SOME NEW OBSERVATIONS BEARING ON THE NATURE OF THE PLEUROPNEUMONIA-LIKE ORGANISM KNOWN AS L1 ASSOCIATED WITH *STREPTOBACILLUS MONILIFORMIS*

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(With Plates 10–13, containing Figs. 1–45)

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Introduction

When the writer first began to study the morphology of the organisms of pleuropneumonia and agalactia (1934) her attention was drawn to an organism of peculiar morphology, usually designated in the English literature as *Streptobacillus moniliformis*. This highly pleomorphic organism which causes rat bite or Haverhill fever in man (Farrell, Lordi & Vogel, 1939; Brown & Nunemaker, 1942) and arthritic abscesses in mice (Mackie, Van Rooyen & Gilroy, 1933) owes its name to the beaded appearance of its bacillary threads. When the development of cultures of *Str. moniliformis* was studied by the agar-fixation technique as described in 1934, it was found that the bacillary chains were interspersed with soft elements resembling those found in cultures of pleuropneumonia (Klieneberger, 1935). Subsequently methods were devised to separate the pleuropneumonia-like growth from the bacillary elements, and cultures were obtained which no longer contained any bacteria, but consisted exclusively of pleuropneumonia-like elements (Klieneberger, 1936). The new organism thus associated with *Str. moniliformis*, but no longer containing bacteria, was designated L1. Several of these L1 strains, the oldest of which was isolated in 1934, have been kept since isolation on artificial media, solid as well as liquid, by an uninterrupted series of passages and have never reverted to the original streptobacillary form of the parent culture. It was further shown that the L1 organism was filterable* through the same filters as the organism of pleuropneumonia, while the streptobacilli were retained by the Berkefeld V filters used for these experiments. Special media were

* It should be mentioned here that filtration experiments can be expected to be successful only with old-established L1 strains under optimal conditions of culture, for newly isolated L1 strains will only grow from mass inocula while pure L1 strains adapted through passages to grow without their bacillary component develop from very small amounts of inoculum. This property of the L1 strains probably accounts for the different results in Brown & Nunemaker's and the writer's filtration experiments (Brown & Nunemaker, 1942; Klieneberger, 1936).

required for the isolation and maintenance of the L1 strain. These also gave optimal conditions for other pleuropneumonia-like organisms. In contrast with this the parent culture was less exacting. The growth type of L1 on liquid and solid media differed from that of the whole culture and resembled the growth of pleuropneumonia-like organisms. For these reasons it was assumed that cultures of *Str. moniliformis* were composed of two different organisms, a streptobacillus and L1, a pleuropneumonia-like microbe. Though the presence of L1 in *Str. moniliformis* cultures was confirmed (Dienes & Edsall, 1937; Dienes, 1938), the conception of the symbiosis of two different organisms was challenged by Dienes (1939 *a*, *b*; 1940), Dawson & Hobby (1939), Heilman (1941 *a*, *b*), Wilson Smith (1941) and Brown & Nunemaker (1942), and the L1 was regarded as a variant rather than as a symbiont of *Str. moniliformis* by these authors. The main difficulty in verifying the symbiosis hypothesis is that so far it has been impossible to free the *Streptobacillus* from the L1 component, while it is fairly easy to derive pure L1 cultures from the mother strains. As the streptobacilli seem to be very delicate, application of physical and chemical agencies have so far always resulted in killing the streptobacillary mother strains first and setting L1 free, and never contrariwise. Dienes (1939*a*), Wilson Smith (1941) and Heilman (1941 *a*, *b*) claim that they have been able to observe bacilli being transformed into globular bodies in *Str. moniliformis* cultures. To the present writer their evidence seems inconclusive owing to the difficulties of following microscopically the development of small granules and elements of a highly attenuated nature present in these cultures. Even by means of stained preparations which show more contrast and detail than living specimens it has not been possible so far to demonstrate the successive developmental stages of L1. Sabin (1941) has called attention to the inconclusiveness of Dawson & Hobby's (1939) serological evidence supporting the variant hypothesis. He points out that it is not surprising that *Str. moniliformis* sera agglutinate L1 strains and vice versa as *Str. moniliformis* always contains L1. Therefore the position at present is that the evidence so far brought forward does not allow us to decide whether the symbiosis or the variant hypothesis explains the more convincingly the true relationship between L1 and *Str. moniliformis*. On the other hand, new evidence in support of either concept would be of importance. *Str. moniliformis* is not unique in bacteriology. Theobald Smith (1918, 1921 *a*, *b*) described a similar organism associated with a pulmonary disease in calves, and Klieneberger (1940) and Wilson Smith (1941) have described an organism of the *Str. moniliformis* type causing an infection in guinea-pigs. From another point of view new evidence would be even more desirable; for if L1 is a variant, claims for the existence of the much-disputed filterable phase in bacteria would receive a fillip; if it is a symbiont related to the pleuropneumonia group our knowledge of this latter group would be enhanced. Helped by greater experience of the morphology of pleuropneumonia-like organisms (Klieneberger & Smiles, 1942), a study of the morphology of *Str. moniliformis* and of L1 has been carried out by improved methods. The results of this investigation together with a serological study of both cultures are set forth in this paper.

A. Morphological studies

Before the actual methods are described it should be pointed out that a peculiar property of the L1 organism as well as of *Str. moniliformis* renders the recognition of the actual elements of the culture very difficult. As shown by Partridge & Klieneberger (1941) these cultures produce in the course of their development large amounts of cholesterol

and cholesterol esters from the medium, which are present in the form of myelin structures (Williams, 1941). Usually 35–40% of the dry weight of the growth consists of these substances. The cholesterol droplets may be small and faint or large and very refractile, they can take different shapes and may be drawn out into fine filaments. They are intermingled with the elements of growth and often extrude beyond it. They are responsible for Wilson Smith's 'streaming effect' and compose the 'thin-walled hollow bodies' and 'tension filaments' mentioned by Heilman (1941). In unstained preparations some of these structures may easily be mistaken, and without doubt have been mistaken in the past by nearly all investigators for actual elements of the organisms. If preparations fixed and stained by the writer's techniques *a* and *b*, described in the next section, are used for the study of the cholesterol-liberating organisms, the task of unravelling their life cycle becomes an easier one, because the 'fatty droplets' do not stain and are merely indicated by spaces of different sizes appearing in the texture of the growth. Therefore dark-ground methods have not been used in the present study and only stained specimens have been examined. Another point should be mentioned here. The growth of the L1 organism is even more liable than that of most pleuropneumonia-like organisms to become distorted when ordinary or impression preparations are made from solid or semi-solid media. Some of these distortions are of a filamentous nature and can be noticed frequently at the edges of colonies as seen from illustrations in the writer's first papers on the L1 organism (1935, 1936). It is therefore essential to grow the organism between the cover-slip and a layer of medium and to fix through the medium. After the fixation the medium can be removed without disturbance of the growth. If for certain purposes a direct fixation of the growth on the cover-glass is required the agar square may be removed before this process; but in this case genuine structures can only be expected at the edge of the preparation where the growth is not covered by the agar, yet is able to develop at its periphery. In liquid medium L1 grows largely in big, solid clumps which are difficult to break up. They seem less liable to distortion, but the examination of the single elements of which they are composed presents a most perplexing task.

