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(PLATES XLIV.-XLVII.)

WITH foot-and-mouth disease and rinderpest, contagious bovine pleuropneumonia is one of the three most important cattle plagues of the world. According to Foster (1934) it first appeared in Switzerland and Germany in 1713 and in England in 1735. By the end of the eighteenth century it had spread over the entire German Empire, France and Italy. It was carried from England to Australia in 1858 and about the same time from Holland to South Africa. At present (Walker 1930), the disease is distributed widely throughout the world with the exception of Western Europe, India and North America. The United States of America were affected in 1843 and it was only by the most energetic and rigid government veterinary measures that the disease was finally eradicated. The history of pleuropneumonia in China is very obscure. As far as we are aware the first outbreak occurred in 1919 when the disease was discovered in a Shanghai dairy, having been introduced by a shipment of milk cows from Australia. In 1920 Hongkong was also reported to be infected from the same source. In 1931 Shanghai was re-invaded by the disease. A certain dairy had three infected animals amongst a consignment imported from Manchuria through Dairen. From this time on the infection has established itself in this virgin soil. Within a few months after the second importation it had spread like fire, involving thousands of cases. According to the Shanghai Municipal Health Report (1932) 10·4 per cent. of the cattle in the Settlement area in that year died from the disease. Since its introduction pleuropneumonia has become endemic in Shanghai and has gradually

invaded neighbouring areas such as Kiangwan, Woosung, Pootung, etc. As the local epizootic offered us an excellent opportunity, we have undertaken an investigation of the virus and this report deals with the results obtained.

Isolation of the virus.

The isolation of the virus may be effected by any one of the three ordinary bacteriological techniques, plating, dilution and filtration. The virus-containing substance, in the case of tissue, was first put in a meat presser and the juice streaked over serum-agar plates or inoculated into a set of 10 serum-broth tubes each containing 9 c.c. of the medium in the following manner. One c.c.

TABLE I.

Strains of pleuropneumonia virus.

Date of isolation.	Specimen.	Source.	Designation of strain.
3rd Feb. 1933	Natural lymph *	Keylock and Pratt	Chinese P.P. virus I.
14th Feb. 1933	,,	,,	Chinese P.P. virus II
29th Jan. 1934	Lung tissue	W. Dairy	W. Dairy
7th April 1934	Natural lymph	M. Dairy	M. Dairy
25th June 1934	,,	F.M.C. Abattoir	Abattoir
?	Culture	Khartoum, Egypt	Khartoum †
?	,,	Lister N.C.T.C. 3720	Lister ‡
?	,,	I.G. Farbenindustrie Aktiengesellschaft Hoechst, Main, Germany	German ‡

* Natural lymph=lung or pleural exudate of diseased cattle.

† Khartoum virus (34th generation) was obtained through the courtesy of Dr S. C. J. Bennett, Veterinary Research Laboratory, Khartoum, Egypt, on 21st January 1933.

‡ We owe both the Lister and German strains to the kindness of Dr J. H. Blakelock of the S.M.C. Health Department. Lister strain was received by Dr Blakelock on 11th November 1932 and the German virus isolated by him from a vaccine in March 1932.

of the virus-containing fluid was introduced into the first tube and mixed with 9 c.c. of the broth, making a dilution of 1 : 10. After thorough shaking, 1 c.c. of the fluid was taken from the first and added to the second tube, making a dilution of 1 : 100. This was repeated for the subsequent tubes until a final dilution of 1 : 1,000,000,000 of the original material was reached. The 10th tube was kept as medium control. The inoculated plates and tubes were now incubated at 37° C. for a week, the former being placed in a jar containing free moisture to prevent too rapid drying. After 3 to 5 days characteristic growth of the virus was usually seen and pure cultures obtained. Fishing of the well-separated colonies on the plates with a platinum needle and subsequent inoculation into broth tubes in the ordinary way generally yielded no growth. On the other hand, if an entire colony was removed with a piece of the agar upon which it grew and placed in broth, growth could constantly

be obtained. If isolation by filtration was preferred, the following procedure was adopted. The fluid was first centrifuged at about 2500 *r.p.m.* for 10 to 15 minutes, the supernatant fluid thus obtained was diluted with hormone broth in the proportion of 1 : 2 or 1 : 3 according to the intensity of turbidity, and the resultant mixture was filtered through a Berkefeld V candle under a negative pressure of 50 cm. Hg for 5 to 10 minutes. Varying amounts (0·25, 0·5, 1·0 c.c.) of the clear filtrate were removed and tested in broth tubes for the presence of the virus.

The various strains of the virus, whether isolated locally or obtained elsewhere, are shown in table I.

As shown in table I three strains of pleuropneumonia virus were obtained from foreign sources and five strains isolated from the local epizootic. The isolation by plating and dilution, though simple in technique, has not always yielded satisfactory results, especially when the virus-containing material was badly contaminated with bacteria. The filtration method has given the best results in our hands.

Methods of cultivation.

The success of cultivation depends on the kind of media employed. No growth was observed in plain broth, blood broth, litmus milk, blood agar, Loeffler's serum and Bordet-Gengou's medium. Martin's broth, which has most frequently been recommended, was found unsatisfactory. On the other hand, ordinary meat infusion broth, hormone broth and Bennett's broth (Bennett 1932) were equally serviceable. However, in the present investigation we used Bennett's broth as a basic medium. It is essentially the same as the hormone broth of Huntoon (1918) described for pneumococcus work except that it has been filtered and that 2 instead of 1 per cent. of peptone has been added in the process of making.

