MEMBRANE SYNTHESIS IN *BACILLUS SUBTILIS*

III. The Morphological Localization of the Sites of Membrane Synthesis

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ABSTRACT

The results of several lines of investigation indicate that membrane growth in *Bacillus subtilis* does not occur at one or a small number of discrete zones. No indications of large regions of membrane conservation were observed. Kinetic labeling experiments of mesosomal and plasma membrane lipids indicate that the mesosomal lipids are not precursors of the plasma membrane lipids. Density shift experiments, in which the changes in buoyant density of membranes were studied after growth in deuterated media, showed no indication of large zones of conservation during membrane growth. Radioautography of thin sections of cells pulse labeled with tritiated glycerol showed no indication of specific zones of lipid synthesis. The consequences of these results for models of cell growth and division are discussed.

INTRODUCTION

An understanding of the manner in which bacterial membranes grow has great relevance to the elucidation of the mechanisms of cell growth and division. Several models of cell division or chromosomal separation (Donachie and Begg, 1970; Jacob et al., 1963) have postulated specific regions of membrane growth and specific zones of conservation of membrane material. Previous work from this laboratory (Mindich, 1970 b; Mindich, 1971) has indicated that proteins of the plasma membrane can be incorporated and can express their functions in the absence of lipid synthesis. These results imply that membrane mass can increase uniformly rather than in specific zones. In order to test whether or not specific growth and conservation zones exist, we have performed three different types of experiments. We have investigated whether the mesosome is a precursor of plasma membrane lipids, by isolating mesosomes and plasma membranes and determining the kinetics of lipid synthesis in these structures. We have studied the conservation of membrane by means of density shift experiments with membranes, in a manner similar to that devised by Meselson and Stahl (1958) for the study of conservation in DNA synthesis. We have studied the possibility of specific localization of lipid synthesis by performing radioautography on thin sections of bacteria labeled for short periods of time with tritiated glycerol.

MATERIALS AND METHODS

Bacterial Strains

Three strains of *B. subtilis* were used: B42 is a derivative of strain 168 and is auxotrophic for glycerol, tryptophan, and histidine and lacks the inducible glycerol phosphate dehydrogenase; B67 is a derivative of strain W23 and auxotrophic for glycerol and defective in the glycerol phosphate

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dehydrogenase (Mindich, 1970 a); strain 168-2 is auxotrophic for leucine, histidine, and tryptophan.

Growth Media

Bacteria were grown at 37°C in a shaking water bath in the minimal medium of Anagnostopoulos and Spizizen (1961) supplemented with an amino acid mixture approximating the composition of 0.05% casein hydrolysate. Deuterated media were prepared according to Crespi et al. (1962) with deuterated sugars and amino acids supplied by Merck Co. (E. Merck AG, Darmstadt, W. Germany). Cells had a 60 min doubling time in normal medium and a 120 min doubling time in deuterated medium.

Chemicals

Glycerol-1,3-^{3}H (1.8 Ci/mmole) was purchased from Nuclear-Chicago (Nuclear-Chicago, Des Plaines, Ill.). Glycerol-2-^{3}H (1 Ci/mmole) was purchased from Tracerlab Div., Richmond, Calif. Both compounds were found to be chromatographically pure. Radioactivity was measured in a Beckman LS2B scintillation counter in a toluene base counting solution for nonaqueous solutions and in Aquasol (New England Nuclear Corp., Boston, Mass.) for aqueous samples.

Mesosome Experiment

ISOLATION OF MEMBRANES AND MESOSOMES: Strain B42 was grown in 100 ml of complete Spizizen medium with 20 μg glycerol/ml until exponential growth was obtained. Glycerol-2-^{3}H (0.1 μCi/ml) was added and the culture was incubated until a doubling of optical density occurred. The culture was then diluted back to its original optical density in medium containing the same specific activity of tritiated glycerol and, in addition, 0.25 μCi glycerol-^{14}C/ml. Samples of 50 ml were removed, placed in an ice bath, and adjusted to 10^{-3} M iodoacetic acid (IAA)[1] and 50 μg glycerol/ml in the cold at 0, 5, 15, and 30 min after the addition of glycerol-^{14}C. The further incorporation of glycerol into lipid was prevented by the IAA. The cells were harvested by centrifugation and resuspended in 5 ml of phosphate buffer (0.1 M pH 7.5) with 0.8 M sucrose, 300 μg lysozyme/ml, and 0.01 M ethylenediaminetetraacetic acid (EDTA). The cells were incubated at 37°C for 30 min. Protoplasts were harvested by centrifugation at 10,000 g for 10 min, resuspended in 5 ml buffer (0.005 M $MgSO_4$ and 20 μg DNase/ml), centrifuged again for 20 min, and suspended in 1 ml buffer. A sample was removed to which glutaraldehyde was added to give a 1% solution; 2% potassium phosphotungstate (KPTA) was added and the sample was inspected as whole mounts in the electron microscope. The remainder of the protoplast preparation was reserved for lipid extraction. The supernatant fluid from the first centrifugation was centrifuged at 105,000 g in the Spinco SW 39 rotor for 45 min and the pellet was suspended in 0.5 ml of phosphate buffer. A sample of 0.1 ml was removed for inspection as whole mounts in the electron microscope, and the remainder was extracted for lipid radioactivity. This procedure follows that of Ferrandes et al., (1966); observation in the electron microscope showed the same tubular mesosomes as found by these authors. The mesosome fraction was also contaminated with flagella as shown by them, and probably contains small amounts of protoplast membrane fragments.

