Morphological Observations on Various Spermatozoa with a New Staining Method

Shunsaku FUJII

Department of Animal Husbandry, Faculty of Fisheries and Animal Husbandry, Hiroshima University, Fukuyama

(Pls. 1-4)

(I) INTRODUCTION

There are many methods for the staining of spermatozoa which are highly stainable. Generally carbol fuchsin, gentian violet and rose bengal are used. Differential staining with a mixture of several acid dyes, such as azan staining, is available for detailed observation of each constituent of the sperm. The author (1956, b) reported that a mixture of three acid dyes, i. e. anilin blue, ponceau PR and picric acid, was useful for this purpose. On the other hand, the silver impregnation method gives excellent results in staining spermatozoa, though it is more or less complicated and the results are not always invariable. Such silver method has been reported by several authors. HARA (1943) observed the fine structure of the sperm of various species with his own silver method. The so-called FONTA method, which is applied to protozoa, is one of the silver methods for the sperm.

The present paper describes a silver method to stain the sperm. This method is especially suitable for observation of the microstructure of the middle-piece of the spermatozoon, as this portion alone is stained selectively dark violet and the remaining portion brown by this method.

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(II) STAINING METHOD

1. The semen is smeared on a slide and, when almost dried, it is fixed with 10 per cent formalin solution for a few hours.

2. The slide is washed with running tap water for about 10 minutes.

3. A dilute gold chloride (add a few drops of 1 per cent solution of gold chloride in 5 cc of distilled water) is mounted fully on the slide at about 37°C for about a few minutes.

4. Without washing or rinsing short with distilled water, the slide is covered with a silver-nitrate gelatin solution (a mixture of equal parts of 1 per cent solution of gelatin and 2 per cent silver nitrate solution) for about 3～4 minutes at 37°C. In this case,

whether the slide is rinsed or not with the distilled water has an important effect upon the staining results. On this point it will be discussed later in detail.

5. The silver nitrate solution is removed and reduction is made with a hydroquinone gelatin solution (a mixture of equal volumes of 1 per cent gelatin solution and 0.5 per cent hydroquinone solution) at 37°C for about 1～2 minutes until the middle-pieces of the spermatozoa become dark violet.

6. The slide is washed thoroughly with tap water, dehydrated with alcohol, cleared and mounted with balsam.

In preparations well stained, the middle-piece is homogeneously shown in a dark violet tint and the head, as well as the tail, in a brown tint. In preparations in which the gold chloride was rinsed with the distilled water, however, the middle-piece is not stained dark violet homogeneously, but only the spiral filament revolving around the middle-piece is stained selectively and distinctly. When specimens are dried too much before fixation, all the portions of the spermatozoon, including the middle-piece, are stained dark violet. On the contrary, when they are washed too long after fixation, the middle-piece is not stained selectively but the whole spermatozoon is stained brown.

All the solutions should be prepared from chemicals of high purity immediately before use and stored in dark. They can be kept only for a few days.

(III) STAINING RESULTS

The findings on spermatozoa of several species of animals stained by the author's method are as follows:

1. Observation on normal spermatozoa

a. Spermatozoa of the mouse

Fig. 1 shows the spermatozoa of the mouse collected from the epididymis and stained by this silver method. The middle-piece or body lying between the neck and the tail is stained intensively dark violet. Frequently, the acrosomes are also stained dark violet.

Fig. 5 shows a specimen in which the gold chloride solution was washed off in a moment. The middle-piece has a distinct helical structure known as the spiral filament or spiral fiber, which is developed around the central core or axial filament. These spiral filaments have already been observed in detail by several investigators, such as JENSEN (1887) with the light microscope, RANDALL (1950) and YASUZUMI (1950) with the electron microscope and NAKANISHI (1955) with the phase-contrast microscope. In this figure, the spiral filament appears to turn about 42 times around the axial filament from left to right or the reverse.

Fig. 9 shows the regularly revolving spiral filament of mouse spermatozoa stained supravitally with Victoria blue 4R by the method of FUJII (unpublished). Victoria blue 4R is a dye recommended by SEKI (1956) for the staining of lipids. Such helical structure has been observed only in the immature sperm collected from the testis but no longer in the mature one from the tail of the epididymis (Fig. 10). As compared with

Fig. 9, the helical structure shown in Fig. 5 must no doubt be the true spiral filament.

