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Study of the Ribosomes of *Mycobacterium lepraemurium*[1]

Seitaro Okada[2]

In a paper on electron microscopic studies of murine leprosy bacilli read by the author ([10]) at the VIIth International Congress of Leprology in Tokyo in 1958, the results of fractionation with centrifugation of destroyed murine leprosy bacilli were reported. In that study, spherical granules 15-20mμ in diameter were found in the sediment obtained after centrifugation at 38,000 rpm for three hours. Considering the chemical composition, shape, and size of the granules, the author presumed that the granules had a significance like that of the fine granules composing the endoplasmic reticulum of various animal cells, viz., the granules later named ribosomes, and that they were related to protein synthesis.

The chemical and physical properties of the "ribonucleoprotein particles" of *Escherichia coli* were determined by the detailed studies reported by Tissières and his collaborators in 1959 ([13]). They believed that the RNA-including particles isolated by Schachman *et al.* in 1952 ([11]) resembled, in chemical composition and size, the ribonucleoprotein particles that had been studied extensively in animal tissues, plants, and yeast, which were believed to be an important site of protein synthesis in those cells. It was proven by McQuillen *et al.* ([7]) in 1959 that the ribosomes were the site of protein synthesis in bacterial cells also.

The author has again taken up the problems of the ribosomes of murine leprosy bacilli and studied their morphologic details and physical properties. The quantity of ribosomes in murine leprosy bacilli has also been measured. As it is very difficult to collect sufficient ribosomes from human leprosy bacilli, murine leprosy bacilli were used for this study.

MATERIALS AND METHODS

Murine lepromas were collected from mice killed 150-175 days after inoculation with the Hawaii strain of murine leprosy bacilli. The bacilli were isolated from these lepromas by the method of Nishimura *et al.* ([9]) without treatment with trypsin and sodium hydroxide, slightly modifying that as shown in Figure A.

The purified bacilli, resuspended in distilled water, were freeze-dried and preserved in a refrigerator. A small number of the purified bacilli were shadow-casted and observed with the electron microscope. Another small quantity of purified bacilli were fixed in a 1 per cent solution of osmium tetroxide in acetate veronal buffer (pH 7.4) to which sucrose was added up to a concentration of 0.25 M. The dehydrated specimen was embedded in methacrylate to which uranyl nitrate was added to give a concentration of 0.2 per cent. The embedded specimen was ultrathin-sectioned and observed with the electron microscope.

The method of isolation of ribosomes from the purified bacilli is shown in Figure B. The dry weight of bacilli used for isolation of ribosomes was 416.7 mgm.

All manipulations were carried out at 0° to 4°C.

The cell walls were isolated from Fraction R_9 by Kanai and Youmans' method ([5]) as indicated in Figure C.

The ultraviolet ray absorption of fractions Cr, S_9 R_{12}, S_{12}, R_{40} and S_{40} was investigated with a Shimadzu self-registering spectrophotometer. The fractions Cr, R_9,

1 Received for publication 21 June 1968.
2 S. Okada, Associate Professor, Leprosy Research Laboratory, Kyoto University School of Medicine, Kyoto, Japan.