Samples were adjusted to 1.6 ml with water and then mixed with 2 ml of chloroform and 4 ml of methanol (Bligh and Dyer, 1959) to form a single phase. After 1 hr, 2 ml of chloroform and 2 ml of water were added with vortexing and the samples were allowed to form two phases. The samples were filtered and the upper layer was removed. The chloroform layer was evaporated in scintillation vials, and radioactivity was determined by scintillation counting. Counts were corrected for spillover and quenching through the use of external standardization. The ratio of ^{14}C counts to ^{3}H counts was determined for all of the samples and is plotted in Fig. 1.

Density Shift Experiment

A culture of strain 168-2 was inoculated from a growing liquid culture onto D_2O medium at a titer of 10^6 cells/ml and incubated overnight. The culture was then diluted in the same medium and incubated further. After one doubling, phenylalanine-^{3}H (3.3 μCi/ml) was added and the culture was incubated for three generations. The culture was then filtered, washed, and resuspended in Spizizen medium containing phenylalanine-^{14}C (10 mμCi/ml). Samples were removed immediately before filtering and at intervals of one generation afterwards.

Cells were centrifuged for 10 min at 10,000 rpm and resuspended in 4.5 ml of buffer containing per ml 2.5 mM $MgCl_2$, 200 μg lysozyme, 20 μg DNase, 20 μg RNase, and 100 μg bovine serum albumin (Armour Industrial Products Co., Chicago, Ill.; Fraction 5) at a concentration of 10^9 cells per ml. Cell concentration was very important for reproducible results. Too high a cell concentration leads to incomplete lysis and multiple peaks upon banding in sucrose. Cells were lysed at 37°C for 40 min. The lysates were cooled, EDTA was added to

[1] *Abbreviations:* EDTA, ethylenediamine-tetraacetic acid; IAA, iodoacetic acid; PTA, phosphotungstic acid.

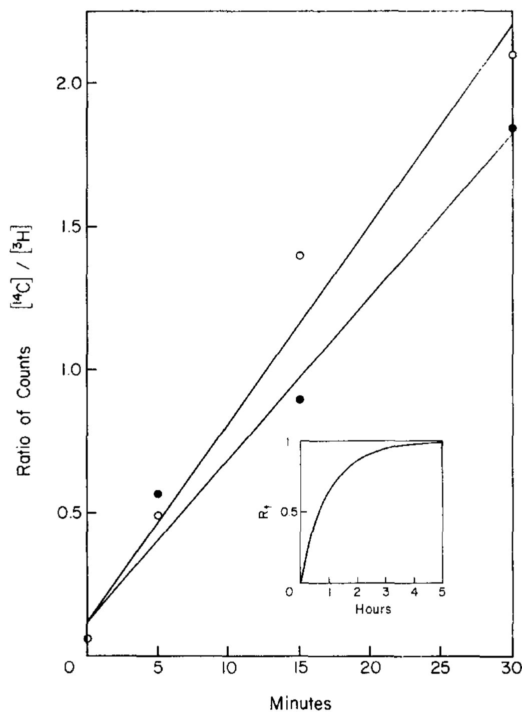

FIGURE 1 Kinetics of labeling mesosomal and plasma membrane lipids. Cells were grown in the presence of glycerol-2-^{3}H. After 1 hr of incubation, glycerol-^{14}C was also added. Samples were taken at the indicated times and mesosomes (●) and plasma membranes (○) were prepared as described in Materials and Methods. Lipids were extracted and the ratio of ^{14}C to ^{3}H radioactivity was plotted. *Inset*: Theoretical curve for equal specific activities of ^{14}C and ^{3}H in a culture growing with a 1 hr generation time. Note that the increase in ratio during the first 0.5 hr is almost linear.