By using this method, a delicate periodical structure was also demonstrated in a cytoplasmic sheath enclosing the axial filament along the whole length of the tail-piece.

b. Spermatozoa of the rat

Rat spermatozoa stained by the author's method are shown in Fig. 6 and the fine structure of the middle-piece in Fig. 2. The photomicrograph in Fig. 11 shows rat spermatozoa stained supravitally with Victoria blue 4R, as mentioned above. The middle-piece is long in proportion to the exceedingly long tail. Therefore, the spiral filaments of rat spermatozoa revolve about 120 times and coil in the same way as those of mouse spermatozoa.

c. Spermatozoa of the rabbit

Rabbit spermatozoa collected from the tail of the epididymis are shown in Fig. 3 and the photograph of the fine structure of the middle-piece is given in Fig. 7. The middle-piece of rabbit spermatozoa is stained selectively dark violet like that of mouse spermatozoa, with a revolving spiral filament. The spiral filament is not so distinct as in mouse spermatozoa and revolves about 20 times. Occasionally, one or two of the granular bodies described by YAMANE (1925) are demonstrated in the center of the head as dark stained structures (Fig. 8).

d. Spermatozoa of other mammals

Spermatozoa of the bull, boar and guinea-pig are shown in Figs. 12, 14 and 16, respectively. The fine structure of the middle-piece of these spermatozoa is illustrated in Figs. 13, 15 and 17, respectively. In these figures, the spiral filament is also visible more or less distinctly in the middle-piece. The spiral filament seems to turn about 30 times in bull spermatozoa and about 20 times in boar and guinea-pig spermatozoa.

e. Spermatozoa of birds

The spermatozoa of birds are remarkably long and slender. It is impossible to differentiate such three typical portions as those of mammalian spermatozoa with an ordinary stain. The spermatozoa of the cock and duck are shown in Figs. 4 and 18, respectively. Their portion corresponding to the middle-piece of mammalian spermatozoon is stained selectively and differentiated clearly from the other portions of the spermatozoon. A detailed observation on the middle-piece disclosed a spiral filament revolving about 5～6 times which no doubt developed from mitochondria as in the mammalian spermatozoon. This filament is not clearly described in BONADONNA's paper (1954) of the electron microscopy of *Gallus gallus*. The acrosome situated at the apical end of the head is stained dark violet.

2. Observation on abnormalities of the middle-piece of spermatozoa

There are many reports on the abnormalities of spermatozoa, most of which are lim-

ited to the head and the tail portions. The abnormalities of the middle-piece are not reported so much because it is difficult to visualize its fine structure by the ordinary staining. However, by means of the author's method, observations can be made not only on the abnormalities at large but also on the special deformities of the middle-peice. As for the deformities of the middle-piece of human spermatozoa, they are divided by WALTER & WILLIAMS (1950) into three principal groups: an enlargement of the middle-piece, a simple deformity of the middle-piece, including a variation in length, and thickening and disorder of the mitochondrial sheath. The abnormalities of mouse spermatozoa observed by the author's silver method are as follows:

The present paper deals only with the abnormalities of the middle-piece. The most frequently observed are spermatozoa with somewhat distinguished spiral structure in the middle-piece. They scatter among those with no distinct helical structure and a homogeneous dark appearance, as shown in Fig. 19. They may not be considered abnormal forms and be classified as immature spermatozoa. This assumption is obtained from the staining results of Victoria blue 4R; that is, the fact that immature spermatozoa collected from the testis show a clear helical structure of the middle-piece and that mature ones from the tail of the epididymis are devoid of such structure entirely, will give a basis for explanation that the helical structure becomes invisible with the maturity of spermatozoa. The second largest group of abnormalities consists of spermatozoa with an abnormal arrangement of spiral filament, such as a little irregular arrangement of the filament (Fig. 20), partial loss of the filament in various places along the middle-piece (Fig. 21), and entire loss of the filament (Fig. 22). The third group is composed of spermatozoa with deformity of spiral filament: partial (Fig. 23) and entire (Fig. 24) deformation of the filament, a massive accumulation of the filament without uniform arrangement around the axial filament (Fig. 25), and a bare axial filament due to lack of mitochondrial sheath (Fig. 26). Moreover, there are spermatozoa accompanied by the dissection of the spiral filament, which consequently forms a shrunk mass of filament (Fig. 27).

