DEMONSTRATION OF CHROMATINIC STRUCTURES IN AVIAN TUBERCLE BACILLI IN THE EARLY STAGES OF DEVELOPMENT

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(With Plates 8–10)

In a previous study Brieger & Fell (1945) described developmental changes of avian tubercle bacilli grown in hanging-drop cultures in chicken-embryo extract. They observed on a warm-stage microscope that after 24–48 hr. filamentous forms were present, which showed two main types of development:

(*a*) The 'standard' type, in which the elongated filaments proliferated and subsequently produced short rods, and

(*b*) A mycelial type which showed true branching and budding.

The present investigation was made:

(1) To find whether similar forms of development are found on standard media, such as the Loewenstein medium.

(2) To see whether the chromatinic structures which are present in ordinary bacteria could also be detected in avian tubercle bacilli, of both 'straight' and 'mycelial' strains.

It has recently been shown that chromatinic structures can be demonstrated in bacteria either by the Feulgen reaction or by staining the bacteria with Giemsa's solution after brief hydrolysis in dilute hydrochloric acid. These methods have revealed discrete chromatinic structures in many species of bacteria (Piekarski, 1937; Neumann, 1941; Robinow, 1944, 1945) and actinomycetes (v. Plotho, 1940; Klieneberger-Nobel, 1947). The chromatinic bodies occur in regular numbers and go through a cycle of growth and multiplication which is correlated with cell division. Their behaviour and staining reactions strongly suggest that they are nuclear structures.

In preliminary experiments we found that after 5–7 min. in warm dilute hydrochloric acid and staining with Giemsa's solution, human and avian tubercle bacilli too showed granular chromatinic material differentiated from a clear cytoplasm. These structures, also briefly referred to in a paper by Porter & Yegian (1945), resemble the chromatinic structures of ordinary bacteria in many respects, but differ from them in characteristic changes which they undergo during the early stages of reproduction.

MATERIAL AND METHODS

The history of the five avian strains used in these experiments has been described in the paper by Fell & Brieger (1947) now in process of publication. Bacterial suspensions were made in saline, and the bacilli were spread on Loewenstein's medium prepared in Petri dishes. Small slabs of medium were cut out at intervals of 24 hr. and impression preparations were made on cover-slips, allowed to dry for a minute and fixed for 1 min. in Schaudinn's sublimate alcohol; they were then rinsed and stored in 70 % alcohol. This method was adopted after it was found that results obtained with osmic vapour fixation were not superior, which was also the experience of Stapp (1942) in cytological work on *Phytomonas tumefaciens*.

To stain the chromatinic structures the preparations were treated for 7–10 min. with N/1-HCl at 57–60° C. and stained with Gurr's Giemsa solution R 66 (2 drops per ml.) for several hours. Differentiation and dehydration were carried out in the following mixtures of acetone (A) and xylol (X): (1) pure acetone; (2) A 14, X 6; (3) A 6, X 14. After two further changes of xylol the stained films were mounted in DPX. Sister preparations were stained by Ziehl-Nielsen's and a few by Feulgen's method.

Preparations were made daily during the first 4–6 days, by which time the mass of growing bacilli had become visible to the naked eye.

The five strains of avian tubercle bacilli examined did not all show the same growth habit during the early development on Loewenstein's medium. The O and W strains, both of moderate virulence, did not produce typical mycelia. The non-virulent L and AF 3 strains, on the other hand, regularly produced many long and branching acid-fast mycelia on the second and third days of incubation, mixed with simple filaments. These mycelia sometimes persisted until the fourth day, when they rapidly disintegrated. Short rods soon overgrew all other forms. Finally, the very virulent B strain recently recovered from a tuberculous bird, showed both

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types of development. An account of the different forms of development in this strain will be given in a separate paper.

RESULTS

Chromatinic matter and cytoplasm could not be differentiated with certainty in the rods and cocci of the inoculum, which were less than 1 μ in length. In all the five strains minute forms elongated during the first 24–48 hr. into bacilli 2–8 μ long. These young organisms, poorly stainable despite the hydrochloric acid treatment, contained a variable number of irregularly spaced more or less spherical chromatinic bodies which in the same bacillus varied in staining, some being deep purple, others red (Pl. 8, fig. 2). At this stage the bacilli resembled some of the human tubercle bacilli figured by Epstein *et al.* (1936).

