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Membrane Synthesis in Synchronous Cultures of *Bacillus subtilis* 168

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Synthesis of bacterial membranes has been investigated in *Bacillus subtilis* by examining incorporation of amino acids and glycerol into the protein and lipid of membranes of synchronous cultures. A simple reproducible fractionation scheme divides cellular proteins into three classes (i) truly cytoplasmic, (ii) loosely membrane bound, released by chelating agents, and (iii) tightly membrane bound. These comprise approximately 75, 10, and 15%, respectively, of cellular proteins in this organism. Incorporation of radioactivity into these fractions, using steady-state and pulse labeling has been followed during the cell cycle. Cytoplasmic proteins and the loosely membrane-bound proteins are labeled at an exponential rate throughout the cell cycle. The membrane fraction is labeled discontinuously in the cell cycle, with periods of rapid synthesis over the latter part of the cycle and a period with no net synthesis during the early part of the cycle. Pulse labeling indicates that synthesis of membrane occurs at a linear rate that doubles at a fixed time in each cycle, which coincides with the period of zero net synthesis. Rates of membrane synthesis measured by pulse labeling during the period of rapid membrane synthesis are significantly less than indicated by steady-state labeling. These discrepancies are consistent with the hypothesis that during the cell cycle certain proteins are added to the membrane from the cytoplasm and that during the period of zero net synthesis there is an efflux of proteins from the membrane. Evidence in favor of this has been presented. The activity of succinic dehydrogenase (a representative of class c) varies in a step-wise manner with periods of rapid increase, approximately coincident with bursts of membrane protein synthesis, alternating with periods without any increase in activity. The activities of malate dehydrogenase (class a) and reduced nicotinamide adenine dinucleotide dehydrogenase (class b) increased throughout the cell cycle. Phospholipid synthesis is continuous throughout the cell cycle.

At a morphological level, synthesis of cell membrane involves (i) continuous extension of peripheral membrane between division, (ii) formation of mesosome and cross-wall membrane, and (iii) separation of deoxyribonucleic acid (DNA)-membrane attachment site into two with apparent extension of the membrane between sites. To relate morphology to the biochemical basis of membrane assembly, component insertion must be considered in relation to cell age (6, 11, 16, 17, 32) and cellular location (1, 3, 5, 9, 12, 19, 24, 26, 28, 38, 43, 47). Most studies of envelope synthesis have been confined to the question of location of sites of synthesis. Membrane synthesis may occur at many dispersed foci or in an equatorial band. The latter possibility, with its corrollary that old envelope components are conserved, is the basis of the replicon theory of genome segregation (13). Incorporation of each membrane component should be considered separately since several studies suggest that incorporation of phospholipid and protein is not mutally dependent (14, 25).

With one exception (28), data for phospholipids (9, 19, 26, 43, 47) favor randomly dispersed incorporation. The possibility that mesosomal phospholipid may be the precursor of plasma membrane has been eliminated (6, 26, 28). Turnover, the thermal mobility of phospholipids, and their chemical heterogeneity, may tend to obscure a specific focus of incorporation.

Autoradiographic or immunofluorescent techniques are probably inherently impossible

with membrane proteins, but indirect experiments in which segregation of certain inducible characters are studied indicate conservation of membrane protein and therefore localized insertion (1). Autoradiographic studies of incorporated ^{3}H-α-aminolaevulinic acid segregation suggest dispersed incorporation of membrane cytochrome proteins but with conservation of quite small membrane subunits (9). Cell wall synthesis must to some extent reflect membrane synthesis, and immunofluorescent techniques indicate that the wall is synthesized in an equatorial band in many strains and that, once synthesized, it is conserved through several generations (5, 12). Chai and Lark (4) claim that a complete round of DNA synthesis is conserved and segregated, with cell wall synthesized one generation later in *Lactobacillus acidophilus*. A specific site of autolysis in *Escherichia coli* has been regarded as evidence of localized wall synthesis (8, 41). Autoradiographic studies of cell wall synthesis favor both models (3, 19, 24).

Very few biochemical studies of envelope synthesis in relation to cell age have been made. Discontinuous synthesis of mucopeptide (11) and phospholipid (6) have been claimed. Recently, Ohki (32) has demonstrated discontinuous increases in cytochrome *b1* and glycerophosphate transport with a simultaneous increase in turnover of phosphatidyl glycerol. Kubitschek et al. (16, 17) consider that uptake sites for all nutrients double in activity at cell division.

This communication describes membrane synthesis in relation to the cell cycle of *Bacillus subtilis*.

MATERIALS AND METHODS

Bacteria, growth conditions, and synchronous cells were described (M. G. Sargent, J. Bacteriol., to be published).

Preparation of cell fractions. Samples of bacterial culture (1–2 ml) were transferred to ice-chilled centrifuge tubes containing chloramphenicol (CAM) (100 μg/ml). Carrier to dilute isotopes was added where required. Cells were sedimented and suspended in protoplast medium (PM) containing sucrose (0.5 M), magnesium sulfate (0.02 M, pH 6.5), malate buffer (0.02 M), and lysozyme (100 μg/ml) and were incubated at 35 C for 15 min. Under these conditions protoplast formation is always complete. Protoplasts were sedimented at 15,000 rpm for 15 min. The mesosomal vesicles (FI) were sedimented at 100,000 $\times$ *g* for 2 h. Since this fraction contains only 1% of cell protein, this step was omitted in certain experiments, in which case, protoplasts were prepared in a small volume (0.2 ml) and were lysed by dilution with tris(hydroxymethyl)aminomethane (Tris)-magnesium (0.05 M Tris, pH 7.4, containing 0.02 M magnesium sulfate). After removal of the mesosomes, protoplasts were suspended in 0.2 ml of PM and were lysed in Tris-Mg in a similar way. Samples were left for 1 h in ice to give maximal lysis, and the membranes were sedimented and washed in Tris-Mg. Supernatant fluids were pooled (FII). Sediments were washed twice in citrate-phosphate (trisodium citrate, 0.01 M, and pH 7.4 phosphate buffer, 0.1 M). Supernatant fluids were pooled (FIII), and sediments (FIV) were suspended either in citrate-phosphate (for enzyme assays) or sodium hydroxide (0.1 M) for estimation of radioactivity.

