ON THE EXISTENCE, MORPHOLOGY, NATURE, AND FUNCTIONS OF THE CYTOPLASMIC MEMBRANE IN THE BACTERIAL CELL

GEORGES KNAYSI

Laboratory of Bacteriology, State College of Agriculture, Cornell University, Ithaca, New York

Received for publication September 22, 1945

Until a few years ago knowledge of the superficial structures of the bacterial cell was in a state of confusion, not only in terminology but also in concept (see the review by Lewis, 1941). In 1930 the author demonstrated simultaneously, by a simple procedure, a differentiated outer layer of the cytoplasm, which he then called "ectoplasm," and a cell wall. In view of the confusing terminology, the author later (Knaysi, 1938) wrote: "Morphologically, the bacterial cell is surrounded by three membranes which may be called: the *cytoplasmic membrane*, the *cell wall*, and the *slime layer*." This statement was confirmed by all subsequent observations (Knaysi, 1941, 1944), and the existence of three membranes was accepted by Mudd *et al.* (1941, 1944), Lewis (1941), and Dubos (1945).

Of the three membranes, the cytoplasmic membrane is probably the most important, and yet it is the one the existence, nature, and properties of which require further consideration. This structure was seen, but usually misinterpreted, by several investigators. Among these was Migula (1897) who, following Bütschli (1890), called it the "Wandbelag" and considered it to be the entire cytoplasm. According to Migula, the cavity limited by this "Wandbelag" is occupied by a relatively huge vacuole, and not by a *central body* as Bütschli believed. Migula's vacuole is what we now consider the cytoplasm proper, in which endospores are formed and vacuoles may appear; for if Migula's concept were correct, we would have to postulate the existence of vacuoles of the second order. Eisenberg (1909) recognized the cytoplasmic membrane as the "Rindenschict," which together with the membrane (our cell wall) constitute the "Ektoplasma." Arthur Meyer (1912) did not recognize any structure which corresponds to the cytoplasmic membrane. He wrote at considerable length of the membrane (our cell wall), but it is evident from his description of its reactions that he, at times, was inadvertently describing the cytoplasmic membrane. He thus attributes to his membrane a high refractive power (p. 151, and figure 34) and states that in bacteria which deposit fat the membrane contains fat (p. 149 and figure 27 of his plate I).

The difficulty in the way of a satisfactory proof for the existence of the cytoplasmic membrane is in the fact that over much of the evidence heretofore presented hangs the shadow of the possibility that it is due to a surface phenomenon (e.g., Henrici, 1934, pp. 122–123). The same difficulties would have been true for the demonstration of the cell wall were it not for the fact

