

THE PLEUROPNEUMONIA-LIKE ORGANISMS:
FURTHER COMPARATIVE STUDIES AND A
DESCRIPTIVE ACCOUNT OF RECENTLY
DISCOVERED TYPES

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(With Plates II–IV)

REPORTS on the isolation of pleuropneumonia-like organisms have been frequent during the last few years, and there can be no doubt that we are dealing here with a widely spread group of organisms. In 1935 the writer reported the occurrence in cultures of *Streptobacillus moniliformis* of a pleuropneumonia-like organism, L1, recoverable therefrom in a pure state and interpreted the association as one of symbiosis. Since then she has found and placed on record several new species of pleuropneumonia-like organisms occurring independently and not in apparent symbiosis with bacteria. New information, collected in both these fields during the past year, forms the subject of this communication and is discussed under the following headings:

A. The relationship of L1 to *S. moniliformis*: symbiont or variant?

B. A new strain resembling *S. moniliformis* isolated from abscesses in the necks of guinea-pigs.

C. Other probable associations from which the symbiont has not so far been obtained in pure condition.

D. Pleuropneumonia-like organisms occurring independently in lesions in rats or mice:

(a) L3—bronchiectatic lesions in rats. (See p. 210.)

(b) L4—abscesses, arthritis and swollen glands in rats.

(c) L5—"rolling disease" in mice.

(d) L6—from brains of mice inoculated intracerebrally with blood of splenectomized mice containing *Eperythrozoon coccoides*.

(e) "M55"—arthritis in mice.

E. Further studies on the filterable organisms isolated from sewage, soil, etc.

A. THE RELATIONSHIP OF L1 TO *S. MONILIFORMIS*: SYMBIONT OR VARIANT?

The four L1 strains described by the writer in the study of 1938 have been maintained and studied for a further period. Attempts to induce them to revert to *S. moniliformis* have been made by the method employed by Dienes (1938), who believes that the L1 is a growth variant of the bacillus and not a symbiont. Dawson & Hobby (1939*a, b*) bring forward evidence pointing, in their view, in the same direction. Small agar blocks covered with colonies

of the writer's four strains have not, however, reproduced streptobacilli if incubated in Levinthal broth enriched with serum as Dienes claimed, but with an L1 growth freshly isolated from *S. moniliformis* streptobacillary elements made their appearance several times under these conditions. The writer is therefore of opinion that Dienes has not dealt with sufficiently purified strains. He purified his strains only five times, while it proved necessary in the writer's experiments to pick isolated colonies 30–50 times in succession before a pure L1 strain was established.

It should be emphasized here that Dienes (1939*a*) and the writer agree in the main points regarding the relationship of the L1 and *S. moniliformis*; but there are some minor points of disagreement which have led the two authors to different explanations of the whole phenomenon.

While Dienes describes the globular bodies as transformed bacilli, the writer finds that in very young colonies of her strains the globular and "balloon-like" forms develop as separate forms detached from the bacilli (see Pl. II, fig. 4). In this photograph (fig. 4) of a living colony which developed in a drop of medium under a coverslip, the single globules can be distinctly seen apart from the granular bacillary chains. On the other hand, from a picture such as that reproduced in Fig. 20 of Dienes's paper (1939*a*) it cannot be concluded that the swollen forms are of bacillary origin, because with all the methods so far described it is not possible to decide if a bacillus is simply swollen or if such a soft globular body as that found in pure L1 growth is lying on top of or surrounding a bacillus. There seems to be a further discrepancy between Dienes's and the writer's observations. On his plates, seeded from a 24 hr. old *S. moniliformis* culture, he observed the bacilli to develop first, while the L1 formed a kind of secondary growth; he interpreted this observation as supporting his opinion that the L1 is produced by the bacilli as a new growth phase. In contrast to this the writer found that if a 2 days old culture was spread on a plate of "special medium"¹ the L1 started to develop after 2–3 hr. of incubation while the bacilli commenced to grow only after a lag period of 5–6 hr. Both elements, bacilli and L1 forms, were always found in the young colonies, while Dienes observed that his young colonies consisted chiefly of bacilli. This difference in the ratio between the numbers of bacilli and L1 forms in young colonies may have been caused by differences of medium or of inoculum; the writer, however, has found that bacillary elements and L1 forms are always mixed up in *S. moniliformis* colonies, no matter if very young or old growth is studied.

It should be mentioned here that the so-called filamentous "network", described by the writer in 1934 for pleuropneumonia and agalactia and in 1935 for the L1 organism, was a misinterpretation of a genuine picture which these organisms often present, for if the globules are tightly packed and if only their contours show up (unstained or stained) a kind of honeycomb structure is presented which in the above-mentioned papers was wrongly interpreted as a network. The interpretation of the structures seen was complicated by the fact that the contours of peripheral globules are often only partly visible in the living as well as in the stained specimen, thus producing a picture which can easily be taken for a meshwork with filamentous extensions. Careful observation of the living young colony will often show that the second part of a half-visible globule becomes visible in later development, thus proving the globular nature of the structure seen. This would explain why Dienes did not find the extended "network" structures described and depicted by the present author in her earlier papers. The writer also agrees with Dienes in the observation that the slightest tearing or distortion applied to colonies of pleuropneumonia-like organisms may produce

¹ Ox heart infusion peptone Levinthal agar (Levinthal & Fernbach, 1922), pH 7.8–8.0 enriched with 30% of horse serum (Klieneberger, 1938).

filamentous formations which are readily stained. It should be emphasized that Dienes and the writer both agree that genuine filamentous forms occur in the young cultures of the L1 organism. The writer is further convinced that filaments also occur as extensions and transformations of globular bodies. There is one more point of disagreement between Dienes and the writer regarding *S. moniliformis* strains which have been in culture for a long period. While Dienes states that such "old" strains show chiefly bacillary forms the writer did not see any noticeable reduction of L1 elements in her "old" strains nor did they quickly lose their pathogenicity for white mice as reported in the literature. The writer is of opinion that *S. moniliformis* strains retain the properties noted at the time of isolation for a considerable time if they are very frequently subcultured.