Radioactive methods: protein labeling. ^{14}C- and ^{3}H-tryptophan or -histidine were used to label protein as shown in text. In the presence of 2 and 5 μg of tryptophan and histidine, respectively, per ml incorporation is proportional to optical density (OD) up to 1.2. Samples for counting (1 ml, in duplicate) were vigorously mixed with 10% trichloroacetic acid (2 ml) after addition of carrier amino acid (200 μg/ml) and bovine serum albumin (100 μg/ml), and were heated at 80 C for 30 min, collected and dried on Whatman GF/A filters, and counted in toluene scintillation fluid (2 ml). Protein synthesis in samples was stopped with CAM (100 μg/ml) and chilling.

Pulse labeling. Samples (1 ml) of a synchronous culture were transferred to prewarmed, boiling tubes containing 1 μCi of ^{3}H-tryptophan. Samples were incubated at 35 C with shaking for 11 min, after which the pulse was terminated by addition of 1 ml of an ice-cold solution containing CAM (200 μg/ml), sodium azide (0.002 M), and tryptophan (200 μg/ml). Cells were sedimented and suspended in citrate-phosphate containing lysozyme (200 μg/ml, 0.25 ml). When lysis was complete, samples were stirred vigorously with a Vortex mixer and were diluted with 2 ml of citrate-phosphate.

The membranes (FIV) were then sedimented and washed once more together with carrier membranes (50 μg of protein). Sediment and supernatant fluid (membranes and cytoplasm, respectively) were processed as above.

Membrane turnover in synchronous cultures. Bacteria (500 ml) were grown in the presence of 0.2 μCi of ^{3}H-tryptophan per ml and 3 μg of nonradioactive tryptophan per ml until cells reached an OD (540 nm) of 2.0. Cells were then diluted into 500 ml of fresh medium. Synchronous cells were prepared immediately and were resuspended in fresh medium.

Lipid labeling. Glycerol-2-^{3}*H* was used to label phospholipid (in the presence of 50 μg of glycerol per ml) as described by Daniels (6). To stop incorporation, 1 ml of an ice-cold solution of GAC (glycerol, 2 mg/ml; sodium azide, 0.002 M; CAM, 200 μg/ml) was added to an equal volume of sample. Cells were sedimented and resuspended in GAC containing lysozyme (100 μg/ml) and then incubated for 30 min at 35 C. Two volumes of ice-cold trichloroacetic acid (10%) were added, and after 30 min the precipitated phospholipids and protein were collected on GF/A filters. Filters were washed with distilled water and then dried. To express tritium counts fully, the filters were immersed in 2 ml of ethanol, incubated at 100 C

until dry, and counted with toluene scintillation fluid. Trichloroacetic acid-precipitable radioactivity prepared by this method represented total phospholipid glycerol and was not significantly contaminated by glycerol teichoic acids. After lysozyme treatment, radioactivity that was not precipitated with ice-cold trichloroacetic acid (10%) was not extracted into chloroform-methanol (2:1, vol/vol). Trichloroacetic acid-precipitated radioactivity was almost completely extracted into chloroform-methanol. The amount of radioactivity in such a trichloroacetic acid precipitate was almost equal to the amount extracted into chloroform-methanol without prior trichloroacetic acid precipitation. Greater precision in measuring phospholipid synthesis in synchronous cells was obtained using the trichloroacetic acid precipitation procedure rather than by extraction into chloroform-methanol.

Enzyme assays: preparation of lysates. Samples (1 ml) were placed in ice-cold centrifuge tubes containing CAM (100 μg/ml). Cells were sedimented and suspended in citrate-phosphate containing CAM (100 μg/ml) and lysozyme (100 μg/ml). Control experiments indicated that when CAM (100 μg/ml) was added to growing cultures at 35 C there was no further increase in succinic dehydrogenase activity after 2 min.

Succinic dehydrogenase (EC 1.3.99.1). Reaction mixtures in 1-ml cuvettes contained 0.2 ml of sodium succinate (0.5 M, adjusted to pH 7.4), 0.05 ml of dichlorophenol-indophenol (1 mM), enzyme preparation, and citrate-phosphate to give a volume of 0.95 ml. Assays were started by using 0.05 ml of phenazine methosulfate (3 mg/ml—freshly prepared) and were read against a blank lacking phenazine methosulfate. A unit of enzyme activity decreases the absorbance of dichlorophenol-indophenol by 0.1 at 600 nm in 1 min at 22 C. The reaction rate was unaffected by cyanide.

Malate dehydrogenase (EC 1.1.1.37). Reaction mixtures in 1-ml cuvettes, contained 0.1 ml of nicotinamide adenine dinucleotide (NAD) (1 mM), 0.05 ml of dichlorophenol-indophenol (1 mM), enzyme preparation, and citrate-phosphate buffer to give a volume of 0.8 ml. Assays were started with 0.2 ml of sodium malate (0.5 M, adjusted to pH 7.4), reading against a blank lacking substrate. A unit of enzyme activity decreases absorbance of dichlorophenol-indophenol by 0.1 at 600 nm in 1 min at 22 C. Cyanide and phenazine methosulfate had no significant effect on the reaction rate.

NADH-dehydrogenase. Reaction mixtures contained 0.2 ml of reduced NAD (NADH) (10 mM), 0.1 ml of potassium cyanide (10 mM), enzyme preparation, and citrate-phosphate buffer to give a volume of 0.95 ml. Assays were started with 0.05 ml of dichlorophenol-indophenol (1 mM), reading against a blank lacking substrate. A unit of enzyme activity decreases absorbance of dichlorophenol-indophenol by 0.1 at 600 nm in 1 min at 22 C.

Chemical assays. Ribonucleic acid (RNA) and protein were determined colorimetrically by the methods of San-Lin and Schjede (40) and Lowry et al. (20), respectively.

Electron microscopy. Samples of membrane, prepared as described above, before and after citrate phosphate treatment, were mixed with an equal volume of 5% glutaraldehyde and left at room temperature for 1 h. The membranes were then sedimented and washed five times with distilled water. Samples were mixed with equal volumes of 2% phosphotungstic acid (pH 7.0), 3% sodium silicotungstate (pH 7.0), or 4% ammonium molybdate (pH 7.0), stained for 5 min, and examined at 60 kV on a Phillips EM 300 electron microscope.

Gel electrophoresis. After dialysis against distilled water and freeze drying, protein samples for electrophoresis were heated for 5 min at 90 C in 1% sodium dodecyl sulfate and 1% mercaptoethanol. Polyacrylamide gel electrophoresis (46) was carried out by using 140 μg of protein on 7.5% gels. Proteins in the gel were stained with amido-black.