From the second or third day onwards, depending on the age of the culture used as inoculum, an increase in the amount of chromatinic matter was noted, and the cytoplasm of the elongated bacilli was now more readily stainable with Giemsa's solution and less easily decolorized than before. During the next few days the bacilli of the non-branching strains began to break up into small rods with one or two central or a single terminal chromatinic granule. These forms were predominant in the layer of confluent growth that had become visible to the naked eye on the fourth to fifth day.

While the initial elongation to multinucleate filamentous forms 2–8 μ long was seen in both the branching and non-branching strains, the fragmentation of the filamentous forms into small bi- and uninucleate cells was delayed in the former. Instead, elongation continued, and on the third and fourth days lateral buds developed, primary and sometimes secondary branches were put out until there were large numbers of mycelia, which proved to be acid-fast, measuring some 15 μ from tip to tip and closely resembling young mycelia of actinomycetes (Pl. 10, figs. 10, 11).

The chromatinic structures in the branched forms went through the same changes that were noted in the 'straight' strains. During the initial elongation of the young bacilli the chromatinic granules were arranged irregularly and showed much variation in the amount of stain absorbed. The cytoplasm at this time was only very faintly stained. Later, there was a great increase in the amount of chromatinic material, and the granules tended to form a single more or less homogeneous column of deeply stained chromatinic matter, extending through most of the length of the branching filaments but usually stopping short some 3–5 μ from the extreme tips. At this stage whole side branches might lose all their chromatinic matter, while the main 'stem' of the mycelium was still filled with it (Pl. 9, fig. 5).

The stage of maximum chromatin content was soon followed by a rapid disintegration of the branched forms, concomitant with the appearance of increasing numbers of small rods which, as in the cultures of the 'straight' strains, rapidly overgrew all other forms.

As in ordinary bacteria there is close parallelism between the results of the Feulgen test and those obtained by the HCl-Giemsa method. The granules giving a positive Feulgen reaction are, however, but faintly stained and much more difficult to see than Giemsa-stained chromatin particles. This is a difficulty already experienced by Epstein *et al.* (1936) in their cytological study of the human tubercle bacillus. According to our present views the chromatinic bodies which have been demonstrated in tubercle bacilli can be interpreted as nuclear structures.

The bacilli of the young cultures which we have examined were acid-fast in Ziehl-Nielsen preparations. We have gained the impression that the pattern of acid-fast granules in the bacilli often followed closely the distribution of the chromatinic material, but we have not attempted to find out the extent and the nature of this correlation.

DISCUSSION

Although the morphological changes in young cultures of growing tubercle bacilli resemble in many ways those of ordinary bacteria, the concomitant changes in the arrangements of the chromatinic structures are unusual.

In ordinary bacteria from young cultures the chromatinic bodies are usually symmetrically arranged, there are rarely more than two or four in a single bacillus of average length, and all the chromatinic structures in a given bacillus are usually equally deeply stained. The chromatinic structures are most sharply differentiated from the cytoplasm and stain most intensely in organisms fixed during the first few hours after subculture, the period during which the bacilli attain their maximum size. Furthermore, the regular spacing of the chromatinic bodies appears to be due to cell boundaries that lie between them, and many bacteria from young cultures consist of two or three separate protoplasts (Robinow, 1944, 1945).

Staining methods for the demonstration of cell boundaries have not given clear-cut results when applied to tubercle bacilli, and there is some evidence to suggest that in its early filamentous stage the avian tubercle bacillus is a single, multinucleate unit in which the nuclear structures divide independently.

In the avian tubercle bacillus, on the other hand, the initial period of elongation is not accompanied by particularly intense staining of the chromatinic

structures, nor are the chromatinic bodies symmetrically arranged. Unusual also is the great variation in depth of staining among the chromatinic bodies in the same bacillus, variations which may represent different stages in a process of multiplication.

There is, lastly, following on the initial elongation of the bacilli, the great increase in the amount of chromatinic matter and its fusion into a single deeply stained column (Pl. 8, fig. 4). This phenomenon, which was regularly encountered in the branching, less consistently in the 'straight' strains, does not fit into the picture of bacterial reproduction. In cells from young cultures of ordinary bacteria this condition is also met with, but it is rare (Neumann, 1941; Robinow, 1944).

