Fundamental Considerations and Experimental Conditions on the Study of the Spiral Direction of Helical Bacteria

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INTRODUCTION

Although the cell bodies of some bacteria, for example, spirilla, spirochetes, saprospira and spiroplasma, show a helical turn, their spiral directions have not been confirmed yet because of their small sizes and the low resolving power of light microscopes (LM). There are some reports[2,4,5,8,9,10] on the spiral direction of spirochetes by LM, however, they are unreliable, because they are lacking in both the description of the experimental conditions and the basic considerations in the study of spiral direction. For example, they fail to discuss the definition of spiral direction, the mechanism of image formation in the electron microscope (EM), the specimen preparation and its setting, or the conditions of microscopy and photography.

Recently, optical instruments including the transmission electron microscope (TEM) and the scanning electron microscope (SEM) were improved and the spiral directions of some spirochetes were described from observations with these instruments[1,3,12,13]. However, these studies were unable to accurately determine the spiral directions of spirochetes, for the same reasons as the previous studies with the LM.

In order to study the spiral directions of bacterial cell bodies, all of the problems mentioned above must be resolved theoretically and experimentally. This paper describes the solution of those problems by making use of some models and examples from experiments.

THEORETICAL PROBLEMS

The problems in studying the spiral directions of helical bacteria may be classified into two major subjects. One is the definition of spiral direction and the other is analysis of the images in the instruments.

I. The definition of spiral direction.

The spiral direction of a helix may be either right-handed or left-handed, however, there is generally confusion on the question of which is which. In the past there has been much confusion among scholars on this matter[6,7]; this will be discussed in another paper by the author. For now, a simple, concise definition of spiral direction is all that is needed.

"If the helix is right-handed, it will advance away from the observer when turned clockwise on its axis. The helix is left-handed, if it advances away from the observer when turned counter-clockwise on its axis."

Fig. 1 provides a concrete example of right-handed and left-handed helices, to eliminate any further confusion.

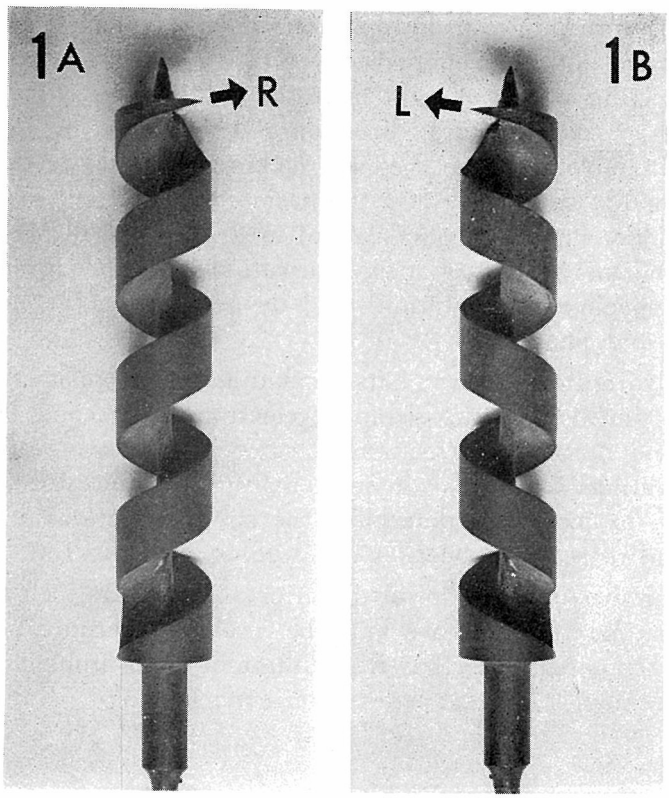

Fig. 1. Photographs of a drill twist for the definition of spiral direction.
A shows an image of the right-handed turn by orthodox printing.
B shows an image of the left-handed turn by reverse printing.

Ⅱ. Type of image formed in the electron microscope.

The type of image formed by the instrument must be well considered, because it is very important in conformational studies to recognize whether the image of a sample is a real image or a mirror image.

Whether the image formed by the SEM is a real or a mirror image is generally of no concern to the researcher, so the type of image in the SEM had to be determined previously by experiment.

For this purpose a nylon string with a right-handed turn was used in the SEM, and a right-handed sample image was obtained as shown in Fig. 2-A. Since our SEM produce images in the same way, it can be concluded from this result that our SEM always show the real image of the sample and not the mirror image.