Methods employed

(*a*) *Agar-fixation technique* for demonstration of growth on solid and semi-solid media. This method has already been described in its present form by Klieneberger & Smiles (1942). It has been used here in the same way with the exception of a slight alteration in staining practice, staining being carried out at room temperature in Gurr's improved Giemsa solution R66 diluted 1 in 20 for from 20 to 30 min. or longer for special purposes.

(*b*) *Osmic acid-Giemsa method.* The treatment of wet films by osmic acid vapours has been extensively used by Robinow (1942) for the fixation of the chromatinic apparatus of various bacteria and spores. It was also recommended by Heilman (1941) in his paper on *Str. moniliformis*. The simple technique used here was as follows. The culture was grown between a cover-slip and an agar layer as described under (*a*). After the required time of incubation the agar layer was removed as gently as possible; the remaining wet film was then quickly placed in osmic acid vapours for 5–8 min. Following this it was dried and stained in Giemsa solution 1 in 20 for 15–30 min. The time varied according to the thickness of the growth, delicate growth being stained longer. As mentioned before only the edges of these preparations were suitable for examination; when successful they showed up brilliantly stained, exhibiting clearly the delicate details of structure. It was of advantage to grind the material up before inoculation.

(*c*) *Azur II method.* Dienes (1939*a*) has studied the morphology of various delicate organisms by placing on the grown agar surface a cover-slip on which alcoholic solutions of different dyes such as methylene-blue and azur II had been dried; he also used watery dye solutions mixed with small amounts of serum

for the staining of growth on the surface of the medium which he covered with a glass slip. The examination took place in transparent light or the cover-slip was removed and an impression made. As already pointed out in my joint paper with Smiles (1942) the disadvantage of the examination of structures on the surface of an agar layer is that it does not allow the use of the high power of the microscope to its full capacity. On the other hand, the placing of a cover-slip on growth already developed produces distortions of delicate material. Therefore these methods could not be adopted here, but Dienes's observation that these simple dyes stain the L1 organism readily without previous fixation was confirmed, and a watery solution of azur II has been used widely for quick information about the development of cultures. It has been found particularly useful for the examination of growth from serum broth cultures whereby it was seen that the thick clumps in which growth of the L1 occurs in the liquid were not much distorted if placed on a slide and covered with a slip. If these preparations were very thin the well-stained and flattened-out edges of colony clumps presented a suitable object for examination with high powers.

By means of the methods described the life cycle of the L1 organism in the pure state as well as in conjunction with *Str. moniliformis* was studied and the result of this investigation is outlined in the following order.

Development of the L1 *organism as observed in pure cultures on solid media*

If old cultures of the L1 organism after three or more days of incubation are examined they seem to consist, apart from the cholesterol droplets, of a slimy material containing numerous granules. These granules are indicated in Fig. 1, outlining the life cycle of the organism. They are seen embedded in the slimy matrix in Fig. 8. The sticky basic substance and the granules are both difficult to demonstrate in the aged culture, as they take stains very reluctantly and are also little refractile in dark-ground preparations. If inoculated on fresh media and incubated for a few hours only the granules seem to pass into another stage in which they have acquired a new affinity for stains. They now show up deeply stained and appear almost black in osmic acid-Giemsa preparations. If incubated further they develop a small fringe or edge of thin, faintly staining material (Figs. 8, 9). They are on their way to develop into small bodies which are present in abundance after about 12 hr. incubation. The time varies according to the age of the inoculum and the quality of the medium. In the osmic acid-Giemsa preparation the small forms show a blue protoplasmatic body surrounding a brilliantly red chromatinic granule or structure (Figs. 2, 10). It seems justifiable to use the expression 'chromatinic' for the granules and structures always present in well-stained L1 bodies, because they take up nuclear stains and give a positive Feulgen reaction. When incubated further the chromatinic centre of the L1 body splits up. It may develop either into a kind of network nuclear structure composed of filaments and granules or into granular structures only (Figs. 3, 10, 11, 12, 13, 14, 25, 26 and 27). Following this a division of these 'nuclei' takes place which precedes the segmentation of the bodies. Many division stages can be found in the photographs mentioned, particularly in Fig. 26. The bodies do not possess a visible membrane, they are extremely soft and are completely deformed by the slightest pressure; their nuclear apparatus is then pressed out and may form thread-like structures. Some slight deformations can be seen in the photograph, Fig. 27. Sometimes segmentation is not complete, and then groups of bodies are seen, the elements of which are connected by thinner protoplasmatic masses (Fig. 10). While growth proceeds, the single bodies grow often to larger dimensions than the initial ones. Their deeply stained chromatinic structures fill the major portion of the elements. In 2-day-old cultures very large bodies may occur which have formed

multiple chromatinic structures and show a superficial similarity to the forms produced by the process of schizogony in some Protozoa (Figs. 4, 13, 14, 25 and 27). As in pleuropneumonia the large elements represent multiple forms and not single units. The large, brilliantly staining chromatinic structures are usually developed in abundance after 2 days' incubation. Later, between the second and third day of development, the nuclear or chromatinic material divides up into small pieces and the protoplasmic substance undergoes the same process of subdivision, until a whole colony consists of granules each of which is surrounded by a small portion of protoplasm (Figs. 5, 6, 15, 16 and 28). At this stage the chromatinic granules take the stain more deeply than before and show up black, instead of red as previously, in the osmic acid-Giemsa preparation, while the protoplasmic material stains more faintly than before. The outer edge of the colony has usually not proceeded quite as far as the centre (Figs. 16, 28), and may still show large bodies with large red chromatinic structures. When incubation is continued the granular centre of each small body reduces its size gradually, while the protoplasmatic zone surrounding it fades away. Thus the small reproductive granules are formed which at first stand out clearly, showing up black, but very soon lose their property of taking the stain deeply (Fig. 7). They are scarcely visible in old cultures in which they are caked together by a material not readily stainable. This substance is probably the remainder of the decomposed protoplasm of the former bodies. It is supposed that the granules formed as described represent the filterable, minimal reproductive units of the L1 organism.