It is of interest to note that the virus did not seem to be particular in regard to the reaction of the medium, for growth has been observed in media ranging from *p*H 6·4 to 8·6. But if consistently good results are to be expected, slightly alkaline media (*p*H 7·2 to 7·6) should be used. Serum was absolutely necessary for the cultivation. Rabbit, sheep and horse sera were all satisfactory but the sera of guinea-pigs, steers and cows were definitely inferior. The addition of horse serum in a concentration of 5 to 15 per cent. promoted growth, but growth was partially inhibited by 20 per cent. and entirely arrested by 50 per cent. As a routine we added 10 per cent. horse serum to all the media employed.

Using Bennett's broth as a basic medium, semi-solid or solid media could easily be prepared by dissolving 0·5 or 2 per cent. of agar in it.

The optimum temperature for the cultivation of the virus is

37° C. Below 30° C. no growth was observed. The virus is usually said to be an aerobe but it has been grown successfully in liquid and solid media in the McIntosh and Fildes jar in the complete absence of oxygen. The growth, however, was less luxuriant than that obtained aerobically.

Cultural characteristics.

When the broth was inoculated with lymph filtrate or a seeding virus, growth usually took place within 48 to 72 hours' incubation. It first appeared either as tiny mucous "islands" and "threads" or as general cloudiness of the medium. The islands and threads were generally seen in the bottom layer of the broth when the culture was kept at 37° C., but when the tube or flask was removed from the incubator these gradually rose towards the surface, forming beautiful silvery flakes, balls or coils of threads suspended in the clear amber-coloured medium (plate XLIV, fig. 1). The elements were extremely delicate and capable of being broken by slight agitation. If the medium was allowed to stand in the incubator, the islands soon became threads, and the threads ultimately ended in general cloudiness. These macroscopic appearances suggested apparent stages of development which will be described in detail in the next section.

The phenomenon of island and thread formation was by no means constant. It seemed to be related to the age of the seeding virus and the size of the inoculum; media appeared to play no important role. The fresher the seeding virus and the smaller the size of the inoculum, the more constant was the occurrence of island and thread formation. If the inoculum was too heavy, even a lymph virus or a first generation culture would only initiate cloudy growth. If an old culture such as Lister or German strain was employed it never showed characteristic growth of this sort, even though the size of the inoculum was extremely small ($1 : 10^{15}$ dilution of a 5th-day broth culture). Leaving aside the influence of the size of inoculum, the phenomenon of island and thread formation seemed to be a sign of youth, which gradually disappeared with advancing age of the culture.

Upon solid and semi-solid media the virus usually developed slowly. Dewdrop colonies (plate XLV, fig. 3) might be seen on the plates on the 5th or 6th day. They began as pin-point growths on the surface, but two or three days later gradually extended down and embedded themselves in the depth of the medium so that the entire removal of a colony was impossible unless a piece of agar was cut out with it. A well-developed colony may attain a size of 2 mm. in diameter and under low power of the microscope may be seen to be composed of a central granular "nucleus," yellowish-

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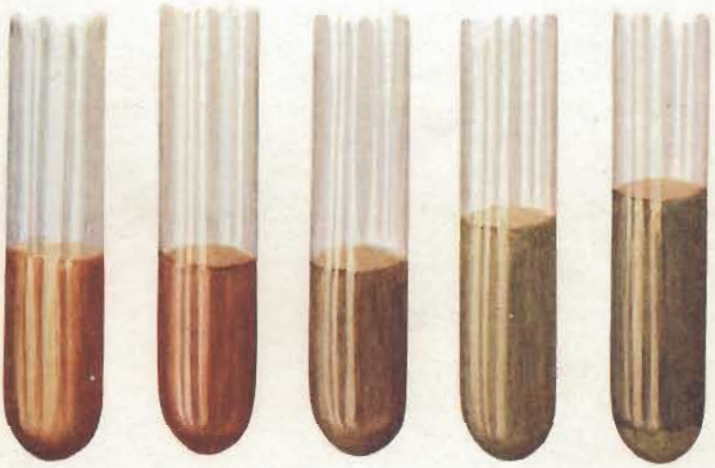
FIG. 1.—Broth cultures of recently isolated strains of pleuropneumonia virus (W. Dairy and M. Dairy strains) showing island and thread formation.



(*a*) (*b*) (*c*) (*d*) (*e*)

FIG. 2.—Cultures of pleuropneumonia virus (henceforth abbreviated as P.P.V. showing degrees of hæmoglobin reduction. (*a*) Media control. (*b*) Lister strain. (*c*) Chinese virus I. (*d*) W. Dairy strain. (*e*) M. Dairy strain.

brown in colour and surrounded by a transparent, refractile and perfectly smooth peripheral zone (plate XLV, fig. 4). Repeated attempts to differentiate the various strains by differences in colony formation have been unsuccessful.

Broth cultures of the virus have been found alive after having been kept at 37° C. for 45 days; at 20° C. and room temperature for 50 days; in the refrigerator (0 to 5° C.) for 98 days. When dried on pieces of filter paper with serum and placed over calcium chloride under partial vacuum in the refrigerator, they were found alive 62 days after the beginning of the experiment.

Morphology and developmental cycle.

The question of the morphology and developmental cycle of the pleuropneumonia virus demands our serious attention because of the existing confusion and perplexity arising from the conflicting reports of work on the subject. As it is generally agreed that for a morphological study of an organism a living specimen in liquid medium is preferable, we have endeavoured to approach the problem by making use mainly of dark field examination of the virus in broth, supplementing this by study of the growth on semi-solid agar and of stained smears (Giemsa's stain: 1 drop to 1 c.c. of a mixture of equal parts of distilled water and tap water; stain overnight, wash and differentiate first with tap water then with distilled water) prepared from broth and impressions from solid media.