The type of image formed by TEM is always the real image because of the nature of its convex lens system. This fact was confirmed in

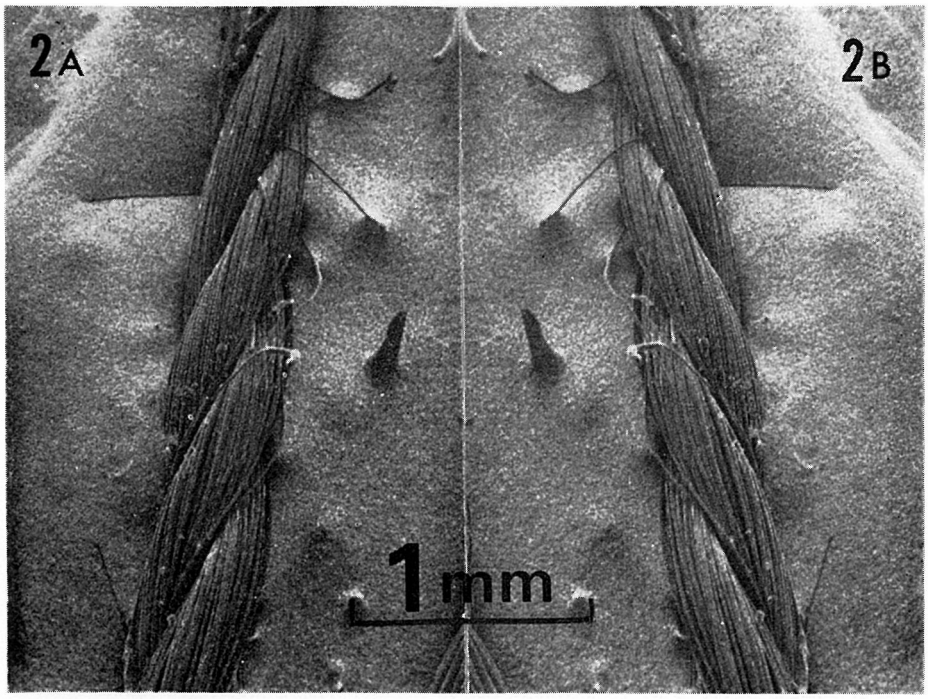

Fig. 2. SEM images of a nylon string with a right-handed turn. They were prepared for the confirmation of image formation in SEM. Magnification: 35×

A : Image by orthodox printing. It shows the right-handed turn plainly.

B : Printed image of the above same film by reverse position and it shows the left-handed turn which is a mirror image of A.

