

PLEUROPNEUMONIA-LIKE ORGANISMS OF DIVERSE PROVENANCE: SOME RESULTS OF AN ENQUIRY INTO METHODS OF DIFFERENTIATION

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(With Plates XVII and XVIII and 8 Text-figures)

THE organism of pleuropneumonia of cattle was first described by Nocard & Roux in 1898. Twenty-five years later, in 1923, a second species of this group, the organism of agalactia of sheep, was discovered by Bridré & Donatien. Two additional types of this group of microbes were isolated from lungs of dogs by Shoetensack in 1934 and 1936. In 1935 and 1936 the writer described another organism, related to pleuropneumonia, and living in symbiosis with a streptobacillus in the association known as *Streptobacillus moniliformis*. Together with Steabben the writer reported in 1937 the occurrence of a similar organism in lung lesions of rats. Recent publications of Laidlaw & Elford (1936), Seiffert (1937 *a, b*), Dienes & Edsall (1937) and Gerlach (1937) have revealed the fact that a number of pleuropneumonia-like organisms, some non-pathogenic, others pathogenic, exists and can be isolated from various materials, such as soil, sewage, or lesions in animals and human beings. Further progress in our knowledge of the group must depend on the elaboration of differential methods, and the writer in the study here presented places on record the results of an investigation of this problem.

THE STRAINS STUDIED

Sixteen strains selected in part from numerous isolations of this group of organisms during the last four years have been studied in detail. In Table I the designation, date of isolation, origin, etc., of the strains examined by different methods are given.

Methods employed in the isolation of the strains studied

The medium of choice for the isolation of strains was a very rich boiled blood agar prepared from ox heart infusion peptone broth. The agar content was 2%, the pH 7.8-8, and 30% of sterile horse serum was added (to be referred to as the special medium) (Klieneberger, 1936). In the writer's experience the medium should possess a semi-solid consistence and a high serum content, and it must not be allowed to dry during the incubation period of 3-6 days. Some strains are more difficult to isolate from the original material than others of the same kind. A device employed if scanty growth is expected, has increased the number of successful isolations. A small piece of medium from an uninoculated plate is cut out and placed on top of the inoculated medium; or if, on the first surface plates, visible colonies have not appeared, it is advisable to transfer a piece of the already incubated medium, upside down, to a new plate. Specially delicate strains like "72 gland" and "L1 M43" required during their first weeks of maintenance special nursing between two agar layers. They would

Table I

Designation	Origin	Isolated by	Date of isolation	No. of passages carried out by the writer
"L1 old"	<i>Streptobacillus moniliformis</i>	Writer	Nov. 1934	338
"L1 rat 30"	"	"	April 1935	182
"59 lung"	Lung lesions of tame rat	"	Nov. 1936	108
"L1 M 43"	<i>Streptobacillus moniliformis</i> "Mackie M43" ("4150 N.C.T.C.")	"	May 1937	64
"Ash"	Lung lesions of tame rat	"	Feb. 1936	150
"5254"	"	"	"	150
"72 lung"	"	"	Oct. 1936	73
"78 lung"	"	"	Nov. 1936	75
"241 lung"	Lung lesions of wild rat	"	June 1937	26
"72 gland"	Enlarged submaxillary gland of tame rat	"	Oct. 1936	73
<i>Asterococcus canis</i> type I	Dog	Shoetensack	1933	45
<i>Asterococcus canis</i> type II	"	"	1933	45
Agalactia ("3722 N.C.T.C.")	Sheep	—	Deposited in N.C.T.C. 1932	—
Pleuropneumonia ("3278 N.C.T.C.")	Cattle	—	Deposited in N.C.T.C. 1931	—
Pleuropneumonia ("4159 N.C.T.C.")	"	—	Deposited in N.C.T.C. 1933	—
Pleuropneumonia ("4732 N.C.T.C.")	"	—	Deposited in N.C.T.C. 1933	—

not grow on the surface for some time, but developed quite well under cover. The same device can also be used for the revival of old strains which have not been subcultured for some time.

The method employed for the separation of the L1 symbiont from the parent *Str. moniliformis* has been described in previous papers (1935, 1936). Two of the old strains then isolated, "L1 old" and "L1 rat 30" (see Table I), have been used in the present study. The isolation of an additional L1 strain was extremely difficult and occupied the writer for some months; it will be recorded in detail. It is of interest, also, as it was obtained from Mackie's *Str. moniliformis*, strain "M43", studied by Mackie *et al.* in 1933 and van Rooyen in 1936; it has been maintained as a pure L1 strain ever since. Its origin therefore merits detailed description:

In 1936 van Rooyen based his studies on *Str. moniliformis* on certain strains from mice, in which he was unable to demonstrate the L1 symbiont which the writer had not failed to find in all strains of this symbiotic culture so far examined (1935, 1936). As it seemed desirable to clear up this discrepancy the writer asked Dr van Rooyen for subcultures of those strains on which his morphological and cultural descriptions were founded, but unfortunately they had all died out. Fortunately one of the old strains with which Mackie and van Rooyen had kindly supplied the National Collection of Type Cultures in 1933 was still preserved there; this strain "M 43" was not only one of those on which the writer's former work was based (1935, p. 94), but had also been used by van Rooyen in his published work of 1936. In January 1937, therefore, the writer commenced a re-examination of this strain in order to see if it might have lost its symbiont after 4 years of subculturing. Dark-ground examination, direct agar microscopy and the agar fixation technique (Klieneberger, 1936) revealed clearly all the characteristic elements of a *Str. moniliformis* culture, viz. bacillary chains, globules of large and small size and the filamentous formations distinguishing the L1 symbiont. The writer then attempted the *experimentum crucis*, viz. the separation of the L1 organism from the parent mass. The first attempts were unsuccessful as the activity of growth of this old strain, which had been subcultured only once a month in the course of 4 years was very low. Consequently the writer submitted the strain to daily serum-broth passages for 4 weeks before attempting again the isolation of the symbiont. After this time the strain grew readily

in serum broth in 20 hr. if one drop was used for the subculture, whereas a similar seeding had required 4 days in the beginning of the month for growth to appear. The method of choice for the isolation of the L1 which among other methods had already been used in 1935 and 1936 was the following: A freshly grown serum-broth culture of a *Str. moniliformis* strain is kept at 37° C. and, after shaking, a drop of this suspension is plated on successive days on the writer's special medium. These plates are incubated for about 6 days and each day examined under the low power of the microscope. When the small L1 colonies are grown up in suitable distribution between the large *Str. moniliformis* colonies, pieces of the medium, containing L1 colonies only, are cut out and transferred to new plates. Many pieces have to be transferred in order to obtain a second passage, and after failures the whole process has to be repeated until a pure L1 strain is obtained. The separation of the L1 growth from its parent mass has been usually achieved in the writer's previous attempts in the course of 3 weeks. In the case of the strain "M43" it took 3 months, for it happened twice that an apparently pure L1 strain reproduced after a number of passages *Str. moniliformis* colonies; after a second and third purification, however, this incident no longer occurred. The pure strain "L1 M43" has now been subcultured sixty-four times. Though growing delicately for a long time and only between two agar layers, it now develops readily on solid and in liquid media so that it could even be used successfully for serological and other tests.

