obtaining growth on the chorioallantoic membranes of chick embryos in the usual manner, but by chilling the embryo to death at 4° prior to incubation they were able to obtain growth and serial passage. Swift (personal

communication) observed that the Type A microörganism of mice is the only one that grows not at all or very feebly on living chorioallantoic membranes of chick embryos, while the Type B microörganism, certain rat strains, and pleuropneumonia grow well; in all instances, however, growth was better with embryos that were killed by chilling. I was able to obtain growth and serial passage of the Type B microörganism in a medium consisting of 0.1 ml. of minced mouse embryo tissue and 4.5 ml. Tyrode's solution with no added serum. The multiplication in this medium appeared to be predominantly intracellular. Sullivan and Dienes (91) using mouse microörganisms demonstrated growth on a minced-chick-embryo-tyrode solution medium as well as on embryo-tyrode-agar (Zinsser, *et al.* 107).

Thermal Death Point. While some members of the pleuropneumonia group are killed by relatively low temperatures, the thermal death points of others are in the same range as those of most viruses and bacteria. Thus the Type A microörganism of mice is killed by a temperature of 45° maintained for 15 minutes (74, 75), while other types isolated from mice (31) are not affected by 45° or 50° in 15 minutes but are killed at these temperatures in 30 minutes, or at 55° in 15 minutes. Laidlaw and Elford (52) reported that the majority of "sewage microörganisms" were killed at 45° in 15 minutes and that none withstood 55° for 5 minutes. The microörganism of agalactia on the other hand is reported (6) to resist 50° for 1½ hours, and 53° for 7½ minutes but not for 10 minutes. The microörganisms of the dog are killed at 48° in 30 minutes, 50° in 10 to 20 minutes, and 55° in 5 minutes (87). The L_1 microörganism associated with *Streptobacillus moniliformis* resists 53° for 15 minutes (45). The filtrable agent of spontaneous polyarthritis of rats (subsequently identified (1, 49) as belonging to the pleuropneumonia group) is killed by 40° in 4 hours, 42° in 60 to 75 minutes, 44° in 30 minutes, 45° in 10 minutes, 46° in 6 minutes, 48° in 4 minutes, and 50° in 2 minutes (Collier, (15)). Collier's data are given here in detail because they are extensive enough to indicate the curve of denaturation of the viable material. In contrast to this marked lability, one may note that the filtrable pyogenic agent

from rat sarcomas (identical with the L_4 microörganism of the rat pleuropneumonia group) was reported (100, 101) to be only attenuated at 56° in 30 to 60 minutes and completely inactivated after one hour at 60°. Another strain of the L_4 microörganism (at first called L_7) isolated from the joints of rats with spontaneous polyarthritis was reported to be killed by heating at 50° for 30 minutes (37). Whether or not this means that different strains of the same serological type can have distinct thermal death points, is, of course, problematical.

Virulence of Cultures. The pathogenic properties of cultures of the various microörganisms will be discussed later on, but it can be stated here that cultures of some of the members of this group lose their virulence after but a few passages *in vitro* while others remain pathogenic for more than a hundred passages. Furthermore, the medium on which a given strain is grown may also modify its pathogenicity (30). While Findlay, *et al.* (37) stated that "it seems that all pleuropneumonia-like organisms require an adjuvant such as agar or cells to start infection, if injected into animals", that has not been found necessary with the microörganisms of bovine pleuropneumonia, agalactia, and those I isolated from mice.

Bovine Pleuropneumonia and Properties of the Etiological Agent

Bovine pleuropneumonia is a highly contagious disease of cattle characterized by extensive consolidation, pleurisy, and subpleural effusion affecting usually one and sometimes both lungs. In young calves there is occasionally also joint involvement. The disease, one of great economic importance, has been recognized in Europe for over 200 years, and at present is distributed throughout the world with the exception of North America, Western Europe, and India (97). It appeared in the U.S.A. in 1843 but was finally eradicated. The infectious agent was early shown to be present in great concentration in the serous exudate or lymph of the lung by subcutaneous inoculation into other bovines which develop after an incubation period of 8 to 15 days an extensive edema spreading from the site of inoculation. Cattle, so inoculated, have fever and often die but never develop

the lung lesions so characteristic of the natural disease or any other lesion[1] excepting that directly associated with the site of inoculation; recovered animals, however, are immune not only to reinoculation but also to the natural disease. Although it is generally assumed that in nature the disease is transmitted by droplet infection through the respiratory tract, it is practically impossible to reproduce it experimentally by this route, even after intratracheal injection of highly virulent lymph, or by any other route. In a renewed attempt to produce pleuropneumonia by intratracheal inoculation, Daubney (20) succeeded only once in 22 trials with highly virulent lymph. In view of the highly contagious character of the disease one wonders if the infectious agent may not have to be carried into the lung tissues by some parasite, a possibility which does not seem to have been investigated thus far, and which might well repay investigation particularly in view of Shope's recent observations on the virus of swine influenza (89).

The infectious agent, which was early shown to pass through filters retaining the known bacteria (29), could not be grown by ordinary procedures or demonstrated in stained preparations of the pathological exudates and tissues, until Nocard and Roux in collaboration with their students (63) adopted the collodion sac technique previously used by Metchnikoff, *et al.* (57) in studies on cholera. The collodion sacs, containing ordinary broth and a "trace" of virulent lymph, were sealed and implanted into the peritoneal cavity of rabbits and guinea-pigs. After 15 to 20 days

[1] An observation by K. F. Meyer (58a) is of great interest with respect to the predilection of so many members of the pleuro-pneumonia group for the joints. He reported: "Lung-sickness virus collected from a sick animal and used for prophylactic inoculations [tail] in cattle, produced in animals of different ages a polyarthritis serofibrinosa. This phenomenon was not due to individual disposition, as is occasionally observed in calves, but it was specific for the strain of virus used by us. The subcutaneous inoculations of synovial liquid of animals affected by this particular strain produced, besides the typical local reaction, a poly-arthritis in all animals [bovines] experimented on. The synovial liquid represented a pure virus, and the micro-organism of pleuro-pneumonia could be cultivated from it. The specific action of the strain became lost in subcultures or by passage through an animal, and no secondary joint affections could be produced subsequently by inoculation of cultures."

growth was present in the sacs implanted in the rabbits but not in the guinea-pigs. Serial passage was accomplished and the cultures were proved to be virulent on subcutaneous injection in cattle which upon recovery were immune to reinoculation with virulent lymph. They soon discovered, however, that this laborious technique was unnecessary, and that they could obtain growth *in vitro* by using an especially rich peptone medium to which was added a small amount of bovine or rabbit serum. They were also first to make use of a step which all new investigators, who find themselves studying a member of the pleuropneumonia group, sooner or later discover for themselves, that the ordinary criteria used for judging growth of bacteria may not suffice; they said: "La culture du microbe de la péripneumonie est abondante; pourtant, elle ne provoque qu'un très léger louche, une opalescence à peine sensible du liquide; *on est obligé, pour se convaincre de la réalité de la culture, d'examiner comparativement, à côté d'elle, un tube de meme bouillon non ensemencé.*" [My italics] Subsequent work by Dujardin-Beaumetz (29) established its growth on solid media, and that of Bordet (3) followed by Borrel, *et al.* (4) revealed for the first time the complex morphology of the infectious agent on artificial media. It was Borrel and his coworkers (4) who first proposed the name of "*Asterococcus mycoides*" for this microörganism because it recalls "les principaux caractères de ce microbe intéressant, gaine muqueuse, filaments pseudo-mycéliens, polarités multiples"; and it is of interest to quote their prophecy, which took 13 years to come true, namely: "Il est difficile en l'état actuel de le comparer à d'autres types puisqu'il est le seul connu de son espèce, mais on peut déjà prévoir qu'il ne restera pas isolé dans ce groupe . . . "

A strain of pleuropneumonia bovis which has grown *in vitro* for some time imparts a distinct turbidity to the fluid medium which upon shaking exhibits silk-like whorls, that are not seen with any other member of the group described thus far. The tendency to form long chains of rings may be responsible for this property. Tang, *et al.* (94) working with strains isolated in China indicated that no growth was observed in plain broth, blood broth, litmus milk, blood agar, Loeffler's serum and Bordet-Gengou's

medium. They found that "hormone" broth with 2 per cent peptone and 10 per cent horse serum was the best. Certain carbohydrates are fermented with the production of acid but not gas. "Glucose, fructose, mannose, maltose and dextrin were strongly fermented, sucrose and trehalose only slightly attacked, while raffinose, inulin, galactose, salicin, xylose, mannitol, arabinose, amygdalin, lactose, dulcitol, iso-dulcitol, sorbitol, inositol, erythritol and adonitol were not acted upon." They also stated that the microörganism was bile-soluble; it reduced hemoglobin in fluid cultures when freshly isolated and for a certain number of passages thereafter, but that old strains lost this property. Five minutes' contact with anaesthetic ether was enough to kill it.

As regards the host range of the infectious agent of pleuropneumonia bovis, Willems (99) established in 1850 that material which infected cattle was innocuous for the goat, sheep, dog, swine, rabbit, guinea-pig, poultry and man. Dujardin-Beaumetz (30) reported that while cultures in bovine-serum broth had the same limited host range as the original lymph, the same microörganisms grown in cultures containing sheep or horse serum, were highly infectious for sheep and goats. In these animals the cultures produced not only the marked swelling at the site of subcutaneous infection but on occasion also fever, polyarthritis, and death. With cultures, presumably grown in horse-serum broth, Tang, *et al.* (94) were also able to infect goats and one water buffalo, but not "white mice, hamsters, albino rats, guinea-pigs, rabbits and cats after subcutaneous, intraperitoneal, intracerebral, intravenous and in some cases intratesticular inoculation." [See also Walker (97)]

Agalactia of Sheep and Goats and Properties of the Etiologic Agent

Agalactia receives its name from the manifestation which first drew attention to the disease, but careful clinical and experimental studies have established that it is a systemic disease, affecting males and females alike, with particular involvement of the joints, the eyes, and in lactating sheep and goats the mammary glands (9). Agalactia has been predominantly a European disease,

being especially prevalent in the mountainous regions of Italy, France, and Switzerland; in recent years it has occurred in Algeria. The course of the disease may be acute or chronic. While death may ensue before the appearance of the usual lesions, that does not occur often and the animals exhibit arthritis, keratitis and occasionally a vesiculo-pustular skin eruption; the lactating females develop mastitis and the scrotum may be inflamed in the males. The infected mammary glands stop secreting milk, develop many indurated nodules, and atrophy. The joints usually affected are those of the carpus and tarsus and less often the femoro-tibial, humero-radial, coxo-femoral and metacarpals. In most animals the arthritis clears up, while in some the process occasionally goes on to abscess-formation with involvement of the articulating surfaces and ultimate ankylosis. In the chronic form of the disease there may be remissions and exacerbations of the arthritis without ultimate deformity. There is usually generalized wasting of the musculature. The pregnant animals abort giving birth to dead fetuses or monsters.

The etiological agent has been shown to be filtrable (9, 10). It is present in the secretions of the infected mammary glands, eyes, and joints, and in the early stages of the disease in the blood. Intravenous inoculation or even feeding of infectious material can produce the typical disease with localization in the mammary glands, joints and eyes, indicating that the agent has a special affinity for these tissues. In 1923, Bridré and Donatien (5) first cultivated the infectious agent (from the fluid of an affected joint) in broth containing 20 to 30 per cent of horse serum. The cultures so obtained and others grown subsequently from infected milk or lymph nodes reproduced the disease in sheep and goats. The cultures were shown to be infective by the cutaneous, subcutaneous, intra-articular, and intravenous routes. Bridré and Donatien recognized the close morphological, cultural, and biological resemblance of their microörganism to that of pleuropneumonia bovis, but were able to show that the two were different in their pathogenic properties and that there was no serological relationship or cross immunity between them (6).