A striking feature which, if further studied, might contribute to a better understanding of the symbiosis problem in *S. moniliformis* is the rapid disintegration of the bacilli. In cultures incubated for 2 days or longer the bacilli are very granular and appear to be degenerate. In older cultures the number of viable bacilli decreases rapidly, while the L1 elements still develop readily if transferred to fresh medium. If evidence could be supplied that bacillary elements may persist in a weakened, latent condition, this would account for Dienes's and the writer's observations that freshly picked and apparently pure L1 colonies revert easily to *S. moniliformis*, whereas thoroughly purified strains have now been kept by the writer for years without ever reverting.

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 L1 form from the parent culture of *S. moniliformis* and have maintained it in pure condition. They both agree that the L1 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 L1 strains reverting into the parent culture under certain conditions, while the writer's strains 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 L1 correspond to 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. Longer experience with *S. moniliformis* and L1 strains and a clearer understanding of the development of pleuropneumonia-like organisms and the L1 form is required before it can be decided if the L1 is an organism different from the streptobacillus or a variant growth phase. The evidence for the second view, based by Dawson & Hobby on serological tests, has not yet been published in full and can therefore not be discussed here.

The reasons why the writer is still in favour 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 L1 growth contains no ordinary bacillary forms, but elements resembling those of the organism of pleuropneumonia bovis. Like pleuropneumonia the L1 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. The experienced student of variation in bacteria will notice that they are far more fragile than these, some being stainable throughout while others appear to contain a watery liquid, and if motile bacteria happen to enter these latter L1 "balloons" they can be watched swimming about inside and impinging on the membrane of the cyst in which they are trapped.

Pl. II, fig. 2, shows a darkground picture of L1 globules. Pl. II, fig. 1, shows a living colony of L1 grown in semi-solid medium in which granules and enormous bodies are to be seen. Pl. II, fig. 3, shows a corresponding colony edge, stained. The membrane itself may appear very refractile and fairly thick in some globules, while it appears very thin in others; it can be drawn out into filaments; in most cases the membrane contains granules resembling those forming a large part of the whole growth. Some of the globules and filamentous appendices resemble in fact myelin structures, and a microchemical test has recently suggested that the L1 and the *S. moniliformis* cultures may contain a considerable amount of sterols. Some of the globular elements in a well-grown culture are doubly refractile. Photographs of stained preparations of some of the main types of pleuropneumonia-like organisms are reproduced in Pl. II, fig. 3 (L1 organism), Pl. II, fig. 5 (organism of agalactia), Pl. III, figs. 13 and 14 (sewage organism "A"), Pl. III, fig. 15 (*Asterococcus canis* II), Pl. IV, fig. 19 (L5 organism), fig. 20 (L6 organism). These photographs demonstrate the characteristic structures common to all pleuropneumonia-like organisms.

Besides possessing a similar morphology, L1 and the organisms of the pleuropneumonia group show also the same colony type, characterized by a central granular part embedded in the agar medium and a flatter peripheral zone. There is further the regular filterability of L1 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 L1 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 L1 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.

B. A NEW STRAIN RESEMBLING *S. MONILIFORMIS* ISOLATED FROM ABSCESSSES IN THE NECKS OF GUINEA-PIGS

A culture resembling *S. moniliformis* was isolated by the writer in the winter of 1938-9, and a note of its occurrence was submitted to the Pathological Society of Great Britain and Ireland in July 1939 and to the Third International Congress of Microbiology in September 1939 (Klieneberger, 1939*b, c*). Among the Institute's guinea-pig stocks were often found individuals presenting large abscesses in the neck. As a rule these abscesses ran a chronic course, but eventually they either burst and cleared up or they regressed and disappeared. The "chronic streptococcus cervical lymphadenitis" of the guinea-pig recently described by Seastone (1939) seems to be of different aetiology, for streptococci were never found in the pus of the swollen lymph glands of our guinea-pigs. The caseous pus did not reveal any formed elements of bacterial nature in an ordinary Gram preparation, but if fixed with methyl alcohol and stained with a weak Giemsa solution over-night or if treated by one of the methods for the demonstration of elementary bodies small bacillary forms and thread-like elements were discovered. On ordinary media this pus yielded no growth; but in rich serum broth and on the writer's special medium growth was obtained in 3-6 days aerobically and in 2-3 days anaerobically. While growth appeared as lumps and flakes in the liquid medium, irregular