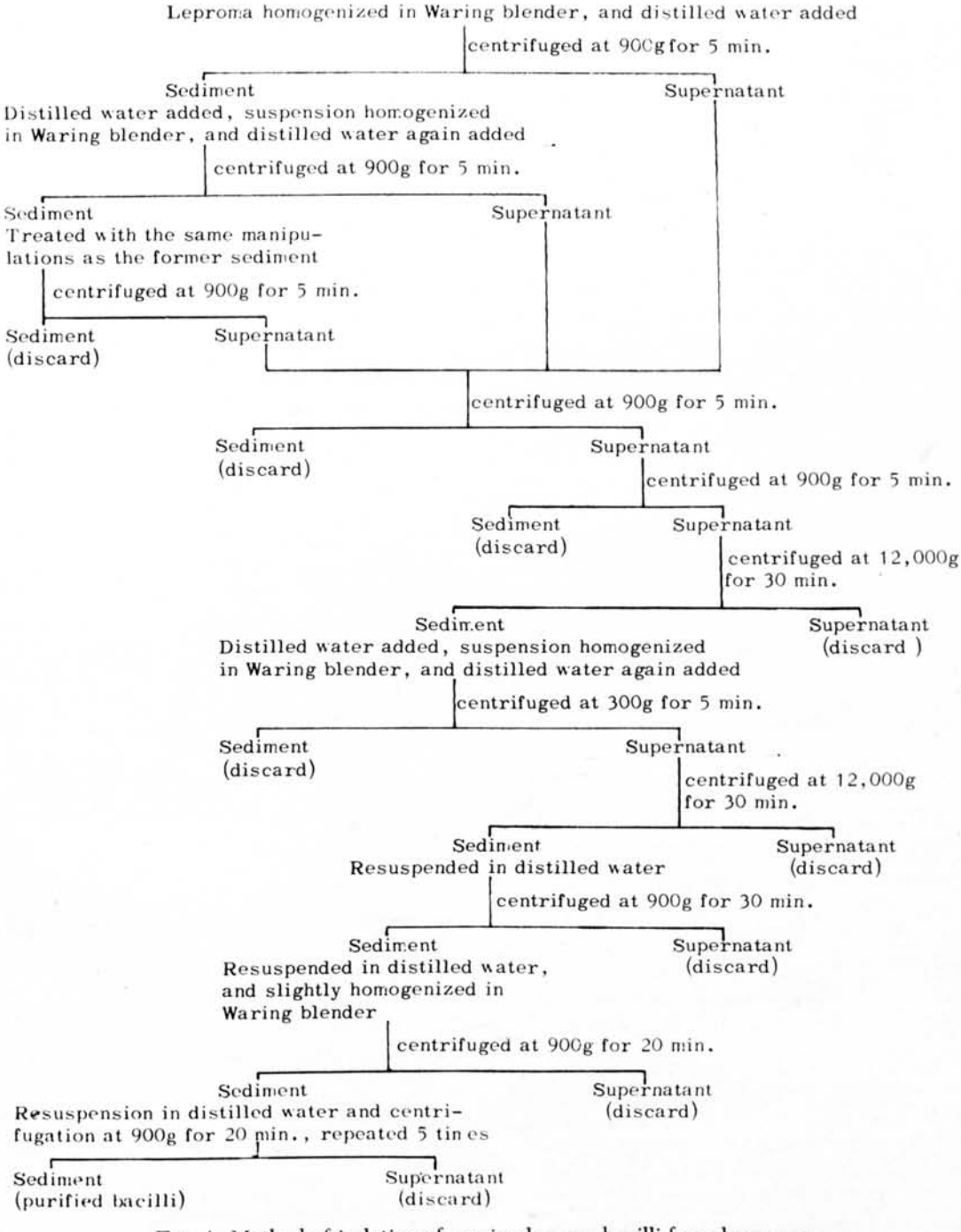


FIG. A. Method of isolation of murine leprosy bacilli from lepromas.

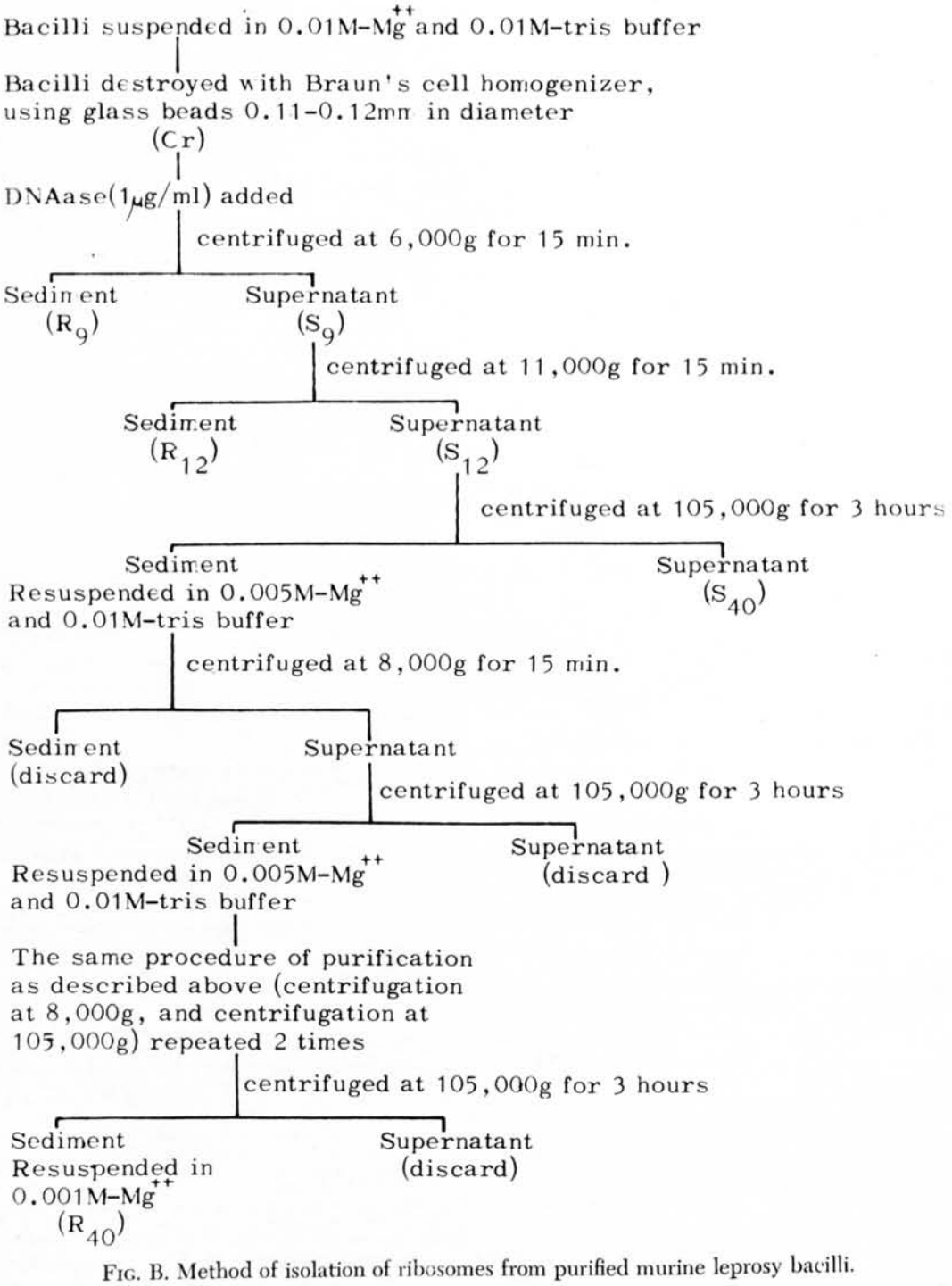


FIG. B. Method of isolation of ribosomes from purified murine leprosy bacilli.