As already pointed out, in the freshly isolated cultures there was island and thread formation which was absent in the old strains. This phenomenon plainly suggested a difference in the mode of development between the two types of cultures, a view supported by dark field examination. For the sake of convenience we shall describe the development observed in the old strains first.

When a broth culture of an old laboratory strain was examined it was seen to be composed of rings, granules and occasionally diplococcoid and cocco-bacillary bodies. When the culture became many days old, it consisted almost entirely of rings; the bacillary bodies might have formed diplococcoid elements and the latter gave rise to granules. The granules were solid spheres but soon the protoplasm in each sphere became eccentric and consolidated at the periphery. This caused a thinning-down at the centre so that when viewed from the side through a binocular microscope the granule appeared as a concave disc or when seen from the plain surface it looked like a ring. Morphologically, therefore, the rings were not rings in the true sense of the word, but were biconcave discs resembling red blood corpuscles. A ring may contain one or more tiny refractile dots of protoplasmic condensation at the

periphery. When two dots were present, they were usually situated opposite to each other.

On transferring the virus to fresh medium, one or all of the dots on the ring began to grow larger and larger (plate XLV, fig. 5) until new granules were formed. These granules or spheres soon became biconcave discs or "rings" which might be connected with or detached from the main body. Protoplasmic dots developed once again on these fresh rings and the process was repeated until the broth was crowded with rings. Essentially, therefore, this method of multiplication was one of simple budding and macroscopically it always gave rise to general cloudiness.

The mode of development of a recently isolated strain was entirely different from what has been described in the case of old virus. It consisted of a complicated process involving cyclic transformations. For convenience of description this process may be divided into the following stages:

Stage of elementary bodies. The four morphological elements (granules, rings and diplococcoid and cocco-bacillary bodies) described above were also seen in an old culture (17 days) of a recently isolated strain. They constituted the elementary bodies (plate XLV, fig. 6). The diplococcoid and cocco-bacillary bodies were only rarely seen, while granules usually predominated. These bodies took the stain intensively. This stage, for convenience, may be also considered as a resting stage.

Filamentous stage. On inoculation into a fresh medium, the granules, rings and bacillary bodies above described soon began to germinate by sending out at first short dendritic processes (plate XLV, fig. 7). These processes, when derived from rings or granules, often had the appearance of sporing tetanus bacilli. They gradually extended and eventually became filaments (plate XLV, fig. 8). These filaments when congregated appeared macroscopically as islands or threads in the broth, and the best preparations for filament examination were obtained from these sources. At the beginning the filaments were rather thick, homogeneous, refractile and highly plastic, showing constant undulating movements. Some were so long as to run through several microscopic fields. They were never segmented and their ends might be round, slightly bulging or terminating in rings. On account of its thin sheath as demonstrated by Giemsa stain (plate XLV, fig. 8), the filament looked like a flexible tubule within which the protoplasm was sometimes seen streaming actively from end to end. The speed of the movement was rather rapid, being about once in every one or two seconds. This process was apparently independent of the difference of light refraction brought about by the undulating movement, because in the Giemsa preparation (plate XLV, figs. 8 and 9) protoplasmic condensation at different regions of the filament due to streaming

Plate XLV.

Fig. 3.—Colonies of P.P.V. on serum agar showing dewdrop appearance. ×24.

Fig. 4. —Colonies of P.P.V. as viewed under the low power of a microscope. ×140.

Fig. 5.—Impression taken from the margin of a colony (German strain) on serum agar. Notice the protoplasmic dots at the periphery of the rings. Giemsa. ×1540.

Fig. 6.—P.P.V. (W. Dairy) in serum broth showing elementary bodies. Dark field. ×1062.

Fig. 7.—P.P.V. (W. Dairy) in serum broth showing germination of elementary bodies. Dark field. ×1595.

Fig. 8.—Plain filaments (W. Dairy) from serum broth showing presence of sheaths. Zigzag arrangement of filaments indicates undulating movements. ×1000.

Fig. 9.—M. Dairy strain showing presence of sheaths and asterococci. Irregular distribution of protoplasm within sheath due to effect of streaming.

Fig. 10.—M. Dairy strain showing chain-formation resembling streptococci. Giemsa. ×1640.

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FIG. 3.

FIG. 4.

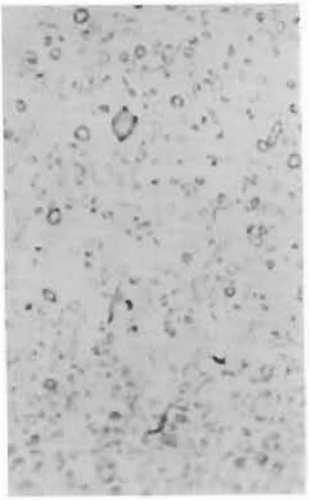

FIG. 5.

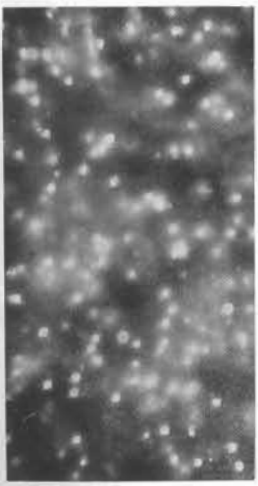

FIG. 6.

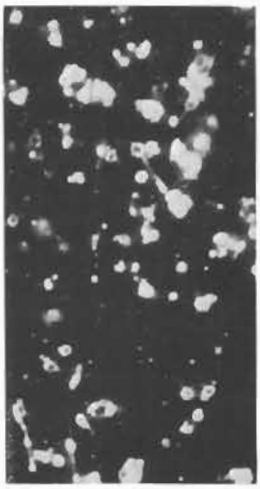

FIG. 7.