dense colonies grew up on the solid medium in which by low magnification some globular elements were discovered (Pl. III, fig. 6). If higher magnifications are used the direct agar method reveals that the whole colony is interspersed with globules of different size and shape. Giemsa-stained colonies show delicate bacillary threads abundantly interlaced at the edges (Pl. III, fig. 9). Between the tangled bacillary chains stained and unstained globules can be seen. A Gram-stained smear of a culture shows Gram-negative clumps of minute bacilli. The structure of the guinea-pig culture resembles in all details that of *S. moniliformis*, the only morphological difference being that the bacillary threads are more delicate and more entangled. The guinea-pig culture differs further from *S. moniliformis* in that it grows at first better anaerobically than aerobically. Evident differences can be demonstrated by animal experiment: *S. moniliformis* has, as we know, no pathogenic effects at all on guinea-pigs and rabbits; it is usually not infective for rats though it inhabits their nasopharynx; the only small laboratory animal for which it is highly infective is the mouse in which it produces arthritis and septicaemia. Prof. Wilson Smith (Sheffield) informed me that he had also independently isolated a culture similar to the guinea-pig strain here described and had studied its pathogenicity extensively. On this work he will doubtless report in due course. The writer has so far been interested chiefly in the morphology of the "guinea-pig culture". Because of its resemblance to *S. moniliformis* it seemed of interest to investigate its possible symbiotic nature. For this purpose old cultures, kept in the incubator for days, or even weeks, were spread on plates of special medium, as described by the writer in 1936. On some plates small colonies came up in which bacilli were no longer seen. From these colonies a growth, different from the parent culture and resembling pleuropneumonia, was obtained. Subcultures at first grew with difficulty and developed on the semi-solid medium only under agar cover. Gradually the growth became better established and a purification process was started. From each subsequent culture a piece of agar was cut out containing as few colonies as possible. It was hardly ever possible to pick a single colony. The agar piece was moved over the surface of a new plate. After the first eight passages some colonies reproduced the whole parent culture, but from others the pleuropneumonia-like culture could be carried on. When this process had been continued thirty times another reversion to the parent culture occurred, but it was again possible to pick pleuropneumonia-like colonies. After twenty passages, during which no reversion took place, this culture was unfortunately lost during the disorganization occasioned by the outbreak of war. The clearing process will again be started with a new strain, and it is hoped that a pure pleuropneumonia-like culture will be obtained. The lost pleuropneumonia-like strain from the "guinea-pig culture" formed a roundish colony with a dark granular centre and a peripheral zone showing a lace-like structure (Pl. III, fig. 7). Pl. III, fig. 8, shows a colony edge at a higher magnification, clearly revealing the globular structure of the peripheral zone. The colony resembled closely that of the L1 organism.

C. OTHER PROBABLE ASSOCIATIONS FROM WHICH THE SYMBIONT HAS NOT
SO FAR BEEN OBTAINED IN PURE CONDITION

During the past year another apparently symbiotic culture was isolated by the writer. It was accidentally found on a plate which had been spread with material from the skin of a pig inoculated with swine-pox. The colonies of this organism consist of Gram-positive cocci interspersed with large, balloon-like forms. This culture grows well on ordinary media and develops at 37° C. as well as at room temperature. The globular forms resemble those of the L1 organism, but attempts to isolate a pleuropneumonia-like microbe from it have failed. The development of peculiar large forms has also been observed in *Fusobacterium nucleatum* (syn. *Bacterium fundiliforme*), in which attempts to separate a pleuropneumonia-like organism have likewise been unsuccessful. Pl. III, fig. 11, shows a darkground picture of a young aerobic serum broth culture of *F. nucleatum* containing many globular forms. As the cultures grow older more bacillary forms develop, and at the same time abundant gas production starts in the liquid medium. On the surface of plates incubated anaerobically numerous globular forms develop. Small colonies often consist chiefly of these forms. It is possible to obtain globular colonies in which bacillary forms are no longer demonstrable, but if transferred they eventually produce a mixture of globular forms and bacilli. Pl. III, fig. 10, shows both forms grown on solid medium (agar fixation Giemsa technique), while fig. 12 shows the edge of a globular colony in which bacilli cannot be detected. Similar phenomena were observed by Dienes (1939*b*) in cultures of a *Flavobacterium*, of *Haemophilus influenzae* and *B. fundiliforme* (syn. *Fusobacterium nucleatum*). It is difficult to decide if these cultures from which Dienes and the writer have not been able to obtain the apparent L1-like elements in pure culture do really contain an L1-like symbiont or growth variant as *S. moniliformis*. 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. Swollen forms have been described in all kinds of cultures, but the separation of the L1 growth from the parent culture has so far only been successful in *S. moniliformis* and the "guinea-pig strain".

D. PLEUROPNEUMONIA-LIKE ORGANISMS OCCURRING INDEPENDENTLY
IN LESIONS IN RATS AND MICE

In previous papers investigations on the organisms of pleuropneumonia, agalactia, *Asterococcus canis* I and II (Shoetensack) and the L1, L3, L4 organisms have been recorded by the writer (Klieneberger, 1938). The more organisms of the pleuropneumonia group that come to light, the more necessary it seems to find differences which may serve for classification purposes. Therefore studies on the growth, morphology, serology, occurrence and pathogenicity of the newly found L5 and L6 organisms (Findlay, Kliene-

berger, MacCallum & Mackenzie, 1938, 1939; Findlay, Mackenzie, MacCallum & Klieneberger, 1939) have been carried out, and new information has been collected about the L3 organism. A number of strains from sewage, soil and water have also been included in these studies. These new types have been compared with those already described (Klieneberger, 1935, 1936, 1938, 1939*a, b*).

(a) *L3—bronchiectatic lesions in rats*

Three different organisms of the pleuropneumonia group have been isolated from rats by the writer. The L1, regularly associated with *S. moniliformis*, was once found independently in the rat lung. The L3 has been isolated by Klieneberger & Steabben (1937) from lung lesions of rats, and some new data regarding its association with the pulmonary disease are recorded in this *Journal*, p. 223, by the same authors.

(b) *L4—abscesses, arthritis and swollen glands in rats*

The third pleuropneumonia-like organism isolated from a swollen gland of a rat (Klieneberger, 1938) was the L4 species. Later in 1938 it was found that Woglom & Warren's pyogenic agent (1938*a, b*) is identical with the L4 organism (Klieneberger, 1939*a*; Woglom & Warren, 1939). The L4 organism grows better anaerobically than aerobically, a feature which distinguishes it from the other members of the pleuropneumonia group so far described. It forms a small, granular colony, consisting mostly of small elements; in the liquid medium it grows with slight opalescence.

