In this issue there appear the last three of a series of five short articles on this subject by Dr. J. H. Hanks, who has dealt with it more broadly and intensively than has been done before, and from a different point of view. The first two articles were in the preceding issue. It seems desirable to condense this important work, to make a continuous story of the findings with a minimum of technical details.

I. The first article [1] summarized studies of the factors which lessen the penetrability of pathogenic mycobacteria by certain dyes. In order that the properties of the bacilli should be as natural as possible, drying and heating of the smears were avoided; they were exposed to the dye after only 3 seconds.

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[1] HANKS, J. H. Significance of capsular components of *Mycobacterium leprae* and other mycobacteria. Internat. J. Leprosy **29** (1961) 74-83.

Staining of *M. phlei* (with safranin 0) occurred quickly, accelerating with time, whereas the staining of pathogens (crystal violet found best for them) proceeds progressively more slowly, and the impermeabilities of individual cells differ very greatly. The murine leprosy bacillus gave the slowest staining rate because of the microcapsules which enclose them uniformly.[2] However, modification of the capsules of the latter, even by brief heating, increased the rate of staining tremendously.

Although *M. phlei,* representing the saprophytic mycobacteria, has in culture an extracellular matrix which promotes clumping, but capsular halos were not demonstrated by the surface coatings of the cells used; they can be demonstrated, however, provided the suspensions are exposed briefly to fresh substrates at 37°C. In the intermediate class represented by BCG (avirulent tubercle bacilli), most of the rods stain readily, but the others may exhibit outlines of capsular material after Congo red coating, and clumps may be so permeated by matrix that the individual organisms cannot be delineated.

Leprosy bacilli from untreated patients which stain readily are narrow; those which resist dye penetration look larger because of capsular halos; and globi may be outlined by the surface matrix as entirely unstained masses. No such appearances are seen in smears from sulfone-treated leprosy patients, in which the bacilli present no capsules and are readily permeable to dyes. The difference may be seen even in ordinary carbol-fuchsin-stained smears; the bacilli in the clumps from untreated patients are usually not in contact with each other, while those from sulfone-treated patients lie closely side by side.

The rugged microcapsules of *M. leprae murium* are associated with infectiousness. The first demonstrations of actual capsular structures were made by electron microscopy of ultra-thin sections, but microcapsules can be demonstrated by light microscopy after proper preliminary treatment.

The extracellular components are very resistant to modification, and alcohol—like formalin—seems to have a fixing and toughening effect. On the other hand they are promptly modified by heating to 98°C, or by shaking in 5-10 per cent chloroform in aqueous suspension.

The differences of penetrability of dyes among the mycobacteria are due to surface characteristics associated with pathogenicity, and these properties are attributable to true capsules and capsular matrices. The bacterial cells which stain readily are devoid of capsules. *M. leprae* may have large capsules, but the nonencapsulated state in sulfone-treated patients is held to indicate senescence rather than lack of viability or a specific effect of the drugs.

II. This paper[3] calls attention to the significance of capsules on *M. leprae* demonstrated by electron microscopy. Certain authors are cited who observed electron-transparent halos surrounding the rods in ultra-thin sections, and in direct views of unsectioned clump an amorphous matrix which tends to keep the rods separated. These are to be seen in material from untreated patients, but are lacking in material from treated patients.

In electron microscopy the electron-transparent zones are demonstrated by negative outlining with the surrounding materials (proteins and debris, and sectioned substances of the cells in which they occur). In light microscopy they can be demonstrated by negative outlining with surface coats. There are, it is suggested, certain advantages in the latter procedure and in the assay of dye permeability, these being simple procedures not requiring an electron microscope.[4]

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[2] A distinction is made between macrocapsules which can be demonstrated by light microscopy, and microcapsules which are less than 0.5 μ wide and hence not visualized in light microscopy.

[3] HANKS, J. H. Capsules in electron micrographs of *Mycobacterium leprae.* Internat. J. Leprosy **29** (1961) 84-87.

[4] Halos may also be well demonstrated in fresh smears from lesions after exposure to osmic vapor until the background material is darkened.—H. W. W.

III. This article[5] deals briefly with the old moot question of the origin of the global matrix of globi of *M. leprae*—whether it arises from the bacillus, or by interaction between the bacillus and host. The electron-transparent zones in ultra-thin sections are interpreted by the author as capsules and matrices which are synthesized solely by the mycobacteria, and which disappear after sulfone treatment—this interpretation being in frank disagreement with those of certain other authors but not of all. Although chloroform coagulates tissue components, it nevertheless promptly penetrates and declumps the globi, showing that chloroform-soluble waxes are the major bonding substance of the electron-transparent material. Treatment with a bile-pancreatin mixture digests and disperses tissue components other than collagen, but it does not declump the bacilli, and it does not alter the dye-impenetrability of the murine bacilli or remove their capsules. Tissue components are absorbed to the capsular surfaces of tissue-grown mycobacteria such as the murine bacilli, and are retained after washing, but they are removed by pancreatin with change of the iso-electric point of the suspension (from pH 4.6 to pH 1.5), without changing the viability and other properties of the bacilli. It is concluded that the tissue components occur only on the outermost (capsular) surfaces of the mycobacteria.

IV. Here[6] is discussed the problem of preserving the internal structure of pathogenic mycobacteria by fixation, which is interfered with by the impermeability of their inert capsules and matrices. These structures may prevent penetration by conventional fixatives, and the fixatives themselves may increase the impenetrability. For example, exposure of smears to the fumes of strong formalin quickly produces excessive basophilism of most of the materials in the smears, but such exposure of the murine bacilli makes them less permeable to dyes than before.