In view of our observations, the inclusion of the mycobacteria into the group of actinomycetales (Topley & Wilson, 1946) seems most appropriate. The mycelial growth habit is known to exist in certain saprophytic mycobacteria (Wyckoff & Smithburn 1933; Jensen, 1934; v. Plotho, 1940). The warm-stage observations of Brieger & Fell (1945) on young hanging-drop cultures of avian bacilli have shown that in avian tubercle bacillus, too, the mycelial growth habit is not a sign of ageing and degeneration—which it has long been regarded in pathogenic species of the mycobacteria—but that it is, on the contrary, the normal mode of development in certain strains at least for a large proportion of the organism. Our present observations made on avian tubercle bacilli grown on Loewenstein's medium fully confirmed this result. The general appearance of the mycelial strains is often strikingly similar to that of certain actinomycetes (cp. Fig. 4, p. 315, Kolle & von Wasserman, 1913, 2nd ed. vol. 5).

The question whether these mycelial tendencies in the early stages of development are related to the mycelial structures described by Vaudremer (1931) and Besançon & Philibert (1924) must remain open at present. The mycelial structures described by the French authors were non-acid-fast (Filamentes de substance non-acido-resistant, 'cyanophile'). The mycelia in our observations were strongly acid-fast. The part these mycelia play in the reproductive cycle of the tubercle bacillus is difficult to assess.

It was difficult to decide whether all the small rods developed from the branching filaments. Appearances suggested that the branching bacilli partially disintegrated into some rods and cocci, possibly by segmentation, as previously described in micromotion pictures of a non-acid-fast mycobacterium by Wyckoff & Smithburn (1933), but most of the small forms were apparently derived from two other sources: (*a*) from minute elements forming already in the early stage of development, and (*b*) from moderately elongated rods not exceeding 2–5 μ which did not develop into mycelia (Pl. 8, fig. 3).

The small size of the tubercle bacillus, the great variety of cocci and minute rods that are present in every preparation, regardless of the age of the culture, the presence of granular forms, the co-existence of acid-fast and non-acid-fast forms, and the peculiar habit of growing in clumps of densely entangled bacilli (Brieger, 1944; Alexander-Jackson, 1945), render the cytological observations difficult to interpret.

It has, however, been possible to dissociate in two of our strains (O and B) a mycelial variant which in all its subcultures shows the mycelial tendencies as a regular part of its life history. A study of the life history of these mycelial variants in hanging-drop and cover-slip cultures in a greater variety of media is now being carried out, which will throw more light on the question of the mycelial stage in the development of avian tubercle bacilli.

SUMMARY

In a cytological investigation of three branching and two non-branching strains grown on Loewenstein medium, it was found that avian tubercle bacilli contain chromatinic material which gives a positive Feulgen reaction and is readily stainable with Giemsa's solution after treatment of the fixed bacteria with hydrochloric acid.

Growing filamentous forms of both 'bacterial' and 'mycelial' strains from 1 to 2 day old cultures contain variable numbers of irregularly spaced, more or less spherical chromatinic bodies which vary in staining in the same bacillus, some being red, others purple. During the third or fourth day the chromatinic material in the bacteria increases very much until most of it is fused into an almost homogeneous deeply stained column. In the *non-branching* strains the filamentous forms with high chromatin content soon break up into small mono- or binucleate elements, and the same holds true for the 'straight' filamentous forms which are also present in cultures of branching strains. The 'mycelial' forms, on the other hand, disintegrate at this time (fourth or fifth day of cultivation), and it is uncertain whether they contribute (by partial fragmentation) to the masses of small mono- or binucleate forms which are the predominant element in old cultures of all the strains investigated.

The chromatinic structures of avian tubercle bacilli have the same staining properties as those of ordinary non-acid-fast bacteria but differ from them in their behaviour during the early development of the bacilli.

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EXPLANATION OF PLATES 8–10

All photographs are from air-dried impression preparations that were fixed in Schaudinn's sublimate alcohol, treated with N/1-HCl at 60° C. and stained for several hours with Giemsa's solution. The scale of magnification for all figures is the same (× 2330).

The photographs illustrate characteristic stages in the early development of the 'Lister' strain. This strain grows as a mixture of 'mycelial' and of short non-branching forms; to some extent the pictures therefore serve also to illustrate the manner of growth of strains entirely composed of non-branching forms.