Serologically (Table 3) it is differentiated from most of the other pleuropneumonia-like organisms. There is some overlapping between the L4 and L5 organisms in so far as the L4 serum agglutinates the L5 suspension to a titre of 1 : 80, while the L5 serum agglutinates the L4 suspension to a titre of 1 : 40. The agglutinin titres of these sera for their homologous strains are considerably higher, viz. 1 : 320 for the L4 and 1 : 10240 for the L5 organism. The L4 serum reacts also slightly with the L1, L5 and L6 suspensions and with some of the saprophytic strains from sewage and soil, but only the L5 serum agglutinates the heterologous L4 suspension.

The L4 causes abscesses in rats if injected subcutaneously or intraperitoneally and if injected intravenously together with cells or agar, produces a severe arthritis to which young animals are specially susceptible. The L4 strains decrease slowly in pathogenicity if kept continuously on artificial media. Recently Findlay, Mackenzie *et al.* (1939) isolated a pleuropneumonia-like organism from cases of spontaneous polyarthritis in rats. They designated this organism L7, because they thought at first that it was different from the organisms so far described. It has since been shown by the writer by cultural and serological tests that this organism is identical with the L4 organism (Klieneberger, 1938, 1939*a*). The writer has also succeeded in culturing a pleuropneumonia-like microbe from swollen joints of rats, kindly supplied by Dr Dyson. These rats had been infected with the agent of Collier (1939) that

had been introduced into this country with rats from Batavia sent directly by Collier to Dr Dyson. It was not possible to investigate if this strain was identical with the L4, for, unfortunately, it was lost at the outbreak of war and the infected rats were destroyed. Rhodes & van Rooyen (1939) do not mention in their recently published paper on an "infective disease affecting limbs and tails of rats" if they have attempted cultivation on the writer's "special medium". From their description of the pathology of the disease it seems that they have been dealing with a condition similar to the L4 infection.

The differences between the three pleuropneumonia-like organisms so far found in rats are outlined in Table 1.

Table 1. *Organisms from rats*

	L1	L3	L4
Growth in liquid	Large clumps	Small clumps	Opalescent
Colony (low mag. of microscope)	Very coarse and large	Fairly coarse and fairly large	Granular and small
Source in rat	<i>S. moniliformis</i> , lung once	Lung lesions	Abscesses, arthritis, swollen glands
Pathogenicity for rats	—	Causes probably "broncho-pneumonia"	Causes abscesses, arthritis, lymphadenitis
Serology, see Table 3	Special type	Special type	Special type, overlapping with L5

(c) L5—"rolling disease" in mice

The first pleuropneumonia-like organism found in mice was the L5. It was isolated from the brains of mice exhibiting nervous symptoms called "rolling disease" (Findlay *et al.* 1938). This characteristic disease has so far been found three times in mice. It was first noted by Findlay in 1933 that mice intracerebrally injected with yellow fever virus developed the nervous symptoms of "rolling disease". He succeeded in separating the agent of "rolling disease" from the yellow fever strain. In 1937 Findlay found that during the course of routine passages of lymphocytic choriomeningitis virus some mice again developed the nervous symptoms. He was then again able to separate the agent of "rolling disease", from the virus and to carry it on in mice. It was proved by Findlay *et al.* (1938) that the cause of the disease was a pleuropneumonia-like organism which was designated L5. In America, Sabin (1938) described the same symptoms exhibited by mice which had been intracerebrally injected with a strain of toxoplasma. Findlay *et al.* (1938) isolated a similar organism from dried mouse brain kindly supplied by Dr Sabin, and showed that the same L5 species was responsible for the nervous disease of the mice in New York and London. Sabin himself was also able to isolate the pleuropneumonia-like organism from his mice. These observations show that during the course of intracerebral passage of different agents in mice the same latent organism became active. The L5 organism seems to have a special affinity for the brain. Not only did it occur three times during the course of intracerebral passages of different materials, but

it multiplied abundantly if injected into the brain and remained alive there for a considerable time. This distinguishes the L5 organism from other members of the group such as the L1, L3 and L4 which do not multiply if injected intracerebrally into mice and cannot be recovered from the brains some days after the injection.

The cultural and morphological features of the L5 organism also distinguish it from other species. The round colony of the L5 organism (Pl. IV, fig. 16) shows a well-marked, small, dark centre and a large peripheral zone in which delicate globular structures can usually be detected by a magnification of 1 : 200, but not by 1 : 80. By high magnification (oil immersion lens) the fairly regular globular structure can be easily demonstrated in unstained and stained colonies. Pl. IV, fig. 19, shows a stained, highly magnified colony of the L5 organism. The thick central part of the colony has stained very deeply, while the thin peripheral layer of globules has stained delicately. Different L5 immune sera prepared in rabbits gave a positive agglutination reaction with the American and English L5 strains. The reaction was obtained in the unusually high titre of 1 : 10240 (Table 3). Though the L5 sera agglutinated the homologous suspensions in these high dilutions, most heterologous species were not agglutinated at all by these sera, with the exception of the L1, L4 and L6 suspensions which showed a positive reaction in the dilutions of 1 : 40 (L1, L4) and of 1 : 160 (L6). There was also positive cross agglutination between the L1, L4 and L6 sera and the L5 suspensions, but the titres of these positive reactions compared with that of the homologous sera and strains were low (Table 3). These tests together with the animal experiments, the morphology and the colonial type, support the view that the L5 is a specific type possessing, however, antigens related to those of L1, L4 and L6.