From van Rooyen's account of his procedure it is easy to understand why he was not able to demonstrate the L1 organism in his cultures. In his description on p. 464 he says: "The subsequent methods of obtaining preparations were similar to those employed by Ledingham (1933) and Klieneberger (1935)"; but if one compares his description and the writer's (1934-6) one will notice that the methods are different in essential points. Van Rooyen has not applied Flemming's weak solution used by the writer which is essential to fix "the filamentous structures"¹ and to reveal the real organization of a *Str. moniliformis* and L1 colony; accordingly these "filamentous formations" cannot be detected at all in the illustrations of van Rooyen's paper. Another difference is that van Rooyen presses the coverslip firmly on the grown agar slab which squashes most of the L1 elements, while the writer takes the greatest care to place the agar slab gently on the slip; finally, instead of immersing medium and adherent coverslip in the fixative as van Rooyen does, the writer uses a specially made glass slab to prevent the fixative from penetrating between coverslip and agar so that fixation takes place only through the agar. In addition, the fixation times which van Rooyen uses are different from those given in the writer's description.

With regard to cultivation methods no indication can be found in van Rooyen's paper of his having tried to culture the L1 colonies according to the writer's method. So far as the writer can see, he has used only Loeffler's serum or blood—or serum-agar. If therefore neither the fixing methods nor the culturing methods recommended by the writer were applied it is not surprising that van Rooyen's results disagree with the writer's and that he was unable to detect the L1 symbiont in *Str. moniliformis* cultures. From the detection of the L1 symbiont in the only one of van Rooyen's strains now available, it cannot be postulated that the other strains employed by him in his study of the morphological cycle of *Str. moniliformis* also contained this symbiont. Nevertheless, it is a reasonable inference that the isolation of the symbiont, even from one of the strains he studied, must vitiate to some unknown degree the interpretation he has placed on his morphological data in so far as they bear on his efforts to define the systematic position of *Str. moniliformis*.

The strains "L1 old" "L1 rat 30" and "L1 M43" are the three strains of *Str. moniliformis* origin studied in this work. The six strains "59 lung", "Ash", "5254", "72 lung", "78 lung" and "241 lung" were all isolated from the lung lesions of rats; five of them from tame

¹ As is apparent from his description and figures van Rooyen uses the expression "filaments" for bacillary chains and long bacillary forms while the writer designated the thread-like structures, developed by pleuropneumonia-like organisms only, as "filamentous formations".

laboratory rats, one, "241 lung", from lesions in a small wild rat. 139 pleuropneumonia-like strains were in all obtained from rats' lungs; of these, the six strains referred to were selected for a more elaborate study on different lines. All the lung strains were isolated in the same way: Pieces of the diseased lungs were minced and spread on plates of the special medium; sometimes exudate or pus was used instead of the minced tissue. The plates were incubated for 4-6 days and each day examined under the low power of the microscope. The subculture was in most cases easily achieved by transferring a piece containing colonies to a new plate. The strain "72 gland" was obtained from the swollen submaxillary gland of an old rat with eye, ear and lung affections. It grew for a long time in very tiny colonies and more slowly than the strains from the rats' lungs.

Sir Patrick Laidlaw kindly supplied the National Collection of Type Cultures with Shoetensack's types of *Asterococcus canis* which grow very well on the writer's special medium. The agalactia strain (No. "3722 N.C.T.C.") and one of the pleuropneumonia strains (No. "3278 N.C.T.C.") have already been used in Ledingham's paper of 1933 and in the writer's study of 1934. The two other pleuropneumonia strains (Nos. "4159" and "4732" N.C.T.C.) have been kept in the National Collection of Type Cultures since 1933. The strains of agalactia and pleuropneumonia grow well on ordinary serum media, but they develop more luxuriantly on the special medium.

Maintenance of the cultures

The writer's whole collection of pleuropneumonia-like strains, which consists now of twenty-five strains, is kept on the special solid medium. While for isolation purposes a very high serum content (30%) is required, a serum content of about 10% is sufficient for maintaining old established cultures. Plates are exclusively used for the subcultures, because this is the easiest way to detect contaminations, even if they form only tiny colonies, before they have overgrown and spoiled the whole culture. The plates are incubated for 3 days and each day examined under the microscope. They are then kept in the cold for about 10 days and again subcultured. A number of the cultures would now keep alive for a considerably longer time, but freshly isolated strains require subculture at shorter intervals; these latter grow only if a large amount of material is transferred while "old" strains can grow up from a very small inoculum.

DIFFERENTIAL CRITERIA INVESTIGATED AND RESULTS OBTAINED

(1) *Growth in liquid media*

All pleuropneumonia-like strains have this in common that they grow at first slowly and scantily and adapt themselves only gradually to artificial media. This adaptation is not a matter of some weeks, but of months or of even longer periods. Neisser stated (1926) that it is necessary for some bacterial cultures to subject them to frequent passages on an optimal medium before they can be studied successfully. He calls this the "norming" ("Normieren") of the strains. Such a "norming" is certainly required for cultures of the pleuropneumonia group. When they have been kept on artificial media for a period of 6 months or a year they are "normed", for they then clearly show their special growth characters which remain constant under constant growth conditions.

The strains isolated by the writer did not at first grow visibly in the liquid medium, but after 10-20 passages on the solid medium they started to grow in the special broth. They grow now as well in the liquid as on the solid medium. The growth in the broth is characteristic for certain groups of strains. "L1

old", "L1 rat 30", "L1 M 43", the three symbionts isolated from *Streptobacillus moniliformis* cultures and the strain "59 lung" found as an independent organism (not associated with a bacillus) in the lung lesions of a rat, show the same type of growth in the liquid. These four strains grow in large colony clumps some of which stick to the wall of the test-tube while others form a sediment at the bottom. The clumps are seen by naked-eye examination after 2-3 days of incubation and may reach half the size of a pin's head; the broth itself remains clear.

The five strains "Ash", "5254", "72 lung", "78 lung" and "241 lung", all originating from lung lesions of rats, grow in colony clumps too, but they are much smaller and more numerous than those of the first group. They stick to the glass walls in the same way, especially to the tilted parts, and form a sediment at the bottom, comparable to a fine layer of sand grains. These clumps or flakes of the second group can be seen only by means of a lens.

A particular strain is "72 gland" recovered from a rat also suffering from lung disease. Inoculated into the special broth it causes a slight opalescence of the medium, but never forms any clumps that can be detected with the lens.