PLATE 8

Fig. 1. Small cocci and rods from a 3 weeks old slant culture on Loewenstein's medium. The succeeding figures show the development of these forms during the first few days on fresh Loewenstein's medium.

Fig. 2. Subculture of (1) after 24 hr. growth. The rods have elongated and contain varying numbers of discrete chromatinic bodies in a faintly stained cytoplasm.

Fig. 3. Three days' growth. The rods have developed into long intertwined filaments, many with short side branches. There are also many short rods. The chromatinic material is much increased.

Fig. 4. Four days' growth. The chromatinic material is now concentrated in deeply stained masses leaving one or both ends of the filaments free.

PLATE 9

Fig. 5. Six days' growth. Long forms have now almost disappeared and masses of small rods are again the prevalent element. To the left of the centre a long filament with side branches in an early stage of disintegration (?).

Fig. 6. Coccoid forms from 5 weeks old slant culture on Loewenstein's medium.

Figs. 7, 8. After 24 hr. the cocci of (1) have grown into slender beaded bacilli. In (7) the cytoplasm is too faintly stained to be visible in the photograph.

Fig. 9. 24 hr. later. Chromatinic material much increased and fused into one or two deeply stained bodies.

PLATE 10

Figs. 10, 11. Branched filaments and 'mycelia' at the height of their development from a 4 days old culture on Loewenstein's medium.

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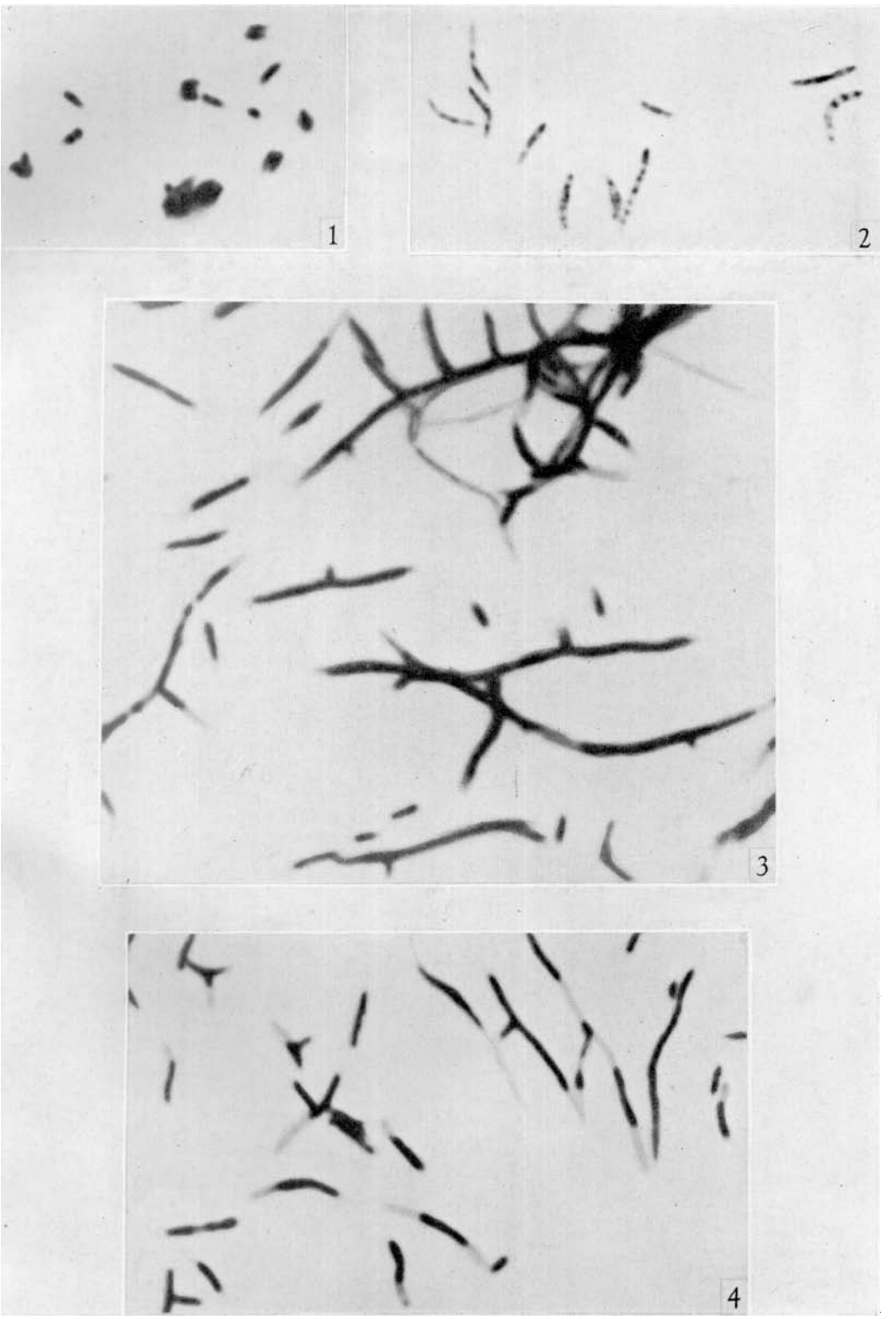