(d) *L6—*from brains of mice inoculated intracerebrally with blood of splenectomized mice containing *Eperythrozoon coccoides*

During work with splenectomized animals carried out in collaboration with Findlay, Klieneberger, MacCallum & Mackenzie (1939) a second pleuropneumonia-like organism was recovered from mice. It was isolated six times from the brains of mice which had been injected intracerebrally with blood of splenectomized mice containing *Eperythrozoon coccoides*. The first suggestion that *Eperythrozoon* and this new organism, L6, was one and the same thing has not been verified. It was not possible to recover L6 regularly from mice injected in the way mentioned and all attempts to isolate it from the *Eperythrozoon* blood failed completely.

The L6 colony can be distinguished from that of the L5. It is usually larger and more irregular, and is further characterized by the presence of very large globules which can be seen by low magnification (1 : 80) and which give the surface of the colony a coarse structure (Pl. IV, fig. 17). The picture of the more highly magnified colony edge is shown in a darkground photograph (Pl. IV, fig. 18; $\times 490$) and stained (Pl. IV, fig. 20; $\times 600$). The L5 growth

is opalescent in liquid medium while the L6 often forms little clumps which are chiefly composed of fairly large globules. The opalescent growth of the L5 is chiefly composed of small granules and small globules appearing as rings in the darkground preparation. The L6 serum seems specific and does not agglutinate any of the other pathogenic species of the pleuropneumonia group. In contrast to this the L6 suspension is influenced to a certain degree by L3, L4 and L5 sera.

(e) "M55"—*arthritis in mice*

In addition to the new types, L5 and L6, a pleuropneumonia-like organism was unexpectedly isolated from a joint of a mouse by Dr H. Jahn, working in the writer's laboratory. This mouse had been used for an experiment with *S. moniliformis* but instead of this organism or the L1 an unknown pleuropneumonia-like organism was cultivated from the swollen joint. This new organism, "M55", which has not been tested serologically and therefore not yet been assigned a number in the L series, causes arthritis and may be related to Sabin's organism from mouse arthritis.

The L5, L6 and "M55" types are the new pleuropneumonia-like organisms isolated from mice during the past year. Table 2 outlines the characteristics of these organisms from mice.

Table 2. *Organisms from mice*

	L5	L6	"M55"
Growth in liquid	Opalescent	In small clumps and opalescent	Slightly opalescent
Colony (low mag. of microscope)	Round, small, dark centre	Flat, irregular, with some large globules	Round, small, centre and peripheral zone granular
Source in mice	Brain in "rolling disease"	Brain after intracerebral injection of <i>Eperythrozoon</i> blood	Swollen joint
Pathogenicity for mice	Causes "rolling disease"	—	Causes arthritis
Serology, see Table 3	Special type, but antigenically related to L1, L4 and L6	Special type, but influenced by L3, L4 and L5 sera	Not yet examined

E. FURTHER STUDIES ON THE FILTERABLE ORGANISMS ISOLATED FROM SEWAGE, SOIL, ETC.

Altogether eleven strains isolated from sewage, soil, etc. were studied. The writer obtained five strains isolated by Laidlaw & Elford (1936) from the National Collection of Type Cultures, which have been kept there since 1936. Dr G. Seiffert kindly sent six other strains to the writer in 1937. The designation of these eleven strains was as follows:

Strain	Isolated by Laidlaw & Elford in 1936
"A"	
"B"	" "
"C"	" "
"Finchley"	" "
"Croydon"	" "

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Strain	"B 10"	Isolated by Seiffert in 1937		
"	"C 15"	"	"	"
"	"P f"	"	"	"
"	"T 22"	"	"	"
"	"K 30"	"	"	"
"	"L 1"	"	"	"

It should be noted here that accidentally one of Seiffert's saprophytic strains has been designated "L 1". Where this strain of Seiffert's is referred to, quotation marks will be used.

GROWTH AND MORPHOLOGY

These eleven strains grow well on liquid and solid media as prepared in this Institute from trypsin digested meat. They do not require special media nor admixture of serum for their maintenance as do the pathogenic strains. The addition of a small amount of serum accelerates their growth however. They develop at 37° C. and also at room temperature, thus differing from the pathogenic cultures. If stored in the cold they keep well for several months, while most of the pathogenic organisms have to be subcultured at shorter intervals. The water and soil strains grow in the liquid medium either with strong opalescence or with a sediment. The kind of growth obtained varies according to the pH of the broth, in the way that these strains tend to form a sediment if the pH is decreased while the same cultures grow with a general turbidity if the pH is increased. They prefer an alkaline medium, but fairly good growth is obtained from pH 7.0 to 9.0, if a small amount of serum is added. The pathogenic strains are less tolerant to changes of reaction and grow best at pH 7.8–8.0.