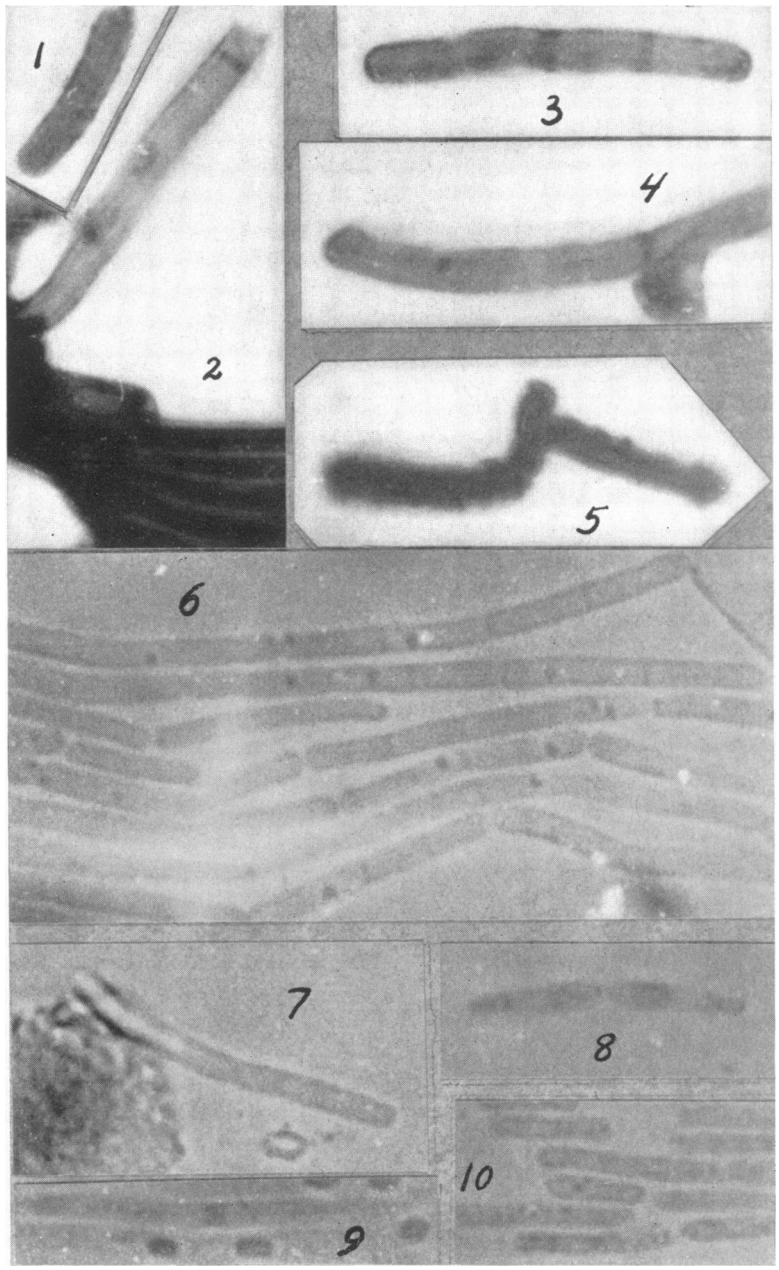

FIG. 1. *Bacillus megatherium*, C_1. From a 5-hour-old culture at 33 C, on a slant of the medium: $\frac{\overline{MITG}^1}{2}$ + 1.5 g agar. Stained by Knaysi's (1941) cell wall method. Early stage of autolysis.

FIG. 2. *Bacillus megatherium*, C_1. Age, medium, and treatment as in figure 1. A ghost cell in which the cytoplasm has completely disappeared but the cytoplasmic membrane is still intact.

FIG. 3. *Bacillus megatherium*, C_1. Age, medium, and treatment as in figure 1. A ghost cell in which the cytoplasm has completely disappeared and the cytoplasmic membrane is partially autolyzed.

FIG. 4. *Bacillus megatherium*, C_1. Age, medium, and treatment as in figure 1. A ghost cell of which only the cell wall and an inclusion remain.

that shrinkage of the cytoplasm leaves the cell wall isolated and makes its demonstration more certain. Consequently, if it were possible to separate the cytoplasmic membrane from the cytoplasm, the evidence for its existence would immediately take the dimensions of proof. This was accomplished by making use of the process of autolysis.

In cultures of *Bacillus megatherium* and of *Bacillus cereus* on agar slant media, particularly in the presence of glucose, numerous ghost cells appear like empty cell walls. However, by using the author's method (Knaysi, 1941), one finds that in different ghost cells autolysis has reached different stages. In some cells, the cytoplasm has disappeared, leaving the cytoplasmic membrane, sometimes intact, still lining the inner surface of the cell wall (figure 2). In more advanced stages of autolysis, fragments of the cytoplasmic membrane may still be seen here and there at the inner surface of the cell wall (figure 3). The cytoplasmic membrane appears dark red, and the cell wall blue. Upon further autolysis, the cell wall takes only a faint purplish color (figure 4). Sometimes the cell wall may be seen surrounded by a bright red layer of variable thickness, the slime layer (figure 5).