Asterococcus canis type I and type II both produce an opalescence in broth more marked than that produced by "72 gland" but not so marked as that by strains of pleuropneumonia.

Pleuropneumonia forms quickly a uniform strong opalescence in the special broth; it grows quite well in ordinary serum broth.

Agalactia, which also develops in ordinary serum broth—but more slowly than pleuropneumonia—forms numerous small flakes which can be seen only with the lens. A sediment may occur, but most of the flakes are suspended in the liquid and never stick to the walls. The growth resembles a suspension in which agglutination has just started.

As we have seen, therefore, from the above description, the strains examined could already be divided up into different groups according to their type of growth in the liquid medium.

(2) *Growth on solid medium (colony type, dissociation)*

Most strains grow in tiny colonies on solid media when freshly isolated. The more they are transferred the quicker they grow and the larger are their single colonies until they have reached their "norm". Well-isolated colonies of one and the same strain show after 3 days' growth on the special medium a characteristic size. Table II gives the average colony sizes for the strains studied. As may be seen from this table the largest colonies are formed by the organism of pleuropneumonia and by the four strains of the first group. The next largest colony is formed by *Asterococcus canis* II, while *A. canis* I develops a slightly smaller colony. Very uniform in size and smaller than all those above mentioned are the average colonies of group II. The smallest colonies are formed by the strain "72 gland" (which, apparently, is a single representative of a special group) and by the organism of agalactia.

Table II

Strain	In culture since	Colony diameter in mm.	Average diameter of group in mm.
"L1 old"	3 years	0.6	0.35-0.6, group I
"L1 rat 30"	2 years	0.55	
"L1 M43"	8 months	0.35	
"59 lung"	1 year	0.4	
"Ash"	2 years	0.2	0.2, group II
"5254"	2 years	0.2	
"72 lung"	1 year	0.2	
"78 lung"	1 year	0.2	
"241 lung"	6 months	0.2	0.12, group III
"72 gland"	1 year	0.12	
<i>Asterococcus canis</i> type I:	Some years		
Coarse colony		0.25	0.15-0.25, group <i>Asterococcus canis</i> I
Granular colony		0.15	
<i>Asterococcus canis</i> type II	"	0.25-0.3	0.25-0.3, group <i>Asterococcus canis</i> II
Agalactia, strain "3722"	More than 5 years	0.1	0.1, group agalactia
Pleuropneumonia, strain "3278"	" 6 "	0.4	0.4-0.6, group pleuropneumonia
Pleuropneumonia, strain "4159"	" 4 "	0.6	
Pleuropneumonia, strain "4732"	" 4 "	0.45	

It appears that if the average colony size after 3 days of incubation is used as a means of differentiation, the groups resulting are the same as those based on growth type in liquid medium. These preliminary groupings are shown in Table II. It will be seen in the course of this paper that not only are these same groups characterized by the tests already described, but that the strains of each group have so many distinctive features in common as to render feasible their classification as different species of the one family of pleuropneumonia-like organisms. For convenience a simple designation has been chosen and will be used in the following. The first group or species, characterized by the formation of large colony clumps in broth and an average colony diameter of 0.35-0.6 mm. after 3 days of incubation is designated as L1 organism, a name that has already been used for the symbiont of the *Streptobacillus moniliformis* culture which belongs to this species. The four strains "L1 old", "L1 rat 30", "L1 M43" and "59 lung" are members of this species. The second group of cultures, all isolated from lung lesions of rats, which form small colony flakes in the broth and have an average colony size of 0.2 mm. belong to a species designated as L3 organism.¹ The single culture, "72 lung", as different from L1 as from L3 by its growth in broth and by its colony size, is called the L4 organism. The other strains which also belong to special types or species are referred to as *Asterococcus canis* I and *A. canis* II (according to Shoetensack) and as the organisms of agalactia and of pleuropneumonia (according to general usage).

Apart from mere size, colony structure after about 3 days of growth can also be used for differentiation, though the colonies in all the groups of pleuropneumonia-like organisms have some striking features in common. They

¹ The name L2 has already been used for the apparent symbiont of a streptococcus culture which however could not be independently isolated (Klieneberger, 1935).

all possess a dark centre and a lighter peripheral zone. This appearance is caused by their tendency to grow into the medium, the centre as the oldest part of the colony embedding itself more deeply than the marginal part. Further, all organisms of the pleuropneumonia family develop a kind of yellowish pigment during a prolonged period of incubation. Finally, they have all very small colonies compared with bacteria and other microbes which repay microscopical study. Though all these colonies have much in common they vary in structure according to the group or species to which they belong. Photographs and drawings of the different types illustrate the various appearances. In the L1 colony (Pl. XVII, Fig. 1) the dark granular centre is relatively small compared with the peripheral zone. The latter shows a very characteristic, coarse structure of lace-like appearance in which large globules and loops can be recognized. The central part of the L3 colony (Pl. XVII, Fig. 2) is less marked than that of all the other species; it shades off gradually into the less dense and less dark peripheral zone; the large colony in the right hand upper corner of Fig. 2, Pl. XVII illustrates this quite well; in addition, the surface of the L3 colony is distinguished by a certain roughness. Sometimes globules and loops can be detected in the peripheral part of one or the other colony, but the lace-like structure of the L1 colony is not produced. The L4 colony (Pl. XVII, Fig. 3) is also very characteristic. In contrast to the L3 colony the central part is always well distinguished; the peripheral zone is relatively dense and uniformly granulated. The L4 colonies show a greater similarity to pleuropneumonia colonies than any of the other types, but they are much smaller and denser. *Asterococcus canis* I presents a very fascinating feature of great interest to students of microbic dissociation. The original *A. canis* I colony (Pl. XVII, Fig. 5) has a relatively large, well distinguished, dark, granular centre and a dense, granular peripheral zone. This granular colony dissociates and throws off a different kind of colony, larger in size. The centre is dark too; the very light peripheral zone shows the same lace-like structure as an L1 colony, only more delicate and more transparent. The following drawings show the appearance of a "granular" (Text-fig. 1) and a "coarse" colony (Text-fig. 2) of *A. canis* I.



Fig. 1.



Fig. 2.