make 0.025 M, and the lysate was centrifuged at 4500 rpm for 5 min to remove debris. The supernatant liquid was then layered over 0.8 ml of 60% sucrose containing 100 µg albumin/ml and centrifuged in the SW 50.1 rotor (Spinco) at 25,000 rpm for 30 min. The material at the interface of the two solutions was removed with a hypodermic syringe and added to 4 ml of 0.1 M phosphate buffer, pH 7.5, with 100 µg albumin/ml. The sample was again centrifuged and the material in the region of the interface was collected. Buffer was added to reduce the density of this material, and 0.4 ml was layered over a 4.5 ml gradient of 27%–63% sucrose containing albumin and 0.05 M phosphate, pH 7.5. The samples were centrifuged in the SW 50.1 rotor for 3 hr at 35,000 rpm. Since the membranes are entire and not fragments, equilibrium banding is attained within 1 hr. No difference is seen between banding patterns after 1, 2, or 3 hr of centrifugation. Fractions were collected through a hole punctured in the bottom of the tube. Radioactivity was determined in a scintillation counter with Aquasol. Sucrose concentrations were determined from the refractive indices of the fractions. No loss of the original label was observed three generations after shifting from deuterated to normal media.

The membranes prepared from cells grown in deuterated media were more fragile than those grown in normal medium. This fragility is manifested by the appearance of bands of lower buoyant density. In the absence of albumin, one finds very irregular banding patterns for the deuterated membranes. Prolonged centrifugation also leads to irregular patterns for the deuterated membranes, especially the appearance of lighter bands. It might be that growth in D_2O induces or activates a protease that is usually not observed in *B. subtilis* membrane preparations.

Preparation of Samples for High Resolution Radioautography

Exponentially growing cultures of B67 were filtered, washed, and suspended in medium without glycerol. After 5 min of incubation at 37°C, radioactive glycerol (100 µCi/ml 1.8 Ci/mmole glycerol-1,3-^{3}H) was added. Cells were harvested by filtering through Millipore filters (Millipore Corp., Bedford, Mass.) in Swinney-type holders. The cells were then washed with 0.1 M phosphate buffer, pH 7.5, followed by washing with 1% glutaraldehyde for 10 min. The filters were placed in 1% osmium tetroxide solution for 30 min, and then dehydrated by serial transfer through 70%, 95%, and absolute ethanols. Samples were then placed in epoxy resin mixtures with propylene oxide and finally embedded in epoxy resin. Approximately 16% of the incorporated radioactivity was lost in the dehydration procedure.

Thin sections about 1000 A thick were cut with a Porter-Blum MT-2 ultramicrotome and were coated with Ilford L-4 emulsion by application with a standard loop, dried, and stored in dessicator boxes for periods of several weeks and developed with Microdol-X (Kodak). Grain density over sections varied from 0.028 grain per cell per day of exposure for 1-min pulses to 0.5 for 60-min pulses. In general, the methods of Caro and van Tubergen (1962) were followed for high resolution radioautography. Samples were stained with alkaline lead citrate after development.

The distribution of grains was evaluated in two ways. (*a*) The relative grain density was determined over the region of the incipient septa and that of the total cell membrane. The length of membrane in the region of the incipient septa was measured with a

planimeter, including the region with a radius of 0.2 μ; the total length of the membrane in the cell section was also measured. The number of grains was determined for the region of the incipient septum and for the whole cell. The number of grains per length of membrane is called the grain density. The ratio of grain density in the region of the incipient septum to that for the whole cell is called the relative grain density. (*b*) The localization of grains with respect to the poles of the cell: the distribution of grains with respect to the distance from the oldest end of the cell was determined in terms of the fraction of the whole length or, if the cell had an incipient septum, the fraction of the distance from the oldest pole to the incipient septum.

RESULTS

Are Mesosomal Lipids Precursors of Plasma Membrane Lipids?

Mesosomes are often observed in the region of incipient septation (Ryter and Jacob, 1963). It has been suggested that lipids are first synthesized at the site of the mesosomes and that this material later becomes the plasma membrane (Fitz-James, 1968). Mesosomes and plasma membrane can be isolated independently (Ferrandes et al., 1966). We can therefore determine the kinetics of labeling of lipids in mesosomes and plasma membranes to determine if the mesosomal lipids are precursors of the plasma membrane lipids. A culture of B42 was grown in medium containing glycerol-^{3}H. After one generation of growth in the presence of the tritium label, ^{14}C-labeled glycerol was also added. At various times afterward, growth was stopped and mesosomes and plasma membranes were isolated and the lipids were extracted. The ratios of ^{14}C to ^{3}H radioactivity were determined. If the mesosome is the precursor of the plasma membrane, then the ratio of ^{14}C to ^{3}H should rise more rapidly and approach its steady state level faster than it does for the membrane. In Fig. 1 we see that the ratio does not increase faster for the mesosomes than for the plasma membrane. This indicates that the mesosomal lipids are not the precursors of the plasma membrane lipids. Similar experiments have been reported by Patch and Landman (1971) for lipids and by Morrison and Morowitz (1970) for the proteins of the mesosome. The conclusion of these authors was similar to ours in indicating that the mesosomal fraction is not a precursor of the plasma membrane.