Investigation of ghost cells accomplishes two purposes: first, it separates the cytoplasm from its membrane, thus isolating the latter; second, it shows that the cytoplasm is much more susceptible to autolysis than the cytoplasmic membrane and, consequently, that the two structures are widely different in chemical composition or physicochemical structure.

Morphology and Properties of the Cytoplasmic membrane

In previous reports (Knaysi, 1938) it was stated that the cytoplasmic membrane is a superficial layer of the cytoplasm characterized by a relatively high refringence and affinity for dyes, that it is the principal (not the only) substrate for the gram and acid-fast stains, retaining the dye after the cytoplasm has been

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FIG. 5. *Bacillus megatherium*, C_1. Age, medium, and treatment as in figure 1. The cell wall and the slime layer.

FIG. 6. *Bacillus cereus*, C_3. A microculture 4 hours and 45 minutes old at 27 C. Medium as in figure 1. Cells living *in situ* showing cytoplasmic membrane and its extensions, which are potential places of division. Note the early plane form of these extensions.

FIG. 7. *Bacillus cereus*, C_3. The first two generations of an endospore; the exine is still visible. Microculture prepared by mixing 13-hour-old culture at 33 C in VFC[2] + 0.1 g of K-phosphate mixture (initial pH = 6.8), with an equal volume of $\frac{\overline{MITG}^{1}}{4}$, and a loopful of this mixture planted on a droplet of 1.5 per cent bacto agar. Age of microculture 2 hours at 28.5 C. It shows uneven thickness of cytoplasmic membrane and two stages of cell division.

FIG. 8. *Bacillus cereus*, C_3. From a microculture prepared as in figure 7. When the microculture was 1 day old at 27 C, it was disconnected and the cover glass dropped in hot, 10 per cent glucose solution for the Sharp test. Note positive cytoplasmic membrane and granules of the two sporangia.

FIG. 9. *Bacillus cereus*, C_3. One-day-old microculture at 30 C prepared as in figure 7. It shows accumulation of cytoplasmic membrane material at a potential place of division.

FIG. 10. *Bacillus cereus*, C_3. One-day-old microculture at 30 C prepared as in figure 7. The cover glass was removed and subjected to the Feulgen procedure (Knaysi, 1942); hydrolysis 10 minutes at 60 C. Note positive membrane and granules.

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[1] MI = 100 ml of meat infusion; T = 1 g of tryptone; G = 1 g of glucose.

[2] VFC = 100 ml of vitamin-free casein hydrolzate.

completely decolorized, and that it contains lipoids and proteins at least partly in chemical combination. We have also described the important role the cytoplasmic membrane plays in the division of the bacterial cell (Knaysi, 1929, 1938, 1941, 1944); indeed, it is the most conspicuous structure during that process, and in this respect the bacterial cell is different from the yeast cell (Knaysi, 1941); this difference constitutes a criterion by which dividing yeasts can be easily distinguished from large bacteria. We have also reported (1944) the surprising observation that the cytoplasmic membrane gives a positive Feulgen reaction.

During the present investigation we have repeated and confirmed most of the observations given above. Very careful study, in bright field, of bacteria in microcultures revealed that the inner surface of the cytoplasmic membrane is usually not a smooth curve but is jagged or wavy, even in actively growing cells (figures 6, 7, 12), and that the inner protoplasm of a single cell is not usually a perfectly homogeneous mass but is frequently segmented by thin, plane films originating from the cytoplasmic membrane and identical with it in physical and chemical properties (figures 6, 12). Some of these films are, potentially, places at which the cell ultimately divides (figures 6, 7). Whether these films are identical with those postulated by Dubos (1945) we are unable to say.

