ON *STREPTOBACILLUS MONILIFORMIS* AND THE FILTRABILITY OF ITS L-FORM

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(With Plate 12)

When I first isolated the L-forms of *Streptobacillus moniliformis* and later of *Fusiformis necrophorus* the relationship between the bacillary form of the organism (A-form) and the non-bacillary, pleuropneumonia-like form (L-form) was explained as one of symbiosis. All other workers in the field opposed my view and regarded the L-form as a phase of the bacillus. In order to show that the L-cycle is an alternative to the bacillary cycle it had to be established (*a*) that the bacillus produces the L-form and how this is achieved, and (*b*) that the L-form reproduces the bacillus and how that is achieved. In respect to (*b*) Dienes & Smith (1944) produced evidence from live material of *F. necrophorus* showing that L-bodies are able to reproduce bacillary forms. Moreover, in a recent paper I (1949) have made a study of the behaviour of the nuclear substance during the development of various L-form-producing organisms. In this work the observations of Dienes & Smith were confirmed and extended by the demonstration of the changes which take place in the nuclear matter when L-forms produce bacilli. In respect to (*a*) a particular effort was made (Klieneberger-Nobel, 1949) to detect the transformations of the nuclear substance during the formation of the L-forms. Evidence has been collected to show that whenever L-forms arise, small chromatinic granules are formed which combine with each other in variable numbers. This process which may be regarded as a 'primitive form of sexuality' (Mellon, 1925), always precedes the development of L-forms. In the light of these new observations the symbiosis hypothesis became untenable, and I adopted the explanation long held by Dienes that the L-form must be a stage of the bacillus.

As long as the L-form could be regarded as an entity of its own, alien to the bacillus, it did not seem remarkable that it contained large quantities of small, granular elements which as in organisms of the pleuropneumonia group could be filtered through Berkefeld-V filters and grown from the filtrate. Yet if the L-form is a stage in the life cycle of the bacillus which, as Dienes has shown (1949*a*, *b*), occurs widely both in Gram-negative and Gram-positive bacteria, this property gains greatly in importance. I have therefore made a new study of *Strep. moniliformis* and its L-form with a view to gathering information about its small elements and to analyse its filtrability.

Two cultures were studied throughout the investigation, a culture of *Strep. moniliformis* kindly supplied by Mr J. Smiles (National Institute of Medical Research, Hampstead), called 'An' and an old L 1 culture separated from a *Strep. moniliformis* culture in 1935, called 'L 1 rat 30'. Both cultures were kept in 20 % serum broth. 'An' was subcultured every day to keep it mostly in the bacillary A-form, 'L 1 rat 30' was transferred every third day.

When strain 'An' was planted on special serum agar it showed at first a regular bacillary morphology consisting mainly of characteristic chains of very small bacilli. After about 15 hr. of incubation the bacillary filaments were no longer so well defined and showed many loops, involvements and twists, as can be seen in the outgrowth of the colony illustrated in Pl. 12, fig. 1. This involved growth is very characteristic for all strains of *Strep. moniliformis* at this young stage. By analogy with the observations on *Fusiformis necrophorus* and other organisms (Klieneberger-Nobel, 1949) it can be assumed that in places where two filaments, or parts of one filament, touch each other, small elements fuse, their nuclear structures combine and in consequence a bulbous swelling arises later. Two such small elements in the process of joining can be seen in Pl. 12, fig. 2. A slightly older stage of development is seen in Pl. 12, fig. 3: wherever bends, loops or twists occurred, swellings have here developed. These swellings are the young L-bodies which increase and multiply as described and depicted in former papers. At the end of their development they disintegrate into numerous granules which are often found in masses at the edges of old L-colonies, as illustrated in Pl. 12, figs. 4, 5. These granules are difficult to demonstrate, and special methods have to be used to make them visible under the light microscope. When whole colonies are fixed through the agar with Bouin's solution and stained by a prolonged Giemsa

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method they will show up if they are present in large amounts (Pl. 12, figs. 4, 5). They also could be demonstrated when viewed under oblique incident illumination with the 'Ultropak'-microscope (Klieneberger & Smiles, 1942). Pl. 12, fig. 6 shows a photograph of an L-colony examined by this method. The granules stand out from the foamy structure of the colony and can be seen particularly well in the outgrowth at the lower edge. Yet the centre of the colony also seems to contain masses of granules.