All these abnormalities of the spiral filament show the disorder at the end of spermatogenesis. As a whole, the abnormalities of the middle-piece are independent or accompanied by deformities of the other portions. The latter cases are found more frequently.

(IV) DISCUSSION

The author (1956, a) reported a new method by which mitochondria are stained selectively and distinctly with the silver method. The method used in the present experiment is a slight modification of the original one in respect to the kind of fixative and the use of gold chloride. When the original method was described, the reasons why mitochondria were demonstrated by means of silver staining were determined as follows: First, when the tissue is immersed in the gold chloride solution, it is impregnated by the solution. When it is dipped in silver nitrate solution long enough, the gold chloride is washed off easily from rough structure, but remains in more densely constructed mitochondria. Accordingly, the combination of cation of silver nitrate and anion of gold

chloride is deposited as insoluble salt. By addition of reducing agents both gold cation and chlorine anion are reduced. An interesting point of this method consists in a dark violet staining of the middle-pieces of spermatozoa. It has been widely accepted that the middle-piece has an axial filament in the center and outside of it and that the spiral filament develops from the mitochondria of the spermatids. Therefore, it is probably due to the same mechanism as considered above that the spiral filament is selectively stained dark violet.

With the maturity of spermatozoa, such a dense substance as mitochondria rich in lipids is accumulated in cytoplasmic crevices of the spiral filament, so that the whole length of the middle-piece stains homogeneous.

(V) SUMMARY

A new silver method was described for the staining of spermatozoa. The method was performed as follows: First, smears of spermatozoa were fixed with 10 per cent formalin and dealt with a dilute gold chloride solution. Then they were impregnated with silver nitrate solution and finally reduced with hydroquinone. The principle of this method is that the middle-piece of the spermatozoa is selectively and distinctly stained in a dark violet tint. Besides, with a slight modification of the original method, the spiral filament in the middle-piece, which was derived from mitochondria and usually is not stained by the ordinary method, may be demonstrated easily. Therefore, it may be said that this method is especially available for the study of fine structure of the middle-piece as well as its general morphology. By using this method, the minute structures of the middle-pieces of spermatozoa of some species were observed and the abnormalities of that portion were demonstrated in photograph.

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EXPLANATION OF PLATES

Spermatozoa stained by the author's silver method. In method A gold chloride was not washed off, while in method B it was washed off before staining.

Plate 1.

Fig. 1. Spermatozoa of the mouse by method A. The middle-piece is stained dark violet homogeneously and selectively and the remaining portion brown.
Fig. 2. Sprmatozoa of the rat by method B. The spiral filament of the middle-piece is distinct.
Fig. 3. Spermatozoa of the rabbit by method A.
Fig. 4. Spermatozoa of the cock by method A. The middle-piece is stained dark violet selectively.

Plate 2.

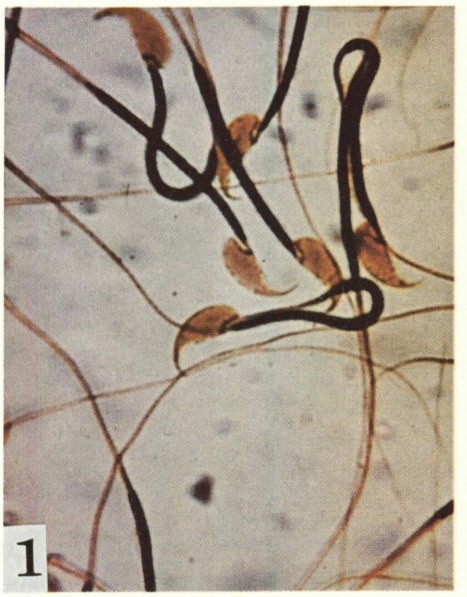
Fig. 5. Spermatozoa of the mouse by method B.
Fig. 6. Spermatozoa of the rat by method A.
Fig. 7. Spermatozoa of the rabbit by method B.
Fig. 8. Rabbit spermatozoa showing the bodies stained dark.
Fig. 9. Mouse spermatozoa collected from the testis stained supravitally with Victoria blue 4R. The spiral filament is shown regularly.
Fig. 10. Mouse spermatozoa collected from the tail of the epididymis stained supravitally with Victoria blue 4R. The spiral filament is not shown.
Fig. 11. Rat spermatozoa collected from the testis stained supravitally with Victoria blue 4R.