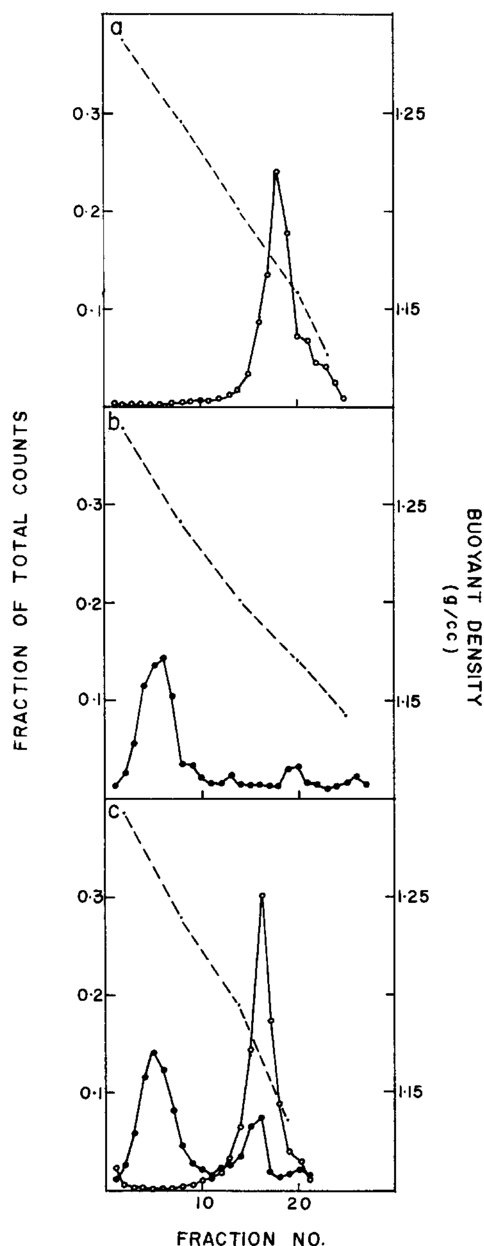

FIGURE 2 Equilibrium banding of membranes in sucrose gradients. Membranes were prepared as described in Materials and Methods and centrifuged at 35,000 rpm for 3 hr. (*a*) Membranes of cells grown in Spizizen medium with phenylalanine-^{14}C. (*b*) Membranes of cells grown overnight in deuterated medium with phenylalanine-^{3}H. (*c*) Membranes of a mixture of cells half of which were grown as in (*a*) and half as in (*b*).

Density Shift Experiment

If the membrane increases in area through growth in discrete zones, and if there remain zones of conserved "old" membrane, then it should be possible to demonstrate this by density shift experiments. In this case, we grow cells in deuterated medium in the presence of phenylalanine-^{3}H. After several generations, membranes are prepared from a fraction of the cells (as described in Materials and Methods) to yield deuterated membranes. These membranes have a buoyant density of 1.26 g/cc which is greater than the value of 1.17 g/cc that is found for membranes from cells grown in normal medium. Fig. 2 shows the banding properties of deuterated and normal membranes and also the resolution obtained when both deuterated and normal cells are mixed together and membranes prepared from the mixture. This "reconstruction experiment" shows that the denser band is resolved from the lighter one, although there is some contamination of the lighter band with material incorporated during growth in deuterated medium.

As part of the same experiment, cells that have grown in "heavy" deuterated medium in the presence of phenylalanine-^{3}H are filtered and washed and suspended in "light" Spizizen medium with phenylalanine-^{14}C. Cells are harvested after one, two, and three doublings of optical density. Membranes prepared from these cells are centrifuged in sucrose gradients and the distribution of both ^{14}C and ^{3}H counts is determined. These membrane preparations are not fragments, but membranes of entire cells. If there is one polar growth zone for the membrane, then one would expect to see the conservation of the density of the deuterated membrane. There should be a separation of ^{14}C and ^{3}H radioactivity, especially in the second and third generations. Newly incorporated ^{3}H radioactivity should be at the normal buoyant density, and the ^{14}C radioactivity, incorporated during growth in D_2O, would be distributed between the position of deuterated membrane and that for intermediate density between deuterated and normal membranes. This is the simplest case and is clearly not consistent with the results shown in Fig. 3 where it can be seen that after one generation the band is rather wide and the counts are somewhat skewed (probably due to the lack of synchronization of division cycles); by the second generation, both ^{14}C and ^{3}H radioactivities are dis-