Figs. 1—4

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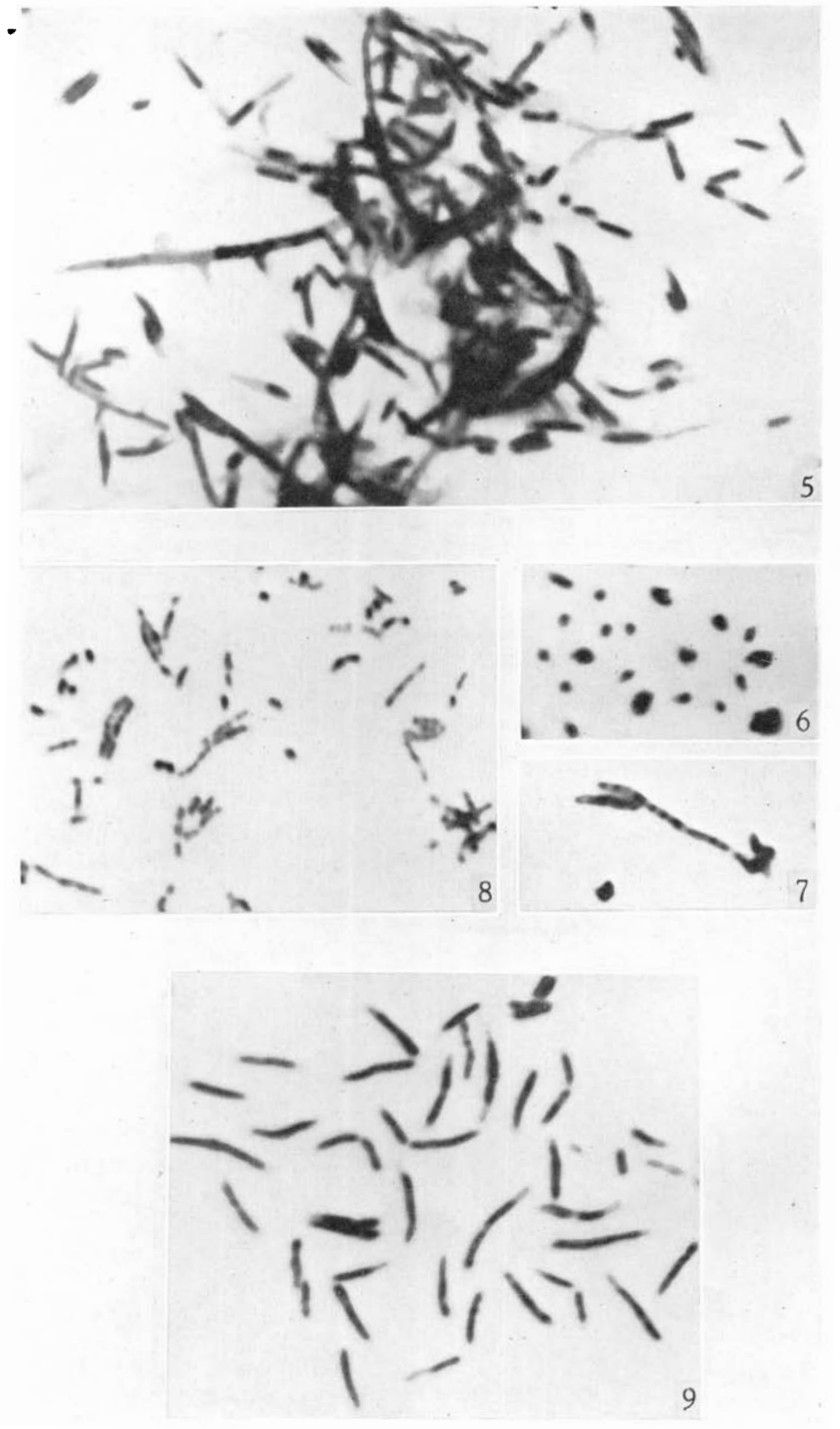