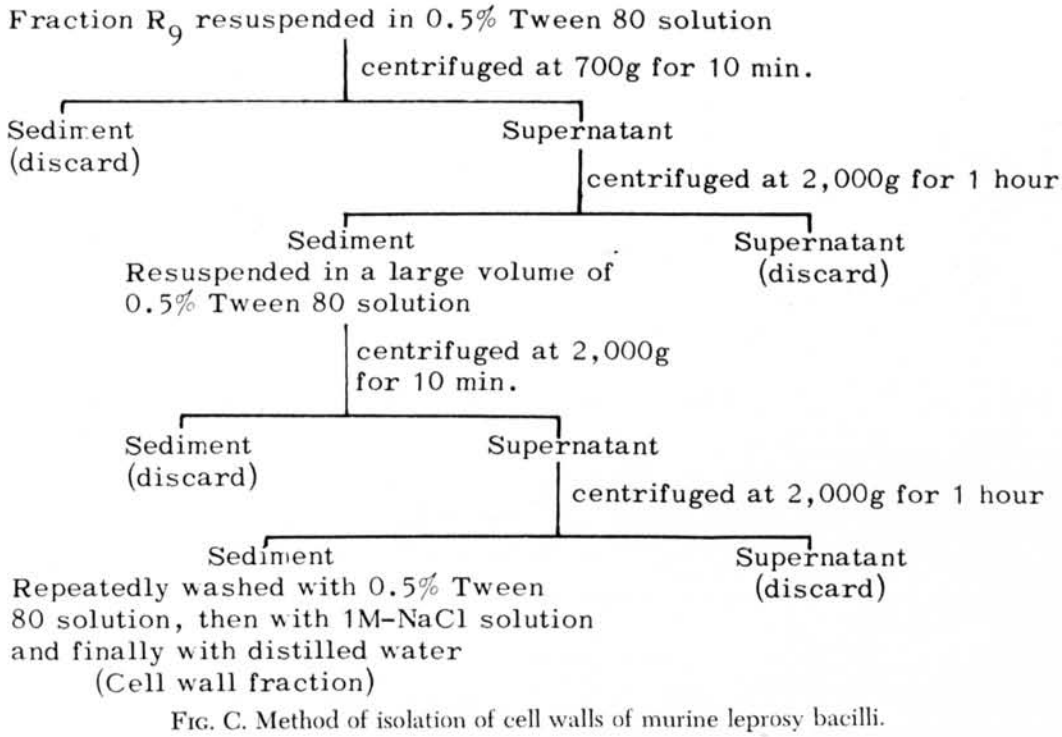


FIG. C. Method of isolation of cell walls of murine leprosy bacilli.

R_{12}, R_{40}, S_{40} and the cell wall fraction were shadow-casted. Fraction R_{40} and the cell wall fraction were negatively stained with phosphotungstic acid solution to be observed with the electron microscope. Fraction R_{40} was centrifuged analytically with a Model-E Spinco ultracentrifuge, and the sedimentation coefficients of particles included in the fraction were calculated.

RESULTS

The electron microscopic features of the shadow-casted specimens made from the suspension of purified murine leprosy bacilli used for the isolation of ribosomes are shown in Figure 8. Although a small amount of irregularly formed fragments can be found, the suspension of murine leprosy bacilli proved to be well purified. In the picture of shadow-casted Fraction Cr, the cell wall and cytoplasm of bacilli are well separated from each other. Prior to this experiment, various methods of destruction of murine leprosy bacilli have been compared, and it was found that Braun's cell homogenizer showed the best destroying effect and separation of cytoplasm from cell walls, and accordingly the best extraction of cytoplasmic substances.

The cell wall fraction contained not only the purified cell wall, but also fragments of bacillary cytoplasm admixed with it. In these admixtures filaments 190-300 Å in width can be found (Fig. 9). Branching can sometimes be seen in these filaments. These filaments may correspond to the filamentous structures observed in ultrathin sections of murine leprosy bacilli showing branching. This presumption should be examined further. Fraction R_{12} contained particles of different size and fragments of cell walls and other structures. Among those, particles of nearly equal size were found, viz., spherical particles 24-28 mμ, 35-38 mμ, and 50-60 mμ in diameter.

Absorption patterns of ultraviolet rays by fractions Cr, S_9, R_{12}, S_{12}, R_{40} and S_{40} are shown respectively in Figures 1, 2, 3, 4, 5 and 6. In Fraction S_9 a peak showing maximum absorption at 260 mμ is found, although no distinct peak can be observed in Fraction Cr. Fraction R_{12} does not show a peak at 260 mμ but absorbs, in a remarkable way, rays of wave length shorter than

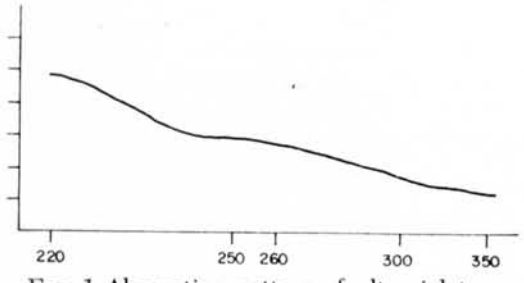


FIG. 1 Absorption pattern of ultraviolet rays by Fraction Cr.

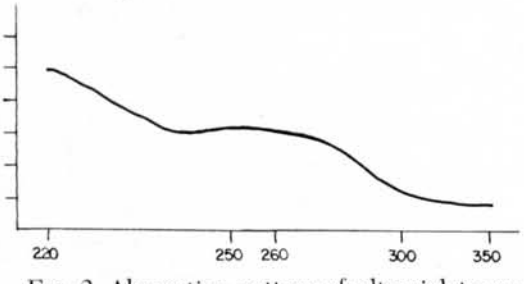


FIG. 2. Absorption pattern of ultraviolet rays by Fraction S_9.

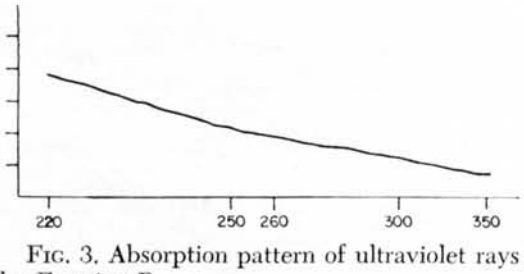


FIG. 3. Absorption pattern of ultraviolet rays by Fraction R_{12}.

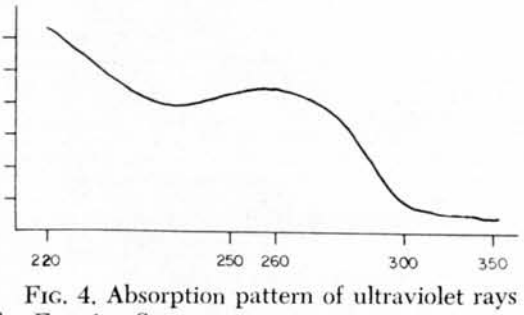


FIG. 4. Absorption pattern of ultraviolet rays by Fraction S_{12}.