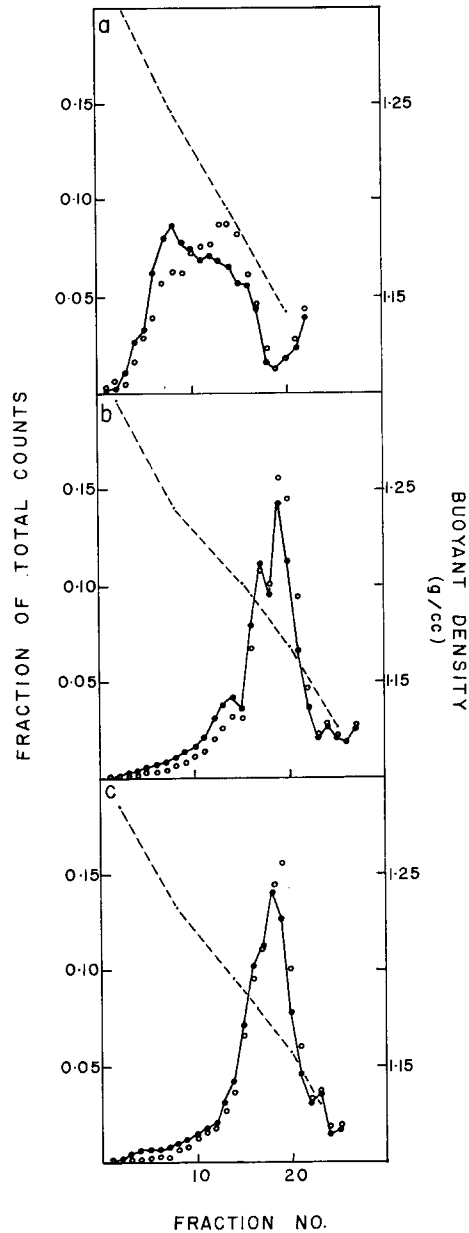

FIGURE 3 Equilibrium banding of membranes in sucrose gradients. Membranes were prepared as described in Materials and Methods and centrifuged at 35,000 rpm for 3 hr. Cells were grown overnight in deuterated medium and for three generations with phenylalanine-^{3}H in the same medium, washed, and grown for one (*a*), two (*b*), or three (*c*) generations in normal medium with phenylalanine-^{14}C. Tritium radioactivity (●); ^{14}C radioactivity (○).