This dissociation and the appearance of both types of colonies (which are called the "granular" and the "coarse" variety) is illustrated by the photographs on Pl. XVIII, Figs. 8 and 9. The "coarse" colony of *A. canis* I resembles an L1 colony as a small child its grown up brother. Single colonies of both types of *A. canis* I have repeatedly been picked in order to discover if they breed true. The "coarse" colony has so far remained "coarse" through twenty-five passages. Pl. XVIII shows in fig. 11 a photograph of this "coarse" strain of *A. canis* I. In

contrast to the "coarse", the "granular" variety does not breed true. The strain selected dissociates again and shows after a time both kinds of colonies. An immune serum has been produced in rabbits from both varieties of *A. canis* I, but no serological difference has been established. *A. canis* II develops fairly large colonies with a small, dark, granulated centre and a large, dense peripheral zone (Pl. XVII, fig. 7).¹ The 3 days old colonies of pleuropneumonia are large on the special medium (Pl. XVII, fig. 4). They possess a small granular centre and a large, light peripheral zone. The very dark and dense central zone occupies a relatively large area of the small colony of agalactia while the small peripheral zone consists after 3 days of a thin transparent layer only. Globules can sometimes be detected in this marginal part by the low power of the microscope. The photograph (Pl. XVII, fig. 6) shows the 3 days old colonies. The growth of agalactia is always accompanied by a production of numerous crystals (Ledingham, 1933), a phenomenon which has not been observed in any other species of the family. The culture of agalactia, No. "3722, N.C.T.C.", the only one in the writer's possession, showed some time ago the same kind of dissociation as that described for *A. canis* I. It threw off colonies of the "coarse" type, much larger and more transparent than the "granular" ones. Globules and loops could be easily recognized in the delicate, lace-like peripheral zone by low magnification. The isolation of both varieties was so far unsuccessful in that they did not breed true. Now they both show again only one kind of colony, the "granular" type.

This dissociation into two types of colonies has only been observed in *A. canis* I and the organism of agalactia.¹ How these different colony appearances are produced is difficult to explain. The differences can already be noticed in an early stage of development. The drawings (Text-figs. 3, 4) show how the "granular" and "coarse" colonies grow up.



Fig. 3.

Fig. 4.

The "granular" kind (Text-fig. 3) can already be seen when still very small; it represents at first a pin point, refractile, greenish dot. This grows into the dark centre; later on the peripheral part appears. In contrast to this the "coarse" colony is at first so transparent that it is only detected when it has already reached some size; there is hardly any contrast between the young structureless colony (Text-fig. 4) and the medium. It is more easily detected on the agar surface when the coarse structure begins to develop in the centre as shown in Text-fig. 4. This structure spreads from the centre to the periphery

¹ Since this was written *A. canis* II has dissociated in a similar way to *A. canis* I.

and becomes more and more marked. It is of a dark and very rough appearance in the L1 organism (Text-fig. 4) and more delicate in *A. canis* I "coarse" (Text-fig. 2). There is some difference in the morphology of both colony varieties in so far as the "coarse" kind contains larger elements than the "granular" kind. Enormous globules, loops and filaments develop in the "coarse" colony, and it is certain that these large elements produce the lace-like appearance, but there is no distinguishing morphological difference between both colony varieties as they both contain the same elements. While some of the enormous elements of the "coarse" colony can already be seen by low magnification ($\times 80$) the mass of the large elements can readily be observed without application of stain by direct agar microscopy (Klieneberger, 1936) and the still relatively low magnification of $\times 200$. Pl. XVIII, figs. 13, 14, 10, 12 show photographs of colonies of L1, *A. canis* I "coarse", agalactia "coarse" and *A. canis* II ("granular" colony type) demonstrated by this method. The differences between the 3 "coarse" colonies (Pl. XVIII, figs. 10, 13, 14) and the "granular" colony (Pl. XVIII, fig. 12) are even more striking when studied by this magnification; but the comparative differences of the colony types in so far as they have a bearing on the differentiation between various species are detected only with the low power of the microscope.

(3) *Morphological differences of the various strains*

All pleuropneumonia-like organisms—so far studied—develop the same kinds of elements, granules, filamentous formations and globular forms. In the writer's former studies of pleuropneumonia, agalactia and the L1 organism three different methods have been used for the demonstration of the morphology, viz. the agar fixation method (Giemsa stain), the direct agar microscopy and the dark-ground illumination method. The first method produces very pretty and reliable preparations, but it is rather laborious; the second method usually does not reveal sufficient detail even with high magnification; the dark-ground method does not always show up all the elements present owing to their varying refractibility. It was not possible to study all the strains by the three methods. The dark-ground was therefore chosen as a quick and convenient method for daily routine examination, and some experience concerning the average morphological appearance of the various strains has been acquired. It is hardly possible to judge from the mere morphological appearance to which species an organism in question belongs. Nevertheless it struck the writer during periods of daily tests that, if grown under the same conditions, regular differences occurred which gave an indication as to which group a strain belonged. Therefore short descriptions of the average dark-ground picture of the different species, grown for 2–3 days in the special broth, are given in the following.

L1 organism. Only large colony clumps showing a bright granular centre and a peripheral zone consisting of globular bodies are seen. Some of the large globules possess very refractile contours. They seem either empty or filled with

granules in active Brownian movement. From the edge of the colony filaments of different width and refractibility may protrude into the surrounding liquid. The filaments may be studded with granules and globules.

L3 organism. Single elements, granules and small filaments and many compact small and large colony clumps containing granules and small globules are found.

L4 organism. A well-dispersed suspension of small elements is seen. Many granules and many small filamentous forms, single and in "asters", are present. Rarely, clusters of elements are found which contain globules of different size some of which may be very large. The picture resembles that of a pleuropneumonia culture, but branched, filamentous forms are not observed.

A. canis I. Many small groups of elements containing granules and small globules are to be seen; small filaments, single and "asters", are found as well. (There is no difference between the "coarse" and the "granular" type in the liquid medium.)

A. canis II. Small elements, chiefly granules, and small groups of elements are to be seen. Globules in groups, more uniform in size and shape than in other species, occur which—but for their shrinking contours—resemble yeast cells.

Pleuropneumonia. The refractile filamentous formations, single or in "asters" and long branched forms, are very characteristic. Groups of elements containing granular masses and small and large globules are found.

Agalactia. The filamentous forms, single and "asters", are smaller than in pleuropneumonia. Many small colony clumps of granular appearance characterize the picture.

(4) *Natural and experimental associations of pleuropneumonia-like organisms with bacteria*

The L1 organism was found in symbiotic association with a bacillus in the cultures of *Streptobacillus moniliformis*. The strain "59 lung" isolated from a rat's lung, shows that it may also occur independently. Apparently *B. actinoides* (Theobald Smith) is an association similar to *Streptobacillus moniliformis*. The writer has so far not been able to separate a second pleuropneumonia-like species from another parent bacterium though the study of certain bacterial cultures such as *B. funduliformis* (syn. *Fusobacterium nucleatum*) raised the suspicion that they contained a second organism. In order to see if there was a tendency in the pleuropneumonia group to associate with bacteria, pleuropneumonia and the L1 organism were combined with different organisms. Most of these attempts turned out failures, for *B. coli*, *B. paratyphosus* B, *staphylococcus*, *streptococcus*, *B. subtilis*, *sarcina* would not combine with the two filterable microbes, which were rapidly suppressed in the mixtures. The same happened if a banal contamination grew up in one of the pleuropneumonia-like cultures; the latter organism was soon lost. In contrast to this, anaerobic bacilli, especially spore-bearing kinds as *B. tetani* and *B. tetano-*

morphus, combine successfully with pleuropneumonia or the L1 organism. Both associates, the anaerobic bacillus and the filterable organism, develop together far more rapidly and luxuriantly than when single. They form overnight an enormous and, in the case of the combination with pleuropneumonia, sticky sediment at the bottom of the tube. The mixtures are kept aerobically in ordinary serum broth; if subcultured a few drops are transferred. Both associations have undergone some hundred passages up to date. If an ordinary smear from these cultures is stained, only the bacilli are seen, but the dark-ground preparation reveals the second filterable organism in both associations. The bacilli are very motile; they swarm round the clusters of the filterable microbes (Text-figs. 5 and 6).