RESULTS

Preparation and characterization of cell fractions. In analyzing the synthesis of the bacterial cell membrane, it was desirable to divide cellular proteins into three classes: (i) cytoplasmic soluble proteins that are released by osmotic lysis of protoplasts, (ii) typical insoluble membrane proteins, and (iii) proteins that are loosely or adventitiously attached to the membrane. Published data suggest that chelating agents would release ribosomes and certain proteins from membranes (29, 30). The effect of repeated washings in Tris-Mg and citrate-phosphate are illustrated in Fig. 1. Protoplasts, prepared as above from bacteria labeled with ^{14}C-tryptophan and ^{3}H-glycerol, were sedimented and suspended in either wash medium to achieve lysis. After 2 h of incubation at 0 C under these conditions to give maximal lysis, the particulate fraction was sedimented at 15,000 rpm for 20 min (supernatant fluid from this step = wash 1). The sediment was then washed three more times. The percentages of total cellular protein, phospholipid, and malate dehydrogenase in the membrane are shown. Although almost all malate dehydrogenase is released at the first step, the amount of membrane protein in Tris-Mg does not reach a constant level until the sediments have been washed four times. Membranes in citrate-phosphate reach constant protein composition after two washes. Phospholipid was released from membrane by successive washes, although with citrate-phosphate the rate of release was three times that with Tris-Mg. Continuous washing of membrane with Tris-Mg apparently ultimately releases proteins that are loosely bound to the membrane.

Table 1 illustrates certain analytical features of the fractionation that support this point of view. Thus, malate dehydrogenase is found in

FII (Tris-Mg-soluble extract) almost exclusively, and succinic dehydrogenase is almost exclusively in FIV (citrate-phosphate insoluble). FIII (extracted from Tris-Mg membranes with citrate-phosphate) has the highest specific activity of NADH-dehydrogenase, although it is predominantly an FII enzyme. Citrate-phosphate treatment evidently removes most of the RNA from the membranes and presumably from ribosomal proteins. When examined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, distinct differences are seen between FIII and FIV, although certain bands are clearly present in both fractions. The staining patterns of gels prepared using the same amount of protein (140 μg) from fractions III and IV are illustrated in Fig. 2. Bands 2, 3, 6, 9, and 11, which are major components of FIV, are present in reduced amounts or are completely absent from FIII. Bands 4, 5, and 7 are present apparently in similar amounts in both fractions. Fraction III is probably slightly enriched in band 8. Differences in minor bands are also evident. The visible bands in FIII do not appear to contribute sufficient protein to compensate for the bands that are absent. The discrepancy may be accounted for by a large number of proteins in low concentrations derived from ribosomes and perhaps contaminating cytoplasmic protein.

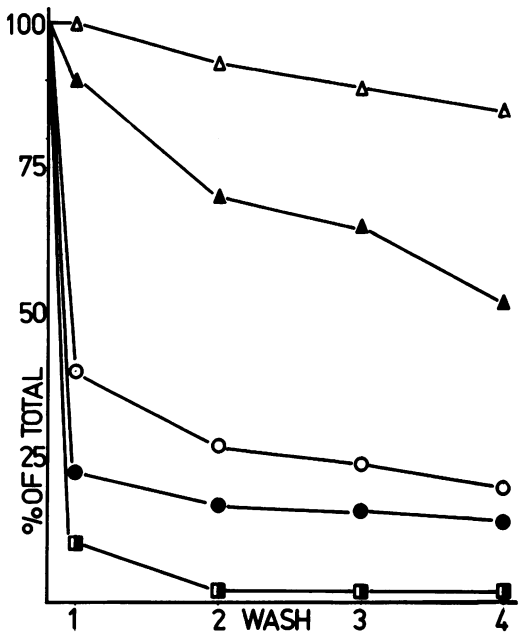

FIG. 1. *Effect of washing on membrane fraction composition. A lysate, prepared as in text, labeled with ^{14}C-tryptophan and ^{3}H-glycerol was washed as shown with either Tris-Mg or citrate-phosphate. Points indicate percentage of component remaining in 15,000 rpm sediment after each wash. Symbols:* □, ■, *malate dehydrogenase in sediment in either Tris-Mg- or citrate-phosphate-washed membrane;* ○, ●, ^{14}C-*protein in Tris-Mg or citrate-phosphate-washed membrane;* △, ▲, ^{3}H-*glycerol in Tris-Mg- or citrate-phosphate-washed membrane.*

The appearance under the electron microscope of magnesium-stabilized and citrate-treated membranes, negatively stained with phosphotungstic acid, is illustrated in Fig. 3. Similar results were obtained with silicotungstate and ammonium molybdate. The protoplast ghost under both conditions is complete with very little indication of fragmentation of its basic structure. The membrane surface, however, appears to become more granular after citrate treatment. After two washings in citrate-phosphate, the membrane forms one major band in density gradients.

Steady-state incorporation of protein label into cell fractions. The relationship between ^{14}C-tryptophan incorporation into each cell fraction and the OD of the culture is illustrated in Fig. 4. In each case, the radioactivity per milliliter of culture increased in proportion to the increase in OD, with no lags apparent.

Incorporation of ^{14}C-tryptophan into synchronous cells is illustrated in Fig. 5. Radioactivity incorporated into FII and FIII increased continuously and in proportion to OD, indicating an exponential rate of increase. However, counts incorporated into FIV, plotted against time,

TABLE 1. *Characteristics of cell fractions*

Fraction	Protein (% of total)	RNA (% of total)	Malate dehydrogenase		Succinic dehydrogenase		NADH dehydrogenase	
			%	Sp act (U/mg)	%	Sp act (U/mg)	%	Sp act (U/mg)
II	73	87	99	1.67	0	0	68	3.03
III	10	11	1	0.01	5.5	0.28	24	8.48
IV	16	2	0	0	94.5	3.02	9.3	1.96