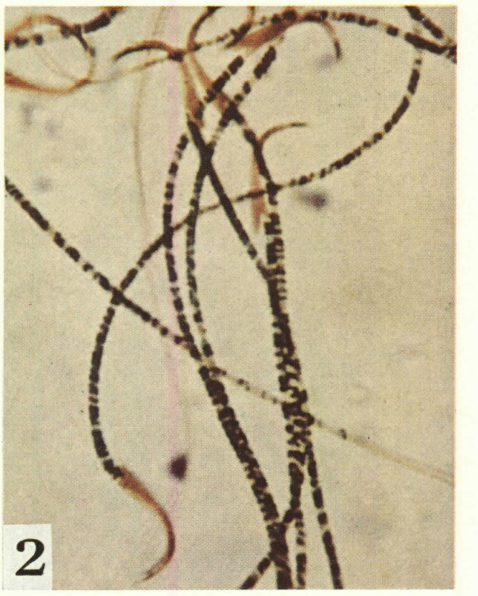
Plate 3.

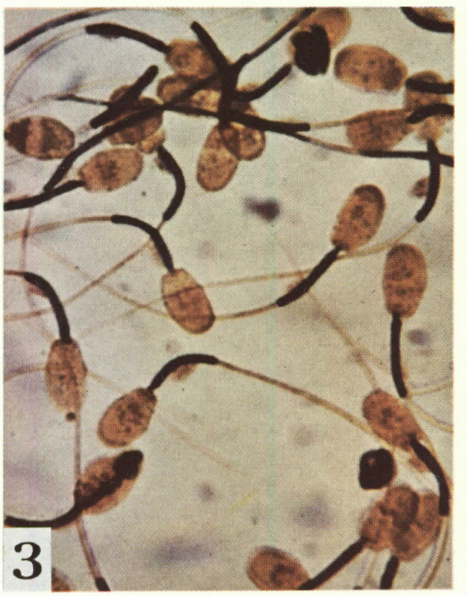
Fig. 12. Spermatozoa of the bull by method A.
Fig. 13. Spermatozoa of the bull by method B.
Fig. 14. Spermatozoa of the boar by method A.
Fig. 15. Spermatozoa of the boar by method B.
Fig. 16. Spermatozoa of the guinea-pig by method A.
Fig. 17. Spermatozoa of the guinea-pig by method B.

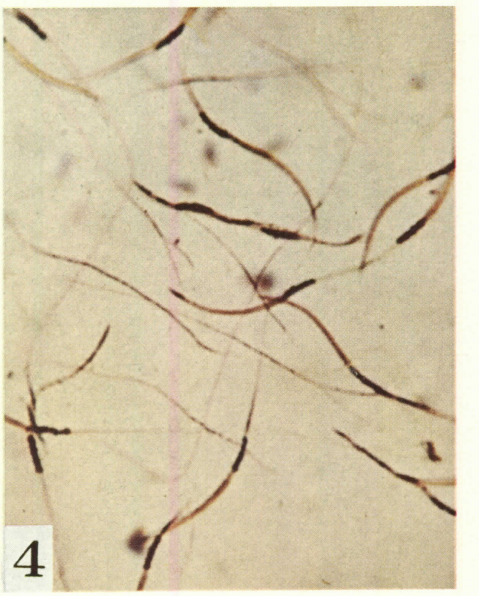
Plate 4.