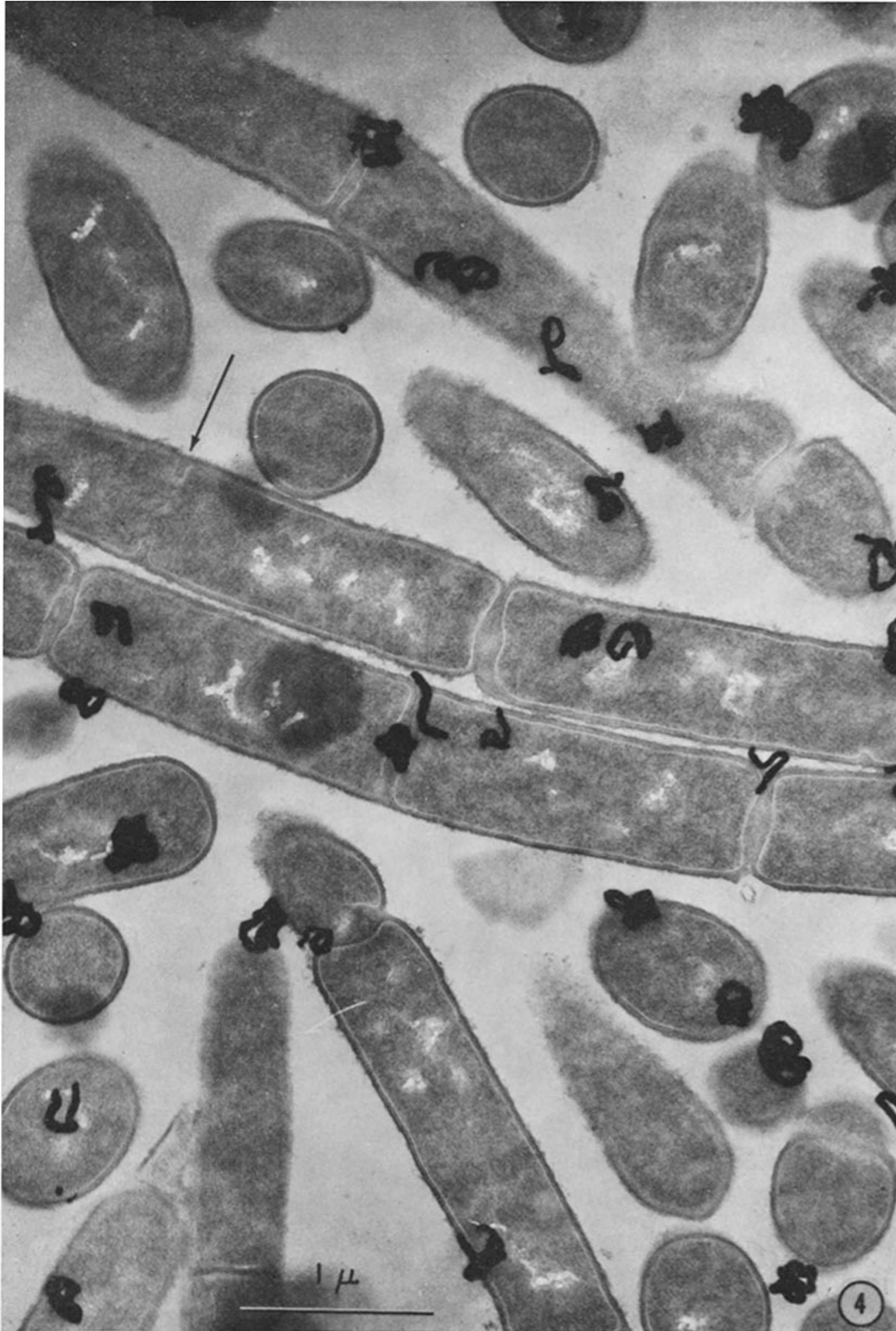

FIGURE 4 Electron micrograph of pulse-labeled *B. subtilis*. Cells were incubated with glycerol-1,3^{3}H for 5 min and prepared as described in Materials and Methods. Arrow, incipient septum. Marker is 1 μ. Grain distribution was determined only for cells showing complete longitudinal sections with entire edges. Tangential sections (see cell in upper portion of plate) were not included. $\times$ 26,000.

tributed very similarly, and by the third generation the distributions are almost identical. The experiments shown in Figs. 2 and 3 are representative of five experiments yielding similar results.

If there exists one central growth zone for the membrane, then one would expect to find a peak of intermediate density labeled with ^{3}H and ^{14}C at the first doubling and the persistence of this peak after additional doublings. At the same time, ^{14}C label should accumulate in the band of normal density. This expectation does not match the results.

If there exist many growth zones for the membrane, or if the growth of the membrane involves uniform expansion of all parts of its surface, we would obtain the results observed in Fig. 3. It remains only to determine how many conserved zones would be consistent with the results. A cell with four conserved zones in the membrane would yield membrane that has a density of half deuterated:half normal membrane after one generation and a density of one-fourth deuterated:three-fourths normal after two generations. This latter band would persist in the third generation, and new label would accumulate in the normal density band. A cell with eight conserved zones would show no separation of label, even at the third generation. We can say therefore that the number of conserved zones in *B. subtilis* is certainly more than four but not necessarily more than eight. Attempts at increasing the resolution of this experiment by fragmenting the membranes were not successful, as both sonication and shearing in a VirTis Omnimixer (VirTis Co., Gardiner, N.Y.) led to the appearance of membrane at hybrid density even in mixtures of pure deuterated and normal membranes. Tsukagoshi and Fox (personal communication) claim to have succeeded in fragmenting *E. coli* membranes to pieces of the order of 400 A without finding evidence of conservation.

Radioautography of Pulse-Labeled Thin Sections

Over 90% of the glycerol incorporated by strain B67 is extractable with lipid solvents (Mindich