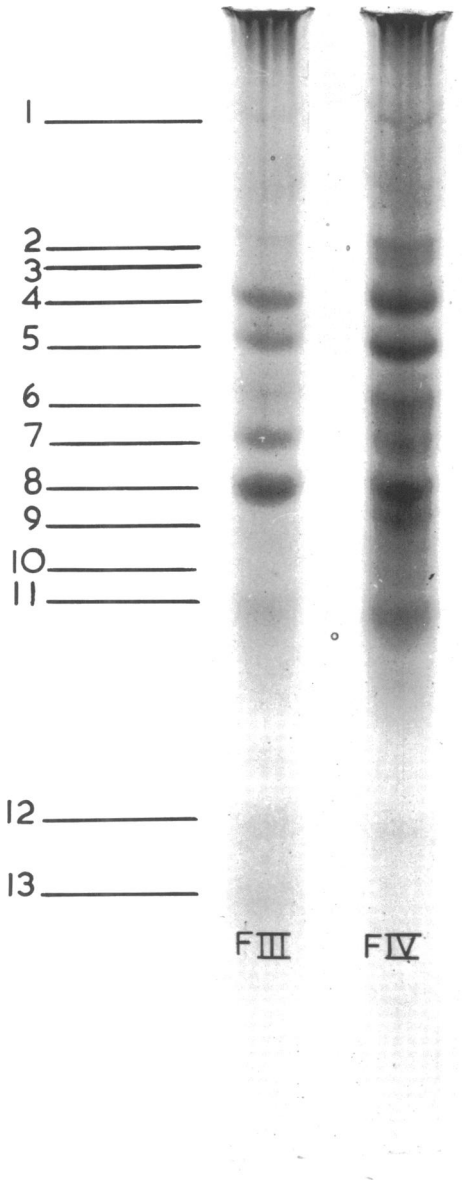

FIG. 2. *Gel electrophoresis of citrate-soluble and -insoluble fractions of the membrane.*

showed periods of rapid incorporation (0–50 and 90–120 min) alternating with periods without or with reduced synthesis (55–90 and 125–145 min) (Fig. 5).

With ^{14}C-histidine as a protein label, similar results were obtained, but the discontinuity of membrane labeling was obscured if the bacteria, from which synchronous cells were selected, were not grown in histidine for several generations.

Rates of membrane synthesis by pulse labeling. Although steady-state measurements of total membrane synthesis (Fig. 5) indicate a period in the cell cycle in which there is no net synthesis of membrane, substantial rates of ^{3}H-tryptophan incorporation into FIV were observed in short pulses. The relationship between the pattern of steady-state and pulse labeling was investigated by using the same synchronous culture. This was divided into two parts (i) which was steady-state labeled by growing cells in the presence of ^{3}H-tryptophan (0.5 μCi/ml) and (ii) which was pulse labeled for 11 min every 5 min (1 μCi/ml). The radioactivity incorporated into membrane (FIV) and cytoplasmic (FII and FIII) protein was determined and is shown in terms of nanograms of ^{3}H-tryptophan. The specific activity of ^{3}H-tryptophan was calculated as described above. The rate of cytoplasmic protein synthesis obtained by pulse labeling indicated an exponential increase in the rate of synthesis comparable with the rate calculated from steady-state labeling, although substantial differences were observed between rates of membrane synthesis obtained by pulse and steady-state labeling (Fig. 6). The rate of membrane synthesis calculated from steady-state labeling, assuming a linear rate of membrane synthesis (shown as a cross-hatched histogram in Fig. 6), was approximately constant during the intervals 0 to 65 min and 100 to 130 min, with no net synthesis in the intervals 65 to 100 and 130 to 160 min. Pulse labeling also indicates an approximately constant rate of membrane synthesis during the intervals 0 to 70 and 100 to 160 min, although at an apparently lower rate of synthesis than indicated by steady-state labeling. In sharp contrast to the steady-state labeling data, the rate of membrane synthesis actually increases during the intervals 70 to 100 and 160 to 190 min. The linear rates are approximately 30, 52, and 110 to 130 ng per min per ml, and these are, relative to the second plateau, in the ratio 1.16:2:4.6 which approximates to doublings in the rate. The pulse data, therefore, suggest that membrane is synthesised at a constant linear rate that doubles at a particular time in the cell cycle (cell age—35 min approximately).

The time course of membrane accumulation, assuming a rate of membrane synthesis equivalent to the pulse rate of membrane synthesis, is shown in Fig. 6 superimposed on the data for steady-state labeling. At the end of the period of

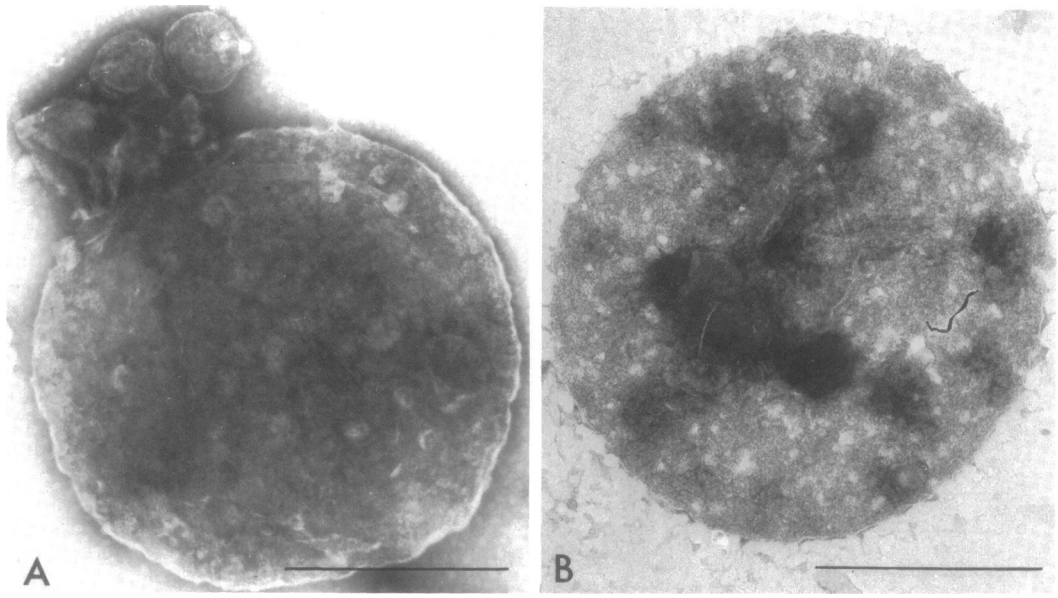

FIG. 3. *Electron micrographs of membrane fractions. (A) Magnesium stabilized; (B) citrate treated. Glutaraldehyde-fixed preparations, negatively stained with phosphotungstic acid. Bar 0.5 μm.*

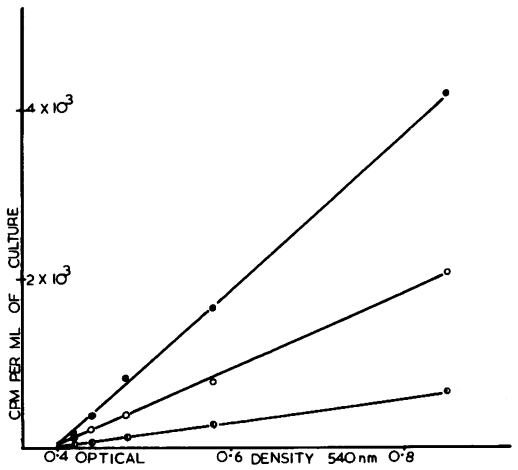

FIG. 4. *Incorporation of ^{14}C-tryptophan into cell fractions relative to optical density. Symbols: ●, cytoplasm (FII); ○, citrate-insoluble fraction (FIV); ◑, citrate-soluble fraction (FIII).*