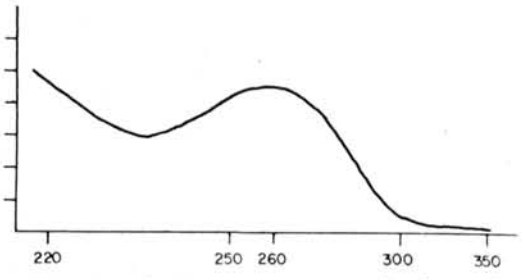


FIG. 5. Absorption pattern of ultraviolet rays by Fraction R_{40}.

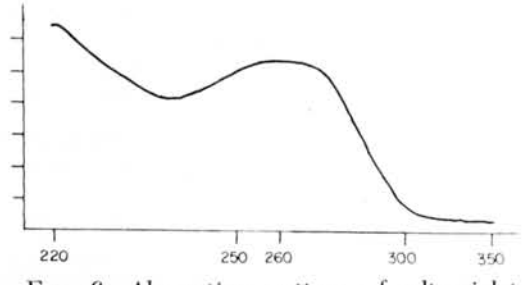


FIG. 6. Absorption pattern of ultraviolet rays by Fraction S_{40}.

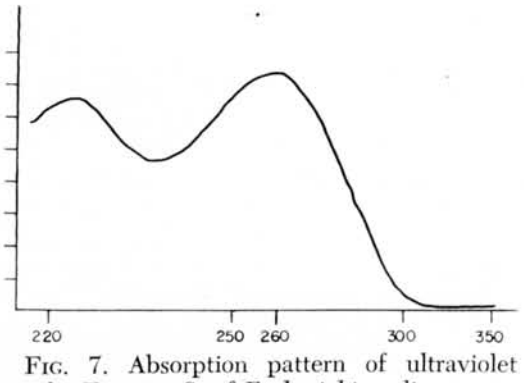
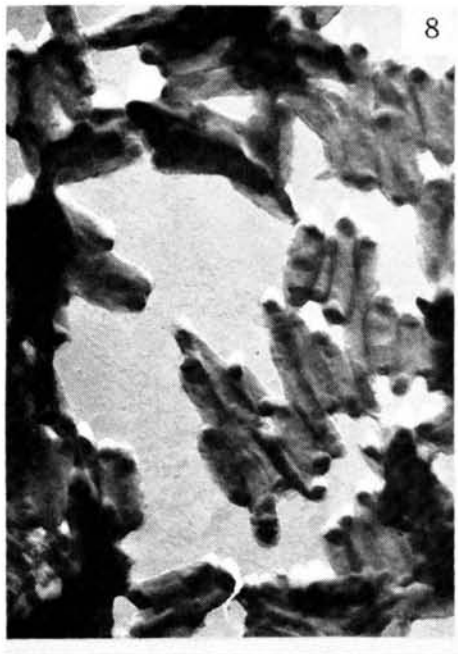
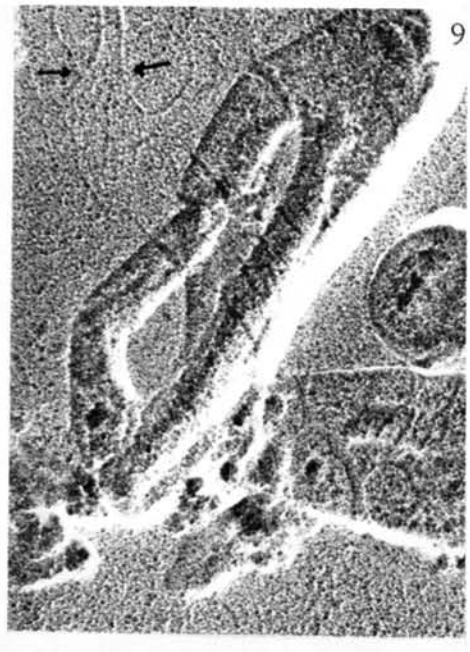


FIG. 7. Absorption pattern of ultraviolet rays by Fraction S_9 of *Escherichia coli*.

260 mμ. In Fractions S_{12} and R_{40}, the absorption of rays of wave length shorter than 260 mμ is decreased by the elimination of Fraction R_{12}, and the absorption that is maximum at 260 mμ stands out. Fraction R_{40} showed a positive orcinol reaction and negative diphenylamine reaction. From these results, the substance showing the absorption pattern of nucleic acid was identified as ribonucleic acid. Fraction S_{40} also showed a remarkable absorption peak, maximum at 260 mμ. Most of the absorption at 260 mμ by Fraction S_{40} is due to ribonucleic acid. Except for the absorption peak at 260 mμ, the specific peak in absorption patterns cannot be observed in every fraction. The absorption pattern of Fraction S_9 of *Escherichia coli* extracted by the same method is shown in Figure 7.

The result of analytical ultracentrifugation of Fraction R_{40}, viz., the ribosome fraction, is as follows. Figure 11 shows the sedimentation pattern with schlieren optics at 18,270 rpm. Figure 12 shows the sedimentation pattern with ultraviolet optics at 20,410 rpm, and Figure 13 that at 40,200 rpm. On the basis of calculations with these data from ultracentrifugation, the sedimentation coefficients of particles included in Fraction R_{40} are 100S, 50S, 70-80S, 30S and 20-23S. The latter three kinds of particles