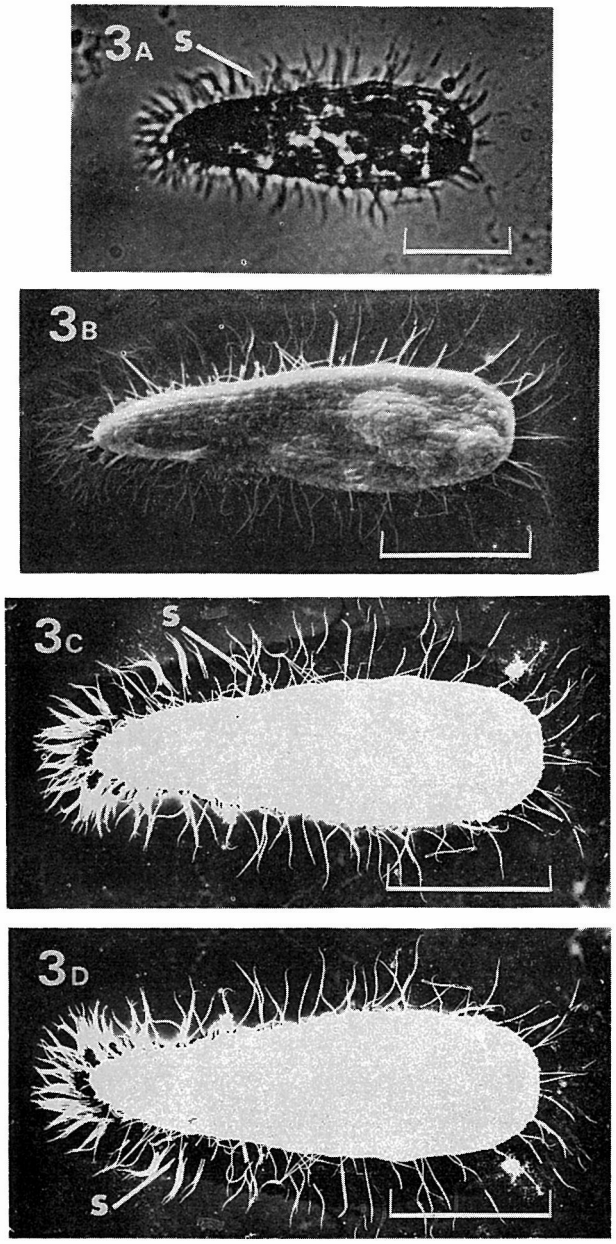

Fig. 3. Images of the same tetrahymena cell on the supporting membrane under LM, SEM and TEM. A is LM image, B is SEM image, C and D are TEM images, respectively. The specimen was observed and photographed after chrome-shadowing, by the different positions of setting, upside position in A, B, and C, and downside position in D. B shows a genuine image of sample as proved in the image of Fig. 2-A. Therefore, A and C can be concluded as a genuine image, because the attitude of each coincides with that of B. On the contrary, D shows a mirror image of the formers. Scale in each photograph shows 10μm. Arrow S shows a shadow of metallic evaporation.

the TEM by using a known material, tetrahymena cell, and the results are shown in Figs. 3-A, -B, -C and -D. It can be confirmed by these results that the TEM shows the real image of the sample when the sample is placed facing the electron beam source, called the "upside". The inverted, mirror image is formed when the sample is placed facing away from the electron beam source, i.e. "downside".

Thus when using the upside setting of sample in SEM and TEM, there is no question about what kind of image is formed. The type of image is always a real image and not a mirror image.

METHODS FOR INVESTIGATION OF SPIRAL DIRECTION

In order to investigate the spiral direction of the microbial cell body, its original three dimensional status must be preserved during the process. Moreover, the real image must be obtained and an intrusion of the mirror image must be prevented during both microscopic and photographic procedures.

Ⅲ. Preservation of helical sample and demonstration of its conformation.

In the case of a bacterial cell body with the native helix, its helical nature must be kept in good condition at the time of specimen preparation. Therefore, a prescribed technique should be followed. For the preservation and demonstration of the helical conformation of a bacterial sample, methods of fixation, drying and metallic coating or shadowing are considered to be very important in the treatment of the sample. Particularly, drying methods must be given special attention. Two ways of drying, air drying (AD) and critical point drying (CPD), are common in specimen preparation today, and these are compared with each other. Fig. 4 shows the drying effects by different methods on leptospira cells in SEM and TEM. CPD shows better results than AD.

In the case of TEM, three methods for contrast making are usually used. They are metallic shadow-casting (shadowing), and positive or negative staining with heavy metals. Among them, shadowing is preferred to be used for the investigation of three dimensional conformations.

In this study, a system of technical procedures, including double fixation with glutaraldehyde and osmium tetraoxide, critical point drying (CPD) and coating or shadowing with some metals, was selected to be used for the preparation of bacterial specimens in SEM and TEM.