The *thickness* of the cytoplasmic membrane is variable even in the same cell (see above, also figures 6, 7, 11, 12). It is logical, however, to consider as normal thickness that observed in cells, or substantial portions of cells, where fluctuations are at a minimum. Measurements at various stages in the development, from germ cells to sporangia, of *Bacillus cereus*, strain C_3, gave the range of 0.21 to 0.35 μ. In view of the fact that the cytoplasmic membrane completely surrounds the cytoplasm, the total thickness of the membrane amounts to 0.3 to 0.4 of the total thickness of the protoplasm (*i.e.*, exclusive of the cell wall). Under certain conditions the membrane extension across the cell, previous to division, may be abnormally thick (figure 9). After division, part of this material remains at each of the contiguous ends of the sister cells as "polar bodies" or "polar caps" which, on account of their behavior toward dyes, etc., have often been mistaken for nuclei.

The *structure* of the cytoplasmic membrane is not always homogeneous. At the time of cell division one observes the formation of a granule at the place where the centripetal growth of the membrane is to take place and a thinning of the membrane immediately beyond that point. It seems as if the granule is the result of the accumulation of the substance of the membrane. In several members of the genus *Bacillus* there is a stage of cultural development, normally preceding that of sporulation, in which the membrane of the living cell becomes heterogeneous throughout (figure 11); this heterogeneity is due to the formation of granules which can be discharged into the cytoplasm under aerobic conditions.

Chemically, the cytoplasmic membrane gives the Sharp test for protein (figure 8), stains well with the dyes of the Sudan series (figure 13), and gives a positive Feulgen reaction (figure 10); these characteristics survive autolysis of the cytoplasm and treatment with fat solvents; the membrane has an isoelectric range