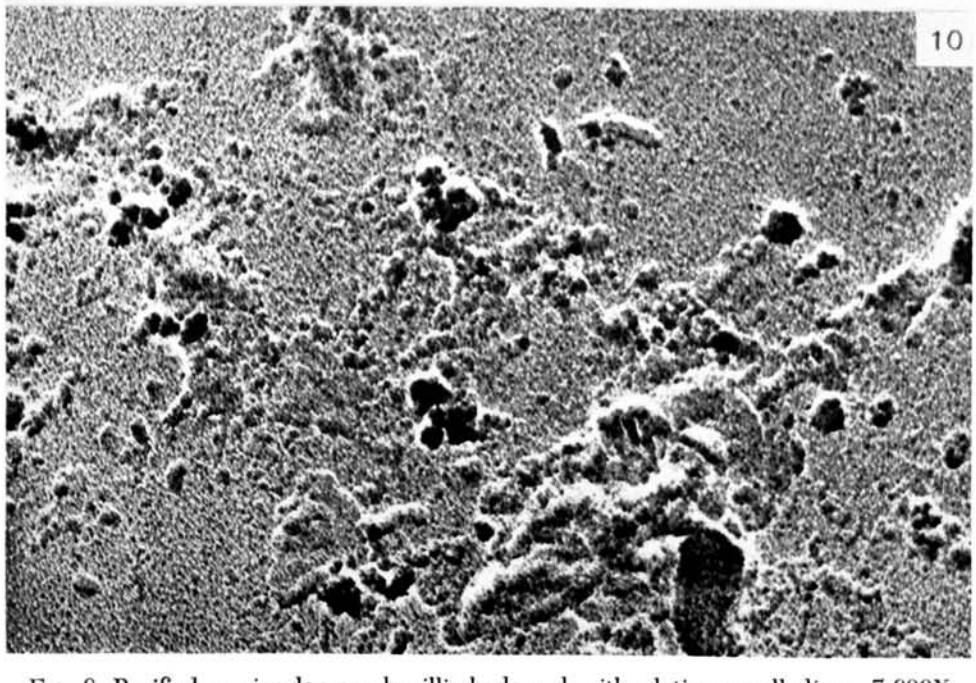
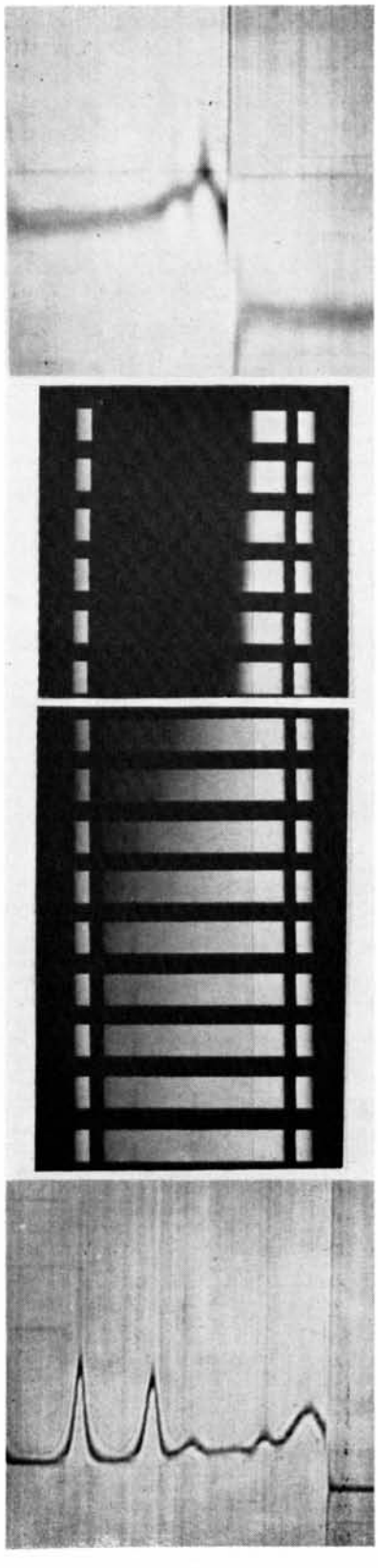


FIG. 8. Purified murine leprosy bacilli shadowed with platinum-palladium. 7,600X
FIG. 9. Cell wall fraction shadowed with platinum-palladium. 42,000X
FIG. 10. Fraction R_{12} shadowed with platinum-palladium. 80,000X



did not show a peak in the sedimentation pattern with schlieren optics, but were detected with ultraviolet optics. The separation of those three kinds of particles in sedimentation patterns is not distinct. It is believed that this result is due to the small quantity present of those three kinds of particles, especially so for 70-80S and due also to the lack of uniformity in size of particles, which will be described later. The 20-23S particles may be RNA of high molecular weight. This problem should be studied further. The sedimentation pattern of Fraction S_9 extracted from *Escherichia coli* is shown in Figure 14. The sedimentation coefficients of ribosomes of murine leprosy bacilli are similar to those of *Escherichia coli,* which are 100S, 70S, 50S and 30S.

The ribosomes of murine leprosy bacilli were fixed with 10 per cent formalin (pH 6.0) including 0.005M-Mg^{++} for one hour, and negatively stained with 1 per cent phosphotungstic acid solution (pH 5.3) including 0.005M-Mg^{++}, to be observed with the electron microscope. The negatively stained ribosomes can be classified as particles 230-245Å, 140-160Å, 170-220Å and 115-120Å in diameter. Particles 230-245Å in diameter seem to correspond to 100S particles, particles 140-160Å in diameter to 50S particles, and particles 115-120Å in diameter to 30S particles. Among the particles 170-220Å in diameter, many particles are distributed in ranges from 175 to 180Å and from 200 to 210Å. But those two peaks in the distribution by size are not sharp. Therefore particles 170-220Å in diameter cannot be classified distinctly in two groups. It is believed that particles 170-220Å in diameter correspond to 70-80S particles, which do not show a sharp sepa-

FIG. 11. Sedimentation pattern of Fraction R_{40} with schlieren optics at 18,270 rpm.

FIG. 12. Sedimentation pattern of Fraction R_{40} with ultraviolet optics at 20,410 rpm.

FIG. 13. Sedimentation pattern of Fraction R_{40} with ultraviolet optics at 40,200 rpm.

FIG. 14. Sedimentation pattern of Fraction S_9 extracted from *Escherichia coli* with schlieren optics at 35,600 rpm.

ration in analytic centrifugation. In addition to these particles, ellipsoidal particles 180-250Å and 240-340Å in size can be found. Most of those particles may be included in the 100S peak in analytic centrifugation. Particles about 100Å in diameter can be observed. These seem to correspond to 20-23S particles.