The microörganism of agalactia grows in cows' and goats'

milk without producing any obvious change in the medium, while at least one strain of bovine pleuropneumonia microörganism tested by Bridré and Donatien (6) failed to grow in milk. Some sugars like glucose, levulose, galactose, raffinose, arabinose, xylose, sucrose, and maltose retard growth while others like lactose, mannitol and erythritol favor it. Good growth occurs in the presence of staphylococci and certain other bacteria and when these organisms are encountered in the first cultures of infectious material, one need only remove them by filtration through a suitable Berkefeld "V" or Chamberland L_1 candle to obtain a pure culture of the agalactia agent.

Pleuropneumonia-like Microörganisms of Dogs and Their Relation to Canine Distemper

In 1934, Shoetensack (86), working in Japan, reported the isolation of pleuropneumonia-like microörganisms from material obtained from dogs with distemper. His own illustrations and the subsequent work done on his strains by Klieneberger (47) leave no doubt that the filtrable microörganisms he called *Asterococcus canis* are indeed, as he himself indicated, members of the pleuropneumonia group. Their role in the etiology of canine distemper is, however, another matter. At first he cultivated these microörganisms from the purulent nasal secretions, and inoculated one-half of an agar slant of a 4-day, 7th generation culture subcutaneously into 4 puppies, 2 weasels, 4 guinea-pigs, and 4 rabbits with negative results. Then from a dog, dying of a spontaneous "distemper-like" disease the same type of microorganism was grown from the purulent secretions of the eye and nose, from the lung, pericardial fluid, and liver. A filtrate from the lung of this animal inoculated subcutaneously into a new dog led to death in 18 days after a severe attack of the respiratory type of distemper. From this dog the same microorganism was again cultured from the lung, pleural exudate, and liver. Inoculation of the 8th generation of this culture presumably produced distemper in 3 puppies. The lungs, spleen, liver, and pericardial fluid of 9 healthy dogs were cultured in a similar manner but in no instance were these microörganisms encountered.

In 1936, Shoetensack (88) reported that in 14 of 15 dogs which died of typical respiratory distemper, *A. canis*, Type I, could be cultured from one or more organs—always from the lungs, and occasionally from the kidneys, brain, and "glands." *A. canis*, Type II (biologically and serologically different from Type I) was found in only 3 of these dogs. He believes that Type II is "seemingly unable to act as the first invader in the case of contagious respiratory type of canine distemper, and can be conceived to be acting only a part of the role in the disease, appearing together with the Type I organism in some cases of canine distemper of the contagious respiratory type. Sometimes it is also found in spontaneous pneumonia in dogs." (87) Concerning prophylaxis with a "dead organism vaccine" prepared from pure cultures of *A. canis*, I and II, Shoetensack states: "The dogs which received two injections of 2 c.c. each of the vaccine have shown results which go to demonstrate the vaccine as a means of prophylaxis against infection from canine distemper." (88) The control dogs all died of severe distemper, and positive cultures of the Type I and II microörganisms were obtained from them. The 5 vaccinated dogs lived longer, died of other conditions, and no Asterococci could be cultured from them.

A critical examination of the data leaves one in doubt as to the relationship between the virus of canine distemper and the microorganisms of the pleuropneumonia group which were so constantly present in the exudates and tissues of the sick dogs. The existing data do not even permit a statement on the pathogenicity of the dog microörganisms, and further study of the entire question, including the possibility of a double etiology of canine distemper, certainly seems indicated. In this respect some recent observations of Pinkerton (70) on mink distemper are of interest. Finely minced lung, spleen, kidney, and bladder tissues from eight minks moribund or dying of distemper were spread over horse-serum-agar slants (Zinsser, Fitzpatrick, Wei medium) and incubated at 37°. While the majority of the cultures remained sterile and no microörganisms or definite inclusions were found in films, several cultures representing lung, kidney, and bladder tissue from one mink and bladder tissue from another, developed irregular focal areas of clouding, 2 to 5 mm. in diam-

eter, in the substance of the medium underlying certain of the tissue fragments. Film preparations revealed a heavy growth of a minute, spiral microörganism which Pinkerton believed to have certain morphological characteristics in common with that of bovine pleuropneumonia. This microörganism was carried for several transfers on the Z.F.W. medium without tissue, and subinoculations on blood agar remained sterile. Intracellular clusters of the organism were observed in the first culture on the Z.F.W. medium, in which the cells were those of mink tissue, as well as in plasma-tissue cultures and in Maitland medium using guinea-pig lung and spleen. It was then found that the microorganism would also grow in blood broth in which it produced a fine clouding. After three transfers in this medium, large amounts of culture were inoculated into five ferrets known to be free of present or past infection with distemper, three subcutaneously and two intranasally. All of them remained well for 6 weeks and subsequent inoculation with moderate doses of the original mink virus produced typical fatal distemper infection in all five of the ferrets. I examined some films of this microorganism which Doctor Pinkerton was kind enough to send me, and my impression is that it cannot yet be included in the pleuropneumonia group chiefly because of the absence of the characteristic polymorphic forms and because not enough is known of its capacity to grow, or its type of growth, on solid media.

Pleuropneumonia-like Microörganisms of Rats

"L_1." Since many rats normally carry *Streptobacillus moniliformis* in their nasopharynx (90), its symbiont or variant designated L_1 by Klieneberger, may be regarded as the first pleuropneumonia-like microörganism demonstrated in this species (44, 45). The peculiarities of this microörganism will be discussed separately later on, but it may be mentioned here that on at least one occasion L_1 has been isolated directly from the lung of a tame rat independently of *Streptobacillus moniliformis* (47).

"L_3." In 1937, Klieneberger and Steabben (46) examined the lungs of 19 rats exhibiting chronic "bronchopneumonia" or "bronchiectasis" and from 17 they isolated pleuropneumonia-

like microörganisms often in pure culture, which they at first called L_1. Subsequent immunologic and other studies (47) revealed however, that, with at least one exception, they were dealing with a distinct microörganism which was given the name L_3. Lungs of normal appearance from 10 rats were also cultured at that time but all with negative results. In 1940, Klieneberger and Steabben (50) reported additional studies on the lungs of rats of different ages comprising a total of 251 laboratory rats and 17 wild ones. Lung lesions were present in 108 laboratory rats but pleuropneumonia-like microörganisms were recovered from the lungs of 138; there were altogether 46 out of 139 rats, 8 months of age or younger, with lungs of normal appearance which yielded these microörganisms on culture. The authors refer to all these microörganisms as L_3, although there is no indication that more than 4 strains were typed serologically. There is still the possibility, therefore, that the pleuropneumonia-like microörganisms in the lungs of rats may belong to a variety of serological and biological types. Among the 17 wild rats they found only one with lung lesions and that one yielded an L_3 microörganism confirmed by serological typing; the lungs of the remaining 16 gave negative cultures.

Although it has not proved possible to produce any pulmonary lesions or any other pathogenic effects in rats with cultures of L_3 microörganisms, Klieneberger and Steabben (50) are inclined to regard them as having some connection with the causation of the natural disease. Subcutaneous or intraperitoneal inoculation of L_3 cultures, particularly when mixed with agar, into mice has produced abscesses but there is as yet no evidence that the microörganism was multiplying in the mouse and not in the implanted agar; no symptoms, however, resulted from intracerebral inoculation in mice (50). Doctor Joel Warren informs me that intravenous inoculation of L_3 cultures in mice produced no apparent disease.

"*L_4.*" Early in 1938, Woglom and Warren (100, 101) reported that under certain conditions suspensions of *sarcoma 39* produced abscesses upon subcutaneous inoculation in rats. These abscesses were transmissible by Berkefeld filtrates containing

no demonstrable bacteria. Upon intravenous injection in rats the filtrable agent produced widespread suppuration involving especially the extremities, the testis, and the soft tissues about the head and larger joints. The mouse was even more susceptible to this agent than the rat, but guinea-pigs and seemingly also rabbits were refractory; it could be cultivated in serial passage on the chorioallantoic membrane of the chick embryo. Cytoplasmic inclusions were reported in the epithelial cells of the skin overlying the abscess.

Somewhat later in 1938, Klieneberger (47) reported the isolation of a new pleuropneumonia-like microörganism from the "swollen submaxillary gland" of a rat. She called it L_4 because it was culturally and serologically different from L_1 and L_3. Upon culturing some of the infective material of Woglom and Warren she discovered that the filtrable pyogenic agent was identical with the L_4 microörganism (48). The L_4 cultures could produce an abscess upon subcutaneous inoculation but had no effect whatever upon intravenous inoculation. However, when some of the material from an abscess produced by a culture was injected intravenously, the effects of the original filtrable agent were reproduced. Woglom and Warren (102) confirmed these findings. In my hands, cultures of an L_4 strain given me by Doctor Warren produced "polyarthritis" and suppuration in the interscapular region upon intravenous inoculation in mice; none of the mice died and the "polyarthritis" disappeared spontaneously.

Still another condition, in which the L_4 microörganism ultimately was shown to be of etiological relationship, came to light in 1938. Collier, (11, 12, 13, 16, 17, 18) working in Java, found that the spontaneous "polyarthritis" (swollen extremities) observed in a wild rat (*Rattus norvegicus*) could be transmitted to white rats in serial passage. The infectious agent, which could not be identified with any cultivable bacteria, was shown to be widespread in the body and capable of producing a polyarthritis regardless of the route of inoculation. Some of the rats died of a systemic infection, and in the survivors the arthritis cleared up spontaneously. Findlay, *et al.* (37) reported finding a similar

spontaneous "polyarthritis" among pure-bred laboratory rats in London. They were able to transmit this condition to other rats with bacteria-free, gradocol-membrane filtrates and showed that the etiological agent is identical with a pleuropneumonia-like microörganism which they at first called "L_7" but which was subsequently found to be serologically identical with L_4 (49). This "L_7" strain was pathogenic for rats and mice but not for pigs, rhesus and cercopithecus monkeys, rabbits, guinea-pigs, voles and hedgehogs. Later, Beeuwkes (1), working in Java in the same laboratory with Collier, reported the isolation of two strains, one from the infectious agent of spontaneous polyarthritis of the rat and the other from a similar agent obtained from rats after inoculation with material from a patient with acute rheumatic fever. These two strains appeared to be identical biologically but since their serological type was not determined, one cannot, of course, regard them as representatives of L_4.

Summary. Three distinct biological and serological types have thus far been found in rats: L_1, L_3, and L_4. L_1 occurs usually in association with *Streptobacillus moniliformis* and is not pathogenic without it. L_3 has been found in normal lungs and in lungs with chronic bronchiectatic lesions, but it has not yet been found to be pathogenic in rats and the extent of its pathogenicity for mice has yet to be elucidated. L_4 has been shown to be the cause of spontaneous polyarthritis of rats and apparently is also latent somewhere in the body, since it has also showed up in a "swollen gland," in transplantable tumors, etc. L_4 is pathogenic for rats and mice. Little is known of the natural habitat of these micro-organisms in rats.

Pleuropneumonia-like Microörganisms of Mice

These mircoörganisms were first encountered in mice in 1938 during the course of experiments with *Toxoplasma* (74, 75) and the virus of lymphocytic choriomeningitis (35); and the first strain was isolated simultaneously in America and England from the brains of mice infected with those agents. During the next two years a number of other biologically and immunologically distinct species were found in mice and it was shown that their natural

habitat was in the conjunctiva, the mucosa of the nose and occasionally trachea, and oddly enough in the brain. Many different stocks of mice were shown to be carriers of these microorganisms (77, 80). Preliminary experiments have revealed that newborn mice do not harbor them, and acquire them only if their mothers or the other mice with which they are in contact are carriers. They make their first appearance in the nose; and the eyes have thus far not been found to be infected until after they have opened. Mice probably remain carriers during their entire lifetime. Thus far, there have not been encountered any spontaneous diseases in which any of these microörganisms are the etiological agents. And yet all of the known strains which appear to be harmless in their natural habitat (unless they are the cause of mouse catarrh) have proved to be highly pathogenic under experimental conditions in which they are brought into contact with the tissues for which they seem to have a special affinity. Work done with at least two types of this group has indicated that while *in vitro*, they can multiply in cell-free media, *in vivo* they grow only in association with or within the cells for which they have a special affinity. These microörganisms of mice vary sufficiently from one another as regards morphology, colony type, pathogenicity or tissue affinities, toxin production and antigenic make-up to permit their classification into a number of distinct species.