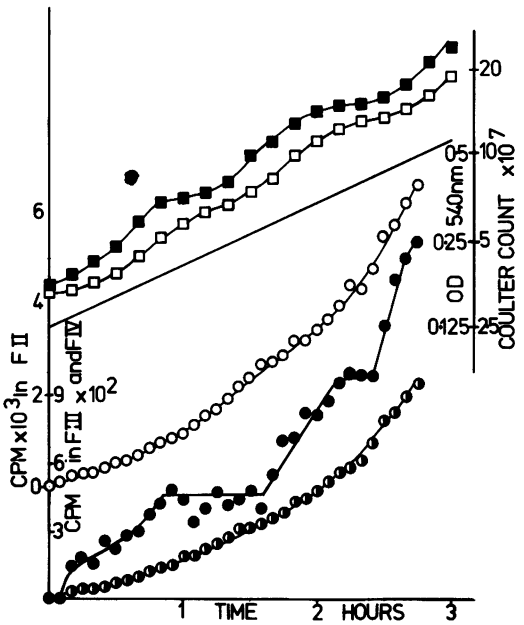

FIG. 5. *Time course of ^{14}C-tryptophan incorporation into cell fractions of a synchronous culture. Symbols: ○, ◑, and ●, ^{14}C-tryptophan incorporated into trichloroacetic acid-precipitable protein in FII, FIII, and FIV, respectively, per ml of culture; —, optical density (540 nm); □, Coulter count per milliliter of culture; ■, Coulter count corrected for numbers of units with septa.*

zero net synthesis, the amount of membrane present in steady-state labeled cells (24 and 56 ng of membrane tryptophan at 90 and 160 min, respectively) is very similar to the amount that would have accumulated on the basis of the rate of synthesis indicated by pulse labeling (27 and 62 ng of membrane tryptophan). These observations are consistent with the following hypothe-

sis. During the period of net membrane synthesis, newly synthesised protein and relatively old proteins from the cytoplasm are incorporated into the cell membrane. At the end of the period of net synthesis, an efflux of protein occurs which is numerically equivalent to the amount of "old" protein added to the membrane from the cytoplasm.

Membrane turnover in synchronous cells. The hypothesis, suggested above to explain the discrepancy between pulse and steady-state labeled data can be tested by pulse-chase studies of membrane synthesis. Two predictions follow from the hypothesis.

(i) If membranes are labeled during the period of rapid synthesis and are then chased with nonradioactive tryptophan at the end of the period of rapid synthesis, there should be an efflux of radioactivity during the period of zero net synthesis. The amount of protein involved is equivalent to the difference between the steady-state level of membrane radioactivity and the value calculated on the basis of linear synthesis at the rate obtained by pulse labeling. In the case of the discontinuities shown in Fig. 6, the amount of protein in the efflux is 29 and 27% of the amount present at 65 and 130 min, respectively.

(ii) If cells are pulse labeled and then chased during the period of synthesis, the radioactivity of the membrane fraction should increase during the chase and then decline when the period of no net synthesis starts.

These postulates were tested as follows. A synchronous culture was labeled with 1 μCi of ^{3}H-tryptophan per ml in the presence of 3 μg of carrier tryptophan per ml for either 50 or 70 min from the time of selection. To terminate the pulse, samples of culture were collected on membrane filters (Millipore Corp.), washed, and suspended in fresh media containing 3 μg of nonradioactive tryptophan. Levels of membrane and cytoplasmic radioactivity were monitored during synchronous growth after either treatment. The level of radioactivity in membrane and cytoplasm in both cases is illustrated in Fig. 7. In the case of cells labeled for 70 min after selection (Fig. 7A), membrane-bound radioactivity falls sharply over the first 10 min, during which approximately 30% of the radioactivity is lost, and then decreases more slowly. Cytoplasmic protein turnover occurs at about 12% per generation. In cells labeled for 50 min (Fig. 7B), the amount of membrane-bound radioactivity rises by about 18% over 5 min, stabilizes for 30 to 40 min and then starts to fall. Cytoplasmic protein turnover is approximately 6 to 8%.

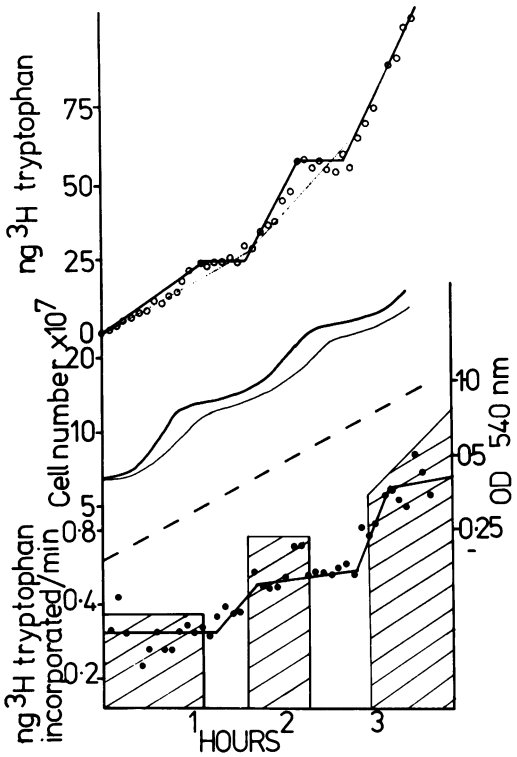

FIG. 6. *Comparison of rates of membrane synthesis obtained from steady-state and pulse labeling. Symbols:* ●, *rate of membrane synthesis obtained by pulse labeling (log scale) (cross-hatched area, rate of membrane synthesis obtained from steady-state labeling);* O, *time course of steady-state membrane synthesis (arithmetic scale);* ·····, *calculated time course of membrane synthesis based on rate of membrane synthesis obtained from pulse experiments;* —, *Coulter count per milliliter;* —, *Coulter count per milliliter corrected for numbers of units with septa;* — —, *optical density at 540 nm.*

These experiments suggest that relatively newly synthesised proteins turn over during the period of no net synthesis, but do not consider changes in older cell proteins. To study these, cells were labeled with ^{3}H-tryptophan prior to selection of small cells (see Materials and Methods) and were washed and resuspended in fresh medium containing nonradioactive tryptophan. The radioactivity of cytoplasmic protein decreases exponentially at the rate of 8% per generation. However, the radioactivity of membrane protein decreases in a step-wise fashion at an average rate of 12% per generation (Fig. 8). The peak rate of membrane turnover is observed midway through the cell cycle (cell age 30 min) during the period of zero net synthesis.