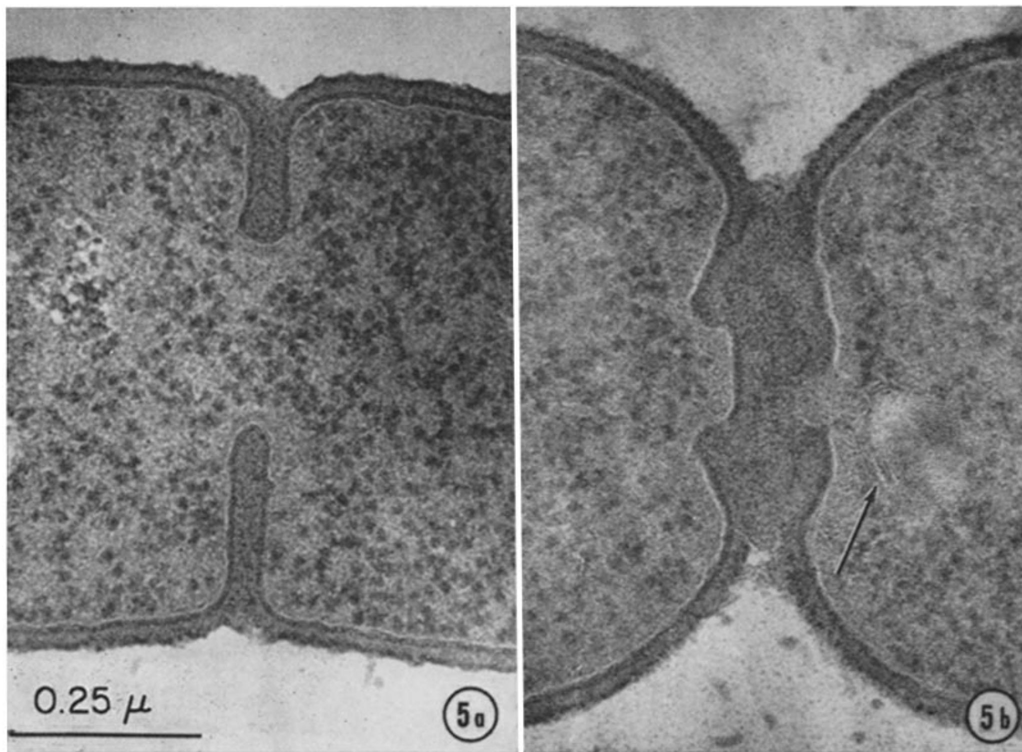

FIGURE 5 Electron micrographs of thin sections of *B. subtilis*. Fig. 5 a, incipient septum is illustrated. Fig. 5 b, septum with attached mesosome (arrow). × 104,000.

TABLE I

Relative Grain Density Over Regions of Incipient Septa

Duration of pulse	Number of cells	Number of incipient septa	Number of grains over total membrane	Number of grains over incipient septa	Relative grain density over incipient septa
min					
1	130	21	97	2	1.3
2	62	35	80	10	1.7
4	53	37	133	27	2.9
4	67	40	75	15	2.3
15	23	12	85	16	1.1
30	55	18	174	15	1.7

Measurements of membrane length and grains were done on radioautographs of cells labeled for various lengths of time. Relative grain density is the ratio of grains per unit length of membrane in the region of the incipient septa relative to the grains per unit length of membrane for the entire cell. Preparations were developed after 27 days (1-, 2-, and 4-min pulses) or 16 days (15- and 30-min pulses).

1970 a). By using glycerol of a high specific activity (1.8 Ci/mmole), it is possible to label cells for very short times in order to localize the sites of lipid synthesis. Experiments of this sort have been done in eukaryotic tissues for the localization of lipid synthesis (Amako and Dales, 1967; Goldberg and Ohad, 1970). The advantage of pulse labeling over studies of conservation is that the problems of turnover and membrane translocations are minimized (Frye and Edidin, 1970). Cells were labeled for periods of 1, 2, 4, 15, 30, or 60 min before fixation (Fig. 4). The distribution of silver grains was determined in two ways.

METHOD (A): The relative grain density was determined for the region of the incipient septum (Figs. 4 and 5) compared to that for the whole cell membrane. Table I shows the results obtained for this experiment; it is clear that the ratio is no higher for short pulses of 1, 2, or 4 min than it is for pulses of 15 or 30 min, indicating that the regions of incipient septation are not regions of preferential lipid synthesis. The relative grain density over the incipient septa was somewhat higher than 1 because the amount of membrane in these areas is underestimated, since only clearly observed membrane was measured and therefore mesosomal membrane was probably not fully accounted for.

METHOD (B): The second method was to determine the distribution of grains within the cell, according to the ratio of the distance from the oldest pole of the cell to the incipient septum or to that of the length of the entire cell if no incipient septa are observed. Cells with more than one incipient septum were very rare. This method should demonstrate polar sites of lipid synthesis or sites

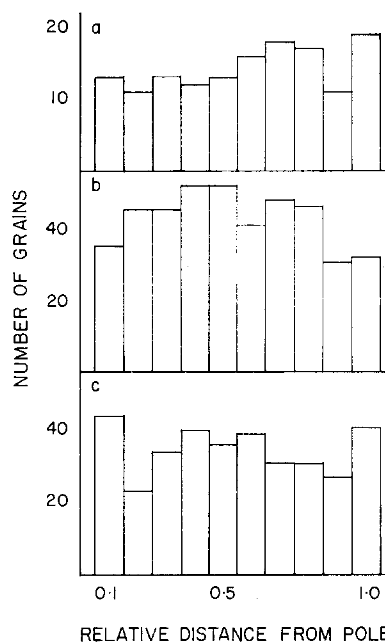

FIGURE 6 Distribution of grains with respect to cell length. The distribution of grains was determined with respect to the distance from the oldest pole of the cell. The cell length was taken to be the distance from the oldest pole to the newest pole or to the nearest incipient septum. Pulse duration was 4 min (*a*, *b*) or 60 min (*c*). In (*a*) only cells containing an incipient septum were utilized.