All the water and soil strains examined form characteristic colonies on solid medium. They are round and granular with a well-marked, dark centre embedded in the agar and a lighter peripheral zone. There is no distinction between the colonies of all these water and soil strains, with the only exception of Laidlaw & Elford's type "C" which develops more slowly and forms smaller colonies than the others. The same can be said about the morphology of these strains, which is very uniform. In darkground preparations from opalescent broth cultures granules, single or in clusters and small globules (rings in the darkground) are observed. If the cultures grow with sediment formation in the broth roundish bodies of different size are also found consisting of granular material; they may correspond to the large forms sometimes found on solid media. In growth from solid medium granules of different size are the main elements, while globules, sometimes large and occasionally drawn out into filaments, are found in older growth. The sizes of the globular structures vary on the same medium and in different colonies on the same plate, but all strains seem to produce, at least occasionally, this peculiar foam-like pattern. These structures are well reproduced in the photographs of impression

preparations in Seiffert's publication of 1937. In Pl. III, figs. 13 and 14, of this paper photographed colony edges of Laidlaw & Elford's strain "A", fixed and stained by the writer's method, are reproduced. The picture resembles those from parasitic species. In older colonies of these saprophytic strains the globules are sometimes very large, but in young colonies the structures seem to be exceedingly delicate and usually only granules can be detected in the young growth, even if all the different methods of demonstration are applied. The mycelium-like structures which Ørskov has drawn in his paper "On the morphology of Seiffert's micro-organisms" (1938), have not been observed by the writer. One of his techniques of examination cannot be recommended, viz. the spreading of an alcoholic solution of victoria blue on an agar surface covered with growth. As mentioned by the writer in 1936, alcohol has a destructive effect on the growth of pleuropneumonia-like organisms.

COMPARATIVE SEROLOGICAL INVESTIGATIONS OF THE SAPROPHYTIC SEWAGE AND SOIL STRAINS AND THE PARASITIC PLEUROPNEUMONIA-LIKE ORGANISMS
(TABLE 3)

Following the methods described in 1938, suspensions have been prepared from the eleven water and soil strains and the corresponding antisera have been obtained from rabbits. Cross-agglutination tests have been carried out with these sera and suspensions. As will be seen from Table 3, ten of these eleven strains are antigenically related, some, such as "A", "Finchley", "T22", "K30", giving practically identical reactions. It is interesting to note that two of these apparently identical strains were isolated in England (Laidlaw & Elford) while two others were isolated in Germany (Seiffert). While ten out of the eleven saprophytic strains show at least some antigenic relationship only one of them represents a special type having no antigens in common with any of the other strains. It is Laidlaw & Elford's strain "C". As mentioned above, it is at the same time the only strain that shows differences in growth. A second culture that is somewhat different, serologically, is Laidlaw & Elford's strain "B"; but while a "C" serum does not agglutinate any of the other strains and the "C" suspension is not affected by any of the other sera, the "B" serum agglutinates most of the suspensions of the other ten strains to a titre varying between 1:20 and 1:80, while the "B" suspension is not affected by any of the other sera. The serological tests show that Seiffert's strains have common antigens with Laidlaw & Elford's type "A"; Laidlaw & Elford's type "B" shows some relationship to "A"; "C" alone represents a special type. This is in agreement with Laidlaw & Elford's results of 1936. They conclude: "There can be no doubt that strains A and C are quite distinct antigenically, and that strain B is more closely related to A than to C."

Cross-agglutination tests have also been carried out between the saprophytic pleuropneumonia-like strains and most of the parasitic strains (Table 3).

Table 3. Results of cross-agglutination tests with all strains of the pleuropneumonia group in the writer's collection

Sera ...	"A"	"Croydon"	"Finchley"	"B"	"C"	"T22"	"K30"	"B10"	"L1"	"C15"	"P F"	Pleuropneumonia	Agalactia	L1 organism	L3 organism	L4 organism	L5 organism	L6 organism	<i>Asteroc. canis</i> I	<i>Asteroc. canis</i> II		
Suspensions																						
"A" Laidlaw	2560	320	160	20		1280	640	40	10	20						10						
"Croydon" Laidlaw	40	320	160			40	40	40	20		20											
"Finchley" Laidlaw	320	640	640	20		320	160	40	40	40	20											
"B" Laidlaw				160												10						
"C" Laidlaw					320										20							
"T22" Seiffert	320	80	40	40		320	160	20	20	40	10											
"K30" Seiffert	640	80	40	20		320	160	10	10	10												
"B10" Seiffert	40	20	80	40		80	20	640	40	40	320					10						
"L1" Seiffert	40	20	40	80		40	20	80	640	20	40											
"C15" Seiffert	40	10	40	40		40	10	320	20	640	160					10						
"P F" Seiffert	40	20	80	40		40	10	320	20	40	320											
Pleuropneumonia												640										
Agalactia													640									
L1 organism														1280		80	40	10				
L3 organism															320							
L4 organism																320	40	10				
L5 organism																	40					
L6 organism																	10,240	160				
L6 organism																	40	1280				
<i>Asteroc. canis</i> I																					80	
<i>Asteroc. canis</i> II																						80

The average end-titres of the tests which were designated "+ " are given in the table.

These reactions gave completely negative results with the one exception of the L4 serum which gave a slight positive result with five water and soil strains including "C"; but the titres of these reactions were very low (1 : 10 for four strains and 1 : 20 for strain "C") and none of the sera prepared with saprophytic strains affected the L4 suspensions. It should be mentioned that the L4 organism is the one growth type of all the pathogenic strains which shows a certain resemblance to the water and soil strains by its very granular colony and by producing chiefly small elements.

None of the saprophytic strains produces lesions in laboratory animals, even if agar is added to the culture injected.

The morphological and serological examinations of all the pleuropneumonia-like organisms in the writer's possession show that with the one exception of Laidlaw & Elford's strain "C" the other ten strains from water and soil are related organisms, while the pathogenic strains can be divided up into species different both from one another and from the saprophytic organisms. Agglutinin-absorption tests have not yet been practised.

DISCUSSION

There exists a group of microbes of which the organisms of pleuropneumonia of cattle and agalactia of sheep are the prototypes. Their morphology, development and possible life cycle have been widely discussed by different authors. Though agreement concerning their nature and development has not yet been reached and we are therefore not able to assign systematic rank to them, it is possible for the student of these organisms, whenever a new type comes to light, to recognize it as a member of the pleuropneumonia group. The author is of opinion that we are dealing with a distinct family whose members resemble each other closely, but differ widely from other known microbes. An attempt is here made to outline plainly the characteristics of the family without attempting to give a definition of their possible nature or a description of their still hypothetical life cycle.