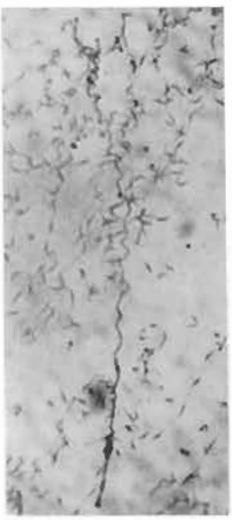

FIG. 8.

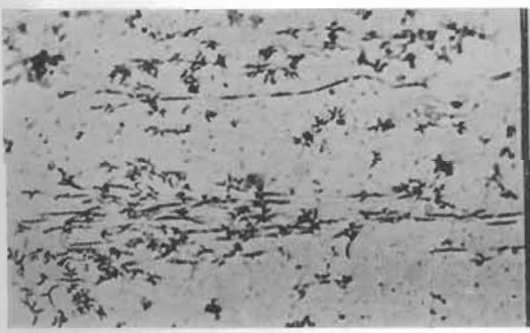

FIG. 9.

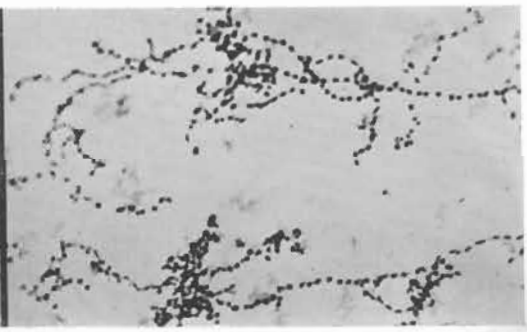

FIG. 10.

could still be obtained. Comparatively speaking, plain filaments were very difficult to stain.

Branching stage or stage of ramification. During the process of streaming areas of protoplasmic consolidation at various points in a filament might protrude to form buds or become granules and rings. The buds gave rise to branch filaments by mere extension. The rings developed peripheral protoplasmic dots which extruded, giving rise to the appearance of asterococci. As the extrusions from each asterococcus developed and extended, these also eventually became branch filaments. Usually four or more branch filaments might grow out from a single asterococcus (plate XLV, fig. 9). If an asterococcus happened to be situated at the end of a filament, a terminal cluster of branches might be developed. This process of ramification continued to develop until a single filament would resemble the twigs of a tree and when many filaments were together the branch filaments would become entangled to form a cobweb structure (plate XLVI, fig. 11). It should be noted here that the process of protoplasmic streaming occurring in the filamentous stage was still going on at this time in the filamentous trunks as well as in the branches.

Stage of chain formation. While streaming, the protoplasm in the filaments might suddenly consolidate at many points, not for the purpose of ramification but for chain formation. The solid portions soon developed into beads and bacillary bodies. Some of the beads might become rings. The rings, beads and bacillary bodies or short connecting filaments might come into contact with one another in series so as to form a variety of chains: some consisted of oval or round beads resembling streptococci (plate XLVI, fig. 10), some of beads interconnected by short filaments (plate XLVI, fig. 12), some of interlocked rings (plate XLVI, fig. 13), some of minute granules linked with rings at regular distances (plate XLVI, fig. 12), and some of rings interconnected by long plain filaments (plate XLVI, fig. 14), etc.

Stage of disintegration. The chains soon broke up into individual granules, rings and diplococcoid and bacillary bodies, thus completing the cycle. In the previous stage a very few of the rings might be unusually large and when they disintegrated they gave rise to such bizarre forms as sausage or balloon shapes (plate XLVII, fig. 15).

The observations described above regarding the modes of reproduction of the two types of cultures in broth were confirmed by dark field examination of semi-solid growths and to a certain extent also by impression preparations. Colonies on semi-solid media were lifted up with a tiny bent scalpel and placed carefully on a slide. A piece of specially thin coverglass was applied in such a way that the weight would fall equally on all sides. After

paraffining the edges of the cover, the preparation was examined. When successful, and if the colony was small enough, the whole structure might be seen under the microscope (plate XLVII, fig. 16). If it was big, details might be obtained field by field.

With an old strain such as Lister or German, the colony consisted almost exclusively of rings and granules. An intact colony was seen to be composed of three strata or zones which became transformed gradually one into another. The central zone was composed of homogeneous, highly refractile material, representing probably the product of autolysis. The middle zone consisted of densely packed rings and granules, the peripheral zone of loosely arranged similar elements. The rings in the peripheral zone were seen, however, to contain refractile protoplasmic dots signifying active growth (plate XLVII, fig. 17).

With a fresh culture the appearance of the colony was quite different from that in the old strain with the exception of a similar central zone. The middle zone consisted of densely packed filaments of various lengths and a few rings and granules while the peripheral zone showed only long radiating filaments with streaming protoplasm (plate XLVII, fig. 16).

Examination of impressions prepared from the colonies of the two types of cultures revealed similar findings. The peripheral zone of the colony of an old strain contained chiefly granules and rings with enlarged protoplasmic dots (plate XLV, fig. 5) while that of a fresh strain consisted of long radiating filaments (plate XLVII, fig. 18). In addition, impressions prepared from both sources showed plaques, quadrangular and vibrionic structures and yeast-like bodies (plate XLVII, fig. 19). These were probably not true morphological elements but mere artefacts produced by squashing of material, as the virus was extremely delicate and readily deformed. Indeed we have shown that by mere spreading with a platinum loop, the virus elements could be transformed into curious shapes.