Type A (Sabin) and L_5 (Findlay, Klieneberger, MacCallum, and Mackenzie). While the Type A microörganism was first isolated from the brains of mice used in experiments with *Toxoplasma*, it has since been found on at least one occasion in the brain of a normal mouse, almost regularly in the eyes (conjunctiva) of carriers, in the nasal mucosa and in the lungs of mice which had received nasal instillations of various materials (77, 91). Regardless of what other type may be carried in the nasal mucosa, the microörganisms isolated from the eyes have, thus far, always been Type A. Upon intracerebral injection of brain or other tissues containing the Type A infectious agent, or of serum-broth cultures, there developed in most mice after an incubation period of 1 to 10 days, but usually on the 2nd or 3rd day, a very charac-

teristic turning or rolling on the long axis of the body with or without other nervous signs. Some of the mice died, some continued with choreiform signs or hydrocephalus for months and years, while the majority recovered in a few days. Two kinds of lesions were observed in the brain after intracerebral injection: the first, consisting of destruction of the periventricular tissue followed by extensive infiltration with polymorphonuclear leucocytes, was present in all mice while the second consisting of almost complete necrosis and lysis of the posterior pole of the cerebellum was found only in mice which exhibited the turning or rolling signs. Intracutaneous, subcutaneous, intramuscular, intratesticular, or intravenous injection as well as nasal instillation under ether anaesthesia, or administration by stomach tube of large amounts of infected mouse brain was without any obvious effect. After intra-abdominal or intrathoracic injection, on the other hand, 20 to 40 per cent of the mice developed convulsions and the characteristic rolling within 17 to 48 hours and died shortly thereafter, revealing the same cerebellar change but not the periventricular destructive and inflammatory lesion found after intracerebral inoculation. Extensive studies disclosed that after intra-abdominal and intrathoracic inoculation the infectious agent multiplied in the mesothelial cells of the peritoneum or pleura but was not present in the brain, suggesting that the lesions in the brain were caused by a toxin. When this infectious agent was grown in glucose-serum broth, the cultures were not only as pathogenic as the infected tissues, but the presence of a true exotoxin, capable of reproducing the same nervous signs and cerebellar lesion after intravenous injection, was demonstrated in them (75).

Primary cultivation of the Type A microörganism in fluid media is facilitated by the addition of 0.5 per cent glucose. About 48 hours after growth first becomes grossly apparent in such a medium, the culture is no longer pathogenic or viable. This is associated with the development of acid, the pH dropping from about 7.8 to about 6.0. In the absence of added sugar this does not occur and subculture is possible for at least a month. Morphologically the elements making up a fluid culture consist

chiefly of elementary bodies, minute rings or ovals with condensed bodies at one or both poles, and occasionally triangular or quadrangular structures in which the elementary bodies are linked by thinner bonds. On solid media good growth is obtained only when the moisture is kept in by sealing the Petri dish. The fully developed colonies vary in size from 20 to 100 μ with only an occasional well-isolated one reaching 200 to 300 μ, and present central areas which are circumscribed, elevated, and darker than the rest. Giemsa-stained impression films of such colonies present in addition to those found in fluid cultures much larger and more polymorphic structures. When fluid cultures are injected intravenously into older mice which are not as susceptible to the toxin as younger ones, or when the microörganisms are injected after being separated from the toxin by centrifugation, approximately 20 per cent of the animals develop a polyarthritis which usually clears up in the course of a few weeks. No cartilage destruction or ankylosis has been observed with this type. During the course of the arthritis an occasional mouse develops choreiform signs. The pathogenicity of this strain has remained even after more than 100 subcultures *in vitro*. Rabbits, guinea-pigs, and rhesus monkeys have not been found susceptible.

The microörganism isolated by Findlay, *et al.* (35) from mice with "rolling disease" and called L_5 was reported by them to be immunologically related to or identical with the Type A microorganism on the basis of cross-immunity tests in mice. However, there are some significant differences in pathogenicity between the two. Neither their original infectious agent in the mouse brains, which had a lower titre, nor the L_5 cultures were pathogenic after intraperitoneal or intrathoracic injection. Not only were nervous signs absent but there also appeared to be no local multiplication since it could not be recovered from the liver or spleen 3 days after inoculation. Furthermore the L_5 culture produced no apparent signs of disease after intracerebral injection unless it was mixed with agar or virus-infected mouse brain. No cerebellar lesion was reported and there is as yet no evidence that L_5 produces the neurotoxin. It would, therefore, be inadvisable to call the L_5 and Type A microörganisms identical until more work has been done,

although Doctor Homer Swift informs me that the two are serologically identical.

In 1939, Sullivan and Dienes (91) produced pneumonia in mice by serial passage of mouse-lung suspensions by means of nasal instillation under ether anaesthesia. From the lungs of such mice they isolated several strains of pleuropneumonia-like microörganisms. They stated that "when injected intravenously, intraperitoneally or subcutaneously in the two strains of mice at our disposal, our cultures failed to produce any clinical-pathological phenomena." Sullivan and Dienes were kind enough to send me two of their strains which I grew in 10 per cent rabbit-serum broth or 30 per cent ascitic-fluid broth with 0.5 per cent glucose added in both instances. After several passages growth appeared in 24 hours, and intravenous injection of 0.5 ml. amounts of 24 or 48 hour cultures into 3- to 4-week-old mice produced within 1 to 2 hours the typical convulsions and rolling characteristic of the neurotoxin effect, with the majority of animals dying in 3 to 4 hours. This toxic effect was completely neutralized by the Type A antiserum, and results of agglutination tests showed conclusively that the two strains of Sullivan and Dienes were identical with the Type A microörganism. While these strains were indeed isolated from lungs with pneumonia, these authors presented no evidence that they were the cause of the pneumonia or that the cultures could either produce pneumonia or immunize against it. The relationship between the experimentally produced pneumonia in mice and the Type A microörganisms is, therefore, still to be investigated.

The Type B Microörganism. This microörganism was first cultured from the brain of a normal mouse (75, 76) and has subsequently been found in the nasal mucosa (77, 80). Morphologically it differs from the Type A microörganism in that it has more complex and polymorphic structures in fluid media. Especially noticeable on dark-field examination are the long undulating and vibrating thin filaments which are attached to the ring structures and which are usually first seen during the second day of grossly apparent growth. The Type B colony differs from that of Type A in not having the central, raised, dark,

nipple-like structure but rather what seems to be a meshwork of vacuoles. Acid is produced in the presence of glucose and the fluid culture loses both its pathogenicity and viability 2 to 3 days after growth becomes grossly apparent. It is further differentiated from Type A by not producing a toxin, by its great affinity for the joints and inability to multiply in most other tissues, and by being immunologically distinct. After intracerebral injection of a culture at the height of growth, the mice remain well and there is not even multiplication of the microörganism. Several rapid brain-to-brain passages lead to the complete disappearance of the microörganism rather than to adaptation and increased virulence. Intracutaneous, subcutaneous, intramuscular or intrathoracic injection or nasal instillation with or without ether anaesthesia induced neither arthritis nor any other local or systemic disease. Arthritis was produced in practically 100 per cent of mice, however, when 0.5 ml. of a 24- or 48-hour culture was injected intravenously and somewhat less often when 1 ml. was injected intra-abdominally. The microörganisms disappear from the peritoneal cavity 24 hours after intra-abdominal injection and there is no multiplication in the viscera after intravenous injection. Swelling of the joints may appear as early as 4 to 5 days, and then the arthritis is migratory, new joints becoming involved as others recede. Fusiform swellings of isolated digits occur frequently. The process is progressive and chronic leading often to ankylosis, especially of the knees, "wrists" and "ankles", after 2 to 5 months. Although it has not yet proved possible to obtain positive cultures from the joints before or during the first day or two of clinically apparent arthritis, there is no difficulty in securing growth in fluid or solid media somewhat later and positive cultures have been gotten as late as 10 weeks after intravenous inoculation. The affected animals appear otherwise healthy. Pathological changes are limited to the joints and consist chiefly of proliferation in the synovial membrane, the capsule, the perichondrium, and necrosis of the articulating cartilage. Cultures have retained their pathogenicity even after 50 to 60 passages *in vitro* but there is an indication that it is diminished after prolonged cultivation *in*

vitro. Inoculation of large amounts of virulent culture into rabbits and guinea-pigs has been without effect.

Types C, D, E and Other Pleuropneumonia-like Microorganisms Reported in Mice. Several strains of the Type C microörganism have been isolated from the lungs of mice which had previously received various materials by nasal instillation under ether anaesthesia (77). Type C produces a progressive polyarthritis in mice following intravenous injection, but differs from Type B in its antigenic make-up and probably also in its capacity to multiply in other tissues in addition to those of the joints. Type C produces no neurotoxin and is also immunologically distinct.

Four strains of Type D were encountered in the nose, lung, and brain of mice (80). It is immunologically distinct from the other types, has no neurotoxin, and produces progressive arthritis in mice after intravenous injection. The same is true of a single strain isolated from the nasal mucosa of a mouse and called Type E because it differed in its antigenic make-up from all the others. Not enough work has yet been done to permit a statement on the tissue affinities of these types.

Findlay, *et al.* (36) inoculated intracerebrally into mice the blood of splenectomized mice containing *Eperythrozoon coccoides.* From the brains of the inoculated mice they isolated on 6 occasions a microörganism which they called L_6. It is not identical with *E. coccoides* and is serologically distinct from L_1, L_3, L_4, and L_5. Its colony has a coarse, globular surface and in liquid medium it forms little clumps which are composed of fairly large globules (49). It is stated (38) that L_6 produces a fatal encephalitis upon intracerebral injection in mice and "arthritic changes" at the site of inoculation in the pad of the foot in 30 to 50 per cent of animals. Klieneberger (49) also reports the isolation by Dr. H. Jahn, from the swollen joint of a mouse, previously inoculated with *Streptobacillus moniliformis*, of a pleuropneumonia-like microörganism which is not L_1, and is called "M55." It is reported to cause arthritis but has not been typed; and its relation to the other members of this group is unknown.

Edward (31) isolated a number of strains of pleuropneumonia-like microörganisms from the pneumonic lesions of mice which

had been given nasal instillations under ether anaesthesia of mouse lung suspensions. These strains did not form distinct colonies on solid medium, but since it is not stated whether or not the evaporation of moisture was prevented, the lack of colonial development might have been due solely to the conditions of cultivation. Fluid cultures, however, presented morphological forms similar to those of other members of the group ("granules, ring forms of various sizes, forms showing filamentous budding, and thin bacillary bodies with several rounded swellings along their length") including reproductive units which passed gradocol membranes with an A.P.D. of 0.45 μ. "All the strains were tested for their ability to ferment glucose, fructose, galactose, xylose, arabinose, rhamnose, maltose, sucrose, lactose, raffinose, dextrin, inulin, salicin, mannitol, dulcitol and glycerol, but production of acid from any of these could not be definitely established. A temperature of 55°C for 15 minutes killed the organisms. They resisted 45° and 50°C for 15 minutes but not for 30 minutes." With respect to sugar fermentation as well as heat resistance, Edward's strains would appear to differ from the Type A and Type B microörganisms, since the latter definitely produce acid from glucose and do not resist 45° and 50° C for 15 minutes. They are also different in their pathogenic properties in that intra-abdominal or intravenous injection of cultures of strains 1, 2, and 4 (all of one serological type) produced neither arthritis nor any nervous signs suggestive of the presence of a neurotoxin. This is in agreement with the fact that strain 4 was not agglutinated by either the Type A or Type B antiserum, but was agglutinated to 1:160 by the Type C antiserum (Personal communication from Doctor Edward). Since the Type C serum had a titre of at least 1:1000 for the homologous microörganism, one cannot regard those strains (i.e. 1, 2, 4, 5, and 6, all of one serological type) as being identical with Type C. Strains 3 and 7 were serologically distinct from the others and Doctor Edward informed me that Type A antiserum agglutinated strain 3 to 1:320 which only suggests antigenic relationship since this serum agglutinated the homologous microörganism to a dilution of 1:6000. Strain 3 was agglutinated to 1:40 by the Type C serum

and not at all by the Type B serum. Although the relationship of these strains to Types D and E is unknown, their failure to produce arthritis would constitute at least one point of difference. Although Edward expressed the belief that the microörganisms he isolated from the lung lesions were actually the cause of the pneumonia, it is to be noted that only the early subcultures could produce pneumonia and that no tests were made to rule out (a) the presence of a virus, or (b) to show cross-immunity between the microörganisms and the pneumonia-producing agent. Horsfall and Hahn (42) were able to show that while pleuropneumonia-like microörganisms were present in the lungs of mice with a spontaneous virus pneumonia, they were not identical with the etiological agent of the pneumonia.

