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CHARACTERISTICS OF *CORYNEBACTERIUM RENALE* PHAGE

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INTRODUCTION

In a previous report YANAGAWA et al. (1968) described the lysogeny of *Corynebacterium renale*. Lysogenic strains were found only in *C. renale* type I, about two-thirds of the type I strains were lysogenic, and it was found that different phage-types existed in Japan and Scotland.

Since phages were thus known in the strains of *C. renale* an effort was made by the authors to clarify the characteristics of the *C. renale* phage. This paper deals with the results obtained from our studies of the *C. renale* phage.

MATERIALS AND METHODS

Phages were isolated from *C. renale* type I strains by ultraviolet irradiation as previously reported (YANAGAWA et al., 1968). These phages were designated by prefixing the word "RP" to the name of *C. renale* strains which produced the phages. In this study we used 10 phages obtained from 10 strains of *C. renale* isolated in Hokkaido, Japan. These were RP 1, 2, 3, 6, 9, 16, 27, 28, 33 and 79. Phage RP 6, obtained from *C. renale* No. 6, was investigated most thoroughly because this phage could be propagated easier than the others, and incidentally, as described under Results the phages used were very similar to each other and phage RP 6 could be regarded as a typical representative of the *C. renale* phages used. In addition phage RP 50 was used only for morphological examination because it could not be propagated easily enough.

Phages were induced by ultraviolet irradiation and propagated by the soft agar method (ADAMS, 1959), then chloroformed and stored as described in the previous report. *C. renale* No. 71, was very susceptible to phage RP 6 as well as other phages, and therefore was used as a propagating strain. The phage titers were expressed as plaque-forming units (PFU). After several propagations, using the soft agar method increasing the volume of the propagating bacteria, the titer of the phages reached $10^9 \sim 10^{10}$ PFU/ml. It was difficult to obtain phage titers of more than 10^{10} PFU/ml.

The rate of phage adsorption by the host cell, *C. renale* No. 71, was calculated by determining the number of unadsorbed phages at varied time, left in the mixture of phage and host cells. The input ratio of phage to bacteria was nearly 1 : 1000. Adsorption was performed at 37°C in a shake flask and 0.1 ml samples were removed at timed intervals and

mixed with 9.9 ml of chilled broth, and then diluted 10 times and immediately centrifuged for 5 min at 3,500 rpm. This supernate was used to determine the unadsorbed phage titer.

A single-step growth experiment was performed principally by the technique described by ADAMS (1959). After 10 min adsorption of phage to the bacteria, anti-phage RP 6 serum (1 : 20) was added to inactivate the remaining free phage. The first and second growth tubes, representing 10^{-4} and 2×10^{-5} dilutions of the adsorption mixture, were held at 37°C for several hours, and samples were removed for phage assay at frequent intervals. The latent period and average burst size were then determined.

The anti-phage RP 6 serum used was the same one prepared and used in the previously reported experiment (YANAGAWA et al., 1968). The neutralization constant (K value) of the immune serum for phage RP 6 was 243.8.

For the determination of the phage nucleic acid, it was necessary to obtain a purified phage preparation. The procedure used for this preparation was as follows. Fully propagated phage RP 6, as described in the previous report, was centrifuged at $50,000\times g$ for 30 min and the resulting sediment was resuspended in a phosphate buffer saline (pH 7.2) containing 0.05% gelatin. This phage concentrate was then purified by differential centrifugation, first, low speed of $5,000\times g$ for 20 min and then high speed of $80,000\times g$ for 30 min. The high speed centrifugation was conducted by placing the phage material on a cushion of 1/5 volume of 15 % sucrose solution. This differential centrifugation was repeated twice, and the resulting phage concentrate was treated with DNase (5 μg/ml with Mg^{++} and Ca^{++}) at 37°C for 1 hr, and then with RNase (40 μg/ml in 0.01 M EDTA) at 37°C for 1 hr. It was then washed twice again by the high speed centrifugation on the sucrose cushion. The purified phage preparation thus obtained was used for extracting the phage nucleic acid.

The phage nucleic acid was extracted from the purified phage preparation by phenol as originally described by MANDELL & HERSHEY (1960). Characterization of the nucleic acid was done by the indol reaction and orcinol test (WATANABE & MIURA, 1957). Acridine orange staining (MAYOR & HILL., 1961) was also applied.