Two-day-old serum broth cultures of 'L1 rat 30' strain were used for the filtration experiments. The growth habit of L1 in liquid medium is to form larger or smaller clumps which settle at the bottom of the tube and stick to the side walls. They are easily shaken off the supporting glass surface and thrown down by centrifuging. The sediment thus obtained was ground in a glass grinder consisting of a tube with a ground glass inner surface and a corresponding pestle carried by a glass rod. Grinding was usually continued for about a quarter of an hour. The ground material was resuspended in serum broth and spun again in an angle centrifuge (4000 r.p.m.) for 10 min. The supernatant which was still slightly opalescent was used for the filtrations. A smear from this supernatant on an agar plate, fixed through the agar and stained overnight with Giemsa solution showed a large number of small, indistinctly stained granules, which were interspersed with occasional larger elements or little groups of elements. Such a stained smear is reproduced in Pl. 12, fig. 7.

The opalescent suspension, prepared as described, was filtered through the following gradocol membranes: A.P.D. = 700, 600, 500, 400, 350 mμ. The positive pressure, approximately 10 cm. mercury, was produced by a pair of hand bellows. The amount filtered each time was 10–15 ml. and the duration of the filtration was 5–15 min. The opalescent suspensions and clear filtrates were titrated by placing measured and diluted amounts into tubes of serum broth. If the result was positive the characteristic colony clumps appeared after 24–48 or 72 hr. of incubation. Thus it was found that the supernatant contained between 1 and 10 million viable particles per ml., while the filtrates which had passed the membranes with an A.P.D. of 700, 600, 500 mμ contained 10,000–100,000 such particles per ml. Consequently the titre of the original material was reduced to 1% by filtration. When a membrane of 400 mμ A.P.D. was used the filtrates were still positive, but a sudden drop in the number of particles to about 10 per ml. of filtrate had taken place. The membrane with an A.P.D. of 350 mμ retained all the particles and a sterile filtrate was obtained. Thus the end-point of filtration was found to be 350 mμ; therefore the particle size would appear to range from 17 250 mμ (see Elford, 1938). Consequently, particles of the L1-form are a little smaller t those of the virus of psittacosis and a little la than the particles of pleuropneumonia of cat agalactia of sheep and vaccinia virus.

The filtration experiments with *Streptobaci moniliformis* were carried out with 18-hr. cultures, sedimented, ground, resuspended finally centrifuged for 2–3 min. at 2000 r.p.m. analysis for the strain 'An' showed that it filterable through gradocol membranes of an A.P of 900 and 800 mμ; but a membrane of an A.P.D 700 mμ retained all the organisms, and the filtr was sterile. Therefore the average size of the bacill A-form of *Strep. moniliformis* is approximat 750 mμ, the size usually attributed to the eleme of a culture of *Bact. prodigiosum*.

Those unfamiliar with the cultivation of t L-form might expect that after filtration of a *Strep bacillus moniliformis* culture through a grado membrane of an A.P.D. less than 750 mμ, growth the L-form might be achieved from the filtra because even at an early stage in its developme a *Strep. moniliformis* culture usually contains so L-elements. This has never been observed so far. has to be realized that the cultivation of the L-for is very difficult in the first instance and that it tak a long series of passages to adapt the L-form to go growth on the boiled blood serum media used, whi it does not grow at all on ordinary media. It therefore concluded that even the best media us so far are not sufficiently suitable to allow t growing out of the minute granules from the filtrat before the L-cultures have been adapted to artifici conditions, and before they will grow from sma inocula or from single granules. Once optimal co ditions for the culture of L-forms of bacteria hav been devised it should be possible to filter th L-elements of a mixed culture through membrane which retain the A-form, to grow the L-form fro the filtrate and to retransform it (also under appro priate conditions) into the A-form.

SUMMARY

It has been shown that the L-form of *Streptobacillu moniliformis* is filterable through gradocol mem branes which retain the bacillary A-form completely From the filtration analysis carried out as described it would appear that the particle size of the L-form of *Strep. moniliformis* ranges with the larger viruses while the bacillary A-form ranges with *Bact. prodigiosum*.