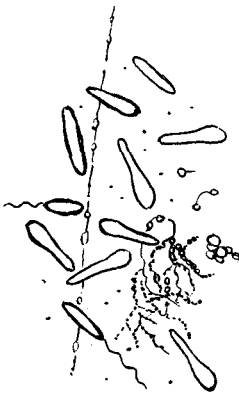


Fig. 5.

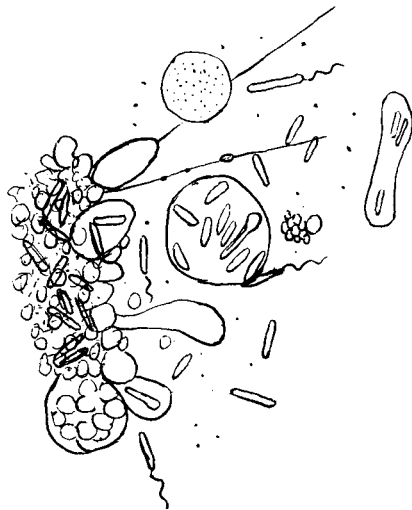


Fig. 6.

They enter the large globules of the L1 and move about inside (Text-fig. 6). The association of L1 with *B. tetani* and *B. tetanomorphus* differs from that of *Streptobacillus moniliformis* in that both members can be recovered from the mixture in a state of purity. It would be of interest to investigate if other pleuropneumonia-like organisms tend to live in nature in association with bacteria. New modes of distinguishing the various species of the pleuropneumonia group might be discovered by studies of this kind.

(5) *Serological examination of the strains*

The serological work met at first with great difficulties. The scanty and irregular growth of the young cultures was the greatest obstacle for some time, but later when the strains had been "normed" it was possible to devise an agglutination technique for all the strains under examination. The method of test and the results obtained are recorded below.

Immunization of rabbits

Suspensions of the living organisms were injected intravenously. They were prepared from cultures grown in the special broth. To avoid the presence in the rabbit immune sera of precipitins for foreign protein, the horse serum used in the preparation of the media for the isolation and maintenance of the cultures was invariably replaced by normal rabbit serum, whenever cultures were grown for the purpose of immunization of rabbits. Flasks containing 85 c.c. of liquid medium plus 15 c.c. of sterile, normal rabbit serum were inoculated with a small piece removed from a well-grown, young plate culture (rabbit serum agar). After 3–5 days of incubation the cultures had grown well; they were centrifuged and the sediments taken up in saline; after shaking, these suspensions were used for the injections. The rabbits showed no reaction during the inoculation period. It was found that a considerable number of large antigen doses given in short intervals of 48 hr. was necessary to produce sera of fairly high titres. The rabbits received usually nine injections of 5 c.c. of antigen of different strengths. The suspensions were standardized by the "Wellcome Opacity Tubes". The first three suspensions used for immunization had an opacity corresponding to that of Wellcome opacity tube No. 3. About 50 c.c. of a well-grown liquid culture were necessary to produce 5 c.c. of such a suspension. The doses given on the three following occasions had twice that strength while those used for the three last injections had four times that strength. Sometimes still higher doses of six or eight times this opacity were given. The optimal time for the collection of the blood was 4 days after the last injection.

Preparation of antigens for the agglutination test.

The preparation of homogeneous and stable suspensions was the most difficult task. Different suspension media (acid and alkaline), heating, shaking and grinding of the material and combinations of these methods were tried. A mere mechanical breaking up of the clumps and the elimination of larger particles if still present by fractional centrifugation proved to be more effective than the other methods tried. The standard method finally devised was the following: The cultures were grown in flasks containing 100 c.c. of liquid medium (85 c.c. of special broth and 15 c.c. of horse serum). 10 flasks (1 litre) was the usual amount from which one batch of suspension was prepared. After 3–5 days of incubation the broth was centrifuged and the sediment washed once with glass-distilled water. After trituration of the fairly thick sediment for about 1 hr. it was resuspended in 5 c.c. of a formolized buffer solution of $pH = 7.1-7.2$.¹ This heavy suspension was centrifuged for 20–30 min. at a speed of 2000 rev./min. and if necessary at a still higher speed. The supernatant represented the stock antigen. For use it was diluted to an opacity corresponding to Wellcome opacity tube No. 1 and equal amounts of this diluted antigen and serum dilutions were mixed for the actual agglutination test. The stock suspensions could be kept for months, if stored in the cold, without any marked reduction of titre. Suspensions of all the L1 and L3 strains of agalactia and *Asterococcus canis* I and II were prepared in this way. Suspensions of pleuropneumonia and L4 could be secured in a still simpler way. Suitable stock antigens of these organisms were obtained by shaking the washed sediment with beads in the formol buffer saline for about half an hour; this procedure was followed by short centrifugation.

Though a fair number of antigens prepared by the methods described were sufficiently homogeneous and stable to give satisfying results, some of them, prepared in the same way, failed to keep stable in the serum and saline controls during 24 hr. Those latter antigens were discarded.

It might be of interest to note that grinding with glass powder or sand, which produced

¹ M/5 citric acid	0.15 c.c.
M/5 Na ₂ HPO ₄ .12H ₂ O	1.85 c.c.
Formalin	0.25 c.c.
Saline	to 100.00 c.c.

very good suspensions with various L1 strains produced considerable deterioration of the antigens of most other organisms, such as agalactia, L3, *A. canis* I and II. Possibly too great a number of small particles of these organisms had been absorbed by the powders. Trituration of these strains was carried out therefore without the addition of any powder.

The agglutination test

The test was carried out in lilliput tubes. To 0.1 c.c. of diluted serum 0.1 c.c. of antigen was added. The serum was diluted with physiological saline. Each test contained controls with physiological saline, 5% NaCl solution and also with the corresponding normal rabbit sera, which had been taken from each individual rabbit before the commencement of the immunization. The controls if negative showed no sedimentation during 24 hr. The tests were kept at 50° C. for 2 hr. and stored in the cold overnight. The reading took place after 24 hr.