Development of the L1 *organism as observed in* Streptobacillus moniliformis *cultures on solid media*

In an old *Str. moniliformis* culture elements of growth are hardly recognizable. It seems to consist of flakes of badly staining material containing faint granular matter, some of which are probably of bacillary, others of L1 nature. If the culture used for the inoculation was not too old and contained still viable bacillary elements new growth may have started after a few hours of incubation and the two different components of the culture can be seen, distinctly stained in their young stages in the osmic acid-Giemsa preparation (Figs. 17, 31). The young bacillus is a slender rod and stains with two dark poles at either end (Figs. 38, 39). The bacilli soon grow into small chains and often show already in an early stage signs of abnormality in shape and staining properties. They are surrounded by numerous small black granules of exactly the appearance as described for the young pure L1 culture (Fig. 31). These granules soon surround themselves with a small and thin protoplasmatic edge. In the 12 hr. culture young bacillary chains and small L1 bodies are found (Figs. 18, 32). As illustrated in these figures the L1 bodies are distinctly stained showing a protoplasmatic blue edge and a red chromatinic corpuscle which enlarges and divides up forming a kind of 'nucleus' of different structure. One distinct L1 element can be seen in Fig. 32 showing a large 'nucleus' in the middle of a protoplasmatic body, the delicate irregular fringe of which can be recognized. It is very striking that the L1 bodies are often situated at the end of a bacillus or in the midst of chains of bacilli (Figs. 19, 20, 21 and 32) as if taking the place of a bacillus. This would suggest that they have either developed from a bacillus according to Dienes's views or on a bacillus which they finally may dissolve or destroy. One characteristic feature of *Str. moniliformis* has to be mentioned here. In overstained Giemsa preparations or in ordinary bacteriological preparations which are overstained from the point of view of

internal structures, the beaded appearance, which has given the name to the organism, is particularly well seen in the places where the bacillary curves turn from one into another direction (Figs. 19, 44 and 45). If the preparations are not overstained, as shown in Fig. 44, but if they are stained to exhibit contrast of structure, as shown in Figs. 19 and 45, the beads can be recognized clearly as L1 bodies with a 'nucleus' and a protoplasmic peripheral portion.

In a similar way as in the pure L1 culture the L1 bodies continue their development when in association with the bacilli. They grow into larger forms with composite chromatinic structures as shown in Figs. 20, 21 and 33. In association with the bacilli they are often scattered about singly or appear in small groups; they do not tend to develop large dimensions as in the pure L1 culture, though occasionally very large elements occur singly or in groups in *Str. moniliformis* growth. Whatever their sizes, their further development is the same. The chromatinic centres and protoplasmatic portions divide up, eventually forming darkly staining corpuscles lying singly in small protoplasmatic bodies. As the cultures grow older the darkly staining corpuscles are reduced in size, while the protoplasmatic parts surrounding them fade away and decay. The bacilli which may be still present at that stage are very small and stain faintly. Finally, in the old culture, both the granules and the remains of the bacilli take the stain very badly. As already mentioned the remaining elements of the culture are held together by a very faintly staining, sticky substance which is presumably formed from the protoplasmatic material originally building up the L1 bodies. The development described is illustrated in Figs. 22, 23, 24, 34 and 35. The last two illustrations show two stages which follow each other closely; in the second the granules are reduced in size, only a few of them are still in the centre of their small protoplasmatic body, most of them are free; the background is very faint.

The development so far described has been followed up mainly in osmic acid-Giemsa preparations which are particularly suitable for the demonstration of the internal structures of the bodies, but the fixation through the agar with Bouin's solution has proved useful also for clearing up some points. It has given valuable information about the arrangement of the growth as a whole. It shows up the 'nuclear' and granular material, but the protoplasmatic portions of the bodies stain faintly, so that there is less contrast and much less brilliance in these preparations. Figs. 29 and 30 show parts of two colonies of the L1 organism fixed through the agar and stained as described. Fig. 29 shows the impression pattern of large L1 bodies in a flat film of growth at the edge of a colony; the bodies seem full of chromatinic structures; the 'holes' were filled with cholesterol bodies in the living state. In Fig. 30 the frequently occurring arrangement of bodies in solid clumps can be observed; the nuclear structures are indicated; some 'cholesterol holes' can be found. The agar-fixation technique would seem to be the only one so far used that will reliably demonstrate how much L1 material is actually present in any stage of a *Str. moniliformis* culture, because at least the surface film of growth will stick to the cover-slip after fixation, while in the osmic acid-Giemsa preparation much of it may be lost, and only the very edge may give an undistorted picture of arrangement and structures. It was found by means of the agar-fixation method that there existed no stage in the *Str. moniliformis* culture, no matter how it was grown, which was free from L1 elements. Even if old stock cultures are transferred at short intervals by means of which the number of viable young bacillary forms increases, numerous L1 elements

are present. When material from these cultures is inoculated on fresh solid media it can be observed how the L1 elements as well as the bacilli multiply from the first hour of growth. Fig. 40 shows a field of very young *Str. moniliformis* growth (agar-fixation method) which is crowded with young L1 elements. The corresponding preparation was made from a densely inoculated plate. In small, young bacillary colonies as they develop when less inoculum is used, L1 material is found in single small elements or often in nests of granular material filling the curves of the bacillary threads. If preparations of young growth are overstained it often looks as if the L1 material was forming a kind of incrustation of the bacillary filaments (Fig. 41), a fact rousing the suspicion that the L1 organism may not be a harmless symbiont, but rather a parasite of the bacillus. When the cultures become older the L1 seems to continue to develop at a stage when the bacilli have stopped growing, except for edges of isolated colonies where they have still fresh nutritive substance at their disposal. Some colonies or parts of colonies then become crowded with L1 bodies, so much so that the bacilli may no more be visible at all (Fig. 43). The pattern of the bacillary filaments can still be recognized; but in their place we now see only chains of bodies. In other cases the bacillary filaments are still faintly preserved as such, but they are covered by an irregular slime which contains masses of darkly and faintly stained L1 granules (Fig. 42). If by accident a slight slipping of the agar square has taken place before fixation the sticky material and granules covering the bacilli may become detached as shown in Fig. 37. The filamentous material which I previously demonstrated was presumably produced in this way. These formations are not artefacts, strictly speaking, but represent deformed and dislocated L1 material.

Development of the L1 *organism in liquid media*

Preparations of L1 from liquid media have been studied in azur II solution in the way described. In an overnight culture usually all the different stages of the organism could be found at the flat edges of the dense colony clumps in which the growth develops. The young growth stains very well; the 'nuclear' structures show up dark blue and the protoplasm takes on a lighter mauvish pink colour. The cholesterol elements take hardly any stain; if they do, they become light blue. Single granules often seem to become liberated and form groups in the liquid. These are probably derived from one mother body. When these granules develop in their turn they grow into aggregates of small round bodies all of which show more or less the same stages. As they continue their development they form packages of larger bodies firmly attached to each other and with their 'nuclear' structures dividing up in similar ways. In liquid media the bodies tend to take the shape of small spheres, but on the surface of solid media they spread as flat, slightly irregular films. Single elements sometimes grow into larger forms in the liquid also and then exhibit distinct granular or network 'nuclei' such as those formed on solid media by corresponding bodies. The chromatinic structures may take all kinds of positions inside the elements developed in the liquid medium; they may be in a central position or on one side of a body as can be clearly observed when the spheres are moved by convection currents.