Fixation of tissues by osmium tetroxide for electron microscopy is conventionally brief, and it suffices for the demonstration of internal structures of ordinary mycobacteria. With pathogenic mycobacteria, however, more time is needed (e.g., 2 days for *M. avian,* 5 days for the H-37Rv tubercle bacillus, and 6 days for the murine leprosy bacillus), much longer fixation than is suitable for tissue cells. This leads to the question of how tissues containing such mycobacteria are to be fixed for study of their structure without over-fixation of the cells and other tissue elements. It is suggested that treatment, as with heat or chloroform, to reduce the impermeability of the bacilli is indicated, but there remains the problem of avoiding destruction of the capsules and distortion of internal structures, and that of simultaneous preservation of the tissue components.

V. In this final article[7] is a discussion of the demonstration of capsules on the leprosy bacillus in smear preparations after carbol-fuchsin staining. Unheated smears, after staining in carbol-fuchsin at 37°C for 5 minutes (and applying the nigrosin coat), showed staining of about ⅔rds of the free bacilli with capsules evident, whereas heating at 98°C for 1 minute and staining in hot carbol-fuchsin for 1 minute showed all of the bacilli stained red but without capsules. Heat and the dilute phenol were found to be the primary capsular solvents in the Ziehl-Neelsen procedure.

Significant as the phenomena reported by Hanks doubtless are, there are workers who hold views that are not in accord with his conclusions regarding the sources of the capsular matrix of the leprosy bacillus. It is true that as far back as 1918 Mitsuda,[8] on the basis of

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[5] HANKS, J. H. The origin of the capsules on *Mycobacterium leprae and* other tissue-grown mycobacteria. Internat. J. Leprosy **29** (1961) 172-174.

[6] HANKS, J. H. The problem of preserving internal structures in pathogenic mycobacteria by conventional methods of fixation. Internat. J. Leprosy **29** (1961) 175-178.

[7] HANKS, J. H. Demonstration of capsules on *M. leprae* during carbol-fuchsin staining. Mechanism of the Ziehl-Neelsen stain. Internat. J. Leprosy **29** (1961) 179-182.

[8] MITSUDA, K. The significance of the vacuole in the Virchow lepra cells, and the distribution of lepra cells in certain organs. Internat. J. Leprosy **4** (1936) 491-508; reprinted in English from Tokyo Iji Shinsi (1918) Nos. 2066 and 2067.

work with fat stains, was inclined to regard the lipoidal substance of vacuolated lepra cells as a product of, or due to, the bacillus—ascribable mostly to its degeneration. However, he pointed out that the lepra-cell colonies found in the viscera show very few bacilli, and that "in such cases lipoid transformation of the leprosy bacillus is unthinkable."

Most recently among the Japanese investigators, Fukushi [9] has concluded from histologic studies that the mechanism of the lepra-cell formation is phagocytosis of lipids from outside the cell. The absorbed lipid envelopes the bacilli and interferes with their metabolism, so that they degenerate and finally disappear. The formation of the foam cells has no connection with the age of the leproma, or with the amount and destruction of the bacilli, nor is it due to degeneration of the lepra cell itself.

Fukushi is also one of those concerned in studies of fixation of mycobacteria for the demonstration of the inner structures.[10, 11] He described a 3-layered cell wall on the tubercle bacillus as seen in ultra-thin sections, but the "slime layer" demonstrable about unsectioned bacilli, especially by metal shadowing, were not seen in the sections.

Other immediately available reports of interest are those of McFadzean and Valentine [12] and of Rees, Valentine and Wong [13] about the evidences of viability of leprosy and rat-leprosy bacilli shown by electron microscopy (the latter group reporting no evidence that the bacilli form capsules); by Malfatti [14] of a study of the role of granules of the leprosy bacilli; and by Chatterjee *et al.*[15] who found that in electron micrographs at least not all gloeal substance is lost from globi in sulfone-treated patients. The vacuoles around groups of bacilli in Virchow cells contain what was thought to be cell debris.

It would seem that the final answers to some of the questions involved are still to be reached. Certainly the matter is a complicated one, especially if one tries to correlate the loss of capsules in the lesions of treated patients with the ordinary histological picture of paraffin sections of such lesions.—H. W. Wade

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[9] Fukushi, K. Histopathological studies on the mechanism of the formation of leprous foam cells. Acta Path. Japonica **9** (1959) 361-422 (abstract in this issue).

[10] Fukushi, K. Electron microscopic studies of tubercle bacilli. V. Studies on fixation in ultra-thin sectioning. Sci. Rep. Res. Inst. Tohoku Univ. Ser. C, **9** (1959) (abstract in this issue).

[11] Shinohara, C., Fukushi, K., Suzuki, J. and Sato, K. Mitochondrial structure of *Mycobacterium tuberculosis* relating to its function. J. Electronmic. **6** (1958) (Annual Ed.) 47-52 (abstract in this issue).

[12] McFadzean, J. A. and Valentine, R. C. The examination and the determination of the viability of *Mycobacterium leprae* by electron microscopy. Leprosy Rev. **31** (1960) 6-11 (abstract in this issue).

[13] Rees, R. J. W., Valentine, R. C. and Wong, P. C. Application of quantitative electron microscopy to the study of *Mycobacterium lepraemurium* and *M. leprae*. J. Gen. Microbiol **22** (1960) 443-457.

[14] Malfatti, M. Aplicación de la técnica del sombreado al estudio del *Mycobacterium leprae*. Semana méd. **115** (1959) 948-957 (abstract in this issue).

[15] Chatterjee, K. R., Das Gupta, N. N. and De, M. L. Electron microscopic observations on the morphology of *Mycobacterium leprae*. Exper. Cell Res. **18** (1959) 521-527 (abstract in this issue).