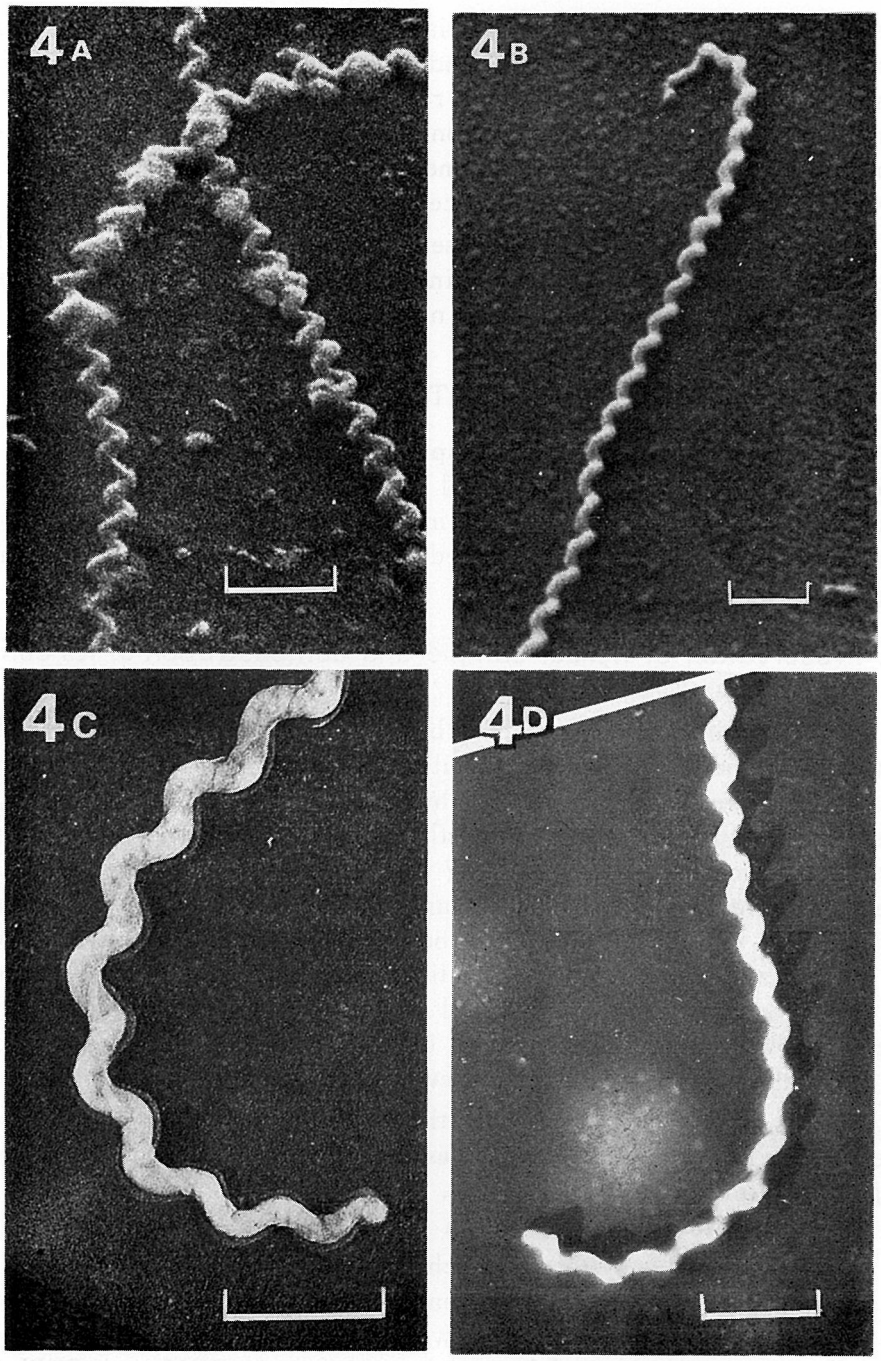

Fig. 4. SEM and TEM images of leptospiral cells (*L. copenhageni*), showing the effects of different drying methods.

A : SEM image, by air drying, showing bad preservation of spirals. Magnification: 18, 000×

B : SEM image, by critical point drying, showing better preservation of spirals. Magnification: 12, 500×

C : TEM image, by air drying, showing bad preservation of spirals. However, a spiral direction can be recognized by observing the interwinning relation between the axial flagellum and the cell body. Magnification: 25, 000×

D : TEM image, by critical point drying, showing better preservation of spirals. Magnification: 15, 000×

Ⅳ. Setting conditions for the specimen in the instruments.

Setting conditions for the specimen, for example, upside or downside in the EM, must be attended to, because the sample images will be opposite with these different settings, as described in the previous section. Therefore, results obtained without taking notice of the sample setting cannot be judged properly.

In the case of SEM, the setting condition of the specimen is always fixed in the upside position, eliminating any confusion about the image. This is illustrated in Fig. 2-A, in which a nylon string with a right-handed turn appears in the orthodox projection as right-handed.

In the case of TEM, there are two different setting conditions for the specimen; the upside position of the sample which faces the electron beam source, and the downside position in the opposite situation. These two settings give opposite results as shown in Figs. 5-A and 5-B, which were the actual images of the sample on the screen in TEM. Since the upside setting in TEM gives the same images as the real image (Fig. 5-A), the upside setting should be used to demonstrate the real image of the sample, and the downside setting should be avoided to prevent confusion.