From this review it is apparent that mice are carriers of a large variety of immunologically and biologically distinct microörganisms of the pleuropneumonia group and also that all the strains which have been tested are different from those that are known in the rat. It is of interest to note, therefore, that in at least two instances mice which had been quartered with rats in the same room or building were found to be carriers of rat microörganisms. In 1939, Dr. Joel Warren isolated an L_4 microörganism from the brains of mice used for passage of herpes virus by the intracerebral route, and Findlay, *et al.* (37) of an L_3 microörganism from the brain of an apparently healthy mouse.

Pleuropneumonia-like Microörganisms of Guinea-pigs

The existence of an independent member of the pleuropneumonia group has not yet been demonstrated in guinea-pigs, but that such may be present in association with some of the bacteria is suggested by the work of Klieneberger. In 1935, she reported the isolation on two occasions of a gram-positive streptococcus or streptobacillus from the nasopharynx of healthy guinea-pigs. Associated with the bacterial colonies were "minute" colonies which she called L_2, but these were never isolated and their essential nature remains obscure (44). In 1940 she described the isolation of a culture resembling *Streptobacillus moniliformis* from large cervical abscesses of guinea-pigs. Since *S. monili-*

formis of rats is not pathogenic for guinea-pigs, it could not be dismissed as merely a cross-infection. From the guinea-pig strain, she obtained a microörganism, resembling L_1, but these cultures were lost with the outbreak of war before any serological work was done with them (49).

Pleuropneumonia-like Microörganisms of Man

In 1937, Gerlach (40) was probably the first to suggest the presence of filtrable microörganisms of the pleuropneumonia group in man. His illustrations and protocols, however, present convincing evidence to the contrary and suggest that he misinterpreted precipitation in the media. In the same year Dienes and Edsall (22) reported the isolation of a pure culture of a pleuropneumonia-like microörganism from a suppurating Bartholin's gland of a laboratory worker. At that time, however, they were inclined to attribute the infection to the patient's contact with rats. While the strain was lost before its biological and serological identity could be established (there was never any reason for calling it L_3), the more recent work of Dienes (28) suggests that contact with rats might have had nothing to do with the infection. Following the demonstration of the special pathogenic properties of the Types A and B mouse microörganisms, Swift and Brown (93) reported the isolation of pleuropneumonia-like microörganisms from patients with rheumatic fever, but they subsequently pointed out that their direct "cultures" were misinterpreted while the ones obtained after passage through mice were biologically and immunologically identical with those normally carried by these animals (77). Many attempts by a number of investigators to cultivate such microörganisms from exudates and tissues of patients with rheumatic fever or rheumatoid arthritis have all been unsuccessful (38, 77, 81, 91).

In view of the fact that mice were shown to carry these microorganisms in their eyes (conjunctiva) and nose, swab cultures from these regions obtained from 100 human beings were studied by Sabin and Johnson (81), but without success. They observed,

however, that when human tonsils were minced and smeared over 30 per cent ascitic-fluid-agar plates, minute colonies (20 to 40 μ in size) resembling those of the pleuropneumonia group sometimes grew out in between the larger bacterial species. These colonies, which they called "X" colonies, occurred in 3 out of 60 cases in which children's tonsils were studied, and on 2 occasions in another group of 103 children. The "X" colonies, however, could not be passaged either *per se*, or in the presence of the associated bacteria or rich filtered extracts of tonsillar tissue, and no studies which would permit a decision concerning their relationship to the pleuropneumonia group were, therefore, possible. In this connection, it is of interest to quote the following sentence from a paper by Seiffert (85) dealing with the saprophytic filtrable microörganisms in soil, manure, etc.: "Es ist noch zu erwähnen dass ich ähnliche Mikroorganismen einmal aus Speichel und dreimal aus Sputum von Bronchiektatikern gewinnen, aber nicht in Passage weiterzüchten konnte." Whether or not there exist in the respiratory tract of human beings microörganisms of the pleuropneumonia group with special requirements for *in vitro* cultivation is a question that is raised by these observations and may well repay further investigation.

A recent brief report by Dienes (28) brings the first evidence that is more than suggestive that such microörganisms exist in human beings. Cultures from the genital tract of female patients who had gonorrheal pelvic infections were studied on an ascitic-fluid-agar medium, and pleuropneumonia-like colonies were encountered in about one-third of the cases. After 48 hours these colonies were often only 10 to 20 μ in diameter, but they developed to considerably larger size on passage. Four strains were thus isolated in pure culture. Since I disagree with Doctor Dienes on some of his morphological interpretations, I was frankly skeptical, until he was good enough to send me not only preparations of these colonies but also a Giemsa-stained film of the growth in a fluid culture. I found these to be morphologically absolutely typical of the pleuropneumonia group and indistinguishable from the forms observed in the cultures of the mouse

microörganisms. Doctor Dienes informs me that his strains grow and survive in broth cultures but the growth is very scanty. Further studies in this new direction are of the greatest interest.

Pleuropneumonia-like Microörganisms as Saprophytes in Sewage and Other Decomposing Matter

In 1936, Laidlaw and Elford (52) reported a new group of filtrable microörganisms, obtained from raw sewage originating in four London districts, but not detected in London tap water, nor in fecal material from man, pig, rabbit, or rat. Sewage was filtered through gradocol membranes with an A.P.D. of 1.0 μ, 0.8 μ, or 0.6 μ and seeded into Fildes's broth or agar. Growth occurred at 22° and at 37° but was optimum at 30°. In broth cultures they found granules, varying in size from small cocci about 0.5 μ in size to small dots which were imperfectly resolved, rings, single or paired, with an occasional one showing a thickening or granule at the periphery or a short filament attached to the margin. The most turbid cultures contained as many as 3 to 10 billion reproductive units per ml. Typical pleuropneumonia-like colonies, i.e. minute, round, granular colonies with well-marked dark centers and lighter peripheral zones, developed on the solid media. The various strains fell into three serological types which they called A, B, and C. Pathogenicity was tested by feeding cultures to rats, by intravenous inoculation in rabbits, inhalation, subcutaneous and intra-abdominal inoculation in mice, all with negative results. Cultures from the peritoneal cavity of inoculated mice were sterile within 24 hours.

Seiffert (84, 85) confirmed these results by showing that similar filtrable microörganisms were present in Germany in soil, compost, decomposing leaves, and manure. At least two of the German strains are serologically identical with and the others are all related to those isolated in England (49). In addition, Seiffert (84) pointed out that: "Diese mehrfach wiederholten Versuche dürften dafür sprechen, dass es in der freien Natur neben filtrablen, an der Grenze der Sichtbarkeit stehenden Mikroorganismen wahrscheinlich auch solche gibt, die nur durch irgendwelche Lebensreaktionen nachweisbar sind." From compost which had

been filtered through membranes of smaller porosity he obtained a *transmissible*, enzyme-like effect, i.e. decomposition of starch, which could be carried on for 5 to 8 passages. There was no decomposition of starch in corresponding dilution experiments, nor after the addition of chloroform. There was no definite turbidity in the cultures and no visible growth on solid media. Liquefaction of gelatin could also be obtained for 6 passages.

L_1 AND STREPTOBACILLUS MONILIFORMIS. QUESTION OF SYMBIOSIS OR VARIATION AND ITS SIGNIFICANCE FOR THE MICRO-ORGANISMS OF THE PLEUROPNEUMONIA GROUP

Streptobacillus moniliformis (54), a gram-negative pleomorphic bacillus, is a normal inhabitant of the nasopharynx of rats (90). While its pathogenicity for rats is doubtful or nonexistent, it produces a highly fatal generalized pyogenic infection in mice (55, 56), which because of the localization of the process, that is essentially one of multiple abscess formation, in the heart muscle, joints, and elsewhere has called forth erroneous comparisons with rheumatic fever (55). In human beings, however, *S. moniliformis* does give rise to diseases (rat-bite fever and Haverhill fever) clinically reminiscent of certain manifestations of rheumatic fever, i.e. recurrent attacks of fever, erythema, and polyarthritis (21, 34, 54, 68).

From the work reported by Klieneberger and others it became apparent that all strains of *S. moniliformis* were associated with or gave rise to another microörganism, called L_1, which possessed many properties in common with the microörganisms of pleuropneumonia and agalactia. Separation of the two microorganisms by the usual bacteriological methods failed and it was not until Klieneberger (45) found that the two were not equally resistant to ageing or heat that such separation was possible. When a 4- to 8-day-old broth culture kept at 37°, or a fresh culture heated at about 53° for 5 to 15 minutes was seeded on 10 per cent horse-serum-agar plates, there developed after a few days' incubation 5 to 300 of the usual, large (3–5 mm.), mixed *S. moniliformis* colonies and in between them a number of minute colonies which could be detected with a magnifier or a micro-

scope. When fully developed after about 4 days' incubation the smaller colonies were about one-fifth the size of the larger mixed variety and were characterized by a dark, granular centre and a clearer ring zone. Subcultures of the small L_1 colonies on the same medium failed, however, and it was not until Klieneberger used a "special medium" [heart-muscle-infusion-peptone broth (pH 7.6–8.0) with boiled blood (5 per cent defibrinated blood), 20 per cent of horse serum, 20 per cent of a sterile filtrate of a *S. moniliformis* serum-broth culture, and 2 per cent agar] that isolation and continued maintenance of L_1 in "pure" culture was possible. After many subcultures old strains of L_1 were able to grow on 30 per cent horse-serum agar without the added filtrate. When it is grown in fluid medium the broth itself remains clear and large colony clumps are found sticking to the wall or as a sediment at the bottom of the tube. Klieneberger has stressed the necessity of repeated purifications of L_1 cultures, since some strains were found often to revert to *S. moniliformis* in the early passages. She has maintained several strains of L_1 cultures for over 100 to 300 passages without reversion to *S. moniliformis* either in her own hands or in anybody else's (personal communication from Doctors L. Dienes and J. Warren). Because of these facts and because a pleuropneumonia-like micoörganism, morphologically, culturally, and immunologically identical with L_1, was isolated on at least one occasion from the lungs of a rat without *S. moniliformis*, Klieneberger has maintained that the *Streptobacillus moniliformis* represents an intimate symbiosis between the streptobacillus and L_1. This concept was challenged by Dienes (23, 24) who suggested that the L_1 microörganism was but a variant of the streptobacillus chiefly on the grounds that the strains of L_1 which he isolated and which apparently grew in pure culture on solid medium reverted to *S. moniliformis* in broth cultures, and partly because, in his opinion, the morphology of the L_1 microörganism as well as of other pleuropneumonia-like microorganisms does not differ materially from that of bacteria. Klieneberger's (49) recent comment on this controversy is very enlightening:

"To make the position quite clear it should be pointed out that Dienes and the writer have both been able to separate the so-called L_1 form from the parent culture of *S. moniliformis* and have maintained it in pure condition. They both agree that the L_1 consists of granules, filamentous forms and pleomorphic bodies, while the *S. moniliformis* cultures contain bacillary chains in addition to these elements. The two chief points of disagreement are the following. Dienes finds his L_1 strains reverting into the parent culture under certain conditions, while the writer's strains [*i.e. the highly purified ones—A. B. S.*] have so far not reverted under similar conditions. Dienes believes further that the globular forms are swollen bacilli and that the filamentous forms in the L_1 correspond to the bacilli, while the writer believes that the globular forms develop independently and that they resemble as well as the filamentous and granular forms elements seen in the cultures of pleuropneumonia and agalactia. . . .