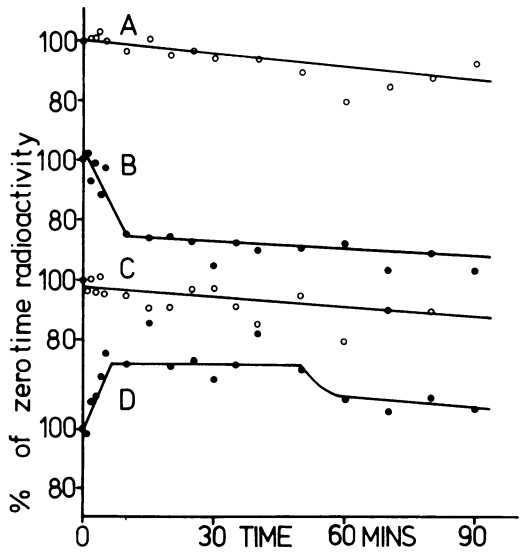

FIG. 7. *Turnover of membrane label during nonradioactive chase of synchronous cells. Synchronous cells were labeled for 70 min (A and B) or 50 min (C and D) with ^3H-tryptophan from time of selection, after which they were collected on membrane filters (Millipore Corp.), washed, and suspended in growth media. Symbols:* O, *cytoplasmic protein;* ●, *membrane protein.*

Enzyme synthesis in synchronous cells. Activities of succinic, malate, and NADH dehydrogenase per milliliter of synchronous culture are illustrated in Fig. 9 and 10, together with data for changes in cell number. NADH dehydrogenase and malate dehydrogenase activities increase throughout the cell cycle (Fig. 9). Assays for these enzymes are not sufficiently accurate to distinguish between continuous, linear synthesis and exponential synthesis. Succinic dehydrogenase activity (Fig. 10) increases in a step-wise fashion with maximal rates of increase at 15 to 55 and 110 to 125 min. The first period of synthesis occurs marginally earlier than septum formation and the second occurs slightly after cell separation. Periods of oxygen deprival (up to 15 min) cause no discontinuity in synthesis of these enzymes in unselected exponentially growing cultures.

Total phospholipid synthesis. Phospholipids were labeled with glycerol-2-^3H. Incorporation was proportional to the OD of cultures while in exponential growth in the presence of 50 μg of nonradioactive glycerol per ml. Daniels (6) has argued that this isotope labels only phospholipids because any reactions involving the 2 position result in loss of the ^3H-label to the cell water pool. However, in *B. subtilis* 168 labeled with glycerol-2-^3H, radioactivity is found in the glycerol teichoic acid and phospholipid. These can be separated analytically by precipitation of phospholipid in a lysozyme lysate with cold trichloroacetic acid (see Materials and Methods). Protein is not labeled under these conditions.

The time course of total glycerol incorporation into the phospholipids of synchronous cells is illustrated in Fig. 11, together with cell number data. Incorporation is continuous and if plotted against OD is seen to be exponential.

DISCUSSION

The significant role played by plasma membrane and mesosome in bacterial cell division indicated by electron microscopy has not been adequately described in molecular terms. The availability of methods for analyzing cell structure (35) and obtaining synchronous cultures

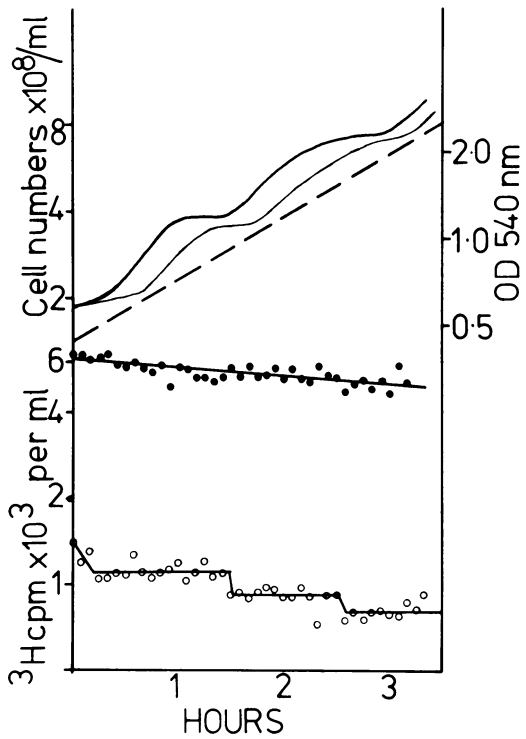

FIG. 8. *Turnover of membrane protein labeled prior to selection of synchronous cells. Symbols:* O, *membrane protein (FIV);* ●, *cytoplasmic protein (pooled FII and FIII);* —, *Coulter units per milliliter;* —, *Coulter units corrected for number of cells with septa;* — —, *optical density (540 nm).*

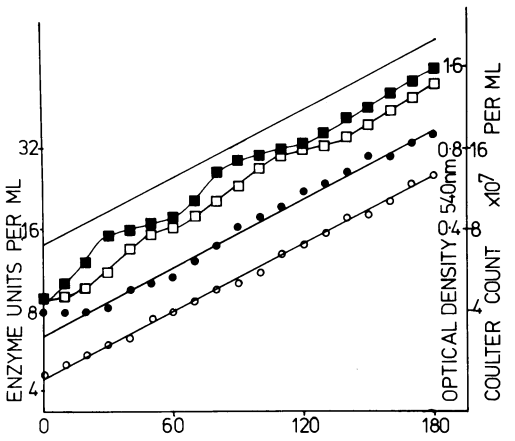

FIG. 9. *Malate and NADH-dehydrogenase activity in synchronous cultures. Malate dehydrogenase* (○) *and NADH-dehydrogenase* ($\times 10^{-2}$) (●) *activities per milliliter of culture. Symbols:* —, *optical density;* □, *Coulter count per milliliter;* ■, *Coulter count corrected for numbers of units with septa.*

(M. G. Sargent, submitted for publication) has made this more feasible with bacilli. A reproducible procedure has been used that separates the cell proteins into three fractions with minimal cross-contamination. Fraction II is cytoplasmic and fraction IV is the residue of the plasma membrane after extraction with chelating agents. Under the electron microscope this appears as a protoplast ghost, and there is no evidence of gross fragmentation, although the surface of the membrane is altered in texture. This is consistent with the view that citrate-solubilized proteins (FIII) may be located on the membrane surface. Enzymes, including NADH dehydrogenase, that are loosely attached to the plasma membrane have been demonstrated by others (7, 29, 30).