For electron microscopy, the purified phage preparations were mounted on carbon coated collodion membrane grids, and after reduction of the material by adsorption with filter paper, stained with either 4 % silicotungstic acid or 2 % phosphotungstic acid which was adjusted with 1 N potassium hydroxide to pH 7.2. The specimens were examined with a JEM-7 electron microscope (Japan Electrical Optics Laboratory Co.) at instrumental magnification up to 100,000×.

RESULTS

1 Plaque morphology

Turbid, circular plaques were visible within 24 hr, these plaques reached their maximal size after 48 hr. The size of the plaques of phage RP 6 was not uniform, they ranged from 0.4 to 1.2 mm in diameter (fig. 1). Phages obtained from both the small and large plaques again formed plaques of various sizes, as shown in figure 1, respectively. When the adsorption time of the phage in a 12 hr broth culture of the host cell, *C. renale* No. 71, was limited to 10 min and unadsorbed phage was eliminated with antiserum, plaques of uniform size of 1.2 mm in diameter were observed (fig. 2). The plaque size of the other phages was essentially

the same as those of phage RP 6. All 10 phages showed the same host range, by lysing *C. renale* Nos. 71, 11, 8, 31, 74, 29 and 49.

2 Phage morphology

Figures 3~7 are the electron micrographs of phage RP 6. No morphological difference was found between RP 6 and other phages (fig. 8). The phage has polygonal head 50 mμ in diameter and tail 260 mμ in length and 10 mμ wide. The tail has fine cross-striations. The empty virion with its transparent head has a hollow tail exhibiting the capsomere structure. In the full phages the tail was also full. The tail tips have a sixfold star-shaped assembly (figs. 6 & 7). A number of very fine fibers could be seen developing from the tail tip holding the cell debris to which the tail tip is attached (fig. 7).

The bacterial site of phage attachment found by electron microscopy was cell wall and not the pili.

3 Stability of phage

The phage RP 6 was examined for stability. At 45°C 20% of the phage were inactivated after 2~20 min, at 50°C 90% were inactivated after 2 min and 99% were inactivated after 30 min, and at 55°C complete inactivation occurred after 30 min. Thus, the phage was heat labile. Mixing of phage with 3% chloroform resulted in 90% inactivation. Treatment of the phage with 0.125% trypsin and 0.15% pronase in a phosphate buffer saline at 37°C for 4 hrs resulted in complete inactivation. The original phage titer of 4×10^9 PFU/ml was kept nearly 10 months stored at −20°C.

4 Adsorption of the phage to the host cell

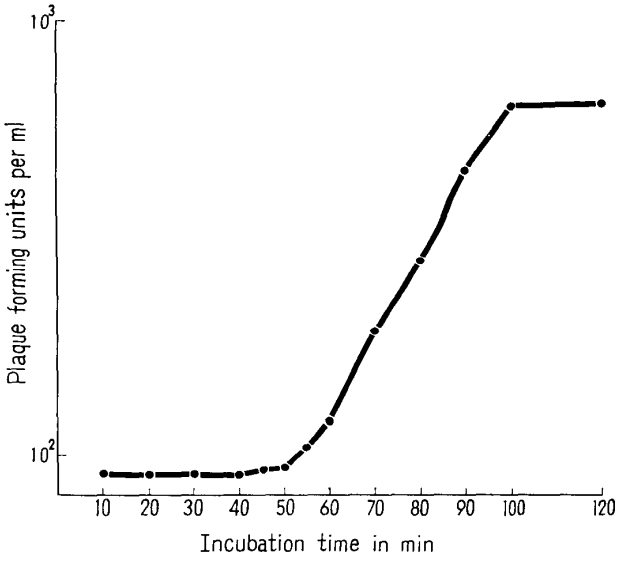
The rate of adsorption of phage RP 6 to the cells of *C. renale* No. 71 is shown in table 1. The adsorption curve consisted of two phases, rapid until 10 min but slowing afterwards. The K value was calculated from the former. The adsorption rate of RP 6 to *C. renale* No. 71 was expressed as the K value $=5.5\times10^{-11}$ ml/min. Therefore, the adsorption rate is slow.