Much the same development of the L1 bodies can be observed by the azur II method in *Str. moniliformis* cultures grown in liquid media, but the entanglement of bacillary threads and L1 elements in dense flakes of material renders the examination even more difficult than in the case of the solid L1 clumps developing in liquid media.

To sum up: While the development of the bacillary elements of *Str. moniliformis* seems to conform with the general idea of the growth and multiplication of bacilli, except that they show abnormal shapes and signs of decay at a very early age, the L1 organism, as observed in pure culture and in association with the streptobacilli, seems to follow another course. A small granule develops into a body which consists of a protoplasmatic and a chromatinic, Feulgen-positive portion and exhibits no membrane. This body, which is of a very soft consistency, divides up by segmentation, preceded by subdivision of the chromatinic material. Finally, the chromatinic structures divide up into small lumps and the protoplasmic material divides up correspondingly, so that each chromatinic element of structure is surrounded by a small portion of protoplasm. The chromatinic granules gradually become smaller and more consolidated, taking the stain more deeply, while the surrounding protoplasm fades away and decays. Thus the cycle is completed. With the improved staining methods the development of the L1 bodies from granules and the eventual formation of the reproductive granules, a process in which chromatinic structures seem to play an essential part, has been followed up for the first time.

B. Serological examination

As shown by different investigators, including the writer, a rabbit immune serum produced with a *Str. moniliformis* strain agglutinates different *Str. moniliformis* strains to approximately the same titre. A similar result is obtained with an L1 immune serum and L1 strains from different sources. If cross-agglutination tests between the two organisms are carried out, both immune sera, viz. that *v. Str. moniliformis* and that *v.* L1, agglutinate both organisms, though not always to exactly the same titre. This might be expected, because all the *Str. moniliformis* cultures contain a large amount of L1 elements which are closely attached to bacillary elements. A serological difference existing between the two organisms could therefore only be revealed by cross-absorption experiments. As more information about the relationship of the two organisms was desired these tests have been carried out. The methods employed for the preparation of the sera and the suspensions used to carry out the agglutination tests were the same as those described in a previous paper (Klieneberger, 1938). For the actual absorption very thick suspensions had to be prepared in order to effect the complete absorption of the homologous antigen. The only alteration was that instead of the buffer solution recommended in 1938 for the making up of the suspensions of pleuropneumonia-like organisms another medium was used which gave better results with *Str. moniliformis*. It consisted of ordinary broth diluted 1 in 3 with saline and formalinized 1 in 500. It should be pointed out that the preparation of suitable *Str. moniliformis* suspensions was not an easy task and could only be achieved by grinding up the sediments of liquid cultures for a considerable time. The ages of the cultures from which suspensions were prepared varied for the two organisms examined, being 2–3 days for the L1 organism and 18 hr. for *Str. moniliformis*. It was noted that *Str. moniliformis* suspensions when prepared as described often consisted of granular particles only on microscopical examination and that bacillary forms could hardly be detected. L1 material consisted always of granular matter exclusively.

The absorption took place in a serum dilution of 1 in 5 and at incubator temperature for 1 hr. The tubes were then centrifuged at high speed until the supernatant was practically clear. The tests were put up in small tubes as described in 1938. The reading was

taken with a magnifier after 24 hr. and repeated after 48 hr. The tubes had been kept previously at 37° C. for 2 hr. and then at room temperature. To control the readings the tests were also read microscopically. The two main strains used for the investigation were a *Str. moniliformis* from the nasopharynx of a rat isolated in 1940 and the old L1 culture separated from a *Str. moniliformis* strain in 1934. This latter *Str. moniliformis* strain had been previously isolated from the same Lister Institute rat stock. The result of the agglutination tests with the unabsorbed and absorbed immune sera are summarized in Table 1.

Table 1. *Cross-absorption tests with* Streptobacillus moniliformis *and* L1 *organism*

(Reading taken after 48 hr.)

Serum prepared with antigen	Unabsorbed or absorbed	Tested against suspension	Final serum dilutions								Controls	
			1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280	Normal serum 1 : 10	Phys. saline
Str. mon.	Unabsorbed	*Str. mon.*	+++	+++	+++	+++	++±	++	+	—	—	—
„	„	L1 org.	+++	+++	+++	++±	++	+	(±)	—	—	—
„	Absorbed with *Str. mon.*	*Str. mon.*	—	—	—	—	—	—	—	—	—	—
„	„	L1 org.	—	—	—	—	—	—	—	—	—	—
„	Absorbed with L1 org.	*Str. mon.*	+++	++±	++	+±	+	±	(±)	—	—	—
„	„	L1 org.	—	—	—	—	—	—	—	—	—	—
L1 org.	Unabsorbed	*Str. mon.*	+++	+++	++	+	(±)	—	—	—	—	—
„	„	L1 org.	+++	+++	+++	+++	+±	±	(±)	—	—	—
„	Absorbed with *Str. mon.*	*Str. mon.*	—	—	—	—	—	—	—	—	—	—
„	„	L1 org.	—	—	—	—	—	—	—	—	—	—
„	Absorbed with L1 org.	*Str. mon.*	—	—	—	—	—	—	—	—	—	—
„	„	L1 org.	—	—	—	—	—	—	—	—	—	—

As will be seen from this table the unabsorbed sera give positive reactions with *Str. moniliformis* and with the L1 organism. The L1 and *Str. moniliformis* sera absorbed with the homologous antigens were completely exhausted and no longer reacted with either of the two strains. Also the L1 serum absorbed with *Str. moniliformis* no longer contained any agglutinins. In contrast to this the *Str. moniliformis* serum absorbed with the L1 organism was only deprived of its L1 agglutinins and still possessed another agglutinin which reacted with the *Str. moniliformis* suspension. This result certainly suggests that the L1 serum contained only agglutinins of one kind directed against the L1 elements, while obviously the *Str. moniliformis* serum contained two kinds of agglutinins, those reacting with the L1 elements and those agglutinating what may be assumed to be the bacillary component of *Str. moniliformis*. Therefore *Str. moniliformis* and the L1 organism seemed to show distinct differences in their serological affinities. This result does not of course either prove that they are symbionts or that they are variants, as symbionts should, and variants might be, different antigenically, but it certainly does not discredit the symbiosis hypothesis.