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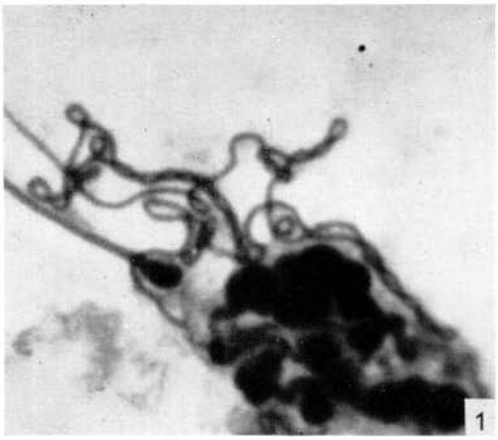

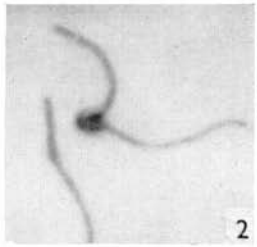

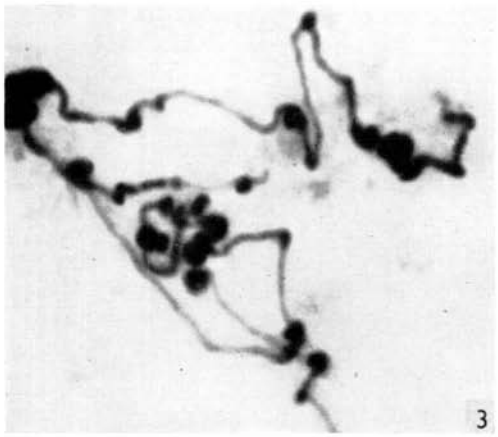

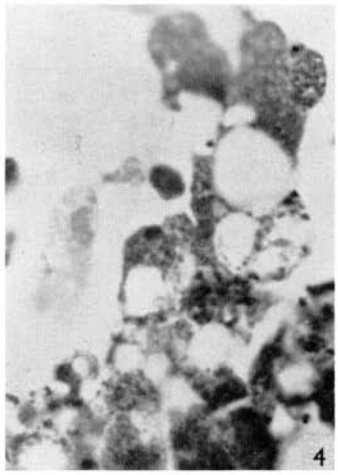

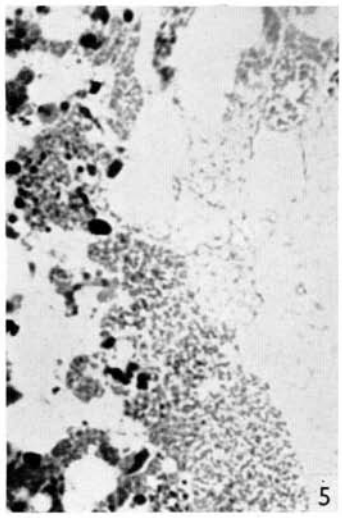

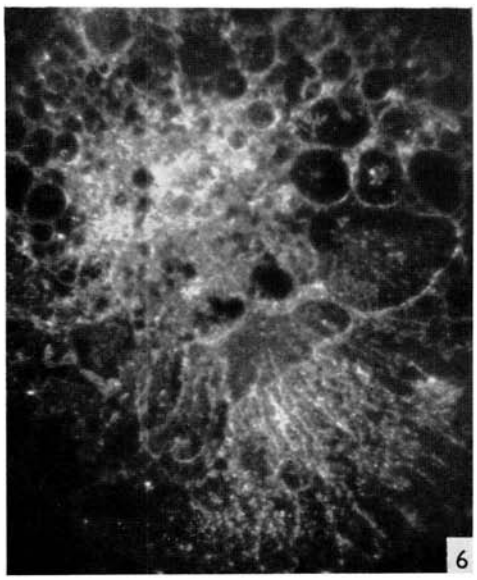

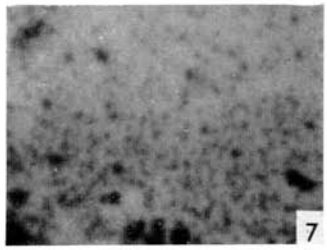

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EXPLANATION OF PLATE 12

Figs. 1, 2, *Streptobacillus moniliformis* 'An', 15 hr. old; stained.
Fig. 3. *Streptobacillus moniliformis* 'An', 24 hr. old; stained.
Figs. 4, 5. Colony edge of 'L1 rat 30' strain, 2 days old; stained.
Fig. 6. Colony of 'L1 old' strain, 2 days old; unstained.
Fig. 7. Smears from suspension of L1 particles obtained by grinding the sediment of a liquid culture and resuspending it; stained.
Enlargements between 2000 and 3000. Photographs 4, 5 and 6 were taken at the National Institute of Medical Research, Hampstead, by kindness of Mr J. Smiles.

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