"The reasons why the writer is still in favor of the symbiosis hypothesis are the following. The bacillary elements of *S. moniliformis* show by their shape, arrangement, multiplication mode and their bacterial "rigidity" that they are true bacilli while the L_1 growth contains no ordinary bacillary forms, but elements resembling those of the organism of pleuropneumonia bovis. Like pleuropneumonia the L_1 cultures contain a large amount of small granules of different shape some of which are not much larger than vaccinia bodies; the globules which form an integral part of both are not to be compared with swollen bacterial forms as Dienes suggests. . . . Besides possessing a similar morphology, L_1 and the organisms of the pleuropneumonia group show also the same colony type. . . . There is further the regular filterability of L_1 and the pleuropneumonia-like organisms through some of the coarser filters such as the Berkefeld V candle. It seems a reasonable conclusion from these data that the L_1 is itself a pleuropneumonia-like organism and consequently unlikely to prove a variant growth form of the streptobacillus. There is the further argument that if the L_1 is a variant form of *S. moniliformis*, the now numerous strains of the L series occurring independently should be derived from streptobacillary mother strains, but evidence of the existence of such is not forthcoming."

One of the cardinal characteristics of the members of the pleuropneumonia group is the possession of reproductive units which are in the range of 125 to 250 mμ in size. Filtration

through Berkfeld candles, which often permit ordinary bacteria to pass and whose pores are large and irregular, cannot be relied upon for this essential information. It is regrettable, therefore, that no filtration experiments with the L_1 cultures through gradocol membranes of known A.P.D. have as yet been carried out or reported. If L_1 cultures could be proved to contain such minute reproductive units, and if these minute units, isolated from early passage cultures by gradocol membrane filtration, were shown to be capable of reproducing *S. moniliformis*, the case for the variant hypothesis would be definitely stronger. Since the bacillary elements in a culture of *S. moniliformis* incubated for 2 days or longer undergo rapid disintegration, it would also be significant to determine whether or not minute elements of the same order of magnitude which could reproduce the original streptobacilli could be obtained by gradocol filtration. Since *S. moniliformis* is a "mixed" culture it is not surprising that a serum prepared against it agglutinates the L_1 microörganisms as well as or better than a homologous serum, and *vice versa*. The preliminary results with agglutinin-absorption reported by Dawson and Hobby (21) do not warrant the conclusion that *S. moniliformis* and L_1 have an identical antigenic make-up. It is perhaps of interest in this respect, that Klieneberger (49) found that immune serums against the L_4, L_5, L_6, and *Asterococcus canis* I microörganisms agglutinated L_1 suspensions in titres varying from 1:10 to 1:80 while the immune serums against other types as well as various bacterial immune serums and normal serums had no effect on these suspensions. There is a suggestion, therefore, that L_1 is not in a class by itself but rather that it may possess a common antigen with some of the other members of the pleuropneumonia group.

To what extent, if any, L_1 contributes to the pathogenicity of *S. moniliformis* is obscure since, in pure culture, it is completely nonpathogenic. It would, therefore, help to elucidate further the relationship between these microörganisms if it were known whether or not immunization with L_1 produced resistance in mice against infection with *S. moniliformis*.

Knowledge of the true relationship between L_1 and *S. monili-*

formis would greatly add to our understanding of the filtrable microörganisms of the pleuropneumonia group. For if L_1 is proved beyond doubt (proper filtration experiments still need to be carried out) to belong to this group, its origin as a variant from *S. moniliformis*, if proved, would be an indication of how the other members of this group might have originated at one time. On the other hand, if it is proved to be a symbiont, the failure, thus far, to find any strain of the streptobacillus, which is free from L_1 or which can be experimentally separated from it, suggests an association by far more intimate than that represented by ordinary symbiosis. The symbiosis of L_1 with *Clostridium tetani* and *C. tetanomorphum* which Klieneberger (47) was able to establish experimentally and to maintain in over 100 passages, differed from that of *S. moniliformis* in that both microörganisms could be recovered in a pure state. Klieneberger has also encountered a gram-positive streptococcus or streptobacillus in the nasopharynx of guinea-pigs, a microörganism grown from an extracted human tooth (44), and gram-positive cocci from the skin of a pig (49) inoculated with swine-pox which gave every appearance of existing in "symbiosis" with microörganisms similar to L_1, but she was unable to separate the L_1-like elements in pure culture. With regard to the peculiar large forms which have on occasion been observed in *Bacterium fundiliforme* (syn. *Fusobacterium nucleatum*), *Hemophilus influenzae* and a *Flavobacterium* (26, 27, 49), Klieneberger (49) remarks that "the mere observation of swollen bodies or globular forms is not sufficient evidence to prove the existence of a filterable, pleuropneumonia-like symbiont or growth phase in these cultures."

FILTRABILITY AND SIZE OF MINIMAL REPRODUCTIVE UNITS

The development of reproductive units or elementary bodies, of the same order of magnitude as that of the larger viruses, in their life cycle constitutes one of the cardinal characteristics of the members of the pleuropneumonia group. Filtration through the ordinary bacteriological filters cannot be relied upon to establish this important property, and only data obtained by filtering suitably prepared material through gradocol membranes

can be used in estimating the size of the minimal reproductive unit. Laidlaw and Elford (52), in writing of the sewage microorganisms, stated: "In its general filtration behavior the organism resembles bovine pleuropneumonia and agalactia and contrasts with viruses and bacteriophages, which, individually, have been found to be relatively uniform in particle size as evidenced by the fact that no appreciable drop in filtrate concentration, as compared with the original, is detected until the porosity of the membrane used is about twice the true end-point value." This marked drop in titre following filtration through membranes of relatively large A.P.D. was also found by Edward (31) in tests on one of the strains he isolated from mouse lungs. The smallest units or elementary bodies actually form only a very small proportion of the total number of reproductive units that are present in a culture.

The larger polymorphic structures in the cultures may be a source of error in filtration experiments and should be removed by centrifugation and preliminary filtration through membranes with average pore diameters of 0.7 to 0.9 μ. As an example may be mentioned the experience with the Type A microörganism of mice. With the infectious agent in the form of mouse-brain suspension in broth which had been highly centrifuged and well diluted, it passed a 980 mμ membrane but was retained by membranes with an A.P.D. of 848 mμ or smaller. When the 980 mμ membrane filtrate was used for further filtration, it passed a 720 mμ but was retained by 628 mμ. When a culture of the Type A microörganism in serum broth which exhibits only a diffuse slight opalescence was filtered without preliminary centrifugation it passed a 584 mμ membrane but was retained by the membranes with an A.P.D. of 500 mμ or smaller; after preliminary, high-speed, horizontal centrifugation of the culture it passed the 500 mμ membrane but was retained by that with an A.P.D. of 396 mμ. On this basis one might estimate the size of the minimal reproductive unit of the Type A microörganism at 200–300 mμ, but further tests need to be carried out with cultures that have been better prepared. Thus the same filtration end-point was obtained with a centrifuged culture of the Type B

microörganism, but when the 584 mμ membrane filtrate was used for further filtration it was found that even the 322 mμ membrane filtrate yielded positive cultures. The filtration end-points reported by Findlay, *et al.* (35) for L_5 both in tissue suspension and in culture are practically identical with those for Type A given above, except that it would appear that they probably did not centrifuge their culture before filtration. Regarding the filtrability of L_7 (the etiological agent of polyarthritis in the rat and serologically identical with L_4) Findlay, *et al.* (37) reported that it passed membranes with an A.P.D. of 560 and 440 mμ but not that of 300 mμ. Since they did not indicate how the culture was treated preliminary to filtration, the final estimate of size must await a more detailed report.

TABLE 1

Data on the size of the minimal reproductive units

MICROÖRGANISM FROM	A.P.D. OF MEMBRANE PERMITTING PASSAGE	A.P.D. OF MEMBRANE WITH COMPLETE RETENTION	ESTIMATED SIZE OF MINIMAL REPRODUCTIVE UNIT
	mμ	mμ	mμ
Pleuropneumonia bovis	250–300	250	125–175
Agalactia	?	300	150–225
Sewage	260	250	125–175
Mouse lung (Edward)	450	330	165–247

The pleuropneumonia, agalactia, sewage, and one of Edward's mouse-lung microörganisms are the only ones on which properly performed filtration experiments have been reported, although additional tests would be desirable on the mouse organism because the gap between A.P.D. 330 mμ and 450 mμ is somewhat too large. Table 1 presents some of the essential data.

TOXIGENICITY AND IMMUNOLOGICAL REACTIONS

Toxin Production

The first suggestion that a microörganism of the pleuropneumonia group may produce an exotoxin came when Nocard, *et al.* (63) observed that the presence of collodion sacs, containing

growing cultures of pleuropneumonia bovis, in the abdominal cavity of rabbits often led to extreme cachexia and death. Since there were no obvious lesions at necropsy and the microörganisms could be cultured neither from the blood nor from the viscera, and since the implantation of many collodion sacs containing only uninoculated medium was not harmful to the rabbits, they concluded that they were dealing in all probability with the diffusion from the collodion sacs of a toxin elaborated by the growing microörganisms. They stated, in fact: "Voilà donc un nouvel exemple d'un animal très sensible aux toxines d'un microbe contre lequel il est pourtant tout à fait réfractaire." In keeping with this is the observation that cows may die after subcutaneous injection of infected lymph or culture, although the only lesion is at the site of inoculation. It is surprising, therefore, that no work appears to have been done or reported on attempts to establish whether or not the microörganism of pleuropneumonia bovis actually gives rise to a true exotoxin.

There is, however, one member of the pleuropneumonia group, i.e. the Type A microörganism of mice, which unquestionably produces a true exotoxin during its growth both *in vivo* and *in vitro* (74, 75). It is called a true exotoxin first because it is present in sterile filtrates during the early period of growth (i.e. even before there is gross evidence of growth), second because it is thermolabile being destroyed at 50° in 30 minutes but not at 45° in the same period (the microörganism itself is killed at 45° in 15 minutes), and third because it is antigenic being specifically neutralized by antitoxin. The existence of this toxin was suspected even before its isolation from cultures, because the extensive brain lesions, which were found in mice dying 17 to 48 hours after intra-abdominal or intrathoracic injection of the infectious agent, were not associated with the presence of any transmissible agent. The production of this toxin *in vivo* is of special interest because all the indications are that under those circumstances the Type A microörganism multiplied only in the mesothelial cells of the peritoneum and pleura. There is thus an example of a toxin elaborated in the cells of one organ producing its effect by acting on those of another (the brain). The evidence

that the toxin produced *in vivo* is identical with that *in vitro* is based on the fact that they both give rise to the same clinical signs, produce the same neurolytic change in the posterior pole of the cerebellum, and are immunologically identical.

The amount of toxin in a culture is relatively small, an intravenous injection of 0.1 to 0.5 ml. being required to demonstrate it. It disappears from the cultures after about 2 days of growth. Three-weeks-old mice are most susceptible and animals older than 2 months usually do not react. After intravenous injection of the whole culture or a Seitz filtrate the first nervous signs, i.e., convulsions or rolling, may appear as early as 5 to 10 minutes, usually within 1 to 2 hours, and in older mice may be delayed for 6 to 9 hours. Most mice die within a few hours or less after inoculation; these show no lesions in the brain or anywhere else. The mice that survive with persistent nervous signs, show the typical neurolytic lesion in the cerebellum as early as 18 hours after inoculation. The toxin continues to be produced in cultures even after more than 100 subcultures *in vitro*. In preliminary tests on rats carried out together with Doctor Joel Warren it was found that intravenous injection of 1 ml. of an active "A" culture produced nervous signs and death within a few hours in four 36-day-old animals but was harmless in adult rats.