DISCUSSION

In the case of *Str. moniliformis* we are dealing with the interesting phenomenon that a bacterial culture isolated from various conditions in man and animals constantly contains two different growth types, a *Streptobacillus* and elements varying from small granules to large, soft pleomorphic bodies. This peculiar fact has been explained in two different

ways, by the conception of a symbiosis or close association between two genetically different microbes (Klieneberger, 1935) and by the 'variant or life cycle theory' (Dienes, 1939*a*; Dawson & Hobby, 1939; Heilman, 1941*a*; Brown & Nunemaker, 1942). In the work here presented, the attempt has been made to collect new evidence pointing in the one or the other direction. The different views will now be discussed in the light of the new information obtained. The main critic of the symbiosis hypothesis, Dienes, holds the view that the elements of which the L1 cultures consist 'are essentially similar to the elements of the bacterium cultures'. He then continues: 'The characteristic properties of the L cultures are produced by the fragility of these elements and their tendency to change into large swollen bodies which sooner or later are vacuolized and disintegrate. A similar transformation occurs in varying degrees in many bacterium cultures, and the excessive development of this process does not separate the L organism from the bacteria.' It has been clearly shown in the present paper by means of new methods that the life cycle of the L1 organism is essentially different from the life cycle of a bacterium. The L1 starts its development from elementary corpuscles which are filterable and which develop first into small and later into larger, soft bodies without membranes which contain striking chromatinic structures. These bodies multiply by segmentation, a process preceded by transformations and multiple divisions of the 'nuclear apparatus'. Eventually reproductive corpuscles are formed in small bodies by a breaking up of the chromatinic and protoplasmatic substances of the larger bodies. Such a development has so far not been observed in bacteria. The bodies do not seem to become vacuolated and then to disintegrate as Dienes believes, but they are the important mother elements which produce the reproductive elementary corpuscles. It may be mentioned here that a foam-like appearance of the whole L1 growth which Dienes has observed is frequently caused by an abundant production of cholesterol droplets which occurs similarly in *Str. moniliformis* cultures. A life cycle resembling that of the L1 microbe has so far only been described for pleuropneumonia-like organisms (Klieneberger & Smiles, 1942). In pleuropneumonia chromatinic structures have similarly been observed before the commencement of granule formation. While in both organisms the chromatinic material is used up in this process, the pleuropneumonia bodies possess at that stage a membrane and the granules form inside these membranous elements. There is no such membrane visible at the periphery of L1 bodies, and each corpuscle is surrounded by a small portion of the protoplasm of the mother body when newly formed. The size of the granules seems to be the same in both organisms, and in both cases the granules form a fairly regular pattern in the texture of the whole growth. Though individual differences of development exist, there are besides the process of granule formation so many similarities between pleuropneumonia and the L1, such as the softness of the elements and their attenuation in certain stages, their colony type and their nutritive requirements, that it seems justifiable to classify the L1 microbe with the pleuropneumonia-like organisms and not with the bacteria. The large forms occurring in bacterial cultures particularly of Gram-negative organisms, which have been described in the past by many authors (Klieneberger, 1930; Dienes, 1939*b*), do not seem to produce reproductive filterable granules. On the contrary the very large ones eventually disintegrate and are often not viable forms, which is in strong contrast to the L1 bodies. These go on to develop as described, but owing to their extreme softness may not always be able to continue their development if transplanted before granule formation has taken place.

Though it is believed that the L1 bodies are of a different nature from that of swollen bacteria, it must be admitted here that there are certain similarities between swollen bacterial forms and L1 bodies. To demonstrate this an illustration of swollen bacterial forms grown overnight on 1% lithium chloride agar has been given in Fig. 36. If fixed with osmic acid vapours and stained with Giemsa solution for 5–8 min. only, the nuclear apparatus of these swollen bacterial forms becomes clearly visible. The 'nuclei' take irregular shapes and in large forms may even split up into filamentous and granular structures. These structures seem coarser than those of the L1 bodies and stain more easily, and a further development similar to that of the L1 has never been observed. This suggests that they are different in nature. The superficial resemblance between the lithium chloride forms of bacteria (see also v. Eisler, 1909; Kuhn & Sternberg, 1931) and some L1 elements, though perplexing at the first moment, may seem less surprising when it is realized that nuclear structures show close similarities as such through the whole realm of living organisms.

The peculiarity of the L1 bodies of being found in close connexion with bacillary elements has led to a second point of divergence between Dienes (1939*a*), Heilman (1941*a*) and Brown & Nunemaker (1942) on one side and the writer on the other. From this observation the authors mentioned drew the conclusion that a transformation of bacilli into bodies had taken place, while the writer is inclined to believe that the L1 bodies have developed on or round bacillary elements which they may dissolve or possibly destroy during their development. This assumption is supported by the observation that the numerous reproductive granules present in old cultures are often seen closely attached to bacilli (Fig. 42) and further by the fact that the bacilli show very early in their development signs of abnormality and decay and die out rapidly if incubation is continued after the first 20 hr. Very frequently *Str. moniliformis* cultures after only 2 days of incubation no longer contain viable bacillary elements.

A third point of disagreement between Dienes and the writer is the following. Dienes records that in young *Str. moniliformis* cultures elements indicating the presence of the L1 have not been found. As has been pointed out here and demonstrated by illustrations, L1 elements are always present in all stages of the cultures, young or old, though not every method is suitable for their demonstration. With regard to Dienes's and Brown & Nunemaker's observations that L1 cultures frequently revert to the bacillary type, it should be mentioned that in the writer's hands freshly isolated strains of L1 have often proved not to be pure cultures, but that five thoroughly purified strains have so far never reverted though they have been propagated for many years in the pure state. This is considered as one of the strongest arguments in favour of the symbiosis hypothesis, while the fact that so far the bacilli have never been freed from the L1 organism is regarded as pointing against this conception; but this fact may be interpreted as an indication that the L1 is not a mere admixture of the culture, but that a closer relationship such as parasitism may exist. This view is supported by the observation that *Str. moniliformis* is a culture of very variable properties. Sometimes it grows with strong turbidity in the liquid and then always contains bacillary forms in abundance. At other times it grows in flakes that form a sediment at the bottom of the tube and leave the supernatant fluid clear. In this case the L1 is present in abundance. The cultures perhaps after a period of very good growth suddenly grow with reluctance and frequently they die out if kept in liquid media and not subcultured every day. This delicacy is always associated with a lack of

viable bacillary forms and is not caused by a weakness of the L1 organisms which survive the bacilli, but do not grow out without them unless special media are used. In contrast to *Str. moniliformis*, L1 cultures once established on the special media grow very regularly and keep well if transferred at weekly or fortnightly intervals.