On account of the criticism of Freiberger (*vide* Ledingham 1933) on the work of Borrel and his associates we examined numerous specimens of uninoculated media controls with and without the addition of serum, centrifuged or otherwise, but were unable to find the long branching filaments with streaming protoplasm and undulating movement, though dancing particles, rings and even short stationary filaments were present. These latter structures however never showed any signs of growth. Therefore the observations reported above cannot be due to artefacts present in the media.

Conditions affecting the morphology.

As we mentioned previously when a culture of the virus of pleuropneumonia was kept under prolonged artificial cultivation

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FIG. 11.—W. Dairy strain showing ramification. Dark field. ×1114.

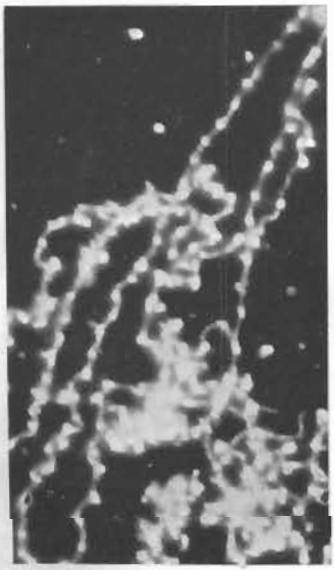

FIG. 12.—P.P.V. recovered from goat no. 1 originally inoculated with Abattoir strain, showing beads interconnected by short filaments; minute granules linked with rings. Dark field. ×1385.

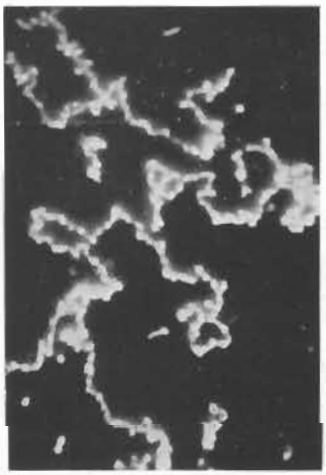

FIG. 13.—W. Dairy strain showing chains of interlocked rings. Due to active Brownian movement these small rings were recorded as granules. Dark field. ×1062.

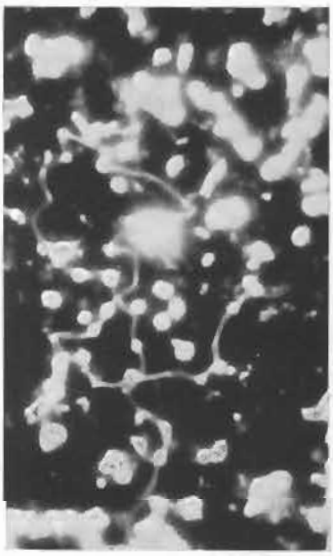

FIG. 14.—Abattoir strain showing rings interconnected by long plain filaments. Dark field. ×1385.

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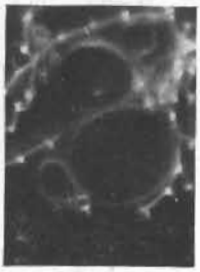

FIG. 15.—Abattoir strain showing balloon-shaped rings. Dark field. ×1330.

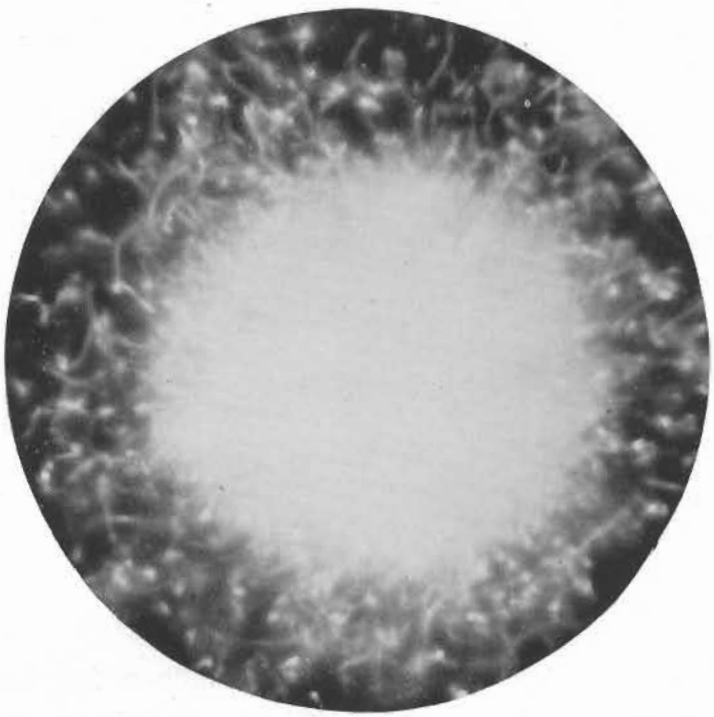

FIG. 16.—Colony of Chinese virus I on semi-solid serum agar showing zones. Note radiating plain filaments in peripheral zone. Dark field. ×1600.

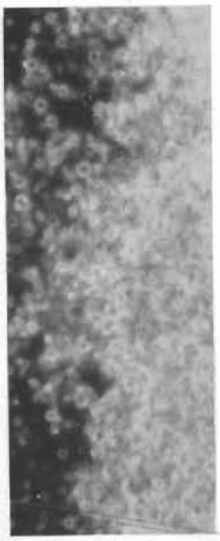

FIG. 17.—Margin of a colony (German strain) on semi-solid serum agar showing multiplication by ring formation. Dark field. ×1600.