at the center of the cells. Fig. 6 a shows the combined results for several 2-min and 4-min pulses in cells containing incipient septa. Fig. 6 b shows the distribution for the same set of pulses for all labeled cells. Fig. 6 c shows the results for a 60-min

pulse. There is, again, no indication of a specific zone of lipid synthesis, or a specific half of the cell that has a greater amount of lipid synthesis.

Donachie and Begg (1970) have proposed that the membrane growth is polar in slowly growing cells and that pieces of specific length are conserved. If this were the case in *B. subtilis*, we might find that the grains are concentrated in the newer pole of the cell. This is not observed in Fig. 6. Their model would predict specific incorporation at the region of incipient septa in faster growing cells. This is not consistent with our observations either.

This method shows that there is not a zone of preferential lipid synthesis at the poles or at the incipient septa of cells of *B. subtilis*. It is still possible that lipid synthesis is not completely uniform over the cell surface. Even a rather small number of sites such as four might easily be missed in these experiments, due to the lack of resolution; however, it seems clear that there are not one or two specific zones of synthesis.

DISCUSSION

The results of this study do not support those models of cell growth and division that entail a limited number of zones of conservation and specific growth zones. Experiments that have been performed recently in several laboratories have given similar results. Tsukagoshi et al., (1971) found no evidence for conserved regions in a series of experiments involving density transfer mediated by the use of bromostearic acid as a replacement for the normal fatty acids of *E. coli*. It was also found that minicell membranes show no evidence of conservation or specific growth zones (Wilson and Fox, 1971). Green and Schaechter (1971) found no evidence for large conserved segments in radioautographic experiments and in studies with minicells. Lin et al. (1971) found no evidence for conservation or specific growth zones in *E. coli* radioautographic experiments by using radioactive oleate or glycerol for studying membrane synthesis. Patch and Landman (1971) found that mesosomes were not precursors of plasma membrane. However, Morrison and Morowitz (1970) claimed that the distribution of radioactive palmitate in cultures of *Bacillus megatherium* showed conservation in long term experiments and specific zones of synthesis in pulse-labeling experiments. In the same study it was pointed out that mesosomal protein does not seem to be precursor material for plasma membrane proteins. We do not understand the discrepancy between the experiments of Morrison and Morowitz and those reported here by us and by the other authors mentioned above. It seems possible that in the former case the label was not completely in phospholipid and that the patterns observed reflect the adsorption of free fatty acids to the cells.

One difficulty in interpreting the conservation experiments performed by us and by other authors is the possibility that lipids and/or proteins are synthesized or inserted in a particular zone and then migrate to other parts of the membrane, either in the plane of the membrane or by means of transport into the cell interior and back to the membrane but at another site. This possibility has apparently been already realized in the experiments of Kornberg and McConnell (1971), who demonstrated high rates of diffusion of phospholipids in artificial membranes, and those of Frye and Edidin (1970) where it was found that mouse-human heterokaryons formed through the use of Sendai virus mix at least two of their protein membrane components in only 40 min. It has not been determined in the latter case whether this mixing is due to the interchange of proteins already in the membrane or to the incorporation of preformed membrane components from the cytoplasm into the membranes; however, if such mixing were to occur in the bacterial membranes, the conservation experiments would be ambiguous. The pulse-labeling experiments would not be affected by these considerations unless the exchange were extremely rapid; the results of our pulse experiments with radioautography are consistent with our conservation experiments.

The lack of zones of conservation in the membrane leaves open the question of how the chromosomes are apportioned to daughter cells during cell division. If the DNA is attached to the membrane (Sueoka and Quinn, 1968; Ganesan and Lederberg, 1965; Ryter and Jacob, 1963) and the membrane grows by uniform expansion, then the growth of membrane cannot be used to effect the separation. In gram-positive bacteria, there is some evidence that wall structures are conserved during growth (Cole and Hahn, 1962; Chung et al., 1964; Briles and Tomasz, 1970). However, in gram-negative bacteria, there is no evidence that the elements of the surface (May, 1963) or the peptidoglycan (van Tubergen and Setlow, 1961; Lin et al., 1971) are conserved. We do not intend to offer solutions to this problem in this discussion,

except to suggest that some internal structure analogous to the eukaryotic mitotic apparatus might be the means whereby the chromosomes are apportioned and whereby the cell measures off the proper site for cell division.

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