Owing to the serious difficulties in obtaining quantitative yields of mesosomal material, a clear-cut pattern of mesosome synthesis has not been obtained. The cause of this probably lies in the heterogeneity of membrane structures within the mesosome. Rogers (36) has emphasized that the mesosome is composed of an intracytoplasmic membrane (that is continuous with the cell membrane) and vesicles and tubules enclosed within it, which may be attached to the membrane to varying extents. Procedures for obtaining mesosomes yield only the vesicular component, and no convincing isolation of the mesosomal "bag" has been described. The yield of mesosomal vesicles and tubules depends on the level of magnesium used (35, 37, 38); maximal yields being favored by low magnesium levels. The level used in these experiments (20 mM) is the lowest commensurate with minimal leakage of cytoplasmic proteins and at which release of vesicles seems to be almost complete morphologically (35). The mesosomal bag in this case is almost certainly in the FIV fraction.

Assembly of the membrane in relation to the cell cycle has been studied by following incorporation of tryptophan or histidine into the proteins of cell fractions in synchronous cells. Tryptophan is of special value for such experiments since it is absent from the flagella of *B. subtilis* (21), and feasible methods of separating flagella from membrane are not available. Using steady-state labeling, fractions FII and FIII were synthesised exponentially, but FIV was synthesised in an apparently discontinuous fashion with periods of incorporation alternat-

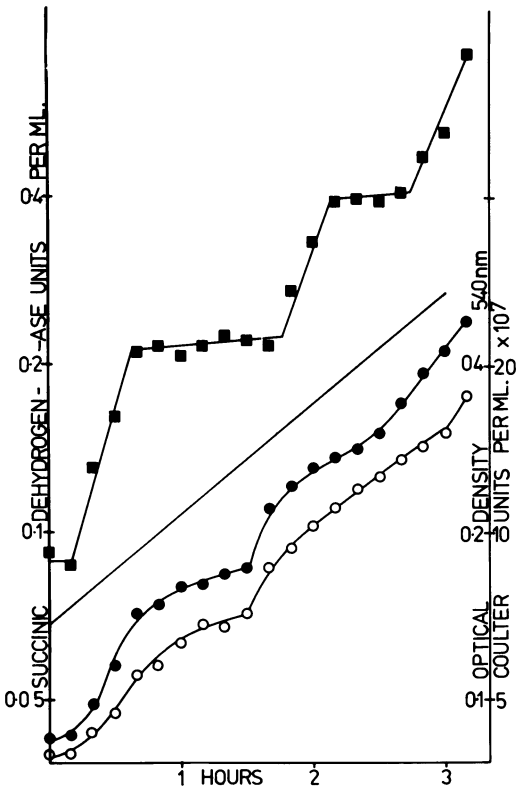

FIG. 10. *Succinic dehydrogenase activity in synchronous cultures. Symbols:* ■, *succinic dehydrogenase activity per milliliter;* —, *optical density;* ○, *Coulter count per milliliter;* ●, *Coulter count corrected for numbers of units with septa.*

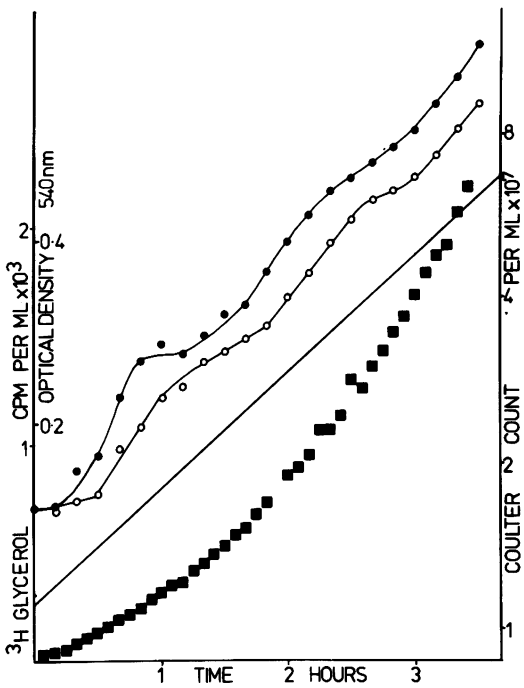

FIG. 11. *Phospholipid synthesis in synchronous cells. Symbols:* ■, *³H-glycerol incorporated into trichloroacetic acid-precipitable fraction of lysozyme lysate per milliliter of culture;* —, *optical density;* O, *Coulter count per milliliter;* ●, *Coulter count corrected for numbers of units with septa.*

ing with periods without net incorporation. In contrast, pulse-labeling experiments indicate that membrane is synthesized continuously at a linear rate, which increases sharply, at a particular cell age and remains constant until the same time in the next cycle. Two kinds of discrepancy are evident. When rates of membrane synthesis, obtained from pulse labeling or calculated from steady-state labeling data, are compared. Firstly, during the period of zero net synthesis, the measured rate of membrane synthesis does not decrease but remains constant and almost doubles in rate towards the end of the period. Secondly, during the period of synthesis, pulse measurements of the rate of membrane synthesis are consistently lower than the rate of synthesis calculated from steady-state measurements. Rates of cytoplasmic protein synthesis obtained by the two methods were virtually identical, suggesting that it is unlikely that the lower rate of membrane synthesis obtained by pulse labeling could be a technical artifact.

To account for the first discrepancy, an efflux of protein-bound radioactivity must be postulated, amounting to approximately 30% of the membrane synthesized in the period of rapid synthesis. This can be demonstrated formally by monitoring the decline in membrane-bound radioactivity in the presence of nonradioactive tryptophan in cells labeled prior to the discontinuity in membrane synthesis. Thus membranes labeled for 70 min after selection of synchronous cells show a considerable amount of turnover which is numerically equivalent to the postulated efflux. Turnover of old membrane labeled prior to selection of cells was also examined. In this case periodic bursts of turnover were observed amounting to approximately 12% per generation, coincident with the period of zero net synthesis. Thus, the postulated efflux of radioactivity from the membrane is derived principally from relatively newly synthesised proteins, although there is a contribution from old membrane. Nishi and Kogoma (31) have demonstrated periodic bursts of protein turnover (10% per generation) coincident with septation in *E. coli*.