Similar tests in mice with cultures of all the other pleuropneumonia-like microörganisms of mice, of L_3 and L_4 of rats, and of an old passage strain from pleuropneumonia bovis yielded no evidence of the presence of a toxin.

Immunological Reactions, Groups, and Types

Immunization of rabbits with cultures of the microörganisms of the pleuropneumonia group leads to the development of agglutinins, complement-fixing and neutralizing antibodies. The development of antibodies during the course of actual infection in susceptible hosts seems less definite, although some animals with pleuropneumonia or agalactia have agglutinins. Also no neutralizing antibodies were found in the serum of recovered animals that were resistant to reinfection with the neurolytic, Type A microörganism of mice (74), the filtrable pyogenic agent

(L_4) of rats (101), and the microörganism of rat polyarthritis (13, 18). Neutralizing or protective antibodies, however, are easily demonstrable in the sera of immunized rabbits (79) and in hyperimmune sera prepared in sheep, goats, and horses (6, 30, 64).

The members of the pleuropneumonia group have been differentiated into a large number of distinct serological types with the aid of the agglutination reaction. All the strains from pleuropneumonia bovis appear to belong to a single serological type (47, 94) which is not related antigenically to any other member of the group. The same is true of all the strains from agalactia. Those found in the dog belong to two distinct serological types which while not related with one another may according to Klieneberger (49) have a common antigen with some of the microörganisms of rats and mice. Three distinct serological types have thus far been identified among the microörganisms isolated from rats, and five distinct types among those isolated from mice. The existing data indicate that many of the murine strains have one or more antigens in common, some being more closely related than others. The microörganisms originally isolated from sewage were found to belong to three serological types: A, B, and C, with A and B possessing an antigenic relationship. Klieneberger (49) compared all the available saprophytic microörganisms from England (Laidlaw and Elford) and Germany (Seiffert) with one another and with the parasitic ones and found (a) that they all fell into the three original types with Type C consisting of a single strain unrelated to any other member of the group, and (b) that the saprophytic and parasitic microörganisms had no antigenic relationship, excepting that the L_4 serum agglutinated many of the saprophytic strains to a titre of 1:10 or 1:20, although none of the saprophytic sera agglutinated L_4.

It is interesting to note (a) that each microörganism that has distinguishing characteristics as regards biological and pathogenic properties is also serologically distinct, and (b) that each animal species seems to carry its own serological types, with the possible exception of some of the rat strains which on rare occasions may be transmitted to mice under natural conditions.

PATHOLOGY

There is probably no pathological picture that is characteristic of effects of the pathogenic members of the pleuropneumonia group. Some of them seem to have distinct tissue affinities while others have not. Thus the microörganism of agalactia, regardless of the route of inoculation, can localize in the joints, the eyes, and the mammary glands of the lactating animals; the Type B microörganism of mice, after intravenous or intraperitoneal injection, will localize and multiply only in the joints; the Type A microörganism of mice will multiply in the cells of the peritoneal or pleural coverings of the organs but not in their parenchymal cells. On the other hand, there are others that will multiply at the port of entry without spreading very far. Thus, when infected lymph or a virulent culture from pleuropneumonia bovis is injected subcutaneously it produces a severe local reaction but not pleuropneumonia; the same is also true, to a certain extent, of the L_4 microörganism of the rat which will produce abscesses at the site of injection and will multiply wherever it is distributed after intravenous injection.

The question of whether or not cytoplasmic or intranuclear inclusions occur is one which requires further study. Meyer (58), Gaiger and Davis (39) found no such inclusions in bovine pleuropneumonia, and Tang, *et al.* (95) could find none in the cells of the chorioallantoic membrane of the chick embryo inoculated with the pleuropneumonia microörganism. Woglom and Warren (101) reported the presence of acidophilic, cytoplasmic inclusions (readily seen in their photographs) in the epithelium overlying the abscesses of 12 to 24 hours' duration which were produced by the filtrable pyogenic agent subsequently shown to be identical with L_4. These inclusions have not yet been reproduced with cultures, but further study of their relationship to the microörganism is definitely indicated. I found the Type A microörganism of mice scattered throughout the cytoplasm of the mesothelial cells of the peritoneum and pleura when Giemsa-stained films of fresh tissue were examined; in sections of fixed tissues, however, I found more compact inclusions in the cyto-

plasm of the pleural cells, but was unable to see them in the peritoneal cells over the liver and spleen. Sections of the joints of mice with experimental arthritis, produced by either type Type A or Type B, showed neither inclusions nor any other intracellular or extracellular structures which could be identified as the microörganisms.

The primary attack of the microörganism of bovine pleuropneumonia appears to be on the connective tissue with the development of marked edema and large amounts of serous exudate. In the natural disease it is the interlobular and subpleural connective tissue network that is affected with subsequent vascular thrombosis and pulmonary necrosis. There may be only a fibrinous pleurisy over the consolidated lung and no effusion in the pleural sac but with large collections of fluid underneath the pleura and along the perilobular connective tissue. At other times, however, the pleura may be covered with a layer of fibrin 1 to 2 cm. thick and there may be anywhere from 1 to 20 litres of reddish-yellow or yellowish clear exudate in the pleural cavity (63, 97). After subcutaneous injection of infected lymph or virulent culture the lesion is one of extensive edema, the connective tissue is gelatinous and masses of clear, amber fluid are found along its extensions. Histologically, K. F. Meyer (58) reported that thick rings of leucocytes and fibrin deposits around the interstitial blood vessels were rather characteristic. According to Ziegler (104) the necrosis, interstitial perivascular and marginal organization processes, and parabronchial changes are diagnostically significant.

In agalactia, the process in the mammary gland is that of an interstitial mastitis characterized by a disappearance of glandular tissue with invasion of young fibrous tissue. Suppuration is extremely rare. The eye lesion is that of an interstitial parenchymatous keratitis with infiltration of small cells and vascular proliferation in the thickened cornea. In the joints the process is usually limited to congestion, cellular infiltration and thickening of the periarticular connective tissue without involvement of the synovia or cartilage; occasionally, however, there is proliferation, thickening, focal necrosis and cellular infiltration of the synovia and erosion of the articulating cartilage and more rarely still

there is formation of fungous polyps consisting of young connective tissue. As a rule, these lesions completely regress and ankylosis is very rare. An endarteritic process can be seen in the joint tissues (9).

The pathological changes produced by the Type A microorganism of mice vary with the site into which it is inoculated. Its multiplication in the peritoneum and pleura gives rise to no local lesions, but the toxin that is elaborated produces not only a distintegration of the posterior pole of the cerebellum but also intense degenerative changes without inflammatory reaction in the neopallial cortex, basal ganglia, and nervous part of the retina (74). When the joints are affected after intravenous injection the process is limited to the periarticular connective tissue and synovia (proliferation and subacute inflammation) without involving the articular cartilage. There is always complete recovery and no ankylosis. With the Type B microorganism, on the other hand, the pathological changes are limited to the joints and involve the capsule, synovia, articulating cartilage and subchondral bone marrow. The essentially proliferative character of the process is apparent as early as the second day after swelling of the joint is discernible and becomes more marked subsequently, affecting the capsule, synovia and perichondrium to such an extent that obliteration of the free joint-space may result. Actual destruction of cartilage has not been seen until about 4 weeks after intravenous inoculation, and at 7 weeks one may find the normal articulating cartilage replaced by undifferentiated cells which may be immature chondroblasts or osteoblasts. In a completely ankylosed joint, at about 5 months, one finds that considerable ossification has occurred in the distorted articulating surfaces which are joined by dense fibrous tissue (76, 78).

The pathological effects produced by the L_4 microörganism of the rat, either as the filtrable pyogenic agent or as the etiological agent of the spontaneous polyarthritis, appear to be quite different from those described above, and are influenced by the fact that the microöorganism has no special tissue affinities. Frank suppuration with the formation of an encapsulated abscess occurs at the site of subcutaneous injection in the rat, while in the mouse

massive edema develops involving the whole side of the animal, microscopic examination of which reveals extensive hemorrhagic necrosis with infiltration of polymorphonuclear leucocytes. After intravenous injection "the suppurative lesions in the feet involved the soft parts only, the bone itself being spared though the marrow was thickly strewn with polymorphonuclear leucocytes" (101). In the spontaneous polyarthritis of rats the process appears to be similar and sometimes "while the joint is still swollen, the skin may ulcerate, and the limb below the joint may be gnawed off by the rat." (37)

EFFECTS OF IMMUNE SERUMS, VACCINES, AND CHEMOTHERAPEUTIC AGENTS

Wherever the effect of immune serums has been studied against natural or experimental infections produced by members of the pleuropneumonia group, the result has generally been that a distinct protective effect could be obtained when the serum was administered before infection but that even early after infection there was either no therapeutic effect or only a doubtful one. Nocard, *et al.* (64) showed this to be the case in pleuropneumonia bovis with hyperimmune bovine serum, and Dujardin-Beaumetz (30) reported a similar result with hyperimmune horse serum. Carré (9) demonstrated the protective effect of hyperimmune serums prepared with agalactia "virus" and Bridré and Donatien (6) obtained similar results with cultures of the microörganism although they found it more difficult to protect against intra-articular than against subcutaneous inoculation; curative tests on a few animals during the first few days of the disease were without effect or inconclusive. Sabin and Morgan (79) showed that potent immune rabbit serum which could completely protect mice against the polyarthritis produced by Type B and the nervous and arthritic manifestations of Type A, had little or no effect when administered after the first clinical signs of disease had appeared.

Bovine pleuropneumonia is among the first diseases whose control was early attempted by protective vaccination. The earliest successful results were obtained by subcutaneous injection

of infectious lymph (serous exudate) into the tail (97, 99). After cultivation of the microörganism the same results were obtained with living cultures, but in both instances the reactions were not only severe but sometimes fatal. Recently, Purchase (73) studying control of the disease in Rhodesia found that while the 11th to the 45th generations of *in vitro* cultures were harmless to cattle, they gave rise to distinct immunity lasting for 2 years, and yielded fairly satisfactory results in the field; he also noted that vaccination was of no value when signs of disease had appeared. Kurotchkin (51), however, reported that in the heavily infected regions of China a field test with 520 animals revealed that "whereas no arrest of epizootics occurred when attenuated culture was used, rapid eradication of the disease was obtained after immunisation with virulent culture." In agalactia, Bridré and Donatien (6) found that: (a) living cultures could not be used because they produced the disease regardless of the route of inoculation, and noninfective doses did not give rise to immunity; (b) even after 100 passages *in vitro* the cultures could not be used because they had not completely lost their virulence; (c) various combinations of serum and cultures yielded no immunity if disease was prevented; and (d) no immunity was produced by cultures killed at 56° for one hour or by formalin. It is of interest, therefore, that Sabin and Morgan (79) found that vaccination with concentrated suspensions of the mouse Type B microörganism killed at 50° (30 minutes) could protect the majority of mice against the experimental polyarthritis although it was practically ineffective when it was begun after the first clinical signs of disease had appeared.

Effect of Chemotherapeutic Agents

In 1928, Bridré, *et al.* (7) reported that the subcutaneous injection of the sodium salt of stovarsol (acetylamino-hydroxy-phenylarsonic acid) exerted both a preventive and curative action on agalactia contagiosa, as it occurs in nature and as it is produced experimentally in sheep and goats. It is interesting that the reason these investigators chose stovarsol for this chemotherapeutic test is that the microörganism of agalactia is

stained by the same dyes as the protozoa but not by the usual (aniline) bacterial stains. In 1938, Pigoury (69) indicated that even a single dose (0.75 gm.) of stovarsol had a distinct therapeutic effect on the course of the mastitis and arthritis in 27 animals, while among 25 others treated with urotropine and in 5 untreated controls the disease pursued its normal course. He also found that a single dose of stovarsol did not protect a well animal for more than a week. Witt is quoted (38) as having observed a beneficial effect of arsenicals in bovine pleuropneumonia.