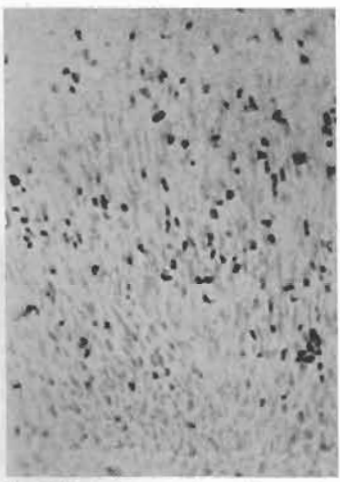

FIG. 18.—Impression of margin of Chinese virus I showing radiating filaments. Giemsa. ×1760.

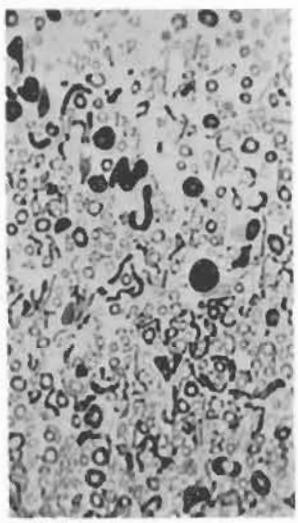

FIG. 19.—Impression of Lister strain showing bizarre forms. Note yeast-like bodies, vibrionic structures, etc. Giemsa. ×1540.

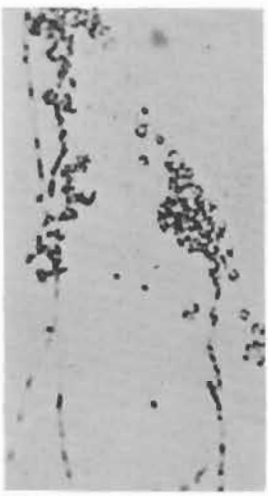

FIG. 20.—Smear preparation of M. Dairy strain (filamentous stage) showing effect of surface tension. Notice rings at edge and filaments in centre of smear. Giemsa. ×1640.

it gradually showed only granular and ring forms. We have also mentioned that slight mechanical pressure may result in the formation of some peculiar forms such as quadrangular or yeast-like bodies. Furthermore, we have found that surface tension also played an important role in the morphology of the virus. For instance, if a drop of glycerol was mixed with a similar amount of filamentous growth, the latter would immediately be transformed into rings and granules. Again, if a drop of filament-containing broth is placed on a slide and carefully allowed to spread itself by tilting the slide, and is then dried in air and stained with Giemsa's stain, it shows numerous rings accumulated at the edge of the smear, with practically no alteration of the filamentous structure at the centre, where the surface tension is presumably much less (plate XLVII, fig. 20).

Biochemical reactions.

Biochemically the virus has been studied as regards its reaction to various carbohydrates, bile and hæmoglobin and its susceptibility to ether. For fermentation tests, 1 per cent. of each of the various " sugars " and 1 per cent. of Andrade's indicator were mixed with the serum broth in which the different virus strains were inoculated. Twenty-three carbohydrates have been tested. Glucose, fructose, mannose, maltose and dextrin were strongly fermented, sucrose and trehalose only slightly attacked, while raffinose, inulin, galactose, salicin, xylose, mannitol, arabinose, amygdalin, lactose, dulcitol, iso-dulcitol, sorbitol, inositol, erythritol and adonitol were not acted upon.

Like the pneumococcus, the virus of pleuropneumonia is bile-soluble. This solubility was best demonstrated by mixing ox bile with the virus during the filamentous stage of the growth in a proportion of 1 : 5 to 1 : 10. On mixing the threads with bile, the medium instantaneously became viscid, and the mucilaginous substance could be lifted up in lines from the glass by means of a platinum loop. On examination, the filaments were all seen to have disappeared. The rings and granules seemed to be less bile-soluble.

If a serum broth containing 10 to 15 per cent. hæmoglobin was inoculated with a virus culture, the brilliant colour was found reduced to dark red within a few days with a greenish precipitate at the bottom. Like the phenomenon of island and thread formation, this property of hæmoglobin reduction seemed to be associated with the age of the seeding virus. In other words, the less the strain had been cultivated artificially, the stronger was the reduction and the earlier its occurrence; a fresh strain may show complete reduction within 24 or 48 hours while an old one may fail to do so in many days (plate XLIV, fig. 2). When a strain

was still older it lost this property entirely. Further, hæmoglobin solution seemed to have an enriching property for the growth of the virus.

The virus of pleuropneumonia was found highly susceptible to ether; it became inert within 5 minutes after coming in direct contact with this anæsthetic.

Filterability.

It has long been recognised that the causal agent of pleuropneumonia was filterable and indeed it was because of this property that it was classified as a filterable virus. The success of filtration depends, however, on a number of conditions, notably the porosity of the filter and the nature of the filtering substance. These points are illustrated in table II.

TABLE II.

Filterability of the virus.

Filter.	Natural lymph.	Artificial lymph.	Broth culture.
Berkefeld V . .	+	+	+
„ N . .	...	+	+
„ W . .	...	+	+
Seitz EK3 . . .	−	+	−

All the filtration experiments were carried out at room temperature under a negative pressure of 50 cm. Hg. The filtering material was diluted with hormone broth in a proportion of 1 : 5 and the turbidity standardised with an excess of broth. It was found that the virus in the artificially produced steer lymph was more filterable than the culture virus. That this difference was due to the presence of filamentous growth in the lymph was confirmed by the following experiment. The filamentous growth (islands and threads) and elementary bodies (10th day culture) from a given strain were separated in hormone broth and their turbidity equalised. The suspensions were then filtered under similar conditions through previously standardised filters. The result is given in table III.

TABLE III.

Filterability in relation to development.

Filter.	Filamentous growth.	Elementary bodies.
Berkefeld V . .	+	+
„ N . .	+	+
„ W . .	+	−
Seitz EK3 . .	−	−

As seen from the table filamentous growths are definitely more filterable than the elementary bodies. The greater filterability is probably connected with the marked plasticity possessed by the filaments. The success of the filamentous growth in the artificial lymph in passing through Seitz EK discs (table II) may therefore be explained by the greater number of filaments present in it.