The electron microscope features of 100S (*a* in Fig. 15), 70-80S (*b* in Fig. 15) and 50S (*c* in Fig. 15) particles are shown in Figure 15. Figure 16 shows the high magnification of 100S particles shown in Figure 15. Some 100S particles show diplococcus-like features, as shown in Figure 16. It appears that two particles attach to each

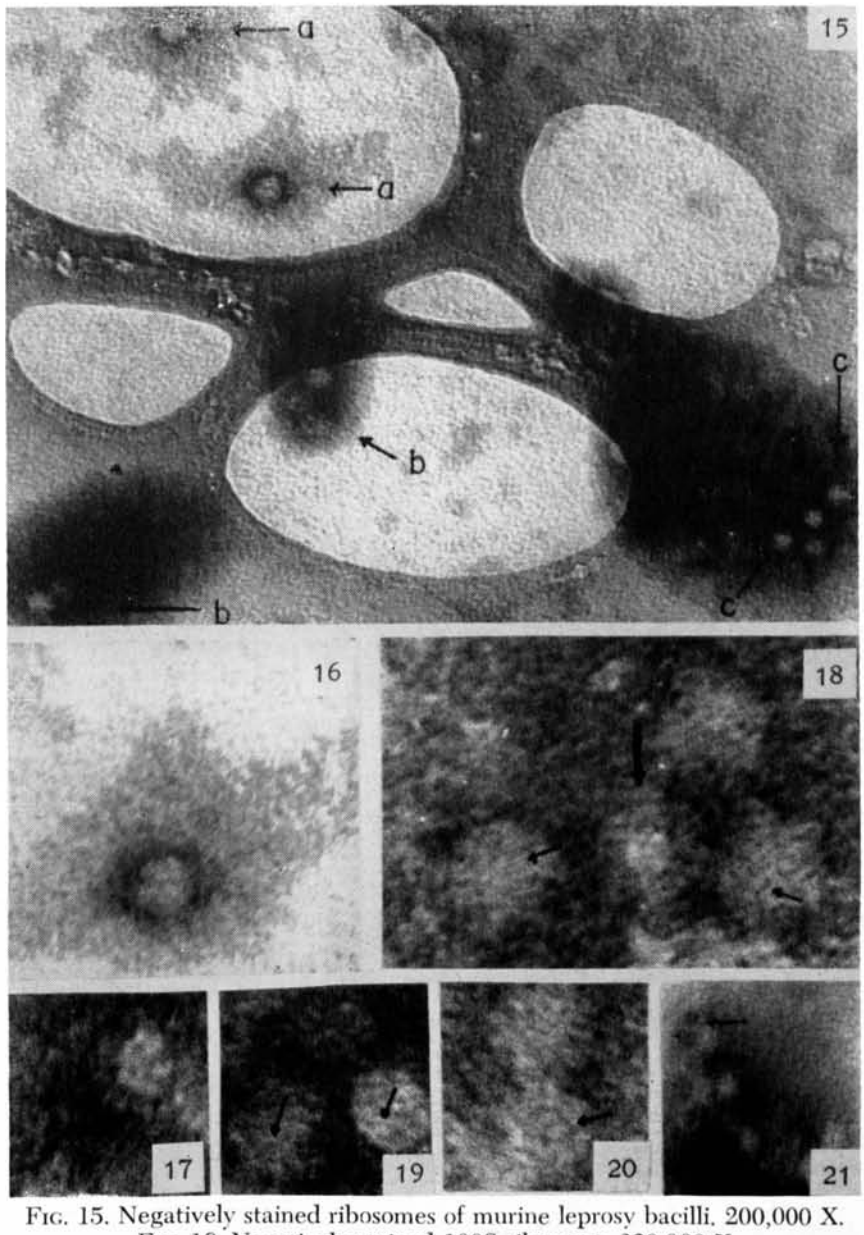


FIG. 15. Negatively stained ribosomes of murine leprosy bacilli. 200,000 X.

FIG. 16. Negatively stained 100S ribosome. 320,000 X

FIGS. 17, 18, 19, 20. High magnification of negatively stained 50S ribosomes. 960,000 X.

FIG. 21. Negatively stained ribosomes. The central parts of some ribosomes represent the dark hollow appearance. 210,000 X