Dawson & Hobby's claim that the L1 organism and *Str. moniliformis* are identical serologically cannot be discussed further, because their experiments have so far not been published in full; but the cross-absorption experiments recorded here seem to indicate that the two organisms are antigenically dissimilar.

For all these reasons the writer is inclined to think that the conception of an association of two organisms explains the relationship between the L1 organism and *Str. moniliformis* better than the variant hypothesis.

SUMMARY

The new data presented show that the life cycle of the L1 organism separated from cultures of *Streptobacillus moniliformis* corresponds in many respects to that of pleuropneumonia. It seems therefore justifiable to classify it with the group of pleuropneumonia-like organisms and not with the bacteria. Its peculiar life cycle, including the formation of elementary reproductive corpuscles, a process previously described for pleuropneumonia, but not occurring in bacteria, is regarded as strong evidence in favour of the conception that *Str. moniliformis* is composed of two genetically different microbes, a *Streptobacillus* and the L1 organism. Cross-absorption tests between *Str. moniliformis* and the L1 organism have shown that these organisms possess different serological affinities.

I should like to express my gratitude to Sir John Ledingham for his constant interest in this work, for his many valuable suggestions and his much appreciated criticism. I am indebted to Mr F. V. Welch (National Institute for Medical Research, Hampstead, Department of Mr J. Smiles) for the photographs.

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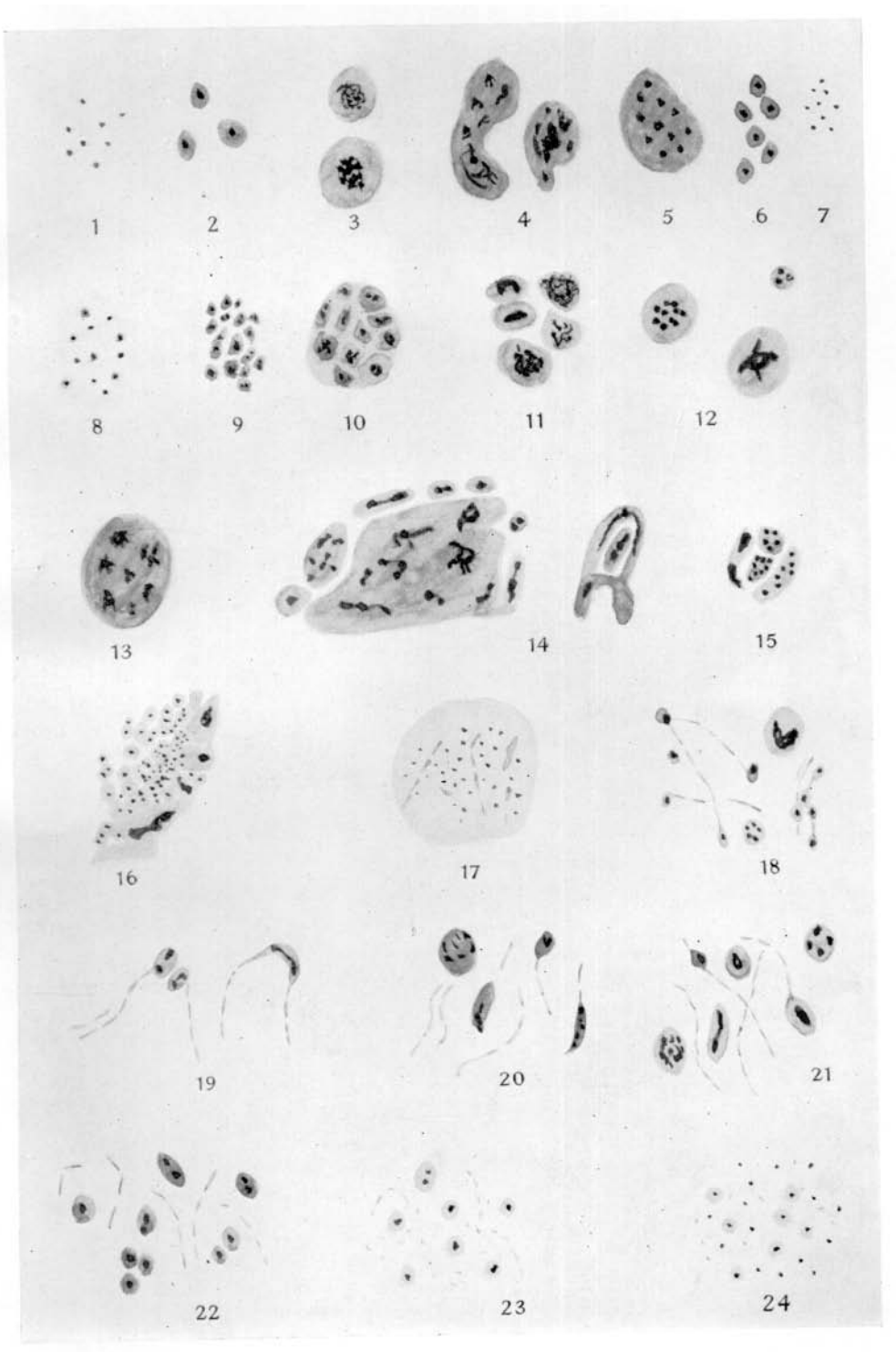

Figs. 1–24

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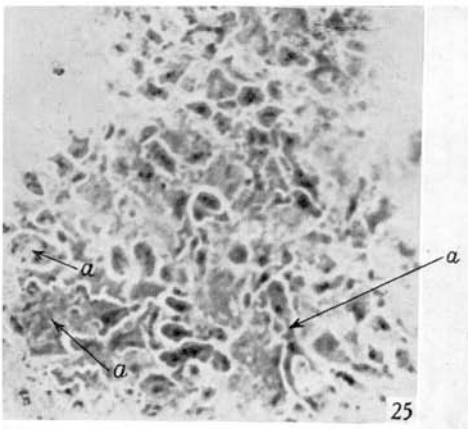