Figs. 5—9

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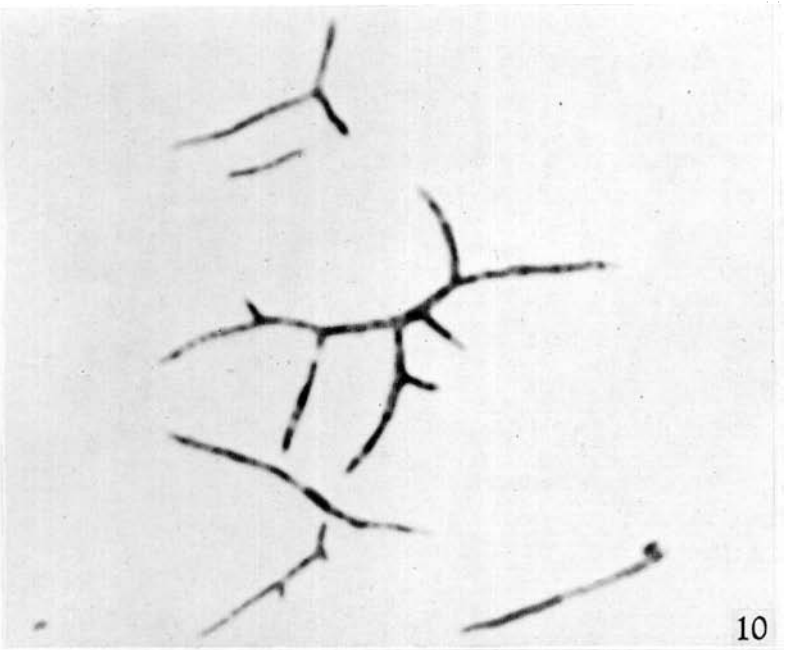

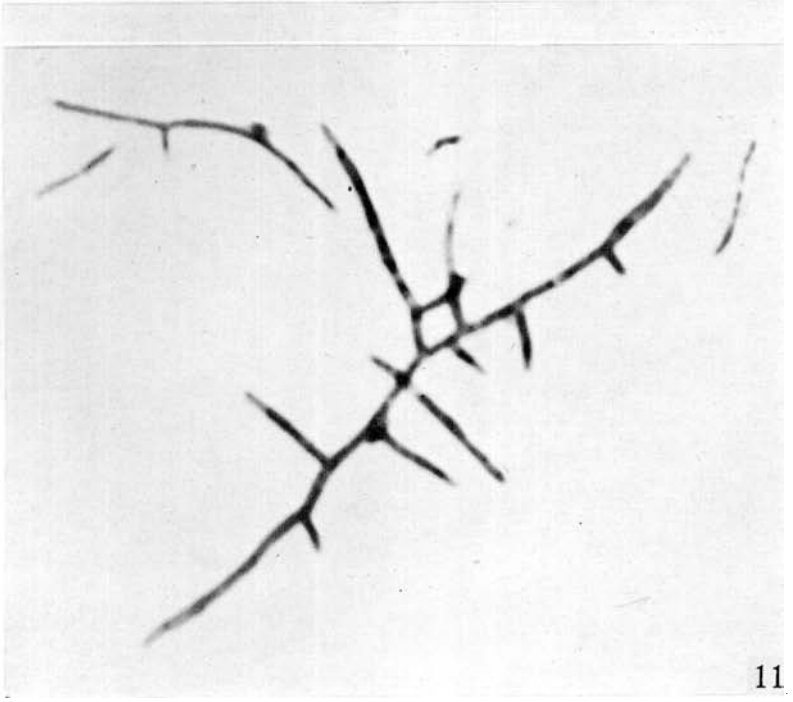

Figs. 10, 11

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