Fig. 18. Spermatozoa of the duck by method A. The middle-piece is stained dark selectively and coils in spiral form.
Fig. 19. The spermatozoon with spiral structure in the middle-piece is an immature one and those stained dark homogeneously are mature ones.
Fig. 20. A spermatozoon showing a slightly abnormal arrangement of spiral filament.
Fig. 21. A spermatozoon showing partial loss of spiral filament (bottom).
Fig. 22. A spermatozoon showing entire loss of spiral filament (right).
Fig. 23. A spermatozoon showing partial deformation of spiral filament (top).
Fig. 24. A spermatozoon showing deformation of spiral filament (left).
Fig. 25. A spermatozoon with a massive accumulation of spiral filament (right), a normal spermatozoon (bottom) and a spermatozoon with a deformation of spiral filament (top).
Fig. 26. A spermatozoon with a bare axial filament, showing loss of spiral filament.
Fig. 27. A spermatozoon with a dissection of spiral filament (left) and one with a bare axial filament (middle).









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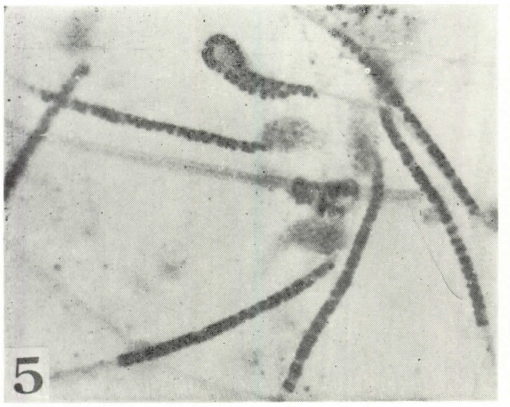
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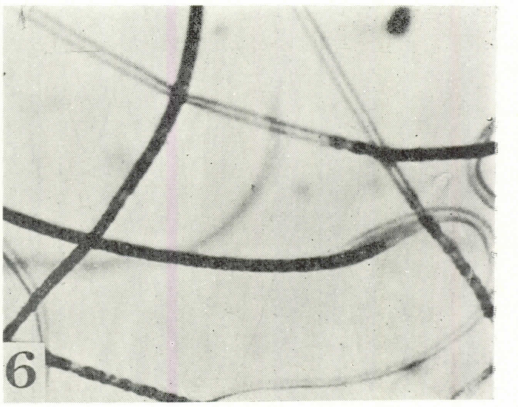
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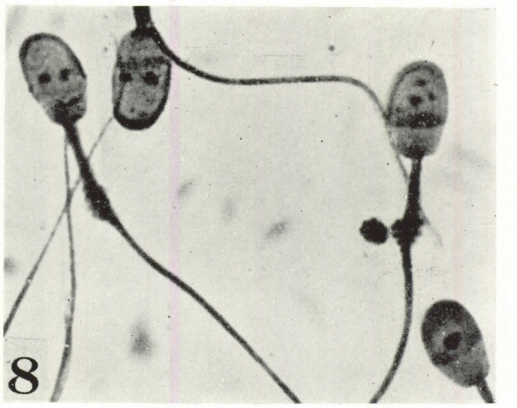
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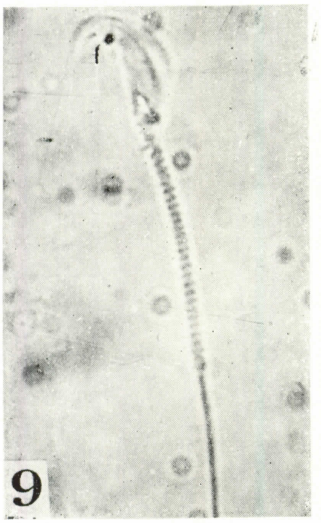
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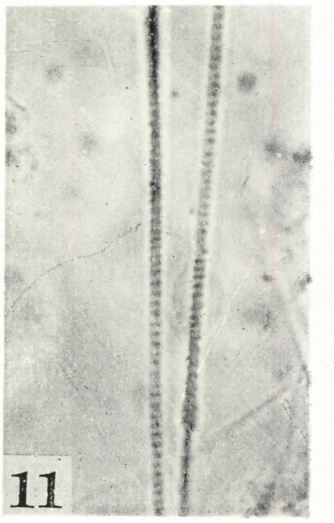
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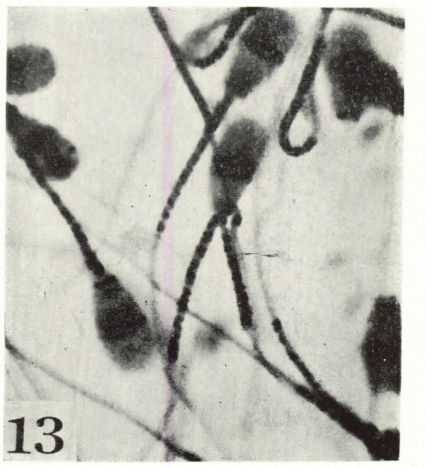
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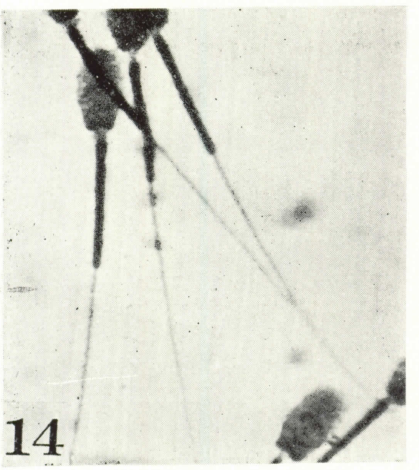
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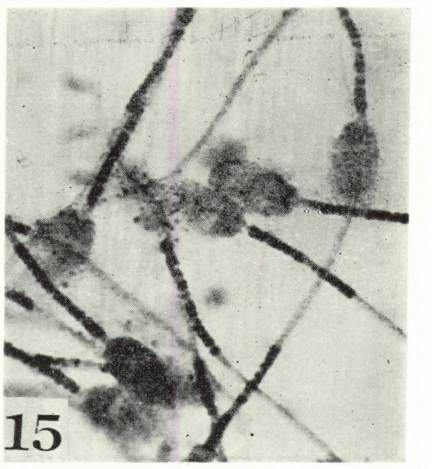
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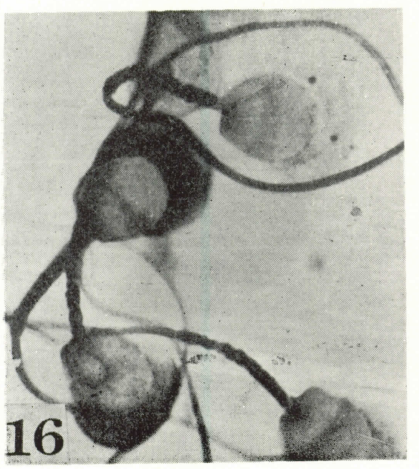
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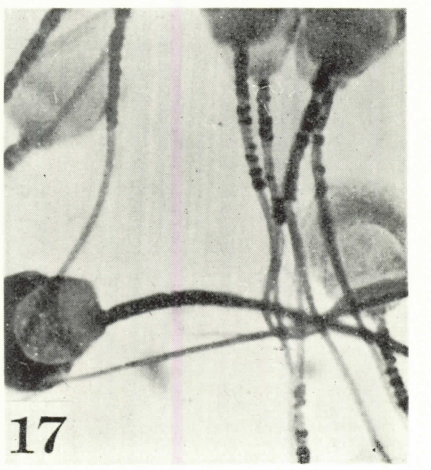
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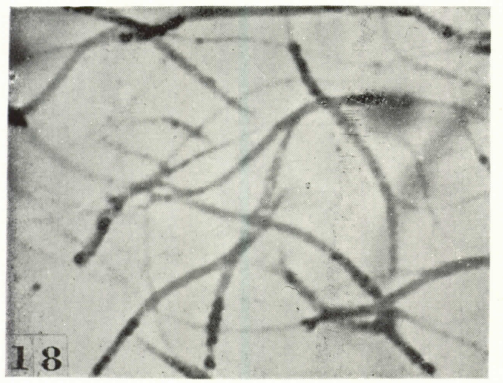