All members of the pleuropneumonia group form characteristic colonies, which grow up slowly and after some days reach a diameter varying between 0.1 and 0.6 mm., the latter size being reached only by some giant colonies and only in a few species. The colonies show usually a darker central zone embedded in the medium and a lighter peripheral zone. If studied under the microscope, actually the only proper means for collecting information about the colonies, at magnifications of 30–200 they show either a granular or a coarser, globular structure which distinguishes them from bacterial colonies. Nearly all of them develop a brownish tinge if they are isolated enough and if incubated for a protracted period.

In an ordinary smear preparation fixed with heat or alcohol and stained with aniline dyes such as fuchsin, methylene blue, gentian violet or by Gram's method formed elements such as bacilli, cocci, commas or spirochaetes cannot be detected. Such a slide shows a cloudy, indistinct, faintly stained material

which looks as if derived from the culture medium. If staining methods are applied which serve for the demonstration of virus particles some granules may or may not be found, but no picture showing large numbers of small elements like the elementary bodies of certain viruses indicates the presence of numerous elements of a micro-organism. Yet each colony of any pleuropneumonia-like organism consists of numerous very small and fairly large elements. Many of them can be demonstrated in a carefully carried out impression preparation, fixed with alcohol after drying, and stained with Giemsa (Ledingham, 1933). The agar fixation method (Klieneberger, 1934) is a modified impression technique. Its advantage is that whole small colonies can be fixed and stained *in situ* without considerable dislocation of single elements so that a fairly true picture of the actual growth is obtained. An even simpler and rather conclusive method, though not revealing the finest details, is the direct agar microscopy (Ørskov, 1927). All magnifications including oil immersion systems can be used to study the growth on top of an agar plate or better on a slide culture. Dienes (1939*a*) used a direct agar microscopy in combination with staining methods, which furnished him with excellent preparations. These methods, together with the darkground observations, show that all pleuropneumonia-like organisms produce a large amount of very small forms which are of slightly different sizes and shapes. These "granules" often stain deeply as if consisting of concentrated material. It has been stated by most authors that the organisms of pleuropneumonia and agalactia form filaments, because granules with filaments attached have often been observed; but the actual germination of granules has so far not been followed up satisfactorily. Filamentous formations occur in all members of the pleuropneumonia group. They are frequent in pleuropneumonia, agalactia and the L1 organism and rare in others, such as the saprophytic strains and the L4 organism; but occasionally they are present in all members of the family. They occur not only in connexion with granules, but also free and in connexion with globules or large bodies, which represent the third type of element composing the growth of pleuropneumonia-like organisms. Two kinds of globules or bodies seem to be present. One kind stains well, sometimes very deeply, purple or bluish with Giemsa solution while the other seems to contain a liquid. Consequently often only its contours can be seen or stained; but sometimes the surface shows up, very lightly stained. If many of these globules are present in a colony a honeycomb or cellular structure is produced which can be mistaken for a meshwork of filaments. This mistake of interpretation has been made in the earlier papers of the present writer on pleuropneumonia, agalactia and the L1. The so-called "network structures" are in reality produced by the contours of globules densely packed together. In the same way the ring forms, so often mentioned in studies on pleuropneumonia and related organisms, must be interpreted as contours of globules. If they turn over in darkground preparations they always show ring forms and therefore must be regarded as outlines of corpuscles or globules.

The different elements present in pleuropneumonia-like organisms can be well demonstrated, but how they grow up and develop is not yet clearly understood. Nevertheless, the members of the group are so well characterized by their morphology and colony type that it seems reasonable to regard them as belonging to a distinct family different from all other Schizomycetales.

Organisms of the pleuropneumonia group have been found in symbiosis with bacteria. In two cases a separation of the pleuropneumonia-like organism from the parent culture has been achieved, viz. in the case of *Streptobacillus moniliformis* and in the case of the so-called "guinea-pig culture", obtained from cervical lymphadenitis in these rodents.

Organisms of the pleuropneumonia group have been found independently and not in association with bacteria in materials such as sewage and soil, showing that these microbes are able to lead a saprophytic existence. Most of the organisms so far found in such materials are related and are represented by Laidlaw & Elford's type "A".

A number of different types of pleuropneumonia-like organisms have been found in lesions of animals. In most cases they have been shown to cause infection in these animals under certain experimental conditions such as, for example, when agar is mixed with a suspension of the organisms. The same is true also of the organism of pleuropneumonia (Mettam & Ford, 1939; Daubney, 1936). In addition to the organisms of pleuropneumonia of cattle and agalactia of sheep the following independent pathogenic types have been described since 1933: *Asterococcus canis* type I and type II (Shoetensack, 1934), the L3 organism in pulmonary lesions of rats (Klieneberger & Steabben, 1937), the L4 organism in swollen glands, abscesses and swollen joints of rats (Klieneberger, 1938, Findlay, Mackenzie *et al.* 1939; Woglom & Warren, 1939), the L5 organism causing "rolling disease" in mice (Findlay *et al.* 1938; Sabin, 1939*a*), the L6 organism in brains of mice after intracerebral injection of blood of splenectomized mice (Findlay, Klieneberger *et al.* 1939), and lastly an organism causing swollen joints in mice (Sabin, 1939*b*, Klieneberger, this paper). These filterable organisms of the pleuropneumonia group are of frequent occurrence in rats and mice and their presence must undoubtedly complicate virus research in these animals.