TABLE 1 *Rate of adsorption of phage RP 6 to C. renale No. 71*

TIME	UNADSORBED PHAGE
min	%
0	100
5	74
10	46
20	36
30	25
40	21

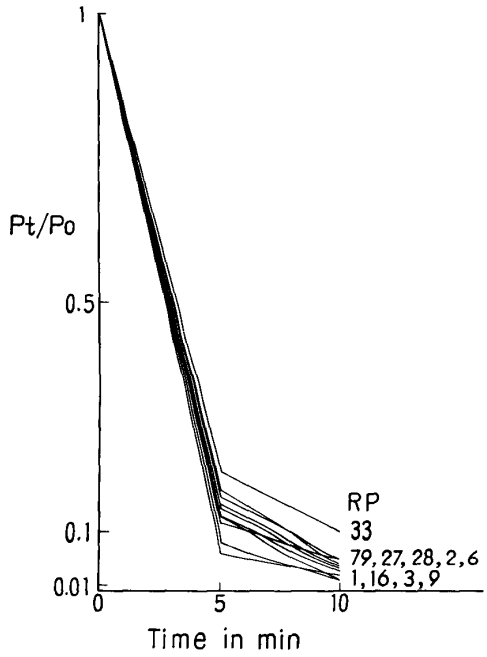
Rate of adsorption (K value) $=5.5\times10^{-11}$ ml/min
Input ratio: 2×10^6 phage particles to 1.4×10^9 bacteria

FIGURE 9 *Single-step growth experiment using a 4 hr broth culture of C. renale No. 71*



Latent period: 50 min
Rise period: 50 min
Input ratio: 4.8×10^7 phage particles to 6×10^6 bacteria
Burst size: 16 phage particles per cell

FIGURE 10 *Neutralization of 10 phage strains after exposure to anti-phage RP 6 serum*



P_0 Phage particles at 0 time
Pt Phage particles at t min after exposure to the anti-phage serum

5 Single-step growth experiment

A typical growth experiment of phage RP 6 on *C. renale* No. 71 is shown in figure 9. A latent period of about 50 min and average burst size of 16 particles were obtained for the 4 hr broth cultures. The burst size is characteristically small.

6 Serological relation of phages

Ten phages, RP 1, 2, 3, 6, 9, 16, 27, 28, 33, and 79, were tested against anti-phage RP 6 serum. The reduction of the phage titer was determined 5 and 10 min after mixing with anti-phage RP 6 serum. As shown in figure 10, all these phages were neutralized quite similarly, indicating their intimate serological relationships.

7 Characterization of phage nucleic acid

The indol test was positive and the orcinol reaction was negative for the extracted phage RP 6 nucleic acid. The acridine orange stain gave the color of yellow-green. These results showed that the nucleic acid was a double-stranded deoxyribonucleic acid (DNA).

DISCUSSION

Investigations of phages from the genus *Corynebacterium* have been focused on the conversion of a nontoxigenic strain of *C. diphtheriae* to toxigenic strain by phage β. Phages from other species of *Corynebacterium* have not been extensively investigated. In the previous report (YANAGAWA et al., 1968) we described the lysogeny of *C. renale* type I and the existence of different phage-types of *C. renale* in Japan and Scotland. We applied the methods of phage characterization on the phages of *C. renale*, of Japanese origin.

It is interesting that the general morphological features of the *C. renale* phage are similar to those of the phage β of *C. diphtheriae* recently reported by MATHEWS et al. (1966). This unexpected similarity of phage morphology in both species of *Corynebacterium* is interesting. The morphological features of the *C. renale* phage also somewhat resembled those of phages λ and T5 of *E. coli*, this includes the capsomere structure of the tail (BRADLEY, 1967).

Our morphological observation also went into detail on the phage's tail. The tail tip had a sixfold star-shaped assembly which was similar to that of the *Staphylococcus* phage (BRADLEY, 1967). The tail fibers which are usually hard to detect, were obvious in a few pictures in that the fine fibers held the cell debris to which the phage tail tip was attached. These tail fibers could not be studied further but generally they resembled those of the T-even phages.

The bacterial site of phage attachment was found by electron microscopy to be the cell wall. Some strains of *C. renale* type I were known to possess pili (YANAGAWA et al., 1968). The cell wall was the site of attachment for the phage of *C. renale*, and not the pili.

Ten phages were almost neutralized by anti-phage RP 6 serum. Thus, the

findings of identical morphology and host range, suggest that these phages belong to the same type.