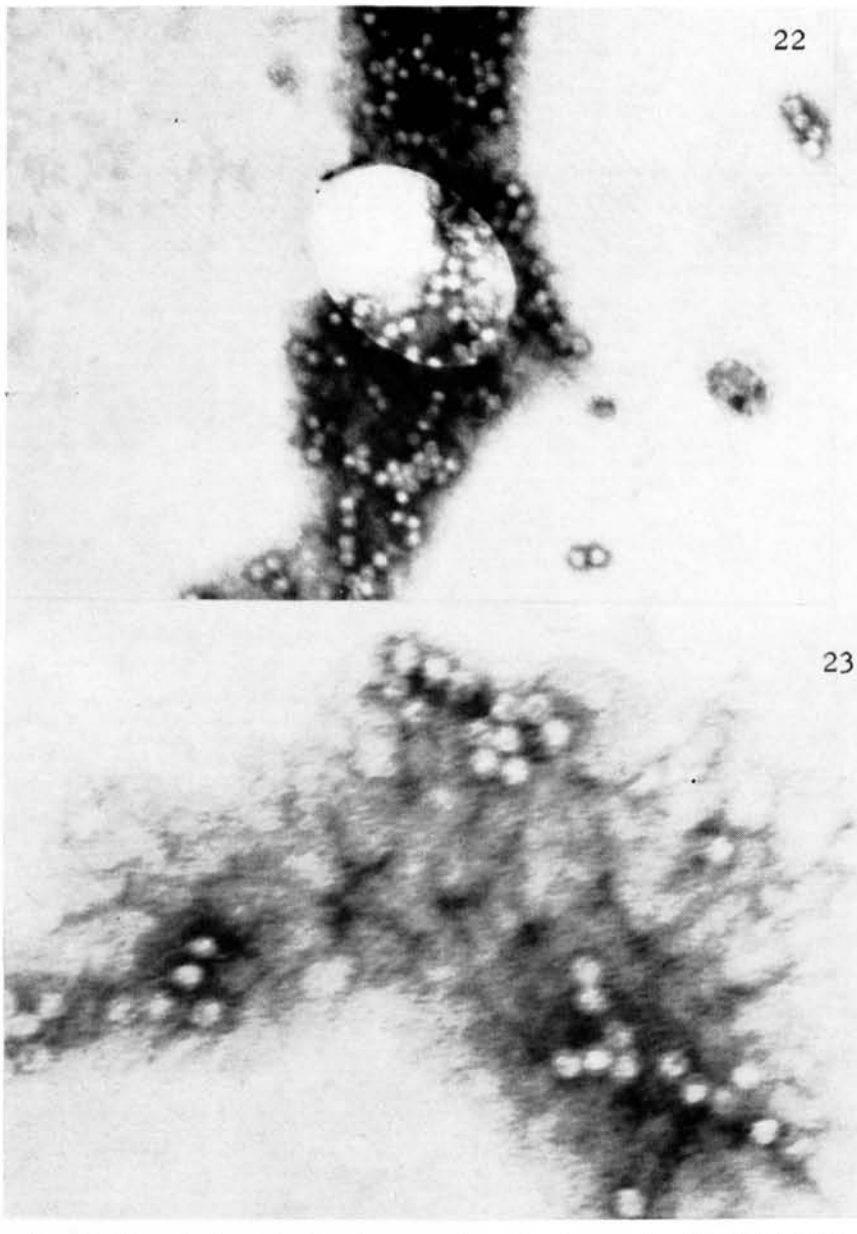


FIG. 22. Negatively stained polysomes of murine leprosy bacilli. 114,000 X.
FIG. 23. Negatively stained polysomes of murine leprosy bacilli. 210,000 X.

other to constitute a 100S particle. Such a diplococcus-like figure is similar to that of the 100S particle of *Escherichia coli*. Figures 17, 18, 19 and 20 show a high magnification of 50S ribosomes. The latter show various shapes, e.g., round, hexagonal or trapezoid shapes. A ribosome in Figure 17 shows a central part in which small particulate subunits are gathered closely together. To this central part of the ribosome, particulate subunits (arrow) are connected with filaments 7-9Å in width, forming a kind of rosette arrangement. Those particulate subunits are 18-25Å in diameter. Also a ribosome in Figure 18 (large arrow) shows a figure resembling that of the ribosome in Figure 17. Such ribosomes as these present an appearance showing the dense central part with a half dense halo in the photograph taken with low magnification. Other

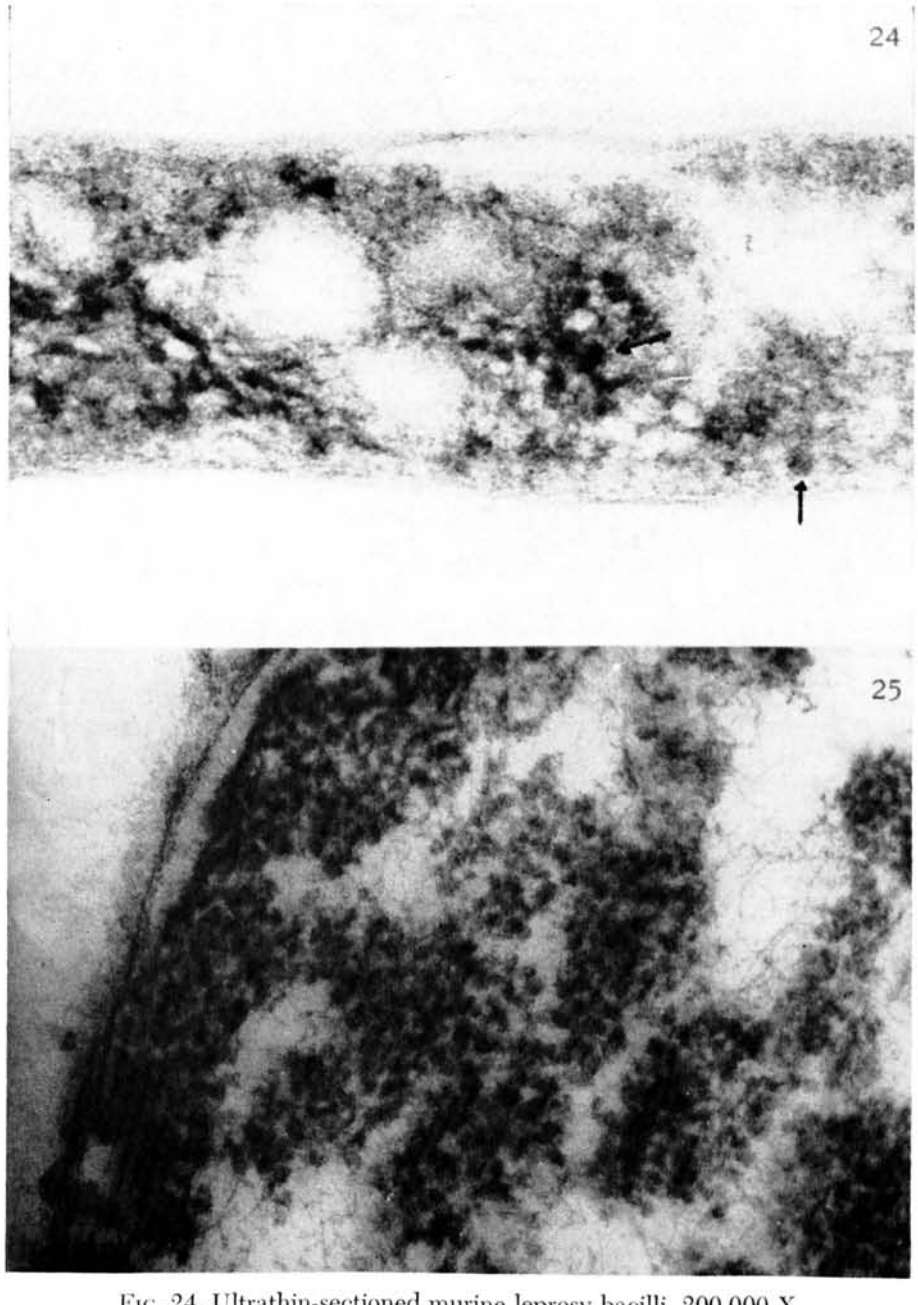


FIG. 24. Ultrathin-sectioned murine leprosy bacilli. 200,000 X
FIG. 25. Ultrathin-sectioned *Escherichia coli*. 185,000 X.