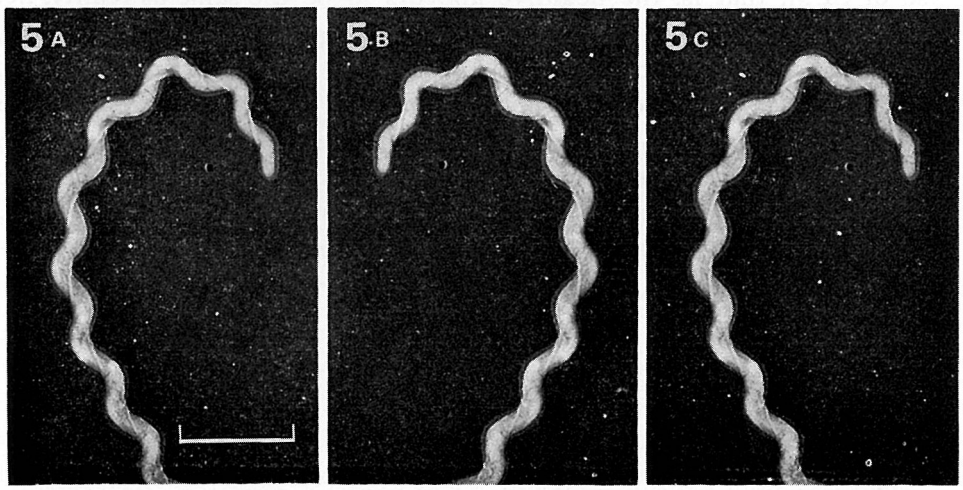

Fig. 5 : TEM images of a leptospiral cell body (*L. copenhageni*) by different positions of specimen setting.

A : Image by the upside setting, light projection is from the posterior side of the positive film at printing.

B : Image by the downside setting, light projection is from the posterior side of the positive film at printing.

C : Image by the downside setting, light projection is from the anterior side of the positive film at printing.

A vs. B and B vs. C are mirror images of each other.

V. Observation and photography of the specimen image in the electron miecroscope.

For the same reasons described previously in the section on setting conditions for the specimen, special attention must be given to the methods for observing and photographing the specimen.

Images in both SEM and TEM can be viewed by two different methods, such as direct or indirect observation. The former is the viewing of the image on the screen of the instrument, and the latter concerns photography. In order to correlate the screen image of the EM with the photographed images on either the negative or prints, the relationship between these methods of image presentation should be understood.

It is possible to coincide the image on the screen of the SEM with the image of the developed negative by looking from the posterior side. Similarly, the SEM screen image will coincide with the print developed by light projection from the posterior side of the negative. Thus, great care should be taken to avoid using the wrong side of the negative when observing or printing the image, in order to prevent the formation of the mirror image.

In the case of TEM, conditions are somewhat different from the case of SEM, that is, the negative appears to be the same as reality, i.e. the shadows are dark and the specimen is bright. If a print is required, the positive film must be prepared by reverse printing on fresh film, then a negative image can be obtained on the photopaper by using the positive film. In this complicated procedure, care must be taken not to change the status of the screen image. So, the final photograph must be printed by light projection from the posterior side of the positive film. Fig. 5 illustrates the results of different settings and different photographic printing methods.

MODEL EXPERIMENT

The first part of this paper presented the theoretical considerations on experimental conditions for the study of spiral direction in helical bacteria. This next part presents an actual experiment performed by the author, to illustrate application of the concepts and techniques described in the first part.

Materials and methods.

A. Strain used: *Leptospira copenhageni* (Shibaura strain)

B. Cultures: In Korthof's medium, 37°C for 7 days.

C. Fixation: Double fixation with glutaraldehyde (2%) and OsO_4 (1%), 3 hours incubation in each.

D. Pretreatment: Washing in phosphate buffered saline (PBS), 3 times.

E. Drying: CPD by Yoshii et al. [14].

F. Metallic coating: Rotary evaporation of Au-Pd for SEM, or Cr-shadowing for TEM.

G. Specimen setting: Upside setting (SEM and TEM) and down-side setting (TEM).

H. Microscopy: By JSM-S1 (SEM-JEOL. Tokyo) at 10 kV and JEM-100B (TEM-JEOL. Tokyo) at 60 kV.

I. Photography (Enlargement and printing): Light projection from the posterior side of both the negative film in SEM, and the positive film in TEM.