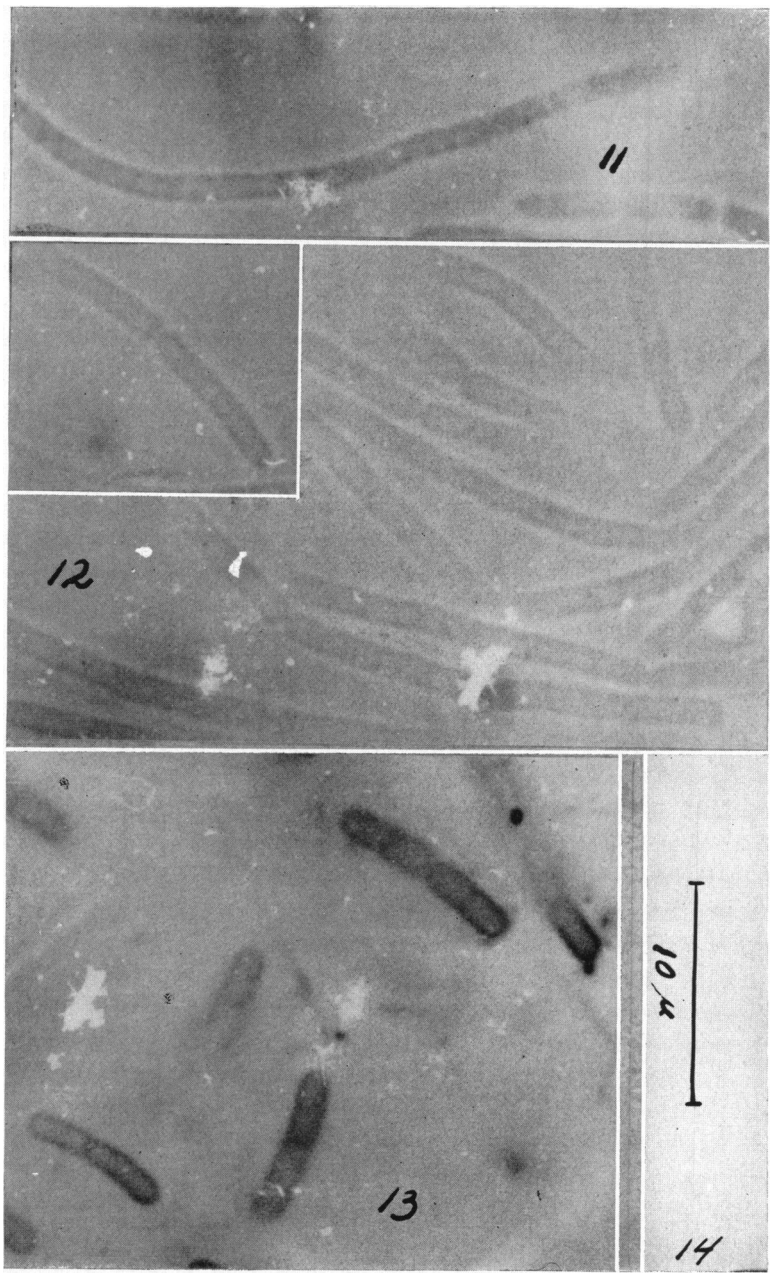

FIG. 11. *Bacillus cereus*, C_3. Cells from one-day-old microculture on $\frac{\text{MITG}^1}{2}$ at 28 C. It shows heterogeneity of cytoplasmic membrane.

FIG. 12. *Bacillus cereus*, C_3. A few-hour-old microculture at 29 C, prepared by placing a loopful of an 18-hour-old culture in VFC[2] + 0.1 g K-phosphate mixture (initial pH = 6.8) on a droplet of bacto agar. It shows uneven thickness of the cytoplasmic membrane and the lamellalike extensions of the membrane which divide the cytoplasm into chambers.

FIG. 13. *Bacillus megatherium*, C_1. Cells from a 7-hour-old slant culture at 33 C on the medium: $\frac{\text{MITG}^1}{2}$ + 1.5 g agar, mounted in Sudan black B. It shows the cytoplasmic membrane and its cross extensions.

FIG. 14. Scale for all illustrations.

[1,2] See page. 115.

much below that of simple proteins, even in gram-negative species. We are, therefore, justified in stating that at least the *principal* component of the cytoplasmic membrane, in gram-positive as well as in gram-negative species, is a highly stable combination of lipoids and proteins, namely, lipoprotein. There is also evidence (see under Discussion and Conclusions) that this lipoprotein molecule may be conjugated with some complex organic radical.

Function of the Cytoplasmic Membrane

At the present time two roles are definitely known to be played by the cytoplasmic membrane. The most conspicuous role is in cell division; the other is in permeability (Knaysi, 1944). Very recently (1945), we observed the formation of lipoprotein granules by the cytoplasmic membrane in a few aerobic sporeformers, and the elimination of these granules into the cytoplasm before sporulation. Having been unable to ascertain the function of these granules, we cannot give this process the proper interpretation.

DISCUSSION AND CONCLUSIONS

The greatest obstacle to a satisfactory demonstration of the cytoplasmic membrane has been the objection that both accumulation of dyes in the surface layer of the cytoplasm and high refringence of that layer may be merely surface phenomena. Admittedly, this objection has been difficult to answer, although one may be convinced, from long and intimate observations of the bacterial cell, that there is more to the cytoplasmic membrane than a mere optical or physicochemical artifact. The author had always considered the possibility that the cytoplasmic membrane, although real, might owe its origin to an accumulation of surface-active material. Recently (1944), however, he presented evidence indicating that, although this hypothesis may be true from the point of view of evolutionary differentiation, this differentiation has already become established in the bacterial cells with which he is familiar. Indeed, in the cells of many common species investigated, the cytoplasmic membrane is in no way a passive structure which merely accumulates on surfaces. It is a permanent, active, even, so to speak, aggressive structure which initiates, rather than follows, the division of the cell; for if the formation of the cytoplasmic membrane followed the division (by constriction) of the cytoplasm, the newly formed membranes would have curved surfaces, normal to the long axis of the cell. Actually, the centripetal growth of the cytoplasmic membrane which initiates cell division is, at first, in a plane normal or *oblique* to that axis (figures 6, 12) and curves only as it splits, thus separating the sister cells.