SUMMARY

1. The four different L1 strains in the writer's possession isolated between 1933 and 1936 have been maintained for a further period without reverting to *Streptobacillus moniliformis*. The supposition that the L1 is a pleuropneumonia-like organism and lives in symbiosis with a bacterium in cultures of *S. moniliformis* is still maintained by the writer and the reasons for it are given.

2. From a culture isolated from lesions in guinea-pigs and pathogenic for these animals called the "guinea-pig strain", an L1-like organism of the pleuropneumonia group has been separated, but not yet been maintained for

a long enough period to ensure irreversibility. The morphology of the "guinea-pig strain" shows that it is closely related to *S. moniliformis* though it can be distinguished from the latter by its cultural and pathogenic properties. The morphology of two other cultures, viz. a saprophytic coccus from the skin of a pig and the organism known as *Fusobacterium nucleatum*, have been described as possibly representing similar symbiotic associations of bacteria and pleuropneumonia-like organisms.

3. Cultural and serological differences between the L4 causing arthritis, swollen glands and abscesses in rats, and the two other pleuropneumonia-like organisms from rats, L1 and L3, are recorded. It has been shown that the organisms occurring in the brains of mice, L5 and L6, and in the joint of a mouse, "M55", are of aetiological significance for the condition in which they occur. At the same time they differ in their colony type, morphology and serological features.

4. Morphological and serological studies of the saprophytic organisms of the pleuropneumonia group isolated by Laidlaw & Elford and Seiffert from water and soil show that Seiffert's organisms are closely related to Laidlaw & Elford's type "A". In agreement with Laidlaw & Elford it was found that their type "B" is slightly different from "A" serologically, while "C" is distinct from "A" and "B" and may be regarded as a special type.

These saprophytic types are not antigenically related to the parasitic varieties.

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EXPLANATION OF PLATES II-IV

PLATE II

- Fig. 1. *L1 organism*, grown on a coverslip in a mixture of 1 part rabbit plasma, 1 part spleen extract and 3 parts of special liquid medium; 4 days 37° C. The globular edge of this colony shows extremely large forms. Photographed alive by Dr Robinow; mag. 1134.
 Fig. 2. *L1 organism*, grown on a coverslip in special liquid medium; 3 days 37° C. Many globular forms. Photographed alive, darkground illumination; mag. 900.
 Fig. 3. *L1 organism*, edge of stained colony, grown on solid medium, showing large globules with their delicate contours at the edge. Agar fixation method, Bouin-Giemsa; mag. 600.
 Fig. 4. *S. moniliformis*, grown on a coverslip in special liquid medium, 3 days 37° C. The bacillary chains containing granular structures and the large globules lying apart can be distinctly recognized. Photographed alive by Dr Robinow; mag. 1134.
 Fig. 5. *Organism of agalactia*, part of stained colony showing globules of different size. Agar fixation method, Flemming-Giemsa; mag. 600.

PLATE III

- Fig. 6. "Guinea-pig strain" colony grown up from pus spread on the surface of special medium after 6 days of incubation. Some big globules can be seen. Photographed alive by Dr Robinow; mag. 90.
 Fig. 7. *L1-like organism* isolated from the "guinea-pig culture". The large, dark, granular centres of the pleuropneumonia-like colonies and the lighter, peripheral zones with their lace-like structures are to be seen. Photographed alive by Dr Robinow; mag. 90.
 Fig. 8. Part of one of these same *L1-like colonies* (see fig. 7) higher magnified; its globular forms can be well distinguished. Photographed alive by Dr Robinow; mag. 486.
 Fig. 9. Colony of "guinea-pig strain" stained. The bacillary filaments, large unstained globules and small stained bodies show up. Agar fixation method, Bouin-Giemsa; mag. 900.
 Fig. 10. *Fusobacterium nucleatum* grown anaerobically on serum agar; a mixture of bacillary chains and globular forms can be seen. Agar fixation method, Bouin-Giemsa; mag. 900.

- Fig. 11. *Fusobacterium nucleatum* grown aerobically in serum broth for 15 hr. The globular forms and a few bacillary chains can be seen. Photographed alive, darkground illumination; mag. 900.
- Fig. 12. *Fusobacterium nucleatum*, edge of an anaerobically grown colony showing only globular forms. Agar fixation method, Bouin-Giemsa; mag. 900.
- Fig. 13. *Sewage organism "A"*. Stained colony edge. Agar fixation method, Bouin-Giemsa; mag. 600.
- Fig. 14. The same, but different colony edge; mag. 900.
- Fig. 15. *Asterococcus canis*, Shoetensack, type II. Stained colony edge, showing large globules, differently stained. Agar fixation method, Bouin-Giemsa; mag. 600.

PLATE IV

- Fig. 16. *L5 organism*, colonies on solid medium, 3 days 37° C. The small, marked dark centre and the delicately structured, peripheral zone can be recognized. Photographed alive by Dr Robinow; mag. 90.
- Fig. 17. *L6 organism*, colonies on solid medium, 4 days 37° C. The globular surface structure shows up. Photographed alive by Dr Robinow; mag. 90.
- Fig. 18. *L6 organism*, edge of a colony grown on solid medium, darkground illumination, photographed without application of a coverslip by Dr Robinow; mag. 490.
- Fig. 19. *L5 organism*, stained, the marked dark centre and the delicate, globular fringe are characteristic for the colonies of this organism. Agar fixation method, Bouin-Giemsa; mag. 600.
- Fig. 20. *L6 organism*, stained colony edge showing a coarser globular structure than the colony of the *L5 organism* (fig. 19). Agar fixation method, Bouin-Giemsa; mag. 600.

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