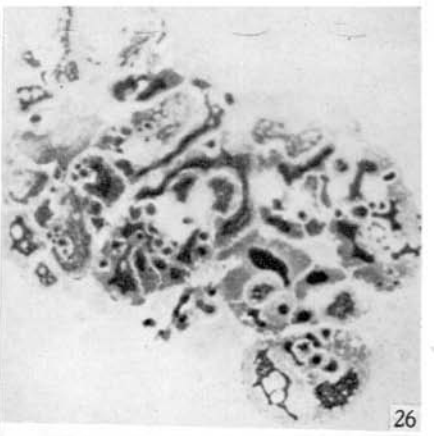

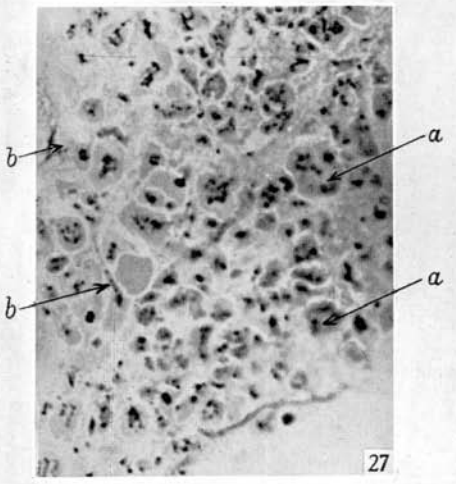

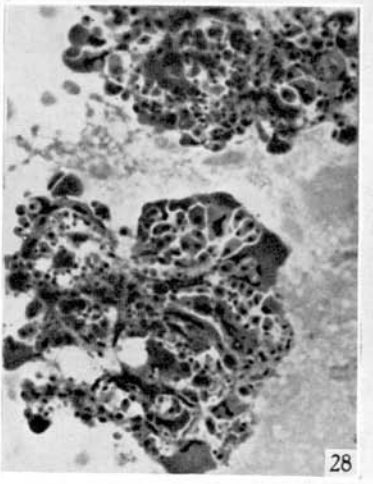

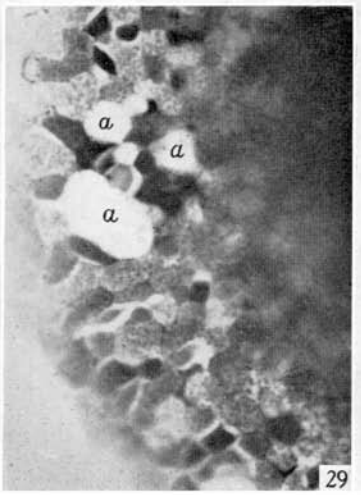

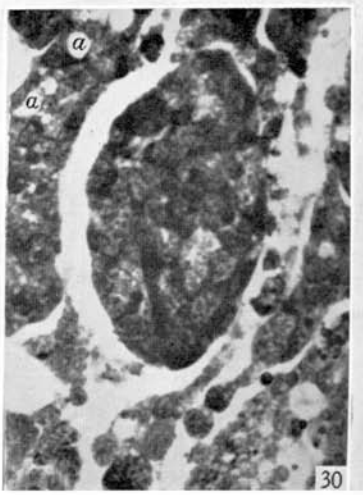

Figs. 25–30

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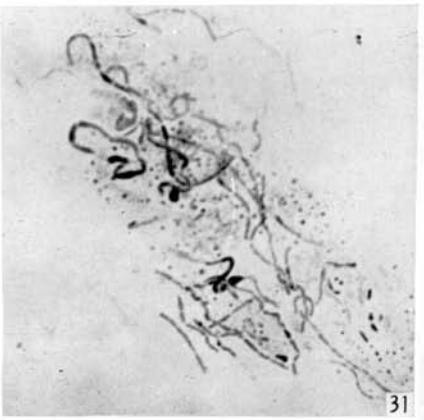

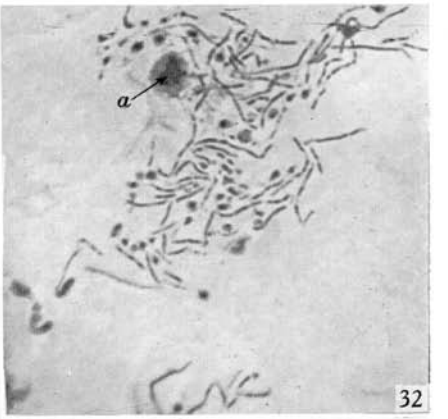

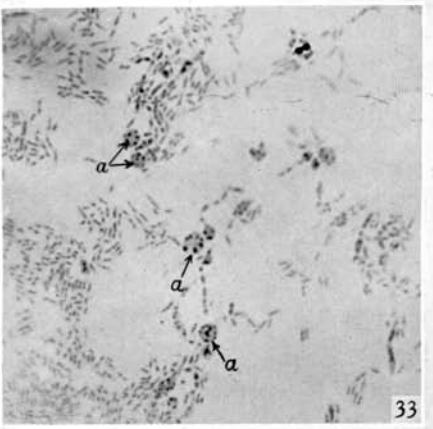

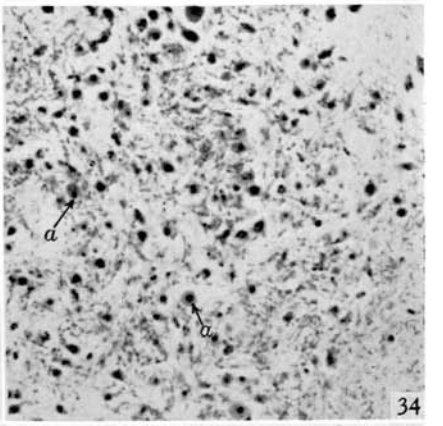

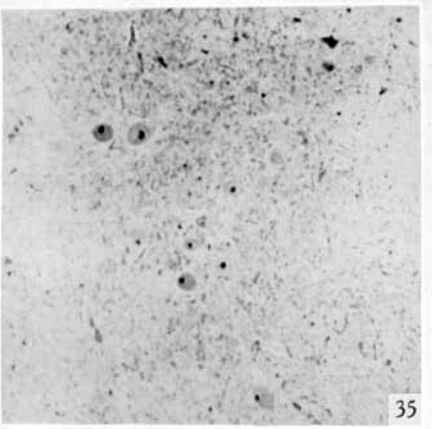

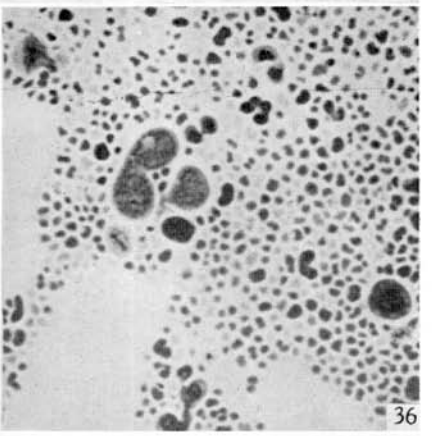

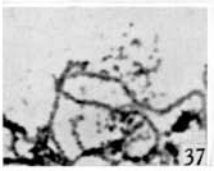

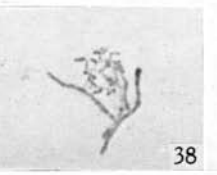

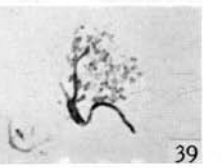

Figs. 31–39

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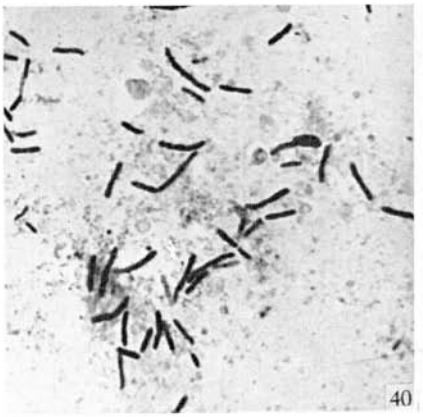