Several years ago (see Knaysi, 1944, p. 35) we made the observation that the cytoplasmic membrane of both gram-positive and gram-negative bacteria gives a positive Feulgen reaction. This means that at least one component of the cytoplasmic membrane contains free, or potentially free, aldehyde groups, and that this component is not removed by hydrolysis with normal HCl for 10 minutes at 60 C. In view of what we know about the chemical make-up of the

membrane and the discovery by Claude (1939) of a lipoid which reacts with Schiff's reagent, the interpretation seems to be obvious. It is necessary, however, to discuss the possibility of the following two hypotheses:

The first is indicated by the observation of Dubos and MacLeod (1938) that treatment of the heat-killed, gram-positive cells of pneumococci with a polynucleotidase obtained from animal tissues renders those cells gram-negative. Henry and Stacy (1943) secured the same result by the action of certain carbohydrate-splitting enzymes or by extraction with a solution of bile. The extraction of gram-positive cells with a 2 per cent aqueous solution of bile removes some carbohydrate and a substance identified as Mg-ribonucleate, leaving a gram-negative skeleton rich in basic protein material. If this skeleton is treated with formaldehyde (or certain other reducing agents) and soaked in the extract, the Mg-ribonucleate recombines with the skeleton and the cells become gram-positive again. Henry and Stacy believe that they have been able to show "that the essential constituent in Gram-positive organisms. . .lies in their having as part of their surface structure the magnesium salt of ribonucleic acid." In view of the fact established by the author (first reported in 1938, p. 94) that the *principal* substrate of the gram and acid-fast reaction is the cytoplasmic membrane, this membrane must be the "surface structure" mentioned by Henry and Stacy. The possibility of the presence of a ribonucleate radical in the lipoprotein complex of the cytoplasmic membrane is in no way contradictory to cytological data and had been suspected by the author (1929) when he wrote: "The membrane (of the tuberculosis organism) stains metachromatically with old methylene blue solutions," and when he further concluded that the membrane and granules of that organism "may be considered as a peculiar combination of fat with protein and other materials." Metachromatism with old solutions of methylene blue is usually considered characteristic of nucleic acids. Ribonucleic acid, free or as nucleoprotein, is known to be gram-positive (Delétang, 1933; Knaysi, 1943) and to react with Schiff's reagent (Knaysi, 1942). Consequently, it would be expected to give a positive Feulgen reaction if it is not removed in the process of hydrolysis; and it is conceivable that, granting its presence in the cytoplasmic membrane, it may be in a stable combination which resists acid hydrolysis as carried out in the Feulgen technique. However, one has also to postulate that it exists in the cytoplasmic membrane of the gram-negative bacteria in a form which resists hydrolysis by acid but which is removed in the gram technique, or that it is so bound that its acidic radicals are not totally free.

The second hypothesis that, in strains without differentiated nuclei, the cytoplasmic membrane contains the nuclear material of the cell at first appears attractive in view of the conspicuousness of the membrane in cell division. However, when one considers that the endospore, which contains nuclear material, is formed free in the cytoplasm, without any apparent co-operation from the cytoplasmic membrane of the mother cell, the soundness of such a hypothesis is questioned.

SUMMARY

The study of spontaneously autolyzed cells in cultures of *Bacillus cereus* and *Bacillus megatherium* made possible the isolation and demonstration of the cytoplasmic membrane. The fact that this membrane is much more resistant to autolysis than the cytoplasm proper indicates considerable chemical or physicochemical differences between the two structures. The cytoplasmic membrane stains with dyes of the Sudan series and gives the Sharp test for proteins and a positive Feulgen reaction. It consists principally of lipoids and proteins in a highly stable chemical combination. The internal surface of the cytoplasmic membrane is jagged and wavy; besides surrounding the cytoplasm, it forms plane films which separate the cells into compartments and which are potential places of cell division; it forms and eliminates into the cytoplasm granules similar to itself in chemical composition; its demonstrated roles are in cell division and in permeability. The thickness of the cytoplasmic membrane varies even in a single cell; in young cells of *Bacillus cereus* it is usually in the range 0.21 to 0.35 μ. The significance of the membrane's positive Feulgen reaction is discussed.

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