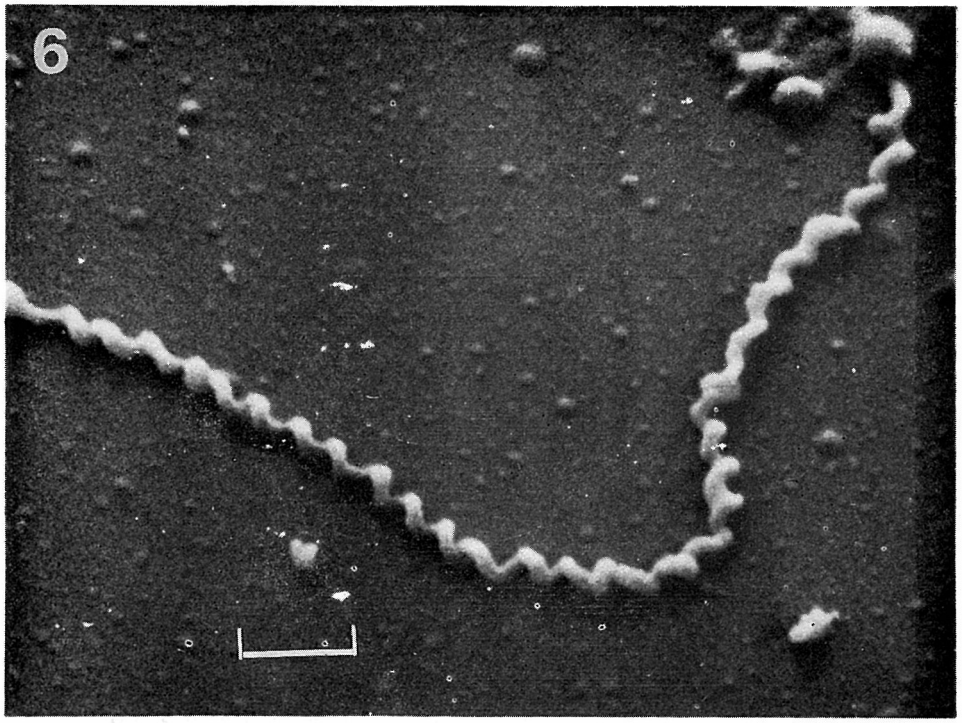

Fig. 6 : SEM image of a leptospiral cell body (*L. copenhageni*), showing the right-handed turn of its spirals. Magnification: 15,000×

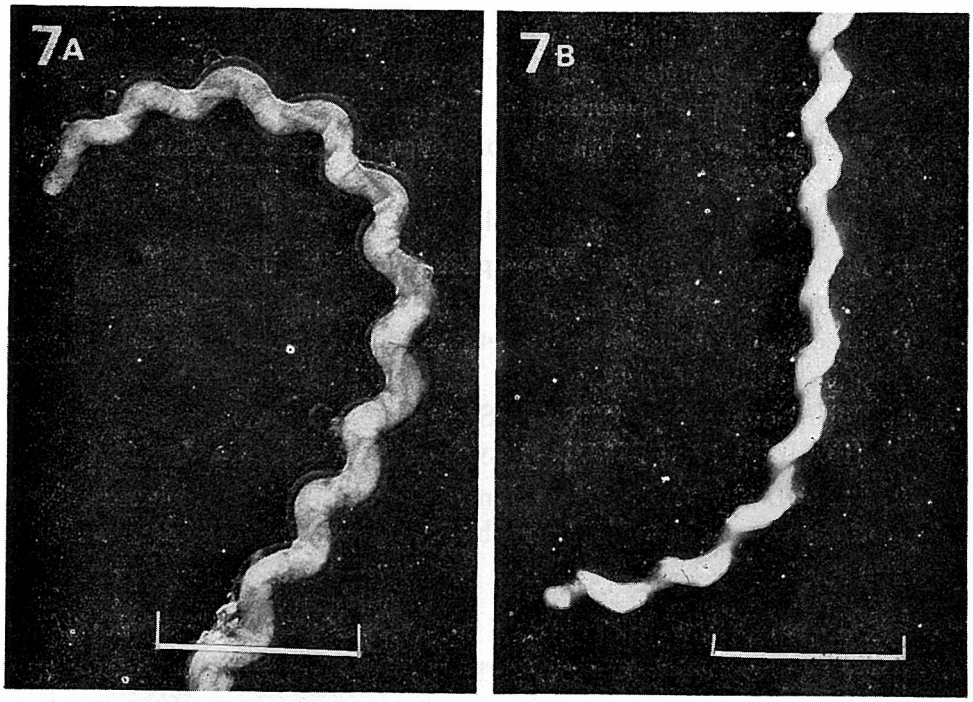

Fig. 7 : TEM images of leptospiral cell bodies (*L. copenhageni*) by different methods of drying.