The immune sera tested had been prepared from three different *Streptobacillus moniliformis* strains (viz. "39 lung", "72 eye", and from Mackie's *Str. moniliformis* strain "M43"), from two L1 strains (viz. "L1 old" and "59 lung"), from three L3 strains (viz. "Ash", "5254" and "78 lung") and from one strain of each of the following species: L4 ("72 gland"), pleuropneumonia (N.C.T.C. "3278"), agalactia (N.C.T.C. "3722"), *Asterococcus canis* I and *A. canis* II. Antigens for agglutination tests were prepared from the 16 strains given in Table I. All of the sixteen antigens were tested against all the sera available. Different normal sera from sheep, horse, cattle, mouse, rat, rabbit and man were tested as controls; they gave no reactions with any of the suspensions; nor had various immune sera prepared against *B. coli*, *B. enteritidis*, *B. typhi-murium*, *B. pestis*, *B. pseudotuberculosis rodentium* and vaccinia virus any effect on the suspensions.

The positive agglutination reactions were usually very marked in the first serum dilutions and the reaction started often very quickly. The first sign of an agglutination was an increase of the turbidity of the tube in question. The agglutination has often been watched microscopically by the dark-ground method and high magnification (7 × 90). The drawings below illustrate the appearance of the unaffected and the agglutinated suspensions of the L1 organism in dark-ground illumination (Figs. 7, 8).

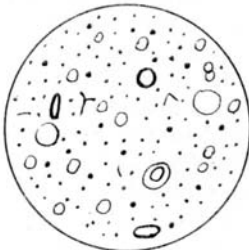


Fig. 7.

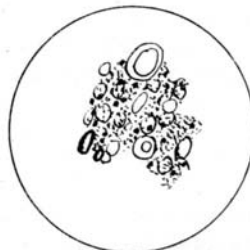


Fig. 8.

Results of the agglutination tests

Agglutination reactions showed that specific agglutinins had been produced in the rabbits. Cross agglutination demonstrated clearly which of the strains were of the same antigenic structure and which were different. Four of the sixteen strains under examination (see Table III) were agglutinated to a fairly high titre (1 : 320 to 1 : 640) by the sera prepared from all the *Streptobacillus moniliformis* and from the L1 strains. The L3, L4, pleuropneumonia, agalactia, *Asterococcus canis* I and *A. canis* II sera had only a very slight effect or none at all on these suspensions of strain "L1 old", "L1 rat 30", "59 lung",

and "L1 M43". The four strains referred to, presenting similar agglutination reactions have already on the basis of other characters been placed in the group of which L1 is taken as the prototype.

The positive reaction of all *Streptobacillus moniliformis* sera with all L1 suspensions confirms the results of previous investigations on the symbiotic nature of the cultures of *Str. moniliformis*.

Table IV shows that the five strains "Ash", "5254", "72 lung", "78 lung" and "241 lung" belong to the same group serologically. They are all agglutinated to a more or less high titre by all the L3 sera prepared, while sera prepared by immunization with *Streptobacillus moniliformis*, L1, L4, pleuropneumonia, agalactia, *Asterococcus canis* I and *A. canis* II had very little or no effect at all on the suspensions of these five strains. The five strains, distinguished by the same antigenic structure, have already been diagnosed as members of one group by other tests. The agglutination reaction proves again the serological identity of cultures of the same origin, morphology and habits of growth.

In the same way the three strains of pleuropneumonia show one agglutination type as can be seen from Table V. The pleuropneumonia serum has a strong effect on the three suspensions (titre: 1:320 to 1:640). None of the other sera gives a reaction with a pleuropneumonia antigen. This shows again that the species pleuropneumonia—well defined by other criteria—is distinguished by its specificity in agglutination test.

The four remaining strains differ serologically from each other as well as from any of the other cultures. Each of them is agglutinated only by its homologous serum and very little or not at all by any of the other sera. These four strains are: one L4 strain ("72 gland"), one agalactia strain (N.C.T.C. "3722") one *Asterococcus canis* I and one *A. canis* II strain. They are single representatives of four different species of the pleuropneumonia family.

To sum up, the sixteen pleuropneumonia-like strains examined belong to seven different agglutination types. According to corresponding differences in other properties their classification into seven different species seems feasible. The seven agglutination types are shown in Table VI.

Table VI shows further that a certain small amount of overlapping occurs in the reactions of the different species. The *A. canis* I serum, for example, agglutinates slightly the L3 strains in dilutions of 1:10 and 1:20, and the L3 sera agglutinate the *A. canis* I suspension to a slight degree. As bacterial immune sera and different normal sera had no effect on any of the suspensions these reactions between sera and strains of different species seem to be due to a group specific antigen. Whether some species of pleuropneumonia-like organisms possess such a common antigen can be decided by cross absorption tests.

(6) *On the pathogenicity of the different species*

The pathogenicity of the organisms of pleuropneumonia and agalactia is well known. Shoetensack (1936) reports experiments with *Asterococcus canis* I

and *A. canis* II from which he draws the conclusion that the first organism has a certain degree of pathogenicity for dogs, but not the second.

The L1 organism has, when combined with a streptobacillus in the cultures of *Streptobacillus moniliformis*, a marked pathogenicity for mice and as reported in the literature also for human beings. Dienes & Edsall (1937) obtained an L1 strain, independently from the bacillus, from an excised suppurating Bartholin's gland from a human patient. They report pathogenicity of their L1 strains (from rats and a human being) for half-grown white mice. If they injected small pieces of serum agar, containing L1 growth, ground and suspended in saline, the mice died after 1-10 days; they showed, if dying after the third day, a clear pleural exudate and sometimes an oedema. When the L3 was first found in the lung lesions of rats its difference from the L1 was not clearly recognized. In the course of the detailed study of the strains recorded here, it has emerged that the L3 is an organism definitely different from the L1. For reasons to be recorded in a later paper it is supposed that the L3 (not the L1) has some causal connexion with the lung disease in which it is found, though it has not so far been possible to produce the condition with the culture. Infection experiments in mice, recently started with the L3 strains, seem to be more promising than those in rats. Intraperitoneally injected into mice, these strains show a tendency to produce abscesses in the place of injection. The reaction is considerably increased, and large abscesses develop, if Dienes' method is used and an emulsion of the culture in serum agar and saline is injected. A number of passages from mouse to mouse have been performed, and it seemed that the pathogenicity of the L3 was thus aggravated. Some of the mice died about 6 days after the injection. The abscesses developed in the others in about 10-14 days. Many colonies of the L3 could be recovered from the pus and often from the spleen as well. The L1 strains, so far tried by the same method, gave in contrast to the results of Dienes and Edsall, either no reaction or, in one case, a very slight one. These experiments in mice show that the L1 and L3 species are distinguished, also, by differences in pathogenicity.

SUMMARY AND CONCLUSIONS

1. Ten pleuropneumonia-like strains isolated from various sources have been selected and studied with a view to discovering reliable criteria for differentiation. Three of them were isolated from the symbiotic association known in the literature as *Streptobacillus moniliformis*, six from lung lesions of rats and one from a swollen submaxillary gland of a rat. For comparison with these, six other strains, viz. three pleuropneumonia, one agalactia and two of Shoetensack's strains from lungs of dogs stated by him to be suffering from distemper and named by him *Asterococcus canis* type I and *A. canis* type II have been included in the inquiry.