Experimental inoculation.

In carrying out immunisation experiments on cattle, the results of which will be communicated later, we had opportunities of observing the reaction to the virus in steers. About 1 to 3 weeks after subcutaneous injection with 0·5 to 1 c.c. of natural lymph or a virulent culture there was a local œdematous swelling which was hot and tender to the touch. It gradually extended downwards involving the whole abdomen or even part of the extremities. This was associated with a rise of body temperature, impairment of appetite and great loss of weight, eventually leading to death. If the skin over the affected parts was incised, a clear straw-coloured lymph oozed out freely, often amounting to several litres. On section adjacent muscle fibres were seen to be widely separated by infiltration. Thoracic and inguinal lymph glands were sometimes affected and the liver and spleen often showed congestion and swelling. Similar experiments were also carried out on goats and one water buffalo with similar results. From one of the goats, which finally succumbed to the infection, we successfully re-isolated the virus from the local lesion three times (7, 14 and 55 days after the subcutaneous injection of 0·5 c.c. of virulent culture).

On the other hand we could find no reaction to the virus in white mice, hamsters, albino rats, guinea-pigs, rabbits and cats after subcutaneous, intraperitoneal, intracerebral, intravenous and in some cases intratesticular inoculation. In rabbits antibodies against the virus may be produced in the sera after a certain number of intravenous inoculations. These are discussed under the next heading.

Serological investigation.

The experience gained from so-called non-protective immunisation by certain culture vaccines or lymph viruses in epizootics suggests that the virus is serologically heterogeneous. The work of Heslop (1924) confirms such a proposition. Heslop has established two serologically independent strains of the virus in Australia. Since we had in hand a number of the virus strains both foreign and local in origin, it was naturally of interest to see whether Heslop's work could be confirmed in China. Antisera were prepared from rabbits by intravenous injection of a 5-day-old serum broth

culture. We gave a series of 6 small doses (0·5 to 1 c.c.), one every other day, followed by 2 or 3 injections of concentrated virus

TABLE IV.

Agglutination test.

Culture.	Sera.							Saline.
	Lister.	German.	Khartoum.	Ch. virus I.	W. Dairy.	M. Dairy.	N.R.S.* 1 : 10.	
Lister . .	1 : 80	1 : 40	1 : 40	1 : 80	1 : 80	—	—	—
German .	1 : 80	1 : 80	1 : 40	1 : 80	1 : 80	1 : 20	—	—
Khartoum .	1 : 80	1 : 80	1 : 160	1 : 80	1 : 160	1 : 20	—	—
Chinese virus I	1 : 40	1 : 40	1 : 40	1 : 80	1 : 80	1 : 40	—	—
W. Dairy .	1 : 80	1 : 80	1 : 160	1 : 80	1 : 160	1 : 80	—	—
M. Dairy .	1 : 80	1 : 80	1 : 160	1 : 160	1 : 80	1 : 160	+	—

The figures indicate the dilution of serum in which definite agglutination took place. Antigen was prepared by concentrating serum broth culture by centrifugation and resuspending the sediment in saline according to the turbidity of Wadsworth $BaSO_4$ standard no. 3. The reading was taken after the tubes had been incubated in the water-bath for 2 hours and refrigerator overnight.

* N.R.S. = normal rabbit serum.

(centrifuged broth culture and the sediment resuspended in salt solution). About 7 to 9 days after the last injection the animals were bled and the sera collected. Agglutination, precipitation and

TABLE V.

Precipitin test.

Serum.	Dilution of antigen.*				
	Undiluted.	1 : 10.	1 : 100.	1 : 1000.	1 : 10,000.
Lister . . .	++	++	+	±	...
German . . .	++	...	++	+	±
Khartoum . . .	++++	++++	++	++	+
Chinese virus I . .	++	...	...	...	...
W. Dairy . . .	++	++	++	+	±
M. Dairy . . .	—	—	—	—	—
Normal rabbit serum .	—	—	—	—	—

* Natural or artificially-produced lymph was used as the antigen.

complement fixation tests were carried out. The results are given in tables IV, V and VI.

TABLE VI.

Complement fixation test.

Immune rabbit serum.		Antigen.	
		Khartoum.	W. Dairy.
Lister	0·2 c.c.	++++	++++
	0·1 „	++++	++++
	0·05 „	++++	+++
German	0·2 „	++++	++++
	0·1 „		
	0·05 „		
Khartoum	0·2 „	++++	++++
	0·1 „		
	0·05 „		
Chinese virus I	0·2 „	+++	+++
	0·1 „		
	0·05 „		
M. Dairy	0·2 „	+++	+++
	0·1 „	++	+++
	0·05 „	±	±
W. Dairy	0·2 „	++++	++++
	0·1 „	++++	++++
	0·05 „	++++	+++
Normal rabbit serum	0·2 „	−	−
	0·1 „	−	−
	0·05 „	−	−

The antigen was prepared by centrifuging a 5th-day broth culture at 3000 *r.p.m.* for half an hour and resuspending the sediment in saline (1 : 4). It was used as the antigen after the anticomplementary and hæmolytic effects had been determined. For the test 2 units of complement contained in 0·5 c.c. of saline were mixed with 0·5 c.c. each of the diluted serum and antigen. After the preliminary incubation in the refrigerator overnight, 1 c.c. of sensitised cells was added. The reading was taken after incubation for one hour in the water-bath at 37° C.