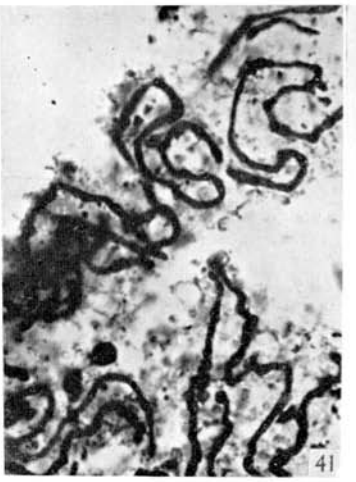

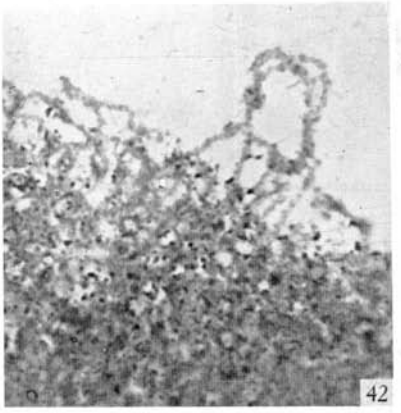

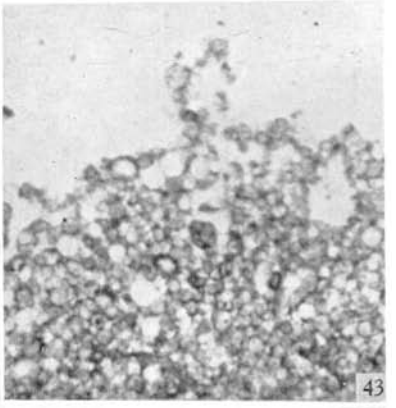

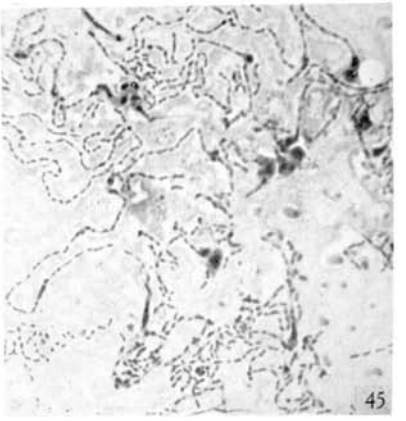

Figs. 40–45

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EXPLANATION OF PLATES 10–13

PLATE 10

Drawings, magnification 1 : 900

Figs. 1–7, diagram of development of L1 organism; 8–16, successive stages of development of L1 organism according to stained preparations; 17–24, successive stages of development of *Streptobacillus moniliformis* according to stained preparations.

PLATES 11–13

Photographs, magnification 1 : 1250

Fig. 25. L1 organism, 18 hr. old, osmic acid-Giemsa preparation. This photograph shows the softness of the bodies, indicated by the lack of proper cell boundaries. Notice the multiple forms in *a*.

Fig. 26. L1 organism, about 30 hr. old, osmic acid-Giemsa preparation. This photograph demonstrates clearly the well-developed chromatinic structures of the protoplasmic bodies.

Fig. 27. L1 organism, 24 hr. old, osmic acid-Giemsa preparation. This photograph shows another type of chromatinic structures. Note the multiple forms in *a* and the slightly deformed chromatinic structures in *b*.

Fig. 28. L1 organism, 3 days old, osmic acid-Giemsa preparation. The granule formation from nuclear structures is very evident.

Fig. 29. L1 organism, colony edge, 2 days old, agar-fixation Bouin Giemsa. This photograph shows bodies which have spread in a very thin layer on solid medium. Note cholesterol 'holes' in *a*.

Fig. 30. L1 organism, 2 days old, part of colony edge, agar-fixation Bouin Giemsa preparation. The growth has developed in densely packed agglomerations on semi-solid medium. Cholesterol 'holes' in *a*.

Fig. 31. *Streptobacillus moniliformis*, 6 hr. old, osmic acid-Giemsa preparation. The first development of L1 granules densely packed round bacillary forms is to be seen.

Fig. 32. *Streptobacillus moniliformis*, 18 hr. old, osmic acid-Giemsa preparation. Note the small bodies with nuclear centres and the large body in *a*.

Fig. 33. *Streptobacillus moniliformis*, about 30 hr. old, osmic acid-Giemsa preparation. Note the L1 forms with multiple chromatinic structures in *a*.

Fig. 34. *Streptobacillus moniliformis*, between 2 and 3 days old, osmic acid-Giemsa preparation. This photograph shows many small L1 forms with dark chromatinic centres; in some of them (*a*) the reproductive granule has already been formed.

Fig. 35. *Streptobacillus moniliformis*, 3 days old, osmic acid-Giemsa preparation. Single reproductive granules in small bodies, free granules and faint bacillary forms are to be seen.

Fig. 36. *B. coli*, grown overnight on lithium chloride medium, osmic acid-Giemsa preparation. Note the chromatinic material in the large forms.

Fig. 37. *Streptobacillus moniliformis*, 2–3 days old, agar-fixation Bouin Giemsa preparation. Note trailing filaments with granules.

Figs. 38, 39. *Streptobacillus moniliformis*, 3 hr. old, agar-fixation Bouin Giemsa preparation. Note small bacilli with granules at either end.

Fig. 40. *Streptobacillus moniliformis*, a few hours old, agar-fixation Bouin Giemsa preparation. Note small L1 forms.

Fig. 41. *Streptobacillus moniliformis*, 12 hr. old, agar-fixation Bouin Giemsa preparation, overstained. Note the L1 structures covering and surrounding the bacilli.

Fig. 42. *Streptobacillus moniliformis*, 3 days old, agar-fixation Bouin Giemsa preparation. Note granular material of L1 organism covering the bacillary threads.

Fig. 43. *Streptobacillus moniliformis*, 3 days old, agar-fixation Bouin Giemsa preparation. Only L1 bodies to be seen. Note the bodies in chains that have taken the place of bacilli.

Fig. 44. *Streptobacillus moniliformis*, grown overnight, osmic acid-Giemsa preparation, overstained, showing the moniliform 'beads'.

Fig. 45. The same as Fig. 44, but stained for a short time only. The 'beads' show up as L1 bodies with chromatinic centre in soft protoplasmatic body (cf. Fig. 19).

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