2. Classification of the sixteen strains has been achieved by a comparative study of type of growth on liquid and solid media, morphology, pathogenicity and serological affinities. Table VII displays these differences.

Table VII. *Differentiation of species*

Isolated from	Growth in special broth	Average diameter of well-isolated, 3 days old colonies on special agar m.m.	Colony type on special agar	Dark-ground picture after 2-3 days in special broth	Agglutinated by	Pathogenicity	Species
<i>Str. moniformis</i> and rat's lung	Large clumps visible with naked eye	0.3-0.6	"Coarse" colony, lace-like peripheral zone	Large clumps containing large globules and filaments of different width	<i>Str. moniformis</i> and L1 sera	—	L1
Lung lesions of rats	Small clumps visible with lens only	0.2	"Granular" colony, no marked centre	Small and large clumps containing granular material and small globules	L3 sera	Produces abscesses in mice	L3
Rat's gland	Slight, uniform opalescence	0.12	"Granular" colony, sharply marked, dense centre	Mostly small elements, rarely clusters of globules	L 4 serum	—	L4
Cattle	Marked, uniform opalescence	0.4-0.6	"Granular" colony, large peripheral zone	Many filaments, single, in "asters" and in long branched formations; clusters of globules occasionally	Pleuropneumonia sera	Pathogenic in cattle	Organisms of pleuropneumonia of cattle
Sheep	Opalescence, flakes visible with lens	0.1	"Granular" colony, large, dense centre, may dissociate in "coarse" type	Filaments smaller than in pleuropneumonia; clumps with granular content and small globules	Agalactia serum	Pathogenic in sheep	Organism of agalactia of sheep
Dog	Slight, uniform opalescence	0.16-0.2	"Granular" colony dissociates into "coarse" type with delicate lace-like periphery	Small elements, single and in groups; clumps with granular content and small globules	<i>A. canis</i> I serum	Pathogenic in dogs	<i>A. canis</i> type I
Dog	Uniform opalescence	0.25-0.3	"Granular" colony, dark centre, dense peripheral zone	Small elements, single and in groups, clusters of globules, uniform in size and yeast-like in shape	<i>A. canis</i> II serum	—	<i>A. canis</i> type II

3. The serological examination was of special importance for the whole problem of classification. For this purpose an agglutination test was devised which clearly revealed that the sixteen strains belonged to seven different serological types. As the strains of the same antigenic structure showed similar growth types in liquid and solid media and similar morphological appearances, seven species of pleuropneumonia-like organisms were set up:

(a) The L1 organism represents the first species. Three of the strains of this type were separated from *Streptobacillus moniliformis* cultures. The fourth culture of the same type was found in the lung of a rat, thus proving that this organism may occur independently as well as in symbiotic association.

New evidence of the symbiotic nature of the *Str. moniliformis* (an association of a streptobacillus with an L1) was given by the agglutination test, in so far as all sera prepared by immunization with *Str. moniliformis* cultures agglutinated to a fairly high titre the four different L1 strains.

(b) The L3 organism represents the second distinct type species. The five strains belonging to it originate from lung lesions of rats, viz. four from tame laboratory rats and one from a wild rat. This demonstrates that tame and wild rats contain the same variety of pleuropneumonia-like organism in the associated lung lesion. It is believed that 138 pleuropneumonia-like cultures isolated from lung lesions of rats by the writer belong to the same L3 species though only five of them have been studied here in detail.

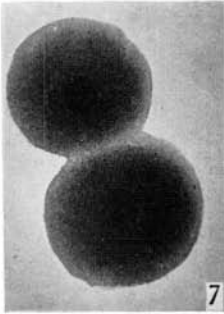
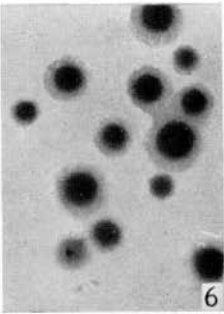
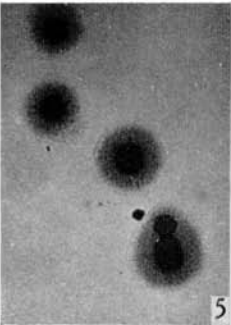
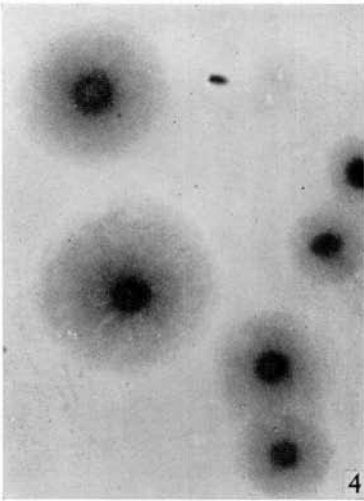
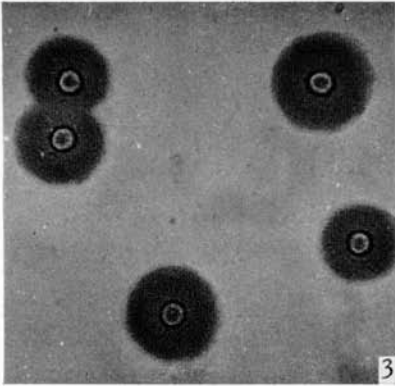
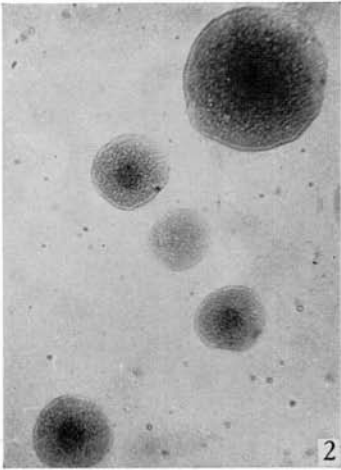
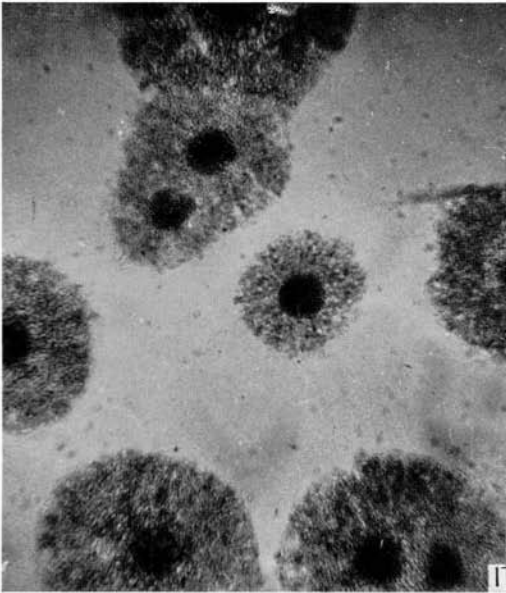
(c) The L4 organism only once isolated from the enlarged submaxillary gland of a rat also suffering from lung lesions was proved to be definitely different from the L1 and L3 organisms by serological and other tests and represents therefore a third new type species.