A : Air drying, the right-handed turn of the cell body can be seen by the interwinning with an axial flagellum. Magnification: 32, 000×

B : Critical point drying, showing the right-handed turn of the cell body. Axial flagellum is difficult to see. Magnification: 30, 000×

Results

Two examples of the results obtained with both SEM and TEM are shown in Figs. 6 and 7. Preservation of the helical cell shape was excellent and the other technical conditions adopted here were found to be useful. The screen image of the samples always showed the spiral direction of the cell bodies to be right-handed. Using the photographic procedures outlined above, the true specimen image was preserved in both SEM and TEM.

Discussion

The technical procedures for specimen preparation proved to be correct in the preservation of the helical conformation of leptospira cell bodies in both SEM and TEM (Fig. 6 and 7). The upside setting of the specimen in TEM proved to be practical, because it provided the

same real image as SEM (Fig. 5A), and prevented the formation of a mirror image as seen in Figs. 5 and 7. The downside setting of the specimen in TEM exhibited an inverted image from the upside setting, which is the mirror image of the real image (Fig. 5B). Therefore, the upside setting must be adopted as the proper setting condition. Regarding photography, light projection from the posterior side of the negative with SEM and positive film with TEM (with the upside setting of the specimen) provided the same image as the EM-screens (Figs. 4B, 4D; 6, 7B).

The spiral direction of the leptospiral cell was right-handed in this preliminary test and this result is regarded as the correct observation based upon the model experiment with the nylon string (Fig. 2 A).

The conditions established in this experiment can be used as a model for future studies on the spiral direction of helical cell bodies.

CONCLUSIONS

Theoretical considerations on the definition of spiral direction, discussions on the experimental conditions for investigations of spiral direction, and confirming tests provided guidelines for the EM study of helical cell bodies, and may be briefly summarized as follows:

1. The definition of spiral direction: "If the helix is right-handed, it will advance away from the observer when turned clockwise on its axis. The helix is left-handed, if it advances away from the observer when turned counter-clockwise on its axis."

2. Mechanism of image formation in EM: No apprehension on the image formation mechanism in both SEM and TEM. of our laboratory Real image can always be obtained in SEM and TEM when the sample is in the upside setting.

3. Specimen preparation: A system of technical procedures, including double fixation with glutaraldehyde and osmium tetraoxide, CPD and coating with Au-Pd alloy- or chrome-shadowing, was adopted as the proper method for preparing specimens.

4. Specimen setting: The upside setting of specimen is fixed in SEM and is prefered in TEM, because this condition always gives the real image on the EM-screens.

5. Photography: Printing must be done carefully to reproduce the same image as shown on the EM-screens. Furthermore, the shadowed image of the sample in TEM is preferred, so it is obtained from a

positive film by reverse printing of the original negative. Light projection printing from the posterior side of the negative (SEM) and positive film (TEM) served the purpose.

Application of these established conditions in electron microscopy, are considered to be useful in the investigation of the three dimensional conformation of biological samples in general.

SUMMARY

For the purpose of studying the three dimensional conformation of samples, such as the spiral direction of the helical cell bodies of some bacteria, the theoretical considerations on the definition of spiral direction and the mechanism of image formation in electron microscopy with SEM and TEM were discussed.

The conventions for classifying spiral direction were set. Concerning image formation, it was found that proper technique eliminated the possibility of mirror images, and assured that the image viewed either directly or indirectly represented the true specimen condition.

Some practical problems on the study of spiral direction, preparation of samples, setting conditions in the EM, and methods for image observation were discussed.

These problems were all resolved in some preliminary tests by using model samples, and the most suitable conditions for the case of leptospira cells were decided. Double fixation, critical point drying, and metallic coating or shadowing, were found to be necessary for the preservation and exhibition of the helical cell body of leptospira. These results provided a theoretical foundation and practical authentication for the morphological study of helical organisms as described in the conclusion.

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