Two organic gold compounds, aurothioglucose (solganal B) and aurodetoxin, were found capable of preventing the development of an arthritis in rats which was subsequently shown to be caused by a microörganism of the pleuropneumonia group (14, 19). When Findlay, *et al.* (37) described the pleuropneumonia-like microörganism which causes the spontaneous polyarthritis of rats in London, they also confirmed the preventive effect of aurothioglucose on the development of the experimental disease. In further chemotherapeutic experiments Findlay, *et al.* (38) studied the capacity of various chemical compounds to *prevent* (a) the "encephalitis" which results from the intracerebral injection of the L_4, L_5, and L_6 microörganisms in mice, and (b) the "arthritis" which develops chiefly in the inoculated extremity following the injection of L_4, L_5, L_6, L_7 (which serologically is identical with L_4), and a guinea-pig strain of *Streptobacillus moniliformis* into the foot-pads of mice and rats. The various compounds were administered subcutaneously immediately after the infectious agents and daily thereafter for 3 days. The authors concluded that (a) *as regards prevention of the encephalitic syndrome and death*, sulfanilamide, sulfapyridine, and sodium salicylate were without effect, neoarsphenamine, acetarsol (stovarsol), and to a less extent tryparsamide were slightly active against L_5 and L_6 but not L_4, while aurothioglucose protected the majority of mice against all three; (b) *as regards prevention of the arthritis*, sodium salicylate, lithium antimonyl tartrate were without effect and neoarsphenamine had a slight effect when tested against L_7, while the organic gold compounds (aurothioglucose, sodium aurothiomalate, lopion, parmanil, and neosolganal) were highly

active against all the microörganisms tested; (c) "organic gold preparations are bactericidal to pleuropneumonia-like organisms both *in vitro* and *in vivo*."

The "arthritis" produced by these microörganisms developed in 2 days and treatment started at that time or later did not appear to result in a disappearance of the lesion in the subsequent 10 days. The authors believed, however, that there "appeared to be some slowing up of the infective process, but since the swelling and arthritis are self-limiting and tend to disappear eventually in the absence of any treatment, it is not easy to judge how far solganal B affects the process once it is fully developed."

The curative effect of certain gold compounds was established in studies carried out by Sabin and Warren (82) on the experimental, proliferative chronic arthritis of mice produced by Type B. They found not only that a curative effect could still be obtained when treatment was delayed as long as 3 weeks, but also that a therapeutically active gold compound had no apparent effect *in vitro* on the microörganism causing the arthritis. Growth, even of minimal inocula, was not inhibited or prevented by concentrations of sodium aurothiomalate varying from 1 to 200 mgm. per cent in the fluid media (a single dose of 2 mgm. can cure mice), and microörganisms grown for several generations in the presence of gold did not lose the capacity to produce arthritis. While the discrepancy between these results on the effect of gold compounds *in vitro* and those of Findlay, Mackenzie, and MacCallum may be due to the fact that different microörganisms were used, it is my opinion, rather, that the conclusion of the latter workers was based on insufficient data (which, as they indicated, they were prevented from enlarging by circumstances beyond their control). Neoarsphenamine, silver arsphenamine, bismuth subsalicylate, sodium salicylate and colloidal gold were without effect on the chronic arthritis in mice. Sabin and Warren have used this experimental disease in mice in a search for chemotherapeutic agents with high curative properties and low toxicity, and have recently reported the preparation of a new compound, calcium aurothiomalate, which is practically nontoxic in mice and yet produces a curative effect after a single dose of 1 mgm. (83).

It is of interest to note that in at least two diseases (agalactia contagiosa and experimental chronic arthritis in mice) caused by members of the pleuropneumonia group, chemotherapeutic agents possess a curative effect which is not exerted by the specific immune serums.

POSITION OF PLEUROPNEUMONIA GROUP IN RELATION TO VIRUSES, RICKETTSIAE, COCCOBACILLIFORM BODIES, AND OTHER FILTRABLE AND NONFILTRABLE BACTERIA

For comparative purposes one may perhaps define the filtrable microörganisms of the pleuropneumonia group as polymorphic forms, which can (a) multiply in cell-free media and produce minute colonies (usually not exceeding 0.6 mm.) on suitable solid media, (b) reproduce in several different ways, and (c) produce, as part of their life cycle, elementary bodies (usually 125–250 mμ in size) capable of reproducing the larger, more polymorphic and complex forms. One may add that while they are best visualized tinctorially by the staining methods used for protozoa, and while in pathological material or in cultures containing a great deal of serum they are not revealed by gram-staining or the usual aniline dyes, they can, nevertheless, be so stained (especially in smears of centrifuged cultures) and are then gram-negative. What they have in common, then, with the viruses is chiefly the small size of one phase of their life cycle. The viruses, however, are characterized not only by their small size but also by the fact that they do not possess in their make-up the constituents which would permit them to exist as autonomous units of life, that is, they must depend upon some more highly organized living cell to supply the materials or functions by which they can multiply (or increase in amount) and thus perpetuate themselves. It has occasionally been assumed that the very small size of even the larger viruses precluded their possession of enough of those enzyme systems which make possible the existence of independent living units. Relatively, therefore, it is of interest that in the microörganisms of the pleuropneumonia group we have examples of minute particles, which, while not exceeding in size many of the typical larger viruses, nevertheless, possess the properties of

independent living units. And yet there appears to be, in addition to size, still another link to the viruses. For with certain members of the pleuropneumonia group, this property of independent existence may be only a matter of adaptation. I am referring to the Type A and Type B microörganisms of mice which in the animal body do not seem to be able to multiply until they locate in the right type of cell where they may be seen to proliferate in the cytoplasm. Upon being transferred to cell-free media, the assumption of independent life by these minute particles seems to depend upon the appearance of a special mechanism which may be so specific that after adapting themselves to the serum protein of one animal, they cannot survive when the protein of another animal species is substituted. (Personal observations on some strains of the mouse pleuropneumonia group). Thus one finds in this group the minutest microbial units endowed with the living functions of independent growth and reproduction, a property which, in the present state of our knowledge, clearly differentiates them from the viruses.

Zinsser (105) defined the rickettsiae, as "minute bacillary or diplococcus-like organisms which are pleomorphic to the extent that range of size and shape may vary from that of minute, just-visible coccobacilli to longer filaments which sometimes approach bacillary size. Although not unstainable by ordinary methods of bacterial staining, as formerly supposed, they are best brought out by special methods—most useful among which are Giemsa and the stain of Castaneda." He further said that "the *Rickettsiae*, in spite of many claims to the contrary, have never been cultivated without the presence of living cells." More recently Zinsser and Schoenbach (106) indicated that the obligate intracellular parasitism of the pathogenic rickettsiae differed from that of the viruses in that multiplication of the former was favored by metabolically inactive cells. This obligate intracellular parasitism as well as the absence of the marked polymorphism thus readily differentiate the rickettsiae from the pleuropneumonia group.

In 1935, Nelson (59) reported certain minute *coccobacilliform bodies* which were associated with infectious fowl coryza, and in

later communications he described morphologically and culturally similar microörganisms as the cause of infectious catarrh of mice (60) and albino rats (62). The *coccobacilliform bodies* as they appear in exudates and in cultures, consisting of minced chick embryo suspended in Tyrode's solution, are gram-negative. While originally, they do not grow on ordinary media with or without blood or serum, Nelson found that they are not obligate intracellular parasites, because they can grow in the Tyrode's solution extract of embryonic tissue, free of cells (61). Furthermore this diffusible growth factor withstands heating at 100° for 60 minutes. Nelson also found that after many passages one strain of fowl coryza bodies could grow in the fluid blood at the base of an agar slant. No colonies were seen on the agar but under similar conditions microörganisms of the pleuropneumonia group are also most unlikely to form colonies. In describing the rat catarrh *coccobacilliform bodies*, Nelson (62) pointed out that they exhibited greater pleomorphism than those of fowl coryza; in cultures "at 48 hours it was customary to find small uniformly stained spherical cells, slightly larger ring forms with a central unstained area, short uniformly stained rods, short forms with a bipolar appearance, and small curved or spindle-shaped rods." The mouse catarrh bodies present in exudate could be filtered through a gradocol membrane with an A.P.D. of 640 mμ (60). Nelson (62) further states that "it is recognized that there is a morphological similarity between the *coccobacilliform bodies*, particularly those of mouse and rat catarrh, and the organisms of the pleuropneumonia group." While the apparent ease with which the bodies are revealed by the Gram's method and the absence of colonies on solid media may be regarded as distinguishing characteristics, it would seem that not enough work has as yet been done by the methods employed in studying the microörganisms of the pleuropneumonia group to permit either inclusion or exclusion of the *coccobacilliform bodies*.

As regards certain well-known filtrable bacteria such as the anaerobic *B. pneumosintes* (*Dialister pneumosintes*), the members of the genus *Veillonella* including *Staphylococcus parvulus* (*Veillonella parvula*), *Staphylococcus* or *Micrococcus minimus*, etc., and

others, almost the only property they possess in common with the microörganisms of the pleuropneumonia group is their relatively small size, lacking altogether the capacity to give rise to the considerably larger and more complex polymorphic forms. Among the other numerous species of filtrable, gram-negative, anaerobic bacteria found in the human nasopharynx, Olitsky (67) has described some as being pleomorphic, tenuous, vibrio-like and forming minute colonies on solid media. Not enough is known about these microörganisms to permit their arbitrary exclusion from the pleuropneumonia group, and it may indeed be worth while to restudy them by the more recently developed methods. Laidlaw and Elford (52) writing of the pleuropneumonia-like sewage microörganisms, stated: "We do not consider that this group is related in any way to the 'filterable forms' of the pathogenic bacteria which have been described by many, e.g., Hadley, Delves, and Klimek (1931), or Kendall (1931 and 1932). The existence of these filterable or 'virus' forms is still contested, but in any case they arise under ill-defined conditions or as the result of special treatment (Hadley *et al.*), or on a particular kind of medium (Kendall). The 'virus' forms are said to multiply for a time, as such, but may, under appropriate conditions, revert to the larger form, which in turn, multiplies as such."

When one inquires in what way, if any, the microörganisms of the pleuropneumonia group differ from all the other bacteria, i.e., the class of *Schizomycetes* one is confronted chiefly with questions concerning the extreme polymorphism and mode of reproduction. On these questions there is not much uniformity of opinion at the present time. As the name itself suggests multiplication among the *Schizomycetes* is characteristically by cell fission, with endospores "formed by some species of the *Eubacteriales*, [and] conidia by some of the filamentous forms." (2) Turner (96) discussing the microörganism of pleuropneumonia bovis in 1935 stated: "What distinguishes it most clearly from other members of the class *Schizomycetes* is 1) its extreme pleomorphism, 2) the characteristic polygenethodism, or provision of alternative modes of reproduction, and 3) its peculiar

protean or amoeboid tendency to change its shape relatively quickly, which is seen nowhere else among the *Schizomycetes.*" When Turner wrote this there were only two species in the pleuropneumonia group, and despite his appreciation of the fundamental difference between them and the *Schizomycetes*, he suggested that they be classified as a separate order under the *Schizomycetes.* It seems to me, however, that if one accepts Turner's viewpoint as applying to the entire group, as I am inclined to do, one cannot classify these microörganisms under the *Schizomycetes* without modifying the definition of that class.

CLASSIFICATION AND NOMENCLATURE

It is not without misgiving that I attempt to classify and name the ever-increasing number of microörganisms in this group. That the job has to be done cannot be denied but it is doubtful that it can be done without having almost everyone else disagree with the result. The very title of this review is in a sense misleading for it pretends to describe a pleuropneumonia group of microörganisms when, with one exception, they have nothing to do with pleuropneumonia. One thing is certain, and that is that we cannot go on calling all the known, as well as yet to be discovered, microörganisms pleuropneumonia-like without twisting our tongues and confusing our minds.