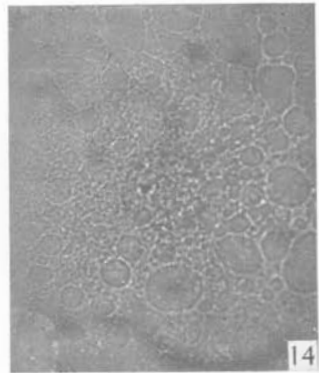
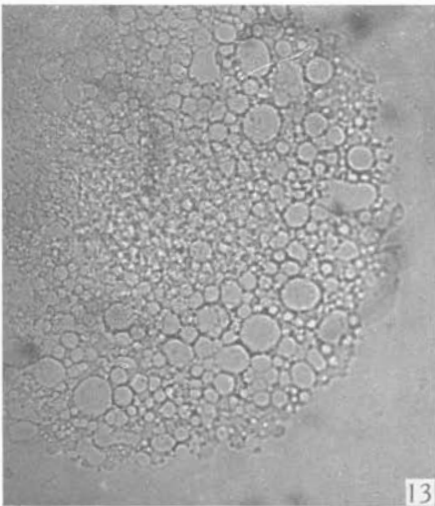
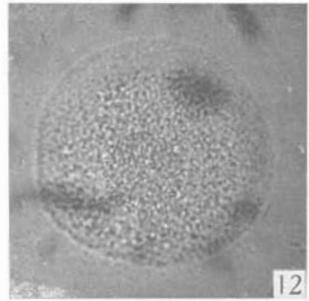
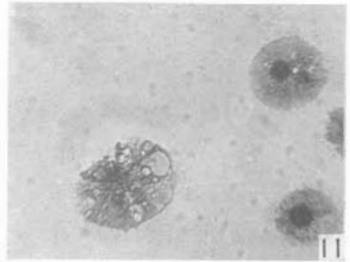
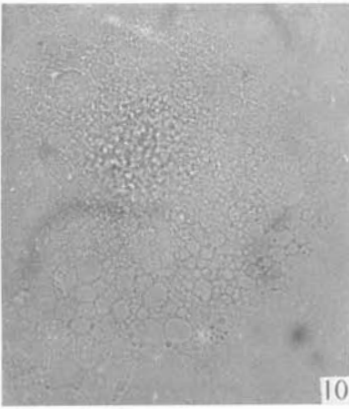
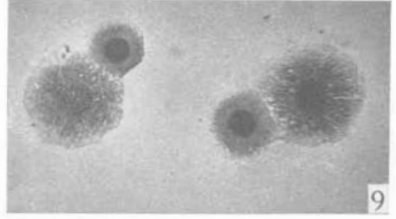
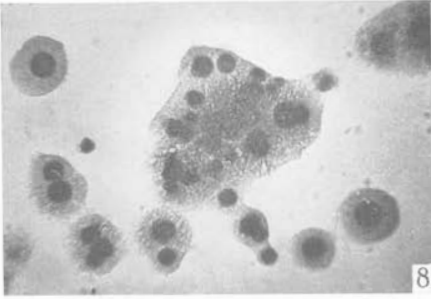
(d) Furthermore the organisms of pleuropneumonia, agalactia, *Asterococcus canis* I and *A. canis* II would appear to be independent species of the same family.

4. Experiments on pathogenicity which are still in progress show so far that the L3 organism is pathogenic for mice. The L1 organism would appear to be devoid of pathogenicity when tested by similar methods.

5. The capacity of pleuropneumonia-like strains for forming associations with bacteria has been tested experimentally, and in a few cases success has been achieved. These associations with *B. tetani* and *B. tetanomorphus*, unlike that of L1 and the streptobacillus in the *Str. moniliformis* combination, though stable, are not such as to preclude the isolation in pure culture of the two anaerobic bacilli.

6. It is likely that the group of pleuropneumonia-like organisms is widely spread in nature. Search for such strains and thorough investigation of their properties would seem to be of prime importance in view of the minute size of the units which can give rise to growth and the light that might be shed by their study on the biological nature of the filterable viruses.





ACKNOWLEDGEMENTS. I have pleasure in recording my thanks to Sir John Ledingham for his constant interest in the work described here and to Dr A. Felix for his suggestions with regard to serological technique.

ADDENDUM. Since this paper was written the writer has succeeded in isolating a pleuropneumonia-like organism from the lesions which develop in rats after injection of Woglom's "pyogenic virus" (*Science*, 22 April 1938, p. 370). The characteristic lesions were produced so far with the fourth passage of the culture from the pus. Detailed examinations of the new strains are in progress. I am indebted to Dr Gye and Dr Knox for kindly supplying a sample of the "pyogenic virus" previously obtained by them from Dr Woglom.

EXPLANATION OF PLATES XVII AND XVIII

PLATE XVII

Colony types

- Fig. 1. L1 organism, 3 days old, $\times 80$.
- Fig. 2. L3 organism, 3 days old, $\times 80$.
- Fig. 3. L4 organism, 4 days old, $\times 80$.
- Fig. 4. Organism of pleuropneumonia, 2-3 days old, $\times 80$.
- Fig. 5. *Asterococcus canis* I "granular", 3 days old, $\times 80$.
- Fig. 6. Organism of agalactia, 3 days old, $\times 80$.
- Fig. 7. *Asterococcus canis* II, 3 days old, $\times 80$.

The photographs have been selected to show the typical structure and do not accurately represent the relative sizes of the colonies. To aid the photographic reproduction of the colonies (figs. 1-9 and 11, Pls. XVII and XVIII) and to display more sharply differences in granularity and contour Unna's methylene blue has been allowed to sink into the colonies for a few minutes (a suggestion of my colleague Dr Gutstein).

PLATE XVIII

Colony types

- Fig. 8. *Asterococcus canis* I, dissociating, 3 days old, $\times 80$.
- Fig. 9. *Asterococcus canis* I, dissociating, 3 days old, $\times 80$.
- Fig. 10. Organism of agalactia "coarse", one colony in direct agar microscopy, $\times 200$.
- Fig. 11. *Asterococcus canis* I, "coarse" 3 days old, $\times 80$.
- Fig. 12. *Asterococcus canis* II, one colony in direct agar microscopy, $\times 200$.
- Fig. 13. L1 organism, edge of colony in direct agar microscopy, $\times 200$.
- Fig. 14. *Asterococcus canis* I "coarse", one colony in direct agar microscopy, $\times 200$.

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