An analysis of the data given in tables IV, V and VI shows that no essential difference in serological behaviour could be demonstrated. All the sera agglutinated and precipitated their own as well as the heterologous antigens. Similar behaviour was also observed in the complement fixation test. The failure of M. Dairy serum to produce precipitation (table V) was probably due to its poor antibody content. This was evidenced by its lower titres in agglutination and complement fixation (tables IV and VI). Among the strains studied, M. Dairy was the least and Khartoum the most antigenic.

DISCUSSION.

The channels of natural transmission of pleuropneumonia are not clearly understood, but it is generally believed that the virus is spread by droplet infection and indirectly through dust particles carrying dried nasal discharge. Locally the "hire cow" system certainly plays an important role. By this peculiar system cows after parturition are leased to dairies and returned to the owners

after the lactation period is over. Organised agents collecting cows often find their way into the most remote villages and towns. There is, therefore, a constant cattle traffic between the urban and rural districts, thus distributing the disease. As noted previously pleuropneumonia first appeared in Shanghai in 1919, but it was probably due to the absence at that time of such a system that the infection was limited to one dairy only. After the " hire cow " system was introduced it was only a few months after its second appearance in this city in March 1931 that the disease found its way into neighbouring areas such as Kiangwan, Woosung, Chenju, Pootung, etc. At present no reliable statistics are available as to how far it has spread, but in all probability it has gained a foothold in the adjacent provinces, as there are no government veterinary measures to cope with the situation.

The real nature of the causal agent of pleuropneumonia has not yet been settled. Bacteriologists in general regard it as a filterable virus because it is readily filterable, preservable in glycerol and non-immunising after being killed. But the facts that it can easily be cultivated in the absence of living cells, that it gives no inclusion bodies in the affected tissue (Gaiger and Davies 1932) and that it is susceptible to ether offer sufficient grounds to justify its removal from the virus category and the classifying of it under the family of frankly microscopic organisms as suggested by Ledingham. Furthermore, the complicated life-cycle of the agent of pleuropneumonia is so definite that it cannot conceivably be regarded as a virus. On the other hand, there are possibilities that filterable viruses may possess life-cycles, as suggested by the observation that the virus of yellow fever has to pass through a latent period in the mosquito before becoming infectious. If it is considered as an organism, it is more a fungus than a bacterium because of the presence of protoplasmic streaming and the ramification and embedding character of its growth upon agar.

Our observations of the two distinct systems of multiplication existing in two kinds of cultures appear to clarify many of the former controversies. For instance, the disagreement between Barnard and Smiles as pointed out by Ledingham is now explainable according to our observations ; for Barnard might have worked with an old culture which multiplied by simple budding while Smiles experimented with a fresh strain which exhibited cyclic development.

The protoplasmic movements of the agent of pleuropneumonia are so striking and fantastic that they should have been noticed by anyone who had a chance to examine young cultures containing the filamentous growth, but so far as the literature at our command goes, no description has been given. Buller (1934) described protoplasmic streaming or translocation as characteristic of certain

groups of fungi such as Ascomycetes, Basidiomycetes, Muscinæ, multicellular algæ, etc. In these plants, the flow of the protoplasm was said to be unidirectional whereas in the agent of pleuropneumonia the movements are backwards and forwards.

Summary.

1. The isolation of the virus of pleuropneumonia was effected by the ordinary bacteriological methods of plating, dilution and filtration, the last being the most serviceable.

2. The virus could be cultivated aerobically or anaerobically in solid, semi-solid and fluid media. Bennett's broth was used as a basic medium in this investigation.

3. After 1 or 2 days of incubation in broth, islands usually first appeared, then threads, and finally general cloudiness in the medium. But with a heavy inoculum only general cloudiness could be seen, the islands or threads being either not present or too transitory to be observed. With the old cultures which we possessed, such as the Lister and German strains, no matter how small the size of the inoculum there appeared only general cloudiness.

4. When cultivation was made on semi-solid medium, colonies of the organism could be observed by dark field illumination. With an old strain the colony consisted almost exclusively of rings and granules arranged in three zones, the innermost being homogeneous and highly refractile, the middle full of rings and granules and the outermost composed of loosely arranged rings and granules.

5. With a fresh strain the appearance of the colony was quite different, with the exception of a similar central zone. The middle zone consisted of some rings and granules but mostly filaments and the peripheral zone of long radiating filaments with protoplasmic streaming within them.

6. Similar to the observations summarised under 4, the broth culture of an old laboratory strain also consisted mostly of rings and granules. From the observations made both with semi-solid and fluid media we conclude that in old strains the reproduction of the virus is merely by simple budding.

7. In the freshly isolated strains the development may be described under five stages. (1) The resting stage or *stage of elementary bodies*, consisting of rings, granules and a few diplococcoid and bacillary bodies. The presence in abundance of these bodies gave rise to general cloudiness of the medium to the naked eye. (2) The *filamentous stage*, consisting of formation of filaments from the dendritic protrusions of the elementary bodies. (3) The *stage of ramification*, consisting of branching and sub-branching of filaments. When filaments and branch filaments congregated, islands and threads were formed. (4) The *stage of chain formation*,

consisting of formation of chains of various types by the consolidation of protoplasm in the filaments. (5) The *stage of disintegration*, in which the chains were broken up progressively into elementary bodies, thus completing the cycle.

8. The virus was bile-soluble, susceptible to ether and capable of fermenting glucose, fructose, maltose, mannose, sucrose, trehalose and dextrin. Fresh cultures reduced hæmoglobin. This property of hæmoglobin reduction decreased as the age of the culture advanced.

9. Goats and a water buffalo were susceptible to experimental inoculation, whereas mice, hamsters, rabbits, rats, guinea-pigs and cats were not.

10. Serologically no distinction could be made out among the different strains studied.

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