C. renale phage is rather labile. They are inactivated considerably by heating at 50°C and also by 3% chloroform. Therefore, heating was not used to kill contaminant bacteria. Chloroform treatment for the same purpose can be used only when the phage titer is high. However, the phage was, stable at −20°C.

The adsorption rate of the *C. renale* phage was slow (5.5×10^{-11} ml/min). The difference in plaque size could be attributed to this slow adsorption rate. A similar phenomenon was observed with *Brucella* phage, by BRINLY-MORGAN et al. (1960) and McDUFF et al. (1962). Slowly adsorbing phage-bacterial systems produce a great diversity in plaque size. The phage adsorbed early produced large plaques, and those adsorbed late produced small plaques.

The burst size of the phage RP 6 was only 16, which is very small compared to other phages. This may be due to the fact that the phage was a temperate one and produced turbid plaques. We considered the difficulty of obtaining a high titer phage preparation as resulting from the small burst size.

SUMMARY

Methods of characterizing phage have been applied to the phages obtained from 10 strains of *Corynebacterium renale*, isolated in Japan. These phages are identical morphologically, serologically and in host range. They have a polygonal head 50 mμ in diameter and a tail 260 mμ in length and 10 mμ wide. The tail is exhibiting a capsomere structure. The tail tips have a sixfold star-shaped assembly and very fine fibers are developed from the tips. Phage titers of $10^9\sim10^{10}$ plaque-forming units per ml were finally obtained after several propagations with *C. renale* No. 71 by the soft agar method of increasing the volume of the propagating bacteria. The following data were also obtained from a representative phage RP 6 and the propagating bacteria. Some loss in activity resulted from heating at 50°C, and in 3% chloroform. All activity was lost by heating at 55°C for 30 min. It had a slow adsorption rate ($K=5.5\times10^{-11}$ ml/min) with a latent period of 50 min and a burst size of 16 particles. Its nucleic acid was a double-stranded DNA.

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EXPLANATION OF PLATES

PLATE I

Fig. 1 Phage RP 6 plaque morphology

Fig. 2 Phage RP 6 plaque morphology when adsorption time is limited to 10 min

Fig. 3 Electron microscopy of phage RP 6

Fig. 4 Electron microscopy of phage RP 6

Scale indicates 100 mμ in figs. 3 & 4.