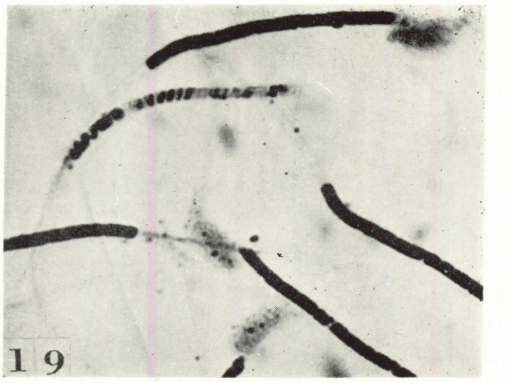

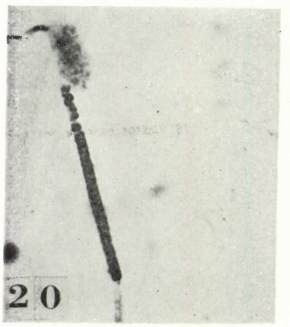

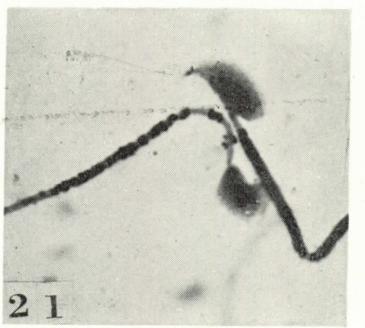

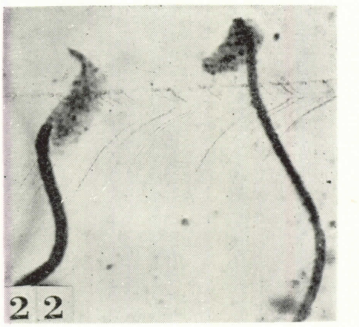

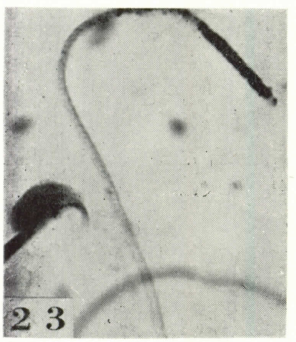

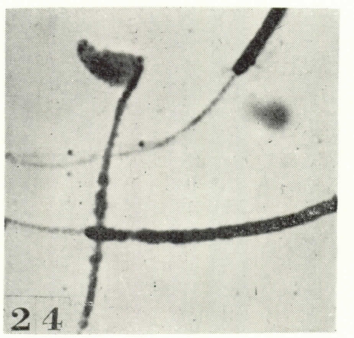

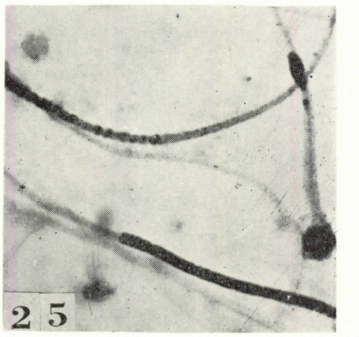

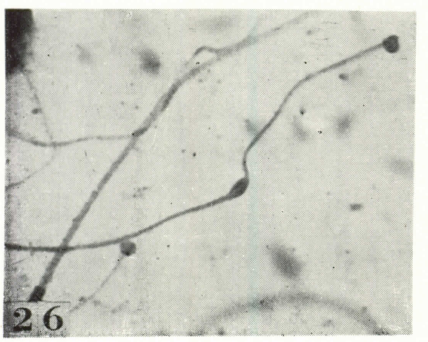

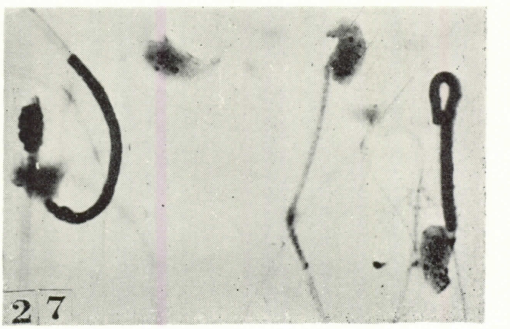

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