An explanation of the second discrepancy (between the steady-state rate of membrane synthesis and the rate of pulse labeling) could be that the steady-state rate of membrane synthesis comprises two contributions: (i) protein that is newly synthesised and (ii) older protein added to the membrane, presumably from the cytoplasm. Since pulse labeling would estimate only the former, the higher rate of synthesis obtained in steady-state-labeled cells could include a contribution from the cytoplasm. On this view, an upward curve in the time course of membrane labeling should be expected and was observed in most steady-state labeling experiments during the period of synthesis, although the line of best fit is uncertain. This hypothesis is also supported by the observation that, if cells are labeled for 50 min during the period of rapid synthesis and are then chased with nonradioactive tryptophan, there is an 18% increase in membrane-bound radioactive protein during the first 5 min of the chase. This observation suggests that proteins synthesized in the cytoplasm may be transferred to the membrane fraction in the manner suggested above. The significance of the two contributions to the net rate of membrane synthesis cannot be assessed at present. However, the amount of protein added to the membrane originating from the cytoplasm is numerically equivalent to the efflux of membrane protein that occurs during the period of no net synthesis, and also the proteins involved in the efflux are labeled principally during the cycle immediately prior to the discontinuity in net synthesis and not in a pre-

vious cycle. The efflux of radioactivity may involve transfer of intact proteins to the cytoplasm or alternatively protein degradation to trichloroacetic acid-soluble products. An unequivocal distinction between the possibilities is not possible as protein degradation clearly occurs under the circumstances of these experiments. Pine (34) has demonstrated a population of rapidly-turning-over proteins within the cell envelope fraction of *E. coli*.

The principle enzymatic functions of the bacterial membrane, nutrient transport, and energy generation are difficult to study in synchronous cultures since large amounts of material are required. Furthermore, the significance of measurements of these activities in terms of amounts of specific proteins is difficult to assess. However, a preliminary approach to this problem has been made in this communication by studying succinic dehydrogenase activity, an example of an extremely insoluble enzyme. Malate and NADH dehydrogenases have also been studied since they are representatives of the cytoplasmic and insoluble fractions, respectively, and are also respiratory enzymes. The activity of succinic dehydrogenase increases discontinuously, with increases at the time of cell division. Such a change in enzyme activity can reflect either alterations in the specific activity of individual molecules or in the amount of enzyme protein. Rigorous discrimination between these alternatives is not possible at present, although inhibition of protein synthesis by CAM stops the increase in enzyme activity (in contrast to the adenosine triphosphatase of *Micrococcus lysodeikticus* [45], suggesting that no activation of the protein occurs subsequent to synthesis. In contrast to succinic dehydrogenase, the respiratory components, NADH-dehydrogenase and malate dehydrogenase, which are not in the citrate-insoluble fraction are synthesised continuously.

The activities of stable enzymes in the bacterial cell cycle follow two major patterns (27), (i) continuous linear synthesis, doubling in rate at the time of duplication of its structural gene (10, 33), (ii) discontinuous synthesis (as shown by succinic dehydrogenase, above), so that enzyme activity increases for only a brief period in the cell cycle (18, 22, 23). "Step" enzymes of this kind seem to appear in a sequence corresponding to the gene order, though not coincident with the time of gene duplication and can appear in the absence of DNA replication. The phenomenon is not well understood but has been explained in terms of oscillatory repression (22) or linear transcription (42). Recently, the suggestion has been made that certain step enzyme phenomena (18) are not observed in synchronous cells obtained by selection methods and that the steps may be caused by the method of synchronization (2). Step enzymes in germinating spores are thought to reflect a developmental process that is controlled by linear transcription (42). The step in succinic dehydrogenase synthesis occurs just before cell separation and, therefore, is added in the course of a linear membrane growth period. Step-wise increases in certain membrane activities have been observed by others. Ohki (32) has shown that cytochrome *b1*, an extremely insoluble membrane protein in *E. coli*, increases in a step-wise fashion in synchronized cells. By measuring rates of uptake of a variety of nutrients, Kubitschek (16, 17) and Ohki (32) claim to have shown that uptake sites (presumably permease molecules) double in number at specific times in the cell cycle of *E. coli*.

These observations give a preliminary outline of membrane assembly in relation to the cell cycle. Five possible processes that may be involved in the insertion of protein into the membrane are indicated in Table 2.

Total phospholipid synthesis measured as glycerol incorporation is apparently continuous in the cell cycle of *B. subtilis*, in agreement with Ohki's (32) observations on *E. coli*. In contrast, with *B. megaterium* synchronized by amino acid starvation, Daniels (6) observed a relatively small acceleration in the rate of phospholipid synthesis at the time of septum formation. Measurements of total lipid synthesis will probably mask specific changes in individual molecular species, and in fact Ohki (32) has shown that phosphatidyl glycerol turnover is restricted to a short period in the cell cycle.

In any study with synchronous cells, the

TABLE 2. *Processes involved in incorporation of proteins into membranes*

Process	Cell age[a]
1. Linear incorporation of newly synthesized protein (pulse labeled) with sharp changes in rate approximately 50 min before cell separation	35 min
2. Addition of proteins from the cytoplasm to the citrate insoluble fraction	35–85 min
3. Efflux of proteins from the citrate insoluble fraction	0–35 min
4. Addition of citrate-extractable protein (including ribosomal protein)	Throughout cycle
5. Discontinuous appearance of succinic dehydrogenase	75–10 min

[a] Cells in which daughter cells have just separated are regarded as at cell age 0. Septum formation occurs 20 min before separation (cell age 65 min). Doubling time 85 min.

possibility exists that phenomena observed are created by the synchronization procedure. Although the method used here is both rapid and gentle, *B. subtilis* 168 nevertheless shows a small reduction in growth rate (up to 15%) after selection. This organism is extremely delicate when grown in minimal media, and reductions in growth rate can be induced by dilution into large volumes of prewarmed media. Published estimates of protein turnover in exponentially growing cultures of bacilli have been quite low (2–7%) per generation (44). However, we typically have found higher and rather variable rates of turnover in *B. subtilis* 168 both in exponential and synchronous cultures. The possibility must be considered that filtration for selection of synchronous cells may induce some turnover. A strain of *B. subtilis* has now been isolated in which there is no reduction in mass growth rate during selection of synchronous cells (unpublished results).

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