In the preceding section on the position of these microörganisms in the microbial world, I set forth the reasons for my belief that they belong in a class distinct from the *Schizomycetes.* It is suggested, therefore, that a new class be established and that it be called *Paramycetes.* Because I could not think of anything as short that would at the same time be descriptive of the cardinal characteristics of the group, and because para (*παρα*) meaning beside or alongside suggests the position of this class as being close to the others, this name was selected to include all the microörganisms which because of morphology or mode of reproduction cannot be included with either the *Schizomycetes* or *Eumycetes.* One order to be called *Paramycetales* is proposed and it is to be characterized by the properties which were previously

given as applying to all the microörganisms of the pleuropneumonia group. Since the parasitic species are quite distinct from the saprophytic ones, it is suggested that two families be established: family I: *Parasitaceae* to include all the pathogenic and nonpathogenic microörganisms found in animals, all requiring a suitable amount of protein for growth; family II: *Saprophytaceae* to include the nonpathogenic microörganisms, requiring no protein for growth, found in sewage, soil, etc. Since the parasitic members are most clearly distinguished from one another, biologically and immunologically, according to the species of animal they inhabit and since a number of distinct species of these microörganisms have been found in the same animal, it is suggested that the names of the animals be incorporated in the names of the genera. Thus:

Family I. *Parasitaceae*

Genus I. *Bovimyces* (microörganisms of cattle)

Species. *Bovimyces pleuropneumoniae*, the etiological agent of pleuropneumonia contagiosa bovis

Genus II. *Capromyces* (microörganisms of goats and sheep)

Species. *Capromyces agalactiae*, the etiological agent of agalactia contagiosa of goats and sheep

Genus III. *Canomyces* (microörganisms of the dog)

Species. *Canomyces pulmonis* I, *Canomyces pulmonis* II } Distinct microörganisms found in the respiratory tract

Genus IV. *Murimyces* (microörganisms of rats, occasionally of mice)

Species. *Murimyces streptobacilli-moniliformis*, usually in association with *Streptobacillus moniliformis* but occasionally independently (L_1)

Murimyces pulmonis, usually in association with certain lung lesions in the rat (L_3)

Murimyces arthritidis, etiological agent of spontaneous arthritis in the rat (L_4)

Genus V. *Musculomyces* (microörganisms found only in mice)

Species. *Musculomyces neurolyticus*, produces an exotoxin with a neurolytic action especially on cerebellum (Type A)

Species. *Musculomyces arthrotropicus*, has an almost exclusive affinity for joints giving rise to a chronic arthritis (Type B)

Musculomyces histotropicus, pathogenic, with broader tissue affinities (Types C, D, E)

When the existence of distinct species of similar microorganisms is established in man, guinea-pig, etc., the genera may be named *Hominomyces, Cavimyces,* etc. The objection may justifiably be raised that it is uncommon to have genera of microörganisms limited to one species of mammal, but thus far that has been remarkably true of this microbial group. In the saprophytic group only two distinct species are recognizable thus far on the basis of both cultural and immunological differences. It is suggested that the genus be called *Sapromyces,* and that the species be names in honor of Sir Patrick Laidlaw who together with Elford first described these microörganisms.

The proposed classification and nomenclature of the known microörganisms of the pleuropneumonia group may thus be summarized as follows:

Class. *Paramycetes*
Order I. *Paramycetales*
Family I. *Parasitaceae*
Genus I. *Bovimyces*
Species 1. *Bovimyces pleuropneumoniae*
Genus II. *Capromyces*
Species 1. *Capromyces agalactiae*
Genus III. *Canomyces*
Species 1. *Canomyces pulmonis* I
Species 2. *Canomyces pulmonis* II
Genus IV. *Murimyces*
Species 1. *Murimyces streptobacilli-moniliformis*
Species 2. *Murimyces pulmonis*
Species 3. *Murimyces arthritidis*
Genus V. *Musculomyces*
Species 1. *Musculomyces neurolyticus*
Species 2. *Musculomyces arthrotropicus*
Species 3. *Musculomyces histotropicus* (mixed species of many types)

Family II. *Saprophytaceae*
Genus I. *Sapromyces*
Species 1. *Sapromyces laidlawi* AB
Species 2. *Sapromyces laidlawi* C

In suggesting this classification I am not unmindful of Ledingham's (53) recommendation in 1933 that the microörganisms of pleuropneumonia and agalactia be grouped together merely as a new genus in the existing family of *Actimonycetaceae*, and of Turner's (96) proposal in 1935 of a new order which he named *Borrelomycetales* in honor of Borrel, who according to Turner first demonstrated the characteristic morphology. Since Bordet (3) was the first to describe the complex morphology of the pleuropneumonia microörganism as Borrel et al. (4) point out in their first communication, and since there already exists a genus called *Borrelia* in the family *Spirochaetaceae*, the name *Borrelomycetales* is an inadvisable choice. If in the course of time the weight of evidence is in favor of these microörganisms belonging to the class of *Schizomycetes*, the remainder of this proposed classification and nomenclature might perhaps still be useful in distinguishing the various members of the so-called pleuropneumonia group.

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PLATE 1*

Figures 1, 2, 9, 10, 11, 12, 13 are reproduced from Ledingham (53) and figure 3 from Klieneberger (48). The others are my own and with the exception of figures 4 and 16, have not been published previously.

FIG. 1. "Fully developed colonies of pleuropneumonia (strain 'Shanghai') and others which have not yet acquired the clear peripheral rings. × 70" (J. Path. Bact.)

FIG. 2. "Colonies of pleuropneumonia. Strain 'PP'. × 70" (J. Path. Bact.).

FIG. 3. L_4 colonies after 10 days' incubation. × 80 (J. Hyg.).

FIG. 4. "X" colonies of Sabin and Johnson (81) grown from human tonsils. × 150 (From Proc. Soc. Exptl. Biol. Med.).

FIG. 5. Type A microörganism of mice. Colonies (3 days' incubation) photographed with transmitted light. × 100.

FIG. 6. Same, photographed with oblique lighting. × 100.

FIG. 7. Type B microörganism of mice. Colonies (3 days' incubation) photographed with transmitted light. × 100.

FIG. 8. Same, photographed with oblique lighting. × 100.

FIG. 9. 4-day culture of pleuropneumonia in serum broth, showing condensed rings and spherical elements. Giemsa. × 1500 (J. Path. Bact.).

FIG. 10. 5-day culture of pleuropneumonia in serum broth, showing predominance of "elementary bodies." Giemsa. × 1200 (J. Path. Bact.).

FIG. 11. Impression of 2-day old pleuropneumonia colony on serum agar showing "moniliform elements" and "yeast-like" bodies. Giemsa. × 2000 (J. Path. Bact.).

FIG. 12. Same. "Active peripheral growth with pseudopodial budding of the large yeast-like bodies. Giemsa. × 2000" (J. Path. Bact.).

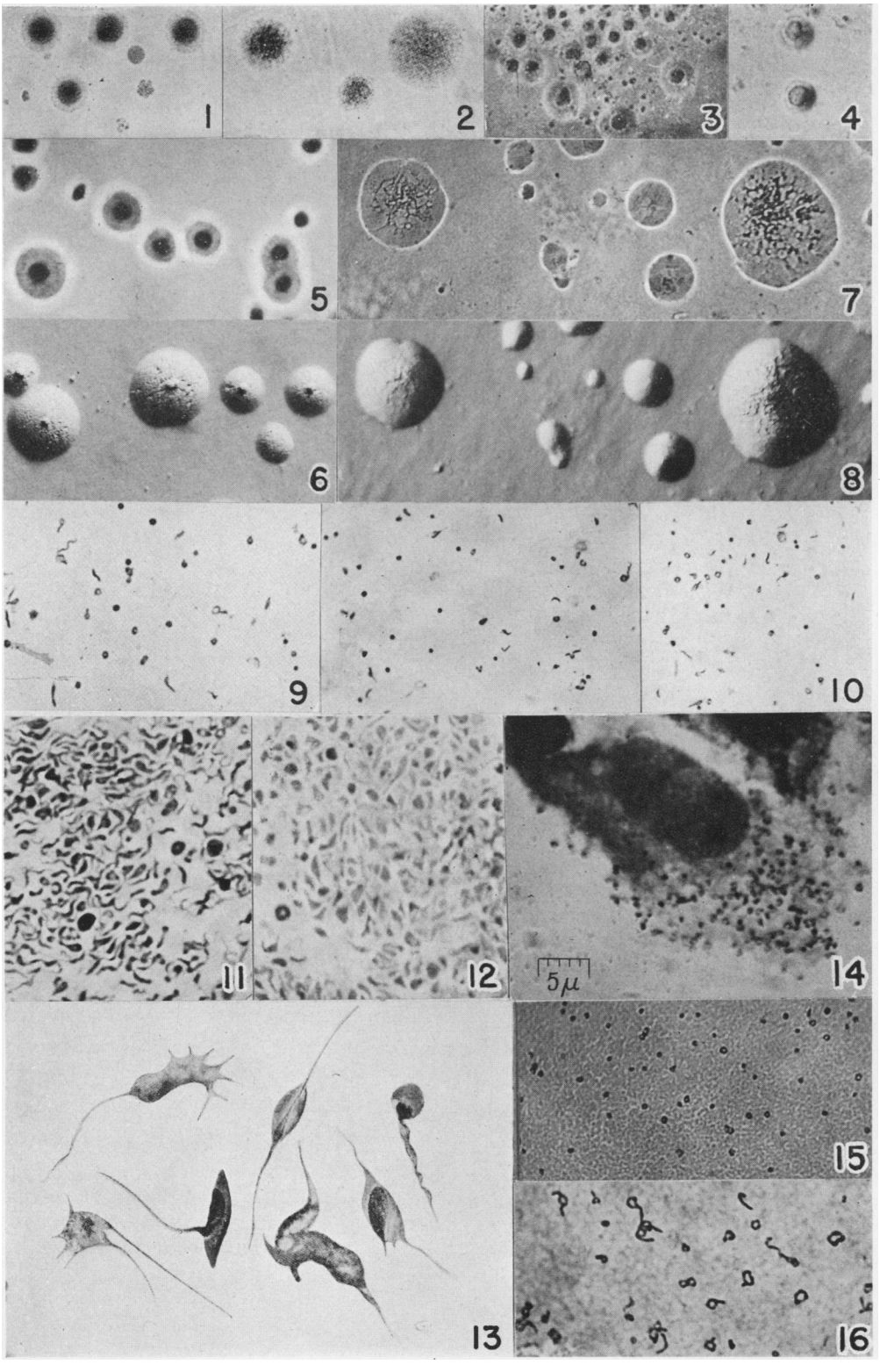
FIG. 13. Impression of 6-day-old pleuropneumonia colony on serum agar. "Composite drawing of 'nucleated' bodies with chromatic elements surrounded by blue-stained sheath. Giemsa. × 1500." (I have seen similar structures in cultures of the Type B microörganism of mice in the early passages after isolation from the animal body; they were present in fluid as well as solid media.) (J. Path. Bact.).

FIG. 14. Mesothelial cell of peritoneum of a mouse inoculated with brain tissue infected with a Type A microörganism, that had been maintained by animal passage and had not been cultured *in vitro*. Compare the typical forms of the microörganism seen in the cytoplasm of the cell with those shown in Fig. 15. Giemsa. × 1600.

FIG. 15. 2-day-old culture in serum-glucose broth of the Type A microörganism of mice. Note relatively simple morphology consisting of elementary bodies and rings exhibiting one or more dense bodies. Giemsa. × 1000.

FIG. 16. 2-day-old culture in serum-glucose broth of the Type B microörganism of mice. Note the more complex morphology especially the filaments "growing out" of the rings. Giemsa. × 1000 (J. Bact., Ref. 82).

* Please note how remarkably different are the forms seen in preparations from fluid cultures as compared with those found in impressions of colonies on solid media.



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