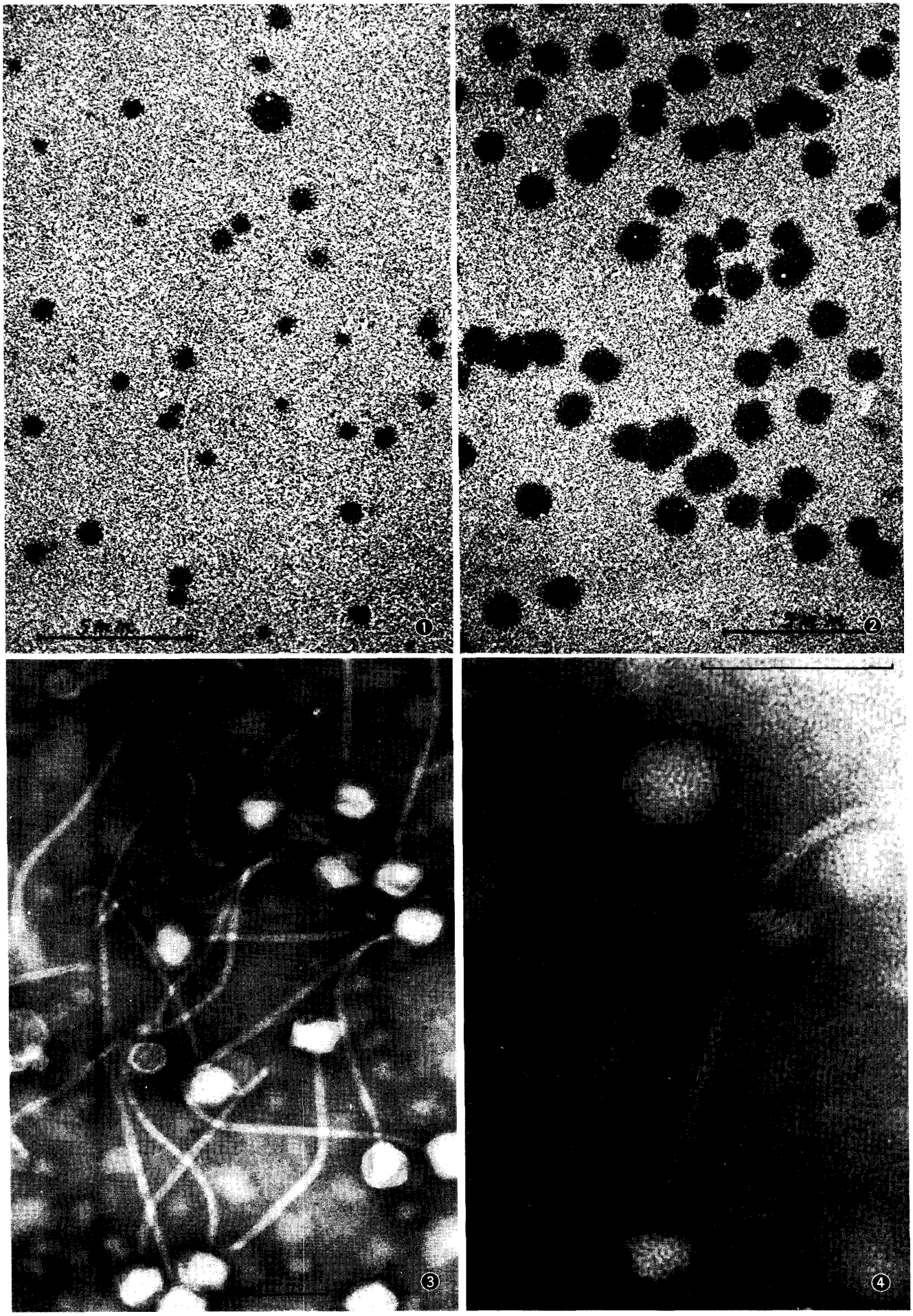

PLATE II

Fig. 5 Electron microscopy of phage RP 6 showing the striation of tail

Fig. 6 Electron microscopy of phage RP 6 showing the tail tip with its sixfold star-shaped assembly (indicated with an arrow)

Fig. 7 Very fine fibers several in number (indicated with arrows) developed from the star-shaped tail tip holding the cell debris to which the tail tip is attached. The phage is RP 6.

Fig. 8 Electron microscopy of phage RP 2

The scales indicate 100 mμ.

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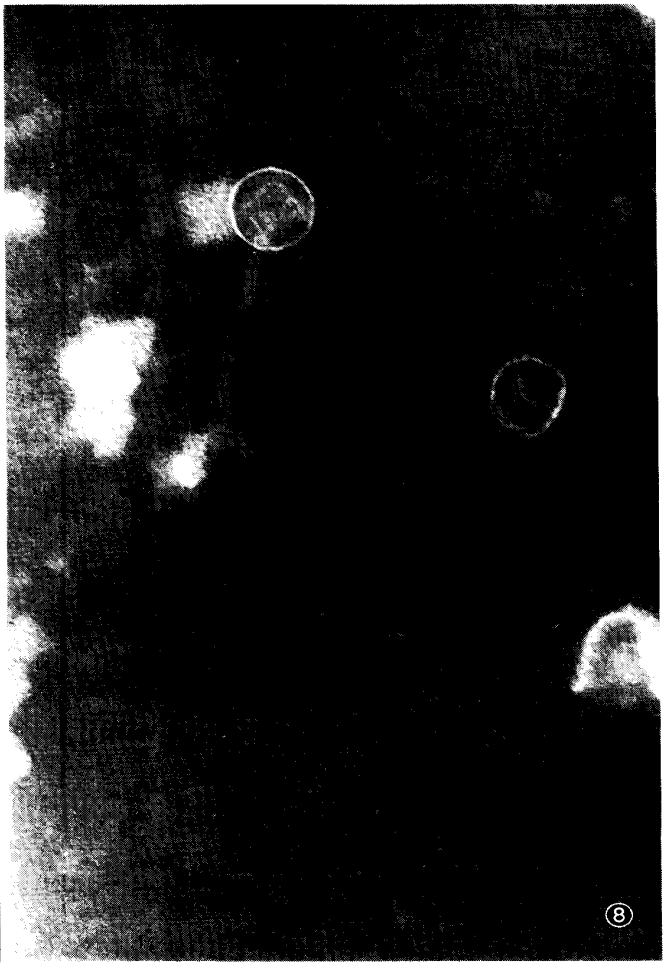

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