ribosomes in Figures 18 and 19 show features that can be observed most frequently. They show also that a ribosome consists of many particulate subunits. Fine filaments 7-9Å in width can sometimes be seen in ribosomes. In a ribosome at the left in Figure 19, such filaments can readily be seen. It is presumed that the ribosome of murine leprosy bacilli is composed of particulate subunits 18-25Å in diameter and fine filaments 7-9Å in width. But this presumption should be examined further. A ribosome in Figure 20 shows an annular dense area (arrow) caused by the penetration of phosphotungstic acid. This feature is similar to that described as a rounded area

in the center of the 50S ribosome of *Bacillus subtilis* by Nanninga (8). In some ribosomes, phosphotungstic acid penetrates remarkably into the center of ribosomes. The central part represents the dark hollow appearance (arrow Fig. 21). Such ribosomes as these may be the degenerated ones. In Figures 22 and 23 several ribosomes are connected, showing a chain-like or flower-like arrangement. Such features as these may represent a type of polysome of murine leprosy bacilli.

The dry weight of ribosomes obtained from the purified murine leprosy bacilli was 1.14 mgm. A comparison of the weight of ribosomes extracted from murine leprosy bacilli with the amount extracted from *Escherichia coli* by Tissières and his collaborators is shown as follows: Amount of ribosomes extracted from bacilli 1 gm. in dry weight, *M. lepraemurium* 2.6 mgm; *E. coli* 306.0 mgm.

The amount of ribosomes included in murine leprosy bacilli is smaller than that in *Escherichia coli*. It may be that a paucity of ribosomes, which play an important part in protein synthesis, is a cause of the long generation time of murine leprosy bacilli.

The electron microscopic picture of ultrathin sections of the murine leprosy bacilli used in this study is shown in Figure 24, and that of *Escherichia coli* in Figure 25. Numerous ribosomes can be seen in the cytoplasm of *Escherichia coli*, but only a small number of ribosomes can be seen in murine leprosy bacilli. Even a crude extract of *Escherichia coli*, corresponding to Fraction S_9 in Figure 2, shows the sharp ultraviolet ray absorption peak caused by RNA. But Fraction S_9 of murine leprosy bacilli shows only a gently-sloping peak, and the prominent peak appears only after repeated purification. Even the crude extract of *Escherichia coli* shows the sharp peaks of ribosomes by analytic ultracentrifugation making the measurement of sedimentation coefficients possible. But the peaks of ribosomes cannot be obtained by analytic centrifugation of Fraction S_9 of murine leprosy bacilli, because of the very low density of ribosomes in the fraction. And in electron microscopic pictures of ribosome fractions of murine leprosy bacilli, small amounts of admixtures can be found. It is evident from these facts that isolation of ribosomes is easier from *Escherichia coli* than from murine leprosy bacilli. It may be that a difference in the constitution of cytoplasm accounts for the variation between *Escherichia coli* and murine leprosy bacilli. The composition of the cytoplasm of murine leprosy bacilli is more complicated that that of *Escherichia coli*, the main components of which are ribosomes, as observed in electron microscopic pictures of ultrathin sections.

The number of ribosomes in ultrathin sections of murine leprosy bacilli differs with the condition of the bacilli. So too, the amount of ribosomes extracted from murine leprosy bacilli may differ with the condition of the bacilli used for extraction. The murine leprosy bacilli used in this study were collected from lepromas of mice killed 150-175 days after inoculation. If the lepromas are harvested earlier, the amount of ribosomes extracted from bacilli may differ from that obtained in this study. This problem should be investigated later.

SUMMARY

The sedimentation coefficients of ribosomes of murine leprosy bacilli are 100S, 70-80S, 50S and 30S. Besides those, 20-23S particles are found. Negatively stained ribosomes were observed with the electron microscope. Some 100S ribosomes are composed of two particles. High magnification of 50S ribosomes shows particulate subunits and filamentous structures. A chain-like or flower-like arrangement of ribosomes, which seems to represent a type of polysome of murine leprosy bacilli, was found. The amount of ribosomes in murine leprosy bacilli is smaller than that in *Escherichia coli*. It is an important cause of the long generation time of murine leprosy bacilli.

RESUMEN

Los coeficientes de sedimentación de ribosomas de los bacilos de la lepra murina son 100S, 70-80S, 50S, y 30S. Además de aquellos se encuentran 20-23S partículas. Ribosomas teñidos negativamente fueron observados con

el microscopio electrónico. Algunos 100S ribosomas están compuestos de dos partículas. Altos aumentos de 50S ribosomas muestran subunidades particuladas y estructura filamentosa. Se encontró un arreglo de los ribosomas en forma de cadena o en forma de flor, lo cual parece representar un tipo de polysoma del bacilo de la lepra murina. La cantidad de ribosomas en el bacilo de la lepra murina es menor que lo en *Escherichia coli*. Esta es una causa importante para el prolongado teimpo de generar del bacilo de la lepra murina.

RÉSUMÉ

Les coefficients de sédimentation des ribosomes de bacilles de la lèpre murine se situent à 100S, 70-80S, 50S, et 30S. En outre, des particules à coefficient de sédimentation de 20-23S ont été trouvées. Les ribosomes colorés par contraste ont été observés par microscopie electronique. Certains des ribosomes 100S étaient composés de deux particules. A un fort grossissement, les ribosomes 50S montraient des sous-unités particulées ainsi que des structures filamenteuses. Un arrangement en chaine ou en fleur des ribosomes a été observé, qui parait représenter un type de polysome chez les bacilles de la lèpre murine. La quantité de ribosomes était plus faible dans les bacilles de lèpre murine que chez *Escherichia coli*. Ceci constitue une cause importante de la durée prolongée de la période de reproduction (generation time) observée chez les bacilles de la lèpre murine.

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